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Transplantation of Ex Vivo Expanded Cord Blood

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ABSTRACT

Umbilical cord blood (CB) from unrelated donors is increasingly used to restore hematopoiesis after myeloablative therapy. CB transplants are associated with higher rates of delayed and failed engraftment than are bone marrow transplants, particularly for adult patients. We studied the ex vivo expansion of CB in an attempt to improve time to engraftment and reduce the graft failure rate in the recipients. In this feasibility study, 37 patients (25 adults, 12 children) with hematologic malignancies (n = 34) or breast cancer (n = 3) received high-dose therapy followed by unrelated allogeneic CB transplantation. A fraction of each patient's CB allograft was CD34-selected and cultured ex vivo for 10 days prior to transplantation in defined media with stem cell factor, granulocyte colony-stimulating factor, and megakaryocyte growth and differentiation factor. The remainder of the CB graft was infused without further manipulation. Two sequential cohorts of patients were accrued to the study. The first cohort had 40% and the second cohort had 60% of their CB graft expanded. Patients received a median of 0.99×10^7 total nucleated cells (expanded plus unexpanded) per kilogram. The median time to engraftment of neutrophils was 28 days (range, 15-49 days) and of platelets was 106 days (range, 38-345 days). All evaluable patients who were followed for 28 days or longer achieved engraftment of neutrophils. Grade III/IV acute GVHD was documented in 40% and extensive chronic GVHD in 63% of patients. At a median follow-up of 30 months, 13 (35%) of 37 of patients survived. This study demonstrates that the CD34 selection and ex vivo expansion of CB prior to transplantation of CB is feasible. Additional accrual will be required to assess the clinical efficacy of expanded CB progenitors.

KEY WORDS

Cord blood transplantation • Ex vivo expansion • Unrelated allotransplantation

INTRODUCTION

Since the first umbilical cord blood (CB) transplantation was performed by Gluckman et al. in 1988, more than 1500 patients worldwide have received related or unrelated CB transplants for a variety of malignant and nonmalignant diseases [1-16]. The majority of CB recipients have been children with an average weight of 20 kg. The event-free survival (EFS) rates reported thus far are comparable or superior to results achieved following unrelated allogeneic bone marrow transplantation [16]. Moreover, there are many reports of what appears to be less graft-versus-host disease (GVHD) than that associated with bone marrow transplantation in children, despite the use of CB grafts with substantially more donor-recipient HLA disparity than that tolerated in recipients of marrow [4,5,9,10,13] or peripheral blood progenitor cell (PBPC) [17-19] allografts.

The major disadvantage of CB is the low cell dose, which results in slower time to engraftment and higher rates of engraftment failure than occur with bone marrow transplantation [20-24]. In published studies of CB transplantation, the median times to an absolute neutrophil count (ANC) of $\geq 0.5 \times 10^9$/L ranged from 22 to 34 days [5,9,10,14,15]. Median times to a transfusion-independent platelet count $\geq 20 \times 10^9$/L varied from 56 to more than 100 days, with engraftment failure rates of 12% to 18%. However, the engraftment failure rate for the adult patients (>18 years old and/or >45 kg) in those series was substantially higher, ranging from 10% to 62%. These larger adult

Table 1. *Patient and Disease Characteristics**

Characteristics	Adults (n = 25)	Children (n = 12)	All (n = 37)
Patient-related			
Age, median (range), y	40 (23-60)	9 (1.3-19)	38 (1.3-60)
Weight, median (range), kg	74 (54-116)	28 (9-84)	61 (9-116)
Male/Female	8/17	8/4	16/21
Diagnosis, n			
AML	7	3	10
ALL	1	9	10
CML/CLL	6	0	6
NHL/HD/breast cancer	9	0	9
Status at transplantation, n			
Stable disease (CML, CLL)	2	0	2
First CR	0	4†	4
Second CR	0	3	3
Third CR	0	3	3
Advanced (refractory/CML-AP)	23	2	25

*AP indicates accelerated phase.

†CR1 patients had monosomy 7 (n = 1), MLL^+ (n = 2), and Ph^+ (n = 1).

patients might benefit the most from the ex vivo expansion of CB progenitor cells. In the majority of those studies, there appears to be a threshold effect in the total nucleated cell (TNC) dose of unmanipulated CB infused and time to engraftment (patients who received doses above the median for that particular study appeared to experience superior engraftment to that of patients who received TNC doses below the median). In a study reported by Gluckman et al., engraftment and survival were superior in patients who received $\geq 3.7 \times 10^7$ TNC/kg [9]. This large a cell dose is not generally available for patients weighing more than 45 kg. For adult patients, it appears that recipients of $\geq 1.0 \times 10^7$ TNC/kg had more favorable engraftment than did recipients of lower cell doses [15]. The delays in engraftment and engraftment failures in the studies referenced above could be due to transplantation of a suboptimal number of progenitor cells and/or to the more immature state of the CB progenitors, requiring additional time for maturation [25]. We have previously demonstrated that human repopulating CB $CD34^+$ cells can give rise to cells that express myeloid markers in the bone marrow within 1 week and in the peripheral blood within 2 weeks following transplantation into nonobese diabetic/severe combined immunodeficiency disease (NOD/SCID) mice [26]. This result suggests that maturation time may not be a factor in delayed granulocyte recovery. Additionally, using the same model system, we have found a dose response that correlates the magnitude of human chimerism in mice with the number of input human CB $CD34^+$ cells transplanted [27]. Although not definitive, together these data suggest that delayed engraftment in patients may be due to a reduction in the number of appropriate CB repopulating cells. We postulated that the ex vivo expansion of CB progenitors prior to transplantation could potentially ameliorate inadequate hematopoietic recovery by generating higher numbers of cells responsible for short-term reconstitution. With that goal, we sought to identify culture conditions that would produce expansion of committed myeloid and megakaryocytic CB progenitors without compromising the long-term repopulating potential of those and other cells in the graft. In preclinical studies, $CD34^+$ progenitors were isolated from CB and cultured for 10 days with stem cell factor (SCF), granulocyte colony-stimulating factor (G-CSF), and megakaryocyte growth and differentiation factor (MGDF) [28]. A median 100-fold expansion of input myeloid and erythroid colony-forming cells in culture and a 500-fold expansion of $CD61^+$ megakaryocyte progenitors were consistently demonstrated. A clinical trial using identical conditions to expand $CD34^+$ PBPCs in the autologous transplant setting demonstrated an improvement in time to neutrophil engraftment compared to that of historical controls [29]. With these data as background, the clinical trial described below was designed and executed, escalating the percentage of available CB cells to be expanded as safety and efficacy permitted.

PATIENTS AND METHODS

Patient Selection

Studies were approved by the Colorado Multiple Institutional Review Board (COMIRB) (protocols #96-154 [transplant] and #97-352 [CB expansion]), as well as the Food and Drug Administration (BB-IND#7166); all patients or their legal guardians gave written informed consent. Patients with hematologic malignancies or breast cancer lacking an HLA-matched sibling donor and willing to have a portion of their CB graft expanded ex vivo prior to transplantation were eligible for enrollment. Patients were required to have normal vital organ function measured within 30 days of study entry.

Between August 1997 and May 2000, 37 patients (21 female and 16 male patients; 25 adults and 12 children) were enrolled in this study. Follow-up for all patients in this study was closed as of October 15, 2001, for purposes of this report. The median age of the patients was 38 years (range, 1.3-60 years). As shown in Table 1, the median weight of the patients was 61 kg (range, 9-116 kg). The diseases for which the patients received transplants included acute myelogenous leukemia (AML) (n = 10), acute lymphoblastic leukemia (ALL) (n = 10), chronic myelogenous leukemia (CML) (n = 3), chronic lymphocytic leukemia (CLL) (n = 3), non-Hodgkin's lymphoma (NHL) (n = 5), Hodgkin's disease (HD) (n = 3), and breast cancer (n = 3). This group of patients had a uniformly poor prognosis and advanced disease according to the International Bone Marrow Transplant Registry criteria [30]. Twenty-three of the 25 adult patients had refractory disease at study entry. The HD, NHL, and breast cancer patients had received a median of 3 (range, 1-5) prior standard-dose regimens. Nine of the 12 pediatric leukemic patients were beyond their first complete remission (CR1); the 3 patients in CR1 all had cytogenetic abnormalities (MLL^+ [n = 2] or Ph^+ [n = 1]).

Selection of CB Grafts

Patient and CB HLA typing was performed by serology or low-resolution molecular testing for class I HLA-A and HLA-B antigens and by high-resolution DNA typing for the class II HLA-DRB1 alleles. Preferred CB units were those that were matched at 6/6, followed by 5/6, and then 4/6 alleles. Nine patients received 6/6, 22 patients 5/6, and 6 patients

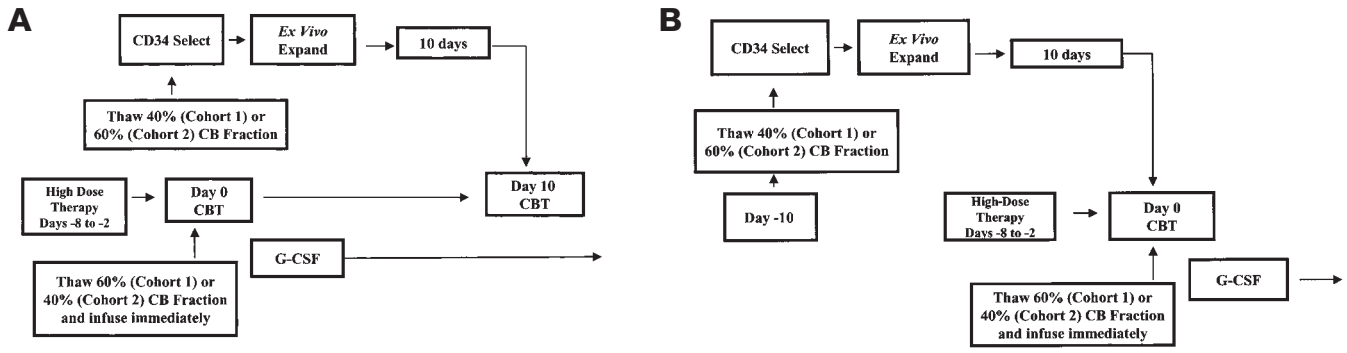

Figure 1. A, Stratum A: cord unit in 1 fraction. Patients received high-dose therapy on days –8 to –2. On day 0, the entire CB unit was thawed; 40% (cohort 1) or 60% (cohort 2) of the CB cells were CD34-selected and placed into expansion culture, while the remaining 60% (cohort 1) or 40% (cohort 2) of the cells were infused immediately without further manipulation. On day +10, the expanded CB cells were washed and infused. Systemic G-CSF was initiated on day 0 and continued until an ANC of 2.5×10^9/L (children) or 5×10^9/L (adults) was reached. B, Stratum B: cord unit in 2 fractions. Patients had 40% (cohort 1) or 60% (cohort 2) of their CB grafts thawed, CD34-selected, and placed into expansion culture on day –10. On days –8 through –2, the patients received high-dose therapy. The expanded CB cells were harvested on day 0, washed, and infused. Approximately 3 hours later, the remaining fraction of the unmanipulated CB cell graft was thawed and immediately infused.

4/6 HLA-matched units. For the 5/6 matches, 10 were mismatched at the A, 8 at the B, and 4 at the DR locus. For the 4/6 matches, 4 were mismatched at A and B and 2 at the B and DR loci. There was no minimal CB cell dose requirement for this study, although attempts were made to obtain CB which had $\geq 1 \times 10^7$ TNC/kg in the prethawed unit. Twenty-five of the 37 patients had their CB units cryopreserved in 1 fraction and were assigned to Stratum A; they received CB units from the St. Louis University Cord Bank (n = 9), New York Blood Center (n = 7), Australian Cord Bank (n = 2), University of Massachusetts (n = 3), University of Dusseldorf (n = 1), and banks in Malaga, Spain (n = 2) and Bergin, New Jersey (n = 1). Twelve patients had their CB units cryopreserved in 2 fractions and were assigned to Stratum B; their units came from the CB banks at the University of Colorado (n = 11) in 40% and 60% fractions and at the University of Dusseldorf (n = 1) in two 50% fractions.

Treatment Schema

For a back-up hematopoietic graft, either an autologous marrow or PBPC fraction was harvested and cryopreserved or a second alternative donor (CB or family member) was identified. As shown in Figure 1A, Stratum A patients (whose CB grafts were cryopreserved in a single fraction) received the high-dose therapy regimens described below on days –8 through –2. On day 0, the entire CB unit was thawed; 40% (Cohort 1) or 60% (Cohort 2) of the CB cells were CD34-selected and placed into expansion culture, as described below, while the remaining 60% (Cohort 1) or 40% (Cohort 2) of the cells were infused immediately without further manipulation. On day +10, the expanded CB cells were washed and infused. As shown in Figure 1B, Stratum B patients (whose CB grafts were cryopreserved in 2 fractions) had 40% (cohort 1) or 60% (cohort 2) of their CB grafts thawed, CD34-selected, and placed into expansion culture on day –10, prior to high-dose therapy administration. On days –8 through –2, the patients received high-dose therapy. The expanded cells that had been in culture for 10 days were harvested on day 0, washed, and infused. Approximately 3 hours later, the remaining fraction of the unmanipulated CB cell graft was thawed and immediately infused. Following the initial CB infusion on day 0, all patients received 10 μg/kg per day of G-CSF systemically until the ANC was $\geq 5.0 \times 0^9$/L (adult patients) or $\geq 2.5 \times 10^9$/L (pediatric patients). The patients were assigned to the strata and cohorts described in Table 2.

Preparative Regimens

One of the 3 high-dose regimens specified in the protocol was administered, based on patient age, underlying disease, and prior therapy. Twenty adult patients with hematologic malignancies received fractionated total body irradiation (TBI) of 1200 cGy total, delivered in 200-cGy fractions twice daily on days –8, –7, and –6, plus melphalan 46.5 mg/m^2 (n = 10) or 33.3 mg/m^2 (n = 10) administered daily for 3 days on days –5, –4, and –3. The melphalan dose was reduced from a total of 140 mg/m^2 to 100 mg/m^2 because of excessive regimen-related toxicity midway through the trial. The breast cancer patients (n = 3) and previously irradiated lymphoma patients (n = 2) received oral

Table 2. *Cohort Escalation Schema**

Stratum	Cohort	% CB Expanded	n (Adults + Children)
A	1	40	14 (10 + 4)
A	2	60	11 (6 + 5)
B	1	40	8† (6 + 2)
B	2	60	4 (3 + 1)
Total			37 (25 + 12)

*Cord unit cryopreserved in 1 (stratum A) or 2 (stratum B) fractions.

†One patient had the cord unit cryopreserved in two 50% fractions and thus received 50% rather than 40% expanded CB.

busulfan every 6 hours for a total of 16 doses, adjusted to provide a cumulative area under the curve exposure over 4 days of 16,000, on days –8, –7, –6, and –5, in combination with melphalan 46.5 mg/m^2 on days –4, –3, and –2. The 12 pediatric patients received 1200 cGy TBI in 200-cGy fractions twice daily on days –8, –7, –6, plus cytosine arabinoside 2000 to 2500 mg/m^2 every 12 hours for a total of 6 doses and cyclophosphamide 45 mg/kg daily for 2 doses on days –3 and–2. All patients received horse antithymocyte globulin (ATG) 30 mg/kg daily on days –5, –4, and –3.

GVHD Prophylaxis Regimens

Cyclosporine and prednisone were administered to all patients for GVHD prophylaxis. Cyclosporine administration began on day –1; the dose was adjusted to maintain a blood cyclosporine level of 350 to 450 ng/mL (adults) or 150 to 250 ng/mL (children). If no GVHD was present on day 100, a cyclosporine taper was initiated and completed over 3 months. The first 8 patients received very high-dose methylprednisolone, 10 mg/kg on day –4, 5 mg/kg on day –3, and 2 mg/kg per day on day –2 through day 2, followed by a taper at 5% of dose per week. A high incidence of infectious complications prompted a reduction in the steroid dose. The subsequent 29 patients received moderate-dose steroids. The adults received methylprednisolone 0.4 mg/kg per day from day 0 through day 4, followed by 1 mg/kg per day from days 5 through 20, with a 5% per dose per week taper initiated on day 21 if no GVHD was documented. The pediatric patients received methylprednisolone 5 mg/kg per day from day –5 through day –2, 2 mg/kg per day from day –1 through day 1, and then 2 mg/kg every other day alternating with 1 mg/kg from day 2 through day 19, followed by a taper at 5% of dose per week.

GVHD Assessment

During the first 100 days, patients were evaluated daily for acute GVHD, which was graded according to standard criteria [31]. After day 100, patients were evaluated for chronic GVHD, which was also graded according to standard criteria, at each visit [32].

CB Manipulations

CB Thawing Procedure. Frozen CB fractions were thawed using the procedure developed by Rubinstein et al. [33]. Briefly, the volume of the CB unit was doubled slowly with a 1:1 suspension of 10% dextran 40 (Braun Medical, Irvine, CA) and 5% human serum albumin (HSA) (American Red Cross, Washington, DC); an additional 50 mL of 10% dextran 40 was then added for a 5-minute incubation, followed by centrifugation at 464g for 10 minutes. The supernatant was expressed and another 50 mL of the 10% dextran 40/5% HSA suspension was added. Prior to infusion or CD34-selection, samples were taken for enumeration of nucleated cells using the Sysmex XE-2100 hematology analyzer, enumeration of CD34$^+$ cells using the flow cytometric procedure described below, viability assessment using trypan blue exclusion dye, and routine bacterial and fungal microbiologic cultures.

CB CD34-Selection Procedure. The fraction of CB cells to be expanded was CD34-selected using the Isolex 300-i device (Nexell, Irvine, CA). The anti-CD34 monoclonal antibody, PR34+ stem cell releasing agent, and magnetic beads were prepared according to manufacturer's instructions. A buffered suspension was made by adding 120 mL of 25% HSA and 360 mL of sodium citrate (Baxter Fenwal, Deerfield, IL) to 3 L of phosphate-buffered saline (PBS) (Nexell). Pulmozyme/$MgCl_2$ was prepared by adding 0.6 mL 500mM $MgCl_2$ to 15 mL Pulmozyme (Genentech, South San Francisco, CA) containing 1500 units of recombinant dornase alpha. CB cells were incubated with 10 mL of 5% immune globulin (Baxter Hyland, Glendale, CO) for 15 minutes at room temperature and filtered prior to being loaded on the selection device. CB cells were CD34-selected following the manufacturer's instructions, except that the Pulmozyme/$MgCl_2$ and magnetic beads were added to the incubation chamber at the "Transfer Cells to Chamber" step following the "Antibody Wash" procedure. The isolated CD34$^+$ fraction was centrifuged in 50 mL sterile conical tubes at 617g for 10 minutes. Supernatant was removed and the cells were resuspended in defined media (Amgen, Thousand Oaks, CA) Samples of the washed CD34$^+$ CB cells were then taken for enumeration of nucleated cells and CD34$^+$ cells, viability assessment, and microbiologic cultures prior to inoculation into expansion culture. The CD34$^-$ (unselected) fraction was cryopreserved in 2 Cryocyte freezing bags (Nexell) for possible future use.

CB Expansion Procedure. The CD34$^+$ CB cells were placed in a 1 L VueLife Teflon fluorinated ethylene propylene bag (American Fluoroseal Corporation, Gaithersburg, MD) with 800 mL of defined media (Amgen) and 100 ng/mL each of SCF, G-CSF, and MGDF (Amgen). The cells were cultured for 10 days in a 5% CO_2 incubator at 37°C without further manipulation or refeeding. On day 10 of culture, the expanded CB cells were transferred into 50-mL sterile conical tubes, centrifuged at 617g for 5 minutes, and then washed twice with the PBS/sodium citrate/HSA buffer described above. Samples of the expanded CD34$^+$ CB cells were taken for enumeration of nucleated cells and CD34$^+$ cells, viability assessment, and microbiologic cultures prior to infusion.

Flow Cytometric Analysis. Small aliquots (1-5 $\times$ 10^5 cells) from the unmanipulated, CD34-selected, and expanded CB fractions were stained with a phycoerythrin-conjugated anti-CD34 antibody (Coulter, Hialeah, FL). Flow cytometric analysis for CD34 expression was performed with a Coulter Epics flow cytometer using the International Society for Hematotherapy and Graft Engineering (ISHAGE) procedure [34]; 100,000 events were counted for the preselected and expanded fractions; 20,000 events were counted for the CD34-selected fractions.

Statistical Analysis

Patients were stratified according to whether their CB unit was cryopreserved in 1 fraction (stratum A) or 2 fractions (stratum B). Exploratory analyses were performed to compare the 2 strata with respect to neutrophil and platelet engraftment, and then the strata were combined for the final analyses. Patients who were followed for a minimum of 28 days without evidence of progressive disease in the marrow were evaluated for neutrophil engraftment, the primary endpoint of this study. Time of neutrophil engraftment was defined as the first of 3 consecutive days with a sustained ANC $\geq$0.5 $\times$

Table 3. *TNC Data*

Parameter	Median	Range
TNC fold expansion*	**56**	**1.03-278**
Pre-thaw TNC/kg, $\times 10^7$†		
All patients	**1.60**	**0.67-9.44**
Adults	**1.43**	**0.67-6.70**
Children	**4.20**	**0.98-9.44**
Post-thaw TNC/kg, $\times 10^7$‡		
All patients	**1.19**	**0.56-8.86**
Adults	**0.95**	**0.56-2.0**
Children	**3.29**	**1.14-8.86**
Infused TNC/kg, $\times 10^7$		
All patients	**0.99**	**0.28-8.50**
Adults	**0.79**	**0.28-2.35**
Children	**2.14**	**0.75-8.50**

*Fold expansion = TNC post expansion/TNC in $CD34^+$ fraction.

†Pre-thaw based on count reported by the cord bank supplying the cord unit.

‡Post-thaw based on University of Colorado Stem Cell Laboratory assays.

10^9/L. Time of platelet engraftment was defined as the first of 3 consecutive days with a platelet count $\geq 20 \times 10^9$/L, without platelet transfusion support in the preceding 7 days. The proportion of patients who achieved engraftment at various time points was based on cumulative incidence, with death (with or without relapse) as a competing risk [35]. Trend tests of cumulative incidence across CD34 and TNC dose categories were based on the methods of Fine and Gray [36]. EFS was estimated by the Kaplan-Meier method, censoring those patients who died early on the day of death [37]. The log-rank test was used for comparing EFS rates between adult and pediatric patients. The Fisher exact test was used to test the difference in the rate of GVHD between pediatric and adult patients. The Fisher exact test and the Mantel-Haenszel (linear-by-linear association) chi-square tests were applied to examine the association between HLA match and the development of GVHD. All statistical analyses were carried out with SAS software (Version 8.1, SAS Institute, Cary, NC).

RESULTS

CB Expansion Data

The pre-thaw TNC doses were based on the nucleated cell counts reported by the CB bank from which the CB unit was obtained. The post-thaw TNC and $CD34^+$ cell doses were determined in the University of Colorado Stem Cell Laboratory using the procedures described above. As summarized in Table 3, the median number of TNC/kg in the pre-thawed CB units was 1.60×10^7 TNC/kg (1.43×10^7 TNC/kg for the adult and 4.20×10^7 TNC/kg for the pediatric patients). Following thawing, a median 74% cell recovery resulted in 1.19×10^7 TNC/kg in the post-thaw CB fractions (0.95×10^7 TNC/kg for the adult and 3.29×10^7 TNC/kg for the pediatric patients). Following the expansion procedure, the median TNC expansion was 56-fold higher and the $CD34^+$ cell expansion was 4.0-fold higher than the preculture values in the CD34-selected fractions. A median of 0.99×10^7 total (expanded plus unexpanded) TNC/kg was infused (0.79×10^7 TNC/kg for the adult and 2.14×10^7 TNC/kg for the pediatric patients). Table 4 shows that a median of 7.35×10^4 $CD34^+$ cells/kg were contained in the thawed CB units prior to further manipulation (6.55×10^4 $CD34^+$ cells/kg for the adult and 16.1×10^4 $CD34^+$ cells/kg for the pediatric patients). Following CD34 selection and expansion, a total (expanded plus unexpanded) of 10.4×10^4 $CD34^+$ cells/kg was infused (8.9×10^4 $CD34^+$ cells/kg for the adult and 21.1×10^4 $CD34^+$ cells/kg for the pediatric patients).

Infusion and Engraftment Data

There was no significant acute toxicity associated with infusion of the expanded or the unmanipulated CB cells. Four patients died of disseminated fungal infections (1 *Aspergillus*, 1 *Chaetonium*, 2 *Candida*) before day 28 on days 14, 18, 24, and 27, prior to engraftment of their neutrophils. Three additional patients developed extensive relapse in the marrow and died, pancytopenic, on days 41, 51, and 78. None of the remaining 30 patients experienced neutrophil engraftment failure. The median time to an ANC $\geq 0.5 \times 10^9$/L was 28 days (range, 15-49 days) (35 days for adult and 25 days for pediatric patients). The median time to an unsupported platelet count $\geq 20 \times 10^9$/L was 106 days (range, 38-345 days) (261 days for adult and 65 days for pediatric patients). As shown in Table 5, there were no significant differences in times to neutrophil or platelet engraftment between Stratum A (expanded CB cells infused on day 10) and Stratum B (expanded CB cells infused on day 0), nor between Cohort 1 (40% CB fraction expanded) and Cohort 2 (60% CB fraction expanded). There was no significant correlation between time to neutrophil engraftment and the number of TNC/kg in the pre-thaw or the post-thaw CB fractions. As shown in Figure 2, for the infused CB fraction ($P = .27$), a delay in engraftment is evident for the lowest TNC dose category, but some delays also occurred in the higher dose categories. As shown in Figure 3, there does appear to be an association between the number of $CD34^+$ cells/kg (expanded plus unexpanded) infused and time to neutrophil engraftment. Patients who received $\geq 5 \times 10^4$ $CD34^+$ cells/kg had a more rapid time to neutrophil engraftment compared to recipients of lower $CD34^+$ cell doses, but because of the small number of

Table 4. *$CD34^+$ Cell Data*

Parameter	Median	Range
$CD34^+$ cell fold expansion*	**4.0**	**0.1-20.0**
Post-thaw $CD34^+$ cells/kg, $\times 10^4$†		
All patients	**7.35**	**0.55-109.0**
Adults	**6.55**	**0.55-19.7**
Children	**16.1**	**4.51-109.0**
Infused $CD34^+$ cells/kg, $\times 10^4$		
All patients	**10.4**	**0.97-311.0**
Adults	**8.9**	**0.97-51.0**
Children	**21.1**	**1.99-311.0**

*Fold expansion = $CD34^+$ cells postexpansion/$CD34^+$ cells in $CD34^+$ fraction.

†Post-thaw based on University of Colorado Stem Cell Laboratory assay.

Table 5. *Time to ANC >0.5 × 10^9/L and Platelet Count >20 × 10^9/L**

	Stratum A (Day 10)	Stratum B (Day 0)	Cohort 1 (40%)	Cohort 2 (60%)
Median days to ANC >500	26	31	26	30
Range	15-49	15-41	15-45	22-49
	P = .8613		*P* = .3192	
Median days to platelets >20,000	126	73	91	126
Range	38-345	56-165	38-345	43-148
	P = .6905		*P* = .9161	

*There were no significant differences in times to neutrophil or platelet engraftment between Stratum A (expanded CB cells infused on day 10) and Stratum B (expanded CB cells infused on day 0), or between Cohort 1 (40% CB fraction expanded) and Cohort 2 (60% CB fraction expanded).

patients the differences were not statistically significant (*P* = .095). There also appeared to be a correlation between TNC dose and time to platelet engraftment. However, more than 60% of the patients had prolonged thrombocytopenia associated with chronic GVHD, and thus the independent validity of this correlation could not be confirmed. At the time of this report, there were no cases of late graft failure or loss of donor chimerism, with a median follow-up of 30 months in surviving patients.

Graft versus Host Disease

Acute GVHD was documented in 20 (66.7%) of the 30 evaluable patients followed 28 days or longer; 8 (26.7%) of the 30 had grade 2, and 12 (40%) of the 30 developed grades 3 to 4 GVHD. One (3%) of the grade 4 patients died from complications of acute GVHD on day 87. Chronic GVHD was documented in 14 (74%) of the 19 evaluable patients followed 100 days or longer. Two (11%) of the 19 patients had chronic GVHD limited to the skin, whereas 12 patients (64%) developed extensive chronic GVHD involving the gastrointestinal tract, liver, and/or lung. One patient (5%) died on day 141 of chronic GVHD of his lung and liver. Both patients who died of GVHD were adults. Table 6 compares the rates of acute and chronic GVHD, respectively, for the adult versus pediatric patients. The proportion of patients with GVHD was higher in the adults (80% acute, 90% chronic) than in the children (40% acute, 56% chronic), but because of the small number of patients the differences were not statistically significant (acute, *P* = .2134; chronic, *P* = .1409). There was no difference in the time of onset of acute GVHD in patients who received the expanded cells on day 0 (Stratum B) versus those who received them on day 10 (Stratum A). There was also no association between the degree of HLA matching and the development of acute or chronic GVHD. The small numbers of patients enrolled in this trial preclude definitive conclusions about these issues.

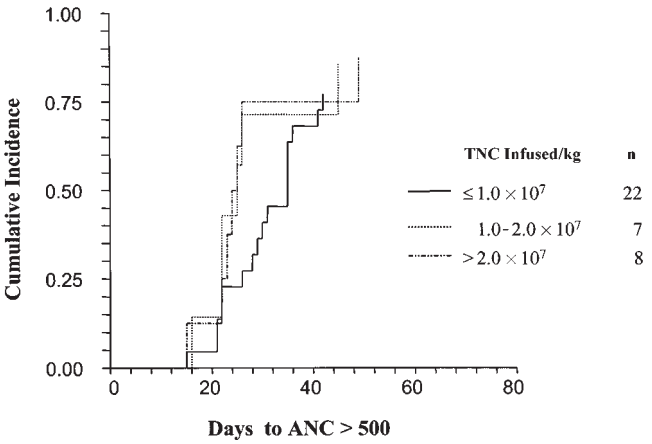

Figure 2. Neutrophil engraftment by TNC infused per kilogram. The kinetics of neutrophil engraftment posttransplantation are plotted against the number of TNC infused per kilogram patient weight (includes the combined expanded plus unexpanded CB fractions). Time to neutrophil engraftment appeared to be longer in the group of patients with the number of TNC/kg in the infused CB fraction <1 × 10^7, but some delays were also seen in those patients infused with 1 to 2 × 10^7 or >2 × 10^7 TNC/kg (Fine and Gray trend test: χ^2 = 1.24 with 1 *df*, *P* = .27).

Current Status

At the time of this report, 12 (32%) of the 37 patients, including 5 of the 25 adults (25%) and 7 of the 12 children (58%), remained alive. All of the surviving pediatric patients and 4 of 5 surviving adults remained free of disease. Eleven of the 37 patients (30%) died as a result of progressive disease; 2 of these 11 were pediatric patients with AML refractory to induction and ALL in second complete remission at the time of transplant, respectively; 9 of 11 relapses occurred in adult patients, all of whom had refractory progressive disease at the time of transplant, with AML (n = 4), HD (n = 2), NHL (n = 1), ALL (n = 1), and breast cancer (n = 1). Fourteen of the 37 patients (38%) experienced fatal non–relapse-related complications. The causes of nonrelapse mortality were GVHD (n = 2), failure to thrive (n = 1; patient died bedridden, anorectic, refusing all medication and nutritional support, with no clinical evidence of GVHD, infection, or relapse), central nervous system bleeding (n = 3), primary central nervous system toxicity in a patient who had received prior brain irradiation (n = 1), pulmonary toxicity (n = 2), resistant disseminated herpes (n = 1), and fungal infections (n = 4).

DISCUSSION

Over the past decade, CB has been used with increasing frequency as a source of allogeneic hematopoietic support for patients lacking HLA-matched sibling donors. The logistical

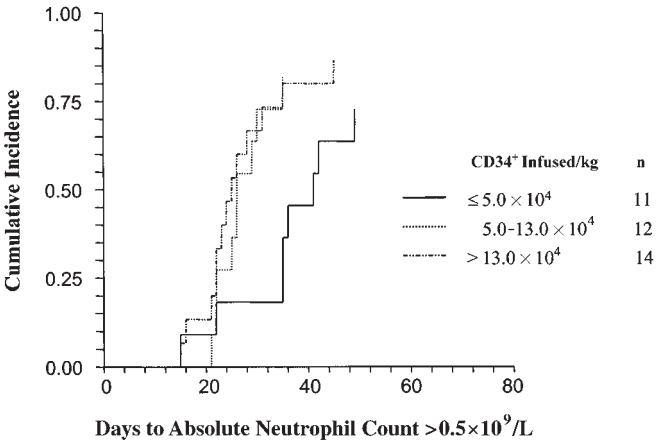

Figure 3. Neutrophil engraftment by $CD34^+$ cells infused per kilogram. The kinetics of neutrophil engraftment posttransplant are plotted against the total number of $CD34^+$ cells infused per kilogram patient weight (includes the combined expanded plus unexpanded CB fractions). There is an association between the number of $CD34^+$ cells per kilogram (expanded plus unexpanded) infused and time to neutrophil engraftment. Patients who received $\geq 5 \times 10^4$ $CD34^+$ cells/kg appeared to have a more rapid time to neutrophil engraftment compared to that of recipients of lower $CD34^+$ cell doses (Fine and Gray trend test: $\chi^2 = 2.78$ with 1 *df*, $P = .095$).

ease and rapidity of procuring CB units that are HLA typed, cryopreserved, and ready to transplant are major advantages over procurement of cells from living unrelated donors, particularly for patients with rapidly progressive malignancies. The common CB bank practice of targeting CB collections from minority donors is making it progressively easier to find appropriately matched CB units for minority patients, who are poorly represented in national and international bone marrow donor registries [38]. Finally, it has become clear that less stringent HLA matching is required for CB compared to unrelated marrow or PBPC transplants [16,39]. The less stringent matching allows many patients who do not have a suitably HLA-matched bone marrow or PBPC donor to receive high-dose therapy and allogeneic CB transplants. Complete remissions and long-term survival can be obtained for such patients who would otherwise certainly succumb to their underlying disease. The major disadvantage of CB compared to bone marrow is the lower available cell dose, with the resultant delays in engraftment and higher rates of graft failure discussed above. The CB expansion strategy described in the report was designed to improve the engraftment results of unmanipulated CB transplants.

Our preliminary results demonstrated that it is feasible and safe to administer expanded CB progenitors to patients with high-risk malignancies following myeloablative therapy. At a median follow-up of 30 months, there were no cases of late graft failure. Moreover, >95% long-term donor chimerism was documented in all patients, demonstrating that sufficient stem cells were in the CB graft to produce long-term engraftment. In this study, we defined patients with graft failure to be patients followed for 28 days or longer who did not have engraftment and did not have extensive relapse in the marrow. We cannot exclude the possibility that the 4 patients in the study who died early might have experienced engraftment failure had they lived ≥28 days. Additional accrual is necessary to assess the impact of the expanded CB cells on times to neutrophil and platelet engraftment and the true engraftment failure rate.

In the current study, following the CD34-selection and expansion procedures, the number of nucleated cells in the CB graft was slightly lower and the number of $CD34^+$ cells slightly higher than the numbers in the thawed unit before the CD34-selection and expansion procedures were performed. We believe that the quality, not the quantity, of CB cells is important for improving engraftment kinetics. A significant number of the nucleated cells in a CB graft, or any hematopoietic graft, are not able to proliferate and are thus incapable of producing the requisite granulocytes needed for rapid neutrophil engraftment. The goal of our expansion strategy is to enrich for a cell population that is highly proliferative and capable of generating rapidly engrafting cells. Thus, if there is an effect of the expansion, allowing smaller cell doses to engraft or reducing the incidence of graft failure, it is likely due to CD34-selection and culture-induced alterations in the engraftment potential of the CB rather than an absolute increase in the total numbers of nucleated or $CD34^+$ cells. Ultimately, we believe that expansion will decrease the risk of delayed myelosuppression and engraftment failure, even though the nucleated cell dose may be lower than that of an unmanipulated graft. Of course, the number of patients accrued to date in this study is too small for any definitive conclusions about efficacy to be made.

The CD34-selection procedure using the Isolex device yields only 30% to 45% of the initial thawed $CD34^+$ cells. Clearly, procedures that limit the TNC and $CD34^+$ cell losses incurred with CD34 selection would result in higher numbers of cells available for expansion. Studies evaluating alternative CD34-selection devices [40] and expansion procedures that do not require CD34 selection [41] are in progress and will be evaluated clinically in the future, in an attempt to improve the magnitude and efficacy of CB expansion.

In previous reports of unmanipulated CB transplantation, there appeared to be a correlation or threshold effect between neutrophil engraftment and the nucleated cell dose [5,9,10,15].

Table 6. *GVHD in Adult versus Pediatric Patients**

	Acute GVHD Summary			Chronic GVHD Summary		
	Adults	Children	Total	Adults	Children	Total
Evaluable, n	20	10	30	10	9	19
GVHD, n	16/20	4/10	20/30	9/10	5/9	14/19
GVHD	80%	40%	67%	90%	56%	74%
P		.2134			.1409	

*The Fisher exact test was used to test the difference in proportion of GVHD between adult patients (16/20 with acute, 9/10 with chronic) and pediatric patients (4/10 with acute, 5/9 with chronic). We note that the proportion of GVHD was higher in adults (80% acute, 90% chronic) than in pediatric patients (40% acute, 56% chronic). However, the difference was not significant ($P = .2134$ acute, $P = .1409$ chronic).

These studies based their analyses on the number of cells in the CB unit before thawing. In our study, there appears to be no correlation or threshold effect between neutrophil engraftment and TNC/kg in the CB unit, either before or after thawing. The latter analyses included the TNC/kg in the unmanipulated fraction, the expanded fraction, and the combination of both. The small numbers of patients preclude making a definitive conclusion about the lack of correlation. There does appear to be an association, however, between the $CD34^+$ cell dose in the thawed CB fractions (unmanipulated, expanded, and both) and time to neutrophil engraftment. The patients in Stratum B who received their expanded cells on day 0 had times to engraftment similar to those of patients in stratum A who received their expanded cells on day 10. Similarly, patients accrued to Cohort 2 (60% fraction expanded) had engraftment times similar to those of Cohort 1 patients (40% expanded). The small size of these subgroups limited the power to make definitive comparisons. Nevertheless, we postulated that an expansion product with more mature committed progenitors and mature cells would be needed to facilitate engraftment to a clinically relevant degree. In a PBPC expansion study conducted at our center during the same time period, patients had their autologous G-CSF–mobilized PBPC fractions expanded for 10 days with the same media and growth factors used for the CB expansion study [29]. The expanded PBPC products contained many more mature-appearing cells of neutrophil lineage, and those patients achieved engraftment more rapidly than did historical controls (within 4-8 days). Based on those data, we have modified our 10-day culture method to generate expanded CB that looked morphologically like the expanded PBPC fractions. In preclinical studies, a modified 14-day method resulted in a median 460-fold expansion of TNCs, compared to a median 98-fold TNC expansion using the standard 1-step 10-day culture conditions [42]. The morphology of the day 14 (2-step) expanded CB cells looked very similar to our cultures of expanded PBPC and included a significantly higher proportion of bands and mature neutrophils (67%) compared to the proportion in the day 10 (1-step) expansion (10%). Therefore, we have now begun accruing patients to Cohort 3 of this study, which uses the new 14-day expansion method, and that data will be forthcoming.

The GVHD rate in this study appears to be higher than that reported by others, particularly for the adult patients [4,5,9,15]. The CD34-selected and expanded CB fractions had no detectable T-cells on flow cytometric analysis. Preliminary morphologic analysis of the expanded products demonstrated the presence of mature dendritic-like cells, which could be contributing to the development of GVHD. Comprehensive studies to identify the phenotypic and functional characteristics of those cells are in progress, and that data will be forthcoming. The GVHD prophylaxis regimen and schedule employed in this study may have contributed to the high rate of GVHD. A day –5 to –3 schedule of ATG was used in the current study, compared to days –3 to –1 in other reports in which less GVHD was documented [4,5,9,15]. We began to taper the cyclosporine at day 100 in the majority of patients, if there was no evidence of GVHD. In initial CB transplantation studies where lower GVHD rates were reported, tapering of cyclosporine was not initiated until 6 to 9 months posttransplantation, even in the absence of GVHD [4,5,9,15]. Our ongoing CB transplantation trials will employ the days –3 to –1 ATG schedule and delay the tapering of cyclosporine until at least 6 months posttransplantation. Once the optimal expansion procedure and time to engraftment are defined, adding methotrexate will be considered to reduce the incidence and severity of GVHD, while monitoring for any engraftment delays.

Although the 20% survival rate for the adult patients is low, they were heavily pretreated, with the vast majority (23 of 25) having had progressive refractory disease at study entry. In contrast, the 58% EFS rate for the pediatric patients is comparable to our results in matched sibling transplantations and other published pediatric CB transplantation reports [4-6,8-10,12,13,15]. Four patients (3 adults, 1 child) died of disseminated fungal infections on days 14, 18, 24, and 27. These patients all had either evidence of fungal colonization immediately pretransplantation (n = 3) or a previously documented fungal infection (n = 1). The reduction in the methylprednisolone and melphalan doses should result in lower infection and regimen-related toxicity rates, respectively, as accrual to the study continues. Once optimal engraftment is achieved, studying CB expansion in adult patients with a better prognosis would also be expected to produce improved outcome. Shortening the time to neutrophil engraftment with improved CB expansion strategies should reduce the infectious and organ-toxic complication rates in the future.

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REFERENCES

1. Gluckman E, Devergie A, Bourdeau-Esperou H, et al. Transplantation of umbilical cord blood in Fanconi's anemia. *Nouv Rev Fr Hematol.* 1990;32:423-425.
2. Wagner J, Kernan NA, Steinbuch M, Gluckman E. Umbilical cord blood stem cell transplantation [abstract]. *Am J Pediatr Hematol Oncol.* 1993;2:169.
3. Wagner JE, Kernan NA, Steinbuch M, Broxmeyer HE, Gluckman E. Allogeneic sibling umbilical-cord-blood transplantation in children with malignant and non-malignant disease. *Lancet.* 1995; 346:214-219.
4. Wagner JE, Rosenthal J, Sweetman R, et al. Successful transplantation of HLA-matched and HLA-mismatched umbilical cord blood from unrelated donors: analysis of engraftment and acute graft-versus-host disease. *Blood.* 1996;88:795-802.
5. Kurtzberg J, Laughlin M, Graham ML, et al. Placental blood as a source of hematopoietic stem cells for transplantation into unrelated recipients. *N Engl J Med.* 1996;335:157-166.
6. Laporte JP, Gorin NC, Rubinstein P, et al. Cord-blood transplantation from an unrelated donor in an adult with chronic myelogenous leukemia. *N Engl J Med.* 1996;335:167-170.
7. Laporte JP, Lesage S, Portnoi MF, et al. Unrelated mismatched cord blood transplantation in patients with hematological malignancies: a single institution experience. *Bone Marrow Transplant.* 1998;22(suppl 1):S76-S77.

8. Cairo MS, Wagner JE. Placental and/or umbilical cord blood: an alternative source of hematopoietic stem cells for transplantation. *Blood.* 1997;90:4665-4678.
9. Gluckman E, Rocha V, Boyer-Chammard A, et al. Outcome of cord-blood transplantation from related and unrelated donors: Eurocord Transplant Group and the European Blood and Marrow Transplantation Group. *N Engl J Med.* 1997;337:373-381.
10. Rubinstein P, Carrier C, Scaradavou A, et al. Outcomes among 562 recipients of placental-blood transplants from unrelated donors. *N Engl J Med.* 1998;339:1565-1577.
11. Locatelli F, Rocha V, Chastang C, et al. Cord blood transplantation for children with acute leukemia: Eurocord Transplant Group. *Bone Marrow Transplant.* 1998;21(suppl 3):S63-S65.
12. Locatelli F, Rocha V, Chastang C, et al. Factors associated with outcome after cord blood transplantation in children with acute leukemia: Eurocord-Cord Blood Transplant Group. *Blood.* 1999; 93:3662-3671.
13. Rocha V, Wagner JE, Sobocinski KA, et al. Graft-versus-host disease in children who have received a cord-blood or bone marrow transplant from an HLA-identical sibling: Eurocord and International Bone Marrow Transplant Registry Working Committee on Alternative Donor and Stem Cell Sources. *N Engl J Med.* 2000; 342:1846-1854.
14. Long G, Madan B, Kurtzberg J, et al. Unrelated umbilical cord blood transplants in adult patients with hematologic malignancies or genetic disorders. [abstract]. *Blood.* 1999;94:570a.
15. Laughlin MJ, Barker J, Bambach B, et al. Hematopoietic engraftment and survival in adult recipients of umbilical-cord blood from unrelated donors. *N Engl J Med.* 2001;344:1815-1822.
16. Barker JN, Davies SM, DeFor T, Ramsay NK, Weisdorf D, Wagner JE. Survival after transplantation of unrelated donor umbilical cord blood is comparable to that of human leukocyte antigen-matched unrelated donor bone marrow: results of a matched-pair analysis. *Blood.* 2001;97:2957-2971.
17. Bensinger WI, Clift R, Martin P, et al. Allogeneic peripheral blood stem cell transplantation in patients with advanced hematologic malignancies: a retrospective comparison with marrow transplantation. *Blood.* 1996;88:2794-2800.
18. Bensinger WI, Weaver CH, Appelbaum FR, et al. Transplantation of allogeneic peripheral blood stem cells mobilized by recombinant human granulocyte colony-stimulating factor. *Blood.* 1995;85:1655-1658.
19. Bensinger WI, Martin PJ, Storer B, et al. Transplantation of bone marrow as compared with peripheral-blood cells from HLA-identical relatives in patients with hematologic cancers. *N Engl J Med.* 2001;344:175-181.
20. McGlave P, Bartsch G, Anasetti C, et al. Unrelated donor marrow transplantation therapy for chronic myelogenous leukemia: initial experience of the National Marrow Donor Program. *Blood.* 1993;81:543-550.
21. Kernan NA, Bartsch G, Ash RC, et al. Analysis of 462 transplantations from unrelated donors facilitated by the National Marrow Donor Program. *N Engl J Med.* 1993;328:593-602.
22. Schiller G, Feig SA, Territo M, et al. Treatment of advanced acute leukaemia with allogeneic bone marrow transplantation from unrelated donors. *Br J Haematol.* 1994;88:72-78.
23. Szydlo R, Goldman JM, Klein JP, et al. Results of allogeneic bone marrow transplants for leukemia using donors other than HLA-identical siblings. *J Clin Oncol.* 1997;15:1767-1777.
24. Berthou C, Legros-Maida S, Soulie A, et al. Cord blood T lymphocytes lack constitutive perforin expression in contrast to adult peripheral blood T lymphocytes. *Blood.* 1995;85:1540-1546.
25. Broxmeyer HE, Hangoc G, Cooper S, et al. Growth characteristics and expansion of human umbilical cord blood and estimation of its potential for transplantation in adults. *Proc Natl Acad Sci U S A.* 1992;89:4109-4113.
26. Hogan CJ, Shpall EJ, Keller G. Differential long-term and multilineage engraftment potential from subfractions of human CD34+ cord blood cells transplanted into NOD/SCID mice. *Proc Natl Acad Sci U S A.* 2001;99:413-418.
27. Hogan CJ, Shpall EJ, McNulty O, et al. Engraftment and development of human CD34(+)-enriched cells from umbilical cord blood in NOD/LtSz-scid/scid mice. *Blood.* 1997;90:85-96.
28. Gehling UM, Ryder JW, Hogan CJ, et al. Ex vivo expansion of megakaryocyte progenitors: effect of various growth factor combinations on CD34+ progenitor cells from bone marrow and G-CSF-mobilized peripheral blood. *Exp Hematol.* 1997;25: 1125-1139.
29. McNiece I, Jones R, Bearman SI, et al. Ex vivo expanded peripheral blood progenitor cells provide rapid neutrophil recovery after high-dose chemotherapy in patients with breast cancer. *Blood.* 2000;96:3001-3007.
30. Sobocinski KA, Horowitz MM, Rowlings PA, et al. Bone marrow transplantation—1994: a report from the International Bone Marrow Transplant Registry and the North American Autologous Bone Marrow Transplant Registry. *J Hematother.* 1994;3: 95-102.
31. Przepiorka D, Weisdorf D, Martin P, et al. 1994 Consensus Conference on Acute GVHD Grading. *Bone Marrow Transplant.* 1995; 15:825-828.
32. Flowers ME, Kansu E, Sullivan KM. Pathophysiology and treatment of graft-versus-host disease. *Hematol Oncol Clin North Am.* 1999;13:1091-1112.
33. Rubinstein P, Dobrila L, Rosenfield RE, et al. Processing and cryopreservation of placental/umbilical cord blood for unrelated bone marrow reconstitution. *Proc Natl Acad Sci U S A.* 1995;92: 10119-10122.
34. Sutherland DR, Anderson L, Keeney M, Nayar R, Chin-Yee I. The ISHAGE guidelines for CD34+ cell determination by flow cytometry. *J Hematother.* 1996;5:213-217.
35. Gooley TA, Leisenring W, Crowley J, Storer BE. Estimation of failure probabilities in the presence of competing risks: new representations of old estimators. *Stat Med.* 1999;18:695-706.
36. Fine J, Gray RJ. A proportional hazards model for the subdistribution of a competing risk. *J Am Stat Assoc.* 1999;94:496-509.
37. Kaplan EL, Meier P. Nonparametric estimation from incomplete observations. *J Am Stat Assoc.* 1958;53:457-481.
38. Freytes CO, Beatty P. Representation of minorities in the national marrow donor program (NMDP) [meeting abstract]. *Proc Ann Meet Am Soc Clin Oncol.* 1995;14:A9.
39. Petersdorf EW, Gooley TA, Anasetti C. Optimizing outcome after unrelated marrow transplantation by comprehensive matching of HLA class I and II alleles in donor and recipient. *Blood.* 1998;92:3515-3520.
40. McNiece IK, Stoney GB, Kern BP, Briddell RA. CD34+ cell selection from frozen cord blood products using the Isolex 300i and CliniMACS CD34 selection devices. *J Hematother.* 1998;7:457-461.
41. McNiece I, Harrington J, James RI, Shpall EJ, Mackay A, Smith A. Ex vivo expansion of CB cells without CD34 selection using coculture on mesenchymal stem cells [abstract]. *Blood.* 2001;98:851a.
42. McNiece I, Kubegov D, Kerzic P, Shpall EJ, Gross S. Increased expansion and differentiation of cord blood products using a two-step expansion culture. *Exp Hematol.* 2000;28:1181-1186.