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Ex Vivo Expansion of Megakaryocyte Precursors from Umbilical Cord Blood CD34$^+$ Cells in a Closed Liquid Culture System

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ABSTRACT

Umbilical cord blood (UCB) provides a rich source of stem cells for transplantation after myeloablative therapy. One major disadvantage of UCB transplantation is delayed platelet engraftment. We propose to hasten platelet engraftment by expanding the number of megakaryocyte (MK) precursors (CD34/CD41 cells) through cytokine stimulation within a closed, pre-clinical liquid culture system. Clinical engraftment data suggest a 5- to 10-fold increase in MK precursors in a UCB unit can accelerate platelet engraftment, so this was our goal. Thirteen UCB samples from full-term births were Ficoll-separated and frozen for subsequent use. On thawing, the mononuclear cell population was positively selected for CD34$^+$ expression. The cells were cultured in gas-permeable Teflon-coated bags in serum-free medium containing the following cytokines: recombinant human interleukin-3, recombinant human Flt3 ligand, recombinant human stem cell factor, and recombinant human thrombopoietin. MK lineage cell expansion was assessed using mononuclear cell count and flow cytometry (CD34/41, CD41, CD34/61, and CD61 expression) on days 7, 11, and 14. Optimal expansion of CD34/41 and CD41 cells was observed at day 11, with a median 6-fold and 33-fold increase in the starting cell doses, respectively. CD34/61 and CD61 cell expansion at day 11 was 7-fold and 14-fold, respectively. MK precursors can be successfully expanded from CD34$^+$ UCB cells in a closed liquid culture system using interleukin-3, recombinant human Flt3 ligand, recombinant human stem cell factor, and recombinant human thrombopoietin to a level that should have a clinical impact in the transplantation setting. Our ex vivo expansion technique needs to be further optimized before it can be used in a pilot UCB transplantation trial.

KEY WORDS

Megakaryocyte • Umbilical cord blood • Expansion • Precursor

INTRODUCTION

Umbilical cord blood (UCB) provides a rich source of stem cells for hematopoietic reconstitution after myeloablative therapy. One major disadvantage of UCB is delayed engraftment of platelets, which can lead to hemorrhagic complications, increased platelet transfusion requirements, prolonged hospitalization, and alloimmunization. Platelet recovery can occur at a median of 71 days after transplantation [1], in contrast to a median of 24 days for allogeneic bone marrow transplant recipients [2], and 10 days for autologous peripheral blood stem cells (PBSC) recipients [3]. Clinically, ex vivo expansion strategies have been found to be safe and effective in both PBSC [4,5] and UCB transplantations [6].

We propose to accelerate platelet engraftment by expanding the number of megakaryocyte (MK) precursors through cytokine stimulation. From PBSC engraftment data, the threshold for significantly faster platelet engraftment has been reported to be between 1 and 3 $\times$ 10^5 CD34/41 cells/kg in the infused stem cell product [7-9]. Less is known about the characteristics of UCB stem cells and their engraftment kinetics. In the largest published series that looked at this subpopulation of cells, patients received only 4 $\times$ 10^4 CD34/41 cells/kg, which may explain their delayed platelet recovery when compared with recipients of other stem cell sources [10]. Therefore, the goal of expansion would be a one-half to one-log increase in this infused cell population. We have previously demonstrated that MK precursors and stem cells can be expanded from UCB CD34$^+$ cells in collagen and small-scale liquid culture experiments [11].

In these experiments, we increased the scale of the expansion and conducted it within a closed liquid culture system, which can be easily adapted for clinical use.

MATERIALS AND METHODS

Cell Source

UCB samples were taken from placentas of women who had normal full-term pregnancies; the placentas were to be discarded. The institutional review board approved and consent was obtained for these collections. A volume of 30 to 100 mL was drained by gravity into a conical tube containing 1000 U of preservative-free heparin, 1 mL of 5% human albumin (Alpha Therapeutics, Los Angeles, CA), and 15 mL of Roswell Park Memorial Institute medium 1640 (Life Technologies, Grand Island, NY). The UCB was processed within 24 hours, with most being processed within 4 hours of collection.

Mononuclear Cell Processing

Mononuclear cells (MNCs) were separated from the whole cord blood by layering over Ficoll-paque (Pharmacia, Uppsala, Sweden) and centrifuging for 35 minutes at 1200 rpm. Once separated, the MNCs were resuspended in 10 mL of phosphate-buffered saline (PBS) and an automated cell count was obtained by Coulter Counter (Coulter Electronics, Hialeah, FL). The MNCs were mixed with Tissue Culture-199 (0.6 of total volume) (Sigma, St. Louis, MO), 5% human albumin (0.2 of total volume), and dimethyl sulfoxide (0.2 of total volume) on ice for a total volume of 20 mL. The cell solution was aliquoted into sterile cryogenic vials (Corning Inc., Corning, NY), placed in a Nalgene Cryo Freezer Container (Nalge Nunc International, Naperville, IL) for freezing, and then stored in liquid nitrogen at −70° C.

Before $CD34^+$ selection, the vials were taken out of the freezer and thawed in a 37° C water bath for 5 minutes. The cells were washed and resuspended in PBS to a total of 30 mL and 200 μL was set aside for flow cytometric analysis. Cell viability was assessed with trypan blue (Stem Cell Technologies, Vancouver, Canada) staining using a hemocytometer. The cells were then washed and resuspended in buffer solution to a volume of 300 μL (buffer consisted of 14 mL of PBS solution, 1 mL of 5% human albumin, and 1000 U of DNAse [ProMega, Madison, WI]).

CD34 Selection

The MNCs were selected based on their expression of the CD34 antigen using magnetic-activated cell-sorting technology (Miltenyi Biotec, Cologne, Germany) according to the manufacturer's recommendations. During this process, the UCB cells were incubated with mouse anti-human CD34 antibodies in the presence of human immunoglobulin G as a blocking reagent. The cells were then incubated with magnetic-activated cell-sorting microbeads and passed over a column in a magnetic field. The $CD34^+$ cells trapped in the column were then eluted with 1 mL of PBS buffer after the column was removed from the magnetic field [12]. The cells were passed through a second column in the same fashion to increase purity.

Flow Cytometric Analysis

Flow cytometry was performed on both the pre- and post-selection cell populations using a FACSort flow cytometer (Becton Dickinson, San Jose, CA) and was analyzed with CELLQuest software (Becton Dickinson). In addition to CD45/CD14 and G1/G2 (isotype), CD34 (FITC and PE), AC133-PE, CD41-PE (gpIIb/IIIa, an MK-specific marker), and CD61-FITC (gpIIIa, an earlier MK marker) were examined (monoclonal antibody for AC133 by Miltenyi Biotec; others by Becton-Dickinson). The mononuclear population was gated for all of our analyses.

Clinical-Scale Culture of $CD34^+$ Cells in a Closed System

CD34-selected cells were seeded in 35-mL Vuelife Teflon bags (American Fluoroseal Corp., Gaithersburg, MD) in serum-free liquid medium (StemSpan SFEM by StemCell Technologies, Vancouver, Canada) at a concentration of 1.0×10^4 CD34 cells/mL. Cytokines (all from Stem Cell Technologies) were added to the medium at the following concentrations: recombinant human interleukin-3 (IL-3) (100 ng/mL), recombinant human stem cell factor (SCF) (50 ng/mL), recombinant human thrombopoietin (TPO) (50 ng/mL), and recombinant human Flt3 ligand (Flt3L) (50 ng/mL). The medium was supplemented with 0.3% human albumin, penicillin (100 U/mL), and streptomycin (50 μg/mL). The bags were placed in a 37° C incubator with ambient O_2 (20%), 5% CO_2, and >95% humidity. Cultures were observed using an inverted microscope at 2-day intervals to determine when maximal expansion had occurred.

Assessment of Cultures for Cell Subpopulation Expansion

At days 7, 11, and 14, the cultures were sampled and a cell count was obtained using a Coulter Counter. Flow cytometry was performed with monoclonal antibodies for CD41, CD61, CD34, and AC133 as described above.. The final cell population for each specific marker was calculated by multiplying the final MNC count by the percentage of MNCs that express the marker (as per fluorescence-activated cell sorter analysis). The MK precursor population was defined as cells co-expressing CD34 and CD41.

Statistical Analysis

Expansion of cell subpopulations was calculated by dividing the final cell population by the seeding dose expressing the specific marker or markers. The magnitude of expansion (or fold expansion) was calculated by dividing the final MNC count by the seeding MNC dose. The data set contains the values of several surface markers (Table 1) measured repeatedly, at days 7, 11, and 14, from each of i = 1,. . .,13 cord blood samples. The surface marker values were evaluated graphically using box plots [13] and plots of the values over time. Regression of each marker value at days 11 and 14 on its previous values was evaluated using the following autoregressive linear model. For a given surface marker, denote by $Y_{j,t}$ the ratio of the percentage of cells having that marker in the sample evaluated at day t divided by the corresponding percentage at time 0. We assume that $Y_{j,t} = \mu+\beta_{7\to11}\, I[t = 11] + \beta_{11\to14}\, I[t = 14] + \epsilon_i$, where I[A] is the indicator of the event A, $\{\mu, \beta_{7\to11}, \beta_{11\to14}\}$ are the model regression parameters, and $\{\epsilon_1,. . .,\epsilon_{13}\}$ are iid Gaussian residuals with mean 0 and constant variance. Thus, each $Y_{j,7}$ has mean $Y_{i,11}$ has mean $\mu + \beta_{7\to11}$, and $Y_{j,14}$ has mean $\mu + \beta_{11\to14}$. Additionally, the term $\beta_{7\to14}\, I[t = 14]$ was included in the

Table 1. *Parameter Estimates of the Fitted Autoregressive Models for Marker Level*

Marker	Variable	Estimated Coefficient (SE)	*P* Value
CD34	Intercept	6.43 (2.72)	—
	7 → 11	0.55 (0.19)	.01
	11 → 14	0.21 (0.12)	.10
CD34/41	Intercept	7.44 (2.31)	—
	7 → 11	0.38 (0.14)	.01
	11 → 14	−0.45 (0.10)	.00
CD41	Intercept	14.61 (4.25)	—
	7 → 11	1.70 (0.24)	<.0001
	11 → 14	0.12 (0.09)	.19
CD61	Intercept	16.09 (5.70)	—
	7 → 11	0.88 (0.14)	<.0001
	11 → 14	0.11 (0.08)	.19
CD34/61	Intercept	24.73 (17.71)	—
	7 → 11	2.28 (0.21)	<.0001
	11 → 14	−0.09 (0.05)	.13
	mnc (7) → 11	−0.73 (0.41)	.09
AC133	Intercept	495.97 (444.05)	—
	7 → 11	0.20 (0.004)	<.0001
	11 → 14	0.68 (0.23)	.009
	7 → 14	−1.82 (0.28)	<.0001
AC133 (excluding 1 patient with AC133 > 4000)	Intercept	48.77 (33.49)	—
	7 → 11	0.48 (0.08)	<.0001
	11 → 14	−3.24 (0.41)	<.0001
	7 → 14	3.87 (0.50)	<.0001
AC133 (excluding 2 patients with AC133 > 350)	Intercept	9.95 (3.64)	—
	7 → 11	0.64 (0.17)	.002
	11 → 14	0.93 (0.16)	<.0001
	7 → 14	−1.12 (0.20)	<.0001

model for AC133 at day 14, and the value of MNCs at day 7 was included as a predictor of CD34/61 at day 11. All model fits were carried out using SAS PROC MIXED [14].

RESULTS

CD34$^+$ Cell Selection

After thawing, all UCB samples had >90% cell viability. The median CD34 purity after immunomagnetic bead selection for all 13 experiments was 84%. Purity was defined as the percentage of MNCs that expressed CD34.

Liquid Cell Culture Expansion of MK Lineage

Maximum expansion of the MK precursor population in the 13 experiments occurred at day 11 of culture with a 6-fold increase of CD34$^+$/CD41$^+$ cells (range: 2 to 41). On day 11 the overall median expansions of different cell subpopulations were as follows: CD41$^+$ cells 33-fold (range: 4 to 99), CD34/CD61 cells 7-fold (range: 2 to 613), and CD61$^+$ cells 14-fold (range: 3 to 104). Expansion of these cell subpopulations at days 7, 11, and 14 are presented in Figure 1 and Table 1. A representative FACS analysis of CD34 and CD41 expression on day 11 of culture is demonstrated in Figure 2.

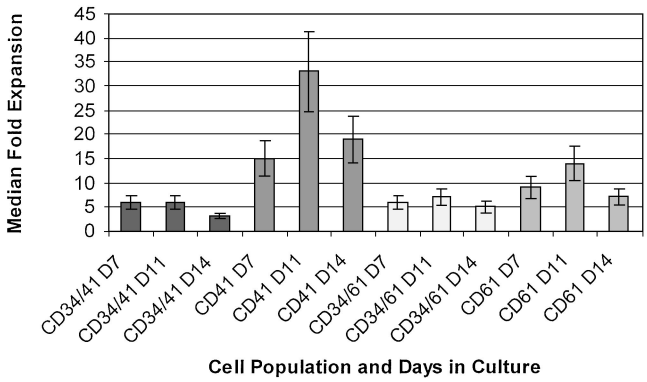

Figure 1. MK lineage expansion in closed liquid culture. Expansion data presented as fold expansion at days 7, 11, and 14 in culture (13 experiments). Note expansion of all subpopulations peaked at day 11. D indicates day.

Liquid Cell Culture Expansion of Stem Cells and MNCs

CD34$^+$ cells expanded 6-fold (range: 2 to 54), AC133$^+$ cells expanded 10-fold (range: 1 to 5911), and MNCs expanded 61-fold (range: 6 to 210). Expansion of these cell subpopulations at days 7, 11, and 14 are presented in Figure 3 and Table 1.

For each of the 6 states of differentiation (AC 133, CD34, CD41, CD61, CD34/CD41, and CD34/CD61), the expression level at day 7 was a strong predictor of the level at day 11 (Table 1). The day 11 levels strongly predicted the day 14 levels for CD34/41 and AC133, with this regression present but weaker for the other markers. In terms of average behavior, CD34, CD41, CD61, and AC133 each increased from day 7 to day 11 and then decreased from day 11 to day 14. The MNC levels showed a monotone increase in both average value and variability over time. The greatest overall variability was seen in AC133. Two of the 13 cases had extreme AC133 values at days 7, 11, and 14, specifically 4926, 5911, and missing for patient number 3 and 367, 510, and 80 for patient number 8. To assess its sensi-

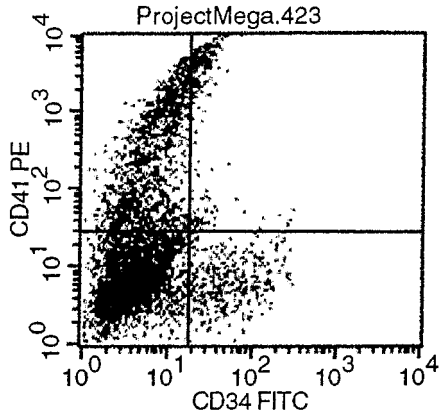

Figure 2. Analysis of CD34 and CD41 expression on a day 11 UCB liquid culture by 3-color flow cytometry. The cells in the upper left quadrant represent cells that have been driven toward megakaryocytic lineage (CD41), the lower right quadrant is the population of expanded, uncommitted (CD34) stem cells, whereas the upper-right quadrant represents the MK precursors (CD34/41 co-expression).

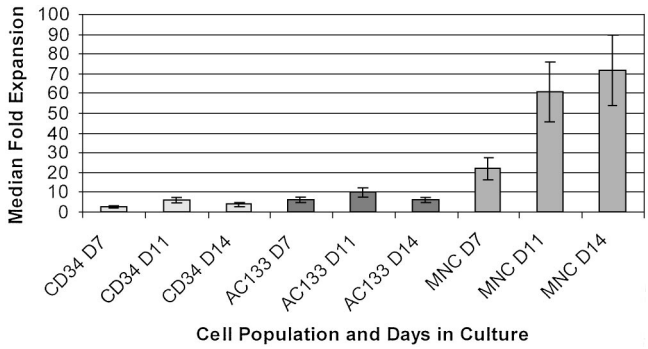

Figure 3. Stem cell and MNC expansion in closed liquid culture. Expansion data presented as fold expansion at days 7, 11, and 14 in culture (13 experiments). Note expansion of all subpopulations peaked at day 11 except MNC. D indicates day; MNC, mononuclear cells.

tivity to these extreme values, the autoregressive model for AC133 was re-fit excluding patient number 3, and then excluding both patient number 3 and patient number 8. The remaining 11 AC133 paths are shown in Figure 4, which illustrates the within-sample variability of the pattern of change from day 7 to day 11 to day 14.

DISCUSSION

One of the greatest obstacles to a safer, more effective, and economical stem cell transplantation is the delay in platelet engraftment. Although neutrophil engraftment has been accelerated through the use of increased $CD34^+$ dosage and/or granulocyte colony-stimulating factor–primed mobilized stem cells with posttransplantation administration of granulocyte-macrophage colony-stimulating factor, little progress has been made in platelet engraftment. This problem is particularly pronounced in UCB stem cell transplantations. We hypothesize that by performing transplantations with a stem cell product enriched for MK precursors we can accelerate the speed of platelet engraftment. To address this hypothesis, we have been identifying the optimal combination of cytokines for ex vivo expansion of MK precursors from $CD34^+$ UCB and PBSCs. Our previous studies in collagen medium and small-scale open liquid culture wells established that the best combination of cytokines for expansion was TPO, IL-3, Flt3L, and SCF [11].

CD34-selected cells were chosen as our source of expandable cells for several reasons. First, CD34 is one of the earliest stem cell markers yet identified, letting us know that we are growing and differentiating a multipotent cell population. Another reason is for the enhanced expandability of this population when purified: Briddell et al. showed that unselected UCB cells incubated with SCF, granulocyte colony-stimulating factor, and TPO for 10 days multiplied 1.4-fold, whereas $CD34^+$ enriched cells multiplied 113-fold under the same conditions [15]. Our CD34-selected cells were cultured in a serum-free medium containing identical concentrations of recombinant human TPO, IL-3, SCF, and Flt3L, as were used in our earlier experiments. These were contained within a closed, pre-clinical system that is a larger expansion system based on our earlier, small-scale liquid well culture work. These data suggest that CD34 selection may eliminate cells that inhibit ex vivo expansion.

To assess the early megakaryopoietic differentiation of a cell, we used flow cytometry. We stained our pre- and post-expanded cell populations with anti-CD61 (glycoprotein IIIa) monoclonal antibody, the first sign of pre-MK development, and CD41 (glycoprotein IIb/IIIa), a glycoprotein expressed later in development, but still not indicative of full MK maturation. We also stained each of these cell populations simultaneously with anti-CD34 antibody to discern which cells were co-expressing this stem cell marker and, therefore, were the desired MK precursor populations.

Calculation of stem cell expansion in our experiments was performed by dividing the total number of cells expressing the CD34 marker by the seeding cell dose. In addition to looking at CD34, we also examined the expression of AC133, a novel early stem cell marker expressed on a subpopulation of CD34 cells that may be an indicator of proliferative potential [16,17]. AC133 selection is now being examined for use in ex vivo expansion [18,19] and retroviral gene transfer experiments [20], whereas its expression is useful in characterizing malignant cells [21,22] and normal stem cells [23] and as a predictor of engraftment [24,25].

We have learned from PBSC engraftment data that the threshold for significantly faster platelet engraftment is in the range of 1 to 3×10^5 CD34/41 cells/kg [7-9], with the small body of UCB data on this subject indicating that this stem cell source contains approximately 1 log less of this subpopulation of cells [10]. The authors acknowledge that hematopoietic stem cells from different sources have different properties and engraftment kinetics, but the 10-fold smaller number of MK pre-

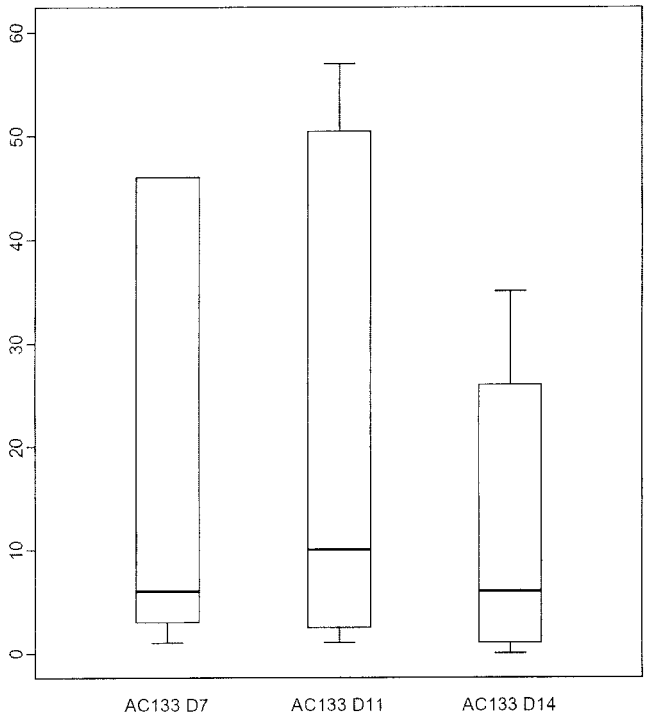

Figure 4. Statistical analysis of AC133 expansion variability. The y-axis is fold expansion of the $AC133^+$ population of cells at days 7, 11, and 14. The heavy line indicates the mean, with each box extending from the 25th to the 75th percentile and the whiskers extending to either the most extreme (minimum or maximum) value or $1.5 \times$ the inter-quartile range.

cursors in UCB when compared with UCB recipients could explain the significantly delayed platelet engraftment seen in these latter patients even with a high $CD34^+$ cell dose. Therefore, the goal of ex vivo expansion of UCB to have a clinical impact on platelet recovery would need to be at least one-half to 1-log increase in this infused MK precursor population.

The data from our experiments demonstrate that the combination of IL-3, Flt3L, SCF, and TPO in a closed serum-free liquid culture system is effective at expanding CD34/41 coexpressing MK precursors as well as more mature and immature cells in the megakaryocytic lineage ($CD41^+$, CD34/61, and $CD61^+$ cells). Although MK precursors increased a median of 6-fold at both days 7 and 11, day 11 was chosen as our optimal day because this was the day that the proliferation of most other subpopulations peaked (Figures 1 and 3). The only exception was MNCs, which had better expansion at day 14 (Figure 3). Although the expansion of MNC, $CD34^+$, and $AC133^+$ cells was not a primary goal of our experiments, we were able to increase the number of these subpopulations, which, when used clinically, should enhance both neutrophil and long-term engraftment [26,27]. The expansion of these cells shows there is no "steal" phenomenon whereby cells are all driven irreversibly to one lineage. It also demonstrates that our technique has the potential to make UCB more applicable as a stem cell source in larger patients, where the finite cell dose was the limiting factor in the past. As far as expansion goals for MK precursors, we have met them by achieving a half-log to 1-log expansion at day 11. Such proliferation of the desired precursors is at the threshold of faster platelet engraftment but ideally would be of an even greater magnitude to consistently provide more than sufficient numbers of these cells to make a clinically significant difference.

Another issue facing ex vivo expansion research and its clinical application is the heterogeneity of response to identical culture conditions seen between stem cell populations from different donors. Additional studies are needed to better understand sample heterogeneity and how this might be exploited for clinical applications.

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