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Ex Vivo expansion of suboptimal (low dose) cord blood products provides sufficient cells for transplantation of adult patients

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Abstract

Background: Recent trials have demonstrated the potential to combine two cord blood (CB) products to increase the cell dose delivered, however often CB products with sufficient cell dose, have a 2 antigen mismatch to each other or the recipient. Better matched products are routinely available however, their use is limited due to low cell content. We have developed *ex vivo* expansion techniques that can provide a 10 to 20 fold increase in cell numbers and evaluated the potential of *ex vivo* expansion to provide sufficient doses for these suboptimal products.

Methods: CB products which were frozen for banking and rejected due to low cell doses (median cells dose 0.7×10^7 total nucleated cells (TNC)/kg for a 50kg individual) were expanded using co culture on allogeneic mesenchymal stem cells (MSC). The CB products were thawed and the mononuclear cell (MNC) fraction isolated by density gradient centrifugation. The CB MNCs were cultured in T162cm^2 tissue culture flasks pre established with MSC in expansion media plus SCF, G-CSF and Tpo. After culture for 2 weeks the TNC/kg were determined.

Results: A median fold expansion of 9 fold was obtained (N=5) resulting in a median cell dose post expansion of 4.3 $\times 10^7$ TNC/kg (for a 50kg individual). In addition, fractionation of the CD34+ve and CD34-ve subsets from the CB MNC, demonstrated that the expansion was almost exclusively from the CD34+cells.

Discussion: These data demonstrate that co culture of CB MNCs on MSC result in significant expansion of suboptimal CB products. The expanded CB products would meet the minimal cell requirements for use of CB in adults and therefore provide the potential use of better matched CB products. This could result in enhanced engraftment and less toxicities such as graft versus host disease that occurs with lower level HLA matched CB products.

Keywords: cord blood, *ex vivo* expansion, CD34+ cells

Introduction

The use of umbilical cord blood (CB), as a source of hematopoietic cells for patients undergoing high dose chemotherapy, has become a standard therapeutic option for pediatric patients with hematologic malignancies.[1–5] However, the low cell doses in CB products has limited the use in adult patients due to delayed engraftment and survival.[6,7] This has led to evaluation of approaches to increase the cell dose by *ex vivo* expansion.[8–10] Several groups have evaluated the potential of *ex vivo* expanded CB units to enhance engraftment,[8–10] however, the results to date have failed to demonstrate significant improvement in the time to neutrophil engraftment. We have proposed that current methodologies employed in these trials, which include selection of CD34+ or CD133+ cells from frozen CB units, are problematic due to significant cell loses during selection and low purities in a number of products.[10,11] Based upon the issues associated with selection of frozen CB units, we have developed culture methods for co culture of CB mono nuclear cells (CB-MNC) on mesenchymal stem cells (MSC).[11] We have demonstrated an expansion of total nucleated cells (TNC) of 10 to 20 fold and clinical trials have demonstrated decreased time to neutrophil and platelet engraftment.[12]

Another approach that has been explored for delivering higher cell doses with CB products is the use of two CB products.[13] Wagner and colleagues have demonstrated the feasibility of this approach for use in adults; however the majority of recipients receive 4 of 6 HLA CB products. A number of patients have better matched CB products available (5 of 6 or 6 of 6), however these products have low cell doses ($<2 \times 10^7$ TNC/kg) and are not suitable for use. We hypothesized that the co culture expansion conditions we developed could be suitable to expand suboptimal CB products, i.e. products with cell doses below 2×10^7 TNC/kg, to provide a sufficient cell dose for adult patient and the use of better matched products would result in decreased immunological complications including graft versus host disease (GVHD).

Methods

Cord blood products

Frozen CB products: Frozen CB products were supplied by Joanne Kurtzberg (Duke University, Durham, NC). The products had been frozen for banking however they were deemed unsuitable due to low cell numbers. All products were collected under a Duke University IRB approved protocol with signed consent. The products were shipped in a dry shipper and stored in liquid nitrogen until used.

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Fresh CB products: Fresh CB products were supplied by Dr. Pablo Rubenstein (New York Cord Blood Bank, New York, NY) and were shipped overnight at room temperature. These products were collected for banking under appropriate IRB approval and signed consent but were deemed unsuitable for banking due to low cell numbers or other issues. CB-MNC was isolated from fresh CB products by density centrifugation over ficoll and the low density cells recovered. CD34+ cells were isolated from the CB-MNC using the Vario MACS selection device (Miltenyi Biotech, Cologne, Germany). Expansion was performed on MSC as described below in Stemline II media plus growth factors.

Isolation of MSC

Bone marrow cells were purchased from Allcells Inc (Emeryville, CA) who obtained the BM aspirates from normal donors under appropriate IRB approvals. The MNC fraction was isolated by ficoll separation and MSC were grown to confluency in T162cm^2 tissue culture flasks (Corning, Acton, MA) in alpha MEM plus 20% FCS.

Ex Vivo expansion of CB-MNC on MSC

CB products were thawed rapidly in a water bath and the MNC fraction isolated by density separation over a gradient and the low density cells collected and resuspended in 500ml of Stemline II expansion media (Sigma Aldrich, St Louis, MO) containing 100ng/ml each of recombinant human (rh) stem cell factor (rhSCF), granulocyte colony stimulating factor (rhG-CSF) and thrombopoietin (rhTpo). Ten (10) T162cm^2 cultureflasks containing confluent layers of MSC were prepared by removing the growth media (alpha MEM plus 20% FCS) and replacing with 50ml of CB MNCs in media plus growth factors, representing 10% of the CB products. The cultures were incubated for 7days in a 5% CO_2 incubator at 37°C. After 7 days the non adherent cells were removed from the flasks and transferred to a 1 liter Teflon bag (American Fluoroseal Corp, Gaithersburg, MD) with an additional 350ml of media and growth factors added. Fresh media and growth factors (50ml) were placed in the T162 flasks. After an additional 7 days (14 days total incubation) both the bags and flasks were harvested and the cells pooled.

Flow cytometric analysis

Cells were analyzed for phenotypic expression of surface proteins specific for subpopulations of hematopoietic cells. Approximately 1 million cells were stained with anti-CD34-PE and anti-CD45-FITC (Becton-Dickinson, San Jose, CA) and a minimum of 50,000 events collected in a list mode file format by flow cytometry (FACS Vantage, Becton-Dickinson). Aliquots of cells were also stained with isotype control antibodies.

Results

Expansion of CB mononuclear cells on MSC

CB products were thawed and washed, resulting in a median of 3.3x10^{8}TNC (range 1.4 to 3.6x10^8). For a 50kg recipient, these CB products would provide only 0.73x10^{7}TNC/kg with zero of 5 products reaching the minimal target dose of 1x10^{7}TNC. These products may be well matched for a patient based upon HLA, but would not be suitable for clinical use due to the low cell dose and so decreasing the number of available grafts for patients. In many cases more mismatched products must be used and so increasing the potential toxicities due to GVHD or the potential of rejection.

Each product was expanded by culturing the MNC fraction on preformed layers of MSC. Ten (10) T162cm^2 flasks were used for each product such that each flask contained 10% of the CB MNC. After *ex vivo* culture a median of 9 fold expansion of TNC was obtained with a range of 6.5 to 24 fold. The median TNC post expansion was 21.6x10^8 (range 11 to 79x10^{8}TNC) (Table 1). For a 50kg recipient, the expanded CB product would be equivalent to 4.3x10^{7}TNC/kg (range 2.2 to 16x10^7), with all 5 expanded products reaching the minimal target of 1x10^{7}TNC/kg (Table 2). In fact all expanded products contained more the 1x10^{7}TNC/kg based upon a 100kg recipient.

Table 1 *Ex Vivo* Expansion of TNC from frozen CB products

TNCx10^8						
Exp #	**1**	**2**	**3**	**4**	**5Median**	
CB-MNC	3.6	2.2	1.4	3.3	3.3	3.3
Post expansion	44.6	19	11	21.6	79.3	21.6
Fold expansion	12	9	7	6.5	24	9

Table 2 Cell doses based upon recipient weight of 50kg (A) and 100kg (B)

TNC x10^7/kg						
A: Recipient Weight 50kg						
Exp #	**1**	**2**	**3**	**4**	**5Median**	
CB-MNC	0.7	0.4	0.3	0.7	0.7	0.7
Post Expansion	8.9	3.8	2.2	4.3	15.9	3.8
B: Recipient Weight 100kg						
Exp #	**1**	**2**	**3**	**4**	**5Median**	
CB-MNC	0.4	0.2	0.1	0.3	0.3	0.3
Post Expansion	4.5	1.9	1.1	2.2	7.9	2.2

Expansion of CB CD34+ Cells on MSC

We hypothesized that the major cell population in the CB units responsible for expansion would be CD34+ cells. To test this hypothesis we fractionated CB MNC into CD34+ and CD34- fractions. CB MNC, CB CD34+ and CB CD34- cell populations were then cultured on preformed layers of MSC. The culture of CB MNC on MSC resulted in a median of 15 fold expansion of TNC with a total of 3.4x10^8 cells harvested after 14 days of culture (Table 3). There was a median of 29 fold expansion of CD34+ cells. Co-culture of CD34+ cells on MSC resulted in a median of 1,352 fold expansion of TNC with a median expansion of 54 fold of CD34+ cells. The CD34-ve fraction resulted in low levels of expansion with a 3 fold expansion of TNC and a 4 fold expansion of CD34+ cells. The levels of TNC and CD34+ cells harvested from the CB MNC cultures was approximately equal to the combination of cells harvested from the CD34+ fraction and the CD34- fraction demonstrating that the primary cell population expanded in the MNC fraction are the CD34+ cells. Although the CD34-ve fraction still contained significant numbers of CD34+ cells, the level of expansion was minimal suggesting subsets of CD34+ cells. We hypothesize that selection of the CB product results in isolation of CD34+ cells expressing higher levels of CD34 and these cells have the highest proliferative potential.

Table 3 Comparison of Expansion from CB MNC, CD34+ and CD34- Cells. The results presented are for triplicate cultures for each culture condition and are from a single representative experiment of 3 separate experiments

		Day 0	Day 14	Fold expansion
MNC	TNC	2.2x10^7	3.4x10^8	15
		2.2x10^7	3.2x10^8	15
		2.2x10^7	4.0x10^8	18
	Median	2.2x10^7	3.4x10^8	15
	CD34+ cells	4.5x10^5	1.1x10^7	25
		4.5x10^5	1.3x10^7	29
		4.5x10^5	2.0x10^7	46
	Median	4.5x10^5	1.3x10^7	29
CD34+fx	TNC	1.7x10^5	2.1x10^8	1,235
		1.7x10^5	2.9x10^8	1,705
		1.7x10^5	2.3x10^8	1,352
	Median	1.7x10^5	2.3x10^8	1,352
	CD34+ cells	8.3x10^4	4.3x10^6	52
		8.3x10^4	6.2x10^6	74
		8.3x10^4	4.5x10^6	54
	Median	8.3x10^4	4.5x10^6	54
CD34-fx	TNC	2.2x10^7	7.1x10^7	3
		2.2x10^7	5.9x10^7	3
		2.2x10^7	8.8x10^7	4
	Median	2.2x10^7	7.1x10^7	3
	CD34+ cells	4.0x10^5	1.6x10^6	4
		4.0x10^5	1.8x10^6	4
		4.0x10^5	1.6x10^6	4
	Median	4.0x10^5	1.6x10^6	4

Discussion

We would propose two potential advantages to the use of co culture for expansion based upon the above results. Firstly the possible enhanced engraftment and secondly the ability to use better matched CB products that may have a low cell dose. Wagner and colleagues have described the use of two CB products to provide an increased cell dose; however, the majority of patients receive a 2 antigen miss matched CB unit.[13] Better matched CB units are routinely identified but are not suitable due to low cell doses. The expansion of the better matched CB units could potentially result in decreased toxicities relating to graft versus host disease (GVHD).

The culture conditions used in this study have been used in previous clinical trials of *ex vivo* expansion of both CB[10] and peripheral blood progenitor cell products (PBPC).[14] In particular the expanded PBPC products resulted in faster neutrophil engraftment which correlated with the total nucleated cells (TNC) infused.[14] In addition, we have previously reported that CD34 selection of frozen CB products results in low purities and low levels of expansion.[11] When CB MNC were cultured for 14days, little if any expansion resulted, however, co-culture on MSC resulted in significant expansion of TNC and progenitor cells (CD34+ cells and CFU).[11] The expanded CB products contain high numbers of neutrophils and promyelocytes and less than 1% T and B lymphocytes.[10,11] Although the focus of this study was expansion of TNC, our previous studies have demonstrated that long term repopulating stem cells are decreased following expansion. When *ex vivo* expanded CB cells were injected into fetal sheep the levels of human engraftment were decreased compared to sheep transplanted with non expanded CB cells.[15] Based upon these data, the clinical trial design currently being undertaken with co-culture of CB MNC on MSC,[12] utilizes two CB products with the expanded CB product providing enhanced short term engraftment while the un expanded CB product provides the long term engraftment. This study reported the median time to neutrophil and platelet engraftment of 15 days and 42 days respectively.[12] The median fold expansion of TNC was 12 fold. This study has led to a phase III trial for evaluation of CB expansion on MSCs.

A second clinical trial of expanded CB cells is being conducted at the Fred Hutchinson Cancer Center by Dr. Bernstein and colleagues.[16] This study targets expansion of CD34+ cells using an engineered form of the Notch ligand, Delta 1. Preliminary data was reported at meeting of the American Society of Hematology on the first 6 patients enrolled in this study and reported rapid engraftment with a median time to neutrophil recovery of 14days (range 7-34days).

Taken together these studies demonstrate the potential of expanded CB cells to provide more rapid engraftment. The data we have presented in this reports expands the range of CB products that may be used for therapy. The clinical trials described above utilize CB products with higher cell numbers and typically CB products with smaller cell doses would not meet minimum cell dose requirements. Testing of this approach will require a clinical trial to evaluate the engraftment potential of these expanded products.

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Conflict of interest

The author declares no conflict of interest.

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