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Mesenchymal Stem Cells in ex vivo Cord Blood Expansion

Simon N. Robinson, Ph.D.[1], **Paul J. Simmons, Ph.D.**[2], **Hong Yang, Ph.D., MD**[1], **Amin M Alousi, MD**[1], **Marcos J. de Lima, MD**[1], and **Elizabeth J. Shpall, MD.**[1]

Simon N. Robinson: snrobins@mdanderson.org; Paul J. Simmons: Paul.J.Simmons@uth.tmc.edu; Hong Yang: hyang@mdanderson.org; Amin M Alousi: aalousi@mdanderson.org; Marcos J. de Lima: mdelima@mdanderson.org; Elizabeth J. Shpall: eshpall@mdanderson.org

[1]Department of Stem Cell Transplantation and Cellular Therapy, University of Texas M. D. Anderson Cancer Center, 1515 Holcombe Boulevard, Houston, Texas 77030. USA

[2]The Centre for Stem Cell Research, The Brown Foundation Institute of Molecular Medicine, University of Texas Health Science Center at Houston, 1825 Pressler Street, Houston, Texas 77030. USA

Abstract

Umbilical cord blood (CB) is becoming an important source of haematopoietic support for transplant patients lacking human leukocyte antigen matched donors. The ethnic diversity, relative ease of collection, ready availability as cryopreserved units from CB banks, reduced incidence and severity of graft versus host disease and tolerance of higher degrees of HLA disparity between donor and recipient, are positive attributes when compared to bone marrow or cytokine-mobilized peripheral blood. However, CB transplantation is associated with significantly delayed neutrophil and platelet engraftment and an elevated risk of graft failure. These hurdles are thought to be due, at least in part, to low total nucleated cell and $CD34^+$ cell doses transplanted. Here, current strategies directed at improving TNC and $CD34^+$ cell doses at transplant are discussed, with particular attention paid to the use of a mesenchymal stem cell (MSC)/CB mononuclear cell ex vivo co-culture expansion system.

Keywords

Cord blood (CB) transplantation; ex vivo expansion; mesenchymal stem cells (MSC)

A. Background

Since the first CB transplant (CBT) was performed by Gluckman et al.[1] in 1988, >20,000 patients have received this procedure to support treatment for a variety of malignant and non-malignant diseases.[2–16] The reported event-free survival rates for such patients are comparable with those achieved following the transplantation of unrelated allogeneic bone marrow (BM), or mobilized peripheral blood progenitor cells (PBPCs).[15] In addition, there are many reports of lower rates of graft versus host disease (GvHD) than are

Correspondence to: Elizabeth J. Shpall, eshpall@mdanderson.org.

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commonly observed with BM and PBPC transplantation, particularly in pediatric patients. This reduced incidence of GvHD is observed despite the use of CB grafts with greater donor-recipient human leukocyte antigen (HLA) mismatching than would be tolerated by recipients of BM,[4;5;9;12] or PBPC allografts.[17–19]

B. Challenges

One major challenge associated with the use of CB for transplantation is the relatively low cell dose available. This is thought to contribute, at least in part, to the slower engraftment and an elevated risk of engraftment failure that is associated with CBT.[20–24] For example, the time required for a patient receiving CBT to achieve an absolute neutrophil count (ANC) of $\geq 0.5\times10^9$/L can range from 23 to 41 days. Similarly, the median time for a CBT patient to achieve a transfusion-independent platelet count of $\geq 20\times10^9$/L can range from 56 to >100 days. Further, while engraftment failure rates for CBT recipients as a whole (pediatric and adult CB recipients) can range from 12–20%,[5;9;13;14] those data for adult patients (>18 years old and/or >45 kg) are particularly poor with engraftment failure rates reaching of 20% or higher having been reported.

C. Evidence Of Threshold Doses For Effective Transplantation

Data from studies performed by Gluckman et al.[9] demonstrated that engraftment and survival were superior in CBT patients who received a transplant dose of $\geq 3.7\times10^7$ TNC/kg. These data suggest that there is a threshold CB total nucleated cell (TNC) dose above which time to engraftment is improved and graft failure rate reduced and below which time to engraftment is prolonged and graft failure rate increased. However, it is rare that a CBT cell dose of $\geq 3.7\times10^7$ TNC/kg is achieved. This is particularly true for CBT patients of >45 kg. Additional analyses of these data revealed that a lower, more readily realized target CBT dose 'threshold' of $\geq 1.0\times10^7$ TNC/kg was still associated with favorable engraftment rates and could be applied for this patient population.[2;9] However, analysis of data from patients who received myeloablative therapy and a single CB unit in North America or Europe through 2005 at the world's three largest CBT registries: Center for International Blood and Marrow Transplant Research (CIBMTR), National Cord Blood Program (NCBP) and Eurocord,[25] revealed that a 100-day treatment-related mortality of approximately 44% was closely correlated with a CBT dose of $<2.5\times10^7$ TNC/kg ($P<0.0001$) despite state-of-the-art care practices. These data underscore the urgent need to improve CBT strategies with the goal of improving neutrophil and platelet engraftment and reducing the risk of engraftment failure, especially for patients >45 kg.

D. Strategies To Enhance CBT Outcome

At the University of Texas M. D. Anderson Cancer Center Transplant Program, PBPC is the most commonly used source of unrelated haematopoietic support for cancer patients. PBPC remain the 'gold standard' against which the efficacy of CBT is compared. For comparison, patients who receive unrelated PBPC transplants achieve an ANC of $\geq 0.5\times10^9$/L at a median of 11 days post-transplant as compared to 23–41 days for CBT recipients. Similarly, while PBPC recipients achieve a transfusion-independent platelet count of $\geq 20\times10^9$/L at a median of 13 days post-transplant, patients receiving CBT might not achieve platelet engraftment until >100 days. Further, while PBPC recipients have an engraftment failure rate of <1%, rates of 12–20% are common for CBT. Given that these differences are likely a consequence of the limited cell dose associated with CBT, two major therapeutic strategies are being explored by different clinical centers to increase the cell dose at transplant and thereby improve time to neutrophil and platelet engraftment and reduce engraftment failure are (i) double CBT[26–30] and (ii) ex vivo expansion.[26,27,31,32]

E. Double Cord Blood Transplantation

While double CBT does provide significantly more rapid neutrophil engraftment (23 days; range 15–41 days),[28–30;33] when compared to single CBT, it continues to be associated with significantly delayed engraftment and elevated engraftment failure when compared to BM or PBPC transplantation.[27] The exact mechanisms by which double CBT improves the rate of engraftment over single CBT remain to be determined, however, it is worthy of note that while the progeny of both CB units are detectable for a short period after transplant, only one CB unit will ultimately predominate.[33] Measures that are predictive of which CB unit will ultimately predominate remain to be determined.

F. Rationale For Ex Vivo expansion

The observation that there is a threshold dose below which CBT recipients have markedly delayed engraftment and elevated risk of graft failure suggests that suboptimal numbers of the cells responsible for rapid engraftment are being transplanted.[34] If this is the case, increasing the dose of those CB cell subpopulations responsible for rapid engraftment, should improve the time to neutrophil and platelet engraftment and reduce the risk of graft failure. This has been the rationale behind the development of ex vivo expansion strategies. However, it is important to emphasize that double CBT and ex vivo expansion are not mutually exclusive, with the hope that a combination of these strategies might provide the greatest benefit to CBT recipients.

E. Concerns Associated With Ex Vivo Expansion

The goal of increasing the numbers of haematopoietic progenitors available for transplant using ex vivo expansion has been explored in a PBPC setting.[35–44] These PBPC studies set the stage for the use of this approach with CB. While there is evidence of functional and phenotypic heterogeneity within the primitive haematopoietic progenitor compartment,[45–48] one concern associated with ex vivo expansion is that short-term reconstituting, lower ‘quality’ haematopoietic progenitors will be expanded at the expense of longer-term reconstituting, higher ‘quality’ haematopoietic progenitors, thereby significantly impacting the haematopoietic reserve of the graft.[49] Evidence primarily in animal models, suggest that this may occur under certain conditions.[50–57] Clinically, while the absence of durable engraftment from ex vivo expanded $CD34^+$ cells has been reported in some cases,[58] durable engraftment has been reported in other patients who received expanded autologous products as the sole source of haematopoietic support following high dose therapy.[51] This suggests that the primitive haematopoietic progenitor cell compartment in general,[59–64] properties of homing[65] and the basic biologic and genetic characteristics of the primitive haematopoietic progenitor cells[66] are preserved following ex vivo expansion.

F. Methodologies

(a) Static Liquid Cultures

Static liquid culture was shown to first require the purification of $CD34^+$ (or $CD133^+$) cells from fresh or frozen tissue.[44;67;68] Although clinical grade immunomagnetic devices can be used for the isolation procedure,[68] the purification process is limited by low recovery of the target cells (median $CD34^+$ cell recovery of 35%, range, 4–70%). Despite this loss, isolated CB $CD34^+$ (or $CD133^+$) cells have been expanded ex vivo and used in clinical trials at the M. D. Anderson Cancer Center.[27;69] Initially, positively selected $CD133^+$ cells were cultured in medium supplemented with 100 ng/ml each of granulocyte colony-stimulating factor (G-CSF), stem cell factor (SCF) and thrombopoietin (TPO),[70] for 10 days which resulted in a 56-fold expansion of total nucleated cells (TNCs) and a 4-fold

expansion of $CD34^+$ cells. A two-step, 14 day ex vivo CB expansion protocol was subsequently developed[71] where the CD133+ cells were cultured for one week with the same SCF-G-CSF-TPO regimen in a small volume of 50 ml and then transferred to a larger 800 ml volume on day 7 with fresh media/growth factors and continued culture for the second week. This procedure yielded a >400-fold increased TNCs and >20-fold increased $CD34^+$ cells. It should be noted that in some cases, due to the upfront cell losses incurred by the immunomagnetic selection procedure, even marked ex vivo expansion only gives rise to a cell product whose numbers are not markedly different from that of the original CB unit. However, the argument can be made that exposure to the ex vivo expansion cytokine milieu generated a cell product that is different in 'quality' to the original CB unit with cells possibly 'primed' by the ex vivo exposure to growth factors and subsequently better able to home, engraft and proliferate when transplanted.

(b) Mesenchymal Stem Cell Based Cultures

Ex vivo liquid culture removes the primitive haematopoietic cells from molecular cues provided by the haematopoietic microenvironment. As a consequence the addition of exogenous cytokines is required to prevent apoptosis and stimulate proliferation. An alternative approach is the ex vivo co-culture of haematopoietic cells with components of their haematopoietic microenvironment. The haematopoietic microenvironment contains the putative stem cell 'niche' and is composed of haematopoietic and non-haematopoietic, cellular and extracellular components thought to provide the complex molecular cues that direct primitive haematopoietic progenitor self-renewal, proliferation and differentiation.[72–80] This would be consistent with the observation that ex vivo contact between primitive haematopoietic progenitors and stromal components of the haematopoietic microenvironment preserve stem cell activity.[81–87] Mesenchymal stem cells (MSC) are one component of the haematopoietic microenvironment and can be isolated from a variety of fetal and adult tissues.(89–92) Phenotypically MSC express CD73, CD90, CD105, CD16, and HLA-ABC(I) and do not express CD31, CD34, CD45, CD80 and HLA-DR(II). MSC can be grown and expanded as adherent, contact-inhibited monolayers in tissue culture flasks, although the primary nature of the cell-type limits the degree of expansion that can be achieved before senescence occurs.

G. Ex Vivo CB MNC/MSC Co-Culture

The CB MNC/MSC ex vivo co-culture technique does not require the isolation of $CD34^+$ (or $CD133^+$) cells from the CB, thereby minimizing the losses associated with this procedure. Using a co-culture strategy with bone marrow-derived MSCs and a supportive growth factor regimen which included Flt3-Ligand (FLT3-L), SCF, GCSF, and TPO, CB MNCs were cultured in 50 ml medium on stroma for 7 days at which time the non-adherent cells were removed in cultured in a larger volume (800 ml) for an additional 7 days. The flasks containing the adherent cells were also re-fed with 50 ml of media and the FLT3L-SCF-GCSF-TPO regimen and cultured for the subsequent 7 days. On day 14 all of the cells from the adherent and non-adherent cultures were pooled for evaluation. Using this strategy a 10–20 fold increase in TNCs, a 7–18-fold increase in committed progenitor cells (colony-forming units, CFU), a 2–5-fold increase in primitive haematopoietic progenitors (high proliferative potential colony-forming cells) and a 16–37-fold increase in $CD34^+$ cells was achieved.[88] Building on this experience, a CB MNC/MSC co-culture strategy was developed to maximize the available expanded cells dose for transplant.[89] In the research laboratory, ex vivo expansion culture was characterized by a 6-fold increase in TNC, 30-fold increase in $CD133^+$ cells, 8-fold increase in $CD34^+$ cells, >200-fold increase in CFU, 50-fold increase in cobblestone area-forming cells persisting in culture for 2 weeks ($CAFC_{wk2}$) and thought to be representative of more mature haematopoietic progenitors, and a reduction in (0.05-fold) cobblestone area-forming cells persisting in culture for 6 weeks ($CAFC_{wk6}$)

and thought to be representative of more primitive haematopoietic progenitors. These $CAFC_{wk6}$ data provide evidence of the expansion of the more mature haematopoietic progenitors at the expense of the more primitive haematopoietic progenitor cell population. The fold increases cited are over those numbers originally present in the CB unit prior to ex vivo expansion and therefore represent true expansion.

H. Ex vivo CB MNC/MSC Co-Culture Expansion Trial at the M. D. Anderson Cancer Center

Clinical-scale CB MNC-MSC ex vivo expansion procedures were developed[89] and validated.[90] A trial was subsequently designed to test the clinical feasibility of transplanting the expansion product from CB MNC/MSC co-cultures into patients with haematologic malignancies. The trial, approved by the MD Anderson Institutional Review Board (IRB) Protocol 05-0781) and the U.S. Food and Drug Administration (FDA) (IND 13,034), details that patients will receive two CB units matched in at least 4/6 HLA antigens, with a minimum dose of 1×10^7 TNC/kg from each unit. For the initial cohort of 12 patients, a family member (matched at ≥2/6 antigens) serves as the third party haploidentical MSC donor.[90] Approximately 100 ml of marrow is aspirated from the donor and MSC isolated by plastic adherence. In preparation for co-culture with CB MNC, the MSC are grown to >70% confluence in 10 x T175 culture flasks. The CB unit with the lowest TNC dose is then thawed and divided equally between each of the 10 MSC layers, each in 50 ml of ex vivo expansion media containing 100 ng/ml each of SCF, Flt-3-ligand (Flt-3L), G-CSF and TPO. After 7 days of co-culture at 37°C in a 5% CO_2-in-air fully humidified atmosphere, non-adherent cells are removed from each flask and each transferred into individual 10 × 1-liter Teflon-coated culture bags (American Fluoroseal) with fresh ex vivo expansion medium added to generate 800 ml. This liquid culture step (in the absence of MSC) is performed for an additional 7 days (14 days total). The original co-culture flasks also receive 50 mls of fresh medium. At the end of the ex vivo expansion procedure, all non-adherent cells from the culture bags and culture flasks are pooled, washed and prepared for transplantation. Samples are removed for prospective flow cytometric analysis and quality assurance testing.

I. Preliminary Clinical Results with Family Member-Derived MSCs

Patients were admitted on day −9 for hydration and received the designated preparative regimen on days −8 through −2. On day 0, the unmanipulated CB unit was thawed and infused, followed by infusion of the expanded CB cells. Median TNC and $CD34^+$ cell expansions were 12-fold (range, 1–13) and 12-fold (range, 1–27), respectively. The mean expanded doses of 5.7×10^7 TNC/kg and 3.8×10^5 $CD34^+$ cells/kg representing important increases compared to those achieved in our previous expansion studies. Furthermore, when the second unmanipulated CB unit is considered, patients on this trial received a total of 9.5×10^7 TNC/kg and 8.2×10^5 $CD34^+$ cells/kg. Recipients of myeloablative therapy, engrafted neutrophils in a median of 14.5 days (range, 12–23) and platelets in 30 days (range, 25–51).

J. Rationale For The Use Of “Off-The-Shelf” MSC For The Clinical Protocol

The complex logistics of generating MSC from a patient’s family member have limited accrual to the clinical trial. Such limitations include:

- **a.** an appropriate family member was not always be available to donate marrow
- **b.** disease progression is rapid for selected patients with for example, acute leukemia who were relapsing during the 3 weeks it took to generate sufficient MSCs and 2 weeks to perform the CB MNC/MSC co-culture expansion (delaying transplant for

5 weeks total after patient enrolled on the trial). We postulated that the availability of an “off-the-shelf” source of Good Manufacturing Practice (GMP)-compliant, allogeneic MSC would alleviate this logistical problem with MSC essentially available for immediate use.

The development of master cell banks from young, healthy volunteers provides an optimal source of MSC and standardization of selection and isolation procedures ensures a reproducible, readily available MSC product. The Stro-1 antibody, developed by Dr. Paul Simmons,[91] allows prospective isolation of human bone marrow MSC without plastic adherence. Angioblast Systems, Inc., acquired the technology and developed the allogeneic MSC product Revascor™ for clinical use primarily in the treatment of ischaemic cardiovascular disease.[92–95] To date, more than 20 congestive heart failure patients have received injections of Revascor™ with no adverse events related to the cells (Dr. Silviu Itescu, Angioblast Systems, Inc., *personal communication*). Angioblast has agreed to supply Revascor™ as an ‘off-the-shelf’ product for the M. D. Anderson CB MNC/MSC co-culture expansion trial.

Pre-Clinical Studies of Angioblast-MSC-CB Expansion

Preclinical studies have confirmed that the 10 flasks of MSC required for the ex vivo CB expansion protocol can be routinely generated in 4 days from a single vial (10^7 cells) of the Angioblast MSC product. Multiple experiments performed to compare the performance of the Angioblast MSC product with that of normal donor-derived MSC have revealed no difference in the expanded CB product generated.[96]

K. Preliminary Clinical Results with Angioblast-Derived MSCs

With the pre-clinical Angioblast data described above, the M.D. Anderson CB MNC/MSC ex vivo expansion Protocol 05-0781 and FDA IND 13,034 were amended to include a separate cohort of patients who would be treated identically to the first cohort, but who would receive CB cells expanded on the Angioblast product. Accrual to that cohort has been initiated. During CB MNC-MSC ex vivo expansion, a median expansion of 14-fold (range 1–30) for TNC and 40-fold (range 4–140) for the $CD34^+$ cells was achieved. At transplant, the contribution of the unmanipulated CB included 2.35×10^7 (range 0.2–8.2) TNC/kg and 0.95×10^5 (range 0–4) $CD34^+$ cells/kg, while the contribution of the ex vivo expanded CB unit was 5.8×10^7 (range, 0.3–14.4) TNC/kg and 8.7×10^5 (range, 0–93.4) $CD34^+$ cells/kg. These were higher doses than we have ever infused into any of our recipients of unmanipulated double CBT, or CB expanded with our liquid culture system. As with the family member-derived MSCs, median time to neutrophil engraftment (500/μl) was 15 days (range 9–42) and platelet engraftment (>20,000/μl) was 38 days (range 13–62) with 26 patients (81%) of patients becoming platelet transfusion independant. On transplant day+21, the chimerism assays revealed that the MSC-expanded unit contributed to engraftment with a mean of 19% of the mononuclear cell, 16% of the T cell, and 14% of the myeloid fractions due to the expanded unit. Subsequently, hematopoiesis was increasingly derived from the unexpanded unit with long-term engraftment provided exclusively by the unexpanded unit by six months posttransplant in the vast majority of patients.

L. Summary

Our initial CB expansion protocol (#02-407, IND#7166) involved culture of CD133+ CB cells in teflon bags for 14 days with media containing 100 ng/ml SCF, G-CSF and TPO. In this system the CD133+ CB cells are initially cultured in bags with 50 ml of media/growth factors for 7 days and then transferred to a bag with 800 ml of fresh media and growth factors for another 7 days at which time they are washed and infused. With this strategy our

patients experienced a modest improvement in engraftment of 20 days for neutrophils and 65 days for platelets compared to recipients of double unmanipulated CB units who engrafted neutrophils in 22 days and platelets in 100 days. However, we experienced a loss of >70% of the CB CD34+ cells following the CD133-selection procedure, which stimulated us to investigate the MSC-based co-culture system, where we could culture the entire CB unit without need for positive selection.

The use of the MSC-based CB expansion protocol (#05-0781) used a similar 14-day strategy where for the first 7 days the cells are cultured in 50 ml, but this time in 10 flasks containing 10% of the CB unit plus MSCs that are 70% or more confluent (rather than the entire CD133+ fraction in one bag with the liquid culture system). The growth factor regimen for this trial included the addition of Flt3-ligand to the SCF-GCSF-TPO regimen. On day 7, the non-adherent cells in each of the 10 flasks are transferred to 10 teflon-coated bags with 800 ml of media and the four growth factors (rather than transfer to a single bag in the liquid culture system with three growth factors). Fifty ml of media/growth factors are added to the 10 flasks containing the adherent layers of MSC-CB, and both those flasks and the 10 bags are cultured for another 7 days. On day 14, all of the cells in the bags and flasks are pooled, washed and infused. This strategy has shown more promising results providing markedly higher TNC and $CD34^+$ cell doses than have ever before been achieved. The improvments are likely due to the use of MSCs to recapitulate the hematopoietic milieu, the ability to culture the whole CB unit rather than the CD133+ fraction, minimizing the large upfront CD34+ cell losses due to the positive selection procedure, and possibly the addition of FLT3-ligand to the cultures. The improvement in median times to engraftment of neutrophils (14 days) and platelets (35 days) are encouraging and accrual to the trial continues.

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