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Umbilical Cord Blood Hematopoietic Stem Cell Expansion *Ex Vivo*

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Abstract

Umbilical cord blood is an attractive source of hematopoietic stem and progenitor cells in the treatment of hematologic diseases, especially in allogeneic hematopoietic cell transplantation. However, due to the low abundance of these cells, the therapeutic use of umbilical cord blood has been limited mostly to the pediatric setting. The strategies for adult umbilical cord blood transplantation have been improved, with the recent development of various approaches for expanding stem cells in vitro and enhancing their long-term homing efficiency. In this brief review, we discuss a number of strategies for stimulating the proliferation of umbilical cord blood hematopoietic stem cells in vitro, including the utility of transcription factors and growth factors (cytokine cocktails), as well as co-culturing with stromal cells. Ultimately, we make the case that improvements in umbilical cord blood stem cell expansion will be critical for enhancing transplantation engraftment efficacy and providing potential cure for hematological diseases.

Keywords: Umbilical cord blood; Stem cell; Hematopoietic; Cytokine

Introduction

Umbilical cord blood (UCB) has been considered as an attractive source of hematopoietic stem cell, which offers an alternative approach to bone marrow transplantation in the treatment of both malignant and non-malignant hematologic diseases [1-7]. As early as 1939, scientists have predicted that UCB could be applied to therapeutic use. However, UCB transplantation was not successful until 1988 [2]. As perhaps the largest source of stem cells available, the UCB, normally discarded, has recently been used worldwide for transplantation, especially allogeneic transplantation, to treat patients. By now, more than 25,000 transplantation procedures have been performed, and approximately 500,000 UCB units have been donated and banked for public use [5,8-10]. Generally speaking, UCB transplantation has at least two advantages over bone marrow transplantation [5,8-12]. First, it has much lower risk of acute and chronic graft-versus-host diseases (GVHD), which could lead to morbidity and mortality. Second, it has a lesser requirement for HLA-antigen matching (e.g., only 2 of the 6 HLA –A or B loci). However, there are also two significant challenges for the UCB transplantation, especially in adult patients [12,13]. First, UCB has a limited number of stem cells and colony-forming activity. Considering an adult's body mass, one single unit of UCB is far inadequate to provide sufficient numbers of stem cells for transplantation in an adult patient. Approximately 2-3 x 10^8 total nucleated cells could be harvested in an unit of UCB, while the recommended total number of nucleated cells for transplantation to an adult is 2 x 10^7/ kg, minimum. Second, UCB transplantation is associated with delayed engraftment and immune reconstitution (as indicated by recovery in neutrophil and platelet counts), as well as delayed post-transplantation recovery, due to a lack of sufficient amounts of progenitor cells to sustain the therapy.

To improve the outcome of UCB transplantation in adult patients, one potential solution is to extensively expand UCB stem cells *ex vivo* [14-18]. Such expansion would not only augment stem cell abundance, but it would also facilitate cell homing in mismatched UCB transplantation, by overcoming HLA mismatch [19]. Either in single or double UCB transplantation trials, clinical data indicated that the greater the mismatch of HLA antigens, the greater the requirement for higher doses of donor total nucleated cells. For example, units of UCB that were 2/6 HLA-mismatched to the recipient required greater than 5.0 x 10^7/ kg total nucleated cells to achieve a comparable outcome to that achieved by units that were 1/6 HLA-mismatched, at 2.5 x 10^7/ kg total nucleated cells [20-22].

Various approaches to achieve higher abundance of hematopoietic stem cells and progenitors for infusion have been explored and tested in in-vivo or clinical trials [23-30]. McNiece and co-workers succeeded in maintaining the long-term repopulation capability of HSC in culture, and achieving hematopoietic recovery when applying *ex vivo* manipulated HSC to a fetal sheep model [29]. Von Drygalski et al. further demonstrated in a mouse model that the bone marrow long-term engraftment potentials could be sustained following *ex-vivo* expansion [30]. Delaney et al. reported even more promising data in a phase I cord blood transplantation trial [31], where the time to neutrophil recovery was substantially shortened by transplanting *ex-vivo* expanded cord blood progenitors along with un-manipulated UCB. Following the primary UCB cell infusion, 9 out of 10 patients who received a secondary infusion of manipulated HSC attained ANC (absolute neutrophil count) >500/ µl with a medium time of 16 days, whereas 20 patients in a concurrent cohort who receive a secondary infusion of non-manipulated HSC attained the same ANC with a medium time of 26 days; the two cohorts were treated under identical conditions in the primary infusion and post-transplant immunosuppressive regimen.

There is strong indication that *ex-vivo* expanded HSCs have the potential to facilitate engraftment of un-manipulated UCB cells, but they are not necessarily the source of long-term hematopoietic recovery

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[25,29,32,33]. Hence, although the main purpose to expand UCB is to provide adequate number of HSC for primary transplantation, the amplified HSC could also be applied for supportive therapy after the primary transplantation, thus further reducing the risk of GVHD associated with infusion of cells from another mismatched donor. Additionally, the combinatorial administration of expanded and non-expanded HSC also improves outcome in clinical trial by accelerating graft homing.

A number of current *ex-vivo* expansion strategies are described below. These approaches have been extensively studied and applied in clinical trials worldwide [23-28].

Methods

Cytokine combination mediated expansion

The methods of culturing UCB cells with addition of various cytokine combinations have been extensively investigated, though an ideal cocktail recipe has yet to be reported. As it is well known, the fates of HSC are determined by a variety of cytokines and growth factors that are generated endogenously by the bone marrow. Research scientists and clinical providers have tried to mimic this endogenous micro-environment, and have succeeded in amplifying the primitive hematopoietic stem cells/progenitors in the presence of various growth factors. In this approach, which has been proven to be a safe and stable way to obtain sufficient number of graft [34], the hematopoietic progenitors (CD34+) were isolated, before *ex vivo* culture, with use of a clinical-grade magnetic beads system (e.g., the Miltenyi CliniMACS system). Generally, a purity of greater than 90% CD34+ cells could be reached on culture day 0, before the HSCs were incubated with various cytokine, either alone or in combination [35]. Factors that have been utilized to promote expansion of HSC include Flt-3 ligand, SCF, G-CSF, TPO, IL-3, IL-6, and IL-11 [25-27,30,36-44]. The mechanisms by which these factors maintain stem cell proliferation potentials are not yet clear and sometimes controversial. Research data indicated that Flt-3 and TPO might prevent telomere degradation [39,45]; whereas SCF and IL-6 stimulate the proliferation of HSCs and maintain their long-term repopulation capabilities [46-48].

To gain a maximal fold of HSC expansion *ex vivo*, various concentrations of cytokines have been tested by our lab [49,50] and several other groups. Shpall et al. reported a clinical study showing that, when HSCs were cultured with SCF, TPO, and G-CSF supplements (each at 100 ng/ml), they underwent a 4-fold (median, range 0.1-20) increase in CD34+ cells, and a 56-fold (median, rang 1.03-278) increase in TNC, by 10 days in culture [24]. McNiece et al. further improved the expansion protocol, achieving a >20-fold increase in CD34+ cells and a >400-fold increase in TNC, by day 14 in culture, with use of the same cytokine supplements (SCF, TPO, G-CSF) [37]. We have also attempted to optimize the *ex vivo* culture system, in order to enhance HSC expansion at even greater efficiency. Cytokines, including Flt-3, SCF, and G-CSF, were included in our custom-made basal medium, and various doses of each of the cytokines were tested over a period of 14 days in culture. Our preliminary data (unpublished) demonstrated that a >60-fold increase in CD34+ cells and a >600-fold increase in TNC could be achieved, thus providing a promising approach to expand CD34+ cells for further pre-clinical studies. The cytokine combinations supported not only a short-term HSC expansion *ex vivo* in an undifferentiated state; they also shortened the UCB engraftment time.

SALL4 signaling pathway mediated expansion

Over-expression of SALL4, an embryonic stem cell factor, has been reported recently as a novel approach to proliferating hematopoietic stem cells *ex vivo*. Originally, SALL4 was found to be expressed in human leukemia cell lines and primary acute myeloid leukemia cells [51-55]. Aguila et al. and others showed that SALL4 plays a critical role in maintaining the multiple potential and in governing decisions affecting the fate of HSCs through transcriptional modulation and interaction with OCT-4 and Nanog [52,56,57]. Lenti-viral SALL4B over-expression lead to a more than 10000-fold expansion of human HSCs in the presence of appropriate cytokines (TPO, FLt-3, SCF), with enhanced stem cell repopulation capacity *in vivo*. To develop a clinically applicable protocol utilizing SALL4B factors, the investigators generated a recombinant TAT-SALL4B protein that is capable of promoting human HSC expansion *ex vivo* [57]. Further pre-clinical studies and clinical trial are required to support these results.

Notch signaling pathway induced expansion

Accumulating evidence from multiple clinic centers has shown that Notch ligand, Delta-1, Jagged-1 and Jagged-2, contribute to a rapid early engraftment of UCB progenitors, as well as regulate HSC proliferation and hematopoietic cell fate decision [58]. Initial studies using soluble or cell-bound Notch ligand indicated limited expansion effects on progenitors in murine model and humans [59-61]. Investigators further revealed that ligand immobilization was required for Notch signaling pathway activation [62,63], which leads to marked HSC proliferation and shortened lymphoid and myeloid repopulation [31]. Based on that knowledge, researchers, by culturing human cord blood C34+CD38- cells with immobilized ligand and cytokines in serum free conditions, were able to gain approximately 100-200 fold increases in CD34+ cells, which were functional for reconstitution in an immune-deficient mice model [64,65]. More recently, based on pioneering studies by Wagner et al., who demonstrated safety of double cord blood unit infusion [66], Delaney et al. applied the Notch ligands-expanded UCB cells along with un-manipulated HSC to patients undergoing myeloablative regimen, and achieved a quite favorable outcome [31, 67]. CD34+ enriched UCB cells were cultured with Notch ligand Delta-1 and serum free media supplemented with SCF, TPO, FLt-3, IL-3, and IL-6 for 16 days, and approximately 164 fold expansion of CD34+ cells (Range: 41-471) and an average of 562 fold expansion of total cells (Range: 146-1496) were achieved. The expanded cells were used for graft infusion in ten patients with high risk acute leukemia and morphological remission. However, it appeared that variations in the densities of Notch ligands might result in different cell-fate outcomes [64]. Low density Delta-1 stimulated proliferation of CD34+ cells, whereas high-dose Delta-1 induced apoptosis of CD34+ precursors, resulting in decreased cell numbers.

Co-culture with stromal cells

Naturally, the hematopoietic stem cells in bone marrow are maintained and regulated by a specific microenvironment referred to as “niche” [68]. Therefore, extensive studies have been carried out to co culture HSCs with stromal cells, in order to recreate their niches and gain UCB stem cell expansion *ex vivo* [69,70]. In addition to providing direct support to HSCs, stromal cells are known to secret a number of cytokines and adhesion molecules at high levels, including osteopontin (OPN), angiopoietin 1(Ang-1), thrombopoietin (TPO), Jagged-1, CXC chemokine ligand 12 (CXCL12, also known as SDF1), G-CSF, GM-CSF, SCF, and IL-6 that can contribute to HSCs’ proliferation and differentiation [71-77]. Dexter demonstrated that an adherent

stromal-like co-culture system was able to sustain the self-renew properties of hematopoietic stem cells as well as early B lymphopoeisis [78]. Researchers revealed that ablation of those stromal cells in bone marrow significantly decreased the HSC numbers and reduced HSC homing to bone marrow by 90% [79].

A variety of stromal cells have been studied as matrices for *ex vivo* expansion of HSC, including MSCs isolated from bone marrow [80-82], adipose tissue [83-85], placenta [86], umbilical cord blood [87-89], umbilical cord tissue (Wharton's Jelly) [90-94], and fetal liver [95]. Bone marrow-derived mesenchymal stem cells have been widely used and clinical-scale UCBMNC-BMMSC in vitro co-culture procedures were well developed [96] and validated [97]. Clinical trials have been designed accordingly utilizing autologous [80,98] or related family member donor [97,99] (matched at ≥ 2/6HLA antigens) bone marrow MSC for UCB co-culture. The MSC were grown to >70% confluence and then co cultured with UCB total nuclear cells for 7 days, in the presence of the following cytokines (100 ng/ml each of SCF, FLt-3 ligand, G-SCF, and TPO). Non-adherent cell were removed from each flask and transferred into individual culture system with MSC conditioned medium for additional 7 days (totally 14 days), achieving approximately 12 fold increase in CD34+ cells and 12-fold increase in total nuclear cells. However, functional and phenotypic heterogeneity have been observed within primitive hematopoietic stem cells/progenitors [100-104]. Most MSC-UCB co-cultures were established on bone marrow MSCs with exogenously added cytokines to promote HSC proliferation; the resulting cells have been referred to as "lower quality" hematopoietic progenitors contributing to short term reconstitution [32,103,104]. Evidence from both animal models and clinical trials indicated that *ex vivo* expanded CD34+ cells sometimes do not support long-lasting engraftment [29,30,33,36,105-109]. Currently, greater efforts are dedicated to maintaining HSC "stemness property", or their CD34+CD38-phenotype, which has been considered to be the marker for the subpopulation that contains the highest clonogenic activity [110-113]. Others have reported that CD34+c-kit+HLA-DR could represent high proliferative potential colony-forming cell and long-term BM culture initiating cells [114-117]. Rodriguez-Pardo et al. reported recently that a higher percentage of CD34+CD38- cells were observed on day 7 in a BM-MSC co culture system, when cultured without, than with, cytokine supplements [82]. Isern et al. described a new method of isolating and culturing human BMMSCs as non-adherent mesenchymal spheres, which presented relatively undifferentiated phenotypes; this method supported high efficiency expansion of HSC through secreted soluble factors, and it did not rely on cell-to-cell contact [118]. Both *in vivo* and *in vitro* studies demonstrated that these MSC spheres yielded a much greater extent of expansion of UCB CD34+ cells (40-fold versus 6-fold), as well as significantly increased long-term human HSC engraftment in murine model, when compared with regular plastic-adherent BM MSCs.

Even though bone marrow derived stromal cells have been extensively used as the feeder cell for HSC Co-culture, the BMMSCs also have limitations [99]. These limitations include: 1), a suitable family member is not always available to donate bone marrow; 2), time taken to generate adequate BMMSCs (about 3 weeks); and 3), time required to produce sufficient amount of HSCs via co-culture (2 more weeks). In addition, BM MSC harvesting is a painful and high risk procedure to donor. Therefore, clinicians and researchers have been seeking alternative and optimal cell sources to be used as "off-the-shelf" MSC for clinical trials. In that regard, the Simmons lab developed the stro-1 antibody, which allowed prospective isolation of human bone marrow cells from young, healthy volunteers, and the development of the angioblast MSC master cell banks [119]. Preliminary clinical study revealed no difference in performance between expanded UCB product and angioblast MSC product from normal donor-derived MSCs.

Another attractive source of MSC is umbilical cord tissue, known also as Wharton's Jelly [120]. The beneficial properties of this latter source of cells include: 1), sufficient amounts of MSCs, at least 4.6 x 10^6 cells, can be isolated from 1 cm of umbilical cord, and the stemness properties of these cells lasted longer than bone marrow MSCs in culture (10 passage versus 3 passage) [94]; 2), they are hypoimmunogenic (which alleviates concerns of GVHD) and thus can be used in autologous or allogeneic settings [91]; 3), they have high thaw-survival rates (~90%), which makes them a reliable MSC cell source [94]. Studies to date have shown that Wharton's Jelly-derived MSC promoted HSC proliferation through secretion of various factors, including interleukin family (IL1a, IL-6, IL-7, IL-8), hyaluronic acid, cell adhesion molecules, cadherins, and growth factors (SCF, HGF), at much higher levels than by bone marrow derived MSCs [121,122]. Being naturally rich and hypoimmunogenic, Wharton's Jelly-MSCs-conditioned medium alone seems to serve as a better support to expanding stem cells than could long term culture-initiating cells and colony forming cells, and can potentially bring about even greater desirable effects in clinical study.

Summary

Umbilical cord blood has been well recognized as an alternative donor source of HSCs for allogeneic transplantation to treat malignant and non-malignant hematopoietic diseases. However, particularly for adult patients, UCB transplantation has been limited by the low dose of HSCs available in each graft, which leads to delayed myeloid and lymphoid engraftment. Current strategies to overcome this limitation include *ex vivo* expansion, infusion of two UCB units, and co-transplantation with mesenchymal stem cells (suitable for immunomodulation and prevention of GVHD), which could be applied individually or in combination to enhance homing and long-term engraftment. Ample clinical trials have shown that expanded UCB cells are safe for transplantation, and favorable outcomes have been achieved due to increased progenitor infusion and facilitated engraftment. Further efforts are being undertaken to identify the optimal expansion conditions, include the use of cytokines, stroma co-culture, and novel signaling pathway stimulation, to preserve primitive hematopoietic progenitor properties. Overall, expectations remain high that eventually *ex vivo* expansion of UCB will be able to improve clinical outcomes and benefit more patients.

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