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SPECIAL ISSUE

Usefulness of Umbilical Cord Blood Cells in Era of Hematopoiesis Research

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Although worldwide experience with umbilical cord blood (UCB) transplantation is still relatively limited, clinical experience with UCB transplantation is encouraging. The use of UCB for hematopoietic stem cell transplantation (HSCT) has advantages and disadvantages. Among the advantages are rapid availability, ability to more rapidly schedule the transplant as the UCB units are stored and ready for use, the apparent reduced need for an exact human leukocyte antigen (HLA) match, and induction of a less severe graft versus host disease (GVHD) compared with bone marrow. The major limitation of reduced numbers of hematopoietic stem cells (HSC) in UCB is being addressed by basic research. It is promising that potential improvements in engraftment efficiency without increased stem cell numbers or actual increased stem cell numbers through dual UCB transplant or ex-vivo expansion might lead to improved treatment approaches. However, its therapeutic potential extends beyond the hematopoietic component suggesting regenerative potential in solid organs as well. Many different stem and progenitor cell populations have been postulated with potential ranging from embryonic like to lineage-committed progenitor cells. UCB derived MSCs have the differentiation capacity and also the therapeutic potential with regard to regenerative medicine, stromal support, immune modulation and gene therapy. Therefore, further advances are eagerly anticipated.

Keywords: Umbilical cord blood cells, Transplantation, Therapeutic potential

Introduction

The first allogeneic umbilical cord blood (UCB) transplantation was successfully performed in order to treat a child with Fanconi's anemia in October 1989 (1). Fifteen years later, this patient is doing well with full donor hematopoietic and lymphoid reconstitution. This first success showed that a single unit of cord blood contained enough hematopoietic stem cells (HSCs) to reconstitute definitely the host lympho-hematopoietic compartment. These days, UCB has been widely accepted as a useful source of alternative HSC for patients who have no readily available human leukocyte antigen (HLA) matched donors. To date, more than 10,000 cord blood transplant procedures have been performed worldwide for pediatric (2, 3) and adult patients (4, 5).

UCB has many compelling advantages as an attractive HSC source for transplantation purposes compared with peripheral blood stem cell (PBSC) and marrow HSC (6). HSC from UCB are enriched in primitive hematopoietic stem cells, which are able to produce *in vivo* long-term repopulating stem cells. The properties of UCB cells should compensate for the relatively low number of cells contained in a single cord blood unit. In the long-term, a higher frequency of reconstitution of early and committed hematopoietic progenitors was observed in children receiving a UCB compared to bone marrow suggesting that the delay in engraftment may reflect the difficulty of UCB progenitors to reprogram themselves toward differentiation. In a clinical aspect, several clinical studies showed that hematopoietic recovery is delayed after UCB engraftment, but improves if a higher number of nucleated and $CD34^+$ cells are infused (2-5). Recent studies have shown

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that the cell dose is the most important factor for donor selection: a minimum cell dose of 3×10^7 nucleated cell/kg is required for successful engraftment. More recently the use of two UCB units seems to improve the engraftment rate (3, 7).

The second advantage of UCB is related to the immaturity of the immune system at birth. This property should decrease the alloreactive potential of lymphocytes and as a consequence reduce the incidence and severity of graft versus host disease (GVHD) after an HLA matched or mismatched transplant (3). These properties should lead to less stringent criteria for HLA donor recipient selection. Owing to the less stringent HLA matching requirements between donor-recipient, nearly all (>95%) patients are able to find at least one potential 4～6/6 matched UCB unit on the National Marrow Donor Program (NMDP) Registry and the majority will find a potential 5/6 match in USA. Recipients who received 4～5/6 HLA matched UCB unit(s) have shown GVHD rates and survival outcomes similar to those who receive 6/6 HLA matched unrelated donor (MUD) marrow transplants (8).

We anticipate that UCB transplantation will continue to grow and will attract more attention in clinical and biological research in the future. This growth will also be stimulated by advances in the basic biology of UCB and new approaches for expansion of absolute HSC numbers and improvement of engraftment ability.

Researches on UCB collection and cryopreservation

UCB can be collected for several purposes. A number of techniques have been proposed for the optimal collection of UCB, considered to be a UCB unit with sufficient volume of blood, total nucleated cells, and $CD34^+$ cells. As engraftment is closely correlated with the number of infused cells, numerous variables in the UCB collection process have been examined in attempts to maximize the cell dose.

Early studies suggested that factors that may increase these numbers include volume of UCB collected, number of nucleated cells or number of $CD34^+$ cells, larger birth weight, fewer prior live births, and birth order, with a larger number of cells in first-born children (9). Other factors include prolonged stress during delivery, placing the infant on the mother, abdomen after delivery, collection prior to delivery of the placenta, cesarean section, early clamping of the umbilical cord, and normal saline flush of the umbilical vessels (10).

More recent analyses from the National Heart Lung and Blood In (HLBI) supported Cord Blood Transplantation (COBLT) study banking program have further identified factors predictive of cell dose (11). Higher cell doses were obtained from cesarean section deliveries as opposed to vaginal deliveries, and in higher birth weight newborns. There was a significant correlation between $CD34^+$ cell dose and colony-forming unit (CFU)- granulocyte macrophage (GM), CFU-granulocyte erythrocyte-macrophage megakaryocyte (GEMM), burst forming unit-erythroid (BFU-E), and total CFUs (12). Despite this information, there remains considerable debate about the optimal collection method.

Efficient clinical banking of UCB requires the volume reduction of cord blood collections to decrease storage space and costs. Because of concerns about the loss of nucleated cells, UCB that was initially collected for transplantation was cryopreserved without removal of red blood cells (RBCs) or further separation. The simplest way to achieve this purpose is to deplete RBCs in the cord blood before cryopreservation. Early study suggested RBC lysis method was effect for the red cell depletion of UCB without major losses of CFC or $CD34^+$ cells (13). However, other studies revealed different result probably due to different study method. Subsequently, numerous investigators explored different methods of UCB separation including the use of Ficoll, Percoll, methylcellulose, gelatin, starch, and lysis to remove RBCs and recover nucleated cells (14). These methods have allowed for more efficient storage of UCB units, although it is not yet clear what the best separation procedure is.

Technically, cryobiology is the study of living systems, at any temperature below the standard physiological range. However, freezing temperature variations above or below the physiological threshold may be very dangerous if not lethal to all types of cells. The therapeutic use of stem and progenitor cells from different organs/systems to treat a variety of human diseases require the development of validated, clinical-grade cellular freeze-thawing procedures to minimize adverse effects or toxicity to patients (15). To achieve successful results of transplantation and treatment of significant diseases, optimum methods need to be developed on how to obtain stem cells, the way in which they are cultivated, how and for how long they are cryopreserved.

Currently the standard method for human cell cryopreservation is slow programmed freezing, using medium containing human serum albumin and cryoprotective molecules such as dextran and dimethylsulphoxide (DMSO) at high concentrations. Freezing procedures and chemical

agents may induce substantial damage due to maintaining the cells at temperature higher than the glass transition temperature of the sample or the toxicity of cryoprotective agents. Trounson's group demonstrated that current methods of freezing, storage and recovery trigger apoptosis and spontaneous differentiation of human embryonic stem cells with consequent loss of pluripotency (16). In a study with using UCB, Woo et al. also reported cryopreserved $CD34^+$ cells had lower clonogenic potential than $CD34^+$ cells of fresh sample. However, UCB had the same frequency of CFU-GM after cryopreservation in long term stromal based culture (17).

In addition, it would essentially be important to develop protocols for freeze-thawing all clinically relevant cells in a state for immediate use on patients and, most of all, establish ways to minimize adverse responses. However, UCB is generally stored overnight and it undergoes a $CD34^+$ selection process the next day for reducing the cost and due to the convenience. Although this procedure may affect the short and long-term repopulating capacity, the number of CFU-GM was similar between the immediate and overnight cryopreservation group. And there was no significant difference in measuring the number of apoptotic cells between both groups. Jung et al (18) concluded the continue use of overnight storage of UCB before cryopreservation was useful as an alternative way to be convenient and inexpensive.

Today, the scientific community is very concerned with the available cryoprotective agents and their effects on the function of the cells. Indeed, a number of non-toxic cryopreservation reagents and protocols have been developed by biotech companies dealing with this aspect of clinical research. Particular attention has been given to hematopoietic stem and progenitor cell conditions since they have been used to treat a number of human diseases. However, one aspect of stem cell research, which has not yet been fully explored, is on the chromosomal stability of these cells as a function of duplication time, cryoprotective reagents and storage conditions. The variation in chromosome number is probably the main type of genomic instability recorded and this is usually manifested as loss or gain of whole chromosomes, generally known as aneuploidy (19). Chromosome instability is a devastating phenomenon underlying several human diseases. Aneuploidy arises from meiotic errors and is almost always found in cancers, but is also associated with aging. Stochastic or spontaneous chromosomal variations in somatic cells appear as low level mosaic aneuploidy which are usually thought to be insignificant and overlooked probably due to unapparent phenotypic effects.

Characteristics of primitive hematopoietic stem and progenitors cells in UCB

The number of nucleated cells available for UCB transplantation is greatly limited compared with that available for bone marrow transplantation. However, the frequency of primitive cells in UCB, as determined by assays of colony-forming cells, is greater than that of bone marrow. Numbers of CFU-GM, BFU-E, and CFU-GEMM of the UCB collections were at least five times more than those of the bone marrow (20). Another similar result was reported by the Broxmeyer laboratory (21). Most of these values fell within the limits of the corresponding numbers present in bone marrow used for successful bone marrow transplantation. It was noted that the progenitor cell content of UCB units was more rigorously associated with major covariant of post-transplantation survival than was the nucleated cell count, and that progenitor cell content was a better indication of UCB grafts (22). As important is the quality of these cells in UCB compared with bone marrow, UCB includes cells with extensive proliferative capacity and replating ability in vitro. Therefore, primitive human UCB cells appear to have great capacity to engraft/repopulate the hematopoietic system of mice with severe combined deficiency syndrome (SCID).

There are a number of cell surface phenotypic markers to define primitive HSCs of UCB. For the most part, these markers, which include CD34, CD38, Thy1, c-kit, fms-related tyrosine kinase-3 (Flt3), and rhodamine-123, are the same for primitive cells found in UCB and bone marrow (23). These primitive cells express CD34 antigens and low or absent levels of CD38, Thy1, c-kit, and rhodamine-123, and are positive for Flt3. Human $CD34^+$ cells contain HSCs and progenitor cells (HPCs), but this marker alone is not a perfect indicator for HSCs or the composition of HSCs and HPCs in a graft. CD133 has also been used to identify populations enriched for HSCs and HPCs. In the human system, $CD34^+$ cells can be sub-typed into a CD38- population, considered to contain the earliest cells including HSCs, and a $CD38^+$ population in which HPCs are highly enriched and HSCs are few or absent.

Today, it is possible to more rigorously define murine than human HSCs phenotypically. Mouse HSC functional assays depend on repopulation of lethally irradiated mice in either a competitive or a noncompetitive situation. Murine HSCs (24) are phenotypically characterized into long-term (Sca-1^{hi}Thy-1^{lo}Lin Mac-1^{lo}c-kit^+) and shortterm (Sca-1^{hi}Thy-1^{lo}Lin-Mac-1^{lo}c-kit^+) repopulating cells. Murine bone marrow HSCs were found to be highly puri-

fied in a phenotypically defined population of $CD150^+$ $CD244^-CD48^-$ cells (25). In contrast, non-self-renewing multipotential HPCs were defined as $CD244^+CD150^-$ $CD48^-$ and most restricted progenitors were designated as $CD48^+CD244^+CD150^-$.

More rigorous phenotyping of murine than human HPCs is also available. A recent review documents myeloid lineage commitment from HSCs with the murine system (26). More definitive phenotypic categorizing of human HSCs and HPCs is needed. In this regard, a clonogenic subpopulation of $CD34^+CD38^-$ cells was identified that expressed high levels of CD7 and possessed only potential for lymphoid cell development (27). These cells also expressed CD45RA and HLA-DP, but were low or absent in expression of c-kit and Thy1.

ex vivo expansion of UCB stem cells

In order to carefully choose the *ex vivo* conditions that will most likely expand the number of long-term reconstituting cell (LTRC), studies will need to carefully evaluate the characteristics of the expanded cells and their interaction with both the soluble and insoluble components of *ex vivo* culture. Investigators have placed extensive laboratory efforts into expanding HSCs of UCB *ex vivo*. Although it is clear that primitive UCB cells can be greatly expanded *ex vivo*, these studies demonstrated greater expansion of the more mature cells than the most immature cells in this primitive population (28). Clearly, loss of these most primitive cells is undesirable.

Isolating cells to expand based on only their surface protein expression is likely to include undifferentiated and mature cells. The surface phenotype can change depending on the activation status of the precursor cells and does not provide information on the functional ability of the cells *in vivo*. Early data suggested that spleen repopulating cells (SRC) were $CD34^+CD38^-$ in contrast to CFC and LTC-IC which were also found in the $CD34^+$ $CD38^+$ fraction (29). Although CD34 expression has been the most commonly selected surface marker for *ex vivo* expansion, CD34 is often expressed on more differentiated cells and large animal models suggest that $CD34^+$ cells are not the cells primarily involved in marrow reconstitution, and following *ex vivo* culture, dissociation between $CD34^+CD38^-$ cell expansion and SCID-repopulating capacity has been observed (30). Preliminary data suggests that as UCB units are made up of progenitor cells that possess both a $CD34^+$ and a $CD34^-$ phenotype, isolating a more primitive marker $CD133^+$ may identify cells that are less mature than those that express CD34 (31). Murine models using this cell type showed excellent engraftment. Functional assays, such as aldehyde dehydrogenase activity, may be a more accurate method to isolate the most effective UCB progenitors.

The proper mixture of growth factors and cytokines used in *ex vivo* culture conditions has not yet been determined. Different cytokine components can affect speed of recovery of white cells and platelets as well as affecting long-term donor engraftment. Most *ex vivo* conditions involve SCF, Flt-3 ligand (Flt3L) and thrombopoietin (TPO). SCF has been shown to improve homing capacity of UCB cells in preclinical models (32). Flt3L leads to short-term expansion and helps regulate the expression of very late antigen (VLA)-4 and VLA-5, adhesion molecules which play a part in proliferation and differentiation either directly or through the modulation of cytokine-induced signals (33). In a study to evaluate cytokine combinations that lead to expansion without change in repopulating potential, Levac et al. (34) cultured $CD34^+CD38^-Lin^-$ cord blood cells in serum-free media with SCF and Flt3L and found that TPO may be able to replace IL-3, IL-6 and G-CSF without changing the number of SRC. In addition the combination of Flt3L and TPO may prevent apoptosis and support the self-renewal of primitive stem cells by preventing telomere degradation (35).

Low numbers of megakaryocyte progenitors in UCB grafts have been implicated in the delay in platelet recovery after UCB transplantation, but the problem may also be in the maturation capacity of the megakaryocytes from UCB (36). TPO acts preferentially on late, $CD41^+$ megakaryocyte progenitors by binding to the Mpl receptor. TPO dose-response curves have been determined for normal human $CD34^+CD41^+$ progenitors. The combination of TPO plus other cytokines, including EPO, IL-3, and SCF, was showed a synergistic effect on increasing the number and size of CFU-megakaryocyte colonies during expansion of $CD34^+CD41a^+$ cell from UCB (37). It had also been established that GATA and cis-acting sequences co-regulate the megakaryocyte progenitor expression of Mpl, CD41, and CD42a. Interestingly, TPO along with erythropoietin may stimulate erythropoiesis by inhibiting apoptosis of erythroid progenitor cells (38). When to add stem cell factor to in vitro culture of $CD34^+$ cells, prominent increment of erythroid colony counts was showed. These effects of TPO might be related to the GATA-1, an important hematopoietic transcription factor, which plays a critical role in differentiation and maturation of erythroid and megakaryocytic cell lines.

Other cytokines, such as stromal cell-derived factor (SDF)-1/CXCL12, have also been implicated as useful

agents for *ex vivo* expansion of HSCs. SDF-1/CXCL12 has recently been found to enhance the replating capacity of multipotential (CFU-GEMM) and macrophage (CFU-M) progenitor cell-derived colonies in vitro (39), a measure of self-renewal capacity of HPCs, and also to greatly enhance the *ex vivo* expansion of human cord blood HPCs. It remains to be determined if SDF-1/CXCL12, in combination with SCF, Flt3 ligand, and TPO or other cytokines, will *ex vivo* expand human HSCs.

Schofield's (40) 'niche hypothesis' suggested that true HSC are in essence fixed tissue cells, existing in association with one or more other supporting cells. It is these microenvironmental cells that were postulated to form the niche that enable HSC to indefinitely self-renew, while effectively inhibiting differentiation and maturation. In vitro and *in vivo* studies have shown that stromal cells provide a rich environment of signals (cytokines, extracellular matrix proteins and adhesion molecules) that control proliferation, survival and differentiation of hematopoietic progenitor and stem cells. It is currently not known whether non-contact conditions are sufficient for *ex vivo* expansion or whether stromal binding is required.

Mesenchymal stem cells (MSCs) have been used in preclinical models as a method to maintain stem cells in an immature state. MSCs give rise to osteoblasts, chondrocytes, adipocytes and myelosupportive stroma. MSCs secrete various cytokines and express adhesive ligands and soluble factors critical for hematopoiesis, and have been demonstrated to support hematopoiesis in vitro in co-culture with HSCs. Co-transplantation of MSCs with UCB has been demonstrated to support hematopoiesis *in vivo* in a dose-dependent fashion in NOD/SCID mice (41).

There is a need for understanding molecular pathways involved in HSC proliferation and maintenance. As current *ex vivo* systems have not been proven to enhance long-term reconstituting cells (LTRC), it will be necessary to further study and manipulate molecular pathways that expand the stem cell niche in culture without impinging on recipient engraftment. Such pathways involve cell signaling with Notch, Wnt/-catenin, the receptor tyrosine kinase Tie2 and bone morphogenetic proteins (BMP) in combination with transcription factors BMi-1 and homeobox gene HoxB4. Cytoplasmic mediators of these pathways such as phosphatase and tensin homolog (PTEN) have become areas of intense investigation as PTEN may be the most important switch between the leukemic and normal stem cell, an exciting arena for translational research as the mTor inhibitor Rapamycin (Sirolimus) has been shown to at least partially make up for the loss of PTEN function (42).

Endothelial progenitor cells and mesenchymal stem / stromal cells in cord blood

The UCB contains multipotent stem cells capable of differentiating into cells of different connective tissue lineages such as bone, cartilage and adipose tissues. These stem cells include endothelial progenitor cells (EPCs) and mesenchymal stem or stromal cells (MSCs). Sometimes these cells are found in low frequency in UCB or are absent; thus, great variability is noted in the frequency of these cells in different cord blood collections. Whether or not this variability is technical or has significance for transplant outcome remains to be determined. EPCs and MSCs are still not well defined phenotypically and enhanced characterization of phenotypes that recapitulate functions, will increase the potential for use of these cells from cord blood.

An immature subset of high-proliferative EPCs has been characterized. This cell achieved at least 100 population doublings and had the capacity to form colonies in secondary and tertiary dishes with maintenance of high telomerase activity. EPCs from UCB had greater proliferative potential than those from adult tissue sources. EPCs were redefined by clonal analysis and using HSC/HPC principles (43).

Erices et al. (44) reported that UCB-derived mononuclear cells appear to have the characteristics of MCSs. MSCs are referred to as highly proliferating and adherent fibroblastic cells that possess a unique expression profile of cell surface molecules. In the past few years, their potential has been rigorously demonstrated not only for producing the various cells of mesodermal origin but also for ectodermal neural cells and even insulin producing cell (IPC). Cheong et al. (45) reported that when cultured under serum-free medium containing DMSO and followed by high-glucose condition, mononuclear cells of human UCB trans-differentiated into insulin-producing cells which are endocrine cells capable of the production and secretion of physiologically active insulin in vitro.

Conclusion

Hematology patients requiring allogeneic stem cell transplant for treatment of life-threatening blood disorders are already benefiting from UCB transplant. Because of many favorable attributes, including reduced GVHD. With recent advances in UCB stem cell biology, it is promising that potential improvements in engraftment efficiency without increased stem cell numbers or actual in-

creased stem cell numbers through dual UCB transplant or ex-vivo expansion might lead to improved treatment approaches.

Future anticipated efforts in the field of UCB transplantation include greater success in efficiently engrafting adults and higher-weight children with a single UCB collection, thus decreasing the potential for enhanced GVHD possible with the use of multiple UCB units and enhanced down-regulation of GVHD, and up-regulation of the graft-versus leukemic effect, perhaps by using MSCs, dendritic cells or other immune cell types. There is also a need to know whether UCB is or is not also efficacious for regenerative medicine in a non-hematopoietic situation.

Potential Conflict of Interest

The authors have no conflicting financial interest.

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