Review Article

Ex vivo-Expansion of Cord Blood Cells and Its Clinical Application

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Abstract

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Cord blood is a rich source of hematopoietic stem cells and could be potentially used for transplantation instead of conventional sources of stem cells (bone marrow or peripheral blood).

Cord blood cells are successfully used in pediatric and adult patients, but their major limitation is the low number of hematopoietic stem cells for patients of large body size.

There are several possible solutions for this problem, including use of third party donor, use of multi-unit cord blood, and finally *ex-vivo* expansion of cord blood hematopoietic stem cells to accelerate engraftment of transplanted cells. In this article we will discuss *ex-vivo* expansion from bench and clinical points of view.

Keywords: Cord Blood, Expansion, Transplantation

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Introduction

Cord blood is a potential source for hematopoietic transplantation. The most important limitation of its use is the low number of hematopoietic stem cells (HSCs) in cord blood samples that might prevent a successful engraftment or cause delayed engraftment and higher morbidity and mortality during pancytopenic period after the transplantation. One of the possible theoretical methods to circumvent this unacceptable mortality rate is *ex vivo* expansion of hematopoietic cells to increase the number of stem cells and then use of this *ex vivo* expanded HSCs as a source of transplantation.

Biology of human umbilical cord blood hematopoietic stem cells

Cord blood stem cells are identified by their immunophenotypic and functional characteristics.

Immunophenotypic characterization of cord blood HSCs:

Certain immunophenotypic markers help define primitive cell populations, such as: CD34, CD38, thy-1, c-kit, HLA-DR, Rhodamine 123, etc.

CD34 antigen, an integral membrane glycoprotein, is a defining hallmark of HSCs. It has been suggested that this molecule functions as a regulator of hematopoietic environment (1). In cord blood, the CD34+ cell content is about 1% of nucleated cells, which is similar to the bone marrow content of 1-3% (2). However, the frequency of CD34+ cells is much higher, up to 11%, at earlier stages of gestation, although this decreases with age of gestation (3).

One of the most frequently used markers is the CD38 antigen, which is absent on the more primitive CD34+ cells (4). The subset of CD34+CD38- cells in cord blood is fourfold higher than in adult bone marrow (5).

Another cell surface marker commonly used in immunophenotyping is HLA-DR. Primitive CD34+ cells in the bone marrow are DR-, while CD34+ cells from cord blood with similar functional properties express HLA-DR (6).

The function of Thy-1 on HSCs is unknown. It might be involved in HSCs development by mediating signals inhibiting the proliferation of primitive cells. Baum and colleagues demonstrated that only CD34+ cells expressing the Thy-1 antigen reconstituted human hematopoiesis in SCID mice (7).

The c-kit proto-oncogene encodes a transmembrane receptor with tyrosine kinase activity and is intimately involved in hematopoiesis (8). The ligand for this receptor is steel factor or SCF. The c-kit antigen is

expressed by 60% of CD34+ cord blood cells (9).

Expression of lymphoid and myeloid associated antigens on umbilical cord blood CD34+ cells has also been documented. Saeland *et al.* found that in contrast to adult bone marrow in which 25% of the CD34+ cells express CD10 and 18% express CD19, these markers are rarely expressed in CD34+ cell population derived from cord blood (10).

The vast majority of umbilical cord blood CD34+ cells (90%) co-express Flt3 (CD135). It is the receptor for early acting cytokine Flt3 ligand (11).

Several cell adhesion molecules are present on umbilical cord blood CD34+ cells, as is observed in bone marrow (BM) CD34+ cells. CD44 and LAM-1 adhesion receptors involved in the homing of hematopoietic cells have been found to be strongly expressed on umbilical cord blood CD34+ cells (10).

Functional characterization of cord blood HSCs.

Different methods are used to assay function of HSCs, including: colony forming cell (CFC) assay, long term culture – initiating cells (LTC-IC) assay, and SCID repopulating cells (SRC) assay.

Published data indicate that there are about 13 to 2400 GM-CFC, 8000 BFU-E, and between 1 and 10000 CFU-GEMM per ml of cord blood. (12). CFC proportion is higher in cord blood compared to BM, and is even higher in cord blood samples from pre-term infants (13).

Pattergell *et al.* compared the results of LTC-IC in BM, cord blood, and mobilized peripheral blood. They showed that mononuclear cells (MNCs) of peripheral blood produce more CFCs than cord blood and BM (14). However, others found significantly more progenitor cells in LTC-IC assay and longer cell production in cord blood MNCs compared with BM ones (15).

Vormoor *et al.* has reported stable human hematopoiesis in SCID mice transplanted with human cord blood cells. It has been suggested that the human cells capable of repopulation in such animals represent more primitive properties than LTC-ICs (16). Studies show that the frequency of SCID repopulating cells (SRCs) in cord blood is three folds higher than BM and six folds higher than peripheral blood (17).

Immunological characterization of cord blood HSCs.

There is less graft versus host disease (GVHD) with cord blood compared to BM transplantation, and we can transplant cord bloods with more HLA mismatches (18). Therefore, cord blood may generate a lower immunological response compared to adult cell transplantation. T lymphocytes and NK cells are the most important cells in this response. Cord blood T cells have significantly less ability than adult T cell to produce IL-2 and express functional IL-2 receptor complexes. Moreover, the potential of cord blood cells to produce helper T cells derived cytokines (INFγ and IL-2) is lower, presumably due to the presence of immature, naive T cells in cord blood (19).

NK cells have been implicated in mediating the graft versus leukemia (GVL) effect. While cord blood generally manifests low NK activity compared to adult BM and blood, cord blood NK activity is readily augmented *in vitro* by cytokines such as IL-2 and IL-12, suggesting that cord blood NK cells should be as effective as adult BM or blood NK cells to mediate a GVL effect (20).

Cell cycle status of cord blood HSCs. Practically, all CD34+CD38- cells in cord blood are in G0/G1 status (21). These cells would present a useful target for retroviral transfection, as they respond to cytokine stimulation and rapidly enter S phase of the cycle. The proportion of primitive CD34+ in G0/G1 decrease from 98% to 55% after 48 hours of exposure to certain cytokines or unknown factors in cord blood plasma (22).

Important factors in cord blood expansion

1. Stromal cells:

Stroma-dependent culture systems have been developed to study long term hematopoiesis *in vitro*. Stromal cells are a source of growth factors and adhesion molecules to support stem cells (23). Cord blood mononuclear cells can not form long term colonies in culture without supportive environment (15). Some murine cell lines have been found to be useful for stroma dependent cultures of HSCs.

Kawada and colleagues (24) used a murine cell line for expansion of cord blood HSCs. They showed that direct adhesion of stromal cells to human progenitors significantly increased the number of CD34+CD38- cells. When cell lines were physically separated from human progenitor cells, they failed to get good results. It is suggested that stromal cells can support proliferation of HSCs by direct cell-cell interaction. It seems that stromal support improve expansion of cord blood hematopoietic stem cells (25).

2. Effect of serum on cord blood expansion:

Unknown factors present in cord blood plasma, probably capable of crossing the placenta, affect both by themselves and in the presence of growth factors, hematopoietic progenitor

cell proliferation and differentiation at all stages of hematopoietic development. Moreover, co-culturing the cord blood cells with mesenchymal cells may increase expansion and also CXCR-4 expression on *ex vivo* expanded cord blood cells (26).
In the absence of growth factors, CFU-GM expansion didn't occur in cord blood cell cultures supplemented with fetal calf serum or peripheral blood plasma (27). Broxmeyer *et al.* noted that cord blood plasma, but not fetal calf serum or peripheral blood plasma, increased both the size of secondary, replated CFU-GEMM and the number of times that CFU-GEMM could be replated to form colonies in culture containing SCF and erythropoietin (EPO) (28). As combining IL-1, IL-3, IL-6, IL-11, G-CSF and GM-CSF couldn't reproduce this effect, it is possible that the cord blood plasma effect may be attributable to a novel growth factor with synergy for SCF and EPO (28). IL-6, Flt3 ligand, and thrombopoietin (TPO) are the possible candidates (29).
Although experiments showed that expansion could be improved using serum, but to transfer expansion to the clinic, good manufacturing practice (GMP) standards are required. 235-fold expansion of cord blood CD34+ cells was obtained with a cocktail containing flt3 ligand, TPO, IL-6, and IL-11 at 5 weeks with serum-free medium [30]. Flt-3 ligand, SCF, and TPO are considered as early acting and indispensable cytokines. By adding IL-3 for these cytokines in serum free culture, the amplification of CD34+ cord blood cells was increased 20.9 fold as opposed to 9.3 fold without IL-3 after 7 days (31).
CD34+CD38- surface phenotype has been used to measure primitive cell numbers. One UK study demonstrated a lack of expression of CD38 on CD34+ cells in serum free cultures. This finding must be considered in *ex-vivo* cord blood expansion in serum free conditions, and CD34+CD38- phenotype should not be used to confirm the presence of primitive progenitor cells (32).

3. Purification:
Cells belonging to the stem cell compartment are rare, representing about 1 in 10,000 mononuclear cells in the adult bone marrow. Purifying the stem cell compartment becomes progressively easier as markers become available to select these cells positively (CD34+Thy-1+) or negatively (CD38-DR-). CD34+ selected cell fraction has been used most frequently as the starting cell population for the expansion of hematopoietic progenitor cells.

CD34+ selection is a time-consuming and expensive process, leading to approximately 50% loss of CD34+ cells (33). In the static culture, the increase in total number of cord blood mononuclear cells is less than CD34+ selected sample. However the CD34+ proportion increases significantly in the mononuclear cell culture and decreases in the CD34+ cell culture. This, in part, compensates the limited increase of cell number in the mononuclear cell culture. [34] In addition, cultures initiated with mononuclear cells have nearly the same performance as CD34+ cells in perfusion cultures (35).

4. Cytokines:
I) Stimulatory Cytokines
Hematopoietic cell proliferation and differentiation is regulated by stimulatory and inhibitory signals that are mediated by cytokines. Cytokines are secretory proteins produced by a variety of hematopoietic and non-hematopoietic cells. Each cytokine alone has modest effect on hematopoietic cell amplification, but they show additive or synergistic effect in combination with other growth factors (36, 37, 38, 39).
Moreover, the effect of cytokines in a culture system is not only a function of their concentration and synergistic interaction, but also other factors like culture conditions (40) and target cells. Cord blood progenitors and/or their progeny can produce cytokines including GM-CSF and IL-3, in culture, leading to autocrine or paracrine stimulation of cells (41).
Stem Cell Factor (SCF) is a potent hematopoietic growth factor [36] produced by bone marrow stromal cells and fibroblasts. It interacts with a variety of other growth factors to influence very early hematopoietic stem cells (36, 42). The c-kit acts as its receptor.
Flt3-Ligand (FL) provides a significant amplification of both committed and early progenitors (43). Flt3-L has been shown to act directly on quiescent cells causing them to enter the cycle (44). In addition, it has been demonstrated to induce the proliferation of CD34+CD38- bone marrow and cord blood cells that are non-responsive to other early acting cytokines (45, 46, 47).

Granulocyte-Colony Stimulating Factor (G-CSF) and **Granulocyte-Macrophage Colony Stimulating Factor (GM-CSF)** are produced by a variety of cell types including fibroblasts, endothelial cells, macrophages, and T lymphocytes**.** G-CSF can act on primitive as well as later hematopoietic progenitors, whereas the action of GM-CSF may be restricted to terminally differentiating cells (48).

Thrombopoietin (TPO) is an early acting cytokine (49). TPO stimulates and supports survival of primitive stem cells. It also regulates megakaryocyte proliferation and differentiation (49, 50, 51, 52). The C-mpl acts as its receptor.

Erythropoietin (EPO) secreted by renal tubular cells has limited proliferative effects; it modulates/stimulates survival and terminal maturation of erythroid progenitors. EPO also increases red blood cell production.

Interleukin-3 (IL-3) is a multilineage stimulator with direct megakaryocyte, mast cell/basophil, B cell, and eosinophil stimulatory activity. There is some evidence that IL-3 increases asymmetric division of the stem cells, leading to stem cell differentiation (53).

There is some evidence that **Interleukin-6 (IL-6)** dramatically stimulates expansion of human hematopoietic progenitor cells *in vitro* in the presence of SCF [54]. IL-6 is active on more immature hematopoietic progenitors (50). Its receptor has two chains: a ligand binding chain (IL-6R), and a signal transducing chain (gp130) (54).

Interleukin-11 (IL-11) is produced by bone marrow stromal cells and induces megakaryocyte colony forming and maturation.

Although different combinations of cytokines have been used, standard expansion protocols have not yet been established due to complexity of cytokine interactions.

Different studies have shown that maximal expansion of hematopoietic cells is generally achieved using one or more cytokines acting on primitive cells in combination with cytokines acting on less-primitive cells (53). In addition, the cytokines acting through separate signaling pathways show more synergism (i.e., IL-6 in combination with SCF) (54). Some of these combinations are presented in Table 1.

II) Inhibitory Cytokines

Macrophage inflammatory protein-1 alpha (MIP-1 alpha) is secreted by monocyte/ macrophage and T cells. It has been shown that MIP-1α prevents *in vivo* murine stem cells from entering cell cycle (62).

Table-1: Effects of cytokines on *ex vivo* expansion of human stem/progenitor cells (53).

Reference	Cells	Culture	Cytokines	Duration	Expansion time		
					TNC	CFC	LTC-IC
Piacibello (55)	CD34+	IMDM/ 10%FCS	FL+TPO	<30 weeks		28 millions	270000
Dening-Kendall (56)	Cd34+	IMDM/ 20%FCS	SCF+ IL-3+ IL-6+ GM-CSF+ G-CSF	14 days	2500	CFU-GM: 49 BFU-E: 1	2.5
Kogler (57)	Cd34+	Serum- Free	FL+ SCF+ IL-3	7 days	138	CFU-GM: 264 CFU-GEMM: 94 BFU-E: 126	6.7
Piacibello (50)	CD34+	IMDM/ 10%FCS	SCF+ IL-3 SCF+ FL	8 weeks 12-14weeks	12000 <20000	36 <300	
Ohmizono (38)	CD34+	Serum- Free	SCF+ IL-3	14 days		BFU-E: 50 CFU-GM: 30 CFU-GEMM: 30	
Traycoff (58)	CD34+	Serum- Free	SCF+ IL-3	5-7 days			Reduced
Ruggieri (59)	CD34+	McCoys/ 10%Cord Serum	SCF+ IL-3 +GM-CSF	7 days	64	11	
Moore (60)	CD34+	Not stated	SCF+ IL-3 +IL-1 + EPO	14 days	2800	600	18
Durand (61)	CD34+	Serum- Free	SCF+ IL-3	21 days	1000	5000	
Migliaccio (42)	CD34+ SBA-	Serum- Free	SCF+ IL-3 SCF+ G-CSF SCF+EPO	3 weeks		20 10 2-3	

IMDM: Iscov's modified Dulbecco's medium; FCS: fetal calf serum; FL: Flt3 ligand; TPO: thrombopoietin; SCF: stem cell factor; IL: interleukin; GM-CSF: granulocyte-macrophage colony-stimulating factor; G-CSF: granulocyte colony-stimulating factor; EPO: erythropoietin; TNC: total nucleated cells; CFC: colony forming cells; LTC-IC: long-term culture-initiating cells; CFU-GM: colony forming unit granulocyte-macrophage; BFU-E: burst-forming unit Erythroid; CFU-GEMM: colony forming unit granulocyte-erythroid-macrophage-megakaryocyte.

Some studies showed that MIP-1 α suppressed the growth of immature hematopoietic progenitors. Nevertheless, recent studies suggest that it has also a stimulatory effect on proliferation of more mature hematopoietic progenitors (63, 64, 65, 66). MIP-1α may have a role in maintaining the long-term culture initiating cells (LTC-IC) (67). There is also some evidence that MIP-1 α inhibits bone marrow granulocyte-macrophage-colony forming cells (GM-CFC), while stimulating GM-CFC from cord blood $CD34^+$ cells over the same dose range (68, 54, 93).

Transforming growth factor-beta (TGF-beta) is secreted by most cell types and affects most cell types. The major biological effect of TGF-beta on hematopoietic cell growth is the reversible inhibition of entry into the cell cycle (69). Many studies have shown the inhibitory activities of TGF-beta on hematopoiesis (70, 73), but recent evidence supports that TGF-beta can have both inhibitory and stimulatory effects, depending on the differentiation state of the target cell and other cytokines interacting with the cell (71, 74). There is also some evidence that the inhibitory effect of FCS and human serum on progenitor cell proliferation is caused by TGF-beta (71, 72).

Leukemia inhibitory factor (LIF) is a glycoprotein affecting a wide spectrum of cells (75). LIF prevents differentiation commitment of normal embryonic stem cells (75, 76). In human, it stimulates IL-3-dependent growth of primitive HSCs (77, 78). LIF induces *in vitro* proliferation of primitive HSCs and appears to be required for the survival of HSCs *in vivo* (79).

Since using a combination of just stimulatory cytokines leads to production of more mature cells rather than primitive stem cells, it seems that an ideal combination of cytokines should include both stimulatory and inhibitory cytokines.

Bioreactor systems

Recently, alternative culture techniques such as bioreactor systems have been developed to maintain growth factors and other required elements such as oxygen as well as waste products in constant and characteristic concentrations. This stirred culture system is particularly required when large-scale expansion culture is planned, especially for clinical purposes. By providing a closed system, this method protects the culture medium from infectious agents, which always threaten the conventional cultures especially during refeeding and other manipulations. Some studies have reported significant improvement in cord blood expansion in bioreactor perfusion culture system, compared with static cultures (80, 81, 82).

Cord blood cryopreservation and expansion

The use of cord blood for transplantation would be much facilitated by banking of cord blood samples. Since cryopreservation remains the method of choice for long-term preservation of progenitor/stem cells, cord blood cryopreservation has become an important issue in banking and transplantation. It seems that the most suitable cryopreservation techniques used for cord blood samples are almost similar to those of bone marrow or peripheral blood progenitor/ stem cell cryopreservation (83, 84).

Although several studies have shown that cryopreservation does not significantly reduce expansion potential (85), colonogenicity, and immunophenotypic properties of cord blood progenitor/ stem cells, most of these studies have focused on quantitative measurements and they have not assessed the quality of recovered progenitor/ stem cells after cryopreservation (86).

Rice *et al.* found that cryopreservation of *ex-vivo* expanded cord blood cells doesn't deteriorate engraftment measures in NOD-SCID mouse model (87). This study raises the question whether expansion of cord blood cells should be done prior to or after cryopreservation to obtain the best clinical results.

Clinical studies of transplantation by ex-vivo expanded cord blood cells

Animal models demonstrate that engraftment of *ex vivo* expanded cord blood cells is possible, but it is delayed (88). Recent data demonstrated that *ex vivo* expanded cord blood cells may be useful in transplantation in adults. These expanded cells resulted in faster neutrophil engraftment (89, 90, 91, 92). Theoretically, transplantation of *ex vivo* expanded cord blood cells at the same time of non-expanded cord blood cells from the same donor could increase the number of immediately available progenitor cells responsible for short term engraftment.

Kogler *et al.* showed that expansion of 1/8 of sibling cord blood in the presence of G-CSF, TPO, and flt3-L and then simultaneous transplantation of a high risk leukemic patient using both expanded and non-expanded cells resulted in rapid and durable neutrophil engraftment (91). Pecora *et al.* evaluated the effect of supplementing unrelated umbilical cord blood with *ex vivo* expanded umbilical cord blood cells from the same donor in two

older adult patients with high risk CML and no alternative donor (92). They used a clinical grade perfusion system (Aastrom Replicell) and observed that *ex vivo* expanded cells facilitate hematopoietic recovery.

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