Expansion of Non-Enriched Cord Blood Stem/Progenitor Cells CD34+ CD38- Using Liver Cells

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ABSTRACT

Many investigators have used xenogeneic, especially murine stromal cells and fetal calf serum to maintain and expand human stem cells. The proliferation and expansion of human hematopoietic stem cells in *ex vivo* culture were examined with the goal of generating a suitable protocol for expanding hematopoietic stem cells for patient transplantation. Using primary fetal liver cells, we established a serum-free culture system to expand human primitive stem/progenitors cells. Non-enriched cord blood CD34+ cells were cultured on a monolayer of mouse primary fetal liver cells in the presence of trombopoietin, flt3/flk2 ligand, and/or stem cell factor, IL-6 and IL-3 under serum-free conditions. After 1 or 2 weeks of culture, cells were examined for clonogenic progenitors and percentage of CD34+ CD38- cells. In the presence of trombopoietin, flt3/flk2 ligand, and stem cell factor, fetal liver cells supported expansion of CD34+ cells more than 10 to 20 fold. In addition, colony forming unit-cell assay was expanded more than 5- and 10-fold after 1 and 2 weeks of culture, respectively. These results strongly suggest that fetal liver cells may be a suitable feeder layer for expansion of hematopoietic progenitors from umbilical cord blood *in vitro*. *Iran. Biomed. J. 9 (3): 111-116, 2005*

INTRODUCTION

Cord blood cell is an attractive source of hematopoietic stem cells for allogeneic transplantation in children and adults with malignant and non-malignant diseases [1, 2]. Compared to bone marrow transplant results, previous studies have shown a delay in kinetics of neutrophil and platelet engraftment after cord blood transplant [3]. This delay shows at least partially, a 100-day increase in mortality. This can be explained by the lower number of nucleated cells (median 2×10^7 /kg) and CD34+ cells (median 2×10^5 /kg) infused compared to a bone marrow transplant (median 2×10^8 /kg nucleated cells and 2×10^6 /kg CD34+ cells) [4]. The lower number of cells infused in a cord blood unit is partially compensated by the intrinsic properties of cord blood cells that are enriched in immature progenitors.

It has been shown that early engraftment is delayed but long-term engraftment measured by the number of long-term culture initiating cells in the bone marrow one year after transplant is superior after cord blood transplant compared to bone marrow transplant [5, 6]. This result shows that long-term engraftment is not impaired and that means of accelerating short-term engraftment might decrease early mortality after cord blood transplant. Eurocord and other studies have shown that the speed of engraftment was correlated with the number of nucleated cells and CD34+ cells infused; the dose recommended is above the median. The number and type of HLA disparities is also closely related to engraftment of neutrophils and platelets, explaining why it is not recommended to use a cord blood unit with more than two HLA mismatches [7, 8].

By *ex vivo* expansion with cytokine cocktails,

Several studies have shown that it was possible to expand progenitors *in vitro* and in NOD/SCID mice models [9-11]. Despite these encouraging results, few clinical studies have been published because of the limitation of access to clinical-grade cytokines and the difficulty of interpretation of the results.

In co-culture systems for human stem cell expansion, murine stromal cell lines have been used by a number of investigators [12-15]. In addition to murine stromal cells, a recent report demonstrated marked supportive effects of porcine microvascular endothelial cells [16]. In the present study, we demonstrate that under serum-free conditions, mouse fetal liver cells supported marked expansions of CD34$^+$ cells, CD34$^+$CD38$^-$ cells, colony-forming unit cell (CFU-C) in culture, and LTC-IC in synergy with trombopoietin (TPO), flt3/flk2 ligand (FL), and stem cell factor (SCF).

MATERIALS AND METHODS

UCB samples. After obtaining consent from patients (n = 11) scheduled to undergo Cesarean section, UCB was collected). On average, 60 ml of UCB was collected by gravity into a sterile container after cutting the distal end of the cord. Heparin (1000 U) was added to prevent coagulation. White blood cell counts were measured with hemocytometer using trypan blue. The sample was diluted with an equal volume of HBSS (Sigma, St. Louis, MO), and layered onto Ficoll-Hypaque (Pharmacia-Amersham, Piscataway, NJ; d 1.077 g/ml) density gradients to deplete red blood cell (RBC). The mononuclear cell (MNC) interface was collected, diluted into three volumes of HBSS, and pelleted at 250 ×g for 10 minutes. The cell pellet was washed two more times and resuspended in either 5 ml of expansion media. When necessary, RBC-depleted UCB cells were stored at -70°C. First cells were suspended in HBSS at a concentration of 2×10^8 cells/ml, and then an equal volume of penta-starch cryopreservative medium [13] containing 10% dimethyl sulfoxide (DMSO)/8% HSA/12% penta-starch in normosol R was added.

Preparation of fetal liver cells from mouse embryo. The liver of 13-15-day mouse embryo (BALB/c, Razi Vaccine and Serum Research Institute, Karaj, Iran) was cut into pieces with scalpel in plates containing DMEM and 10% FCS. The pieces were flushed several times by a 19 G-syringe into 15 ml tubes. Cells were washed three times by HBSS and cultured in DMEM with 2% fetal bovine serum (FBS) for 24 h in 5% CO$_2$ and

37°C. After incubation for 24 h, the liver parenchymal cells were adhered to plate and other cells were suspended in culture medium. Medium exchanged with DMEM containing 20% FBS (Fig. 1A).
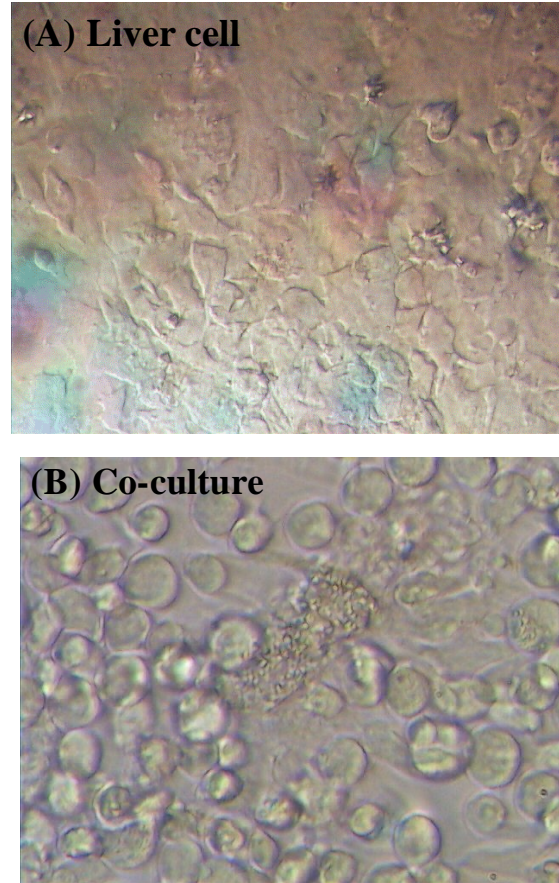

Fig. 1. (**A**), Phase microscopy of fetal liver cells; (**B**), Appearance of proliferating cells in the co-culture of human cord blood CD34$^+$ cells on a monolayer of fetal liver cells in the presence of TPO, SCF, IL-3, IL-6 and FL.

Cytokines. Recombinant human flt3 and recombinant human TPO and recombinant human SCF (also known as mast cell factor or c-kit ligand), recombinant human IL-6 and IL-3 were used in this study. All these cytokines were purchased from Sigma (USA).

Co-culture of hematopoietic cells and primary fetal liver cells. Liver cells (2×10^3) were plated in 25-cm^2 flasks, in 5 ml of DMEM with 10% FBS supplemented with antibiotics and left for one week at 37°C in a humidified atmosphere of 5% CO$_2$ and 95% air. On the day of co-culture, the liver cells were washed with PBS and re-cultured in the serum-free Stemspan medium (Stem cell Company, Canada) and then gamma irradiated with a ^{137}Cs

source at a dose of 15 Gy. Irradiated feeder cells were trypsinized and 10^5 cells were transferred into a 24-well microplate. The next day, 5×10^5 cord blood MNC were cultured on monolayer pre-established in a 24-well microplate using 1 ml of Stemspan medium supplemented with combinations of cytokines included TPO (50 ng/ml), SCF (10 ng/ml), FL (100 ng/ml), IL-6 (30 ng/ml) and IL-3 (50 ng/ml) at 37°C in a humidified atmosphere of 5% CO_2/95% air for 2 weeks. Culture medium was replaced after 1 week with fresh medium containing the same concentration of cytokines.

Clonal cell culture. Methylcellulose clonal culture was performed in 35-mm suspension culture dishes. The culture medium consisted of IMDM, 1% 4,000-centipoise methylcellulose (Sigma, USA), 30% FCS, 1% BSA; (Sigma, USA), 10 ng/ml IL-3, 10 ng/ml SCF, 10 ng/ml granulocyte-colony stimulating factor, and 2 U/ml erythropoietin. After 14 days of incubation at 37°C in a humidified atmosphere of 5% CO_2 in air, the colonies were scored with an inverted microscope. Densely packed colonies that reached >1 mm in size were scored as high-proliferative potential colonies (HPP-CFC) [17].

Flow cytometric analysis. Aliquots of cells were stained with FITC- and PE-conjugated monoclonal antibodies in PBS/0.1% BSA at 4°C for 30 minutes. The analysis was performed using an EPICS XL flow cytometer (Coulter, Tokyo, Japan). Antibodies used were as follows: FITC-conjugated CD34 antibody and PE-conjugated CD38 (DAKO, Denmark). FITC- and PE-conjugated mouse IgG1 antibodies (DAKO, Denmark) were used as isotype-matched controls. Dead cells were gated out with a forward vs. side scatter window and propidium iodide staining.

Statistical analysis. Student's *t*-test was used to calculate statistical differences.

RESULTS

Synergistic effects of fetal liver cells and early-acting cytokines on ex vivo expansion of human CB progenitors. MNC (5×10^5) from cord blood were plated on a liver cell layer under serum-free conditions with or without combinations of TPO, SCF, IL-3, IL-6 and FL. Non-adhering cells and cells adhering weakly to liver cells were collected by gentle pipetting after 2 weeks of culture for analysis. Without cytokines, the mean number of

total nucleated cells, $CD34^+$ cells, and $CD34^+$ $CD38^-$ cells after 2 weeks of culture was 2-5 times the initial input number. In contrast, in the presence of TPO not only total nucleated cells, $CD34^+$ cells, and $CD34^+CD38^-$ cells, but also CFU-C, CFU-Mix and HPP-CFC were significantly expanded. The addition of SCF and/or FL to TPO further enhanced the expansion of nucleated cells and progenitors. Representative photomicrographs of liver cells and growing hematopoietic cells in culture supplemented with TPO, SCF, IL-3, IL-6 and FL are shown in Figure 1. Although there were some differences among experiments as to the degree of expansion, the maximum output of progenitors was consistently observed when stimulated with TPO, SCF, IL-3, IL-6 and FL. The results of four experiments are shown in Figure 2.
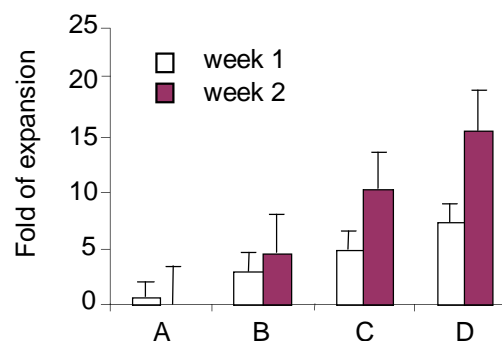

Fig. 2. Expansion of human cord blood progenitors. **(A)**, Control (without feeder and cytokines); **(B)**, Feeder; **(C)**, SCF, TPO, flt3, IL-3, IL-6; **(D)**, Feeder + cytokines. Data represent mean ± SD of the fold increase compared with the initial value in four experiments performed on four separate cord blood donors.

Dependency of progenitor expansion on liver cells. We examined whether liver cells are required for progenitor expansion. MNC were cultured with TPO, SCF, IL-3, IL-6 and FL in the presence or absence of liver cells for 2 weeks and the growing cells were collected by gentle pipetting for analysis. The cellular proliferation was 2-fold and the expansion of $CD34^+$ cells was 10-fold in the presence of fetal liver cells. Furthermore, $CD34^+CD38^-$ cells were only scarcely recovered without liver cells, indicating important roles for liver cells in progenitor expansion. The results are presented in Figure 3A. To assess whether cell-cell contact between liver cell and progenitors are important, we examined the effects of separation of liver cells and MNC by a 0.45-μm microporous filter. The filter separation suppressed not only total cellular proliferation, but also the expansion of
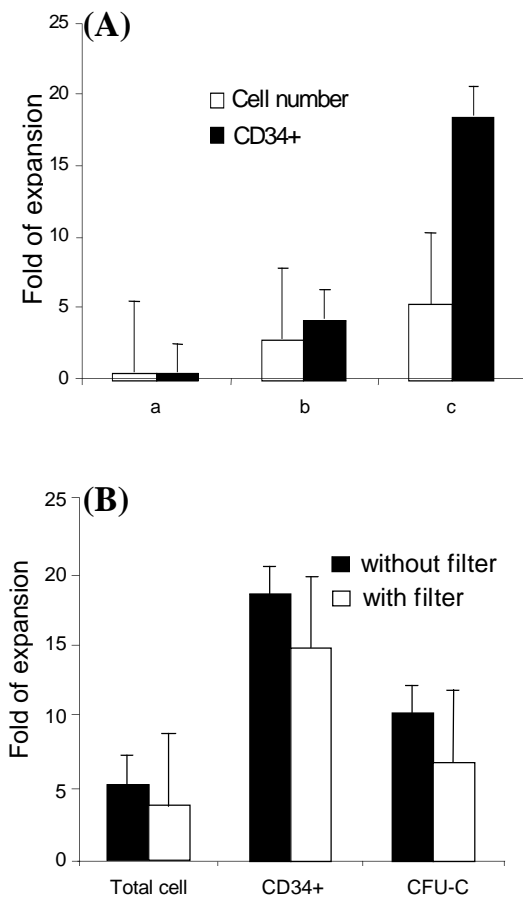
CD34^{+} cells, CD34^{+}CD38^{-} cells, and CFU-C (Fig. 3B). These results suggested that diffusible soluble factor(s) generated by liver cells were not enough for the maximum expansion of progenitors.

Representative data of flowcytometric analysis of the cells at the start of culture and after 2 weeks of expansion culture are shown in Figure 4.

DISCUSSION

Preparation of liver cells as monolayer co-culture helps the expansion of primitive cord blood stem cells. Our results suggest that the insertion of a micropore filter between primary liver cells and progenitors suppressed the expansion of progenitors and diffusible soluble factor(s) generated by liver cells were not enough for the maximum expansion of progenitors. One possibility is that membrane-bound cytokines or those sequestrated in the extracellular matrix of liver cells are responsible. For better expansion, cytokines secreted by liver cells only upon contact with progenitor cells may have been essential. Koibuchi *et al.* [18] investigated the role of HGF in blood formation and demonstrated that HGF is necessary for primitive hematopoiesis by regulating the expression of stem cell leukemia. Another possibility is that cell-cell contact of stromal cells and progenitors was essential. Multiple signaling pathways known to affect stem cell-fate decisions have been implicated in guiding hematopoietic development, suggesting the potential value of exploiting these pathways in enhancing *ex vivo* expansion [19, 20]. Brandt *et al.* [16] have demonstrated the safety and effectiveness of rescuing myeloablated non-human primates with expanded grafts derived from CD34^{+} marrow cells.

Fig. 3. Effects of fetal liver cells on progenitor expansion. (**A**) a, Control (without feeder and cytokines), b, SCF, TPO, flt3, IL-3 and IL-6, c, Fetal liver feeder + Cytokines. 5×10^5 human cord blood mononuclear cells were cultured with TPO, SCF, FL, IL-3 and IL-6 in the presence or the absence of liver cells (one culture for each group). Data represent mean ± SD of fold increase compared with the cells at the start in three experiments performed on three separate cord blood samples. (**B**) Effect of separation of fetal liver cells and Cord blood mononuclear cells by filter.
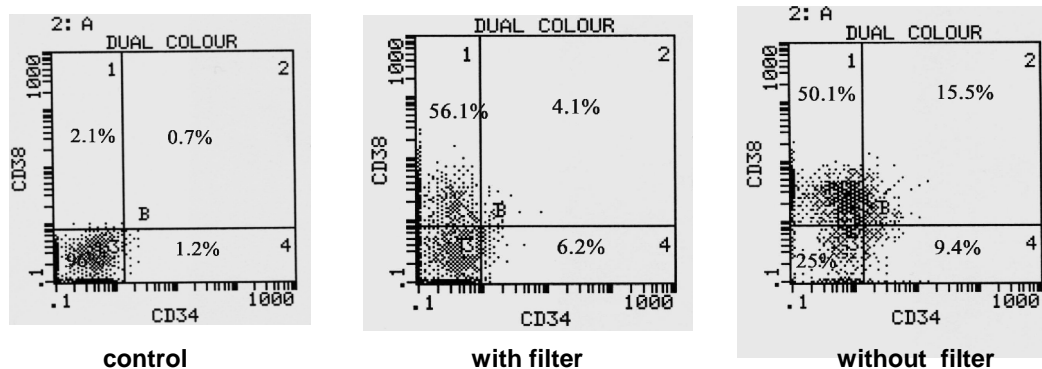

Fig. 4. Flow cytometric profile of expanded cells. Expanded cells, non-adherent hematopoietic cells, and cells attached weakly to liver cells were collected after 2 weeks of culture with fetal liver cells, TPO, SCF, FL , IL-3 and IL-6 for flow cytometric analysis (n = 6).

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These studies show the ability of micro vascular endothelial cells to maintain the marrow engraftment capacity of marrow-derived stem cells during cycling of the cells [16]. Various strategies have been evaluated for their ability to overcome cell dose limitations and reducing the time to engraftment. These strategies are: simultaneous transfusion of two UCB units from different donors [21], *ex vivo* expansion of cord blood stem cells [22, 23] as well as *in vivo* stimulation of UCB stem cells using growth factors or agents that up-regulate the expression of intercellular adhesion molecules and vascular cell adhesion molecule-1 [24]. These methods are still under investigation.

The main obstacle to UCB transplantation in adult recipients is the insufficiency of hematopoietic progenitors. Hence, in this investigation we explored fetal liver cells as the feeder layer for UCB CD34$^+$ cells and found that it could, in synergy with extra cytokines, dramatically expand CD34$^+$ cells by 18.6 ± 3.9 fold, CFU-C by 10.3 ± 2.1 fold, and LTC-IC by 7.43 ± 2.66 fold over a 2-week period. Detection and characterization of the molecule(s) responsible for the interaction may further our understanding of the regulatory mechanisms in the self-renewal of human stem cells and provide a novel strategy for *ex vivo* expansion of these cells.

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