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A Co-culture System for Expansion of Nonenriched Cord Blood Stem/Progenitor 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 (HSC) in *ex vivo* culture was examined with the goal of generating a suitable protocol for expanding HSC 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 more than a 10- to 20-fold expansion of $CD34^+$ cells. In addition, CFU-C assay were 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*.

Key words: *Ex vivo* expansion, stem cells, umbilical cord blood, fetal liver cells

INTRODUCTION

Cord blood is an attractive source of hematopoietic stem cells for allogeneic transplantation in children and adults with malignant and nonmalignant diseases[1,2]. Compared to bone marrow transplant results, previous studies have shown a delay of the kinetics of neutrophil and platelet engraftment after cord blood transplant[3]. This delay explains, at least partially, increased 100 days mortality. It can be explained by the lower number of nucleated cells (median 2×10^7 kg^{-1}) and $CD34^+$ cells (median 2×10^5 kg^{-1}) infused compared to a bone marrow transplant (median 2×10^8 kg^{-1} nucleated cells and 2×10^6 kg^{-1} $CD34^+$ 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]. These 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 using *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.

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In coculture 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 units in culture (CFU-C) and LTC-IC in synergy with TPO, FL and SCF.

MATERIALS AND METHODS

Umbilical Cord Blood (UCB) samples: UCB was collected after obtaining consent from patients (n=11) scheduled to undergo Cesarean section. 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 (WBC) counts were measured with hemocytometer using trypan blue. The sample was diluted with an equal volume of Hank's Balanced Salt Solution (HBSS) (Sigma, St. Louis, MO) and layered onto Ficoll-Hypaque (Pharmacia-Amersham, Piscataway, NJ; d 1.077 g mL^{-1}) density gradients to deplete Red Blood Cells (RBC). The mononuclear cell interface was collected, diluted into three volumes of HBSS and pelleted at 250×g for 10 min. 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 suspending the cells in HBSS at a concentration of 2×10^8 cells mL^{-1} and then adding an equal volume of pentastarch cryopreservative medium[13] containing 10% dimethyl sulfoxide (DMSO)/8% HSA/12% pentastarch in normosol R.

Preparation of fetal liver cells from mouse embryo: The liver of 13-15 day mouse embryo (Balb/c, Razi Institute, Karaj, Iran) is cut into pieces with scalpel in plates containing DMEM and 10% FCS. The cut pieces were flashed several times by a 19G syringe into 15 mL tubes. Cells washed three times by HBSS and cultured in DMEM with 2% FBS for 24 h in 5% CO_2 and 37°C. After a 24 h incubation the liver parenchymal cells were adhered to plate and other cells were suspended in culture medium. Medium exchanged with DMEM contain 20% FBS (Fig. 1A)

Cytokines: Recombinant human Flt-3 ligand (FL) and recombinant human thrombopoietin (TPO) and recombinant human stem cell factor (SCF, also known as mast cell factor [MGF] or c-kit ligand [KL]), recombinant human interleukin-6 and interleukin-3 were used in this study. All these cytokins were purchased from Sigma company, USA.

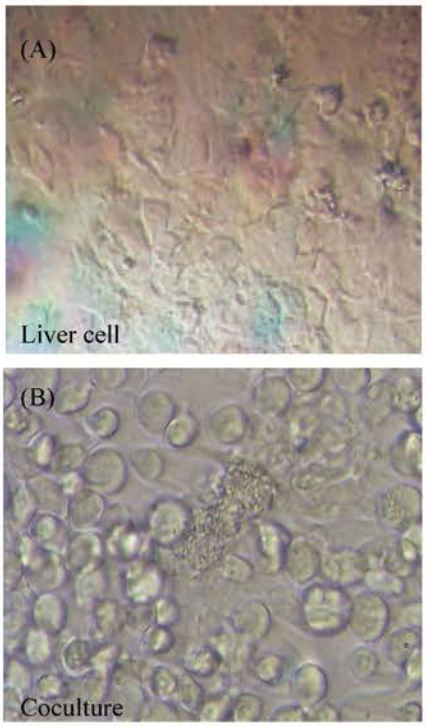

Fig. 1A: Phase microscopy of fetal liver cells,
B: Appearance of proliferating cells in the coculture of human cord blood $CD34^+$ cells on a monolayer of fetal liver cells in the presence of TPO, SCF, IL-3, IL-6 and FL

Coculture 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 antibiotics and left for one week at 37°C in a humidified atmosphere of 5% CO_2/95% air. On the day of coculture, the liver cells were washed with PBS and recultured in the serum-free Stemspan medium (Stemcell Company, Canada) and then gamma irradiated with a ^{137}Cs at a dosage of 1,500 cGy. Irradiated feeder cell trypsinated and 10^5 cells wee transfer to 24-well microplate (nunc). The next day $5x10^5$ cord blood mononuclear cells were cultured on monolayer preestablished in 24-well microplate using 1 mL of Stemspan medium supplemented with combinations of cytokines included TPO (50 ng mL^{-1}), SCF (10 ng mL^{-1}) and FL (100 ng mL^{-1}), IL-6 (30 ng mL^{-1}) and IL-3 (50 ng mL^{-1}) at 37°C in a humidified atmosphere of 5% CO_2/95% air for 2 weeks. Culture medium was replaced after 1 week of culture 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, 1.0% 4,000-centipoise methylcellulose (Sigma, USA), 30% FCS, 1%

BSA; (Sigma, USA), 10 ng mL^{-1} IL-3, 10 ng mL^{-1} SCF, 10 ng mL^{-1} G-CSF and 2 U mL^{-1} 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 fluorescein isothiocyanate (FITC) and phycoerythrin (PE)-conjugated monoclonal antibodies in PBS/0.1% BSA at 4°C for 30 min. Analysis was performed by 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: 5x10^5 mononuclear cells (MNCs) 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. Cells not adhering and 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 Fig. 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 Fig. 2.

Dependency of progenitor expansion on liver cells: We next examined whether liver cells are required for progenitor expansion. Mononuclear cells were cultured with TPO, SCF, IL-3, IL-6 and FL in the presence or the absence of liver cells for 2 weeks and 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 Fig. 3A. To assess whether cell – cell contact between liver cell and progenitors are important, we examined the effects of separation of liver cells and mononuclear cells 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.

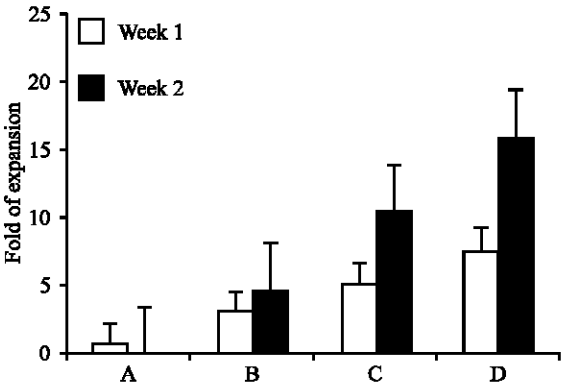

Fig. 2: Expansion of human cord blood progenitors, A: Control (without feeder and cytokines), B: Feeder, C: SCF, TPO, Flt-3, IL-3, IL-6, D: Feeder+cytokines. Data represent mean ± SD of the fold increase compared with the initial value in four experiments performed on four separate cord blood donors

Representative data of Flow cytometric analysis of the cells at the start of culture and after 2 weeks of expansion culture are shown in Fig. 4.

DISCUSSION

Preparation of liver cell as monolayer coculture help the expansion of primitive cord blood stem cells. Present result suggested 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

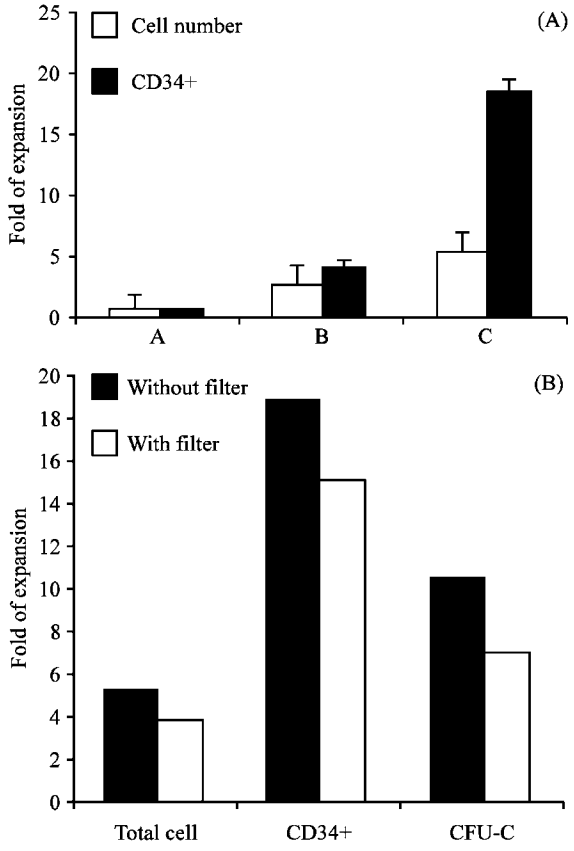

Fig. 3: Effects of fetal liver cells on progenitor expansion. (3A) A: Control (without feeder and cytokines), B: SCF, TPO, Flt-3, IL-3 and IL-6, C: Fetal liver feeder + Cytokines. $5x10^5$ human cord blood mononuclear cells were cultured with TPO, SCF, FL, IL-3 and IL-6 in the presence or the absence 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. (3B) Effect of separation of fetal liver cells and Cord blood mononuclear cells by filter

extracellular matrix of liver cells were responsible. Cytokines secreted by liver cells only upon contact with progenitor cells may have been essential. Koibuchi *et al.*[18] investigated the role of Hepatocyte Growth Factor (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 nonhuman primates with expanded grafts derived from $CD34^+$ marrow cells. These studies show the ability of microvascular endothelial cells to maintain the marrow engraftment capacity of marrow-derived stem cells during cycling of the cells. Various strategies have been evaluated for their ability to overcome cell dose limitations and to reduce the time to engraftment, including simultaneous transfusion of two UCB units from different donors[21,22] *ex vivo* expansion of cord blood stem cells[23-25] as well as *in vivo* stimulation of UCB stem cells using growth factors or agents that up-regulate the expression of intercellular adhesion molecules (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1)[26]. 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 of 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

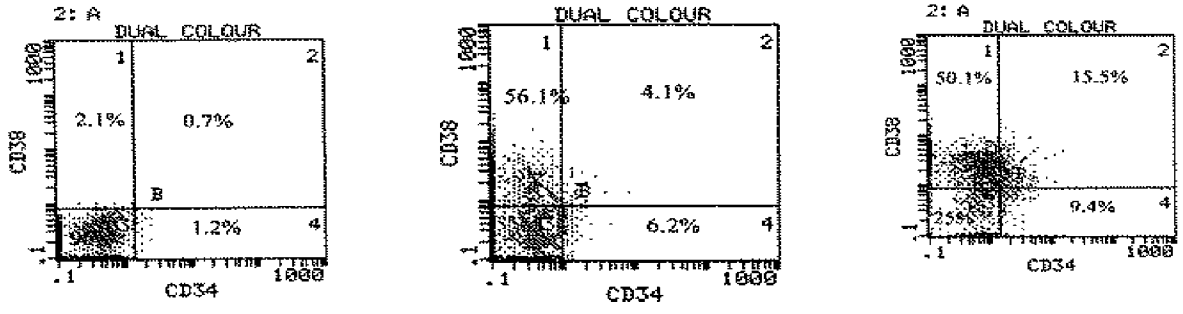

Fig. 4: Flow cytometric profile of expanded cells. Expanded cells, nonadherent hematopoietic cells and cells attached weakly to liver cells were collected after 2 weeks of culture with fetal liver cells, TPO, SCF, FL , IL-3 and IL-6 for flow cytometric analysis (n = 6)

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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