Apoptosis, DAP-Kinase1 Expression and the Influences of Cytokine Milieu and Mesenchymal Stromal Cells on Ex Vivo Expansion of Umbilical Cord Blood-Derived Hematopoietic Stem Cells

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Abstract Expansion of umbilical cord blood-derived CD34⁺ cells can potentially provide them in numbers sufficient for clinical applications in adult humans. In this study apoptosis rate of expanded cells, mRNA expression and promoter methylation status of DAPK1 were evaluated during cord blood hematopoietic stem cell (CB-HSC) ex vivo expansion using cytokines and a co-culture system with mesenchymal stromal cells (MSCs). Ex vivo cultures of CB-HSCs were performed in three culture conditions for 14 days: cytokines with MSCs feeder layer, cytokines without MSCs feeder layer and co-culture with MSCs feeder layer without cytokine. Total number of cells, CD34⁺ cells and colony forming unit assay were performed during expansion. Flow cytometric analysis by propidium iodide was performed to detection of apoptosis rate in expanded cells. Methylation status of the DAPK1 gene promoter was analyzed using methylation specific PCR, and DAPK1 mRNA expression was evaluated by real time-PCR. Maximum CB-CD34⁺ cells expansion was observed in day 10 of expansion. The highest apoptosis rate was observed in cytokine culture without feeder layer that showed significant difference with co-culture condition. The data showed that ex vivo expansion of CD34⁺ cells in all three culture conditions after 10 days resulted in, significant increased expression of DAPK1, also a significant difference between co-culture without cytokine and two other cytokine culture was observed ($p < 0.01$). DAPK1 gene promoter of expanded

CD34⁺ cells at days 5, 10 and 14 of culture remained in unmethylated form similar to fresh CD34⁺ cells. Expression of DAPK1 in hematopoietic cells was increased during 10 days expansion of CD34⁺ cells. Also no methylation of DAPK1 promoter was observed; otherwise it would be capable of initiating some leukemic cell progression or disruption in hematopoietic regeneration.

Keywords Hematopoietic stem cells · Cord blood expansion · Mesenchymal stem cells · Apoptosis

Introduction

Umbilical cord blood (UCB) has been used as a source of hematopoietic stem cells (HSCs) for complete or partial human leukocyte antigen-matched allogeneic transplantation in treatment of hematologic diseases. Cryopreserved UCB has been used to reduce the risk of GVHD and treatment-related mortality (TRM) and to improve survival [1, 2]. However, there are approximately 10^7 CD34⁺ cells in one unit of cord blood, whereas for a successful transplantation, $2.5 \times 10^5/\text{kg}$ CD34⁺ cells are required [3]. Ex vivo expansion of UCB-HSCs is the best way to increase the number of CD34⁺ cells and improve the kinetics of UCB-HSCs engraftment [1, 2]. But HSCs differentiation to precursor cells during ex vivo expansion leads to decreased numbers of primitive HSCs [4, 5]. Life, death, self renewal and differentiation of HSCs are the major processes that regulate the numbers and lifespan of the HSCs pool and defects in these processes can contribute to haemopoietic insufficiencies and development of haemopoietic malignancies [6]. In the normal hematopoiesis, a balance between cell loss (apoptosis and differentiation) and cell gain (proliferation and mitosis) can determine HSC population size [7].

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Several factors have been identified as potential mechanisms underlying the self renewal and proliferation of HSC. Some external factors are including GM-CSF, Epo, Flt3, and Notch, also tumor suppressors like p21 influence stem cell division, regulating self renewal [7]. DAP-kinase is a Ca_2^+ calmodulin-regulated serine/threonine kinase that participates in apoptotic systems initiated by interferon- γ , TNF- α and activated Fas [8]. DAPk is a novel protein family involved in apoptotic and autophagic cell death, tumor suppression and metastasis [9]. Moreover, DAP-kinase1 was found, by several criteria to be involved in the multi-step process of tumor genesis [10]. The methylation status Analysis of Dapk DNA extracted from tumor samples, showed high incidence of hyper methylation in the several human carcinomas and B cell malignancies [8]. But, there were no studies about epigenetic changes that would probably occur during culture and in vitro expansion of hematopoietic stem cells with cytokines. Therefore, in this study we analyzed the expression and methylation status of DAPK1 during ex vivo cord blood hematopoietic stem cell expansion using cytokines (FLT3L, SF, and TPO). On the other hand, mesenchymal stromal cells (MSCs) which form a major part of the bone marrow microenvironment playing an important role in hematopoiesis regulation; homing and HSCs maintenance in quiescence phase [11] were used as the feeder layer. So DAPK1 gene evaluation and apoptosis rate of expanded cells was analyzed in co-culture and cytokine condition and our results from expanded CD34^+ cells in co-culture system without cytokine were compared with those from cytokine cultures.

Materials and Methods

Isolation of CD34^+ Cells

HSCs were collected from fresh human UCB ($n = 3$) after written informed consent from normal full-term pregnant women according to guidelines approved by the Ethics Committee at the Iranian Blood Transfusion Organization.

Due to high red blood cells in cord blood, the RBCs were precipitated using hydroxyl ethyl starch (Stem cell technologies—Ottawa, Canada). Mononuclear cells were isolated by density gradient centrifugation on Ficoll-Hypaque (Bio sciences, Sweden). The MNCs incubated with anti-human CD34 conjugated with Micro Beads (Miltenyi Biotec, USA) for 30 min at 4 °C. CD34 cells were isolated using an affinity column.

Feeder Layer Cultivation

MSCs were isolated from fresh bone marrow after informed consent was received from one healthy donor

(Taleghani hospital, Tehran). Briefly, MNCs were collected from a 10 mL bone marrow aspirate by density gradient centrifugation on Ficoll-Hypaque (Bio sciences, Sweden) and were washed once in MSCs culture medium: low glucose DMEM (Dulbecco's Modified Eagle Medium) supplemented with penicillin and streptomycin. The MNC were cultured in a 75 cm^2 culture flask at 37 °C. After 2–3 days, non-adherent cells (hematopoietic cells) were removed and adherent cells (MSCs) were left in the culture medium until the cells achieved 80 % confluences. MSCs were characterized by flow cytometric analysis using monoclonal antibodies against CD105, CD44, CD166, CD90, CD34, and CD45 (Dako, Denmark). Differentiation potential of MSCs was investigated by Osteogenic differentiation kit (Chemicon, USA). To be used as a cell feeder layer, MSCs were harvested with 0.25 % trypsin–EDTA solution (Stem cell technology, Denmark), 1×10^4 cells were plated in 6-well plates. When the cells reached more than 90 % confluence in DMEM, they were washed once with PBS and were replaced by serum free medium (stem span) to co-culture with HSCs. The remaining cells saved at -70 °C for later use.

MSCs Osteogenic Differentiation Assay

Osteogenic differentiation was performed on bone marrow derived MSC. For this purpose, 5×10^4 cells were seeded in collagen/Vitro nectin coated 24-well plates. After 100 % confluences DMEM enriched with FBS (Fetal Bovin Serum) were replaced with differentiating medium containing 100 μM L-ascorbic acid-2-phosphate and 1 m β -glycerophosphate with 1 mM dexamethasone (Chemicon, USA). On day 14, cells were fixed in 70 % ethanol for 1 h at 4 °C and stained for 15 min with alizarin red-S (Sigma) at room temperature (RT). Alkaline phosphatase staining was performed with alkaline phosphatase kit (Sigma-Aldrich). Differentiated cells in the 24-well plate were fixed with acetone and Fast Blue RR salt in Naphthol AS-MX Alkaline solution was use for staining. Mayer's hematoxylin was used as counterstaining.

Ex Vivo Expansion of CD34^+ Enriched Cells

1×10^5 CD34^+ enriched cells ($n = 3$) were cultured in six well plates (Nunc, Denmark) for 14 days (Duplicate, $n = 6$) in the serum-free medium (Stem cell technology, Denmark) at 37 °C under 5 % CO_2 humidified air in the three culture conditions: cytokines culture supplemented with SCF (Stem cell factor); 50 ng/mL, TPO (Thrombopoetin); 50 ng/mL and FLT3L; 40 ng/mL (Stem cell technology, Denmark), co-culture with MSCs feeder layer and above mentioned cytokines and co-culture with MSCs without any cytokine.

Colony-Forming Cell Assays

Fresh CD34⁺ cells and expanded cells at day 10 of culture ($1\text{--}2 \times 10^3$) were seeded in the semisolid culture (MethoCult GF H4434, StemCell Technologies) following the manufacturer's instruction. Methylcellulose-based media were aliquoted in 35-mm² Petri dishes and incubated at 37 °C, 5 % CO₂ and humidified incubator. After 14 days of culture, the number of colony was counted under the inverted microscope.

Proliferative and Phenotypic Analysis

Cells viability was determined at days 0, 5, 10, and 14 of culture using trypan blue staining (StemCell Technologies, USA), and the cell number was determined via counting with Neuobauer. The stem cell and lineage markers were analyzed by flow cytometry (4-color, SY-Space Partec, Germany) using monoclonal antibodies. CD2 and CD19 (Dako, Denmark) were used to evaluate lymphoid lineage differentiation; CD14, CD15, and CD13 for myeloid differentiation; Glycophorin A for erythroid differentiation and CD34 and CD38 to assess the percentage of stem cells. Flow cytometric analysis was performed by incubating harvested cells with different fluorescent conjugated monoclonal antibodies at 4 °C for 30 min. Then the cells were washed in PBS and fixed with 2 % paraformaldehyde (Sigma). Isotype controls were used in every experiment.

Apoptosis and Cell Cycle Distribution Analysis by Flow Cytometry

Apoptosis rate and cell cycle distribution were evaluated at day 10 of culture by flow cytometry (Partec, Germany). Prior to staining, 200 µL of 1×10^6 cells/mL were washed by phosphate buffered saline (PBS) and suspended in 200 µL of PBS. Cells were treated with 50 µL of RNase (1 mg/mL) and 100 µL propidium iodide (PI, 400 µg/mL) (Sigma-Aldrich, Spain) for 30 min at 37 °C in the dark. The fluorescence of stained cells (Intra cellular staining) was analyzed in a flow cytometry (Partec, Germany) and relative gated cells in each cell cycle were determined. Data acquisition and analysis were performed using flow Macs 2.0 software.

Samples and Extraction of Total RNA and DNA

DNA was extracted from fresh cord blood CD34⁺ cells and expanded cells in all the three culture conditions at days 5, 10, 14 of culture, while total RNA was just extracted at days 0 and 10 of culture. They were immediately frozen and kept at −70 °C until use. DNA was isolated by QIAamp DNA Mini-kit (QIAGEN, USA) and RNA was extracted using Trizol reagent (Roche, Germany).

Bisulfite Modification and Methylation-Specific PCR (MSP)

Genome DNA was subjected to bisulfite modification using an EpiTect Bisulfite kit (Qiagen). Modified DNA was purified, resuspended in distilled water and stored at −20 °C until use. Human genomic DNA was used to determine the bisulfate conversion efficiency.

MSP Modified DNA template was used to amplify the DAPK1 promoters with methylated and unmethylated-specific primer pairs of which the sequences have been chosen for promoter regions containing frequent cytosines, and CpG pairs near the 3' end of the primers to provide discrimination in the PCR between methylated and unmethylated DNA [12, 13]. Primer sequences for the DAPK1 gene, unmethylated reaction was as follows:

5'-GGATAGTTGGATTGAGTTAATGTC-3' (sense),
5'-CAAATCCCTCCCAAACACCAA-3' (antisense);

and for methylated reaction as follows:

5'-GGATAGTCGGATCGAGTTAACGTC-3' (sense),
5'-CCCTCCCAAACGCCGA-3' (antisense);

The PCR mixture contained EpiTect Master Mix, 2× (QIAGEN, USA), 10 pmol of each primer and bisulfite treated DNA (approximately 50 ng) in a final volume of 25 µL. PCR amplification of the modified DNA samples consisted of one cycle of 95 °C for 10 min; 40 cycles of 95 °C for 30 s, 56 °C for 45 s, and 72 °C for 30 s; and 1 cycle of 72 °C for 10 min. Each modified DNA sample was amplified by unmethylated and methylated primers with 100 bp PCR products length band. Bisulfite converted methylated and unmethylated EpiTect PCR DNA (QIAGEN, USA) was used as controls. Peripheral lymphocyte DNA from healthy donors was also used as the unmethylated control. The products were separated by electrophoresis on 2 % agarose gel. Results were always confirmed by repeated MSP analysis.

Expression Analysis and Quantitative Real-Time PCR

Reverse transcription was performed on 1 µg total RNA, with AccuPower CycleScript RT PreMix reverse transcription Kit (Bioneer, USA). The reaction was carried out in 12 cycles of 22 °C for 30 s, 45 °C for 4 min; 55 °C for 30 s, and one cycle of 95 °C for 5 min. Products were analyzed in 1.5 % agarose gel under UV light. Quantitative RT-PCR was performed with the LightCycler technology, using 3 µL cDNA in 25 µL reaction volume with 0.4 µM of each primer and 12.5 µL of 2× Fast Start DNA Master SYBR Green I (Roche Molecular Biochemicals, Germany). The forward primer for DAPK1 was 5'-CAC GGC CGC

GGC CCG GGG TC-3' and reverse primer was 5'-GGC CCG GTG CAG CAC CAC CA-3' and PCR product was 180 bp [14]. Thermal cycling was initiated at 95 °C for 5 min followed by 40 cycles of PCR (95 °C, 30 s; 60 °C, 30 s; 72 °C, 20 s). GAPDH was used as an endogenous control. Fold change ratio was calculated via $2^{-\Delta\Delta C_t}$ method.

Statistical Analysis

Results obtained from multiple experiments are expressed as the mean $\pm$ standard deviation (SD). The data were analyzed using the *t* test. Probability values <0.01 defined significant differences between test points.

Results

Mesenchymal Stromal Cells Characterization

MSCs Immunophenotyping

MSCs isolated from bone marrow were characterized by flow cytometric analysis of specific surface antigens. MSCs were found to be positive for the following adhesion molecules: CD44, CD166 and CD105, CD90 which together were considered as markers for MSCs. As shown in Fig. 1, the MSCs were negative for haematopoietic lineage markers, namely, CD34, and CD45 (Fig. 1).

Osteogenic Differentiation Assay

Osteogenic differentiation potential of bone marrow derived MSCs was assayed by alizarin red staining and evaluation of alkaline phosphatase activity. Both Alizarin red staining and alkaline phosphatase activity showed a positive reaction.

Ex Vivo Expansion of CD34⁺ Enriched Cells with Selected Cytokines, in the Presence and Absence of Human Mesenchymal Stromal Cells

Purity of separated CD34⁺ cells determined by flow cytometric analysis were 88 % $\pm$ 12. Moreover, 31.45 % $\pm$ 7 of them were positive for the CD38 marker and lineage specific CD markers expression was low ($n = 3$; Fig. 2).

Ex vivo expansion of human cord blood CD34⁺ enriched cells in serum-free medium supplemented with SCF, TPO and FLT3L was evaluated either with or without feeder layer using flow cytometric analysis. Figure 2b–d shows the percentage of CD34⁺, CD38⁺ cells in expanded cells in the co-culture system without cytokine, cytokine cultures without MSCs and with MSCs after 10 days, respectively.

Figure 3a, b shows the fold increase in total number of cells (TNC) and number of CD34⁺ cells during 14-days liquid cytokine culture in the presence and absence of human mesenchymal stromal cells. Maximum expansion was observed at day 10 of culture in all conditions, which the mean fold change of TNC was 32 ± 2 in cytokine culture without MSCs feeder layer and 46.6 ± 5 in the co-culture system with cytokines. The mean fold change of CD34⁺ cells was 20.5 ± 17.8 in cytokines supplemented culture and 43.25 ± 28 in the co-culture system with cytokines ($n = 6$; Fig. 4).

However, in co-culture system without cytokine, TNC and CD34⁺ cell numbers were increased up to 4.88 ± 1.5 and 2 folds respectively. Expansion of CD34⁺ selected CB cells at day 14 of both cytokine culture and co-culture with cytokines was decreased and relative differentiation was also observed.

So the mean fold change of TNC and CD34⁺ cells at day 10 in the co-culture system with cytokines was higher than the cytokine culture without MSCs feeder layer and co-culture system without cytokines significantly ($n = 6$, $p < 0.05$). However, in the co-culture system without cytokine TNC and CD34⁺ cell numbers were increased up to twofolds, the cell viability was remained 80 % after 14 days.

Colony-Forming Cell Assays

The highest CFU fold change was also observed in cytokine culture with MSCs at day 10 (90 ± 24) (Fig. 3). The CFU increase in cytokine culture without MSCs at day 10 was 87 ± 13 , and in co-culture with MSC was 52 ± 9 . So The mean fold change of CFU at day 10 in both cytokine cultures with and without MSCs feeder layer was higher than co-culture system without cytokines significantly ($n = 3$, $p < 0.05$), but there was no significant differences between two cytokine cultures with and without MSCs feeder layer (Fig. 5).

Cell Cycle Distribution Analysis

Cell cycle analysis was performed by PI staining on expanded cells cultured in all three culture condition at day 10 of culture. Distribution percentage of expanded cells was 56.5 ± 3.02 G0/G1, 9.46 ± 1.69 G2 m and 25.61 ± 2.52 S phase of cell cycle in co-culture without cytokine. In co-culture with cytokine 42.65 ± 2.33 percent of expanded cells was G0/G1, 13.07 ± 0.77 percent G2 m, 26.69 ± 1.08 S phase of cell cycle and in cytokine culture, distribution percentage of expanded cells was 33.09 ± 2.77 G0/G1, 7.62 ± 0.59 G2 m and 20.32 ± 3.2 S phase of cell cycle.

Fig. 1 CD marker expression on fresh MSCs. **a** CD 105, 93 %, **b** CD 166, 98.8 %, **c** CD 45, 3.8 %, **d** CD44, 99.5 %, **e** CD 34, 2.8 %

Our results showed population of cells in G0/G1 phase in two co-culture condition was higher than cytokine culture significantly and also in co-culture without cytokine was higher than co-culture without cytokine ($p < 0.005$). The population of cells in G2/M phase in co-culture with cytokine was higher than another condition ($p < 0.05$). But there was no significant differences between population of cells in S phase of cell cycle (Fig. 6).

Apoptosis Analysis

Apoptosis analysis was performed by PI staining on expanded cells cultured in all three culture conditions at day 10 of culture. Apoptotic cells percentage was 7.67 ± 1.29 in the co-culture without cytokine, 17.18 ± 0.35 in the co-culture with cytokine and 38.10 ± 0.94 in the cytokine culture. Our results showed in the cytokine culture apoptosis rate was higher than two co-cultures conditions

Fig. 2 Flow cytometric analysis of fresh CD34⁺ enriched cells (a) and expanded cells in the co-culture system without cytokine (b) cytokine culture without MSCs (c) and with MSCs (d) at day 10 of expansion: Specific staining was performed with anti-CD34-FITC and anti-CD38-PE antibodies, FL1: CD34, FL2: CD38 (a) CD34: 70 % CD38: 14.31 % (b) CD34: 70.90 % CD38: 58.31 % (c) CD34: 60.40 % CD38: 54.21 % (d) CD34: 79.54 % CD38: 66.68 %

Fig. 3 a Mean fold change of TNC. b Mean fold change of CD34 cells in cytokine culture and co-culture with MSCs supplemented with cytokine and without cytokine

Fig. 4 **a** Co-culture of HSCs with MSCs after 10 days expansion in serum free media supplemented with cytokine cocktail. **b** CFU assay of expanded HSCs at day 10 of culture

Fig. 5 CFU assay of expanded HSCs at day 10 of the cytokine culture and co-culture system with and without cytokines. Apoptosis analysis at day 10 of three culture conditions ($p < 0.005$) DAPK1 mRNA expression fold change ratio, in the expanded cells to the fresh $CD34^+$ cells at day 10 of three culture conditions ($p < 0.01$)

significantly ($p < 0.005$) and apoptosis rate in the co-culture with cytokine was higher than co-culture without cytokine ($p < 0.005$; Fig. 5).

mRNA Expression of DAPK1 After 10 day Ex Vivo Expansion of $CD34^+$ Cells

Expression of DAPK1 in fresh $CD34^+$ cells and expanded cells after 10 days in all the three culture conditions was evaluated by real time-PCR (Fig. 7). The results showed that the fold change ratio of DAPK1mRNA expression in the expanded cells to the fresh $CD34^+$ cells was 9.49 ± 1.79 ($n = 3$), at the co-culture system without cytokines, 21.32 ± 2.82 ($n = 3$), at the co-culture system with cytokines and at the cytokine culture without MSCs feeder layer 24.03 ± 1.39 ($n = 3$; Fig. 5). So, ex vivo expansion of $CD34^+$ cells in all the three culture conditions resulted in, high significant increased expression of DAPK1 and there was significant differences between co-

culture system without cytokine and two cytokine cultures ($p < 0.01$). However, there were no significant differences between two cytokine cultures.

Methylation Status of the DAPK1 During Ex Vivo CB-HSCs Expansion

We examined the methylation status of DAPK1 gene promoter in fresh $CD34^+$ cells and expanded cells in all the three culture conditions at days 5, 10 and 14 of culture with MSP analysis. Fresh $CD34^+$ cells samples only showed an unmethylated band. Methylation of this gene did not occur in any of the eight samples taken from the cytokine supplemented culture with and without MSCs feeder layer and co-culture with MSCs without cytokine at different days of expansion. In samples with methylated primer no PCR products were detected. However, in samples with unmethylated primer, 100 bp bands were detected (Fig. 8). So, methylation status of DAPK1 gene promoter during

Fig. 6 Percentages of cells in each quadrant is representative of **a** control, **b** co-culture without cytokine, **c** co-culture with cytokine, **d** Cytokine culture and in each part (RN1) represent G0/G1 phase, (RN2) G2M phase, (RN3) S phase and (RN4) late apoptosis phase

Fig. 7 Amplification curve of DAPK1 gene: fresh CD34⁺ cells, expanded cells in three culture conditions and standards

ex vivo expansion of CB-HSCs in all the three culture conditions was not changed.

Discussion

Ex vivo expansion of UCB-HSCs at different combinations of recombinant stimulatory cytokines is one way to increase the number of CD34⁺ cells and the kinetics of

engraftment of UCB-HSCs [15]. Another way is expansion of HSCs in co-culture with mesenchymal stromal cells that produce a more “natural” haematopoietic microenvironment for proliferation, self renewal and differentiation of HSCs [16]. However, it has been clearly shown that cord blood CD34⁺ cells with a high number of cell divisions in short-term cultures are associated with an increase in the percentage of apoptotic CD34⁺ cells [17].

But prevention of apoptosis is an important strategy to improve stem cell engraftment in preclinical and clinical settings [18]. In this study, effects of hematopoietic cytokines (SCF, FL3L, and TPO) and mesenchymal stromal layer on expression and promoter methylation status of DAPK1 (a serine/threonine kinase that participates in apoptotic systems) during CB-CD34⁺ cells expansion was evaluated.

Maximum expansion was observed at day 10 of culture in the two cytokine supplemented cultures. Moreno et al [19] also reported that UCB derived CD34⁺ cells showed significant proliferation after 14 days in cytokine culture and maximum expansion was observed at day 7 of culture.

The mean fold changes of CD34⁺ cells at day 10 of expansion in the two cytokine cultures with and without MSCs were higher than co-culture system without

Fig. 8 Methylation specific PCR of bisulfite-modified DNA using primer pairs specific for methylated and unmethylated DAPK1 promoter sequences in all the three culture condition, *M* primers specific for methylated DNA; *U* primers specific for unmethylated DNA. *1* DNA ladder *2* methylated DNA control with the methylated primer pairs. *3* unmethylated DNA control with the unmethylated primer

pairs, *5*, *7*, *9* and *11* illustrates DNA amplification at days 0, 5, 10 and 14 of culture respectively with unmethylated DAPK1 primers specific for unmethylated DNA. *4*, *6*, *8* and *10* have no band that illustrate lack of DNA amplification for expanded cells at days 0, 5, 10 and 14 of culture respectively with methylated DAPK1 primers specific for methylated DNA

cytokine. In keeping with our results (Jang et al. [20]) also showed that proliferation capacity of HSCs in recombinant cytokine culture with UCB-MSCs feeder layer was higher than the cytokine culture without MSCs, and also in the co-culture system without cytokine CD34⁺ cell numbers were increased up to folds. However, in comparison to cytokine cultures, lineage differentiation rate was low in the co-culture system without cytokine (results were not shown).

Silvaa et al. [21] cultured CB CD34⁺ enriched cells in serum-free medium supplemented with SCF, bFGF (fibroblast growth factor), leukemia inhibitory factor (LIF), and Flt-3, in the presence or absence of stromal layer, and they observed the stromal layers would support process of expansion without the exhaustion of the more primitive stem cells and differentiation induction.

MSC have been demonstrated to serve as a feeder layer and to maintain HSCs in an undifferentiated state [22]. The rate of expansion in two our cytokine cultures decreased at day 14 of expansion, but in the co-culture system without cytokine fourfold expansion constantly was observed during 14 days. Song et al. [23] claimed that co-cultures of hematopoietic cells with bone marrow derived mesenchymal stromal cells without added cytokines, was able to preserve repopulating HSCs for several weeks. HSC differentiation during expansion increases HSC senescence and cell death possess [24], but direct contact between stem cells and cellular microenvironment has been shown to play an essential role in self-renewal versus differentiation [25].

In this study expanded HSCs in the cytokine supplemented culture compared to the co-culture system without cytokine have the highest DAPK1 mRNA expression and the highest apoptosis.

Goldenberg et al. [18] reported that SCF under serum-free conditions induces apoptosis measured by annexin V-PI cell staining in spite of increasing CD34⁺ proliferation. Wei et al. [26] claimed when K562 cells were co-cultured with normal MSCs, anti apoptotic genes was up regulated and apoptosis rate was decreased. In our study the least DAPK1 expression and apoptosis rate was observed in the co-culture system without cytokine. Also the highest percentage of G0/G1 cells was observed in the co-culture system without cytokines. Therefore, HSCs in this condition had the least proliferation and were maintained in the quiescent phase. But in co-culture system with cytokine the highest proliferation capacity was observed and apoptosis rate compare to cytokine culture decreased significantly.

So because the less rate of apoptosis and highest rate of proliferation, the best strategy for HSC expansion is co-culture with MSCs with minimum concentration of cytokines.

DNA methylation, an important mechanism for gene suppression, is one of the earliest processes in tumor cells initiation [27, 28]. Issa and Greco et al. [29–31] reported the tumor suppressor gene, DAPK1, is one of the most common, and earliest, epigenetically mediated losses of tumor suppressor function events in myelodysplastic syndromes (MDS) and acute myeloid leukemia (AML).

The normal epigenetic modulation of these genes allows them to prevent stem/precursor cells from becoming immortalized and acquiring infinite cell renewal capacity and the inappropriate silencing of these genes blocks their activation and allows for abnormal survival and clonally expansion and prevents differentiation [8].

In our study after screening for methylation changes in expanded CB-HSCs at several time points, we have demonstrated that methylation of DAPK1 promoter in these cells during in all the three culture conditions has not occurred. Therefore, FLT3L, SCF and TPO showed no effects on methylation status of DAPK1 gene in expanded CB-CD34⁺ cells after 14 days of treatment.

Conclusion

Expression of DAPK1 in CD34⁺ cells was not decreased during ex vivo expansion on human bone marrow mesenchymal stromal cells feeder layer. Also, no methylation of DAPK1 promoter, that may initiate some leukemic cell progression or disruption in hematopoietic regeneration, was observed.

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