Adipose Stem Cells as a Feeder Layer Reduce Apoptosis and p53 Gene Expression of Human Expanded Hematopoietic Stem Cells Derived from Cord Blood

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ABSTRACT

Introduction: Human hematopoietic stem cells (hHSCs) have been used for transplantation in hematologic failures. Because the number of hHSCs per cord blood unit is limited, the expansion of these cells is important for clinical application. It has been reported that cytokines and feeder layer provide a perspective to in vitro expansion of hHSCs. In this regard, cord blood CD34+ cells expanded after co-culture with adipose-derived stem cells (ADSCs) feeder layer and cytokine conditions were analyzed in terms of apoptosis and the expression level of p53, a tumor suppressor gene.

Methods: Three cultures of cord blood CD34+ cells were prepared ex-vivo for 7 days, including cytokines stem cell factor (SCF), thrombopoietin (TPO), and fetal liver tyrosine kinase 3 ligand (Flt3L) with ADSCs feeder layer, cytokines without feeder layer, and a culture on micro porous membrane with cytokines. Apoptosis rate and the expression level of P53 gene in all groups were analyzed by real-time PCR.

Results: The data showed that p53 expression and apoptosis rate in CD34+ fresh cells were higher than other culture systems. P53 gene expression in the expanded cells at both cytokine cultures with and without ADSCs feeder layer decreased, but it was lower than other culture systems at co-culture system with cytokines ($P < 0.05$). Apoptosis rate in expanded cord blood CD34+ with feeder layer was significantly lower than the group without ADSCs feeder layer.

Conclusion: It could be concluded that direct culture of CD34+on feeder layer ADSCs affects the expansion of HSCs.

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1. Introduction

HSC (hematopoietic stem cell) is an adult stem cell that has the ability to self-renewal and it differentiates to blood cells and other cell type such as adipocyte cells, cardiomyocytes, and endothelial cells (1). The balance between self-renewal and differentiation of HSCs is important for the preservation of stem cell pool (2). Inadequate number of HSCs in the cord blood limits its use for transplantation. Therefore, the expansion of cord blood HSCs is important for clinical application. To solve this problem researchers have used a cocktail of growth factor, which causes ex vivo expansion of hHSCs.

This rapid expansion may increase the percentage of hHSCs in transplantation, however, it is associated with differentiation too (2). Therefore, besides the growth factor, using feeder layer has been reported for hHSCs expansion (2). Feeder layer with cytokine secretion and direct connection with hHSCs can affect the cell cycle inhibitor genes and increase self-renewal of hHSCs (3). One of the cell cycle inhibitor genes is tumor suppressor p53. Defection in this suppressor gene is associated with hematologic malignancies. P53-dependent cell cycle control, senescence, and apoptosis functions are actively involved in maintaining hematopoietic homeostasis under normal or stressful conditions (4). Although, loss of p53 function promotes leukemia and lymphoma development in humans and mice, increased p53 activity inhibits hematopoietic stem cell function and results in myelodysplasia (5). As exquisite regulation of p53 activity is critical for homeostasis, we evaluated expression of p53 in human cord blood CD34+ expanded cells following co-culture with adipose derived stem cells.

2. Material and Methods

2..1. CD34+ Cells Isolated from Umbilical Cord Blood

CD34 HSCs were collected from fresh human umbilical cord blood after obtaining written consent from normal full-term pregnant women and according to the ethics committee guidelines, at the Iranian Blood Transfusion Organization. The cord blood was obtained with consent and mononuclear cells with Ficole (Sigma, 1.077 ± 0.001 kg/L) were separated. Then, these cells using anti-CD34 antibody labeled with nanoparticles of Fe (America Milton Biotech) were incubated and $CD34^+$ cells using column MACs (America Milton Biotech) were separated. To confirm CD34 marker for cells isolated from umbilical cord blood, the antibody anti-CD34 and anti-CD38 were used and cells were obtained with an average of 70%.

2.2. Isolation of Mesenchymal Stem Cells from Adipose Tissue

After obtaining written permission from patients, the adipose tissue samples were obtained during liposuction surgery and transported to the laboratory in sterile conditions. Then, type IV collagenase (250 U/ml) was added to the sample, which was incubated for 60 minutes at 37˚C with 5% CO2. Afterward, the samples were centrifuged for 5 min at 1500rpm. Cell pellet with Dulbeccos Modified Eagle's Medium (DMEM) medium containing 10% fetal bovine serum (FBS) were suspended and cultured. Mesenchymal stem cells (MSCs) from passage 4 were used to differentiate into fat and bone cells.

2.3. Evaluation of the Differentiation Potential of Mesenchymal Stem Cells

To differentiate into fat cells, a medium containing 10 nm dexamethasone, 5 mM NaCl, 10 mM IBMX, and indomethacin was prepared. And to differentiate into osteoblast cells, a medium of DMEM, high glucose with 10% fetal bovine serum and 10 nM dexamethasone, 35mg/mL of ascorbic acid, and 1 mM β-glycerophosphate was used.

Cells were incubated in %5 CO2, at 37˚C for 21 days. To demonstrate differentiation into fat cells and osteoblast cells, Alizarin Red and Oil Red staining was used. Also, for providing feeder layer, the cells were treated with mitomycin.

2.4. $CD34^+$ Cells Cultured in Different Conditions

Isolated $CD34^+$ cells were cultured for 7 days in different conditions, which were as follows:

1. They were cultured in the presence of a 100ng/ml cytokines such as stem cell factor (SCF), thrombopoietin (TPO), and fetal liver tyrosine kinase 3 ligand (Flt3L). In all groups, the cytokine and serum-free medium (Stemspan) were used.

2. In this group the cells were cultured directly on the feeder layer and in the presence of mentioned cytokines.

3. In this group, the cells were cultured on the microporous membrane with a pore size of 0.4 µmol in the presence of mentioned cytokines. In addition to the mentioned groups, another group of cells were tested immediately after extraction and without being cultured.

2.5. Apoptosis Analysis by Annexin *V* and Propidium Iodide

Apoptosis kit (Bioscience, USA) was used for apoptosis analysis. At 14th day of culture, 1×10^4 cells after resuspended in 1x binding buffer were treated with fluorochrome- conjugated Annexin V for 10 min and then washed and resuspended in 1x binding buffer. Next, propidium iodide solution was added and fluorescence of the stained cells was analyzed by flow cytometry.

2.6. Analysis of Real-Time PCR

Using TRIzol (Sigma), total RNA of the cells were extracted. The cDNA for the reverse transcriptional reaction synthesis and real-time PCR was performed with an EvaGreen and data were analyzed.

The sequence of p53 and GAPDH are as follows:

Forward: 5′ - TCCTCAGCATCTTATCCGAGTG 3′

Reverse: 5′ AGGACAGGCACAAACACGCACC- 3′

GAPDH,

Forward: 5′- ATGGGGAAGGTGAAGGTCG-3′

Reverse: 5′ GGGGTCATTGATGGCAACAATA- 3′

2.7. Statistical Analysis

Data were analyzed using SPSS P values <0.05 are considered as significant between the test points.

3. Results

Isolated ADSCs were certified by flow cytometric analysis of surface antigen markers. ADSCs were positive for CD45, CD73, CD105, and CD90 antigens, which together were considered as markers for ADSCs. ADSCs were negative for hematopoietic lineage markers, CD34, and CD45 (Figure 1). Potential osteogenic differentiation of adipose derived stem cells was assayed by Alizarin Red and Oil Red staining. Both activities showed a positive reaction (Figures 2, 3). Separated CD34 + HSCs analyzed by flow cytometry were 88.8%. %22.2 of them was positive for CD38 marker. Ex-vivo expansion of human cord blood (enriched CD34+ cells in serum-free medium supplemented with SCF, TPO, and FLt3L) was evaluated either with or without feeder layer using flow cytometry analysis (Figure 4).

Annexin V and PI staining was performed for apoptosis analysis of the cultured expanded cells. The percentage of apoptotic cells for PI and Annexin in co-culture with and without feeder layer was shown in Figure 4.

The highest CB-HSC expansion was found at day 14. The expression level of p53 gene in co-culture of CD34+ hHSCs with ADSCs feeder layer in the presence of the cytokines group was significantly lower than other groups ($P < 0.05$). The highest expression level of p53 gene was observed in CD34+ hHSCs ($P < 0.05$). The direct contact between HSCs and feeder layer was prevented by microporous membrane and consequently the expression of p53 gene increased compared to direct contact of feeder layer with hHSCs group (Figure 5).

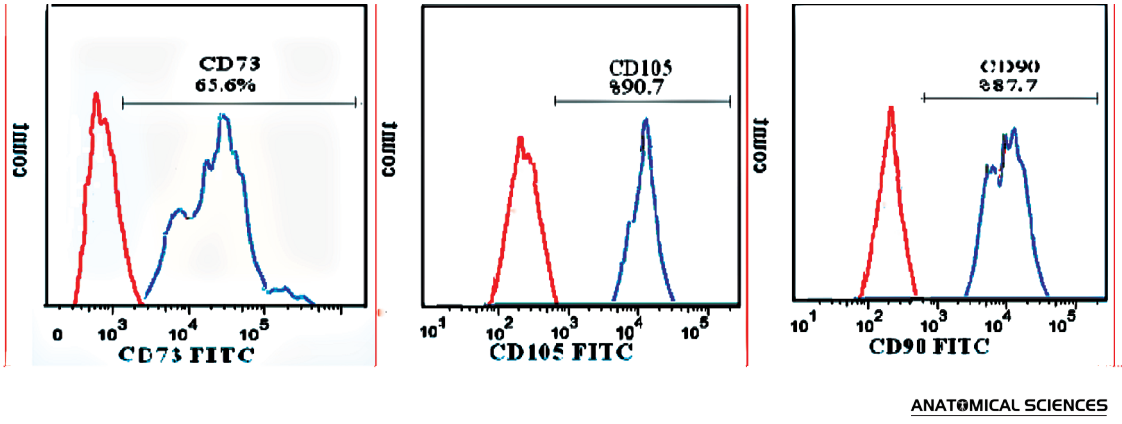

Figure 1. Flow cytometric analysis of percentage of CD105, CD90 and CD73 markers in ADSCs.

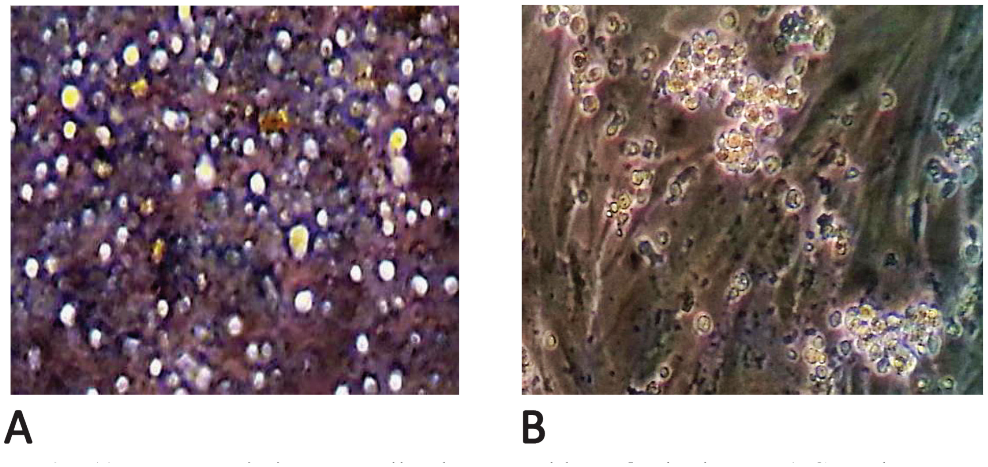

Figure 2. A) Hematopoietic stem cell cultured without feeder layer. **B)** Co-culture hematopietic stem cells on ADSCs feeder layer after 7 days of expansion.

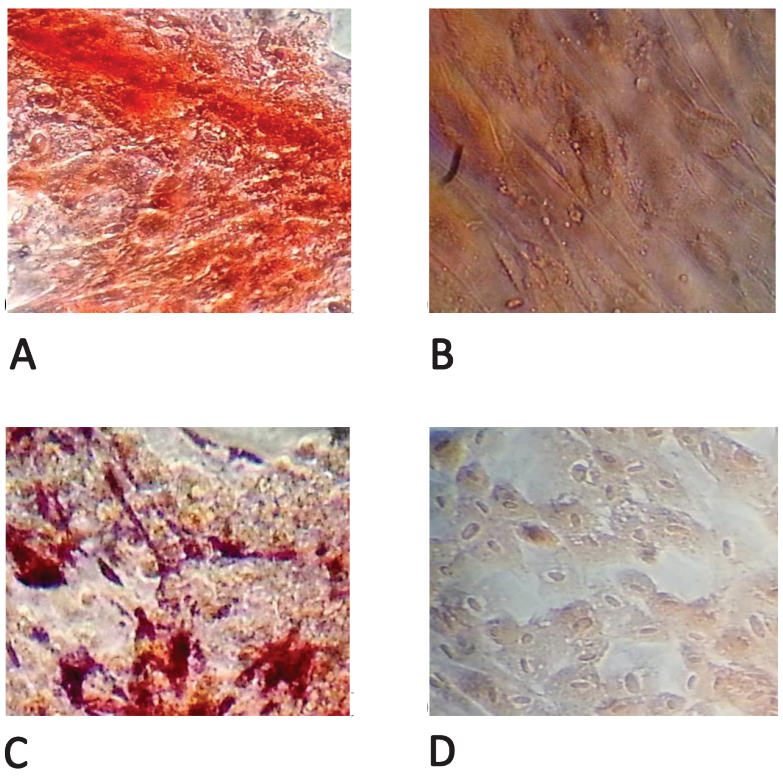

Figure 3. Osteogenic differentiation of ADSCs: **A)** positive reaction of osteoblastic differentiated cells with Alizarin Red staining, **B)** undifferentiated cells, **C)** Increased alkaline phosphatase staining in osteoblastic differentiated cells, and **D)** Undifferentiated cells.

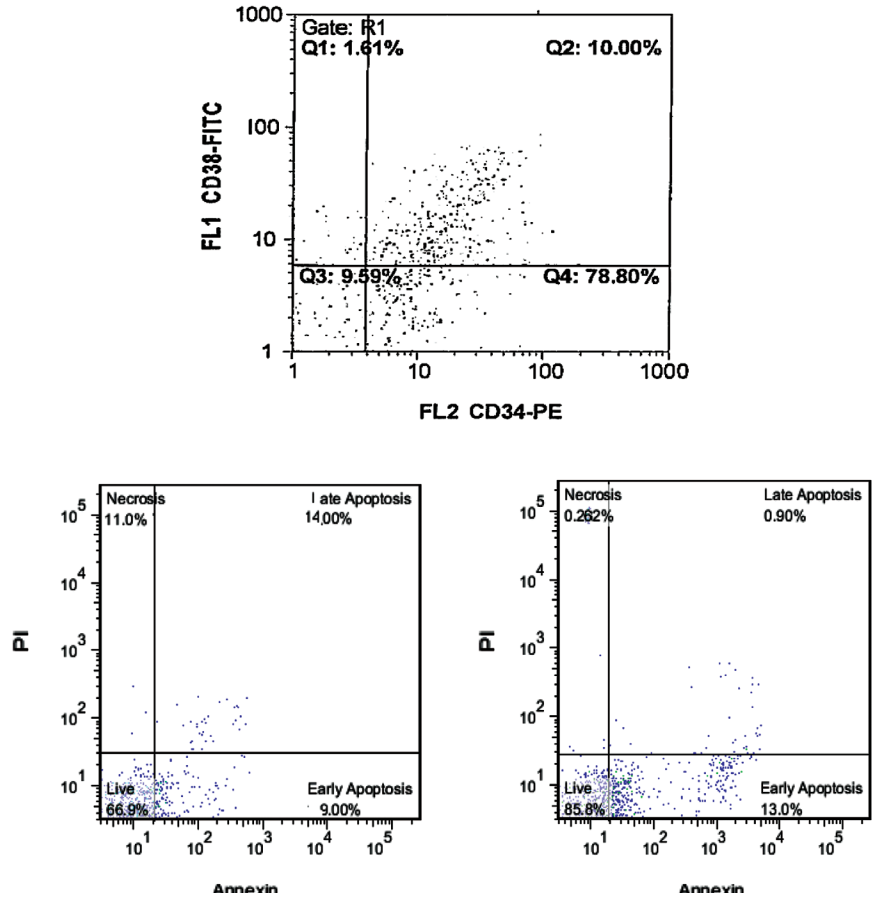

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Figure 4. Flow cytometric analysis of cord blood HSCs: **A)** Flow cytometric analysis of fresh CD34⁺ enriched cells. Specific staining was performed with anti-CD34 FITC and anti-CD38 PE antibodies and **B)** Flow cytometric analysis of apoptosis at day 14 in different culture condition of CD34⁺ with and without ADSCs feeder layer by PI and Annexin V staining, 1) Cord blood CD34⁺ in co-culture with ADSCs, and 2) Cord blood CD34+ without ADSCs feeder layer.

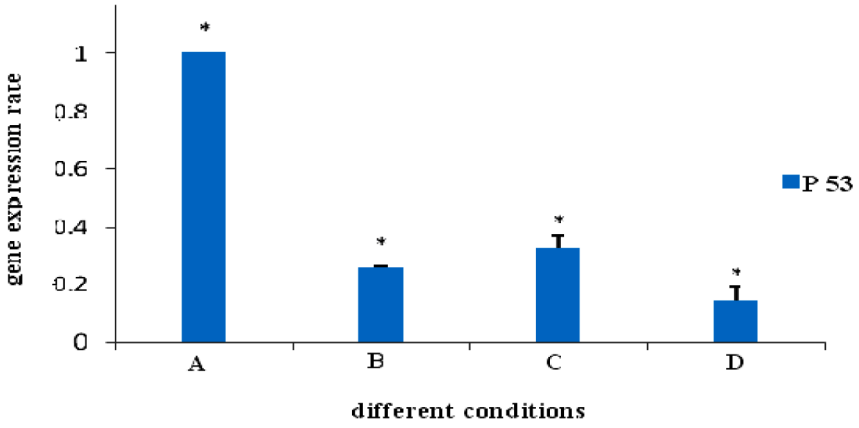

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Figure 5. Comparative and real-time PCR analysis of p53 gene expression in fresh cord blood CD34⁺ **(A)**, cytokine culture without feeder **(B)**, direct contact between CD34⁺ HSCs, and feeder layer prevented by microporous membrane **(C)**, and co-culture of CD34⁺ hHSCs with ADSCs feeder layer in the presence of the cytokines **(D)**. The expression level of p53 gene in co-culture of CD34⁺ hHSCs with ADSCs feeder layer in the presence of the cytokines group was significantly lower than other groups ($P < 0.05$). The highest expression level of p53 gene was observed in fresh CD34⁺ hHSCs ($P < 0.05$). The direct contact between HSCs and feeder layer was prevented by microporous membrane and consequently the expression of p53 increases compared to direct contact of feeder layer with hHSCs group.

4. Discussion

Ex-vivo expansion of cord blood HSCs at different combinations of recombinant stimulatory cytokines is one way to increase the number of $CD34^+$ cells and the kinetics of engraftment of cord blood HSCs (6). Jang et al. showed that the proliferation capacity of HSCs in recombinant cytokine culture with cord blood MSCs feeder layer was higher than the cytokine culture without MSCs,besides in the co-culture system without cytokine $CD34^+$, the cell number increased up to 3 fold (7). However, in comparison to cytokine cultures, lineage differentiation rate was low in the co-culture system without cytokine. Also in 2013, Gleting and colleagues used the various feeder layers such as bone marrow MSCs, osteoblast, and adipocyte to show that the feeder layer has a significant role in enhancing HSC self-renewal (2). The results of the present study showed that.

In the presence of ADSCs feeder layers, the expression of p53 has been reduced.

P53 as a cell cycle inhibitor has an important role in the inhibition of the proliferation and increases the differentiation of hHSC. It seems that the decreased expression of p53 gene in the presence of feeder layer prevents hHSCs differentiation. In 2009, Alakel and colleagues showed that adhesion and direct cell-to-cell contact with MSCs feeder layer supports ex vivo expansion, migratory potential, and stemness of $CD133^+$ hematopoietic progenitor cells (8). According to them, the expression of genes involved in adhesion, cell-cycle control, motility, and self-renewal were more highly in feeder layer groups. Our data were consistent with Alakel's findings. The direct contact between HSCs and feeder layer was prevented by microporous membrane and consequently the expression of p53 gene increased compared to direct contact of feeder layer with hHSCs group.

In conclusion, in co-culture system with microporous membrane, differentiation rate of hHSCs increases, so direct contact between HSC and feeder layer cells is essential for hHSCs expansion. In 2012, Oodi and colleagues showed that the gene expression of p16 as an HSC cell cycle inhibitor, in the presence of the cytokines, with and without MSCs feeder layer decreased (9). In 2013, it has been reported that the gene expression of p15, other cell cycle inhibitor, during ex vivo expansion did not decrease (10). Based on our findings, in the presence of the cytokines and ADSCs feeder layer, there is a significant reduction in the expression of p53 that indicates the importance of the ADSCs feeder layer. Although, loss of p53 function promotes leukemia and lymphoma development, it is essential to study expression of some oncogenes such as c-myc in similar conditions with and without ADSCs feeder layer.

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