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Lower Oncogenic Potential of Human Mesenchymal Stem Cells Derived from Cord Blood Compare to Bone Marrow and Adipose Tissues

KEYWORDS: mesenchymal stem cells, bone marrow, adipose, cord blood, P53, OCT4, sox2, cmyc

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ABSTRACT *Objective: Human mesenchymal stem cells (MSCs) derived from bone marrow (BM), cord blood (CB) and adipose (AD) tissues have potential application in cell therapy. In perspective of regenerative medicine, each of these three sources has advantages and disadvantages. The aim of the present study was comparison of re-programming and pluripotent markers OCT4, Sox-2, c-Myc and also tumor suppressor gene P53 in MSCs derived from BM, CB and AD tissues.*
Methods & Materials: We analyzed the expression level of OCT4, Sox-2, c-Myc and P53 genes in hMSCs derived from BM, CB, and AD tissues by cell culture, real time RT-PCR and immunocytohcemistry.
Results: Our data revealed that the expression level of pluripotent genes OCT4 and Sox-2 in MSCs derived from CB and BM were higher than AD significantly. In contrast to OCT-4A and Sox-2, the expression level of oncogenic factors c-Myc was significantly higher in MSCs from AD tissue than CB and BM. Also a significantly much lower level of P53 gene expression was detected in the MSCs from AD tissue as compared to the other sources. Our data demonstrated that human MSCs derived from CB have lower oncogenic potential compared to bone marrow and adipose tissues.
Conclusion: we concluded mesenchymal stem cells derived from human cord blood have lower oncogenic potential compared to bone marrow and adipose tissues

Introduction

Two types of stem cells are currently recognized, adult stem cells and embryonic stem cells (ESCs). Adult stem cells are harvested from different tissue sources and variously called multipotent mesenchymal stromal cells or mesenchymal stem cells [1-5]. MSCs could differentiate into osteoblast, condroblast, cardiomyocyte or even cells of nonmesodermal derivation including hepatocytes and neurons (6).Although mesenchmal stem cells are originally isolated from bone marrow, similar populations have been reported in other tissues such as adipose tissue and unbilical cord blood. Adult stem cells have generated great interest because of their potential application in regenerative medicine. In perspective of regenerative medicine, each of these sources has advantages and disadvantages. Unlike bone marrow MSCs, however, AD stem cells (ADSCs) can be obtained in large quantities at low risks (7). In addition to being more abundant and easily accessible, the adipose tissue yields far more stem cells than bone marrow on a per gram basis (5,000 vs. 100-1,000) (8). Indeed adipose tissue provides a readily accessible and affluent deposit of stem cells due to the liposuction procedure epidemic. Biopsy of adipose tissue for extraction of stem cells is also less harsh on patients as compared to procedures involving bone marrow tissue. ADSCs are also a more reliable source for auto graft transplants as compared to the bone marrow MSCs. These are capable of autologous and alogenic tissue grafting, and there is a minimal immunogenic response to such a procedure (9,10). ADSCs have notable expression of pluripotent factors such as FGF, TFG, and fibronectin. They also provide a higher level of active biological factors such as IL-6 and HGF (11). Bone marrow MSCs require a higher density of cells for the initial culturing (>50000 cells/cm2) than the ADSCs (3000 cells/cm2) (12). The high accessibility of adipose tissue along with their remarkable properties presents them as a suitable candidate for regenerative medicine application. However, adult stem cells have limitations in their application because they cannot be propagated indefinitely in culture; number of these cells also decrease with aging and there is evidence that these cells may exhibit reduced proliferation and differentiation with aging (13-17).

ESCs are considered to be pluripotent stem cells and are derived from the inner cells mass. These cells are capable of differentiation to any cell. In contrast to adult stem cells, ESCs can be cultured indefinitely while maintaining their pluripotency (18, 19, 20). Because of ethical concerns association with the application of ESCs in mediciene applications, there is paucity of information regarding their applicative potentials for tissue regeneration. On the other hand, Yamanaka and Takahashi managed to reprogram the mouse somatic cell to pluripotent ESC-like by the simple retoviral over expression of four transcription factos: OCT-4, Sox-2, Klf-4, and c-Myc (21). Stem cells derived from this process are named induced pluripotent stem cells (iPSCs) and closely resemble ES cells since they restore a genome associated with a pluripotent marker expression and fulfill all major biological criteria for pluripotency such as the in-vitro differentiation into cell types of all three germ layers.

In the present study we have isolated MSCs from bone marrow, adipose tissue, and umbilical cord blood sources. Considering the pros of MSCs derived from adipose in comparison to bone marrow and umbilical cord blood MSCs, the aim of this study was comparative analysis of expression of pluripotency markers of sox-2, OCT-4, and c-myc in MSCs from human adipose, bone marrow, and cord blood. Since P53 gene well known as a tumor suppressor, expression of P53 gene will further compared in all of sample cells by comparative real time PCR analysis.

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Materials and Methods

Cell culture

Mesenchymal stem cells from adipose tissue: Human subcutaneous fat was obtained from healthy patients undergoing abdominal dermolipectomy in the department of Plastic Surgery of Erfan Hospital (Tehran, Iran). The patients were between 20 and 50 years old.The adipose tissue was rinsed with PBS containing 1% penicillin and streptomycin, minced into small pieces, and then incubated in a solution containing 0.075% collagenase type IA (Sigma-Aldrich, St. Louis, MO) for 1 h at 37°C with vigorous shake. The top lipid layer was removed and the remaining liquid portion was centrifuged at 220g for 10 min at room temperature. The pellet was treated with 160 mM NH4Cl for 10 min to lyse red blood cells. The remaining cells were suspended in DMEM supplemented with 10% fetal bovine serum (FBS), filtered through a 40-µm cell strainer (BD Biosciences, Bedford, MA), and plated at a density of 1 × 106 cells in a 10-cm dish. After reaching 80% confl uence, the cells were used for experiments as needed.

Mesenchymal stem cells from cord blood: Human umbilical CB was collected after informed consent of the mother using the guidelines approved by the Ethics Committee on the use of Human Subjects by a standardized procedure using syri containing L-heparin as anticoagulant. After 2:1 dilution with PBS, mononuclear cells were obtained by Ficoll density-gradient centrifugation (400g for 25 minutes). The cells were washed twice in PBS und seeded at a density of 1 to 3 3 106 cells/cm2. Growth of adherent cells was initiated in myelocult medium (StemCell Technologies) with dexamethasone (1027 M; Sigma-Aldrich), penicillin (100 U/mL; Gibco), streptomycin (0.1 mg/mL; Gibco), and glutamine (2 mM; Gibco). Nonadherent cells were removed after 72 hours, and the adherent cells were fed weekly with culture medium. Expansion of the cells was performed in Mesencult basal medium (M3; StemCell Technologies) with additive stimulatory supplements.

Mesenchymal stem cells from bone marrow: Human MSC from the bone marrow aspirates were obtained from the iliac crest of healthy donors aged 25–35 years and then were collected in a syringe containing 10,000 IU heparin to prevent coagulation. The mononuclear cell fraction was isolated by Biocoll density gradient centrifugation (d 5 1.077 g/cm3; Biochrom, Berlin, Germany). In brief, mononuclear cells were plated in expansion medium at a density of 105 cells/cm2 in tissue culture flasks (Nunc, Wiesbaden, Germany) coated with 10 ng/mL fibronectin (Sigma, Deisenhofen, Germany). The expansion medium consists of 58% Dulbecco's Modified Eagle's Mediumd-Low Glucose (DMEM-LG, Cambrex, Apen, Germany) and 40% MCDB201(Sigma), 2% fetal calf serum (FCS; StemCell Technologies, Vancouver, BC, Canada), supplemented with 2 mM L-glutamine, 100 U/mL Pen/Strep (Gibco, Eggenstein, Germany), 1% insulin transferrin selenium, 1% linoleic acid bovine serum albumin, 10 nM dexamethasone, 0.1mM L-ascorbic acid-2-phosphate (all from Sigma), platelet-derived growth factor, and epidermal growth factor (10 ng/mL each, R&D Systems, Wiesbaden, Germany). On reaching 80% confluency, cells were trypsinized with 0.25% trypsin / 1mM EDTA (Invitrogen, Karlsruhe, Germany) and replated at about 9000 cells/cm2. Cells were expanded for 2 to 6 passages. The plastic adherent cell fraction was reseeded at a density of about 9000 cells/cm2. Cells were expanded for 2 to 6 passages.

Quantitative real-time PCR: RNA of treated and non-treated MSCs, CB and adipose tissue derived stem cells were extracted using Trizol reagent (Invitrogen) according to the-manufacturer's protocol. RNA was analyzed with quantitative real-time PCR (qPCR).

Melting curve analyses and PCR product sequencing were performed to verify primer specificities. RT-PCR was repeated at least three times using the following conditions. Each of the reaction mixtures contained 10 µl of SYBR Green master mix (Applied Biosystems), 5 pmoles each of forward and reverse primers and 5 µl of 100 times diluted cDNA. The primer sequences used for qPCR were as follows:

OCT4 A, forward : 5′-GAAACCCACACTGCAGATCA-3′
reverse: 5′-CGGTTACAGAACCACACTCG -3′
c-Myc, forward: 5′-CACCAGCAGCGACTCTGA -3′
reverse: 5′-GATCCAGACTCTGACCTTTTGC-3′
SOX 2 forward : 5′-TGCTGCCTCTTTAAGACTAGGAC-3′
reverse:: 5′-CCTGGGGCTCAAACTTCTCT -3′
P53, forward : 5′ - TCCTCAGCATCTTATCCGAGTG 3′
reverse: 5′ AGGACAGGCACAAACACGCACC- 3′
GAPDH, forward: 5′- ATGGGGAAGGTGAAGGTCG-3′
reverse: 5′ GGGGTCATTGATGGCAACAATA- 3′

Results

Several studies have reported that over expression of OCT-4, Klf-4, Sox-2, and c-Myc is sufficient to induce cellular reprogramming. In the present study we compared the expression levels of these genes in human MSCs derived from CB, BM, and adipose tissues. All types of MSCs used in the present study were in the third passage as confirmed by adult stem cell markers such as CD-34, CD-45, CD90, CD105, CD73 and CD38. Our data showed a significantly higher level of OCT4 expression in MSCs from CB compare to the other cell types (Fig 2a). The expression level of this gene in MSCs of BM was higher than ADSCs significantly. As it is shown in Fig. 2b, CB stem cells expressed significantly higher level of the Sox-2 pluripotent marker than other cell types. The expression level of these genes was zero or low in ADSCs. In contrast to OCT-4A and Sox-2, the expression level of oncogenic factors c-Myc was significantly higher in MSCs from AD tissue than CB and BM (Fig 2c and 2d). Also our findings showed that the expression level of c-Myc in MSCs of CB were lower than BM. Since c-Myc gene has been introduced as oncogennic genes and P53 well known as tumor suppressor gene, the findings of c-Myc genes was further confirmed by comparative real time PCR analysis of p53. As expected, a significantly much lower level of P53 gene expression was detected in the ADSCs as compared to the other cell types (Fig. 4). Our results showed a significantly much higher level of P53 gene expression in the MSCs from CB and BM than in ADSCs.

Immunostaining analysis of OCT-4A gave nuclear signals in all groups. Although nuclear signals could be detected in all cells, a high expression of OCT4 was observed in CB stem cells compared to other cells. The expression of OCT-4 in ADSCs (d) was also very low. There was also a strong widespread nuclear signal of OCT4 from labeled CB (b) and MSCs (c).

Discussion

The present study showed that the expression of pluripotent genes and reprogramming factors OCT-4 and Sox-2 in MSCs derived from CB were higher than BM and AD significantly. OCT-4 and Sox-2 are widely accepted as markers for embryonic stem cells as well as the reprogramming factors (22). OCT4 expression has already been reported in

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several adult somatic cells (22). Afterwards it was reported that OCT-4 expression in adult human differentiated cells challenges its role as a pure stem cell marker (23). Wagnera believed in the expression of OCT4 in adult stem cells is less certain [24]. Tai and colleagues reported that OCT4 expression in somatic cells is restricted to small populations of multipotent cells with high self-renewal capacity, namely the adult stem cells in normal tissues (22). Recently, researchers succeeded to induction of pluripotent stem cells from primary human fibroblasts with only OCT4 and Sox-2 factors (25, 27).

In relevance to Ratajczak and colleagues suggestion, OCT-4 is an embryonic transcription factor which incurred at low concentrations within somatic cells (28). The present experiment has shown that all of sample cells not only expressed OCT-4 gene but also the expression of OCT4 transcriptional factor was higher in MSCs derived from CB than bone-marrow and ADSCs significantly. In 2008 Guiting and colleagues reported that the expression of this gene in adipose-derived stem cells is very low (29).In contrast to the finding of other researchers, Izadpanah and colleagues came to the conclusion that OCT-4 is not specific to embryonic stem cells (25). Conjunction with Izadpanah and colleagues finding, our results showed OCT- 4 is not specific to embryonic stem cells (25). One possible explanation could be that MSC derived from CB and BM has some properties of embryonic stem cells while being considered adult stem cells. Our data showed CB stem cells express the main pluripotent stem cells markers OCT4 and Sox-2 more than ADSCs and BM stem cells. This might confer a more reprogrammable state for MSC from CB. The other possible explanation could be based on Bhartia hypothesis. He and colleagues in 2012 reported that the true stem cells in adult body tissues are the very small embryonic-like stem cells (VSELs), whereas the MSCs are actually progenitor stem cells that arise by asymmetric cell division of VSELs (30). According to this hypothesis, cord blood contains VSELs that are possibly lost during cord-blood banking, therefore adult autologous stem cell trials efficacy is low. It seems cord blood derived stem cells have higher number VSELs than bone marrow and ADSCs. In view of our data it can be concluded that cord blood derived stem cells are considered as a source of pluripotent stem cells that have a more regenerative potential than bone marrow and adipose derived MSCs. On the other hand, our findings revealed that the expression level of c-Myc well- known as reprogramming factors, in cord blood stem cells were significantly lower than the other cell samples. The c-Myc gene in mammals is positioned on chromosome 8 and has been considered as a regulator of %15 of the entire gene pool. It is well known that the reprogramming factors Klf4 and c-Myc are more oncogene as compared to other pluripotent genes (31-35). It means omission of c-Myc and Klf4 from the reprogramming process is important since reactivation of the c-Myc virus can cause tumor formation (36). The increased expression of c-Myc is observed in %70 of human tumors because it is a notorious oncogene in human cancers (37). Observation indicates that there is an association between this gene and colon, breast, lung, and gastric cancers. Tsai and colleagues reported that overexpression of only OCT4 and Klf4 genes were sufficient to induce reprogramming without exogenous or endogenous c-Myc (26). Here we report that MSCs derived from AD endogenously express high levels of c-Myc therefore we propose that these cells can be reprogrammed into iPS cells mere by OCT-4 expression. Since the reprogramming factors Klf-4 and c-Myc are induced as oncogene, high level expression of these genes in MSCs derived from AD indicate that use of these cells in regenerative medicine may be followed by carcinoma. In contrast to stem cells derived from AD, our data revealed MSCs from CB and BM expressed c-Myc genes at a lower level. It seems there is relationship between oncogenic property of ADSCs and Gletting' data. Gletting and colleague found that adipocyte assist in suppressing human hematopoietic stem cells differentiation and aid in prolonging their survival in vivo (38). We know hematopoietic stem cells cannot be maintained in vitro for extended time periods because they rapidly differentiate or die. To extend in vitro culture time, researchers have made attempts to use human mesenchymal stem cells in addition adipocyte to create feeder layers that mimic the stem cell niche (38). It seems OCT-4 and Sox-2 act as reprogramming genes and c-Myc function by aiding in the activation of pluripotent genes. It may be concluded due to the tumorogenic properties of Klf4 factor, its increased expression in ADSCs could be catastrophic.

Based on the present data considering the high expression of OCT-4 embryonic stem cell marker, MSCs from CB are propose as the more appropriate candidate for cellular therapy as compared to MSCs derived from the BM and AD.

Conclusion

Mesenchymal stem cells derived from CB are the more appropriate candidate for cell therapy when compared to BM and adipose tissues. Based on our data, adipose tissue derived stem cells express high endogenous levels of oncogenic factors c-Myc while represent a low level of pluripotency and reprogramming factors OCT4 and Sox2 as compared to the other groups. It could be concluded that MSCs derived from human cord blood have lower oncogenic potential compared to Bone Marrow and Adipose Tissues

Figure legend

Figure1: phase contrast microscopy image of mesenchymal stem cells derived from cord blood in the second passage.

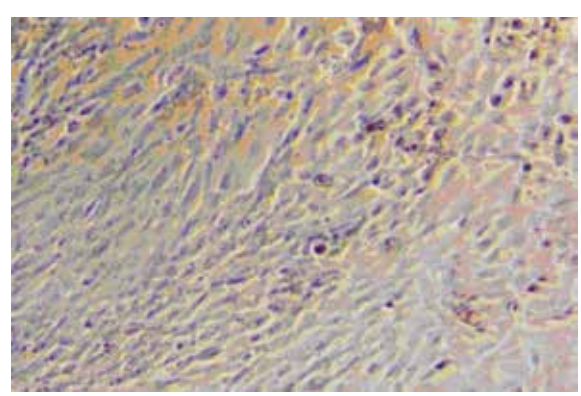

Fig 2: Comperative real time PCR analysis of OCT4, Sox-2, c-Myc and P53 genes expression in MSCs derived from BM, CB, and AD tissues (A): Expression of OCT-4 gene in MSCs derived from CB is significantly higher than BM and adipose tissue. Also the expression level of OCT4 gene in MSCs of BM was higher than ADSCs significantly. The expression level of c-Myce was significantly higher in ADSCs than CB and BM. The most lower of expression of c-Myce was detected in MSCs of BM. A significantly much lower level of P53 gene ex-

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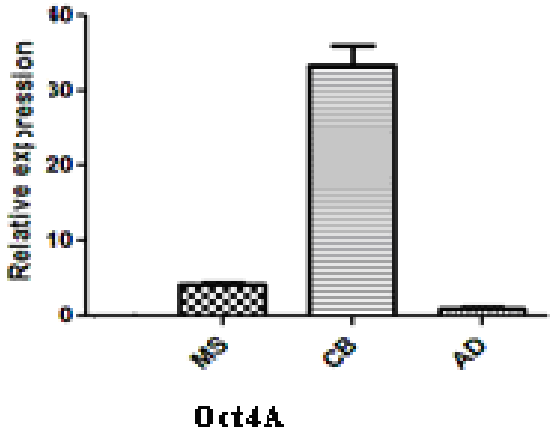
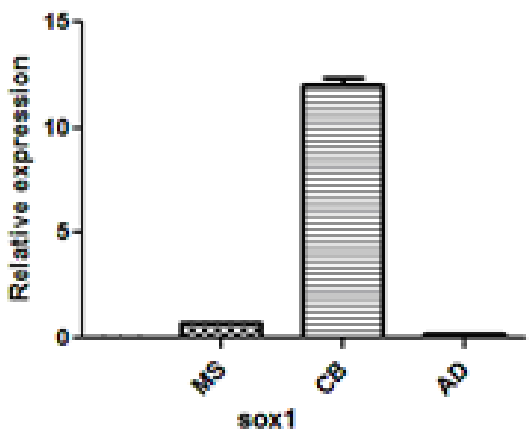
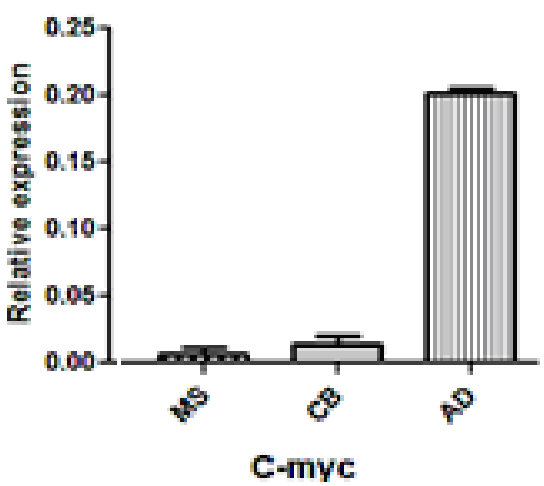
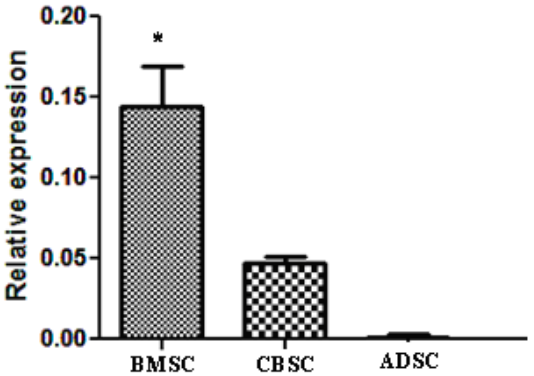
pression was detected in the ADSCs compared to the other cell types. Much higher level of P53 gene expression was found in CB than BM mesenchymal stem cells significantly. *P<0.05



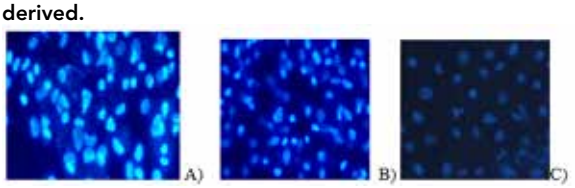






P53

Fig3. Immunostaining analysis of OCT4A in MSCs derived from CB (a), BM (b) and AD (c) tissues. Immuno cytochemistry was performed using a specific monoclonal antibody raised against the OCT4A peptide (sc-5279) antibody that can recognize OCT4A. The antibody gave a nuclear signal in all of sample cells, which colocalized with the Hoechst 33258 staining (blue signals). Although nuclear signal could be detected in each of three group cells, a widespread signal was observed in MSCs of CB compare to the other cells (a). Widespread staining intensity of OCT4 in ADSCs (d) was no or very low. Abbreviations: MSCs, mesenchymal stem cells;CB, cord blood; BM, bone marrow; AD, adipose derived.



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