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Original Article

Comparison of the Differentiation Potential and Characteritics of Mesenchymal Stem Cells Isolated from Human Umbilical Cord Blood and Bone Marrow to Hepatocyte-like Cells

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Abstract

Background: In recent years we have successfully adopted an in vitro hepatogenic differentiation of mesenchymal stem cells (MSCs). In this protocol the biologically active hepatocyte-like cells were differentiated from the stem cells isolated from either bone marrow or umbilical cord blood (UCB) samples. The aim of the present study was to compare the efficiency of the hepatogenic differentiation of MSCs isolated from UCB and MSCs .
Methods: Differentiation process of MSCs was carried out in a selective medium supporting hepatogenic differentiation for 3 weeks. Then using specific markers we have examined the hepatocyte formation following hepatogenic differentiation of the stem cells. Hepatogenic markers namely albumin, α-fetoprotein (AFP) and cytochrome P450 3A4 (CYP3A4) were monitored at different time intervals during differentiation.
Results: Transdifferentiation of the UCB and bone marrow MSCs was also characterized by measuring albumin, AFP and CYP3A4 at mRNA levels using reverse transcription polymerase chain reaction (RT-PCR). AFP was expressed in the undifferentiated UCB-MSCs and increased on day 21 of differentiation. However, AFP was not detected in the undifferentiated bone marrow MSCs. But, AFP expression started during the first week of differentiation. Albumin expression was detected in hepatocytes from UCB as well as bone marrow. The expression of albumin and its secretion from hepatocyte prepared from bone marrow appeared earlier compared to the cells derived from UCB .
Metabolic function of the hepatocytes evaluated by secretion of albumin in the culture media was also similar in the cells isolated from both the sources.
Conclusions: The differentiation potential of MSCs derived from human UCB and bone marrow under in vitro condition is comparable. However, it appears that there is time-dependent difference in the onset of expression of liver specific markers particularly albumin synthesis in hepatocytes derived from different stem cells.

Keywords: Stem cells; Hepatocyte; Differentiation; Umbilical cord blood; Bone marrow; Albumin.

Introduction

Mesenchymal stem cells (MSCs) were first introduced by Friedenstein et al (Friedenstein et al., 1970), when they isolated these cells from human bone marrow. Later on the capacity of MSCs to differentiate into mesoderm-derived tissue and their role in controlling the hematopoietic niche has also been reported (Friedenstein., 1974).

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In 1980s, the multi-potential capacity of MSCs was demonstrated by showing their ability to differentiate into osteoblasts, chondrocytes and adipocytes (Piersma et al., 1985; Caplan et al., 1986).The ability ofMSCs to transdifferentiate into ectoderm-derived tissue has also been reported by Kopen et al (1999).

MSCs have been isolated from different sources such as bone marrow (Pittenger et al., 1999), adipose tissue (Zuket al., 2001), synovial tissue(De Bari et al., 2001), lung tissue (Sabatini et al., 2005), umbilical cord blood (Erices et al., 2000), and peripheral blood (Zvaifler et al., 2000). Regardless of the source, the MSCs are heterogeneous and have variable growth potential, but their surface markers and differentiation potential into mesoderm are similar (Baksh et al., 2007). More recently it has been reported that MSCs isolated from various adult tissue sources have different morphology, differentiation capabilities, and gene expression (Nombela-Arrieta et al., 2011).

MSCs have been used for liver regeneration in a chemically-induced liver damage in an animal model (Piryaei et al., 2011). The clinical application of human MSCs in treatment of chronic liver diseases has been reviewed (Allameh and Kazemnejad, 2012).The multipotency of MSCs with unlimited potential are believed to be a suitable source of cells promising for liver regeneration(Banas et al., 2007). In recent years we have developed a 2-step protocol for trans-differentiation of human bone marrow MSCs to functionally active hepatocyte-like cells (Kazemnejad et al., 2008, Kazemnejad et al., 2009).The protocol used in our laboratory for MSCs isolation from human bone marrow as well as UCB and hepatogenic differentiation is a modified procedure worked out in other laboratories(Snykerset al., 2011; Lee et al., 2004).This protocol has been successfully applied for isolation of MSCs from human cord blood (Esmaeili et al., 2014), as well as from bone marrow (Ghaderi et al., 2011).

The differentiation potential of the MSCs is often checked by the ability of cells to differentiate into oseteoblasts and adipocytes. Hepatogenic differentiation of MSCs is usually characterized by showing expression of liver-specific markers hepatocytes following differentiation induction. However, the time-course difference in expression of liver specific markers in hepatocytes derived from MSCs of different sources has not been investigated. The aim of the present study is to compare time-dependent expression of liver markers after differentiation induction MSCS derived from two sources.

Materials and methods

Chemicals and reagents

Ficoll–Hypaque (density,1.077 g/ml), bovine serum albumin (BSA), agarose, ethidium bromide, Tris base, disodium ethylene diamine

tetra-acetate (Na2EDTA), dimethyl sulfoxide (DMSO), Triton X-100 , DEX, were the products of Sigma–Aldrich (USA).Hepatocyte growth factor (HGF), and oncostatin-M (OSM) were obtained from the Chemicon (USA). FBS, DMEM, trypsin-EDTA, L-glutamine and penicillin–streptomycin were supplied by Invitrogen-Gibco (USA). Hydroxyl ethyl starch (HES) was obtained from Fresenius (Germany). RNA extraction kit was from Qiagen (USA), cDNA synthesis kit from Bio-Rad (USA) and reagents used for polymerase chain reaction (PCR) Fermentas (Canada), albumin secretion ELISA kit from the Bethyl Laboratories, USA.

Isolation and culture of MSCs from BM and UCB

BM aspirates (10 ml) were obtained from iliac crests of human donors (aged 19- 32 years) at the Bone Marrow Transplantation Center, Shariati Hospital, Tehran, Iran. The BM samples were obtained after the informed consent of the donors according to the guidelines of the Medical Ethics Committee, Ministry of Health, Iran. The UCB sample used in this study was obtained from UCB collected from a full-term infants with the informed consent of the mother. BM or UCB mononuclear cells (MNC) were isolated by 30-min centrifugation (400*g*) on Ficoll density gradient 1.077 (Ficoll-HyPaque). The collected ring of MNCs was washed with PBS and the cells were recovered by centrifugation at 400×g for 10 min. The isolated MNC layers were then washed in PBS, resuspended in growth medium containing DMEM-low glucose supplemented with 15% (v/v) FBS, 2-mM glutamine, 100 µg/ml of streptomycin, 100 U/ml of penicillin and plated in 75-cm^2 polystyrene plastic tissue-culture flasks. The cell cultures were maintained at 37°C in a humidified 5%CO_2 incubator. Following 3 or 4 days of incubation, the non-adherent cells were washed away leaving behind the adherent cell population that was growing as fibroblastic cells in clusters. The hematopoietic stem cells and non-adherent cells were removed with every 3-4 days change in medium. When the cells reached 70-90% confluence, the cultures were harvested with 0.25% (w/v) trypsin-EDTA solution and plated in 25 Cm^2 plastic cell culture flasks at a density of 10^4 cells/Cm^2.

Characterization of MSCs

Flow cytometric analysis of hBMSCs:

The hBMSCs were detached from the tissue culture flasks after 14-33 days *in vitro* with trypsin/EDTA and counted. About 2×10^5 cells were divided into aliquots and centrifuged at 1000 rpm for 5 min at RT. The pellet was resuspended in human serum and incubated for 30 min on ice. After centrifugation at 1000 rpm for 5 min, the pellet was resuspended in

3% (v/v) human serum albumin (HSA)/PBS and incubated with appropriate antibodies including Fluorescent isothiocyanate (FITC)-conjugated mouse anti-human CD44 (H-CAM), CD13, CD105 (Endoglin or SH2), CD34 and Phycoerythrin (PE)-conjugated CD166 (ALCAM), CD45 (leukocyte common antigen) for 1 hour on ice, washed twice in PBS and centrifuged for 5 min. The cells were resuspended in 100 µl of PBS and studied by a Coulter Epics-XL flow cytometer (Beckman Coulter, CA). An isotype control with FITC- or PE-labeled antibodies was included in each experiment, and specific staining was measured from the cross point of the isotype using a specific antibody graph. The corresponding histograms were created by Win MDI 2.8 software (Scripps Institute, CA).

Osteogenic and adipogenic differentiation of hBMSCs:

The potential of the isolated cells to differentiate into osteogenic and adipogenic lineages was confirmed. For osteogenic differentiation, the hBMSCs were induced for two weeks by Modified minimum essential medium (α-MEM) supplemented with 10% (v/v) FBS, 0.1 µMDEX, 10 µMβ-glycerophosphate, and 50 µMascorbate-phosphate (Covas*et al*., 2003; Kim *et al*., 2004). The culture medium was changed twice a week for up to two weeks. The cells were fixed with methanol (90%) for 10 min at RT and identified by specific histochemical staining for calcium, using the Alizarin red staining kit. The stained material was examined with a phase contrast microscope (Nikon, Japan). For adipogenesis, the cultured cells were incubated in the adipogenic medium DMEM supplemented with 10% (v/v) FBS, 1 µMDEX, 200 µM indomethacin, 1.7 µMinsulin, 500 µM isobutylmethylxanthine, 0.05 U/ml penicillin, and 0.05 µg/ml streptomycin for two weeks. After 14 days, the cultured cells were detected for the presence of adipocyte, using the oil red o-staining procedure. Briefly, the cells were fixed in a 10% (v/v) solution of formaldehyde in aqueous phosphate buffer for 1 hour, washed with 60% isopropanol and stained with oil red o-solution for 10 min, followed by repeated washings with distilled water prior to destaining in 100% (v/v) isopropanol for 15 min (Janderovaet al., 2003; Ramriezet al., 1992).

Induction of *in vitro* hepatogenic differentiation of BM and UCB MSCs

The MSCs from two sources BM or UCB at third passage (2×10^4 cells/cm^2) were seeded on a 24-well tissue culture plate and incubated for 48 h at 37 $^{\circ C}$ in a humidified 5% CO_2 incubator. Hepatocyte differentiation and characterization were performed using a two-step protocol employing HGF, DEX and OSM, as described in our recent publication (Kazemnejad et al., 2007). Briefly, the cells were cultured in a culture medium consisting of low-glucose

DMEM supplemented with 20% FBS, 20 ng/ml HGF and 0.1 mM DEX for 7 days. Then OSM was added at a concentration of 10 ng/ml and maintained for 14 days in order to promote hepatocyte differentiation.

Characterization of hepatocyte-like cells

Expression of liver markers in hepatocyte-like cells

Albumin, AFP, CYP3A4 in differentiated hepatocyte-like cells from BM-MScs and UCB-MSCs were measured byRT-PCR. Briefly, total RNA was isolated from the cells using RNasy Mini kit. The cDNA was synthesized using the iScript™ cDNA Synthesis Kit. PCRs were conducted using 1µl of cDNA to amplify a number of the target genes by using specific primers (Table1).

PCR reaction comprised of : 2.5µl10X buffer, 1.5 mM $MgCl_2$, 1mM deoxynucleotide triphosphate, 1pmol reverse and 0.2 pmol forward primers, 3 U TaqDNA polymerase, 1µlcDNA, and water to a volumeof25µl.

PCR amplification program was an initial denaturation 3 min at 95$^{\circ C}$, followed by 35 cycles of 30 s at 95$^{\circ C}$, 45 s at 59-63$^{\circ C}$ and 1 min at 72$^{\circ C}$ and a final extension 7 min at 72$^{\circ C}$ (annealing temperature was set for each primer as shown in table1). The amplified DNA fragments were electrophoresed on a 2% (w/v) agarose gel. The gels were stained with ethidium bromide (10 µg/ml) and photographed on a UV transilluminator (Uvidoc, UK).

Metabolic activity of the hepatocyte-like cells

Albumin secretion in culture media was used as an index of metabolic function of the hepatocyte-like cells. For this purpose, at different stages of differentiation (days 0, 7, 14, 21 and 35), culture media of hepatocyte differentiating cells was collected and analyzed for albumin secretion by using ELISA kit. This analysis was performed according to manufacturer's instruction using a microplate reader ELISA (TECAN, Austria).

Statistical analysis

All the statistical analyses were performed using the SPSS statistic version 17 software. Data were analyzed using one-way ANOVA in cell samples collected at time intervals during hepatocyte differentiation. All experiments were carried out in triplicate and the data are presented as mean ± standard deviation (SD). Wherever indicated P value <0.05 is considered as significant.

Results

Morphology of MSCs

The MSCs isolated from human bone marrow as well as those separated from UCB were examine under microscope for their morphological characteristics. The MSCs appear as cylindrical in shape regardless of the

source of the cells. The cells were cultured routinely on DMEM-LG culture media and cells in passage 3-5 when reached confluency were used for hepatogenic (Fig. 1 section A).

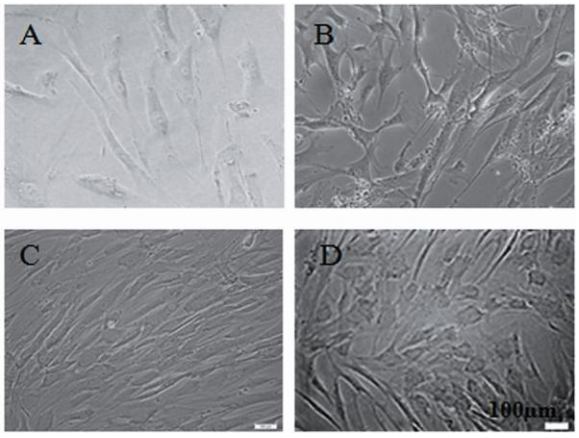

Fig.1. Morphology of cells during in vitro hepatocyte differentiation from UCB-MSCs. Undifferentiated MSCs (A), day7 (B), day14 (C) and day21 (D)

Characterization of MSCs

Morphological studies of differentiated hepatocyte-like cells

Hepatocyte-like cells differentiated from MSCs either bone marrow or UCB showed that the cells were changed from original cylindrical shape to spherical-shaped cells. These morphological changes were often observed in cells from day 14 of differentiation (Fig. 1). The hepatocyte-like cells from both the sources could be maintained in culture for up to 35 days.

Expression of liver markers during the differentiation

The liver specific markers measurement in hepatocyte-like cells from UCB- MSCs showed that in case of bone marrow stem cells there was no detectable expression of albumin, AFP and CYP3A4 at mRNA levels in undifferentiated MSCs (Fig. 2, section A). However, the cells collected 10 days after hepatogenic differentiation expressed a considerable amount of albumin and AFP specific mRNA. Expression of albumin and AFP were further increased in cells collected on day 21 of differentiation.

In case of MSCs isolated from UCB, the undifferentiated cells expressed small amount of AFP specific mRNA. However very little albumin and CYP3A4 expression was detected as measured by RTPCR (Fig. 2, section B).The hepatocytes differentiated from UCB expressed considerable amounts of AFP, albumin and CYP3A4 at their mRNA levels after 21 and 35 days of differentiation.

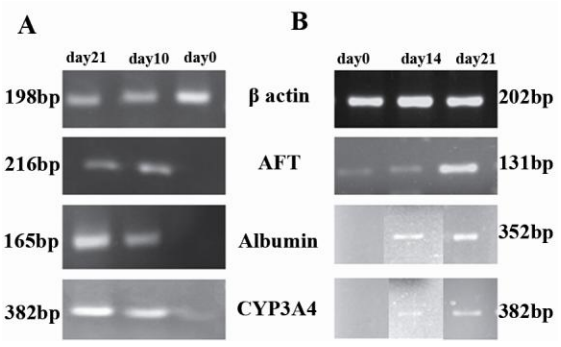

Fig. 2. RT-PCR analysis of liverspecific genes expression in hepatocyte-like cells from BM-MSCs (section A) and UCB-MSCs (section B). Expression of α-fetoprotein (AFP), CYP3A4 during in vitro hepatocyte differentiation.βactin used as internal control.

Metabolic function of hepatocyte-like cells

The ability of cells to synthesis albumin, the culture media was collected on days 0, 7, 14and 21 of differentiation and then albumin level was determined. As shown in figure 3 the albumin secretion in the media by hepatocyte-like cells differentiated from MSCs from either UCB or bone marrow started from the second week of differentiation (Fig.3).As shown in figure 3, hepatocyte-like cells derived from BM-MSCs begin to secret albumin on day7 and increase on day 21 but the cells differentiated from UCB-MSCs begin to secret albumin on day 14 and increase on day 21.

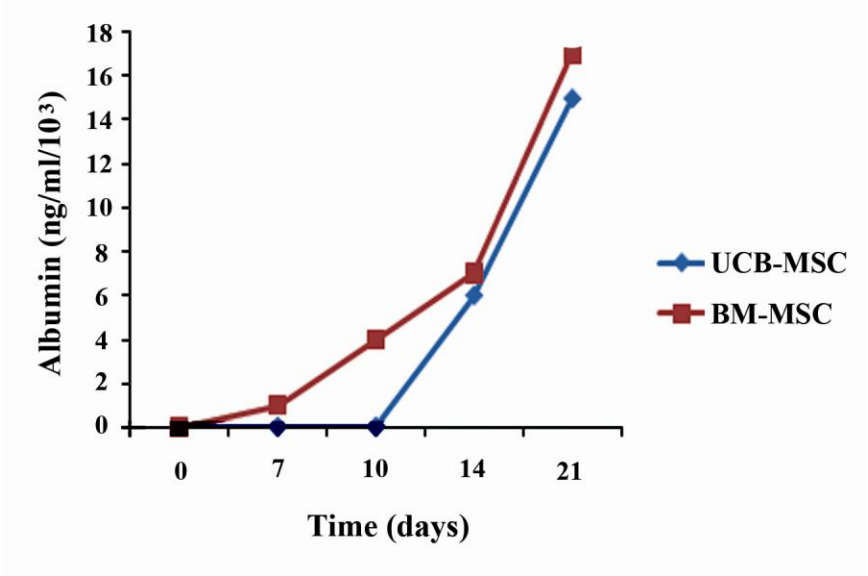

Fig.3. Comparison of the rate of albumin secretion during in vitro hepatogenic differentiation.

Discussion

The stem cell therapy for chronic liver disease is a challenging issue and the safety issues are the major limitation for treating human subjects with the stem cells. The source of the cells, the stage of differentiation and the expansion and transplantation of the cells are major factors in clinical application of the stem cells (Allameh and Kazemnejad 2012).

In the present study MSCs from two different sources were expanded and differentiated into hepatocyte-like cells to compare their morphological and biochemical behavior during a long-term culture. It was observed that hepatocytes derived from both the sources express liver markers such as AFP, albumin, CYP3A4. However, there was a difference in the expression time of these markers in hepatocytes derived from bone marrow or umbilical cord blood. As shown in figure 3, this

difference in albumin synthesis and secretion in was significantly different in both the preparations. In this connection it was demonstrated albumin synthesis starts earlier (day 7) compared to the cells derived from UCB. Under these conditions, the morphological changes of hepatocyte-like cells from UCB-MSCs and BM-MSCs were comparable in both cells. Both the cells showed modifications from original cylindrical shape to spherical shape resembling hepatocytes.

The hepatocytes derived from MSCs of different sources exhibited common characteristics during differentiation. For instance, morphological changes started on day 14 and completed after 3 weeks. The number and the appearance of granules in the cytoplasm were comparable suggesting that the absolute hepatocyte differentiation (Lee et al., 2004).

In this study, comparison of AFP expression at mRNA level in MSCs differentiated from BM and UCB showed considerable difference. This difference was mainly in the time of expression of AFP during 3-week hepatogenic differentiation. In MSCs derived from UCB, expression of AFP in mRNA level was detectable prior to differentiation. Following hepatocyte differentiation induction, AFP expression was elevated until day 21. MSCs derived from BM expressed very little AFP at mRNA level. However AFP expression in these cells started during the first week after hepatogenic induction.

According to Kosmacheva et al. (2011)the AFP expression is absent during differentiation of MSCs derived from UCB and bone marrow to hepatocytes suggesting that differentiated from these stem cells is complete mature in comparison with fetal liver cells. However our experience shows that the cells express AFP as well as albumin during the differentiation. The expression of AFP during hepatogenic differentiation may indicate the presence of hepatic progenitors such as oval cells (Thorgeirssonet al., 1996).

Expression of liver specific markers in hepatocytes during differentiation has been confirmed by biochemical and molecular techniques. However, very little expression of liver albumin and AFP was detected in undifferentiated MSCs. Comparison of liver specific proteins revealed that possibly the time of albumin expression differs depending on the cell source. For instance, we observed that albumin expression appears earlier (at least 3 days) in case of bone-marrow derived cells when compared to that obtained from UCB.

Like albumin, the expression of CYP3A4 was detected in differentiated hepatocytes on day 14. CYP3A4 expression started in hepatocyte-like cells differentiated from bone marrow derived MSCs earlier than thoseisolatedfrom UCB. Regardless of the cell source, the

expression of CYP3A4 implies that the cells gain liver function after 14 days of differentiation onset.

Based on this limited information it appears that there was no noticeable differences in the morphological characteristics of hepatocytes derived from either UCB or bone marrow. However, there was a time-dependent difference in the onset of expression of liver markers particularly albumin expression in both the cells.

Acknowledgment

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