Differentiation Potential of Breast Milk-Derived Mesenchymal Stem Cells into Hepatocyte-Like Cells

Mahsa Sani¹ · Sepideh Ebrahimi² · Fatemeh Aleahmad¹ · Mahin Salmannejad¹ ·
Seyed Mojtaba Hosseini^{3,4} · Gelareh Mazarei⁵ · Tahereh Talaei-Khozani¹

Received: 14 March 2017 / Revised: 17 May 2017 / Accepted: 16 June 2017 / Published online: 27 July 2017
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Abstract Human breast milk stem cells (hBSCs) contain a population of cells with the ability to differentiate into various cell lineages for cell therapy applications. The current study examined the differentiation potential of hBSCs into hepatocytes-like cells. The cells were isolated from the breast milk and were treated with hepatogenic medium containing hepatocyte growth factor, insulin-like growth factor and dexamethasone for 7 days subsequently; Oncostatin M was added to the culture media. RT-PCR and immunocytochemistry were performed to detect the hepatogenic markers. The glycogen storage and the ability of the cells to absorb and release indocyanin green were also tested. The data showed that most of the differentiated cells formed cell aggregates after the 30th day, with more cells accumulated to form spheroids. RT-PCR revealed the expression of the hepatic nuclear factor, albumin, cytokeratin 18 and 19, cytochrome P2B6, glucose-6-phosphatase and claudin. The functional assays also showed glycogen storage and omission of indocyanine green. Our study demonstrated hBSCs are novel population that can differentiate into hepatocyte-like cells.

Keywords Cell culture · Differentiation · Hepatocyte · Human breast milk · Stem cell

1 Introduction

To overcome the shortage of liver donors, cell therapy could be an alternative for people on transplant waiting lists. Along with other sources of stem cells, breast milk is

introduced as a new source of cells for therapeutic application. The human breast milk contains a heterogeneous cell population. Although, there is a controversy about the origin and the type of cells isolated from the breast milk, the presence of the mammary stem cells (MaSCs) [1] and stem cells from other origins, such as the mesenchymal stem cells (MSC) [2, 3] in the human breast milk, has been proposed. The Pluripotency, as an important property of stem cells, has been demonstrated by showing their differentiation potency into mesenchymal (3), ectodermal [1, 2] and endodermal [1]. Cell lineages of human breast milk cells show differentiation potential into adipocytes, osteocytes, and neurons *in vitro* [3, 4]. The use of human breast milk as a source of stem cells not only does help to avoid invasive procedures and the ethical controversy, but is also considered as an abundant source of pluripotent stem cells. CD marker profile of breast milk-derived cell displays expression of mesenchymal stem cell (MSC) markers such as CD90, CD44, CD271, and CD146. Also a subpopulation of these cells is known to express the

✉ Tahereh Talaei-Khozani
talaeit@sums.ac.ir

¹ Laboratory for Stem Cell Research, Anatomy Department, Shiraz Medical School, Shiraz University of Medical Sciences, Shiraz, Iran

² Department of Biochemistry, Shiraz Medical School, Shiraz University of Medical Sciences, Shiraz, Iran

³ Student Research Committee, Shiraz University of Medical Sciences, Shiraz, Iran

⁴ Cellular and Molecular Research Club, Shiraz University of Medical Sciences, Shiraz, Iran

⁵ Department of Medical Genetics, Centre for Molecular Medicine and Therapeutics, University of British Columbia, Vancouver, BC, Canada

embryonic stem cell markers, such as TRA 60-1, Oct4, Nanog, and Sox2 [3]. With regard to the pluripotent capability of the hBSCs, it can be proposed that at least a subpopulation of the cells isolated from breast milk can be considered as stem cells. An ongoing investigation has been to differentiate hepatocytes from stem cells. Various sources of stem cells such as human bone marrow [5], Wharton's jelly derived-MSCs [6], adipose-derived MSCs [7], menstrual blood derived-MSCs [8] and embryonic stem cells [9] have been used to differentiate into hepatocytes. The potential of hBSCs to differentiate into hepatocytes was also shown in a previous study [1]; however, the study just showed the expression of the hepatocyte markers including alpha-fetoprotein and albumin, that could be expressed by endoderm as well [10]. Some hepatocyte-specific genes such as albumin, alpha-fetoprotein (AFP), cytokeratin-19 (CK-19) express in the early stage of hepatocyte differentiation, meanwhile, the others including CK18, cytochrome P2B6 (CYP2B6), and glucose-6-phosphatase (G6P) were expressed in mid- and late stages [11]. Hepatic nuclear factor 4 (HNF4), as a key regulator of hepatocyte-specific genes, also plays a pivotal role in hepatocyte differentiation [12]. At this point, no comprehensive data are available on the potential of hBSCs to hepatocytes. Therefore, we conducted a research on hepatic differentiation ability. The objective of the present study was to examine the differentiation potential of the hBSCs into functional hepatocytes *in vitro*.

2 Materials and methods

2.1 Chemical and reagents

Dulbecco's modified Eagle's medium (DMEM, pH 7.4), fetal bovine serum (FBS), trypan blue, knock out serum replacement (KOSR), 2 mM L-glutamine, non-essential amino acids, and Fangizone were prepared from Gibco Co. penicillin and streptomycin and Trypsin–EDTA 0.05% were obtained from Bio Idea. Basic-fibroblast growth factor was provided from Stem Cell Technologies Co. and β -Mercaptoethanol was purchased from Sigma. Antibodies for flowcytometric and Immunofluorescence analysis were prepared from Abcam, Dako, Santa Cruz Biotechnology, and Sigma-Aldrich.

2.2 Sample collection

Breast milk samples were collected from 15 healthy volunteers with a mean age of 22–30, from the day of delivery to 6 months postpartum. All volunteers signed the informed consents approved by the Shiraz University of Medical Sciences Ethics Committee for providing the samples. The samples were collected in sterile tubes

(5–50 ml) and then transported to the laboratory in sterile condition to isolate the cells.

2.3 Isolation and culture of human breast milk-derived cells

Each sample was diluted 1:2 with Dulbecco's Modified Eagle's Medium (DMEM) (Gibco, USA) (pH 7.4) and centrifuged at 300 g [2] for 20 min at room temperature. The cell pellet was washed twice with DMEM containing 7% fetal bovine serum (FBS). The Trypan blue exclusion assay was performed to evaluate the cell viability. Thereafter, the pellets were re-suspended in DMEM/F12 supplemented with 15% knock out serum replacement, 2 mM L-glutamine (Gibco), 100 U/mL of penicillin (Bio Idea) and 100 μ g of streptomycin (Bio Idea), 10 ng/ml basic-Fibroblast Growth Factor (Stem cell Technologies), 1% non-essential amino acids (Gibco), 0.1% fangizone (Gibco), 0.1 mM β -Mercaptoethanol (Sigma). Cells were then cultured at 37 °C in 95% humidity and 5% CO₂. The culture medium was refreshed every 24 h. After 2–3 days, the cultures were washed with phosphate buffer saline (PBS) to remove the non-adherent cells. Contaminated cells were omitted by sequential passaging.

2.4 Hepatogenic differentiation

In order to evaluate the hepatogenic differentiation of hBMSc, the cells at passages 2–3 were divided into two, hepatogenic-treated and control cultures; and were seeded at 5×10^4 /well in a 24 well plate where they were exposed to hepatogenic medium. In the first phase, the hepatogenic-treated cells were exposed with DMEM supplemented with 10% FBS, 20 ng/ml hepatocyte growth factor (HGF), 10 ng/ml insulin-like growth factor-1 (IGF-1), 10^{-7} M dexamethasone for 7 days. In the second phase, Oncostatin M (OSM) was added at a concentration of 10 ng/ml and the culturing was continued up to 30 days [13]. The culture medium was replaced every three days. The control cells were cultured in the absence of hepatogenic media. The experiment was performed in triplicate for each sample.

2.5 Immunofluorescent staining

The differentiated cells were fixed with 4% paraformaldehyde at room temperature for 20 min. The fixed cells were permeabilized with PBS containing 0.1% triton X-100. The non-specific binding sites were then blocked with PBS containing 10% goat serum. The washed cells were incubated for 1 h at room temperature with primary antibodies against FITC-conjugated anti-albumin (ALB, 1:50, Dako) and non-conjugated anti-cytokeratin 18 (CK-18, 1:100, Abcam, UK, Cambridge), -cytokeratin 19 (CK-19, 1:100 Abcam, UK, Cambridge) and -alpha

fetoprotein (AFP, 1:100, Abcam, UK, Cambridge) primary antibodies. The cells were washed and subsequently incubated with FITC-conjugated donkey anti-rabbit IgG (1:100, Santa Cruz Biotechnology) for CK 18 & 19 and goat anti-mouse IgG (1:160, Sigma-Aldrich) for AFP secondary antibodies at 37 °C for 45 min in the dark. The cells were counterstained with Hoechst at a ratio of 1:1000 (Sigma-Aldrich). The data were obtained by using a Niconfluorescent microscope (Nicon, ECLPSE E600, Tokyo, Japan).

2.6 Reverse transcription–polymerase chain reaction (RT–PCR)

RT-PCR was used to evaluate the expression of ALB, AFP, CK19, CYP2B6, HNF4, G6P, and claudin in the differentiated cells. Total RNA was isolated from the cultured cells with Biazol isolation reagent (Bioflux). The quantity of RNA was estimated by a spectrophotometer (Eppendorf, Germany). Standard RT was performed using a Reverse RevertAid First Strand cDNA Synthesis Kit (Thermo scientific), 1 µg of total RNA and 0.5 µg oligo (dt18) per reaction, according to the manufacturer's instructions. The cDNA samples were subjected to polymerase chain reaction (PCR) amplification using human specific primers (Table 1). Amplification conditions were as follows: Initial denaturation at 95 °C for 3 min followed by 30 cycles of denaturation at 94 °C for 30 s, annealing at 60 °C for 30 s, extension for 45 s at 72 °C. The amplified DNA fragments were electrophoresed on a 2% agarose gel and visualized using a UV transilluminator (Uvidoc, Cambridge, UK). As a positive control, human hepatoma cell line, HepG2, (Pasteur Institute, Tehran, Iran) was used. Syngene software was applied to quantify the gene expression.

2.7 Glycogen storage assessment

Intracellular glycogen was detected by periodic acid–Schiff (PAS) staining. The differentiated cells were fixed in 4% paraformaldehyde. The slides were oxidized in 1% periodic acid for 5 min and washed three times with deionized water. Subsequently, the plates were treated with Schiff's reagent for 15 min [14]. Finally, to remove the excess dye, the cells were washed with deionized water.

2.8 Indocyanine green test

To determine the cellular uptake of Indocyanine green (ICG), 1 mg/ml of ICG (Sigma-Aldrich) was dissolved in William's medium (PAA, Cambridge, England) containing 10% FBS and the solution was added to the differentiated cells. The cells were incubated at 37 °C and 5% CO₂ for 30 min. Subsequently, the cells were washed three times with PBS and the cellular uptake of ICG was examined by an inverted microscope (Olympus, CKX41). To determine the ICG release, the medium was replaced with a fresh one and re-incubated for an additional 6 h [15]. The optic density of the collected culture medium was evaluated at 820 nm. Human hepatoma cell line (HepG2) and undifferentiated cells were used as controls.

2.9 Statistical analysis

The data were analyzed using Mann–Whitney test. The subsequent analyses were performed by LSD. All graphs were depicted by a prism. A *P* value less than 0.05 was considered as significant.

Table 1 The primer sequences used for RT-PCR

Primer	Sequence	Annealing temperature (°C)	Product size (bp)	NCBI accession no.
ALB	F 5'-ACAGAGACTCAAGTGTGCCAGT-3' R 5'-GCAAGGTCCGCCCTGTCATC-3'	60	198	NM_000477.5
CK-19	F 5'-ACTACACGACCATCCAGGAC-3' R 5'-CCGTCTCAAACCTGGTTCGGA-3'	60	126	NM_002276.4
CYP2B6	F 5'-TTCTTCCGGGGATATGGTGT-3' R 5'-TCCCGAAGTCCCTCATAGTG-3'	55	91	NM_000767.4
G-6-P	F 5'-CGACGAAGCGCAGACAG-3' R 5'-GTATCCGACTGATGGAAGGC-3'	60	113	NM_000402.4
HNF4	F 5'-AAGAAATGCTTCCGGGCTGG-3' R 5'-GACGGGGGAGGTGATCTGTC-3'	60	156	NM_001287184.1
Claudin	F 5'-CTTCTTGCAGGTCTGGCTAT-3' R 5'-AGGTTGTTTTTCGGGGACAG-3'	60	196	NM_021101.4
GAPDH	F 5'-CTCTCTGCTCCTCCTGTTTCG-3' R 5'-ACGACCAAATCCGTTGACTC-3'	60	114	NM_002046.5

F forward, R reverse

3 Results

3.1 Morphology of stem cells derived from breast milk

A heterogeneous cell population of breast milk-derived stem cells was detected in the culture flasks one day after isolation. Some cells formed colonies while others were fibroblast-like. After 10 ± 2 days, the size of the colonies got smaller and two types of cells, fibroblast-like and round cells were observed predominantly. The fibroblast-like cells comprised the majority of cell types (Fig. 1A, B).

Fig. 1 The morphology of breast milk-derived cells. **A** Human breast milk-derived stem cells formed small colonies along with some fibroblast-like cells. **B** The cells expanded from the colonies and produced more fibroblast-like cells. **C** The cell morphology was

3.2 Hepatogenic-treated cells

As shown in Fig. 1C, the cell morphology changed after exposing the cells to hepatogenic medium. The frequency of the round and flat cells increased in cell cultures; however, no morphological changes were observed in the control cultures. Some cells formed large aggregates with multinucleated cells among the ordinary cells.

3.3 Immunocytochemical staining

The production of ALB, CK-18, CK-19, and AFP was assessed by immunostaining and fluorescent microscopy.

Fig. 2 Expression of hepatogenic markers such as ALB, AFP, CK-18 and CK 19 in differentiated hBSCs was examined by immunofluorescence staining. The cell aggregates, hepatosphers, were formed by hepatocyte-like cells. As positive and negative controls, HepG2 cell line and undifferentiated cells were used, respectively. Scale bar for all figures is the same (50 µm)

Fig. 3 The expression of hepatocyte-specific genes showed by RT-PCR. The level of expressions were semiquantified by syngene software and the graphs compare the expression level of the genes with positive (HepG2 cell line) and negative (undifferentiated cells) controls

The hepatocyte-like cells were stained positively for CK-18, CK-19 and AFP on the 30th day. The percentages of the differentiated cells and the intensity of reactions to antibodies increased significantly compared with those in the undifferentiated cultures. The ALB was detected in both differentiated and undifferentiated cells (Fig. 2).

3.4 Hepatic specific gene expression

The mRNA expression of hepatocyte-specific genes, such as HNF4, ALB, CK19, CYP2B6, G6P and Claudin was detected in hepatocyte-like cells differentiated from hBSCs (Fig. 3). In this study, the undifferentiated hBSCs did not express mRNA of the hepatocyte genes except for ALB. The semi-quantitative RT-PCR showed that the amount of

CK19, CYP2B6 and G6P was comparable with that of the positive control. The hepatocyte-like cells expressed a lower amount of HNF4 mRNAs compared with the positive control. On the other hand, claudin was expressed in a higher amount in hepatogenic-treated cells compared with positive control. All cultures, including experimental, negative and positive control ones, were found to express albumin.

3.5 Functionality assay

Indocyanine green (ICG) is a cyanine dye which is used for evaluating the hepatic function. ICG uptake was shown by differentiated hepatocyte-like cells; however, the undifferentiated cells could not absorb and omit ICG. Hepatoma

Fig. 4 **A** The functional test of differentiated hepatocyte-like cells showed they performed indocyanine green uptake, an organic anion that is clinically used as a test substance to evaluate liver function; **B** HepG2 cell line as positive control uptake and released ICG, while the undifferentiated cells could not absorb and omitted it. **C** The

graph shows a significant increase in indocyanin release in differentiated cells compared with negative control. **D** Differentiated hBSCs in the presence of hepatogenic medium at day 30 displayed glycogen granules storage; **E** whereas undifferentiated cells had a weak reaction to PAS

cells were used as a positive control. The ICG release of hepatocyte-like cell compared to the undifferentiated one was significantly (Fig. 4A, B, and C). Glycogen granules were detected in the cytoplasm of the cells exposed to hepatogenic medium. A very weak reaction to PAS was detected in the undifferentiated cells (Fig. 4C, D).

4 Discussion

The most essential metabolic processes of the human body, such as regulation of blood glucose level, metabolism of proteins and lipids, and detoxification of the urea, takes place in the liver [16]. In our previous studies, we had presented anon-invasive source and a new population of cells isolated from human breast milk [3, 4]. The current study investigates the hepatogenic potential of hBSCs within a 2D monolayer culture system, supplemented with exogenous growth factors. Our data showed the expression of endodermal markers such as albumin by naive hBSCs. Besides, it was shown that a subpopulation of these cells could express some embryonic stem cell markers such as TRA-60-1, Oct4, Sox2 and Nanog that indicate higher pluripotency of hBSCs [3]. Therefore, the expression of both endodermal and pluripotency markers make these cells as an appropriate source for hepatocyte differentiation.

Our data indicated that the differentiated cells acquired a cuboidal to polygonal shape and some of them formed cell aggregation. Cell aggregate formation by some hepatocyte

cell lines was previously detected when they were cultured in appropriate conditions. They showed that the hepatocytes initially formed a monolayer, but later they aggregated to form spheroids called hepatospheres; then, they were detached from culture dishes [16]. It has been also reported that the differentiated hepatocytes from human MSCs cultured on a biocomposite formed hepatospheres [16, 17]. Hepatosphere formation from hepatocytes has been suggested to mimic the liver tissue *in vitro*, which are attached to form bile canaliculi [18]. The current study also illustrates the formation of hepatospheres in the 2D culture system. At the same, time this study showed a significant increase in claudin expression compared with both negative and positive controls that may indicate the formation of the bile canaliculi. MSCs show a form of hepatosphere just when they are co-cultured with hepatocytes [19].

Some hepatocyte-specific markers, such as HNF4, play a vital role in hepatocyte differentiation and functions. Knockout of HNF4 led to disruption of many proteins needed for adult liver functions [12]. Our data exhibited the expression of HNF4 by hBSCs in the presence of hepatogenic medium. Cyp2B6 is one of the major cytochromes which are present in the hepatocytes and involved in the metabolism of 25% of drugs [20]. Both Cyp2B6 [21] and G6P [9, 22] can be considered as late hepatic markers. The current study also showed the expression of these late hepatic markers. These cells were also able to store glycogen and participate in the uptake and release of ICG and this indicates that the differentiated hBSCs were functional *in vitro*.

Although the fate of hBSCs in the infant's intestine remained to be clear, mouse breastmilk stem cells has been shown to migrate through the epithelium of the digestive system and settle down within neonate organs [23]. Despite pluripotency, these cells are not tumorigenic [1]; and therefore, provide a non-invasive autologous source of stem cells without any ethic concerns. hBSCs has also been suggested to preserve in cell bank for liver therapeutic application [24]. To be used as a cell source in regenerative medicine, there is a necessity to find the differentiation potential of this novel source of stem cell; and the current study showed hepatogenic potential of the hBSCs.

In conclusion, this study demonstrated the differentiation potential of hBSCs into hepatocyte-like cells; they displayed the capability to express many key factors that are important in liver functions. The current study introduced a non-invasive method to a source of stem cells that can be useful as potentially an efficient treatment in regenerative medicine.

Acknowledgements The authors wish to thank the research deputy of Shiraz University of Medical Sciences for offering the Grant no. 6810.

Compliance with ethical standards

Conflict of interest The authors indicate no potential conflicts of interest.

Ethical Approval This project was approved by the ethic committee of Shiraz University of Medical Sciences (Ec-92-6810).

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