Research Article

Neural Differentiation of Human Umbilical Cord Blood-derived Mesenchymal Stem Cells

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Abstract

Background: Cell therapy is a potential therapeutic approach for neurodegenerative disorders. Human umbilical cord blood-derived mesenchymal stem cells (hUCB-MSCs) are an appropriate source of stem cells for use in various cell-based therapies.

Objectives: In this study, we examined a real-time PCR approach for neural differentiation of hUCB-MSCs in vitro.

Materials and Methods: MSCs were cultured in DMEM medium supplemented with 10% FBS in a humidified incubator equilibration at 5% CO_2 and 37°C. For the neural differentiation of MSCs, the DMEM was removed and replaced with pre-induction media (retinoic acid [RA], basic fibroblast growth factor [bFGF], and epidermal growth factor [EGF]) and basal medium for two days. They were then cultured in nerve growth factor (NGF), 3-isobutyl-1-methylxanthine (IBMX), ascorbic acid (AA), and basal medium for six days. We also monitored the expression of markers for neural differentiation with real-time PCR.

Results: The results of real-time PCR showed that the expression of the GFAP, MBP, and MAP-2 genes after two-step induction was significantly increased compared to the common induction protocol. In addition, our study showed that RA should play the main role in the neural differentiation and fate of MSCs compared to other neural inducers.

Conclusions: Taken together, the combination of chemical and growth factors in the two-step induction protocol may improve the efficiency of MSC differentiation into neural progenitor cells, and may be a new method for the easy and fast application of MSCs in regenerative medicine in the future.

Keywords: Mesenchymal Stromal Cells, Neuron-Like Cells, Cell Differentiation

1. Background

The development of stem cells for the treatment of neurodegenerative diseases is currently the subject of intensive research efforts. Embryonic stem cells (ESCs), neural stem cells (NSCs), bone marrow (BM)-derived mesenchymal stem cells (MSCs), and adipose-derived adult stem (ADAS) cells have been shown to generate differentiated neural cells both in vitro and in vivo, which can be used as substitute therapies for various neurodegenerative diseases (1-5). However, despite the differentiation capability of these cells, ethical conflicts, legal restrictions, the invasive procedures required to obtain them, and graft-versus-host disease (GVHD) are major challenges in their development for clinical applications that emphasize exploring and evaluating different sources for use in various cell-based therapies (1, 6). MSCs have been shown to be ideal candidates for regenerative medicine (7).

In the present study, human umbilical cord blood-derived mesenchymal stem cells (hUCB-MSCs) were selected due to advantages such as lower immunogenic potential, lack of GVHD, higher capacity for differentiation into neural cells, quick accessibility, and easier and non-invasive collection after delivery compared to that of BM-MSCs (8). Furthermore, hUCB-MSCs are more primitive than BM-MSCs and MSCs from other sources. Moreover, in contrast to BM-MSCs, the differentiation potential of hUCB-MSCs does not change during frequent passages (9).

In recent years, more attention has been paid to MSC-derived NPCs (MSC-NPCs) for the treatment of neurodegenerative diseases (10-13). To date, it has been reported that MSCs could induce neural differentiation through many in vitro methods, such as chemical inducers, growth factors, and co-cultures with neural cells (14-17). However, due to differences in MSC isolation, MSC culture conditions, MSC sources, and other factors, the results of the previous studies are not compatible with each other.

2. Objectives

Based on previous reports that showed transdifferentiation of MSCs, we designed a real-time PCR approach for the neural differentiation of hUCB-MSCs in vitro.

3. Materials and Methods

3.1. Isolation of MSCs from Human UCB

The collection, isolation, and propagation of hUCB-MSCs was performed as described in the literature ([18-20]). To summarize, the mononuclear cell (MNC) fraction was obtained by Ficoll-Hypaque low-density gradient separation (mononuclear cells < 1.077 g/mL; Cedarlane, Hornby, Ontario, Canada), followed by ammonium chloride lysis of red blood cells. After being washed twice in phosphate-buffered saline (PBS; Gibco, USA), the collected MNCs were re-suspended in high-glucose Dulbecco's modified Eagle medium (DMEM; Gibco), and supplemented with 10% fetal bovine serum (FBS; Gibco), L-glutamine (Gibco), 100 U/mL of penicillin, and 100 mg/mL of streptomycin (Gibco). MSCs were cultured in 25 cm² tissue-culture flasks (Nunc, USA) in a humidified atmosphere of 95% air with 5% CO_2 at 37°C.

3.2. Flow Cytometry Analysis

After the third passage, the cells were trypsinized with 0.05% trypsin-ethylenediaminetetraacetic acid (EDTA), washed twice with PBS, then stained on ice with phycoerythrin (PE)-conjugated mouse anti-human CD44, CD45, and CD105 antibodies, and fluorescein isothiocyanate (FITC)-conjugated mouse anti-human CD34 antibodies (BD Biosciences, USA), according to the manufacturer's instructions. The cells were then incubated in the dark for 30 minutes at 4°C. To remove the unlabeled antibodies, the cells were washed with PBS containing 2% FBS (stain buffer) with centrifugation at 1300 rpm for 5 minutes. In the control group, PE-IgG1 and FITC-IgG1 were used. The stained cells (10,000 events counted) were analyzed with flow cytometry (Partec Flomax, ver 2.4e).

3.3. Neural Differentiation

The differentiation potential of the cells was examined on the fourth passage of the hUCB-MSCs. For the induction of neurogenic differentiation, 20,000 cells per well (on a 24-well plate) were cultured in DMEM supplemented with 10% FBS in a humidified incubator equilibration with 5% CO2 at 37°C. For the neural differentiation of MSCs, first the DMEM was removed and replaced with pre-induction media containing basal medium, then supplemented with L-glutamine, 5 μM of retinoic acid (RA, Sigma), 10 ng/mL of

basic fibroblast growth factor (bFGF, Sigma), and 10 ng/ml of epidermal growth factor (EGF, Sigma) for two days. After 48 hours, induction was improved by adding 10 ng/mL of nerve growth factor (NGF, R&D Systems, USA), 0.5 mM of 3-isobutylmethyl-xanthine (IBMX, Sigma),100 μM of ascorbic acid (AA, Sigma), and basal medium for six days.

3.4. RT-PCR and Quantitative Real-Time PCR Analysis

In brief, total RNA was isolated from undifferentiated and differentiating hUCB-MSCs using an RNA isolation kit (Qiagen, USA). Synthesis of cDNA was carried out with the Moloney murine leukemia virus (M-MuLV) reverse transcriptase (RT) and a random hexamer as primer according to the manufacturer's instructions (Invitrogen), in order to confirm the expression of neural-specific genes by RT-PCR. PCR amplification was conducted using a standard procedure with Taq DNA polymerase, with denaturation at 94°C for 15 seconds, annealing at 55°C or 60°C for 30 seconds based on the primer, and extension at 72°C for 45 seconds. The PCR products were separated by gel electrophoresis on 2% agarose gel in 1 × tris-acetate-EDTA buffer and visualized with SYBR Safe staining, and then images were captured using the Bio-Rad Gel documentation system. The experiments were generally conducted in triplicate. The nucleotide sequences and the amplicon sizes of the designed primers are listed in Table 1. In order to confirm and evaluate the expression levels of neural-specific genes by quantitative real-time PCR, the cDNAs were used for a 40-cycle PCR in a Corbett Rotor-Gene 6000 Analyzer (Corbett, Germany). Quantitative real-time PCR was performed in triplicate by SYBR Green Real-Time Master Mix (Takara, Japan) in the Rotor-Gene 6000 system, followed by a melting curve analysis to confirm PCR specificity. The cycle threshold (Ct) was calculated automatically and normalization was carried out against the β-actin Ct value. Relative expression was quantified using REST 2009 software (V2.0.13).

3.5. Statistical Analysis

Two-sided paired t-test for related samples and Friedman's two-way analysis of variance by rank were used to analyze the flow cytometry. All data were analyzed using SPSS software.

4. Results

4.1. Fibroblastic Morphology and Surface Markers of hUCB-MSCs

A fibroblast-like phenotype was obtained from cord blood after three passages in vitro (Figure 1). The flow cytometry analysis of cell-surface markers in the MSCs (10,000 events) showed the expression of CD105 (84.85 $\pm$

Table 1. Amplification Cycles, Sequences, and Amplicon Size of the Specific Primers Designed for Assessing Expression of Neural Markers

Cycles	Amplicon Size, bp	Reverse Primer	Forward Primer	Marker
35	97	CAT TCT CTC TTC AGC CTT CTC	AGT TCC AGC AGC GTG ATG	MAP2
35	127	ACT CCT TAA TGA CCT CTC CAT C	GCA GAC CTT CTC CAA CCT G	GFAP
35	179	ACT CCC TTG AAT CCC TTG TG	ACC CCG TAG TCC ACT TCT TC	MBP
35	96	CCT CTT CTT CCC ATA TTT CCT G	GAA GGT GAA GGG CAA ATC TG	Nestin
35	85	GGG GTC TTT GCG GAT GTC CAC	CTT CCT TCC TGG GCA TG	β-actin

9.40, n = 3) and CD44 (94.45 $\pm$ 4.9, n = 3) (P < 0.05), but not of CD34 (1.80 $\pm$ 0.35, n = 3) or CD45 (2.5 $\pm$ 1.40, n = 3) (P < 0.05). The surface-marker patterns corresponded to UCB-MSCs. As was evidenced by flow cytometry, the isolated cells were positive for CD105 and CD44, but negative for CD34 and CD45 (Figure 1).

4.2. Differentiation Studies of MSCs

Neural morphologies (a sign of neural differentiation) were observed on the second day, when some cells were stretched in one and/or two directions. The control samples showed no changes in shape. The MSCs were differentiated into MSC-NPCs, with this characteristic being identified through morphology, RT-PCR, and quantitative real-time PCR assays (Figure 2).

4.3. Neural-Specific Gene Studies

Neural-specific gene expression levels with real-time PCR showed that Map2 was upregulated in the sample group compared to the control group (P = 0.000). GFAP was also upregulated in the sample group compared to the control group (P = 0.000). The nestin sample group was not different from the control group (P = 0.680), while MBP was upregulated in the sample group compared to the control group (P = 0.000).

The report produced by REST 2009 indicated that the neural markers in the differentiated cells were upregulated. In this study, the maximum level of gene expression was related to GFAP and the lowest level was related to nestin (Figure 2).

5. Discussion

Despite the advantages of ESCs, they are not practical for neurodegenerative diseases due to several problems (6, 21). Recently, MSCs have been the focus of intensive investigations because of their relative advantages (22-24). The generation of MSC-NPCs from hUCB-MSCs can be used for basic research in order to develop effective cells for regenerative therapy (11). In addition, recent reports have documented that MSCs can differentiate into MSC-NPCs (16,

17). These studies generated MSC-NPCs under culture conditions. In the present study, consistent with previous reports, the expression pattern (Figure 1) on flow cytometry over 10,000 events showed that CD105 and CD44 markers were positive, while CD45 and CD34 markers were negative (25, 26).

There are different methods of inducing MSC-NPCs. Tio and Wang used the same culture protocol, with some differences (27, 28). Woodbury used a culture protocol by adding in the first week of culture followed a simple medium with serum (16). The present study was conducted with a two-step induction protocol. The first step was pre-induction with basal medium, RA, bFGF, and EGF; the second step was induction with NGF, IBMX, AA, and basal medium. The time-duration for the emergence of MSC-NPCs and various types of neural markers depends on the culture system used. Tio and Wang's method showed the longest duration for which no basal medium was provided (27, 28). In contrast, we found that if B-ME was used immediately from the start, MSC-NPCs failed to form. In agreement with previous studies, in the present study, using the most common growth factors, as well as the selection of basal medium instead of FBS, was found to be an efficient method for inducing cells to be selected.

In our protocol, a significant increase occurred in GFAP, MAP2, and MBP expression, especially GFAP. Undifferentiated hUCB-MSC cells did not express neuron-specific genes and did not stain positively for neuro-specific proteins on quantitative real-time PCR and ICC, respectively.

Previous studies showed that RA combined with other factors, such as NGF, β-ME, BDNF, Forskolin, and IBMX, is necessary for the neural differentiation of MSCs in vitro (29-31). In the present study, it was found that after the combined treatment with a low concentration of RA, more than 30% of hUCB-MSCs were differentiated into GFAP-expressing cells. RA should be a main factor in the neural differentiation of MSCs compared to other inducers.

In brief, the importance of our simple method will be clear when it is compared with other methods that are complex and time-consuming. MSC-NPCs share many molecular and cellular characteristics with neural stem

Figure 1. Flow Cytometry Analysis of Cell-Surface Markers in MSCs Showed Expression of CD105 (84.85 $\pm$ 9.40, n = 3) (P < 0.05) but not of CD34 (1.80 $\pm$ 0.35, n = 3) (P < 0.05)
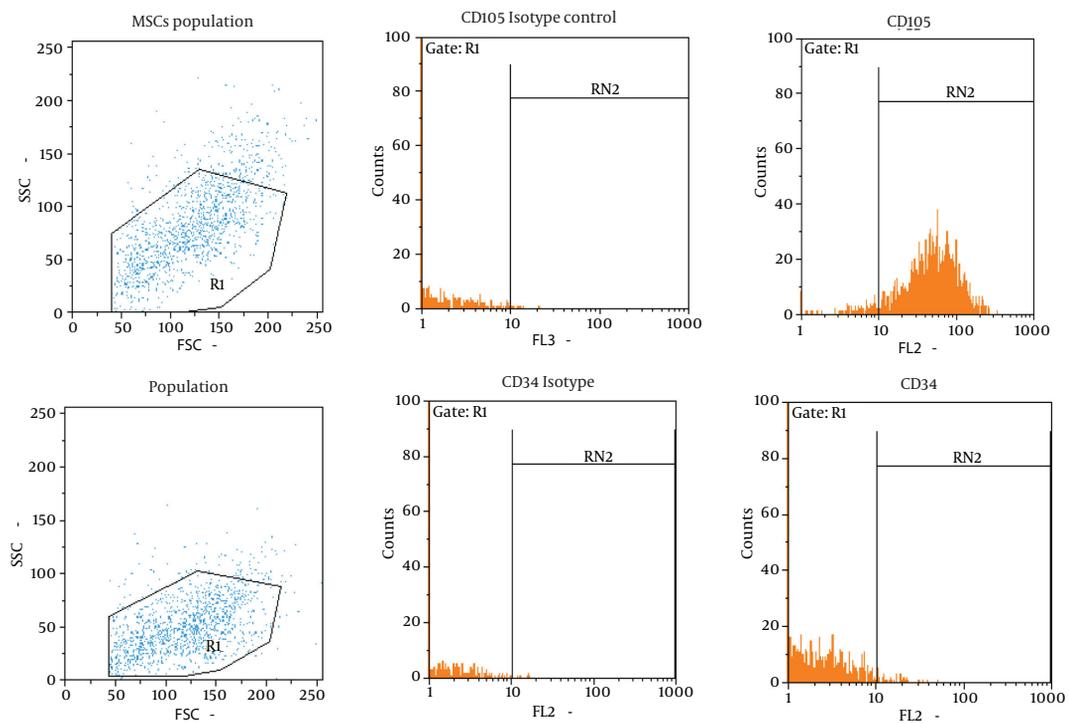
The surface-marker patterns corresponded to UCB-derived MSCs.

cells, and on a cellular level they have the same morphology, with the formation of a spheroid body structure that is stretched in one and/or two directions after in vitro culturing. In contrast to MSCs, the use of MSC-NPCs for cell transplantation results in more effective cell-based therapies; in addition, the tumor-formation problem is avoided. These characteristics suggest therapeutic uses for MSC-NPCs as a new and unlimited source in regenerative medicine. Finally, further and complementary studies are required that include identification of neural proteins with western blot. The functionality of MSC-NPCs must be carefully assessed in animal experiments before being used in clinical applications.

Footnotes

Authors' Contribution: Hassan Rafieemehr and Maryam Kheirandish designed the study, performed the data analysis, prepared the manuscript, approved the final version, and supervised the study. Masoud Soleimani collected the data, prepared the manuscript, and performed the cell differentiation.

Figure 2. Neurogenic differentiation Capacity of UCB-MSCs and Morphological Appearance of Neural-Differentiated UCB-MSCs
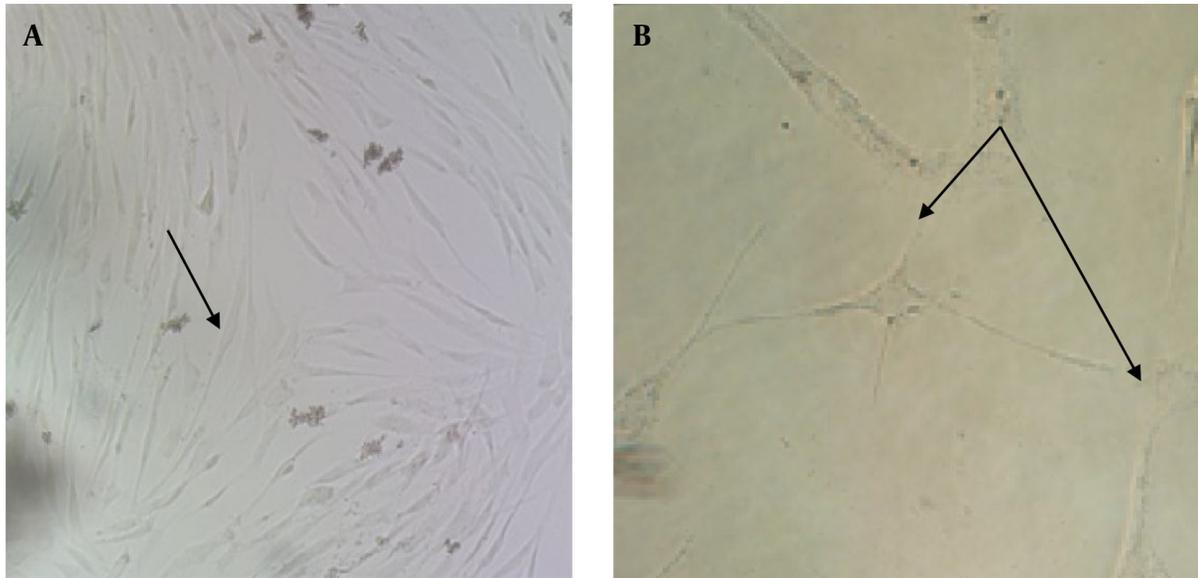
A, Before differentiation, the UCB-MSCs showed fibroblast-like-shaped cells; B, The UCB-MSCs progressively acquired a neuron-like morphology, a large nucleus, and long processes. Magnification = x200.

Figure 3. Neural-Specific Gene Expression Levels With Real-Time PCR
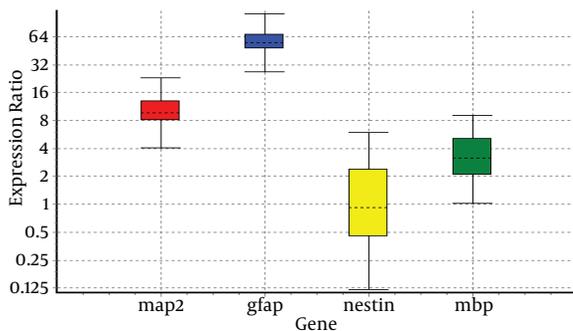
Map2 was upregulated in the sample group compared to the control group by a mean factor of 9.714 (SE range 6.611 - 16.880). The Map2 sample group was different from the control group (P = 0.000). GFAP was upregulated in the sample group compared to the control group by a mean factor of 54.569 (SE range 39.909 - 84.713). The GFAP sample group was different from the control group (P = 0.000). The nestin sample group was not different from the control group (P = 0.680). MBP was upregulated in the sample group compared to the control group by a mean factor of 3.063 (SE range 1.692 - 6.649). The MBP sample group was different from the control group (P = 0.000). The boxes represent the interquartile range, or the middle 50% of observations. The dotted line represents the median gene expression. Whiskers represent the minimum and maximum observations. Report produced with REST 2009 V2.0.13.

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