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A New Two Step Induction Protocol for Neural Differentiation of Human Umbilical Cord Blood-Derived Mesenchymal Stem Cells

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Abstract

Background: In this study, we examined a new two step induction protocol for improving the differentiation of human umbilical cord blood-derived mesenchymal stem cells into neural progenitor cells.

Materials and Methods: Human umbilical cord blood-derived mesenchymal stem cells were first cultured in Dulbecco's modified eagle medium supplemented with 10% fetal bovine serum in a humidified incubator equilibration with 5% CO2 at 37oC. To stimulate neural-differentiation of human umbilical cord blood-derived mesenchymal stem cells, Dulbecco's modified eagle medium was removed and replaced with pre-induction media including retinoic acid, basic fibroblast growth factor, epidermal growth factor, and basal medium for 2 days. Then, we used nerve growth factor, 3-isobutylmethyl-xanthine, ascorbic acid, and basal medium for 6 days. Real-time PCR was performed to analyze the expression of neural specific genes such as glial fibrillary acidic protein, microtubule-associated protein 2, major basic protein, and Nestin.

Results: Real-time-PCR showed that the expression of glial fibrillary acidic protein, major basic protein, and microtubule-associated protein 2 genes, after two step induction, significantly increased compared to common induction protocol. In addition, our study showed that retinoic acid might play a main role in neural-differentiation and fate of mesenchymal stem cells compared to other neural inducers.

Conclusion: The combination of chemicals and growth factors in a two step induction protocol may improve the efficiency of differentiation of human umbilical cord blood-derived mesenchymal stem cells into neural progenitor cells and provide a new method for easy and fast application of human umbilical cord blood-derived mesenchymal stem cells in regenerative medicine.

Keywords: Umbilical cord blood, mesenchymal stem cell, neural progenitor cells, neural-differentiation.

Introduction

The development of stem cells for treatment of neurodegenerative diseases is currently the subject of intensive research efforts. These cells such as embryonic stem cells (ESCs), neural stem cells (NSCs), bone marrow-derived mesenchymal stem cells (BM-MSCs), and adipose-derived adult stem cells (ADAS) have shown to generate differentiated neural cells both in vitro and in vivo, which can be used for substitute therapy in various neurodegenerative diseases [1-6]. Mesenchymal stem cells (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 reasons such as lower immunogenic potential, lack of graft-versus-host disease (GVHD), higher capacity for differentiation into neural cells, quick accessibility, as well as easier and non-invasive collection of them after delivery compared to that of BM-MSCs [8]. Furthermore, hUCB-MSCs are more primitive than BM-MSCs and other sources and their differentiation potential

does not change with frequent passages [9].

In recent years, more attention has been paid to mesenchymal-derived neural progenitor cells (MSCs-NPCs) for treatment of neurodegenerative diseases [10-13]. To date, it has been reported that MSCs could go through neural-differentiation using different methods in vitro, such as using chemical inducers, growth factors, and co-culture with neural cells [14-17]. But, due to the differences in MSCs' isolation methods, culture condition, and sources, the results of the previous studies are not compatible.

Considering the previous reports showing the transdifferentiation of MSCs, we designed a method to improve the efficiency of neural-differentiation of hUCB-MSCs cultivated under appropriate conditions in vitro.

Materials and Methods

Isolation of MSCs from the Human Umbilical Cord Blood

Collection, isolation and propagation of human hUCB-MSCs were performed as described previously [18-20]. To summarize, mononuclear cells (MNCs) fraction was obtained using Ficoll-Hypaque low-density mononuclear cells <1.077 g/ml (Cedar Lane, Canada) gradient separation, followed by ammonium chloride lysis of red blood cells. After washing in phosphate-buffered saline (PBS; Gibco, USA) for two times, the collected MNCs were re-suspended in high glucose-Dulbecco's modified eagle medium (DMEM; Gibco, USA), supplemented with 10% fetal bovine serum (FBS; Gibco), L-glutamine (Gibco), 100 U/ml penicillin and 100 mg/ml streptomycin (Gibco). MSCs were then cultured in 25cm^2 tissue culture flasks (Nunc, USA), in a humidified atmosphere of 95% air with 5% CO_2 at 37ºC.

Flow Cytometry Analysis

In brief, for performing the flow cytometry, hUCB-MSC samples at third passage were harvested by treatment with 0.05% trypsin/EDTA, washed, and re-suspended in PBS. Human AB serum (Sigma, USA) was added to 1×105 cells to block FC receptors. Then the cells were

stained with selected monoclonal antibodies and incubated for 30 minutes at 4ºC in a dark place. Afterwards, the cells were washed and fixed using paraformaldehyde (Merck, Germany). The samples were stained with PE-conjugated mouse anti-human CD44, CD45, CD105, and FITC-conjugated mouse anti-human CD34 (BD Biosciences, USA). The results of the flow cytometry were analyzed using Partec Flomax software (Version 2.4e). Immunoglobulin isotype controls were also evaluated in each run.

Neural Differentiation

For the induction of neurogenic differentiation, 20000 cells were cultured in DMEM supplemented with 10% FBS in a humidified incubator equilibration with 5% CO2 at 37oC. To stimulate neural-differentiation of hUCB-MSCs, first DMEM was removed and replaced with pre-induction media containing basal medium supplemented with L-glutamine, 5µM retinoic acid (RA, Sigma), 10 ng/ml basic fibroblast growth factor (bFGF, Sigma), and 10 ng/ml epidermal growth factor (EGF, Sigma) for 2 days. Then after two days, induction was improved using 10 ng/ml nerve growth factor (NGF, R&D Systems, USA), 0.5 mM 3-isobutylmethyl-xanthine (IBMX, Sigma), 100µM ascorbic acid (AA, Sigma), and basal medium for 6 days.

RT-PCR and Real-time PCR Analysis

In brief, total RNA was isolated from undifferentiated hUCB-MSCs and differentiated hUCB-MSCs using an RNA isolation kit (Qiagen, USA). Synthesis of complementary DNA (cDNA) was carried out using Moloney-murine leukemia virus (M-MuLV) reverse transcriptase (RT) and random hexamer as the primer, based on the manufacturer 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 and denaturation at 94ºC for 15 seconds, annealing at 55ºC or 60ºC for 30 seconds based on the primers, and extension at 72ºC for 45 seconds. PCR products were separated by gel electrophoresis on 2% agarose gel in 1×tris-acetate-ethylenediamine tetraacetate buffer and visualized using SYBR® Safe Gel Stain, and then images were captured using a Bio-Rad Gel documentation system. Experiments were generally conducted in triplicates. Nucleotide sequences and the amplicon size of the designed primers are listed in Table 1. In order to confirm and evaluate the expression level of neural-specific genes by quantitative real-time PCR the cDNAs

were used for a 40-cycle PCR in a Corbett 6,000 Rotor-gene analyzer (Corbett, Germany).

Quantitative real-time PCR was performed in triplicate using Sybr Green Real-Time Master Mix (Takara, Japan), in a Rotor-gene 6000 system (Corbett), followed by 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 (Version 2.0.13).

Statistical Analysis

Two-sided paired t-test and Friedman's two-way analysis of variance by rank were used to analyze the flow cytometry results. All data were analyzed using SPSS software version 21.0 (Armonk, NY: IBM Corp.).

Results

Fibroblastic Morphology and Surface Markers of hUCB-MSCs

Fibroblast-like phenotype was obtained from cord blood after three passages in vitro (Figure 1). Flow cytometry analysis of cell surface markers in hUCB-MSCs (10000 events) expressed CD105 (84.85±9.40, n=3), and CD44 (94.45±4.9, n=3) (P< 0.05), but did not express CD34 (1.80±0.35, n=3), and CD 45 (2.5 ±1.40, n=3) (P< 0.05).The surface marker patterns corresponded to UCB-derived MSCs. As was evidenced by flow cytometry, the isolated cells were positive for CD105, and CD44, while negative for CD34 and CD45 (Figure 2).

Differentiation Studies of hUCB-MSCs

Neural morphology (sign of neuro-differentiation) was observed on the second day. Some cells were stretched in one and/or two directions. The control samples showed no changes in shape.

Neural -Specific Gene Studies

Study of neural specific genes expression levels using real-time PCR showed that, Map2 was UP-regulated in sample group in comparison to control group (P value <0.0001). Also GFAP was up-regulated in sample group in comparison to control group (P value <0.0001). Nestin in sample group was not different compared to control group (P value =0.680). MBP was also up-regulated in sample group in comparison to control group (P value<0.0001). In the present study the maximum level of gene expression was related to GFAP and the lowest level of gene expression was related to Nestin.

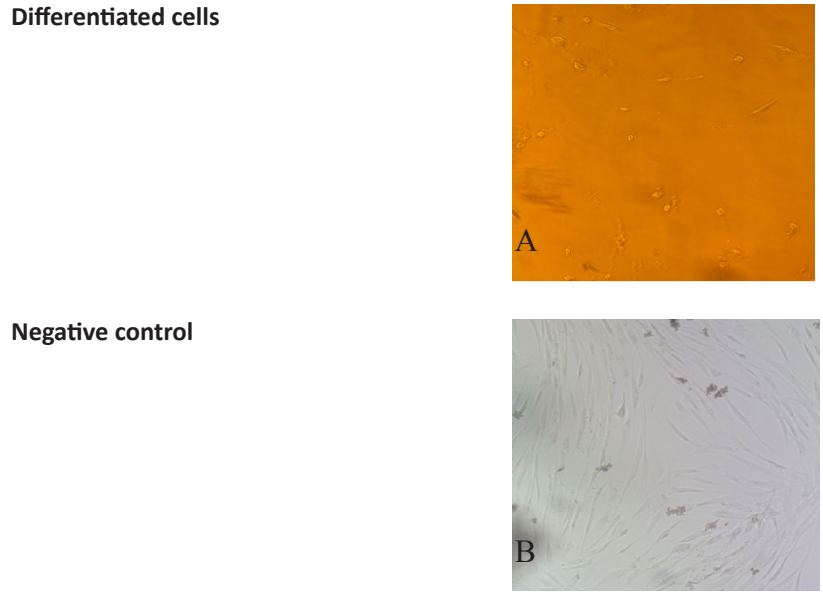

Figure1 (A,B): Neurogenic differentiation capacity of hUCB-MSCs. Magnification=x100.

Table 1: The number of amplification cycles, sequences, and the amplicon size of the specific primers designed for assessing expression of neural markers.

Cycles	Amplicon size (bp)	Reverse primer	Forward primer	Markers
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

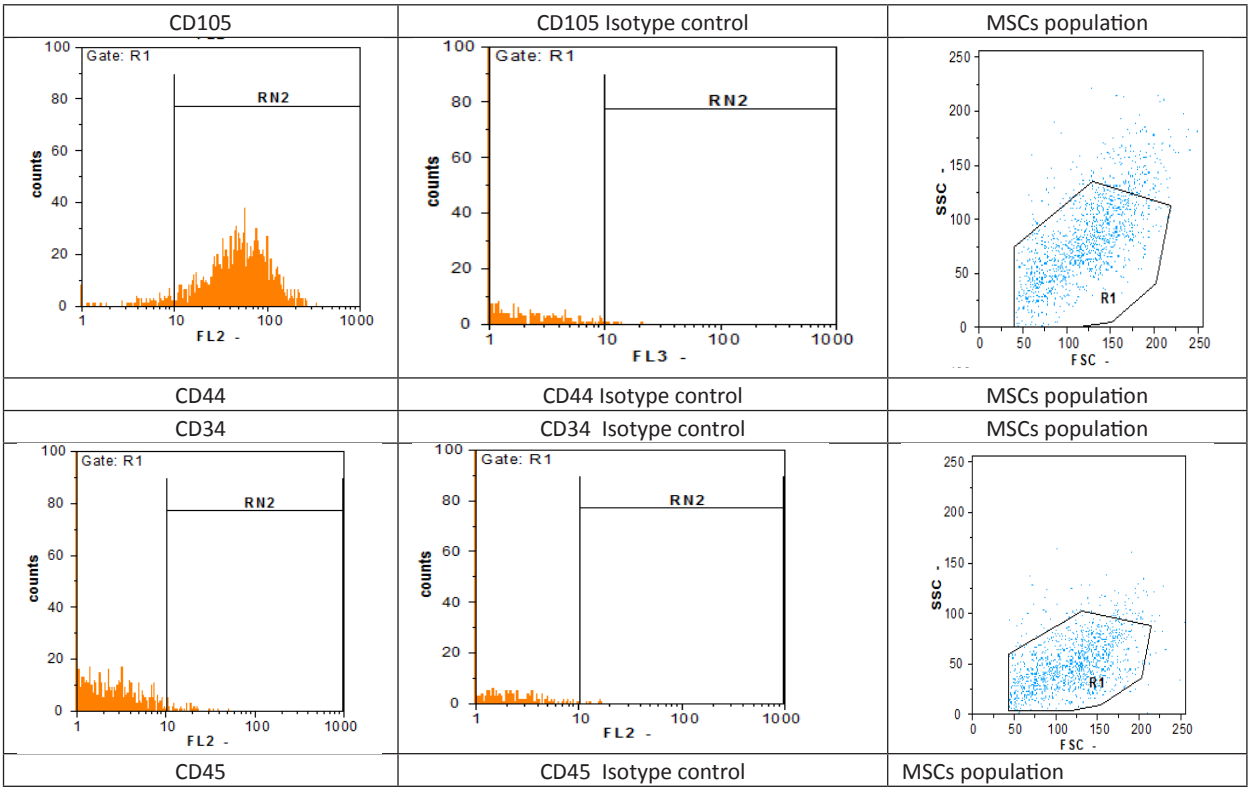

Figure 2: As was evidenced by flow cytometry, the isolated cells were positive for CD105, and CD44, while negative for CD34 and CD45

Discussion

In our study, consistent with previous reports, an expression pattern of flow cytometry showed that markers including CD105 and CD44 were positive and markers such as CD45 and CD34 were negative [21].

There are different methods to induce MSCs-

NPCs; Tio et al. [22], and Wang et al. [23], have used a similar culture protocol, with some differences. Woodbury et al.[17], have used a simple

culture protocol by adding a simple medium with serum. The present study was conducted utilizing 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.

Time duration for the emergence of MSCs-NPCs and the kind of neural markers depend on the used culture system. Tio et al.[22], and Wang et al. [23], methods in which no basal medium is provided take the longest time. It seems that in the present study using the commonest growth factors as well as the selection of basal medium instead of FBS were efficient methods for inducing the cells.

In our protocol, a significant increase occurred in GFAP, MAP2 and MBP expression, especially the GFAP expression. Undifferentiated hUCB-MSCs did not express neuron-specific genes and did not stain positive for neuro-specific proteins by quantitative real-time PCR and integrated cell culture respectively.

Previous studies have indicated that RA combined with other factors such as NGF, β-ME, BDNF, Forskolin, and IBMX are necessary for neural-differentiation of MSCs in vitro [24-26]. In the present study it was found that after the combined treatment with low concentrations of

RA, greater than 30% of hUCB-MSCs differentiated into GFAP-expressing cells, so RA might play a main factor in neural-differentiation of MSCs compared to other inducers.

In brief, the importance of our simple method will be clear when we compare it with other complex and time consuming methods.

MSCs-NPCs share many molecular and cellular characteristics with neural stem cells, on

cellular level; these cells have the same morphology with formation of spheroid body structure and are stretched in one and/or two directions after culture in vitro. In contrast to MSCs, the use of MSCs-NPCs for cell transplantation is a more effective cell-based therapy; and also the tumor formation problem is avoided. These characteristics propose the therapeutic use of MSCs-NPCs in regenerative medicine as a new and unlimited source. Further studies including identification of neural proteins using western blots are suggested.

Conclusion

The combination of chemicals and growth factors in a two step induction protocol may improve the efficiency of differentiation of human umbilical cord blood-derived mesenchymal stem cells into neural progenitor cells and provide a new method for easy and fast application of human umbilical cord blood-derived mesenchymal stem cells in regenerative medicine.

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