Original Article

Changes of neural markers expression during late neurogenic differentiation of human adipose-derived stem cells

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Abstract

Background: Different studies have been done to obtain sufficient number of neural cells for treatment of neurodegenerative diseases, spinal cord, and traumatic brain injury because neural stem cells are limited in central nerves system. Recently, several studies have shown that adipose-derived stem cells (ADSCs) are the appropriate source of multipotent stem cells. Furthermore, these cells are found in large quantities. The aim of this study was an assessment of proliferation and potential of neurogenic differentiation of ADSCs with passing time.

Materials and Methods: Neurosphere formation was used for neural induction in isolated human ADSCs (hADSCs). The rate of proliferation was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay and potential of neural differentiation of induced hADSCs was evaluated by immunocytochemical and real-time reverse transcription polymerase chain reaction analysis after 10 and 14 days post-induction.

Results: The rate of proliferation of induced hADSCs increased after 14 days while the expression of nestin, glial fibrillary acidic protein, and microtubule-associated protein 2 was decreased with passing time during neurogenic differentiation.

Conclusion: These findings showed that the proliferation of induced cells increased with passing time, but in early neurogenic differentiation of hADSCs, neural expression was higher than late of differentiation. Thus, using of induced cells in early differentiation may be suggested for *in vivo* application.

Key Words: Adipose-derived stem cells, neural differentiation, neurogenesis, proliferation

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Received: 22.10.2014, **Accepted:** 04.03.2015

INTRODUCTION

The brain and spinal cord injury can have destructive results on cognitive, sensory, and motor impairments in patients.[1] Stem cell therapy is a great hope in the treatment of neurodegenerative diseases.[2] Commonly, the cell sources include embryonic stem cells (ESCs), and neural stem cells (NSCs) from embryonic or adult brain tissue have been proposed for neuronal cellular therapies.[3-7] However, ethical opposition

Access this article online	
Quick Response Code:	Website: www.advbiores.net
	DOI: 10.4103/2277-9175.166146

How to cite this article: Razavi S, Khosravizadeh Z, Bahramian H, Kazemi M. Changes of neural markers expression during late neurogenic differentiation of human adipose-derived stem cells. Adv Biomed Res 2015;4:209.

has impeded the use of ESCs and NSCs for clinical application.[8,9] Studies have shown that mesenchymal stem cells (MSCs) are an autologous supply of stem cells for therapeutic purposes.[10-13] Adipose tissue is a source of stem cells that isolated by less invasive methods and in larger quantities as compared with bone marrow. The number of stem cells extracted from adipose tissue is higher than those of bone marrow tissue (2% vs. 0.002%).[14-17]

Adipose-derived stem cells (ADSCs) can differentiate towards adipocytes, osteoblasts, chondrocytes and neuron-like cells.[15,18-21] Recent studies showed that human ADSCs (hADSCs) express neurotrophic factors include nerve growth factor, brain-derived neurotrophic factor (BDNF), and glial-derived neurotrophic factor, also these cells might express surface molecules to regulate neurite outgrowth and neural differentiation of ADSCs and indicated that hADSCs have the ability to neural induction.[18,22,23] In addition, ADSCs could differentiate into Schwann-like cells and these cells were immunopositive for Schwann cell markers.[24] Some of the profits of hADSCs for neural induction may be due to their production of extracellular matrix molecules such as laminin[25] and neurotrophic factors,[22] also hADSCs express metabolic genes and neuroprotective molecules.[26]

In these studies, different protocols and times were used to neural induction of hADSCs. Different time points such as sometimes,[18] 5 days,[27] 2 weeks[28] were considered to neural induction. However, whether the passing time has effects on the rate of neural differentiation not been clearly evaluated. High induction rate of hADSCs neural differentiation in a short time could promise sufficient neuron-like cells for treatment of neurodegenerative diseases. In this study, hADSCs induced toward a neuronal phenotype via neurosphere formation then the viability of induced hADSCs evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, and neurogenic differentiation was detected by immunocytochemical and real-time reverse transcription polymerase chain reaction (RT-PCR) analysis after10 and 14 days post induction.

MATERIALS AND METHODS

Human adipose-derived stem cells isolation and culture

All procedures were conducted according to Isfahan University of Medical Sciences, Medical Faculty Ethic Committee approval. HADSCs were isolated from subcutaneous adipose tissue from 3 female donors during abdominal surgery upon collecting a written consent. Adipose tissue was washed 3 times with sterile phosphate buffer saline to eliminate red blood cells and debris. Samples were digested with % 0.01 collagenase type I (Sigma, St. Louis, Mo, USA) for 30 min at 37°C, after neutralization of enzyme with the same volume of Dulbecco's modified eagle's medium (DMEM-F12) (PAA Laboratories GmbH Austria) containing 10% fetal bovine serum (FBS) (Gibco BRL, Paisley, UK). The cell suspension was centrifuged for 10 min at 1600 rpm. The suspended pellet cells were plated in 25 cm^2 tissue culture flask using DMEM-F12, supplemented with 10% FBS and 1% penicillin/streptomycin at 37°C and 5% CO_2 incubator. After cells reached nearly 90% confluency, they were trypsinized and subcultured. HADSCs for this study were used at passage 3–5.

Induction of neurogenic differentiation

The isolated cells were dissociated with 0.25% trypsin-ethylene diamine tetraacetic acid (EDTA) and counted. HADSCs were placed on culture dish with 5 cm diameter at a concentration of 1×10^6 in DMEM-F12 supplemented with 2% B27, 20 ng/ml basic fibroblast growth factor (bFGF), 20 ng/ml human epidermal growth factor (EGF). After neurosphere formation, neurospheres were singled with 0.25% trypsin-EDTA and used for terminal differentiation.

For terminal differentiation, singled neurosphere cells plated in 24-well plates and in neurobasal medium supplemented with 5% FBS, 1% penicillin/streptomycin, 1% L-glutamine, 1% N_2, 1% non-essential amino acids, 2% B27 for 3 and 7 days (the growth factors, and supplements are all from Gibco BRL, Paisley, UK). The induced cells were divided two groups 10 and 14 days after neural differentiation.

MTT assay

In order to determine the effect of past time on the cell viability and proliferation, induced cells were seeded into 24 well plates.

Neural induction medium of each well was aspirated and then, 200 µl of DMEM-F12 and 20 µl of MTT solutions were added. The cell culture plates were incubated at 37°C in 5% CO_2 for 4 h. The supernatant was discarded and 200 µl of dimethyl sulfoxide (DMSO) was added. After pipetting by microplate reader at the wavelength of 540 nm, the absorbance of each well was determined.

Immunocytochemical analysis

Differentiated cells were fixed in 4% paraformaldehyde for 30 min. Samples were permeabilized with 2% triton X-100 for 30 min. Blocking in 1 mg/ml

bovine serum albumin and primary antibodies incubation against mouse anti nestin (1:300, Abcam, Cambridge, MA, USA), mouse anti glial fibrillary acidic protein (GFAP) (1:600, Abcam, Cambridge, MA, USA) and mouse anti microtubule-associated protein 2 (MAP2) (1:300, Abcam, Cambridge, MA, USA) were performed overnight. The secondary antibodies, anti-mouse fluorescein isothiocyanate-conjugated IgG antibody (1:500, Abcam, Cambridge, MA, USA) were used for 2 h at 37°C. For nucleus visualization, the cells were stained with 4', 6-diamidino-2-phenylindole (DAPI) (1:1000, Sigma-Aldrich, St. Louis, MO, USA). Experiments were performed in triplicate and samples were observed using a fluorescence microscope (Olympus BX51, Japan). To merge the pictures, image J 1.42 software (Wayne Rasband, National Institutes of Health, Bethesda, MD, USA) was used. At least 100 cells were counted on per sample.

Real-time reverse transcription polymerase chain reaction analysis

Total RNA was isolated from induced cells using RNeasy mini, RNA isolation kit (Qiagen) according to manufacturer's protocols. The complementary deoxyribonucleic acid was synthesized using total RNA, oligo-dt, primers and reverse transcriptase (fermentas). The real-time PCR was performed with gene-specific primers and the SYBR Green PCR Master Mix (Qiagen) using a thermal cycler rotor-gene 6000 (Qiagen).The primer sequences (forward, reverse) are shown in Table 1. The gene of interest was normalized against the reference gene glyceraldehydes-3-phosphate dehydrogenase (GAPDH). The expression level of each target gene was calculated by $2^{-\Delta\Delta Ct}$.

Statistical analysis

The data collected from MTT, immunocytochemistry, and real-time RT-PCR were analyzed by one-way ANOVA. Data were expressed as mean ± standard error and statistical significance was considered when $P \leq 0.05$. The experiments were replicated at least 3 times.

Table 1: The list of primer sequences (forward, reverse) used in real time RT-PCR analysis

Gene	Forward (top) reverse (bottom)
Nestin	5´-AACAGCGACGGAGGTCTCTA-3´
	5´-TTCTCTTGTCCCGCAGACTT-3'
MAP2	5´-TCAGAGGCAATGACCTTACC-3´
	5´-GTGGTAGGCTCTTGGTCTTT-3´
GFAP	5´-CCTCTCCCTGGCTCGAATG-3´
	5´GGAAGCGAACCTTCTCGATGTA-3'
GAPDH	5´-ACCACAGTCCATGCCATCAC-3´
	5´-TCCACCACCCTGTTGCTGTA-3'

RT-PCR: Reverse transcription polymerase chain reaction, MAP2: Microtubule-associated protein 2, GFAP: Glial fibrillary acidic protein, GAPDH: Glyceraldehydes-3-phosphate dehydrogenase

RESULTS

Morphological features of human adipose-derived stem cells during cultured and neural induction

The isolated hADSCs were observed by a phase contrast microscopy throughout the culture and differentiation. The isolated hADSCs presented a mono-layer of spindle-shaped cells, looking like fibroblast cells after 2 passages. Flow cytometric analysis showed that hADSCs expressed surface markers of CD44, CD90, and CD105, but were negative for hematopoietic lineage markers CD14, CD45, and CD34 (data were not shown). These cells formed neurospheres as spheres of floating cells after culture in preinduction medium. Induced ADSCs exhibited cytoplasm retraction and bi-or tri-polar, spindle-like shapes in induction medium [Figure 1].

Proliferation rate of human adipose-derived stem cells during neural induction

The viability of differentiated ADSCs was determined at 10 and 14 days after induction. The mean optical density in late differentiation of hADSCs at day 14 (1.0522 ± 0.17529) was significantly increased as compared with differentiated ADSCs at day 10 (0.4707 ± 0.09279) ($P < 0.02$).

Immunocytochemistry of induced human adipose-derived stem cells

Ten and 14 days after the neural differentiation, differentiated hADSCs were labeled with nestin, GFAP, and MAP2 and cell nuclei were counterstained with DAPI. Primary antibodies were eliminated for negative control.

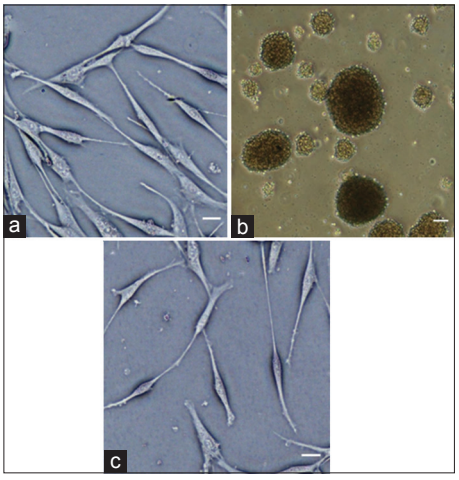

Figure 1: Morphological characteristics of human adipose derived stem cells (hADSCs) following neural induction. (a) Undifferentiated hADSCs cultured in Dulbecco's modified eagle's medium. (b) hADSCs cultured for 7 days in neural induction medium and neurosphere could be observed. (c) Induced hADSCs exhibited cytoplasm retraction and bi-or tri-polar, spindle-like shapes in induction medium. Scale bars in (a) and (c) are 50 μm and in (b) is 200 μm

The mean percentage of positive cells for neural markers of nestin (progenitor neural marker), GFAP (astrocyte marker), and MAP2 (mature neural marker) was evaluated at 10 and 14 days after induction [Figure 2].

Immunocytochemistry analysis showed that the mean percentage of nestin positive cells in late neural differentiation was significantly decreased relative to these cells at day 10 after induction (29.033% ±3.73% vs. 54.35% ±12.48%) ($P < 0.05$), also the mean percentage of GFAP positive cells at day 14 (35.233% ±6.093%) was decreased as compared with these cells at day 10 (52.23% ±9.92%). While the mean percentage of MAP2 positive cells at day 14 (47.2% ±13.30%) was higher than these cells at day 10 (42.90% ±4.99%) after induction [Figure 3].

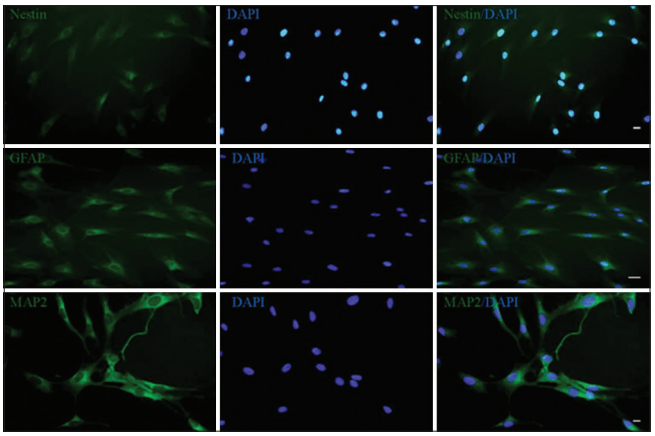

Figure 2: Immunocytochemical staining for neural markers nestin, glial fibrillary acidic protein (GFAP), and microtubule-associated protein 2 (MAP2) in induced human adipose derived stem cells at day 14. All nuclei were counterstained with DAPI. Scale bars: Nestin = 100 μm, GFAP = 50 μm, MAP-2 = 50 μm

Real-time reverse transcription polymerase chain reaction analysis

In order to determine the effect of passing time on the expression of neural markers at the level of mRNA in the differentiated hADSCs, real-time RT-PCR analysis was assessed and expression detectable levels of nestin, GFAP, and MAP2 in differentiated hADSCs were measured at 10 and 14 days after induction.

The result of real-time RT-PCR showed that all the neural markers down-regulated in differentiated hADSCs at day 14 compared to day 10. The levels of expression of nestin as well as GFAP in differentiated hADSCs at day 14 were down-regulated as compared with these cells at day 10 (1.6612 ± 0.35681 vs. 3.0702 ± 0.69694) and (1.2005 ± 0.19956 vs. 1.5112 ± 0.10453), respectively, also the levels of expression of MAP2 in differentiated hADSCs at day 14 (0.7529 ± 0.17885) was down-regulated relative to day 10 (1.1495 ± 0.1514) [Figure 4].

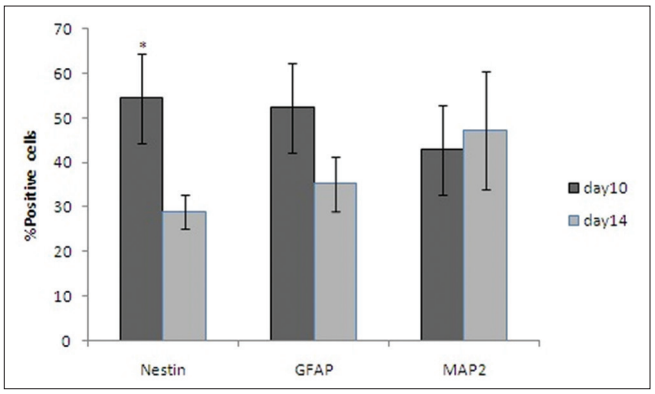

Figure 3: Comparison the mean percentage of nestin, glial fibrillary acidic protein, and microtubule-associated protein 2 positive cells in induced human adipose derived stem cells at 10 and 14 days. Immunocytochemistry showed that the mean percentage of nestin positive cells at day 14 was significantly decreased relative to these cells at day 10 after induction. The positive cells were shown as mean ± standard error (*$P < 0.05$)

DISCUSSION

Our study demonstrated that the proliferation potential of induced hADSCs was increased with passing time while, the levels of expression of nestin, GFAP, and MAP2 at day 14 was decreased relative to day 10. In addition, compared to day 10, the processes of induced ADSCs were not grown much longer at day 14.

Zuk *et al*. showed that neurogenic induction for 30 min result in a change in hADSCs morphology and cells exhibited a neuronal-like phenotype, while no significant increase in neuronal-like phenotype was observed after 3 h post induction.[18] Safford *et al*. indicated that the viability of differentiated ADSCs was decreased at day 5 relative to day 1.[29] Ahmadi

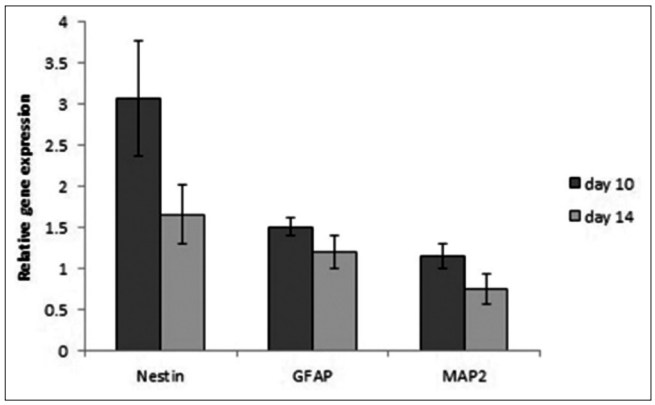

Figure 4: Real-time reverse transcription polymerase chain reaction analysis of the induced adipose-derived stem cells (ADSCs) at 10 and 14 days post induction. The expression level of nestin, glial fibrillary acidic protein, and microtubule-associated protein 2 in induced hADSCs at day 10 was up-regulated as compared with expression of these markers at day 14. Values are mean ± standard error

et al. (2012) showed that induced hADSCs at neural medium similar to our neural induction medium, expressed nestin in 1-week over than MAP2 and GFAP, but expression of late markers up-regulated after 3 weeks.[30] The variety of results in neural cell morphologies and the different expression of neural protein observed in different researches are probably due to some factors like differences in the protocols, cell passage numbers, cell densities, donor's age, duration of neural induction, etc.[30]

The viability of induced hADSCs was increased at day 14, may be due to the presence of EGF and bFGF in neural induction medium as important factors for cell proliferation.[31]

The neural induction and neuronal differentiation look to be related to the cell cycle control system. Neuronal differentiation induces G1 synchronization and growth arrest, resulting in progress terminal differentiation. The inadequate survival of differentiated ADSCs for 1-week may be due to cell cycle arrest and loss of self-renewal capability after early differentiation. However, after intracerebral transplantation, ADSCs can survive for at least 30 days in the brain and stain positively for neuronal and glial cells markers.[13] BDNF has most important roles in the brain development and adult neuroplasticity include regulation of neuronal survival, neurogenesis, neurite outgrowth, and synaptic plasticity.[32,33]

In recent study, comparison of neural markers in induced MSCs derived adipose and bone marrow tissues with immunohistochemistry, real-time RT-PCR, and western blotting analysis have shown that both MSCs can express nestin, MAP2, and GFAP markers and morphologic and phenotypic changes consistent with neural cells after 14 days post induction. Similar to this study, the level of expression of nestin was higher than GFAP and MAP2 genes at late differentiation.[21]

Zemelko *et al*. demonstrated neurogenic potential of human MSCs isolated from bone marrow, adipose tissue, and endometrium and they reported all of MSCs are disposing to neurogenic differentiation. However, their induction protocol was different to our procedure.[34]

In addition, hADSCs express BDNF, therefore; the decrease of neural differentiation of hADSCs with passing time may be related to change the level of neurotrophic factor during neural induction. Although, it appears that 10 and 14 days is not enough time for evaluation effect passing time on neural induction and more time and interval are needed. However, it seems differentiated ADSCs in early stage of neural differentiation may be favorable time for obtain adequate rate of neural-like cells to support regeneration of neurons after neural injuries.

CONCLUSION

Taken together, the results of this work showed that morphology and phenotype of induced hADSCs were similar in early and late of neural differentiation, but the viability and specific neural markers were changed with passing time. The viability of induced hADSCs significantly increased with passing time, while, expression neural markers of differentiated hADSCs were decreased. Quantification of neurotrophic factors released from induced hADSCs such as BDNF at day10 and 14 could provide valuable information for applications *in vivo* application.

Acknowledgments

This research was supported by Isfahan University of Medical Sciences.

Financial support and sponsorship

This research was supported by Isfahan University of Medical Sciences.

Conflicts of interest

There are no conflicts of interest.

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