Iranian Journal of Basic Medical Sciences
Vol. 13, No. 3, Summer 2010, 76-84
Received: Oct 21, 2009; Accepted: Feb 21, 2010

IJBMS

Original article

Differentiation of Adipose-derived Stem Cells into Schwann Cell Phenotype in Comparison with Bone Marrow Stem Cells

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Abstract

Objective(s)
Bone marrow is the traditional source of human multipotent mesenchymal stem cells (MSCs), but adipose tissue appears to be an alternative and more readily available source. In this study, rat adipose-derived stem cells (ADSCs) were induced to differentiate into Schwann-like cells and compared with rat bone marrow stem cells (BMSCs) for their Schwann-like cells differentiation potential.

Materials and Methods
BMSCs and ADSCs were characterized for expression of MSCs-specific markers, osteogenic and adipogenic differentiation. They were induced to differentiate into Schwann-like cells and analyzed for expression of the Schwann specific markers. The immunocytochemical differentiation markers were S-100 and real time quantitative Real-time polymerase chain reaction (RT-PCR) markers were S100, P75 and glial fibrillary acidic protein (GFAP). 3-(4, 5-Dimethylthiazol- 2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay and Annexin V-Fluorescein isothiocyanate (FITC)/ Propidium iodide (PI) double labeling method were employed to detect early stage cell apoptosis.

Results
BMSCs and ADSCs showed similarities in expression of the MSC-specific markers, osteogenic and adipogenic differentiation. Both quantitative RT-PCR and immunocytochemical analysis demonstrated that BMSCs and ADSCs had equal expression of the Schwann-specific markers following Schwann-like cells differentiation. However, gene expression of P75 was higher in BMSCs compared with ADSCs. MTT assay and flow cytometry found that of the total BMSCs and ADSCs in the culture medium, 20% to 30% of the cells died, but the remaining cell population remained strongly attached to the substrate and differentiated.

Conclusion
Comparative analysis showed that Schwann-like cell differentiation potential of ADSCs was slightly decreased in comparison with BMSCs. Therefore, BMSCs are more favorable choice than ADSCs for tissue engineering.

Keywords*:* Adipose tissue, Bone Marrow, Differentiation, Stem Cells

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Introduction

Peripheral nerve injuries are common and often result in incomplete or no functional recovery, particularly after a complete transection (1). Functional recovery usually requires guided axonal regeneration into the growth environment of the distal nerve stump (2). Key regulators of the regeneration process of the injured nervous tissue are Schwann cells (SCs), which provide structural support and guidance for peripheral nerve regeneration following injury by releasing neurotrophic factors (3). SCs thus become the most important and commonly used seed cells for developing "tissue engineered nerves". It has been shown that an involvement of SCs enhances the yields of nerve scaffolds to guide nerve regeneration, especially promoting axonal regeneration (4). However, the culture of sufficient numbers of autologous SCs is time consuming, limiting the usefulness of this technique as delay compromises axonal regeneration (5). Use of allogeneic cells elicits an intense immune response (4), which clinically would require immune suppression, difficult to justify within the context of peripheral nerve repair.

Therefore, considerable efforts are focused on inducing other cells to turn into SCs so as to expand their source. Recent studies showed that bone marrow stem cells (BMSCs) can be differentiated into Schwann-like cells (5-9). However, the harvest of BMSCs frequently required general or spinal anesthesia, which was a highly invasive and painful procedure (10).

It is reported that adipose-derived stem cells (ADSCs) could be isolated from adipose tissue (11). The phenotypic and gene expression profiles of ADSCs are similar to BMSCs (12) and these cells can be expanded in culture for extended periods (11). Both ADSC and BMSCs are derived from embryonic mesoderm; ADSCs and BMSCs have similar phenotype and gene expression profiles (13); and both of them can differentiate along several mesenchymal tissue lineages, including adipocytes, osteoblasts, chondrocytes, myocytes, and so on (11). To our knowledge, however, a few recent reports have shown the Schwann-like cells differentiation potential of ADSCs (10, 14-16) by using a method similar to the method to induce BMSCs into Schwann like cells. In addition, a comparative study of the Schwann-like cells differentiation potential *in vitro* of BMSCs and ADSCs has not yet been performed. In this study, ADSCs were induced to differentiate into Schwann-like cells and their Schwann differentiation potential was compared with BMSCs.

Materials and Methods

Isolation and culture of BMSCs and ADSCs

About 6- to 8-week-old male Wistar rats of the Albino strain were killed using diethyl ether and the bones were collected under sterile conditions; then all the bones were cut at both ends. The bone marrow from each bone was collected by flushing the bone with α Minimum Essential Medium Eagle (αMEM) (Sigma, USA) containing 1000 U/ml Penicillin G. After filtering, the cells were centrifuged at 1000×g for 5 min. The purified cells were finally dispersed in αMEM with 15% fetal bovine serum (Sigma, USA) containing 100 U/ml penicillin and 100 µg/ml streptomycin (14). Primary ADSC were harvested from scrotal fat pad of the same rats. Epididymal adipose tissue was excised, placed on a sterile glass surface, and finely minced. The minced tissue was placed in a 50 ml conical tube (Greiner, Germany) containing 0.05% tissue culture grade collagenase type 1 (Sigma, USA) and 5% bovine serum albumin (Sigma, USA).The tube was incubated at 37 °C for 1 hr and shaken every 5 min. After filtering through a sterile 250 µm nylon mesh, the tube contents were centrifuged at 250×g for 5 min. The cell pellet was resuspended in ADSCs medium: Dulbecco's Modified Eagle Medium (DMEM/F12) (Sigma, USA), 10% fetal bovine serum (Gibco, USA), 100 U/ml penicillin and 100 µg/ml streptomycin (Sigma, USA). Cell count was determined with a hemacytometer (17).

Cell culture and expansion

The isolated stem cells were plated in T75 tissue culture flasks containing appropriate stem cell medium at a density of 10×10^5 cells per flask. The flasks were maintained in a tissue culture

incubator at 37 °C and 5% carbon dioxide. The medium was replaced every third day afterwards. Cell viability was confirmed by continued cell division and the cells were subcultured using 3 ml of trypsin/ ethylenediaminetetraacetic acid (EDTA) (Sigma, USA) when the flasks reached 90% confluence.

Primary culture of Schwann cells

Primary Schwann cells (SC) were obtained by methods similar to those first described by Brockes *et al* (18). Briefly, the sciatic nerves of 2 neonatal rats (1–2 day old) were exposed, removed, and placed in chilled DMEM containing 1% penicillin streptomycin. Nerves were digested with 37.5 U/ml collagenase Type 1 (Sigma, USA) and 0.03% trypsin-EDTA (Gibco) for 15 min at 37 °C. The medium was aspirated and the enzyme digestion cycle repeated a further three times. Fetal bovine serum (10% in DMEM) was added to neutralize the enzymes. Nerves were mechanically triturated, then passed through a 70 μm Falcon filter to remove residual debris. The resulting cell suspension was centrifuged at 600 g for 5 min, the supernatant gently aspirated from the cell bolus and fresh cell growth medium added. Cells were then plated onto poly-D-Lysine coated 25 cm^2 flasks and incubated in 5% CO_2 at 37 °C overnight. Twenty four hr after plating, the medium was removed and replaced with fresh cell growth medium containing 100 μM cytosine-b-D-arabinoside (Sigma-Aldrich, UK). This antimitiotic agent effectively reduces the numbers of the more rapidly proliferating fibroblasts relative to the SC population. After a further 48 hr, the medium was replaced with cell growth medium supplemented with 4.1 μg/ml forskolin (Sigma-Aldrich) and 63 ng/ml epidermal growth factor (EGF) (Sigma, USA).

Characterization and purification

Rat BMSCs and ADSCs within 3–5 passages after the initial plating of the primary culture were harvested by trypsinization, and then the cells were fixed in neutralized 2% paraformaldehyde solution for 30 min. The fixed cells were washed twice with phosphate buffer saline (PBS) and incubated with antibodies to the following antigens: Cluster of differentiation molecule 31 (CD31), (CD44), CD45 and CD90 (all from Chemicon, CA) for 30 min. Primary antibodies were directly conjugated with Fluorescein isothiocyanate (FITC). Flow cytometry was performed with a FACscan flow cytometer (Becton Dickinson, CA) (19).

BMSCs and ADSCs multilineage differentiation potential

Cultured stem cells of passage 2 were disseminated at a density of 5000 cells/cm^2 and maintained in growth medium for 3 days. Then medium was replaced by differentiation medium with slight modifications (7, 17). Differentiation medium contained αMEM, 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin and 100 mg/ml streptomycin and additionally either 10 nM dexamethasone, 50 mg/ml L-ascorbic acid and 10 mM b-glycerophosphate (osteogenic differentiation) or 10 nM dexamethasone, 200 mg/ml indomethacin, 5 mg/ml insulin and 0.5 mM IBMX (adipogenic differentiation). Media change was performed every 3–4 days. After 21 days, osteogenic deposits and adipocytes were visualized by alizarin red and oil red O staining respectively.

Transdifferentiation of BMSCs and ADSCs to SC-like cells

BMSCs and ADSCs at passage 5 were detached from culture dishes and plated at the concentration of 10×10^5 cells/cm^2 on 24-well culture plates, both types of cells were incubated in culture medium containing 1 mM beta mercaptoethanol without serum for 24 hr. The culture media was then replaced with new media containing 10% FBS and 35 ng/ml all-trans-retinoic acid (Sigma, USA). Three days later, cells were finally transferred to culture media containing 10% FBS and trophic factors of 5 mM forskolin (FSK) (Calbiochem, CA), 10 ng/ml recombinant human basic fibroblast growth factor (bFGF) (Peprotech, UK), 5 ng/ml platelet-derived growth factor-AA (PDGF) (Peprotech, UK), and 200 ng/ml heregulin-b1-EGF-domain (HRG) (R&D systems, USA) and cultured for 10 days.

Immunostaining of cultured cells

Differentiated BMSCs and ADSCs cultured on chamber slides (Lab-Tek, Denmark) were fixed in 4% (w/v) paraformaldehyde at 4 °C for 20 min. Cell nuclei were labelled with 6 diamidino-2-phenylindole dihydrochloride (DAPI, Sigma-Aldrich) (1 µg/ml) for 60 min at room temperature. Cells were then incubated overnight at 4 °C with primary antibodies to S100 (rabbit polyclonal; 1:200; Dako, Denmark). The following day, slides were incubated for 2 hr with FITC-conjugated secondary antibody (horse anti-mouse or goat anti-rabbit; 1:100; Vector Labs., USA). Slides were examined under a fluorescence microscope (Olympus BX60). Cultures of Schwann cells were similarly stained as positive controls according to the antibodies used.

Cell viability assay

The 3-(4, 5-Dimethylthiazol- 2-yl)-2, 5-diphenyltetrazolium bromide (MTT) (Sigma, USA) test measures the mitochondrial (metabolic) activity in the cell culture, which reflects the number of viable cells. In brief, the cultures (10×10^5), seeded to a 96-well plate, were washed with PBS, and 100 µl of culture medium containing 50 µl MTT reagent was added. Following incubation in the incubator at 37 ºC for 1 hr (in 5% CO_2), absorption of the medium was measured in ELISA Reader (Anthos 2020) at 540 nm.

Annexin V/PI assays

To confirm apoptosis induction, annexin V, and propidium iodide (PI) double staining was performed by using the Annexin V-FITC Apoptosis Detection Kit as described by the manufacturer (BD Biosciences, CA). The cells stained with annexin V-FITC were analyzed with a flow cytometer (FACScan; Becton Dickinson, CA), using CellQuest Software (BD Biosciences), which was also used to determine the percentage of apoptotic and/or necrotic cells.

RNA extraction and quantitative RT-PCR

Total (Ribonucleic acid) RNA was isolated with Trizol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Isolated RNA was dissolved in RNase-free water and the amount of RNA was determined by measuring absorbance at 260 nm with a spectrophotometer. RNA quality was verified by agarose electrophoresis and the measurement of OD260/OD280. After that, the RNA samples were treated with DNase I (Invitrogen). The complementary deoxyribonucleic acid (cDNA) was prepared from 2 mg of total RNA with cDNA synthesis kit (Bio-Rad, Hercules, CA) in a final volume of 20 ml. The primers for all assayed genes were designed, using the Oligo 6.0 program. Primers are listed in Table 1. For a single PCR reaction amounting to 20 ml, 0.1 ml of cDNA was used. To make the visualization of PCR products possible in real time, the SYBR Green I supermix (Bio-Rad) was used. A two-temperature cycling, consisting of a denaturation step at 95 °C for 10 sec and annealing/ extension step at 56–68 °C for 30 sec, was carried out in an iCycle instrument (Bio-Rad). The optimal annealing temperature was determined for each gene and the lengths of the PCR products were verified by means of agarose electrophoresis. In addition, the specificity of each PCR reaction was assessed by performing melting curve analysis after each reaction. The relative expression levels were analyzed, using the $2^{-\Delta\Delta Ct}$ method by normalizing with β-actin housekeeping gene expression, and are presented as fold increase relative to the control group. Amplified PCR products were separated on a 2% agarose gel by electrophoresis and the bands were visualized by ethidium bromide and photographed with an UVP Imaging System (UVP Company, USA).

Statistical analysis

The results are listed as the mean±SD. The statistical difference was analyzed by two-way ANOVA followed by Bonferroni post hoc test. $P< 0.05$ was considered to be significant. All assays were performed in triplicate.

Table 1. Primer sequences used for the RT-PCR

Gene		Primers
P75	Forward	5'-CATCTCTGTGGACAGCCAGA-3'
	Reverse	5'-CTCTACCTCCTCACGCTTGG-3'
S100	Forward	5'-ATAGCACCTCCGTTGGACAG-3'
	Reverse	5'-TCGTTTGCACAGAGGACAAG-3'
GFAP	Forward	5'-GGTGTGGAGTGCCTTCGTAT-3'
	Reverse	5'-TACGATGTCCTGGGAAAAGG-3'
Beta actin	Forward	5'-CTGGCACCCAGCACAATG-3'
	Reverse	5'-AGCGAGGCCAGGATGGA-3'

Results

Rat BMSCs and ADSCs characterization

Rat BMSCs and ADSCs appeared as a monolayer of large, fibroblast like flattened cells (Figure 1A, B) which when treated with appropriate induction media were able to adopt an osteogenic (Figure 1C, D) or adipogenic (Figure 1E, F) phenotype.

Flow cytometry analysis of rat BMSCs and ADSCs within 3–5 passages showed that both of them were CD44 and CD90 positive, but CD31and CD45 negative (Figure 2).

Immunocytochemistry of BMSCs and ADSCs after induction

We detected the expression of SC marker S-100 to evaluate the nature of SC- like cells. Almost all the differentiated rat BMSCs, ADSCs and Schwann cells were also positive to the S-100 antibody (Figure 3).

Cell viability

The effect of sequential administration of β- Mercaptoethanol (1st day), all-trans-retinoic acid (3rd day) and a mixture of FSK, bFGF, PDGF and HRG (10th day) was analyzed in BMSCs and ADSCs during 1st, 3rd, 7th and 14th day of transdifferentiation period into Schwann-like cells. When the cells viabilities of BMSCs and ADSCs were compared at the same experimental times, the BMSCs presented significantly higher MTT activity on 3^{rd} and 7^{th} day of transdifferentiation period than the ADSCs ($P< 0.05$) (Figure 4).

Quantification of apoptosis by flow cytometry

By staining cells with annexin V-FITC and PI, FACS was used to distinguish and quantitatively determine the percentage of viable cells (lower left quadrant), apoptotic cells (lower right quadrant), late apoptotic necrotic cells (upper right quadrant) and necrotic cells (upper left quadrant) after sequential administration of β- mercaptoethanol, all-trans-retinoic acid and a mixture of FSK, bFGF, PDGF and HRG which can induce differentiation of BMSCs and ADSCs into Schwann-like cells (Figure 5). The proportions of viable cells, early apoptotic cells, late apoptotic/necrotic and necrotic cells for the control group were 94.34%, 2.34%, 1.62% and 1.7% respectively. For BMSCs after transdifferentiation into Schwann-like cell, the proportions were 80.84%, 13.93%, 5% and 0.23% respectively. For ADSCs after transdifferentiation into Schwann-like cell, the proportions were 67.07%, 27.70%, 5.07% and 0.16% respectively.

Real time quantitative PCR (RT-PCR) analysis of Schwann gene expression of differentiated BMSCs and ADSCs

We confirmed the Schwann-like differentiation of differentiated cells, using real time quantitative PCR for specific markers of schwann cells differentiation (S100, P75, GFAP) relative to the housekeeping gene ß-actin (Figure 6). The expression levels of S100 and GFAP mRNA in differentiated BMSCs were not significantly higher than those in differentiated ADSCs on day 14 following differentiation ($P< 0.05$). However, the expression levels of late schwann marker gene (P75) was significantly higher in BMSCs than ADSCs on day 14 following differentiation ($P< 0.05$).

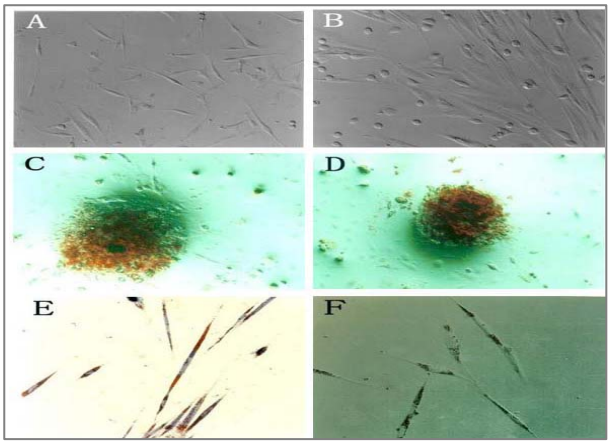

Figure 1. Undifferentiated BMSCs (A) and ADSCs (B), under phase contrast microscopy, display a flattened fibroblast-like morphology. Alizarin red staining of mineralised bone tissue and oil red O positive intracellular lipid droplets indicates that BMSCs (C, E) and ADSCs (D, F) can differentiate to osteoblasts and adipocytes respectively.

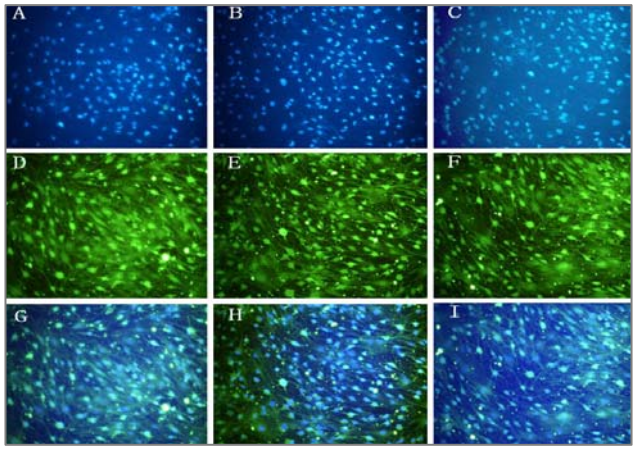

Figure 3. Immunocytochemistry of S100 in differentiated rat BMSCs (A, D, G), ADSCs (B, E, H) and rat Schwann cell-line (C, F, I). After the induction, differentiated BMSCs and ADSCs became positive for S100 antibody. Rat Schwann cell-line was used as positive control. DAPI staining (A, B, C); S100 antibody (D, E, F); merge (G, H, I).

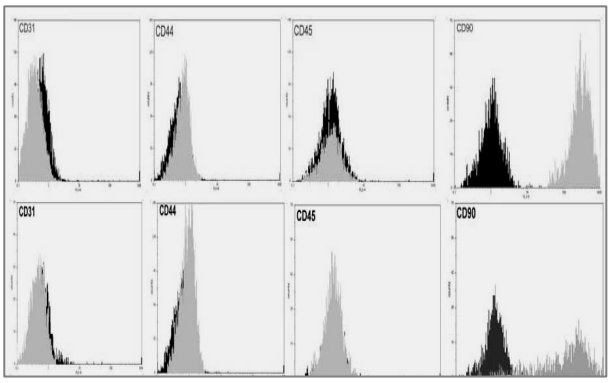

Figure 2. Flow cytometric analysis of rat BMSCs (upper row) and ADSCs (lower row). Rat BMSCs and ADSCs within 3–5 passages were harvested and detected of specific cell surface antigens. Cells stained with a FITC-conjugated nonspecific IgG were examined as a control (FITC). Flow cytometric analysis shows that rat BMSCs and ADSCs do not express CD31and CD45, but express CD90 and CD44.

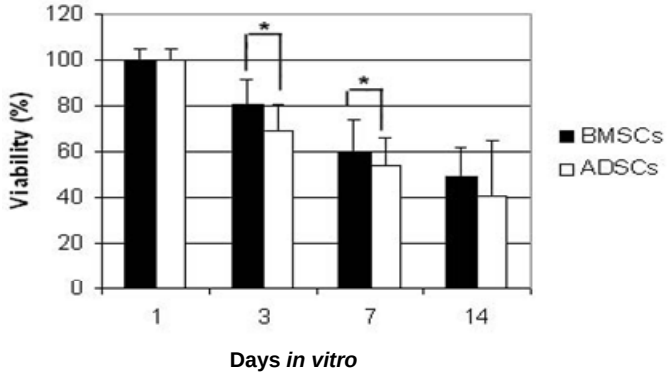

Figure 4. Determination of the percentage of viable cells after sequential administration of β- mercaptoethanol, all-trans-retinoic acid and a mixture of FSK, bFGF, PDGF and HRG which can induce differentiation of BMSCs and ADSCs into Schwann-like cells. The cell viability on 1st, 3rd, 7th and 14th day was quantified, using the reagent MTT. Values are means±SD (*P< 0.05).

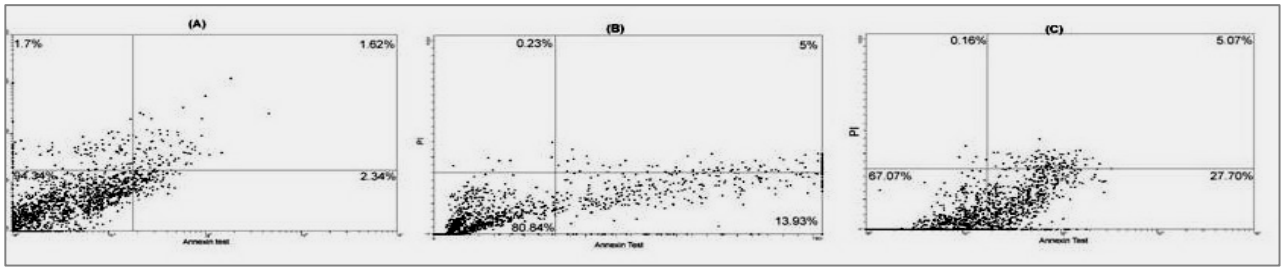

Figure 5. Analysis of BMSCs and ADSCs apoptosis after transdifferentiation into Schwann-like cells by flow cytometry. FACS analysis after staining with Annexin V/PI. Four distinct cell distribution patterns are visible: normal viable cells (lower left quadrant), apoptotic cells (lower right quadrant), late apoptotic or necrotic cells (upper right quadrant) and necrotic cells (upper left quadrant). (A) Nontreated cells (control), (B) BMSCs were differentiated by differentiation medium into Schwann-like cells, (C) ADSCs were differentiated by differentiation medium into Schwann-like cells.

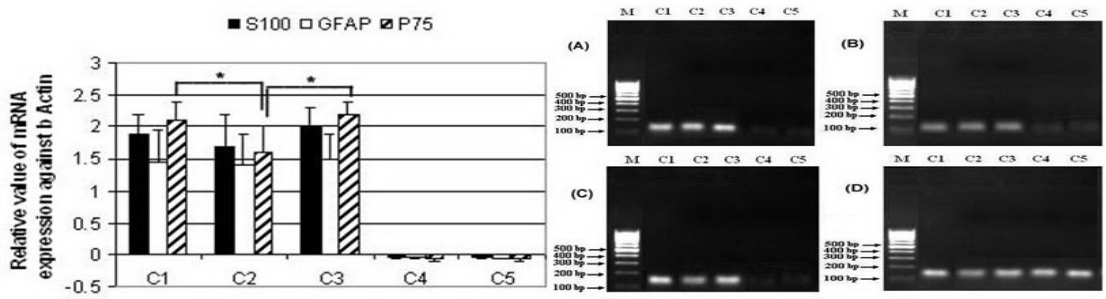

Figure 6. (Left) Expression of three representative genes by real-time quantitative PCR product of mRNA extracted from BMSCs and ADSCs induced into Schwann-like cells. The BMSCs (C1), ADSCs (C2), rat Schwann cell-line was used as positive control (C3), undifferentiated BMSCs (C4) and undifferentiated ADSCs (C5) were used as negative controls. The levels of gene expression (S100, GFAP, and P75) were calculated after normalizing against ß-actin in each sample and are presented as relative mRNA expression units. Values are mean±SD (*****P< 0.05).
(Right) Electrophotograms of RT-PCR product of mRNA extracted from BMSCs and ADSCs induced into Schwann like cells. The mostleft lane represents the DNA ladder (M), BMSCs (C1), ADSCs (C2), rat Schwann cell-line was used as positive control (C3), undifferentiated BMSCs (C4) and undifferentiated ADSCs (C5) were used as negative controls. (A) S100, (B) GFAP, (C) P75 and (D) ß-actin.

Discussion

Regarding comparison between BMSCs and ADSCs there are similar findings (20). The overall results of the present study corroborate the results of the previous studies that suggested equal or comparable capacity of BMSCs (5-9) and ADSCs (10, 14-16) for differentiation into Schwann-like cells.

This study clearly shows the plasticity of BMSCs and ADSCs by their differentiation into Shwann-like cells with the typical spindle-shaped Shwann cell morphology. In this study we have confirmed that the BMSCs and ADSCs express characteristic mesenchymal cell surface markers (CD44 and CD90 positive but CD45, CD31 and CD45 negative), also, demonstrated the multilineage potential of BMSCs and ADSCs (21). Clear evidence is provided to show that rat BMSCs and ADSCs have the ability to differentiate along a glial lineage and express cell markers (S100, P75 and GFAP) which are typical for glial cells including Schwann cells. Similar results have been reported for rat BMSCs and ADSCs separately (5-10, 14-16).

In this study we have shown that sequential administration of β- Mercaptoethanol, all-trans-retinoic acid as preinducer factors and a mixture of FSK, bFGF, PDGF and HRG as inducer factors can induce differentiation of BMSCs and ADSCs into Schwann-like cells. The differentiation media and protocols used in our study are modified from previous studies on Schwann differentiation of BMSCs (5-9).

β-mercaptoethanol was used to promote formation of neurite-like outgrowth (6, 7). Retinoic acid is known to induce differentiation of embryonic stem cells into neural-lineage cells (6). An increase in cAMP, and thus, an elevated expression of mitogenic genes can be achieved when cells are treated with forskolin (22, 23).

Although Schwann differentiation potential of ADSCs is remarkable, but RT-PCR showed that expression levels of investigated cell marker (P75) were lower than in differentiated BMSCs. Moreover, the rate of positive staining for markers of ADSCs transdifferentiated into Schwann-like cells was equal to BMSCs. We speculate that predifferentiation of ADSCs towards a Schwann-like phenotype may enhance the cells' ability to resist the chemical compound of the differentiate media, although this hypothesis will need to be tested in future studies.

MTT assay and flow cytometry found that of the total BMSCs and ADSCs in the culture medium, 50% to 60% of the cells died, but the remaining cell population remained strongly attached to the substrate and differentiated. Although Keilhoff (8) had pointed out to the incidence of more than 50% cells death during differentiation of BMSCs into Schwann-like cells but there is no report in relation with the type and amount of cell death in BMSCs and ADSCs during differentiation into Schwann like cells. As a ‘‘side effect’’ of differentiation

an increased cell death rate was noted. This seems to be in contrast to the presumed cytokine effect, but there is evidence indicating that either direct contact to axons or survival factors secreted by neurons are necessary for the survival and development of SC precursors as well as of mature SC (24).

Conclusion

Comparative analysis showed that Schwann-like cell differentiation potential of ADSCs was slightly decreased in comparison with BMSCs. Therefore, BMSCs are more favorable choice than ADSCs for tissue engineering and help development of cell-based therapeutics for various nervous diseases.

Acknowledgment

This study was supported by Tehran University of Medical Sciences and Health Services, Tehran, Iran (grant No. 8382-30-01-88).

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