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Original Article

Apoptosis of Rat Adipose-Derived Stem Cells during Transdifferentiation to Schwann-Like Cell

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Abstract

Background: Adipose-derived stem cells (ADSCs) are a population of pluripotent cells used for tissue engineering purposes. The main purpose of the present study was to transdifferentiate the ADSCs to Schwann-like cells and to determine the intensity of apoptosis in ADSCs during the transdifferentiation process.

Methods: ADSCs were isolated from the inguinal adipose tissue of adult rats and the identity of the undifferentiated ADSCs was confirmed by the detection of specific cells surface markers. The ADSCs were transdifferentiated by sequential administration of beta mercaptoethanol, all-trans retinoic acid and a mixture of forskolin, beta fibroblast growth factor, platelet derived growth factor and hergulin. The immunocytochemical properties of transdifferentiated Schwann-like cells were examined at specified time point. RT-PCR was used to investigate the gene expression of the undifferentiated and transdifferentiated ADSCs**.** Cell apoptosis was assessed with annexin/propidium iodide staining and 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay.

Results: Expression of Schwann cell marker S100 was determined by immunocytochemical staining. RT-PCR analyses revealed that the induced ADSCs exhibited Schwann cell-specific markers such as S-100, P75, and glial fibrillary acidic protein on the 14th day. MTT assay and flow cytometry studies showed that of the total ADSCs in the differentiation medium, 50% of the cells died by apoptosis, but the remaining cell population remained strongly attached to the substrate and continued to differentiate.

Conclusion: ADSCs could differentiate to Schwann-like cells in terms of morphology and phenotype. An increased cell death rate was noted and the principle mode of cell death was apoptosis.
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Keywords ● Adipose tissue ● stem cells ● apoptosis ● Schwann cells

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Introduction

Key regulators in regeneration process of the injured nervous tissues are Schwann cells.[1] Even though cell-based therapy using these cells seems to be effective and promising, peripheral nerve must be sacrificed for the cultivation

of Schwann cells.[2] It is desirable to harvest highly proliferative cells from easily accessible sources other than peripheral nervous system to produce the cells with Schwann cell characteristics.[3]

An alternative candidate may be the mesenchymal stem cells.[2] These cells which are isolated from adult bone marrow and adipose tissue (ADSCs) have shown a great potential for cell therapy because of their multipotent capabilities.[4] For cell-based regenerative therapies, mesenchymal stem cells are expanded in vitro and differentiated to Schwann-like cells by applying culture media of different compositions.[5] The components of the applied media can influence cell proliferation and viability including replicative senescence and apoptosis.[6]

However, recent studies have indicated that mesenchymal stem cells can transdifferentiated to Schwann-like cells in vitro by media containing β-mercaptoethanol and all-trans-retinoic acid.[6-15] β-mercaptoethanol,[16] and all-transretinoic acid (RA),[17] are toxic chemical that were used as pre-inducer factors.[6-15] Moreover, at specific concentrations in culture medium, β-mercaptoethanol increases mesenchymal stem cell apoptosis.[17] RA is a more active inhibitor of proliferation and induces differentiation in relatively undifferentiated systems, i.e. neuroblastoma and embryonic stem cells.[18] In contrast, in other systems, such as keratinocytes,[19] and embryonic lung cells,[20] stimulation of proliferation is dominant. While the use of specific chemical compounds to induce neurogenic differentiation of ADSCs in culture has been well documented, the potential effects on cell death have not been elucidated.[7,15]

The main purpose of the present study was to transdifferentiate the ADSCs to Schwann-like cells and to determine the effects of RA and β-mercaptoethanol on ADSCs apoptosis during transdifferentiation process.

Materials and Methods

Isolation and Culture of Adipose Derived Stem Cells

Four- to 8-week-old, male, Sprague–Dawley rats were used. After sacrificing the rats, the inguinal fat pad was harvested. The adipose tissue was carefully dissected. The adipose tissue was digested using collagenase type I (Gibco, USA) and then dissociated mechanically. The suspension was centrifuged to separate the floating adipocytes from the stromal vascular fraction. Then the cells in the stromal vascular fraction were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco, USA), supplemented with 10% fetal bovine serum (FBS, Gibco, USA). Twenty-four hours later, the culture medium was changed to eliminate the non-adherent cells. The cells were passaged 3–5 times before being used for the experiments.

Primary Culture of Schwann Cells

Primary Schwann cells were obtained by methods similar to those first described by Brockes and colleagues.[21]

Flow Cytometry

Rat ADSCs within 3–5 passages after the initial plating of the primary culture were harvested by trypsinization, and then the 10^6 cells were fixed in neutralized 2% paraformaldehyde solution for 30 minutes. The fixed cells were washed twice with phosphate buffer saline (PBS) and incubated with antibodies to the following antigens: CD31 (1:300), CD44 (1:500), CD45 (1:300), and CD90 (1:300) (Chemicon, CA) for 30 minutes. Primary antibodies were directly conjugated with Fluorescein isothiocyanate (FITC). The cells stained with FITC rat anti-mouse IgG served as controls. The cells were examined by FACSCalibur Flow Cytometer (Becton Dickinson, Canada) and analyzed using cell quest software. WinMDI 2.8 software (Scripps Institute, Canada) was used to create the histograms.

ADSCs Multilineage Differentiation Potential

The adipogenic and osteogenic differentiation capacities of ADSCs were confirmed according to the previously published protocols.[7]

Transdifferentiation of ADSCs to Schwann Cell-Like Cells

After sub-culturing at the concentration of 10^6 cells/cm2, ADSCs were incubated in DMEM containing 1 mM beta-mercaptoethanol (BME; Sigma, USA) without serum for 24 h. The culture media was then replaced by DMEM containing 10% FBS and 35 ng/ml all-trans retinoic acid (RA; Sigma, USA). Three days later, the cells were finally transferred to inducer medium containing DMEM, 10% FBS and trophic factors of 5 μM forskolin (FSK; Calbiochem, Canada), 10 ng/ml beta fibroblast growth factor (bFGF; Peprotech, UK), 5 ng/ml platelet derived growth factor (PDGF; Peprotech, UK), and 200 ng/ml hergulin (HRG; R&D Systems, USA) and cultured for 10 days.

Immunostaining of Cultured Cells

Differentiated ADSCs cultured on chamber

slides (Lab-Tek, Denmark) were fixed in 4% (w/v) Para formaldehyde at 4°C for 20 min. Cell nuclei were labeled with 6 diamidino-2-phenylindole dihydrochloride (DAPI, Sigma-Aldrich; 1 µg/ml) for 60 min at room temperature. The cells were then incubated overnight at 4°C with primary antibodies to S100 (rabbit polyclonal; 1:200; Dako, Denmark). On the next day, the slides were incubated for 2 h with fluorescein isothiocyanate (FITC)-conjugated secondary antibody (horse anti-mouse or goat anti-rabbit; 1:100; Vector Labs., USA).

The slides were examined under a fluorescence microscope (Olympus BX60). Cultures of Schwann cells were similarly stained as positive controls according to the used antibodies.

RNA Extraction and Reverse Transcription (RT)-PCR Analysis of Gene Expression.

After pre-induction and the induction periods, the total RNA was extracted from the induced ADSCs, the non-induced cells, and Schwann cells using RNA extraction solution **(**RNX™, Cinnagen, Iran), and was stored at −70°C. cDNA synthesis was carried out from 5 µg total RNA using Fermentas kit (Fermentas, Canada) according to the manufacturer's instructions. The cDNA was amplified (the primers of the genes in the present study are listed in table 1). The 25 µl of PCR product contained the following components: 0.2 pM of each primer, 0.3 mM dNTP, 1.5 mM MgCl2, 1U taq DNA polymerase, and 1×PCR buffer (Fermentas, Canada). The PCR reactions were conducted in a programmable thermocycler (Biorad, USA) with the following temperature profile: 94 °C for 5 min, 35 cycles at 94 °C for 45 s, 55 °C for 45 s, 72°C for 45 s, and a final extension at 74°C for 10 min. Ten µg of the PCR product were separated, ran on a 1.5% agarose gel, and stained with ethidium bromide.

Cytotoxicity 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 (6).

ADSCs were incubated with β-mercaptoethanol, all transretinoic acid (RA, Sigma, USA) and inducer medium respectively. Prior to measuring viability, treatment media were removed and replaced with 100 µl fresh 10% FBS/DMEM medium and 100 µl of 3-(4,5 Dimethylthiazol-2-yl)-2, 5 diphenyltetrazolium bromide (MTT; Sigma, USA). The cells were then returned to the incubator for an additional 4 hours before adding 50 µl of 10% sodium dodecyl sulfate (SDS, Sigma-Aldrich, USA) to stop the reaction. Optical density (OD) at 570 nm was read on a microplate reader to indirectly reflect the number of viable cells.

The inhibition rate (IR, IR = [1 OD test/OD control] × 100) was used to evaluate the cytotoxicity of pre-inducer and inducer factors.

Annexin V/Propidium Iodide 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 (Cat. No.556420; BD Biosciences Pharmingen, San Jose, California, USA).

The cells stained with annexin V-FITC were analyzed with a flow cytometer (FACScan; Becton Dickinson, Mountain View, California, USA) using CellQuest Software (BD Biosciences), which was also used to determine the percentage of apoptotic and/or necrotic cells. By staining cells with annexin V-FITC and PI, FACS was used to distinguish and quantitatively determine the percentage of viable cells, apoptotic cells, late apoptotic/necrotic cells and necrotic cells after sequential administration of β-mercaptoethanol, RA, and a mixture of FSK, bFGF, PDGF, and HRG can induce differentiation of ADSCs into Schwann-like cells.

Statistical Analysis

Statistical analysis was performed with the non-parametric Kruskal–Wallis test. The pairwise comparisons were conducted by using Mann–Whitney U-test. Values of $P<0.05$ were considered statistically significant.

Table 1: The primer sequences used for the RT-PCR

Gene	Primers	Annealing temp.(°C)
P75	Forward 5'-CATCTCTGTGGACAGCCAGA-3'	60.7
	Reverse 5'-CTCTACCTCCTCACGCTTGG-3'	
S100	Forward 5'-ATAGCACCTCCGTTGGACAG-3'	54.8
	Reverse 5'-TCGTTTGCACAGAGGACAAG-3'	
GFAP	Forward 5'-GGTGTGGAGTGCCTTCGTAT-3'	60.7
	Reverse 5'-TACGATGTCCTGGGAAAAGG-3'	
Beta actin	Forward 5'-CTGGCACCCAGCACAATG-3'	63.2
	Reverse 5'-AGCGAGGCCAGGATGGA-3'	

Results

Rat ADSCs Characterization

ADSCs appeared as a monolayer of large, fibroblast like flattened cells (figure 1A) which upon treatment with appropriate induction media were able to adopt an osteogenic (figure 1B) or adipogenic (figure 1C) phenotype. Ten days after administration of FSK, bFGF, PDGF, and HRG, the ADSCs became spindle-shaped with two or three processes (figure 1D).

Flow cytometry analysis of rat ADSCs within 3–5 passages showed that rat ADSCs were CD44 (10%) and CD90 (95%) positive, but CD31 (0%) and CD45 (0%) negative (figure 2).

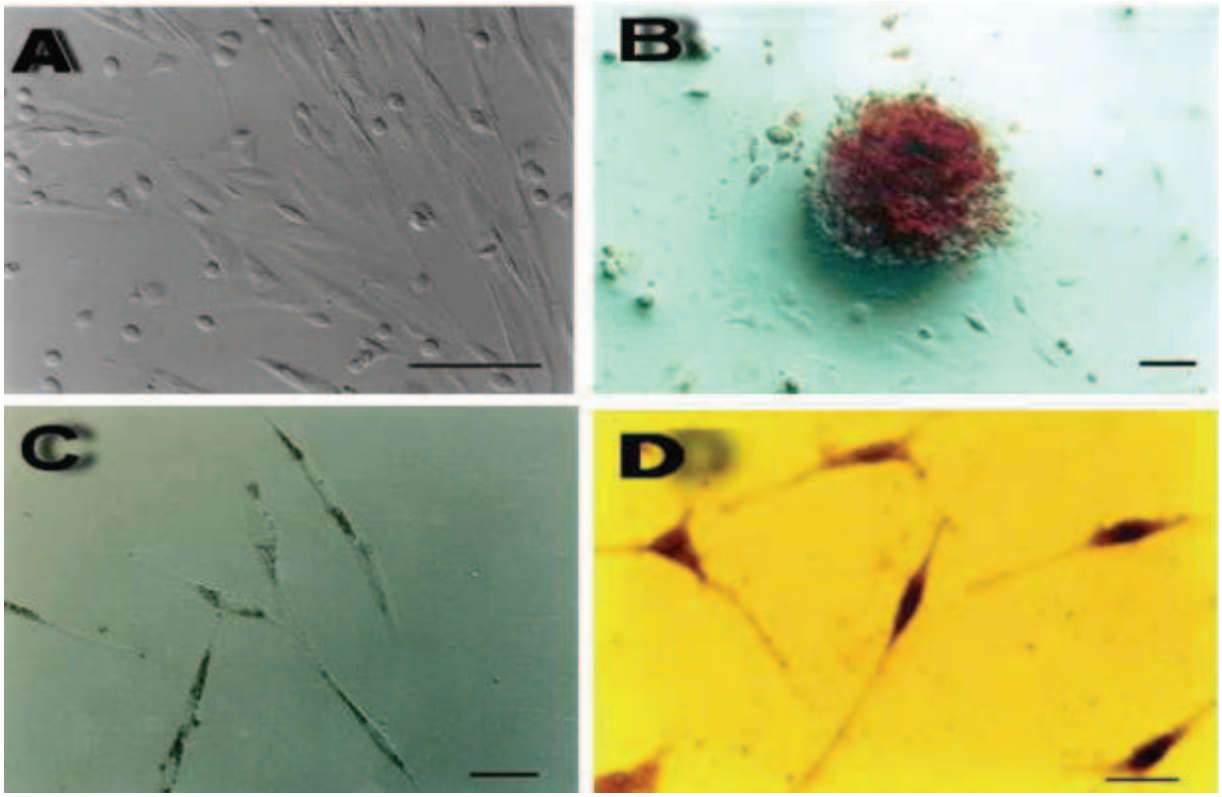

Figure 1: Undifferentiated ADSCs. Under inverted microscope, the cells display a flattened fibroblast-like morphology (A). Alizarin Red staining of mineralized bone tissue (B) and Oil Red O positive intracellular lipid droplets (C) indicate that these cells can differentiate to osteoblasts and adipocytes. Morphology of in vitro cultured Schwann-like cells (D); the cells are spindle-like with two or three processes. Schwann-like cells were stained for S-100 (light microscope). Scale bar 100 µm.

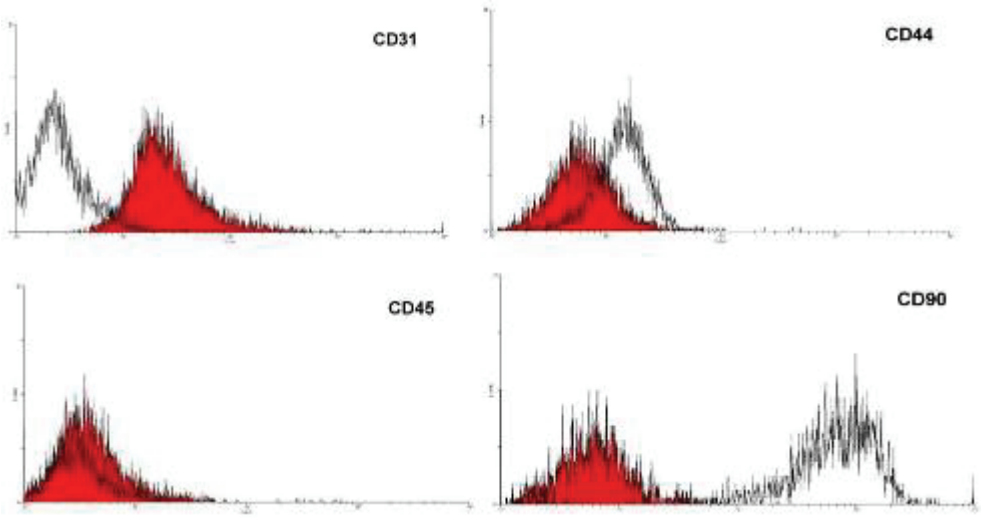

Figure 2: Flow cytometric analysis of rat ADSCs showing that they do not express CD31 and CD45, but express CD44, and CD90.The respective isotype control is shown as red.

Immunocytochemistry of ADSCs after Induction

We detected the expression of Schwann cell marker S-100 to evaluate the nature of Schwann cell-like cells. Almost all the transdifferentiated ADSCs and Schwann cells were also positive to the S-100 antibody (figure 3). After 14 days of induction, expression of S-100 protein was demonstrated in the cytoplasm of Schwann cells-like cells.

RT-PCR Results

The presence of the glial cell marker gene transcripts was detected by RT-PCR methodology (figure 4). This showed that ADSCs, after transdifferentiaion into Schwann-like cells, express the S100, P75, and Glial fibrillary acidic protein compared to the undifferentiated ADSCs. RT-PCR amplification efficacy of the mRNA was confirmed by the amplification of the constitutively expressed beta actin housekeeping gene (figure 4).

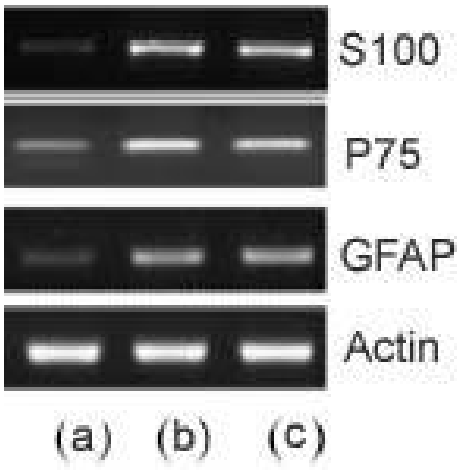

Figure 4: Transdifferentiated ADSCs were assayed by RT-PCR for S-100, P75, and GFAP mRNA. Undifferentiated ADSCs (column a), transdifferentiated ADSCs (column b) and rat Schwann cell-line was used as positive control (column c).

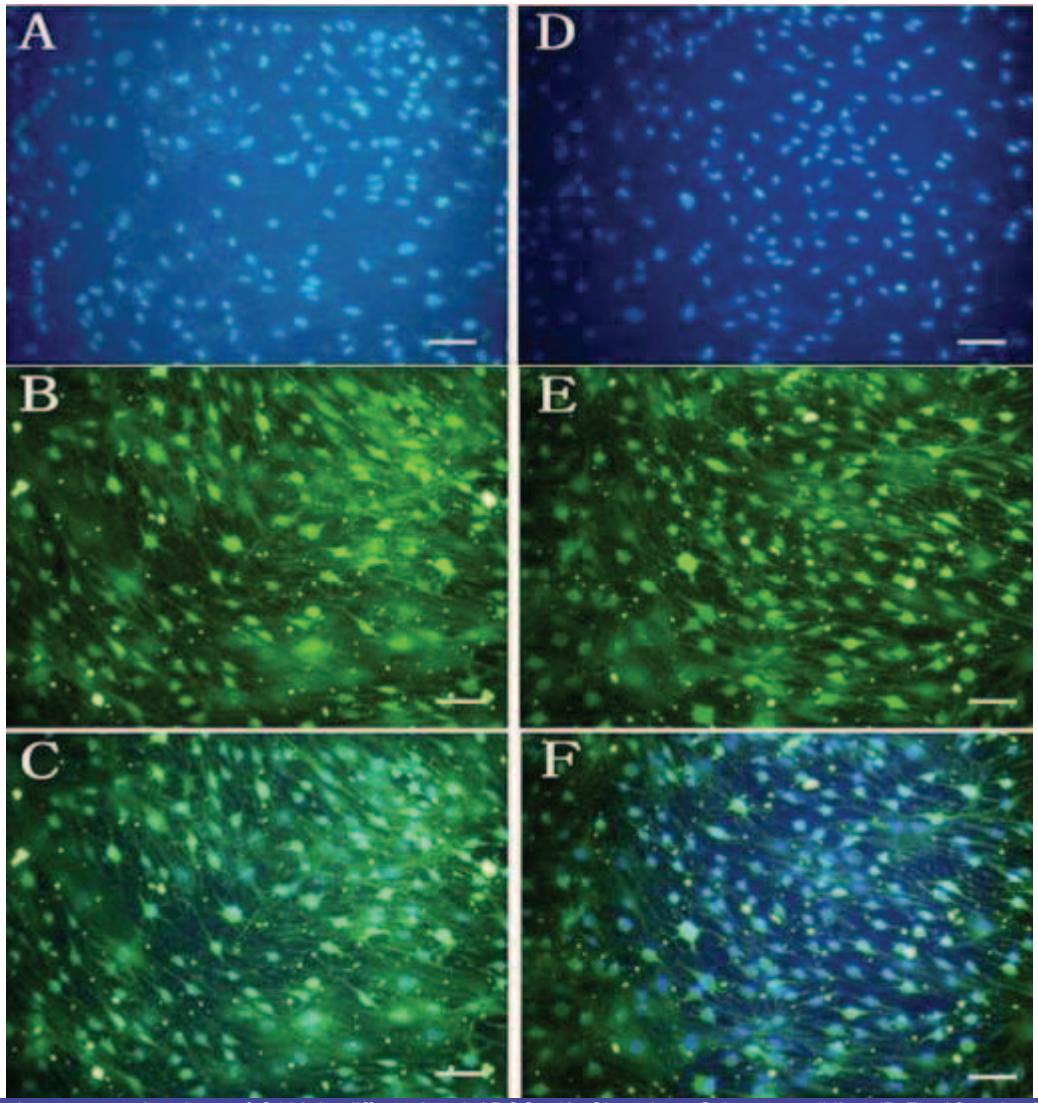

Figure 3: Immunocytochemistry of S100 in differentiated ADSCs (A–C) and rat Schwann cell-line (D-F). After the induction, differentiated ADSCs became positive for S100 antibody. Rat Schwann cell-line was used as positive control. DAPI staining (A,D); S100 antibody (B, E); merge (C, F). Scale bar 150 µm.

Cytotoxicity Evaluation

To study the cytotoxic effect of β-mercaptoethanol, RA and inducer medium on ADSCs during differentiation into Schwann-like cells, we evaluated the viability of ADSCs by MTT assay. This assay usually shows high correlation with number of living cells, cell proliferation and release of mitochondrial matrix enzymes. The cytotoxic activity of β-mercaptoethanol, RA, and inducer medium on ADSCs were 75.6%±5.5%, 60.16%±7%, and 49.3%±12%, respectively, which was lower than in the control group (P=0.0325; figure 5).

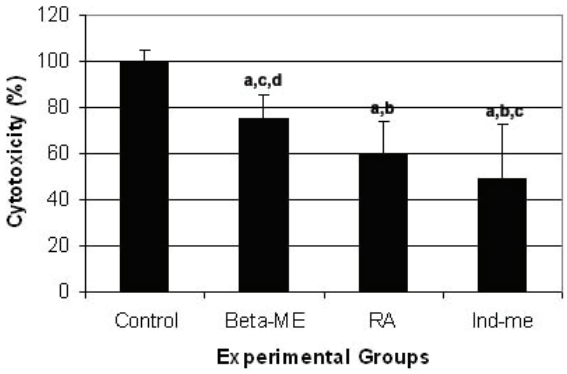

Figure 5: A histogram shows the cytotoxicity of β-mercaptoethanol, RA and inducer medium on ADSCs.
a: Significant difference with control (untreated) group. b: Significant difference with β-mercaptoethanol group. c: Significant difference with RA group (P=0.0325).

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, RA and a mixture of FSK, bFGF, PDGF and HRG can induce differentiation of ADSCs into Schwann-like cells (figure 6, left). The proportions of viable cells, early apoptotic cells, late apoptotic/necrotic and necrotic cells for the control group were 95.28%, 3%, 1.2%, and 1.34%, respectively. For cells treated with β-mercaptoethanol, the proportions were 76.35%, 14.1%, 5.99% and 3.67% respectively. For cells treated with all-trans-retinoic acid, the proportions were 48.95%, 19.45%, 3.83% and 4.32%, respectively. For cells treated with inducer medium, proportions were 39.61%, 8.41%, 1.29% and 1.72 % (P=0.0284; figure 6, right).

Discussion

Mesenchymal stem cells are multipotent cells, which can give rise to mesenchymal and non-mesenchymal tissues in vitro and in vivo.[11] Postnatal tissues have reservoirs of such cells, which contribute to maintenance and regeneration. Examples include bone marrow stem cells in bone marrow and ADSCs in adipose tissue.[4] Over the past decade, stem cells from adult bone marrow and adipose tissue have been exploited as therapeutic vectors in the treatment of a wide variety of diseases.[11] In the present study we confirmed that ADSCs express characteristic mesenchymal stem cells surface markers (CD44 and CD90 positive, but CD31and CD45 negative) and demonstrated the multilineage potential of ADSCs.[22] Clear evidence shows that rat ADSCs have the ability to

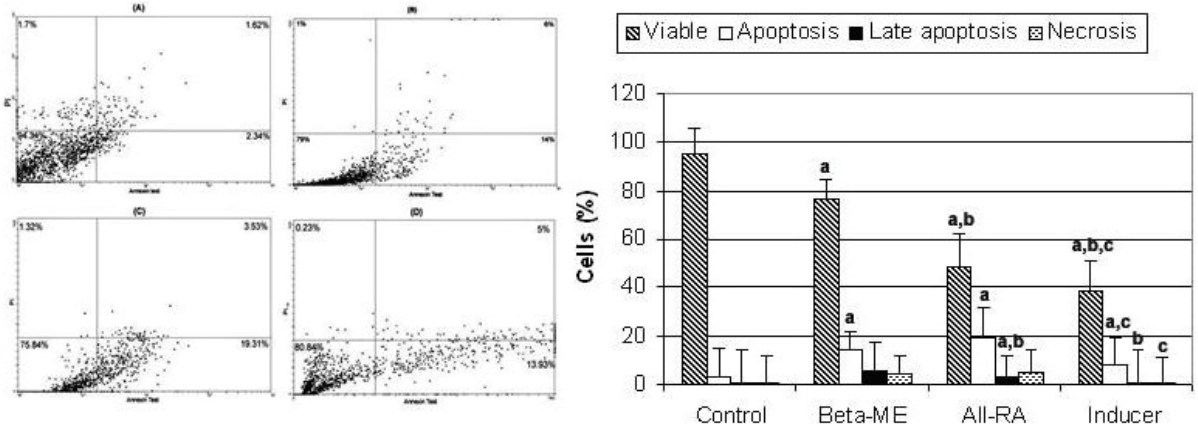

Figure 6: Analysis of ADSCs apoptosis before and after differentiation into Schwann- like cells by flow cytometry (Left).
FACS analysis after staining with Annexin V/PI. Four distinct cell distribution patterns are visible: normal viable cells (lower left quadrant), apoptotic cells (lower right quadrant), late apoptotic/necrotic cells (upper right quadrant), and necrotic cells (upper left quadrant). (A) Non-treated cells (control), (B) induction by β-mercaptoethanol, (C) induction by RA, (D) induction by differentiated medium.
ADSCs apoptosis before and after differentiation to Schwann-like cell phenotype (Right). a: Significant difference with control group. b: Significant difference with β-mercaptoethanol group. c: Significant difference with RA group (P=0.0284).

differentiate along a glial lineage and express cell markers (S100, P75 and GFAP), which are typical for glial cells including Schwann cells.[11-14] In our study, obvious change in the morphology of ADSCs, which tended toward the morphology of Schwann cells, was observed after induction with β-mercaptoethanol, RA, bFGF, HRG, and PDGF.

On the basis of our results, the sequential administration of various factors such as β-mercaptoethanol and RA, followed by a mixture of FSK, bFGF, PDGF, and HRG effectively induces the differentiation of ADSCs into Schwann-like cells. The phenotype of the differentiated ADSCs resembled that of Schwann cells. The previous reports showed that the addition of β-mercaptoethanol to cultured ADSCs induced the formation of neurite-like processes.[23] RA is a morphogenic factor, and was reported to induce the differentiation of neural stem cells into nerve cells.[11] Therefore, β-mercaptoethanol and RA are presumed to work as triggering factor and induce changes in the morphological and transcriptional characteristics of ADSCs.[11-14] Previous studies showed that bFGF acted as a mitogen for bone marrow stem cells, and that Mitogen-activated protein kinases are activated by PDGF and bFGF in mesenchymal stem cells.[24] HRG, a subtype of neuregulin, instructively influences the decision of cell fates and has been reported to induce neural crest cells to develop Schwann cells selectively.[25] FSK increases the level of intracellular cyclic adenosine monophosphate (cAMP), which increases mitogenic responses at an early time.[26] As intracellular cAMP elevation enhances the responsiveness of cells to trophic factors,[25] FSK together with bFGF, PDGF, HRG could exert synergistic effect on ADSCs.[11]

MTT assay and flow cytometry analysis for Annexin V and PI indicated that during the chemical induction process there was an increase in cell death. Measures of cell death during this specific induction process are indicative of toxicity of the used chemical compounds. In agreement with other studies, we showed almost 50% of the cells died within 48 hours of chemical induction by β-mercaptoethanol and RA.[7,17,27]

Our results indicated that RA exerts two apparently opposite effects on cultured ADSCs, i.e., differentiation and induction of cell death.

Cell death can either be the consequence of a passive, degenerative process termed necrosis, or the consequence of an actively driven cell process termed apoptosis.[28] These two distinct modes of cell death can be distinguished based on differences in morphological, biochemical, and molecular changes.[29]

Flow cytometry analysis for Annexin V and PI indicated that putative early apoptotic cells increased after exposure to β-mercaptoethanol and RA.

The mechanism of the apoptotic effects of β-mercaptoethanol and RA are unknown and there are several pathways that need to be investigated.

Oliva and co-workers reported that antiproliferative activity of retinoic acid is associated with (and may be caused by) an up-regulation of two pivotal cdk inhibitors.[30] However there is evidence indicating that either direct contact to axons or survival factors secreted by neurons are necessary for the survival and development of Schwann cell precursors as well as of mature Schwann cell.[6]

In conclusion, our findings indicated that ADSCs could differentiate to Schwann-like cells in terms of morphology and phenotype. As a side effect of differentiation, an increased cell death rate was noted and the principle mode of cell death was apoptosis. The main limitation of our study was the absence of ADSCs viability results after flow cytometry analysis for cell surface antigens.

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Conflict of Interest: None declared

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