Different methods for inducing adipose-derived stem cells to differentiate into Schwann-like cells

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Abstract

Introduction: The aim of the study was to explore an effective method to induce adipose-derived stem cells (ADSCs) to differentiate into Schwann-like cells *in vitro*.
Material and methods: Reagents were applied in two different ways (Dezawa inducing method and modified inducing method) in which inducers including β-mercaptoethanol (β-ME), all-*trans*-retinoic acid (ATRA), type I collagenase, forskolin, heregulin, basic fibroblast growth factor (BFGF) and brain-derived neurotrophic factor (BDNF) were used in different ways to induce ADSCs of rats to differentiate into Schwann-like cells. After induction, the cell morphologic characteristics and the cellular immunohistochemical staining positive rate of anti-S100 and anti-GFAP (glial fibrillary acidic protein) antibodies and the gray value of immunocytochemical dye with anti-S100 and anti-GFAP antibodies and cell activity measured by the MTT method were compared with each other to evaluate the induction effects.
Results: Both methods can induce differentiation of ADSCs of rats into Schwann-like cells, but the cellular morphology of the modified method was more similar to Schwann cells than that of the Dezawa inducing method, there was a higher cellular immunohistochemical staining positive rate and staining grey value in immunocytochemical dye with anti-S100 and anti-GFAP antibodies, and less damage in the cell activity of the modified inducing method than that of the Dezawa inducing method.
Conclusions: The effect of the modified method to induce ADSCs to differentiate into Schwann-like cells *in vitro* is superior to that of the Dezawa inducing method.

Key words: adipose-derived stem cells, Schwann-like cells, MTT, S100, glial fibrillary acidic protein.

Introduction

Peripheral nerve injury is more frequent in clinical practice. Due to the mature neurons' disability of cell division, the effect of peripheral nerve regeneration after injury was not ideal. At present, tissue engineering provides a new method for peripheral nerve regeneration [1]. As the best seed cells for peripheral nerve tissue engineering, Schwann cells play an important role in peripheral nerve regeneration [2, 3], but owing to

the limited source and proliferation ability and the difficulty of cell culture *in vitro* [4, 5], looking for a new method to obtain enough Schwann cells for nerve regeneration was the primary target of the present study.

Adipose-derived stem cells (ADSCs) have the advantages of being easily obtained with an extensive source, easy to cultivate and causing little injury to the donor [6, 7]. Under certain conditions, ADSCs can differentiate into a variety of tissues such as osseous tissue [8], cartilaginous tissue [9], adipose tissue [10], myoid tissue [11] and nervous tissue [12], so they are ideal seed cells for tissue engineering.

This research intended to isolate and culture the ADSCs of rats and induce the cells to differentiate to Schwann-like cells *in vitro* by two different methods, so as to provide an effective method of seed cells induction for peripheral nerve tissue engineering.

Material and methods

Materials

Adult male Sprague-Dawley rats (provided by the animal experimental center of Zhengzhou University) were used, weighing around 250 g. All animals utilized in this research were cared for according to the policies and principles established by the animal welfare act and the NIH guide for care and use of laboratory animals. β-Mercaptoethanol (β-ME), all-trans-retinoic acid (ATRA) and type I collagenase (Sigma Chemicals, USA), forskolin (Alexis, Switzerland), heregulin (Neomarker, USA), basic fibroblast growth factor (BFGF) and brain-derived neurotrophic factor (BDNF) (Peprotech, USA) were used for this experiment. The primary antibody of rabbit anti-rat S-100, rabbit anti-rat GFAP and SABC immunohistochemical staining kit (Boster, China), Dulbecco's Modified Eagle Medium (DMEM) of low glucose and fetal bovine serum (Gibco, USA) were used for this experiment.

Methods

Isolation and culture of ADSCs

Rats were killed by intraperitoneal anesthesia with 10% chloral hydrate solution (0.5 ml/100 g). After immersion sterilization in 75% alcohol, bilateral inguinal fat pads were harvested for experiment under aseptic conditions, minced after washing with phosphate buffer solution (PBS), and dissociated by 0.075% collagenase type I for 90 min. The solution was passed through a 75 μm filter to remove undissociated tissue, then neutralized by the DMEM of low glucose containing 20% (v/v) fetal bovine serum and centrifuged at 1000 × g for 8 min. The stromal cell pellet was resuspended in DMEM of low glucose containing 20% (v/v) fetal bovine serum with 1% (v/v) penicillin/streptomycin solution, and inoculated in 25 ml culture bottles at a density of 4×10^5/ml. The media were renewed after 3 to 4 days, and the nonadherent cells were removed. When the cell fusion rate was up to 90%, the cells were passaged with trypsin/EDTA and inoculated in 50 ml culture bottles. Cultures were maintained in a 37°C incubator with 5% CO_2. The fourth generation cells were induced to differentiation [13].

Cell induction by two different methods

DEZAWA method for cell induction – The ADSCs under sub-fused status of the fourth generation were used for induction according to the Dezawa method [14] for inducing bone marrow stromal stem cells to differentiate into Schwann-like cells *in vitro*: the medium was removed and pre-induction solution (DMEM of low glucose containing 10% (v/v) fetal bovine serum and 100 μl/ml (β-ME)) was added. Cultures were maintained in a 37°C incubator with 5% CO_2 for 24 h. The pre-induction solution was removed and washed with PBS 3 times, pre-induction solution (DMEM of low glucose containing 10% (v/v) fetal bovine serum and 10 μl/ml ATRA) was added. Cultures were maintained in a 37°C incubator with 5% CO_2 for 72 h. The pre-induction solution was removed and washed with PBS 3 times, pre-induction solution (DMEM of low glucose containing 10% (v/v) fetal bovine serum and 20 μl/ml forskolin, 400 μl/ml heregulin, 20 ng/ml bFGF, 10 ng/ml BDNF) was added, cultures were maintained in a 37°C incubator with 5% CO_2 for 24 h, then replaced with DMEM of low glucose containing 10% (v/v) fetal bovine serum.

Modified method for cell induction – ADSCs under sub-fused status of the fourth generation were used for induction. The medium was removed and pre-induction solution (DMEM of low glucose containing 10% (v/v) fetal bovine serum and 50 μl/ml β-ME, 5 μl/ml ATRA, 10 μl/ml forskolin, 200 μl/ml heregulin, 10 ng/ml bFGF, 5 ng/ml BDNF) was added, cultures were maintained in a 37°C incubator with 5% CO_2 for 24 h, the induction solution was removed and washed with PBS 3 times, replaced with DMEM of low glucose containing 10% (v/v) fetal bovine serum for 24 h, then used as the pre-induction solution for 24 h, and replaced with DMEM of low glucose containing 10% (v/v) fetal bovine serum.

Comparison of two different induction methods

Cells of two different induction methods were compared and the undifferentiated cells were used as a control group.

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Growth and differentiation of cells – the growth and morphological change of the two groups were compared.

Immunocytochemical staining and counting – at the same time (the 5th day of the Dezawa method and 7th day of the modified method, same time of every control group), the ADSCs of different groups were digested by 0.25% trypsin and 0.02% EDTA, dropped on an aseptic glass slide coated with polylysine after centrifugation and collection, cells were incubated at 37°C for 2 h, washed with PBS for 5 min, dried and fixed with 4% paraformaldehyde, then the cell of all groups were stained by S-100 and GFAP, PBS were used as the first antibody only in the negative control group. Ten visual fields of every slide were observed randomly under light microscopy (400×), the positive cell ratio of every 50 cells in a visual field was counted, and the mean gray scale of positive staining area was analyzed by an image analysis system.

Activity of cells by MTT method – ADSCs (180 μl of 1×10^6/ml) of every group were placed in a 96-well culture plate which contained DMEM of low glucose containing 10% (v/v) fetal bovine serum, 20 μl MTT of 5 mg/ml was added to each well for 4 h, the liquid were aspirated and 200 μl of dimethyl sulfoxide (DMSO) were added on an oscillator for 10 min, and the absorbance of each pore was detected at the wavelength of 490 nm in an enzyme-linked immunosorbent assay (ELISA) meter.

Statistical analysis

We analyzed the results by SPSS 10.0, the main statistics method including one-way analysis of variance (ANOVA) which was performed to determine the statistical significance between groups. The *t*-test was used to determine whether the difference between the averages of the data sets was statistically significant. A significance level of $p < 0.05$ was used as the cutoff.

Results

Growth and induction of cells

The cells of both groups were decreased in volume, shrunken and spindle-shaped after induction, the cubic configuration of the cell was more obvious than before, a hyperlucent zone and 2 or 3 slender processes surrounded the cell body, then cells continued to shrink into slender processes, the processes were more slender than before and morphology of cells was similar to Schwann cells, and the cell nuclei were round and located on one side of the cell body. There were no morphological changes of the control group (Figure 1 A–C). After induction, there were more necrotic cells and lower cellular plating density of the Dezawa method

compared with that of the modified method. The cells of the modified method grew more rapidly than the cells of the Dezawa method.

Immunocytochemical staining and counting

After being stained by S-100 and GFAP, the cytoplasm of the positive staining cells was dyed yellow. The morphology of positive staining cells was consistent with that of living cells observed under an inverted microscope. The undifferentiated cells of the control group showed negative staining, i.e. they had no expression of S-100 and GFAP. The staining intensity of S-100 and GFAP of cells in the modified method is more strongly positive than that of cells in the Dezawa method (Figure 1 D–I). In cells of the modified method, the staining positive ratio and gray value of S-100 were 67.3 ±5.1% and 95.3 ±4.81; the staining positive ratio and gray value of GFAP were 65.5 ±5.8% and 94.71 ±4.14. In cells of the Dezawa method, the staining positive ratio and gray value of S-100 were 57.2 ±4.5% and 121.21 ±4.32; the staining positive ratio and gray value of GFAP were 54.2 ±4.2% and 123.44 ±4.18. The staining positive ratio and gray value of S-100 and GFAP of the modified method were superior to those of the Dezawa method; there was a significant difference between them ($p < 0.05$) (Table I).

Activity of cells by MTT method

There was no significant difference of cell activity between the control group (6.12 ±0.35) and the modified method (5.36 ±0.41) ($p > 0.05$), but there was a significant difference between the Dezawa method (5.83 ±0.21) and the modified method ($p < 0.05$) (Table I).

Discussion

The ADSC is a kind of multipotent stem cell originating from adipose tissue. It possesses morphology and multilineage differentiation potential similar to bone marrow stromal stem cells (BMSCs) [15, 16]. Compared with BMSCs, the extensive sources and superficial location of subcutaneous adipose tissue, and abundant cells easily obtained from waste adipose tissue (about 1×10^9 stem cells from 10 ml of adipose tissue) without complicated anesthesia and operation, and low incidence of infection to the donor are the advantages of ADSCs. In addition, the culture conditions of ADSCs are not as strict as those required for BMSCs, and the ADSCs possess strong capacity for proliferation (average passage time about 60 h) and stable multiplication ratio for 13–15 generations. Furthermore, the proportions of aging and dead cells during cell proliferation are low [17], so ADSCs are gradually becoming an optimum selection for seed cells of tissue engineering.
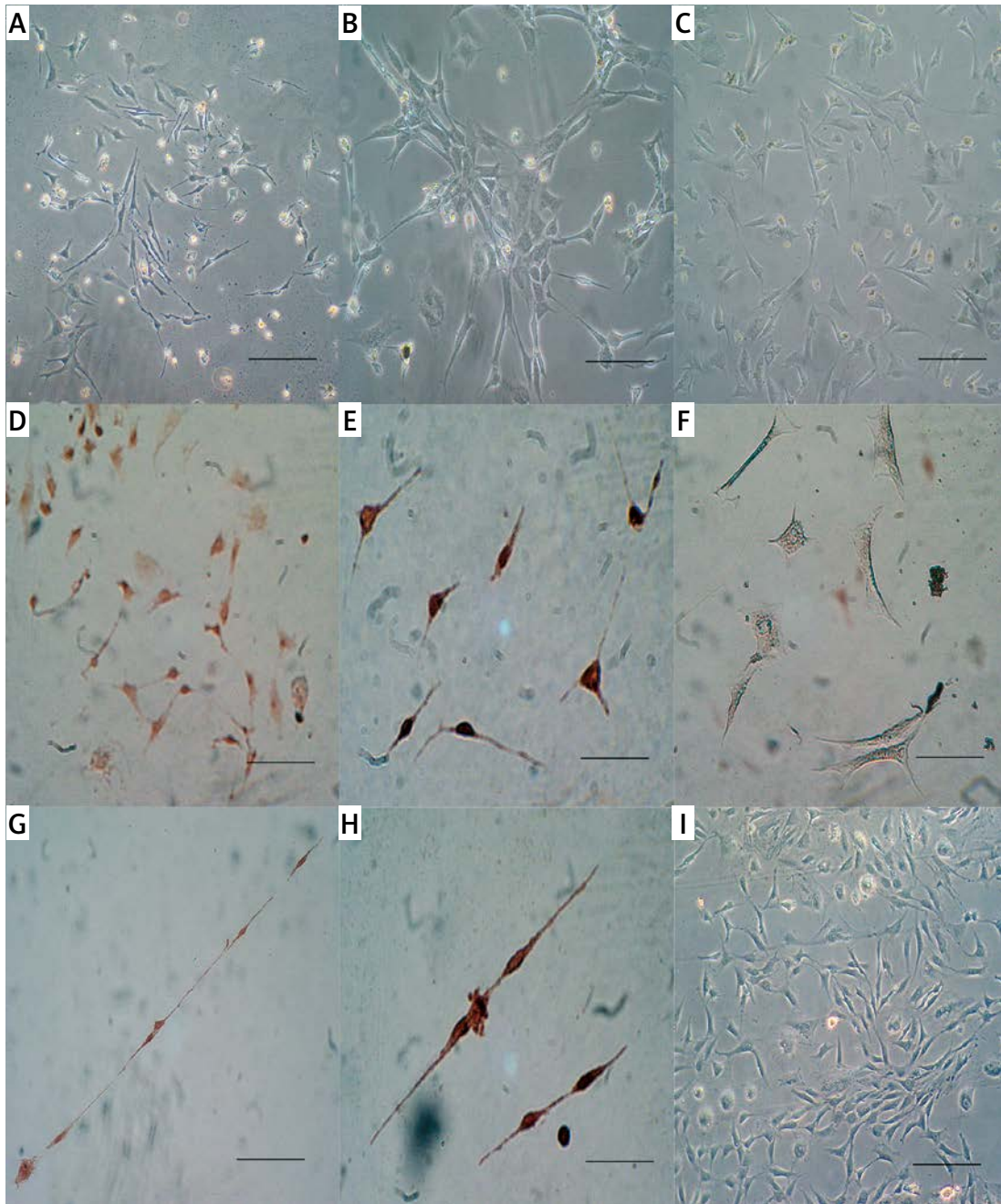
Figure 1. A, B – The cells of Dezawa and modified method showed shrinkage with spindle shape, the cubic configuration of cells was more obvious, and morphology of cells was similar to Schwann cells. Magnification 200× and scale bar is 100 for A, magnification 400× and scale bar is 50 for B. **C** – There were no morphological changes of the control group. Magnification 200× and scale bar is 100. **D** – Immunocytochemical staining of GFAP in cells of Dezawa method. Magnification 200× and scale bar is 100. **E** – Immunocytochemical staining of GFAP in cells of modified method, staining intensity of cells was more strongly positive than that of cells in Dezawa method. Magnification 400× and scale bar is 50. **F** – Cells of control group (undifferentiated cells). Magnification 400× and scale bar is 50. **G** – Immunocytochemical staining of S-100 in cells of Dezawa method. Magnification 400× and scale bar is 50. **H** – Immunocytochemical staining of S-100 in cells of modified method, staining intensity of cells was more strongly positive than that of cells in Dezawa method. Magnification 400× and scale bar is 50. **I** – Cells of control group (undifferentiated cells). Magnification 200× and scale bar is 100

Adipose-derived stem cells can differentiate into neural cells under the appropriate inducing conditions. Ferroni *et al.* [18] found that ADSCs can expressing nestin, glial fibrillary acidic protein (GFAP) and neuronal nuclear protein (NeuN) under induction of valproic acid, butylated hydroxyanisole, insulin and hydrocortisone, confirmed by Western blot. Other research showed that murine ADSCs can express the protein phenotype of neuron or glia cells under the use of inducers *in vitro* [19].

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Table I. Comparison of staining positive ratio and gray value in different groups ($x \pm s$, $n = 10$)

Variable	S-100	GFAP	MTT
Dezawa method:			
Staining positive ratio	57.2 ±4.5%	54.2 ±4.2%	5.83 ±0.21[‡]
Gray value	121.21 ±4.32	123.44 ±4.18	
Modified method:			
Staining positive ratio	67.3 ±5.1%*	65.5 ±5.8%*	5.36 ±0.41[#]
Gray value	95.3 ±4.81*	94.71 ±4.14*	
Control group:			
Staining positive ratio	0	0	6.12 ±0.35
Gray value	0	0	

Compared with Dezawa method, $p < 0.05$, ‡compared with control group, $p < 0.05$, #compared with control group, $p > 0.05$.

Owing to the complexity of orientational differentiation of stem cells and because the effect of a variety of inducers used at the same time is better than that used one by one, we changed the Dezawa inducing method in which single inducers are used several times to one in which combined inducers are used several times.

S-100 is a kind of acid calcium binding protein, and GFAP is a kind of intermediate filament protein; both of them exist in Schwann cells. Gray scale is a transparent degree index of translucent medium. Its value is divided into 0 to 256 grades and is inversely proportional to the strength of the positive immunohistochemical reaction product. The result shows that the staining positive ratio and gray scale of S-100 and GFAP in Schwann-like cells of the improved inducing method were superior to those of the Dezawa inducing method, which means that the induction effect of the former was better than that of the latter. β-mercaptoethanol (β-ME) is an antioxidant that increases intracellular cyclic adenosine monophosphate (CAMP) and prevents the peroxide from causing damage to cells and promotes adhesion and proliferation of stem cells. It was shows that β-ME can protect neurons in serum-free medium and improved the growth of neuritis [20]. It was also shown that β-ME promotes neural differentiation of stem cells [21]; the principle was to accelerate glutathione synthesis and reduce the cell response to oxygen tension. At the same time, the increase of the concentration of intracellular CAMP can promote axonal elongation, probably owing to the activation of protein kinase A (PKA) or the p38 signal pathway originated by CAMP.

Forskolin is an adenylate cyclase activator which can increase the intracellular CAMP content and improve the cells' response to neurotrophic factor. Kim *et al.* [22] found that forskolin could induce the glial cell differentiation of neural stem cells through activation of the PKA signal pathway or increase the concentration of intracellular CAMP.

All-trans retinoic acid (ATRA) is critical for the development, growth and essential physiological activities of vertebrates. A certain concentration of ATRA can promote cell proliferation, differentiation and maturation. Disorders of ATRA might not only cause malformations and genetic mutations in a mouse embryo, but also induce the differentiation and gene expression of human teratocarcinoma cells. It has a strong promotability in mitogenesis and differentiation mainly through the cell surface receptors RARs and RXRs, and also regulating the expression of some encoding factors, which is very important for the early differentiation of neural cells [23]. Research shows that the receptor of ATRA exists in the nucleus. All-trans retinoic acid combines with the receptor in chromatin, then regulates a series of gene expression and changes the phenotype and differentiation of cells after entering the cell [24].

Heregulin is a subtype of neuregulin. Neuregulin is a neural gene regulator and an important axon-derived signal which can prevent apoptosis of Schwann cell precursors and selectively induce neural crest cells to differentiate into Schwann cells through a signaling system. During this process, the main function of neuregulin-1 is participation in the differentiation of glial cells, regulation and control of the formation of myelin sheath and Schwann cells [25, 26].

The bFGF is a kind of polypeptide factor that promotes the growth of cells and mitogen of glia cells. It promotes the proliferation and differentiation of glia cells and neuronal precursor cells, plays an important role in the early development of the nervous system and has a nutritional role in neurons. BFGF also promotes cell differentiation and decides the direction of differentiation *in vitro*. It promotes proliferation of multipotent neural stem cells that can differentiate into neurons and glia cells. Qian *et al.* [27] found that the proliferation or differentiation of neural stem

cells of embryonic brain was dependent on the concentration of bFGF: the cellular mitogenic signal was initiated under low concentration of bFGF, while the cellular mitogenic and differentiation signals were initiated under high concentration of bFGF.

The BDNF is a kind of neuronal protective agent, and its mechanism is probably related to the inductive effect on the upregulation of TrkB mRNA. Its signal transduction pathway begins with receptor tyrosine phosphorylation and finishes with the Ras-MAPK pathway. The BDNF can activate the signaling pathways (including the pathway of G protein and protein kinase C) through tyrosine kinase receptor B, so as to promote neural stem cells to differentiate to glia cells [28]; this process is probably related to its binding to the receptor in stem cell membrane and activation of mitogen-activated protein kinase.

Through the MTT method in the research, we found that the activity of cells in the modified method was enhanced when compared with cells in the Dezawa method when the dosage and induction time of the inducer were decreased, the probable reasons including the following: Firstly, the small amount of inducer has less effect on the activity of cells; secondly, the time interval between application of different inducers in the modified method was 24 h, so the damage of some cells caused by the inducer can be repaired and the activity of cells were improved, which more conform to the growth cycle of cells. This modified method of inducer application probably provided a more effective method to induce ADSCs to differentiate into Schwann-like cells *in vitro*.

In conclusion, although we have induced the rat ADSCs to differentiate into Schwann-like cells *in vitro* successfully, owing to a lack of specific molecular markers for identification of the adult stem cells [29], there were still some limitations in identification of the cellular differentiation effect only depending on the morphology and specific marker of cells. Hence further research on the induction mechanism and expression of cell function is required.

Conflict of interest

The authors declare no conflict of interest.

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