Purity determining of cultured OECs from olfactory mucosa of rats' pups

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Received; 2015/08/23 revised; 2015/09/7 accepted; 2015/09/12

Abstract

Introduction: Cell transplantation is one of the main strategies for spinal cord injury repair. As OECs of the olfactory mucosa can be obtained by simple biopsy in all individuals without affecting their smell sensation, OECs considered as a promising candidate for autologous transplantation in the nervous system injury, especially for spinal cord repair. Thereby in the current study OECs were cultured from olfactory mucosa of 7 days old rats' pups and their purity was examined by flow-cytometry after simultaneous double staining for p75 and GFAP markers.

Materials and methods: 7 days old Wistar rats' pups were deeply anesthetized by ketamine / xylazine (60/6mg/Kg). Then the nasal cavity was opened sagittally and the olfactory mucosa was separated from posterior part of nasal septum and at last OECs were obtained from lamina properia of olfactory mucosa and were cultured. The cultured cells were simultaneously immunolabeled for p75 and GFAP markers and finally purity of cultured cells assessed by flow-cytometry.

Results: cultured OECs demonstrated two different morphologies, a spindle shape Schwann-like and an astrocyte-like OECs with flat sheet-like morphology. In addition, simultaneous immunolabeling for p75 and GFAP markers of OECs exhibited OECs were positive for both markers at the same time. The flow-cytometry results displayed that 87.9±2.4% of cells were p75/ GFAP double positive cells,1.05±0.4 only p75 positive and 5.8±1.5% were single positive for GFAP.

Conclusion: Purity of cultured OECs in our study is probably more than 87.9% by flow- owing to p75+/S100+ and GFAP+/S100+ olfactory ensheathing cells were not counted. Thus the culture procedure of this study seems to be a good protocol for OECs purifying and cell therapy in CNS damages.

Keywords: OECs, Cell therapy, Spinal cord injury, Flowcytometry

Introduction

Spinal cord injury (SCI) is a major cause of disability but there is no universally accepted treatment yet. Recently, cell transplantation, has become one of the most promising therapeutic approaches for SCI, and was strongly examined by numerous investigators. A number of different types of stem cells, neural progenitors, and glial cells have been examined via experimental researches, but several reasons including safety and

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efficacy, have excluded them from clinical uses (1).

Some animal studies have reported that transplantation of bone marrow stromal cells, neural progenitor cells or olfactory ensheathing cells (OECs) can promote functional improvement following SCI (2-7).

Olfactory ensheathing cells (OECs) are considered as a specialized population of glial cells of the primary olfactory system which are existed in both PNS and CNS (8, 9).

They ensheath olfactory axons in the olfactory bulb (OB), the region of termination of the olfactory sensory axons in the CNS, as well as the olfactory mucosa of the nasal cavity(9), hence the olfactory system has unique properties which consists of both PNS and CNS tissue and it is known, for its capacity, to support neurogenesis throughout life (10).

OECs have the unique property of enfolding olfactory axons along the whole axonal path from the olfactory mucosa to the outer layer of olfactory bulb and prevention of their exposures to inhibitory molecules (11). These non-myelinating glial cells play a role in guiding the axons that grow from the olfactory mucosa through the lamina propria to the OB. Therefore, they have properties of both astrocytes and Schwann cells (8, 12-14) owing to their ability to live within the CNS as well as axonal growth. So OECs have been suggested for cellular therapy in the nervous system, mainly for spinal cord lesions due to their properties to support axonal growth (12-14).

Functional improvement has been reported after transplantation of lamina propria into the whole thoracic spinal (15). Transplantation of olfactory mucosa in clinical trials has revealed interesting results (16). Several studies indicated that transplanted OECs could experimentally myelinate, in the right environment, the demyelinated axons (13, 17).

Lu et al and Plant et al, reported an improvement of locomotor function following delayed transplantation of OECs into the complete or incomplete thoracic spinal lesions (15, 18).

Preclinical studies exhibited that OEC's transplantation following spinal cord injury, such as corticospinal tract lesions, were associated with myelinating regrowing axons and functional improvements in the locomotor abilities (19).

Human OECs have been reported to remyelinate spinal cord injuries in rodents after transplantation, and accordingly in clinical trial autologous OECs were transplanted in the injured spinal cord of paraplegic humans (20).

OECs are able to stimulate tissue sparing and neuroprotection, increase outgrowth of both intact and injured axons, activate angiogenesis and remyelinte the demyelinating axons (21).

According to the previous reports, other advantages of using OECs are as follow: first, there is evidence that OEC's transplantation reduces the proteoglycans expression in the reactive astrocytes after photochemical lesions to spinal cord (22, 23). Second, OEC's reduce sprouting at the lesion area but increase the axonal growth into the distal part of transected spinal cord (24). It is believed that the role of OECs in the olfactory system is to direct the newly generated axons to their correct position in the CNS environment of the olfactory bulb (10). Third, it seems the growth factors produced by OECs play important roles in CNS regeneration. In vitro cultured rats' OECs could express NGF, GDNF, BDNF and their receptors. NGF and BDNF are important factors for survival and regrowth of damaged neurons. In addition, NGF, BDNF and PLP are the growth factors of OECs which are known to support neurite outgrowth (25).

In the other hand, OECs of the olfactory mucosa can be obtained by simple biopsy through the external nares in all individuals without affecting the smell sensation. OECs from olfactory mucosa (OM-OECs) represent a promising

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candidate for autologous transplantation to avoid graft rejection, in the nervous system specialy for spinal cord repair (26, 27).
In addition, mitotic activity rate and migration OECs from the lamia propria of olfactory mucosa (OM-OECs) is higher than OECs from the OB-OECs (28). Therefore, the mentioned advantages of OM-OECs prompt us to choose OM-OECs for study. So in this research OECs were separated from olfactory mucosa of 7 days old rats' pups and cultured, followed by immunolabeling for p75 and GFAP markers of OECs, and then their purity were examined by flow-cytometry after simultaneous double staining for p75 and GFAP markers.

Materials and methods

OECs culture: All cultures have been performed in the sterile conditions with autoclaved all surgery tools. Primary cultures of OM-OECs were prepared as described previously with slight modifications (29). Briefly, 7 days old wistar rats' pups were deeply anesthetized by ketamine /Xylazine (60/6mg/Kg) and the nasal cavity was opened sagittally. Care was taken to avoid taking nasal septal cartilage. The OM of the rat is easily identified by its one-third posterior position on the nasal septum and by yellowish appearance of the epithelial surface (27). Respiratory mucosa was carefully removed from the post-anterior part of the nasal septum. After several times washing in the PBS with and then without 1% penicillin/streptomycin samples from each rat were digested enzymatically in 1 ml of trypsin 1% for 45 min at 37˚ C. Trypsinization was stopped by adding Dulbecco's Modified Eagle's/Ham's F12 medium (D.M.E.M/F12, Invitrogen), supplemented with 10% Fetal Bovine Serum (F.B.S, Invitrogen) and 1% penicillin/streptomycin (Invitrogen) (DF-10S). Then the olfactory epithelium of OM was removed and underneath lamina properia was triturated and cut in some small pieces using a micropipette, until a homogenous soup (cell suspension) was obtained .The obtained soup was centrifuged at 1200 rpm for 10 minutes, followed by resuspention in DF-10S and the centrifugation was repeated again. Then, cells were plated in 25 cm^2 flasks pre-coated with poly-L-Lysine (50 mg/ ml, Invitrogen) containing 5ml of DF-10S plus 2µM of forskoline mitogenic factor (30, 31) and 1% penicillin/streptomycin. The flasks were incubated at 37℃, 5% CO2.
Most of the fibroblasts and astrocytes were attached to the flask surface after 24h and 48 hours respectively, while OECs remained unattached (32). Therefore, after 72h OECs were collected from the supernatant and replated onto another flask and were left to grow to confluency for 14 days .The medium was changed daily.
Immunocytochemistry (ICC) of OECs: Some markers used for identification of OECs are p75, GFAP and S100 (33-35).
To determine OECs by p75 and GFAP markers in the culture, additional cell culturing was performed on cover slips pre-coated with poly-L-Lysine (50 mg/ ml, Invitrogen) and was processed for immunocytochemistry. The simultaneous double immunostaining was performed for p75/GFAP, and only double positive cells in these staining were considered as OECs (33-35). The primary antibodies used were as follow: anti-p75 (Abcam,rabbit, 1:200) and anti-GFAP (Abcam ,mouse,1:200).
The secondary antibodies used for the immunostaining were Alexa flour488 goat anti mouse and Alexa flour 568 goat anti rabbit (Abcam, 1:1000 for both).
The procedure briefly is described as follows: 5×10^2 cells were cultured on the cover slips pre-coated with poly-L-Lysine (50 mg/ ml, Invitrogen) for 72h. Cell cultures were primary fixed in fresh 4% phosphate-buffered paraformaldehyde for 2 minutes. Then a secondary fixation was carried out in fresh 2% phosphate-buffered paraformaldehyde for 10 minutes at ambient temperature, followed by fixative washing and incubating with triton X-100

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for 10 minutes to cells become permeable to primary antibodies. Nonspecific binding was minimized with goat serum 10% for 1h followed by incubation with primary antibodies overnight at room temperature. Secondary antibodies were incubated for 1h at room temperature in dark.

Cells were counterstained for 5min by DAPI (1µg/ml) in dark. Then the samples were washed three times with PB (.1 M, PH=7.4) and one time with Diionized water and then were mounted with glycerol buffer and were observed by fluorescent microscope (Olympus AX70). It should be noted that all steps after secondary antibodies, incubation was performed in dark. Between each step, cover slips were washed tree times in PB (.1M, PH=7.4) for 10 minutes. Additionally, negative controls were stained in parallel but primary antibodies were omitted. Double positive cells for p75/GFAP were considered as OECs.

Purity evaluation of cultured OECs: For purity determining of cultured OECs Flow-cytometry was carried out. Simultaneous double staining for p75/GFAP was performed. The primary antibodies used for this procedure were anti-p75 (Abcam, rabbit, 1:200) and anti-GFAP (Abcam, mouse, 1:200).

The secondary antibodies used were as follow: Alexa flour488 goat anti mouse and Alexa flour 568 goat anti rabbit (Abcam, 1:1000 for both).

The cells were washed with PB (.1 M, PH=7.4) and treated with trypsin/EDTA. Then, cells were washed with PB and double immunolabeled, during 1h at ambient temperature, with the anti-p75/anti-GFAP. At the end of incubation, 2 ml of PB .1 M (PH=7.4) was added and the cells were centrifuged (1200rpm for 10 min). Cells were incubated with the secondary antibodies (1:1000) for 1h in the dark. At the end of the incubation, 2 ml of PB .1 M (PH=7.4) was added and the cells were centrifuged (1200rpm for 10 min). Then, cells were resuspended in 1 ml of PB (.1 M, PH=7.4). The total number of 10,000 events was analyzed on a flow-cytometer (FACS, USA) directly after the staining procedure. The experiments were triply repeated and the results were expressed as mean ± standard deviation (SD).

Statistical analysis

Statistical analysis was performed using MiniTab software (release 11.12, Minitab Inc., State College, PA, USA). The experiments were all repeated three times and the results reported as mean ± standard deviation (SD) at a significance level of $p \leq 0.05$. Differences were considered statistically significant when the p value was equal or less than 0.05.

Results

As Figure 1 indicates the cultured OECs demonstrated two different phenotypes after attaching to flask surface, including a spindle shape Schwann-like and an astrocyte-like OECs with flat sheet-like morphology In addition, simultaneous immunolabeling for p75 and GFAP markers of OECs demonstrated OECs positivity for both markers at the same time (Figure 2).

Furthermore, the flow-cytometry results displayed 87.9±2.4% of cells were p75/ GFAP double positive cells, 1.05 ±.4 only p75 positive and 5.8±1.5% were single positive for GFAP (Figure 3). The experiments for flow-cytometry were repeated three times and the results were demonstrated as mean ± standard deviation (SD).

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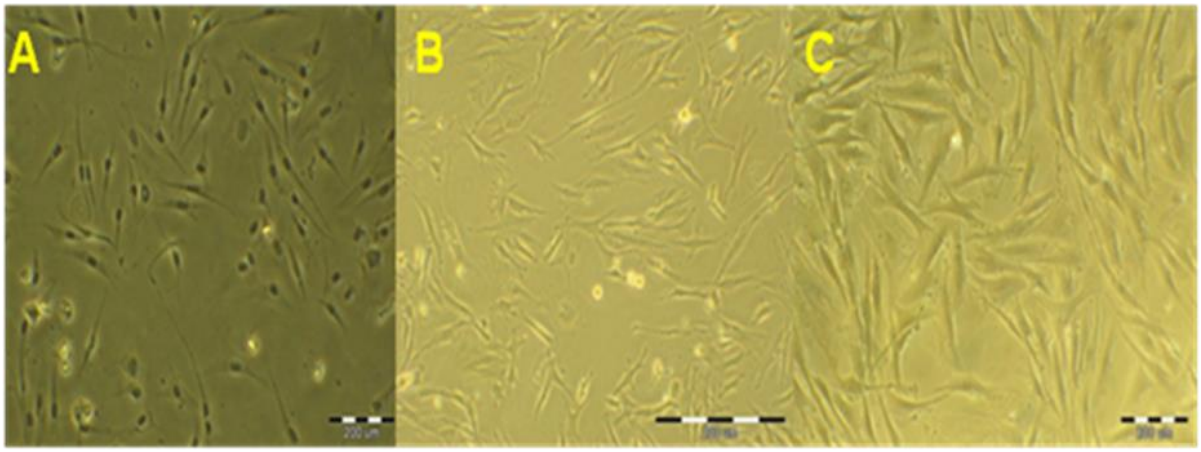


Figure 1. Phase-contrast images of primarily cultured OECs for 10 days**.** Both morphology of cultured OECs defined as Schwann-like and Astrocyte-like (fibroblast like)**: A)** Schwann-like with a long fusiform bipolar morphology, **B** and **C)** both types of OECs observed with different magnifications. Astrocyte-like with a flat sheet-like morphology and short randomly oriented processes.

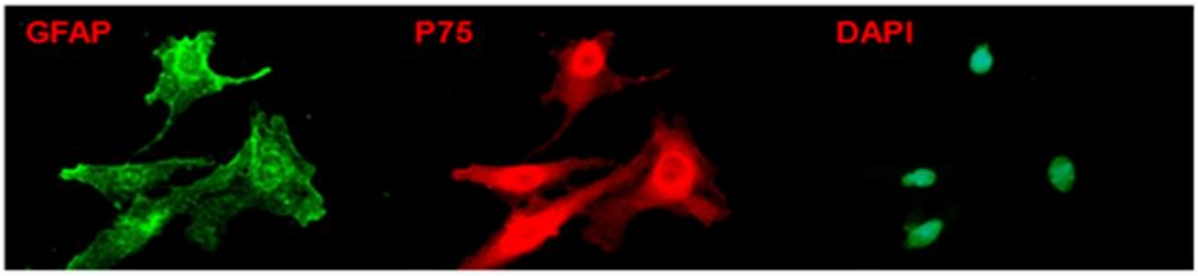


Figure 2. Simultaneous double staining (Immunocytochemistry (ICC)) for p75 and GFAP markers of cultured OECs **,** $p75^{+}/GFAP^{+}$ are double positive cells for p75 and GFAP markers at the same time. Counterstaining (nuclear staining) was performed by DAPI in all samples.

Discussion

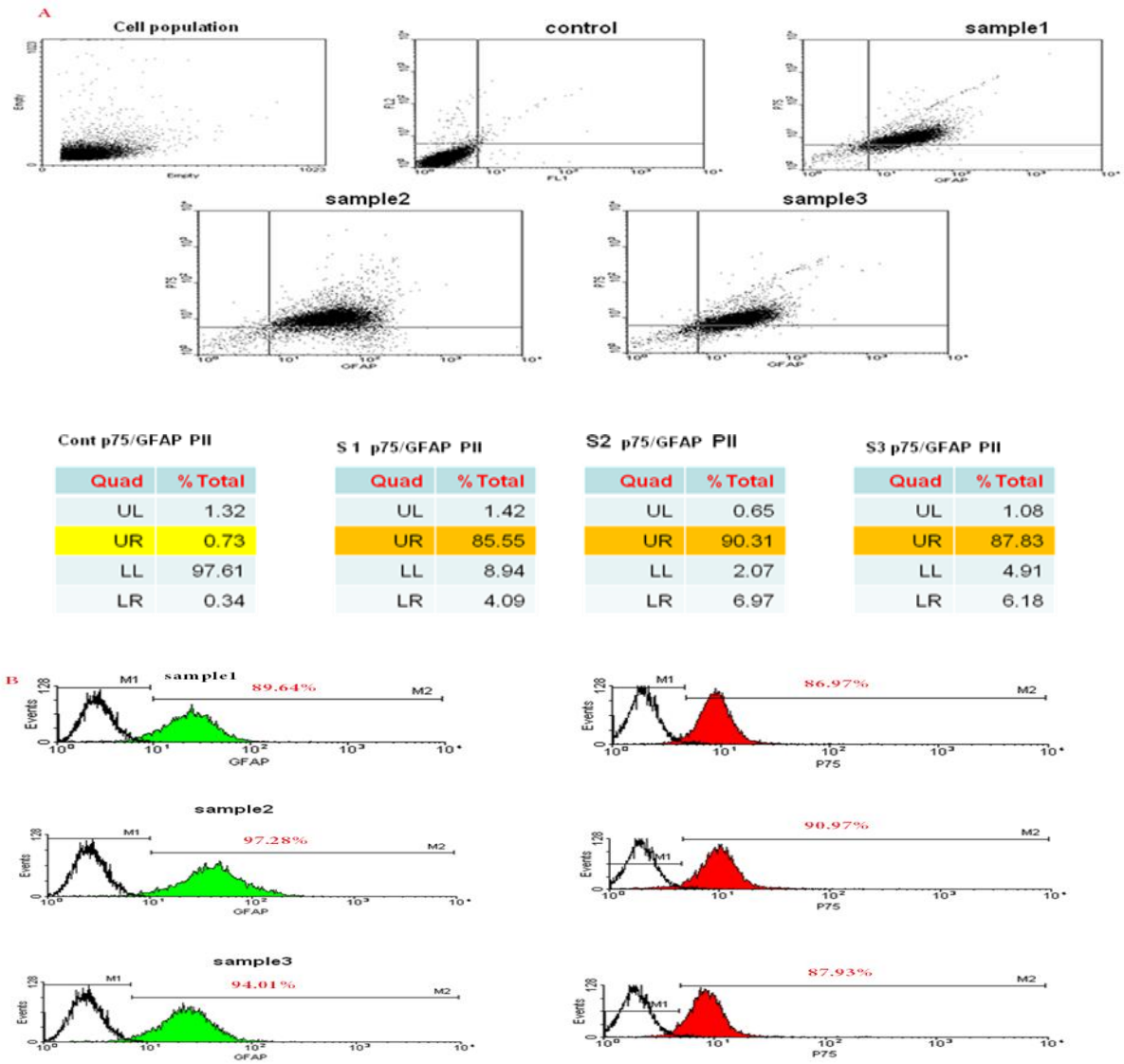
Cell transplantation is one of the most popular strategies for SCI repair. So, the main objective of the current research was OECs purifying in the primary cultures by simple method and determining their purity. Hence, OECs were isolated from olfactory mucosa (OM) of 7 days old wistar rats' pups and were cultured. For determining of OECs and their purity Immunocytochemistry and Flow-cytometry were performed respectively. Cultured OEC's by our team demonstrated two different phenotypes after attaching to flask surface which was in accordance with other researchers who also reported two subpopulations of OEC's (31, 36).

Barnet and Riddle exhibited two subtypes of OEC's that were purified from the olfactory bulb of 7-day old Sprague-Dawly rats' pups after 12 days that was in agreement with our findings (37).

Ensheathing cell morphology is categorized as flat or process-bearing. Flat OEC's (schwann-like) characterized by their long fusiform bipolar morphology (35). Process-bearing (Astrocyte/or fibroblast-like) OEC's are identified with very little cytoplasm and some fine randomly oriented processes longer than the width of the cell body (35) .Vincent and coworkers proposed that the morphological plasticity of OEC's is related to the culture conditions and is rapid and reversible (35).

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Cont p75/GFAP PII

Quad	% Total
UL	1.32
UR	0.73
LL	97.61
LR	0.34

S 1 p75/GFAP PII

Quad	% Total
UL	1.42
UR	85.55
LL	8.94
LR	4.09

S2 p75/GFAP PII

Quad	% Total
UL	0.65
UR	90.31
LL	2.07
LR	6.97

S3 p75/GFAP PII

Quad	% Total
UL	1.08
UR	87.83
LL	4.91
LR	6.18

Figure 3. Cell population graph, A) Dot Plot, B) Histogram from flow-cytometry of OECs for p75/GFAP markers after second passage of cultured cells. In the Dot plot UR demonstrates cells that were double positive for two markers simultaneously, but UL and LR demonstrate cells that were single positive for one marker only. Experiment was performed three times (sample1, 2, 3) to confirm it's reproducibility and results were expressed as mean ± SD. Control group (Negative control) was the group in which primary antibodies were omitted and were stained in parallel.

Vincent and his research group showed that cultured OEC's undergo rapid and reversible changes between flat morphology in the presence of serum and process-bearing morphology in the absence of serum (35).

Purity of OEC's was examined after simultaneous double staining for p75/ GFAP, by flow-cytometry. The cell markers p75 and GFAP were chosen to identify OEC's owing to no coexpression of these proteins by other cell types from the olfactory mucosa. Only cells, immunopositive for both markers were defined as OEC's (35). Our flow-cytometry results showed that 87.9±2.4% of cells were p75/ GFAP double positive cells, 1.05 ±0.4 only p75 positive and 5.8±1.5% were single positive for GFAP only. But p75+/GFAP+ and p75+/S100+ or GFAP+/S100+ were olfactory ensheathing cells. In the culture procedure used in this study, 87.9% of cells were the population of p75+/GFAP+ OEC's alone. Thus purity of our culture was probably more than 87.9%, because some of single

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p75 and GFAP positive cells (p75+ and GFAP+) in this immunostaining could be as p75+/S100+ and GFAP+/S100+ OEC's. Hence triple immunolabeling for these three markers of OEC' was more efficient to complete determining of OEC's purity, amounts of astrocytes and other cells which could contaminate our culture. Schwann cells from the trigeminal nerve are considered as bipolar p75-positive and GFAP-negative and cells that labeled strongly with GFAP but not p75-positive (p75-/ GFAP+) are considered as astrocyte. Astrocytes are abundant in the olfactory nerve layer (ONL) culture whereas they are absent in the OM culture (35).

In agreement with our results, there are findings of investigators that reported the percentage of OM-OEC's after single labeling for p75 marker by flow-cytometry for 85% (38).

But these investigators, in another report, reported that approximately 10% of OM-OEC's were p75-positiveby immunocytochemistry and flow-cytometry (39).

Another research by Vincent and his coworkers cultured OEC's from both olfactory mucosa (OM) and olfactory nerve layer (ONL), in unpurified separated cultures for 2 days in serum-containing Dval-10dS medium and reported that in both cultures, up to 70% of OEC's were process-bearing (astrocyte-like) and 30% were flat(Schwann-like), and both morphologies displayed GFAP staining. In this study p75/GFAP –positive cells were approximately 14% of total population in the OM culture and approximately 6% in the olfactory nerve layer (35). Some possible explanations that account for this discrepancy probably are: the time difference (2 days in Vincent study and 14 days in our study) and method and supplement used for OEC's culture as well as kind of the technique used for purity assessment (imunolabeling in Vincent study but flow-cytometry in our study).

The different experimental procedures used by investigators to purify and culture OEC's seem to partially contribute the varied descriptions of this cell type (35). Additionally, in the culture, the proportion of each subtype changes with the length of culture time (31).

Conclusion

Purity of cultured OECs for p75+/GFAP+ cells of the culture was 87.9±2.4% by flow-cytometry and it's percentage may be more than this if the p75+/S100+ and GFAP+/S100+ cells were immunostained and counted. Thus the culture procedure of this study seems to be a good protocol for OEC's purifying and cell therapy.

Acknowledgments

This research was supported by a grant from the Iran National Science Foundation (INSF) (no. 89003650). The authors would like to thank Anatomy Department and Cellular - Molecular research center of Iran University of Medical Sciences for their technical support. We also thank Atousa Hayat and Maryam Soleimani for their helpful assistance in flow-cytometry.

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