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Novel method to obtain highly enriched cultures of adult rat Schwann cells

Ali Niapour · Fereshteh Karamali · Khadijeh Karbalaie · Abbas Kiani · Mohammad Mardani · Mohammad Hossein Nasr-Esfahani · Hossein Baharvand

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Abstract Schwann cells (SCs) can be used to repair both the peripheral and central nervous systems. Therefore, establishment of a procedure to obtain activated, highly proliferative SCs, in an appropriate time for clinical applications, is a prerequisite. Purification is complicated by contamination with fibroblasts which often become the predominant cell type in an in vitro SC culture. This study describes a novel and efficient method to enrich SCs by utilizing the differential detachment properties of the two cell types. In culture, cells were treated with two different media and the chelator, EGTA, which detached SCs faster than fibroblasts and allowed for easy isolation of SCs. Within seven days, high yields of SCs with a purity of greater than 99% were achieved. This was confirmed by immunostaining characterization and flow-cytometric analyses using an antibody against the p75 low affinity nerve growth factor receptor (p75LNGFR). The entire procedure was completed in approximately 21 days. This method has the advantage of being technically easier, faster, and more efficient than other previously described methods. An SC culture that was about 99% homogenous was achieved.

Keywords Sciatic nerve · Predegeneration · Schwann cell · Detachment property · Purification

A. Niapour · F. Karamali · K. Karbalaie · A. Kiani · M. H. Nasr-Esfahani (✉)
Department of Cell and Molecular Biology, Royan Institute for Animal Biotechnology, ACECR, P.O. Box 815896-8433, Isfahan, Iran
e-mail: mh.nasr-esfahani@RoyanInstitute.org

A. Niapour · M. Mardani
Department of Anatomy, Isfahan University of Medical Science, Isfahan, Iran

H. Baharvand (✉)
Department of Stem Cells and Developmental Biology, Royan Institute for Stem Cell Biology and Technology, ACECR, P.O. Box 19395-4644, Tehran, Iran
e-mail: Baharvand@RoyanInstitute.org

H. Baharvand
Department of Developmental Biology, University of Science and Culture, ACECR, Tehran, Iran

Introduction

Schwann cells (SC) are the main glial cells of the peripheral nervous system which can promote neural regeneration by at least three routes: (i) an increase in cell surface adhesion molecular synthesis (Ide 1996), (ii) production of a basement membrane which consists of extracellular matrix proteins (Rothblum et al. 2004), and (iii) production of neurotrophic factors and their corresponding receptors. SCs have been extensively investigated for cell therapy in a variety of CNS injury models, including the spinal cord (Pearse et al. 2004; Barakat et al. 2005), brain (Kromer and Cornbrooks 1985), and optic nerve (Harvey et al. 1995). Moreover, autologous nerve

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grafting or biodegradable conduits are the gold standard for peripheral nerve repair (Mackinnon and Dellon 1990). In this category, an active area of research involves the development of a biodegradable conduit which contains SCs for the promotion of axonal regeneration. Therefore, mass production of pure SCs within a short period of time is a prerequisite to achieving this aim.

Isolation and purification of SCs is usually a complex process. This complexity is caused by fibroblast contamination which is due to the higher and faster proliferation rate of fibroblasts than SCs. Two main approaches have been implemented for the isolation of SCs: pre-degeneration (Keilhoff et al. 2000; Verdu et al. 2000; Vroemen and Weidner 2003) and intact nerve (Weinstein and Wu 1999). The pre-degeneration approach results in a higher yield of SCs relative to the intact nerve approach (Keilhoff et al. 2000; Mauritz et al. 2004). However, to obtain purified and homogenous SCs different modifications to the pre-degeneration approach have been applied which include: (i) antimitotic treatment such as the arabinoside chlora toxin (Calderon-Martinez et al. 2002), (ii) a combination of antimitotic treatment and antibody-mediated cytolysis which employs the use of complements (Assouline et al. 1983), (iii) repeated explantation methods (Oda et al. 1989; Morrissey et al. 1995a, b), (iv) differential adhesion and detachment methods (Mauritz et al. 2004; Pannunzio et al. 2005; Jin et al. 2008), (v) immunoselective methods (Manent et al. 2003; Vroemen and Weidner 2003), and (vi) serum tapering (Komiyama et al. 2003; Hedayatpour et al. 2007).

Each of these methods have disadvantages: the need for special equipment, high cost, low cell yields, utilization of antimitogens that are not clinically acceptable, and requirement of long time for cell growth. This study now introduces a simple, fast, inexpensive, and efficient method that results in the production of both homogenous and purified SC enrichment.

Materials and methods

Cultivation of SCs

Nerve harvesting and predegeneration

All animal care and surgical processes were undertaken in strict accordance with the approval of the Institutional Review Board and Institutional Ethical Committee of Royan Institute. Sciatic nerve fragments with lengths of approx. 20–25 mm were taken bilaterally from deeply anesthetized (ketamine 80 mg/kg and xylazine 7 mg/kg i.p.) adult Wistar rats (average weight 200–250 g) under aseptic conditions and transferred to a sterile work bench. Nerves, in a sterile environment, were weighed and twice washed in phosphate-buffer saline (PBS; Gibco) and the epineurium was stripped off by fine forceps. A sterile scalpel was utilized to cut the tissues into short segments of 2–4 mm in length. The protocol of SC isolation and purification was summarized in Fig. 1. Sciatic nerve pieces were kept for 15 days for in vitro predegeneration. To allow fibroblasts to migrate out of the explants, we incubated them for 2 weeks in a predegeneration solution comprised of DMEM supplemented with 10% (v/v) fetal calf serum (FCS) and 1% (w/v) penicillin/streptomycin. Simultaneously, in

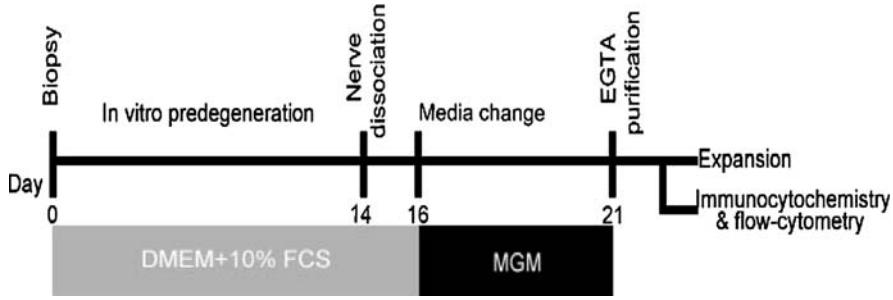

Fig. 1 Depiction of the various treatments used to purify SCs in culture. In brief, after 2 weeks of predegeneration, sciatic explants are dissociated and plated in DMEM + 10% (v/v) FCS onto laminin-coated tissue culture dishes for 24 h. For next 5 days, The DMEM is replaced with MGM media to promote SC proliferation and reduce fibroblast expansion. Purification is fulfilled via EGTA treatment

order to elevate SC proliferation, 2 μM forskolin, as a SC specific mitogen, was added. The medium was refreshed biweekly.

Dissociation and primary plating

Pre-degenerated adult rat sciatic nerves were exposed to supplemented DMEM and an enzyme mixture consisting of 0.125% collagenase Type IV and 1.25 U dispase/ml at 37°C and 5% CO_2 for 20 h (Mauritz et al. 2004). After incubation, the cell mixture was recovered by centrifugation at 1,800 g/w for 10 min at room temperature. The pellet was subsequently resuspended in supplemented DMEM and plated on to 2 μg laminin/ml (Sigma)-coated dishes at 75,000 cells/cm^2 for 24 h. Finally, the media was changed with melanocyte growth medium (PromoCell) to which 2 μM forskolin (Calbiochem), 10 ng FGF-2/ml (Sigma) and 5 μg bovine pituitary extract/ml (BPE-26, Promocell) were added in order to prevent fibroblast proliferation. As a control, the media was changed with fresh supplemented DMEM.

SC purification

Cells were purified at approximately 75% confluence, which occurred within 5 or 6 days post plating. To enrich adult rat SCs, initially the dishes were washed with 2 ml Ca^{2+}- and Mg^{2+}-free PBS followed by 2 ml 1 mM EGTA (Sigma). The dishes were agitated for 2–4 min to release rounded-up or detaching cells. The detachment was monitored with phase contrast microscopy. The suspension of floating cells that consisted of mainly SCs were collected into a centrifuge tube and centrifuged at 1,800 g/w for 10 min. After removal of the supernatant, the pellet was resuspended and plated as described.

Characterization of SCs

Morphology

SCs were marked as phase bright, bi-, tri- or multipolar with a small cytoplasm to nucleus ratio as seen under phase-contrast microscope; while fibroblasts were identified by a much more flattened polymorphic shape with larger rounded nuclei (Morrissey et al. 1995a, b). The total cell numbers and number of SCs were counted from six random fields (magnification 10×) with a phase-contrast microscope) by two independent investigators.

Immunofluorescent staining

SCs were plated on laminin (5 μg/ml, Sigma) and poly L-ornithine-(15 μg/ml, Sigma)-coated glass coverslips. The cells were washed in PBS and fixed with 4% (v/v) paraformaldehyde (Sigma) in PBS for 30 min. Fixed cells were washed with PBS. Blocking was carried out in a blocking buffer that consisted of 10% (v/v) goat serum and 1 mg BSA/ml in PBS for 1 h. Primary antibodies against p75 low affinity NGF receptor (p75LNGFR, 1:500, Abcam; ab6172) were applied in dilute buffer consisting of 3% (v/v) goat serum and 1 mg BSA/ml in PBS at room temperature for 2 h or overnight at 4°C. Then cells were washed and the secondary antibody, goat anti-mouse IgG conjugated-fluorescein isothiocyanate (FITC, 1:50), was applied for 45 min at 37°C. Cells were counterstained with DAP I for 2 min and observed under fluorescence microscope. For negative controls, the primary antibody was excluded.

Flow-cytometry analysis

The harvested cells were analyzed on a FACS Calibur flow-cytometer (Becton-Dickinson, Germany) directly after the staining procedure. Data were processed with WinMDI 2.8 software. The percentage of SCs in various samples was determined by measuring the fraction of p75LNGFR positive cells in the fluorescence intensity dotplot as compared to the total amount of intact cells. A histogram was subsequently prepared. The percentage of putative SCs among total cells was obtained based on three independent experiments.

Purity and yield of SCs

The purity and cell yields were determined by flow cytometry and cell counts with a hemocytometer following trypsinization, respectively. Purity was defined as percentage while yields were determined as the mean of 10^6 cells per biopsy.

Results

Net weight of sciatic nerve

The lengths of six sciatic nerve fragments varied between 20 and 25 mm and their average weights were 12 ± 3 mg.

Primary culture and SC enrichment

Figure 2a shows the phase-contrast image of sciatic nerve explants following 2 weeks of in vitro predegeneration. An average of $4.1 \pm 0.71 \times 10^6$ cells/biopsy were obtained from enzymatic digestion of the two week cultures of predegenerated explants which were resuspended in DMEM supplemented with 10% (v/v) FCS (Table 1). The majority of suspended cells adhered to laminin coated dishes within 24 h and developed into two distinct shapes that represented fibroblasts and SCs. Fibroblasts were characterized by a flat and polymorphic shape with an ovoid nucleus and blunt cytoplasmic processes; while SCs were identified as having small, bright and bipolar or tripolar cell characteristics (Fig. 2b). At this stage, cellular purity was 64.3 ± 0.8% (Table 1). Following replacement of the medium with MGM, the number of fibroblasts substantially reduced (Fig. 2c). Since a high percentage of fibroblasts were observed in the control group, these cells were not used for further characterization (data not shown). Following treatment with EGTA, most bipolar and tripolar cells rounded-up, detached, and were easily released; whereas flat and polygonal-shaped cells did not show these changes (Fig. 2d). Cells obtained from each dish were collected and reseeded respectively into a new laminin-coated dish (Fig. 2e). Seven days after culture, predegeneration and enrichment; the purity of SCs reached 99.3 ± 0.4 with a yield of $2 \pm 0.3 \times 10^6$ cells/biopsy (Table 1). Figure 2f–h shows cellular immunostaining and flow-cytometry, revealing greater than 99% positivity for p75LNGFR, a marker of SCs.

Discussion

SCs are valuable for peripheral nerve repair as well as for spinal cord injury or myelin defects. Therefore, obtaining pure sources and large scale SC production is a prerequisite. The main obstacle for purification of SCs is fibroblast contamination. This study describes an efficient laboratory procedure for purification and production of SCs, however it is important to note that this is not a comparative study.

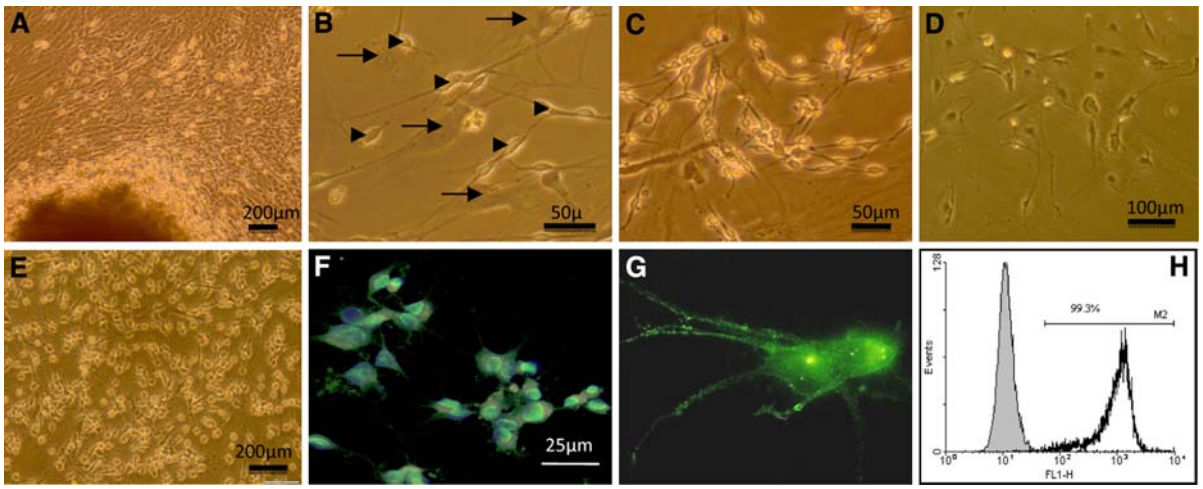

Fig. 2 Purification of SCs and fluorescent immunostaining. **a** Phase-contrast photomicrograph of sciatic nerve explants after 2 weeks of in vitro predegeneration. **b** Culture with DMEM. Fibroblasts (*black arrows*) can be morphologically identified and are clearly differentiated from SCs (*arrow head*). **c** Primary culture with MGM. **d** Remaining fibroblasts after purification. **e** Population‘ of SCs after purification. **f** Immunocytochemical identification of adult rat SCs by p75LNGFR and counterstained by DAPI. **g** Higher magnification of an immunofluorescent stained SC with anti-p75LNGFR. **h** Histogram by flow-cytometry for p75LNGFR positive SCs

Table 1 Schwann cell yield and purity from various biopsies of adult rat sciatic nerves

Wet sciatic nerve with epineurium (mg)	Primary cell yield (million cells/biopsy)	SC cell yield (million cells/biopsy)	(%) Purity before enrichment	(%) Purity after enrichment
12 ± 3	4.1 ± 0.71	2 ± 0.33	64.3 ± 0.8	99.3 ± 0.4

Data represent the mean of results from n = six experiments (±SD)

To increase SC yield, the predegeneration method was used. In vivo and in vitro pre-degeneration of peripheral nerves presents a convenient and effective method to obtain activated SCs and an enhanced cell yield when compared to untreated nerves (Mauritz et al. 2004). The yield, purity, and proliferation rate of SCs increases upon exposure to heregulin/forskolin during the two week predegeneration process (Casella et al. 1996). Therefore, in this study, we also implemented this procedure.

The new approach implemented in this study involved the use of differential detachment time windows between SCs and fibroblasts during exposure to EGTA, in order to purify primary cultures. The key point to this approach is the use of a suitable extracellular matrix (ECM). An ideal matrix allows for differential separation of SCs from fibroblasts. Therefore, in this study we tested various concentrations of laminin and poly-L-ornithine as ECMs, which have been suggested in previous studies. Our observation revealed that implementation of 2 μg laminin/ml led to a higher purification as compared to poly-L-ornithine or the combination of both.

Cell seeding density is an important factor in purification efficiency since at a high cell density, SCs will adhere to fibroblasts. The cell density implemented in this study was 75,000 cells/cm^2, which was lower than previously reported and did not allow intermingling of fibroblast with SCs.

Another factor implemented in this procedure was the use of media that allowed for differential cell proliferation. SCs proliferate faster than fibroblasts in the first 48 h, but after 72 h fibroblasts are the dominating cell type in cultures, overlapping onto SCs. To overcome this problem, DMEM was exchanged with MGM which has been designed to reduce fibroblast proliferation while maintaining melanocyte proliferation. In addition, MGM mitigates the SCs process, thus reducing the adhesion of SCs to ECM which aids in their isolation during EDTA treatment. Culturing SCs in the absence of serum lead to detachment of these cells which allowed the cells to undergo anoikis or cell detachment and death. For this reason, a minimum of 2.5% (v/v) serum was added to DMEM. This has been a common approach for maximizing SC proliferation and minimizing fibroblast expansion, according to the literature (Komiyama et al. 2003; Hedayatpour et al. 2007). One innovation of the presented method is that if this enrichment method (DMEM with 2.5% serum) were implemented, the SCs would adhere firmly amongst proliferative fibroblasts and we would have been unable to use the EGTA treatment for further isolation. The only drawback in the use of good manufacturing practice (GMP) is the presence of bovine pituitary extract in its supplement, which can be replaced by an animal-free product.

Morphological observation in conjunction with cellular characterization showed that greater than 99% purification of SCs can be obtained by following this procedure. Similar results have been reported when the cold jet approach (Mauritz et al. 2004) or collagenase NB (Jin et al. 2008) were used as detachment methods. However when we implemented the cold jet procedure in our initial setup, we were unable to obtain a high purification yield.

In conclusion, our study shows that the use of differential detachment properties of SCs against fibroblasts could be considered to be one of the procedures for obtaining a large quantity of homogenous SCs within a plausible time frame.

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