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A method for obtaining Schwann cell cultures from adult rabbit nerve based on "in vitro" pre-degeneration and neuregulin treatment

Isabel de la Fuente, Ignacio Alcalde, Olga L. Gamboa, Manuel Garrosa and Manuel J. Gayoso

Department of Cell Biology, Histology and Pharmacology, Institute of Neurosciences of Castile and Leon, (INCYL), University of Valladolid, Spain

Summary. Schwann cells (SCs) are basic elements for cell therapy and tissue engineering in the central and peripheral nervous system. Therefore, the development of a reliable method to obtain SC cultures is required. For possible therapeutic applications the cultures need to produce a sufficiently large number of SCs with a high level of purity in a relatively short period of time. To increase SC yield and purity we pre-degenerated pieces of 1-2 mm of adult rabbit sciatic nerves by incubating them for seven days in Dulbecco's Modified Eagle's Medium supplemented with 10% fetal bovine serum, penicillin/streptomycin and NRG1-ß1. Following pre-degeneration the nerve pieces were dissociated and then cultured for 6 or 15 days in the same culture medium. After 6 days of culture we obtained around $9.5x10^3$ cells/mg with approximately 94% SCs (S-100 positive) purity. After 15 days of culture the yield was about $80x10^3$ cells/mg and the purity was approximately 75%. Pre-degeneration and subsequent culture of small pieces of adult nerve with NRG1-ß1 supplemented medium increased the number of SCs and restricted the overgrowth of fibroblast-like cells.

Key words: Schwann cell culture, Nerve pre-degeneration

Introduction

Schwann cells (SCs) provide a structural and trophic support for development and maintenance of neurons and their processes in the Peripheral Nervous System . In addition, SCs play a fundamental role in the regeneration of injured nerves (Bunge, 1993; Diaz-Flores et al., 1995; Bhatheja and Field, 2006). After nerve injury, such as total section, the distal stump degenerates and cellular and myelin debris are removed by macrophages and SCs (Fawcett and Keynes, 1990). SCs become de-differentiated, proliferate and constitute the Büngner's bands, which are the scaffolds for nerve regeneration. Under ideal conditions, the promptly sutured sectioned nerve may recover almost completely. However, in human surgery an acceptable functional outcome has been obtained in 30-93% of the suture-repaired nerve lesions depending on the nerve and level of the lesion (Kim et al., 2004). In contrast, when there is a considerable gap between the proximal and distal stumps the capability of SCs to support axonal regeneration can be surpassed. Therefore, a biological bridge is required in order to achieve nerve regeneration. The gold standard for this bridge is autologous nerve grafting, with the obvious drawbacks, such as morbidity and limited availability (Bellamkonda, 2006).

Functional recovery after autologous graft repair of human sciatic nerve lesions has been established between 24 and 80% (Guertin et al., 2005), the longer the graft the worse the outcome. Nerve allografts could be useful but immunosuppressive treatment is necessary (Scharpf et al., 2006). Artificial bridges have been made with biological structures such as vein and muscle (Berenholz et al., 2005; Raimondo et al., 2005) or synthetic materials (Gibson et al., 1991; Chen et al., 2005; Ciardelli and Chiono, 2006; Yoshitani et al., 2007; Bettinger et al., 2009). These artificial bridges are frequently seeded with cultured SCs which are autologous in the most common therapeutic models (Ansselin et al., 1997; Sinis et al., 2005; Keilhoff et al., 2006; Li et al., 2006).

In the Central Nervous System, glial cells (astrocytes and oligodendrocytes) do not support neural regeneration. Thus, SC grafting has been used to

Offprint requests to: Prof. Manuel J. Gayoso, Dpto. de Biología Celular, Histología y Farmacología, Facultad de Medicina, Universidad de Valladolid, Av. Ramón y Cajal 7, E-47005 Valladolid, Spain. e-mail: gayoso@med.uva.es

facilitate regeneration and remyelination of lesioned axons (Rasouli et al., 2006; Li et al., 2007; Oudega, 2007).

A key point for the success of all these therapies is the availability of SCs, and therefore, a reliable method for acquiring SCs in culture is required. It is easier to obtain them from nerves of neonatal rather than adult animals, probably because neonatal nerves have a population of non-myelinating SCs, which are able to proliferate (Dong et al., 1997; Haastert et al., 2007). However, in most useful therapeutic approaches autologous SCs have to be obtained from the nerves of adult animals.

Nerve dissociation yields a mixed cell population of SCs, as well as perineurial and endoneurial fibroblast-like cells. To purify SC cultures, fibroblast-like cells have to be eliminated because they grow very fast and overtake the population of SCs. To remove this fast growing cell population different strategies, such as antimitotic drugs (Kreider et al., 1981; Morrissey et al., 1995; Calderon-Martinez et al., 2002), differential adhesion (Kreider et al., 1981), magnetic sorting (Manent et al., 2003) or immunopanning (Haastert et al., 2009) have been developed. Other methods to obtain SC-enriched cultures are based on SC-de-differentiation and proliferation caused by Wallerian degeneration in the distal stump of a sectioned nerve (Stoll and Muller, 1999). In some of these methods the distal stump of the sectioned nerve is left in the animal for several days ("in vivo" pre-degeneration) before being harvested for implant or culture. The "in vivo" pre-degenerated nerve segments are more effective than freshly obtained grafts for nerve repair (Danielsen et al., 1994; Bertelli et al., 2006), possibly due to SC proliferation and the secretion of growth factors, although the actual biological mechanisms remain unknown. "In vivo" pre-degenerated nerve segments also produce, after dissociation and culture, a greater number of SCs with more purity and proliferative capability than non-pre-degenerated ones (Keilhoff et al., 1999; Komiyama et al., 2003; Mauritz et al., 2004; Pannunzio et al., 2005). Another means of obtaining pre-degenerated nerve segments is to culture them "in vitro" following neurectomy. After seven or more days of pre-degeneration the explanted pieces of nerve can be dissociated and cultured (Keilhoff et al., 1999) or, in different techniques, be submitted to multiple explants before cell culture (Morrissey et al., 1991). Thus, whether it be "in vivo" or "in vitro", nerve segment pre-degeneration is the starting point of several methods to obtain SC-enriched cultures. Obviously, "in vitro" pre-degeneration will be more useful in clinical practice.

In order to improve SC cultures, several substances, such as pituitary extracts (Paivalainen et al., 2008; Haastert et al., 2009) and neuregulins (Honkanen et al., 2007; Dreesmann et al., 2009) have also been used. In nervous tissue, NRG-1-ß1 (Heregulin1-ß1) is believed to participate in the control of myelination, in the proliferation, survival and differentiation of SCs, and also in demyelination caused by nerve injury (Guertin et al., 2005). Experimental models of therapeutic uses of SC cultures for nerve or spinal cord repair have been performed mainly on small animals, such as rats (Li et al., 2007) and mice (Goto et al., 2010; Xu et al., 2011), while those dealing with larger animals such as cats (Wrathall et al., 1982; Pannunzio et al., 2005; Raimondo et al., 2005), dogs (Dong et al., 1997; Pauls et al., 2004; Rasouli et al., 2006) or rabbits (Strauch et al., 2001; Young et al., 2002; Mohanna et al., 2003) are few in number.

The aim of this study was to obtain highly enriched SC cultures from adult rabbit nerves using "in vitro" pre-degeneration and NRG-1-ß1 as a specific growth factor. We chose rabbits for this study, since their size and availability makes them very useful animals for experimental studies of nerve and spinal cord lesion repair. The method we described is easier and more reproducible than previous methods used in other mammal species.

Materials and methods

Ten male and female 6-8 month old New Zealand rabbits (4.2 Kg to 5.1 Kg) were obtained from the Animal Centre of the University of Valladolid. Care and manipulation of the animals followed the guidelines of the European Union (2010/63/EU) for Laboratory Animal Care and Experimentation. The animals were anesthetized with ketamine (80 mg/Kg, Ketolar®, Pfizer) and xylazine (20 mg/Kg, Rompun®, Bayer Health Care). The sciatic nerves were exposed through a dorsal incision and a 2-cm segment of approximately 0.3 g in weight was removed under aseptic conditions. Sciatic nerve segments were washed in cold L-15 (Leibovitz medium, Sigma, St. Louis, MO, USA) with penicillin/streptomycin (GIBCO, Carlsbad, CA, USA). The epineurium was stripped off with a pair of fine forceps. The sciatic nerve segments were cut into pieces 1 mm in length and placed free floating in 3.5 cm-diameter culture dishes with 3 ml of culture growth medium (CGM). This CGM consisted of Dulbecco's Modified Eagle's Medium (DMEM, Sigma) supplemented with 10% fetal bovine serum (FBS, Sigma), penicillin/streptomycin (GIBCO) and Recombinant Human NRG1-ß1 / ß1 EGF domain (10 nM, 8 μl/ml, Sigma). The culture dishes with the sciatic nerve pieces were kept for seven days (Kraus et al., 2010) in an incubator at 37°C in a humidified atmosphere containing 5% CO_2 with a change of CGM on the fourth day.

Samples for light and electron microscopy were taken at the end of the pre-degeneration period. Pieces were fixed in buffered glutaraldehyde (1%)-paraformaldehyde (1%) and subsequently processed for electron microscopy. Semithin (1 μm) sections were stained with toluidine blue and examined by light microscopy, and ultra-thin sections were observed under a Jeol 100B transmission electron microscope.

After 7 days in culture, sciatic nerve pieces were washed with cold Hank's balanced salt solution, minced with two sterile scalpels and incubated for 4 hours in 2 ml of enzymatic solution at 37°C. This enzymatic solution was prepared by dissolving 5 mg of type II trypsin (1800 unit/mg, Sigma) and 2.5 mg of type II collagenase (GIBCO) in 2 ml of L-15 medium (Kreider et al., 1981). After enzymatic incubation the pieces were disaggregated by passing through a 1ml micropipette tip. The cell suspension was centrifugated at 1800 g for 3 min at room temperature, and washed twice in DMEM with FBS and antibiotics. The resulting cell suspension was counter-stained with trypan blue in a hemocytometer and seeded in three culture dishes with CGM. After 3 days, the culture medium was replaced to remove the non-adhered cells. Microphotographs were taken with a digital camera (Nikon DXM 1200) connected to the phase contrast microscope (Nikon Eclipse TE300). The cell cultures were kept in CGM until becoming sub-confluent, usually seven days after primary culture. On the same day, cells from one of the dishes were washed three times in Ca^{++} and Mg^{++} free Hank's solution, detached with 0.05% trypsin and 0.02% EDTA in the same medium, washed three times in DMEM with FBS and antibiotics, counted in a hemocytometer and replated in two new dishes in CGM. The two remaining dishes were fixed with 4% buffered para-formaldehyde for 15 min at room temperature and processed for immunocytochemistry.

Subsequent cell cultures were kept in CGM medium in an incubator, and the culture medium was changed every 3 or 4 days. When dishes of the primary culture were sub-confluent, one was fixed for immunocytochemistry and the cells from the other were detached, counted and expanded in two new dishes for the first passage. A similar procedure was adopted for the following passages.

A morphological study of SCs was carried out under phase contrast microscopy and immunohistochemical characterization was conducted by using rabbit polyclonal antibodies against S-100 calcium-binding protein (Dako, Glostrup, Denmark) and mouse monoclonal antibodies against the low-affinity nerve growth factor receptor $p75^{LNGFR}$ (Chemicon, Billerica, MA, USA) (Dong et al., 1997; Mauritz et al., 2004). Nuclei of all cultured cells were stained with 4', 6-Diamidino-2-phenylindole (DAPI, Sigma).

After fixation, cells were washed twice in PBS and incubated overnight at 4°C in a double antibody solution: anti S-100 protein (1:300) and anti $p75^{LNGFR}$ (1:50) with 5% goat serum in PBS-Triton X-100 (0.2%) (Fluka, St. Louis, MO, USA). The cultures were then immediately washed with PBS and incubated for 2 h at room temperature with secondary antibody solution: goat anti-rabbit IgG (1:100) (Invitrogen-Molecular Probes Carlsbad, CA, USA), and goat anti-mouse IgG (1:100) (Invitrogen-Molecular Probes) in PBS.

Labelled cells were visualized and photographed in an epifluorescence microscope (Zeiss Axiophot) with a digital camera (Spot RT Color).

Quantitative results were expressed as mean ± standard error of the number of cells obtained from each series of plates counted at 13, 16, 19 and 22 days. Data analysis was done by one way ANOVA and Tukey and Scheffé post hoc test, with $p<0.05$ taken as the significance limit.

Results

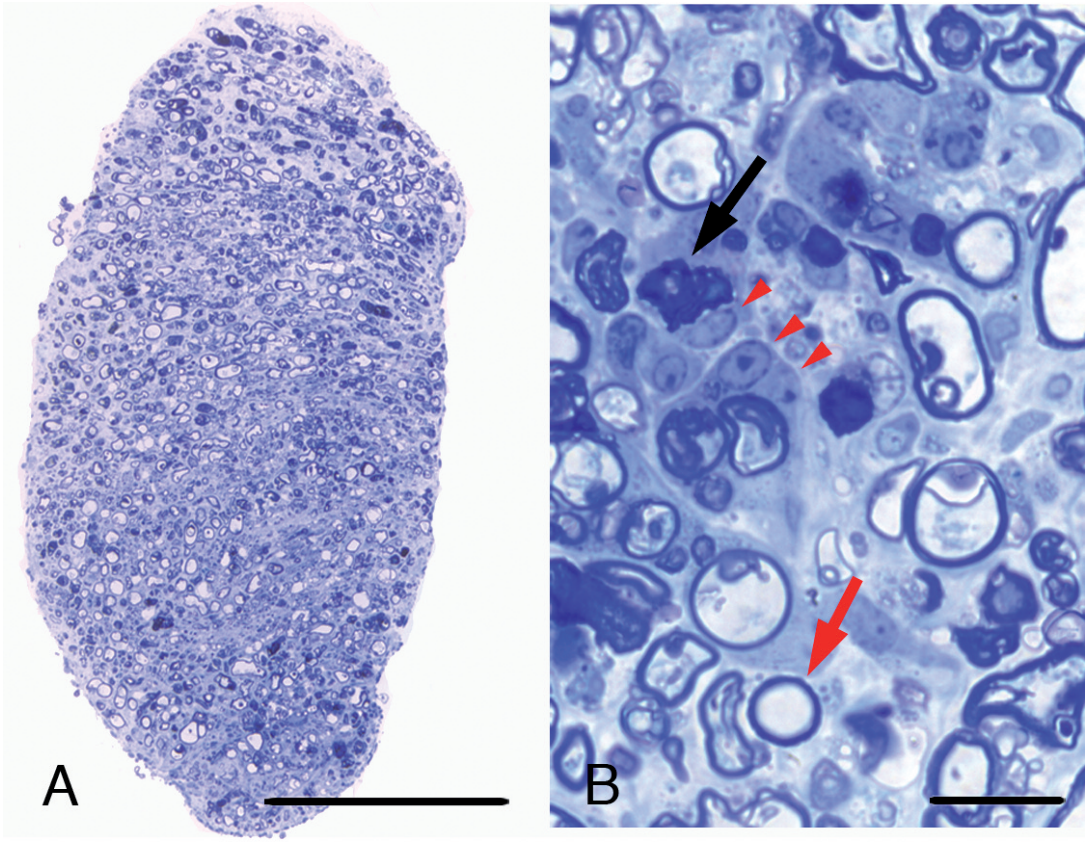
Sciatic nerve fragments showed at the end of the "in vitro" pre-degeneration period (7 days) histological features similar to those characteristic of Wallerian degeneration in the distal stump of a sectioned nerve (Fig. 1). These nerve fragments contained different degrees of myelin sheath disorganization, ranging from relatively well-preserved sheaths to completely compacted and unwinding ones (Fig. 1B,C). SCs were distinguished by their relationship with the degenerated myelin sheath and were characterized by the largely dispersed euchromatin nucleus, a well defined nucleolus and, compared with the normal nerve, a relatively abundant cytoplasm (Fig. 1B,C). In the SC cytoplasm numerous small lipid droplets were distinguished. Fibroblast-like endoneurial and perineurial cells can be identified by their slender morphology and more heterochromatic nuclei (Fig. 1B,C). The most external perineurial cells were rounded, whereas the internal ones maintained their flattened appearance.

After enzymatic disaggregation and 6 days in culture (13^{th} day), the nerve segments, of about 0.30 g each, produced $2.85\pm0.11x10^6$ viable cells (Table 1), corresponding to approximately $9.5x10^3$ cells/mg. In the primary culture most cells formed parallel bundles and showed a typical elongated, fusiform, bipolar or tripolar morphology characteristic of cultured SCs. A small number of flattened cells with rounded and larger nucleus were observed among cell bundles (Fig. 2A). The immunocytochemical study showed that almost all the cells were S-100 (94.34±1.43%) (Fig. 2B) and $p75^{LNGFR}$ (88.03±5.49%) positive (Fig. 2C). Both antibodies labelled cells with the same spindle-shaped morphology, which were mainly double labelled (Fig. 2D). A few cells were negative for both antibodies and showed flattened cytoplasms and a large round nucleus.

At the end of the first passage (16^{th} day)

Table 1. Summary of the processes and cell yield during Schwann cell obtaining.

Day	Culture	Cells/nerve segment	S-100 (%)	p75 (%)
1	Nerve pre-degeneration			
7	Primary culture start			
13	1^{st} passage	$2.85\pm0.11x10^6$	94.34±1.43	88.03±5.49
16	2^{nd} passage	$6.45\pm0.11x10^6$	76.66±11.89	71.01±8.70
19	3^{rd} passage	$11.34\pm0.13x10^6$	83.62±5.64	74.67±3.29
22	End of 3^{rd} passage	$23.99\pm0.5x10^6$	86.77±5.79	74.78±4.02



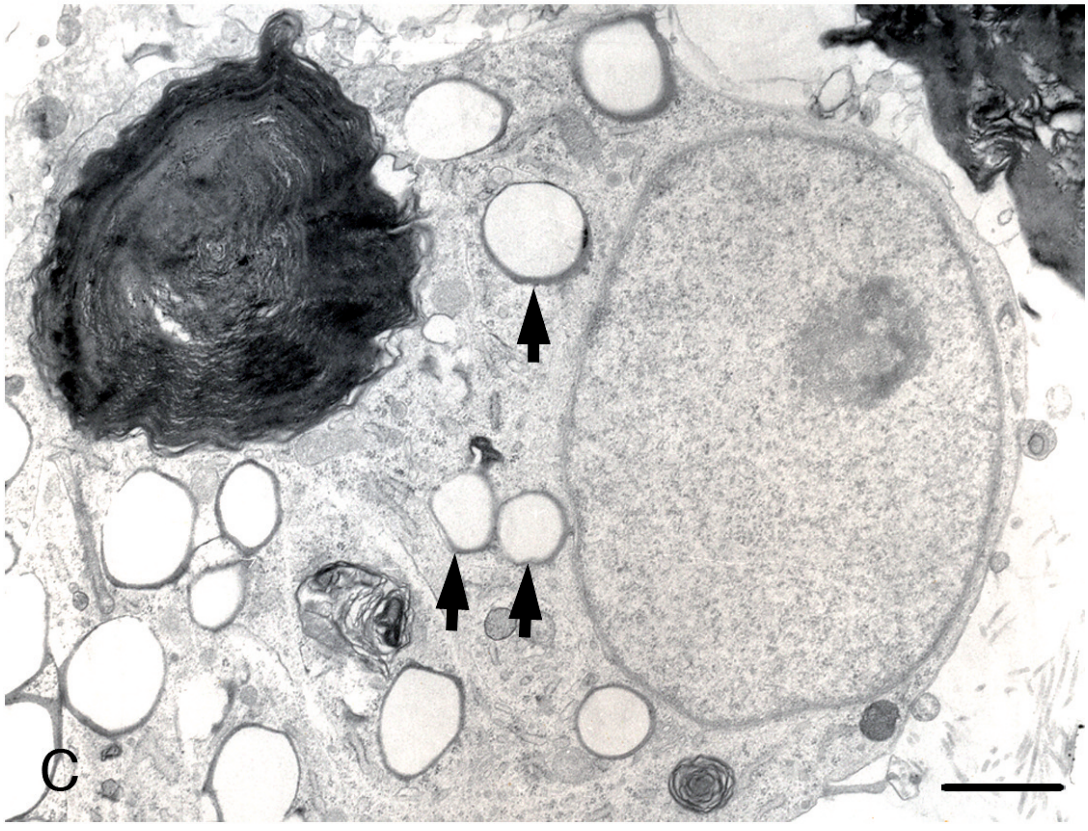


Fig. 1. Sections of sciatic nerve piece at 7 days of pre-degeneration. **A.** General aspect showing loss of fascicle organization due to perineurium and endoneurium stromal de-arrangement. Fibers appear at different degrees of disorganization. Toluidine blue. **B.** Close-up view where some fibers show smooth and regular myelin sheaths (red arrow), whereas others appear completely unwound (black arrow). SCs (arrowheads) keep these myelin debris inside them. Toluidine blue. **C.** Electron-micrograph of SC, where numerous lipid droplets (arrows) and degenerating myelin figures can be seen. Scale bars: A, 100 μm; B, 10 μm; C, 1 μm.

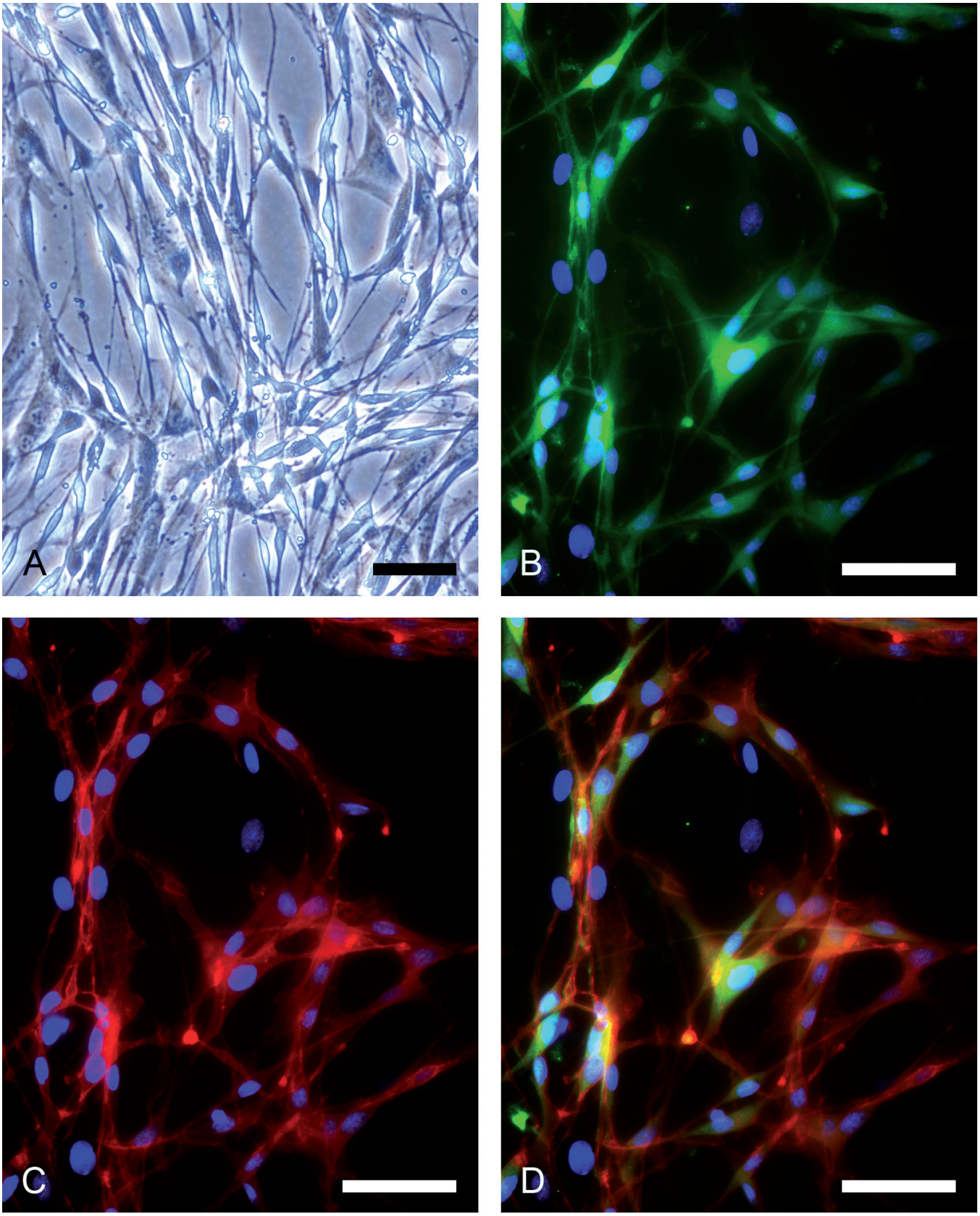


Fig. 2. SCs after 13 days of culture. SCs show their characteristic bipolar or tripolar morphology under phase contrast microscopy **(A)** and positivity to S-100 **(B)** and $p75^{LNGFR}$ **(C)** immunolabelling, with a high degree of co-localization **(D)**. Scale bars: 20 μm.

$6.45\pm0.08x10^6$ viable cells were obtained from each nerve segment (Table 1). The cell morphology was similar to that observed in the primary cultures. The proportion of S-100 (76.66±11.89%) and $p75^{LNGFR}$ positive cells (71.01±8.70%) was smaller than in primary cultures but the differences were not statistically significant. The second culture passage (19^{th} day) yielded $11.34\pm0.13x10^6$ viable cells per nerve segment (Table 1). We did not detect changes in the morphology of these cells. The percentages of S-100 (83.62±5.64%) and $p75^{LNGFR}$ positive cells (74.67±3.29%) decreased, but these changes were not statistically significant. The third culture passage (22nd day) yielded $23.99\pm0.5x10^6$ viable cells per nerve segment (about $79.97x10^3$ cells/mg) and showed cellular morphology and percentages of S-100 (86.77±5.79%) and $p75^{LNGFR}$ positive cells (74.78±4.02%), similar to the two previous passages (Table 1).

Discussion

The method we have described is based on pre-degeneration together with a specific mitogen (NRG1-ß1). When "in vitro" pre-degeneration is carried out with nerve pieces fastened to the culture plate, the fibroblasts proliferate and migrate from nerve pieces to the culture plate. After about 10 days the nerve pieces are re-plated four or more times to deplete them of fibroblasts and then dissociated to obtain SCs (Morrissey et al., 1995). In contrast to these authors, we use "in vitro" pre-degeneration with free floating nerve pieces in a culture medium. When fibroblasts are cultured in floating collagen matrices there is decreased signalling through the ERK pathway (Rosenfeldt and Grinnell, 2000), down regulation of cyclin D1 (Fringer and Grinnell, 2001) and fibroblasts can undergo degeneration instead of proliferation. This situation could be similar to the "in vitro" free floating pre-degeneration that we used, which would cause fibroblasts to remain quiescent while SCs are responsive to mitogens.

The morphology of pre-degenerated nerve pieces at day 6 is similar to the distal stump of a sectioned nerve under Wallerian degeneration, but without the removal of axon and myelin debris by macrophages. "In vivo" myelin degradation is considered to be initiated at the Schmidt-Lantermann clefts by the SCs within 24 h of axonal loss and to continue from 5th day onwards by macrophages (Williams and Hall, 1971). In our experiments myelin sheaths start to be degraded by the SCs without macrophage collaboration and seem to be suspended, waiting for retrieval by the "in vivo" macrophages. The lipid droplets seen in the SC cytoplasm could be derived from myelin degradation.

Our results are similar to those obtained in mouse pre-degenerated nerves, by adding to the culture medium 10% FBS, NRG-1 and forskolin (Manent et al., 2003). In our experiments we used only NRG-1 without forskolin or any other cAMP-elevating agent, as in a possible clinical application setting forskolin could be potentially dangerous. Forskolin seems to be necessary to attain the maximal level of SC proliferation after heregulin stimulation (Schworer et al., 2003). Heregulin, together with forskolin, increases the level of expression of a group of genes, many of them related with cell division, but these genes are not up-regulated by heregulin or forskolin alone (Schworer et al., 2003). Some members of the heregulin family have been considered, in contrast to other growth factors, not in need of the concurrence of cAMP-elevation factors for producing mitogenic effects (Casella et al., 1996; Kim et al., 2004). The reason for this discrepancy might lie in the use of serum in the culture medium (Schworer et al., 2003). When a serum-free medium is used, cAMP elevating agents could be necessary to obtain the mitogenic effect of neuregulin, but when 10% of FBS is used some serum factors could replace the forskolin effect, allowing neuregulin to act. Therefore, when a low concentration of FBS is used, the addition of forskolin could increase the rate of cell culture growth.

Our observations (data not shown) support the importance of fetal serum concentration in the evolution of SC cultures, since cultures with 10% FBS, compared with those of 3% FBS, showed a greater number of cells but lower SC purity. Komiyama et al. (2003) observed that 10% FBS favours SC migration from the nerve explants, while 0% FBS suppresses fibroblast overgrowth and favours SC predominance, and therefore proposed serial changes of FBS concentration to optimize SC cultures.

Maintaining pre-degeneration for 1 or 2 weeks is considered to produce an increase in cell yield of approximately 50% more than when the nerve is immediately dissociated (Morrissey et al., 1991; Casella et al., 1996; Kraus et al., 2010). After pre-degeneration, 1 cm of human intercostal nerve may provide approximately 10^6 cells after immediate dissociation and could generate $3x10^9$ cells in a total culture period of 10 weeks (Casella et al., 1996). We obtained from each 2-cm nerve segment approximately $2.4x10^7$ cells at day 22 ($2x10^5$ cells/mg), a number similar to that obtained by Casella et al. (1996) and higher than that obtained by Morrissey et al. (1991) ($2x10^4$ cells/mg), after multiple explants over 5-6 weeks.

We used nerves from adult animals despite the fact that those taken from newborn animals are much more efficient in terms of cell yield and viability (Brockes et al., 1979; Hou et al., 2006). However, in clinical practice neonatal involvement in nerve repair is rather occasional, whereas the most severe nerve injuries take place in the adult, and the best choice for implants are autologous SCs (Bellamkonda, 2006).

Our procedures yielded a SC purity of between 94% (day 7) and 71% (day 16) comparable to those reported by other authors (Morrissey et al., 1991; Verdu et al., 2000; Mauritz et al., 2004; Jin et al., 2008). By means of differential adhesion to laminin during exposure to a chelating agent (EGTA) and a very low cell seeding density, Niapour et al. (2010) obtained more than 99%

SC purity.

The present method, using "in vitro" nerve predegeneration and NRG-1 as a trophic factor, is a reliable and relatively simple method to obtain high and pure yields of SCs in SC cultures from adult nerves, and may be of benefit for nerve repair.

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