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Interferon beta and glatiramer acetate induce proliferation of Schwann cells *in vitro*

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Techniques as well as substances capable of stimulating cultured Schwann cell (SC) proliferation are needed for future therapeutical applications. In this work, the effects of interferon beta (IFNbeta) and glatiramer acetate (GA) on SC cultures were tested, with interest on the growth curve and potential proliferative effects. Primary cultures were prepared from the sciatic nerves of neonatal rats and seeded onto culture plates. Such cells were then subjected to treatment with different doses of IFN beta (100, 500 and 1 000 IU/ml) and of GA (1.2, 2.5 and 5.0 μg/ml) for five consecutive days. S100β and DAPI double labeling was used in order to confirm the purity of the cultures. Both treatment with IFN and GA resulted in an increased number of cultured SCs. However, only IFN beta induced a statistically significant proliferative outcome. Such results indicate that addition of IFN beta to the culture medium is efficient in order to improve SC proliferation *in vitro*.

Key words: Schwann cell, nerve regeneration, interferon beta, glatiramer acetate, cell culture

Schwann cells (SCs) are the myelinating glial cells of the peripheral nervous system (PNS), which support and ensheath axons with myelin to enable rapid saltatory action potential propagation in the axon. Following a peripheral lesion, the SCs acquire proliferative and fagocytic characteristics, developing a critical role during the Wallerian degeneration process (Ide 1996). Such cells support and provide substrate for the axonal growth (Garbay et al. 2000, Schmidt and Leach 2003). SCs also synthesize and release several neurotrophic factors such as NGF, BDNF, CNTF and GDNF that are fundamental during development and regeneration of the PNS (Jessen and Mirsky 1999, Zochodne 2000). Also, the inefficiency or absence of such cells results in the lack of axonal regeneration (Hall 1986, Berry et al. 2003). On the other hand, several studies with the tubulization technique using SCs provided evidence that such cells contribute to and accelerate the regenerative process (Evans et al. 2002, Schlosshauer et al. 2003). In this sense, new strategies that enhance the proliferation of SCs may positively contribute to the peripheral nerve regeneration process as well as to treat neurodegenerative diseases involving demyelination (Zujovic et al. 2007).

In the present work, two drugs that have been used to treat the remittent/recurrent form of multiple sclerosis (MS) were tested. These drugs, namely interferon beta and glatiramer acetate (GA), are both efficient in hampering the progress of the disease, although GA has shown better local tolerance. IFN beta is a pro-inflammatory cytokine that plays an immunomodulatory action on MS, such as the reduction of the expression of the major histocompatibility complex of class II molecules (MHC) and the increase in IL-10 (Arnason 1996). GA is a random mixture of synthetic polypeptides whose mechanism of action in MS includes high-affinity binding to various class II major histocompatibility complex (MHC) molecules. This efficient MHC binding results in both competition with myelin antigens for T cell activation and induction of specific regulatory T cells (Ziemssen and Schrempf 2007).

The exact mechanism of action of IFN beta and GA is not fully understood, nor are their interactions with nervous or glial cells, in particular with cells of the PNS. A recent study performed in the authors' laboratory showed that IFN beta influenced synaptic plasticity in the central nervous system (CNS) (Zanon and

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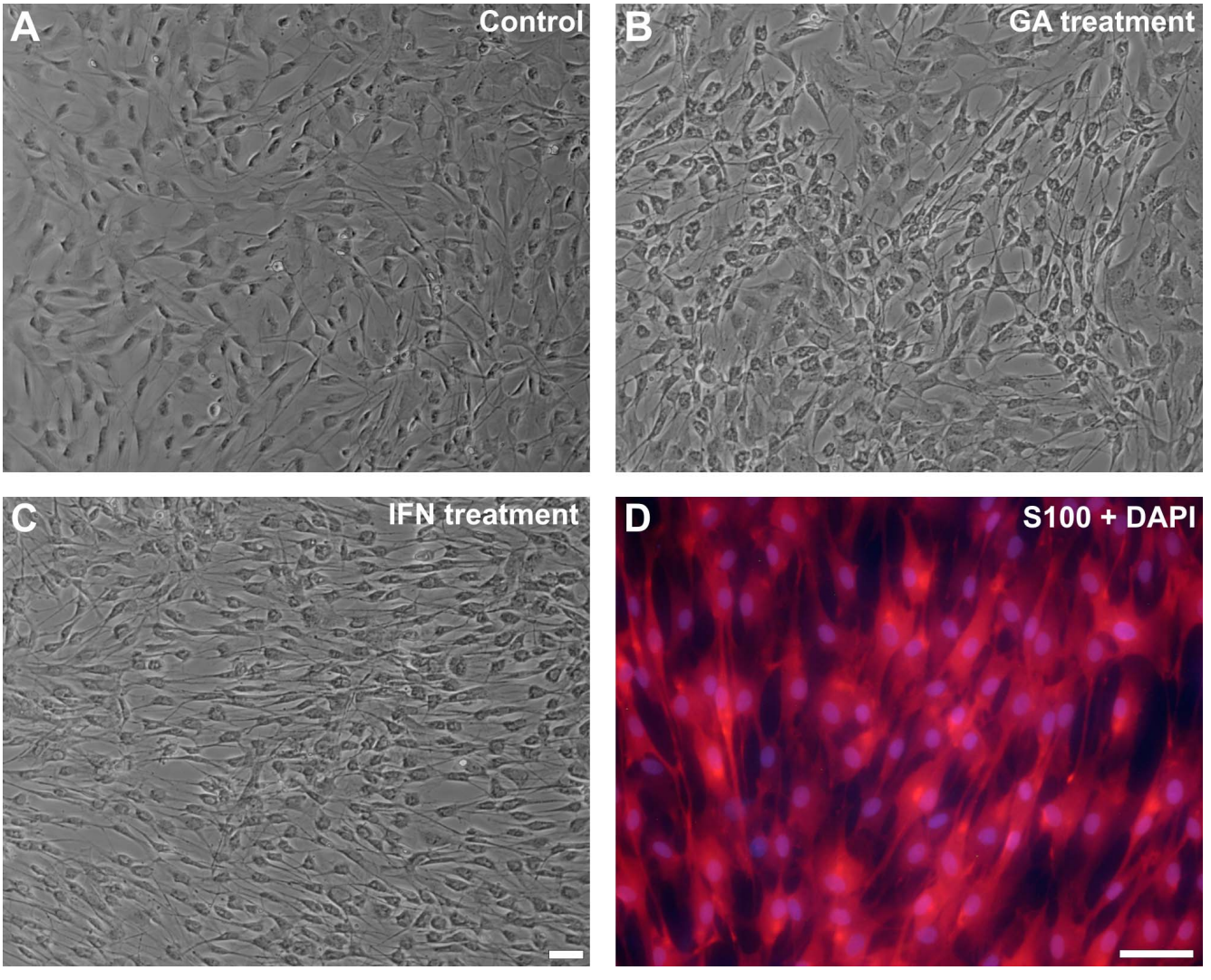

Fig. 1. (A–C) Schwann cells 5 days after culture: (A) Control culture with no drug added, (B) and (C) GA and IFN beta treated cells. Note the higher number of cells in comparison to the control. The micrograph in (D) represents DAPI and S100 double staining showing the purity of the Schwann cell culture. Scale is 50 μm.

Oliveira 2006). In the present work both drugs were tested on Schwann cell cultures, with special interest in the proliferative effects of such substances, which may in turn be used in future therapeutic approaches.

All experiments were carried out in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23). Cultures of the SCs were prepared by a slight modification of the methods of Brockes and coauthors (1979) and Assouline and colleagues (1983). Sciatic nerves from 1 or 2 day-old decapitated Sprague Dawley pups were aseptically removed and dissected free from the epineurium and contaminating tissue. Nerve segments were incubated in 0.05% collagenase for 30 minutes at 37°C and further incubated in 0.15% trypsin for 20 minutes. After incubation, the enzymes were inactivated by adding fetal calf serum, and 0.01% DNAse also added, all in hepes buffered Hanks' balanced salt solution without calcium and magnesium (HBSS). The cell mixture was recovered by centrifugation in 3% BSA (bovine serum albumin, Sigma) at 1 300 rpm for 10 minutes and resuspended in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal calf serum (glia medium, GM). The GM was supplemented with glucose (1 g/ml), insulin (1 ng/ml), nerve growth factor (NGF, 10 ng/ml), pituitary extract (18 ng/ml) and antibiotics as described by Hammarberg and others (1998).

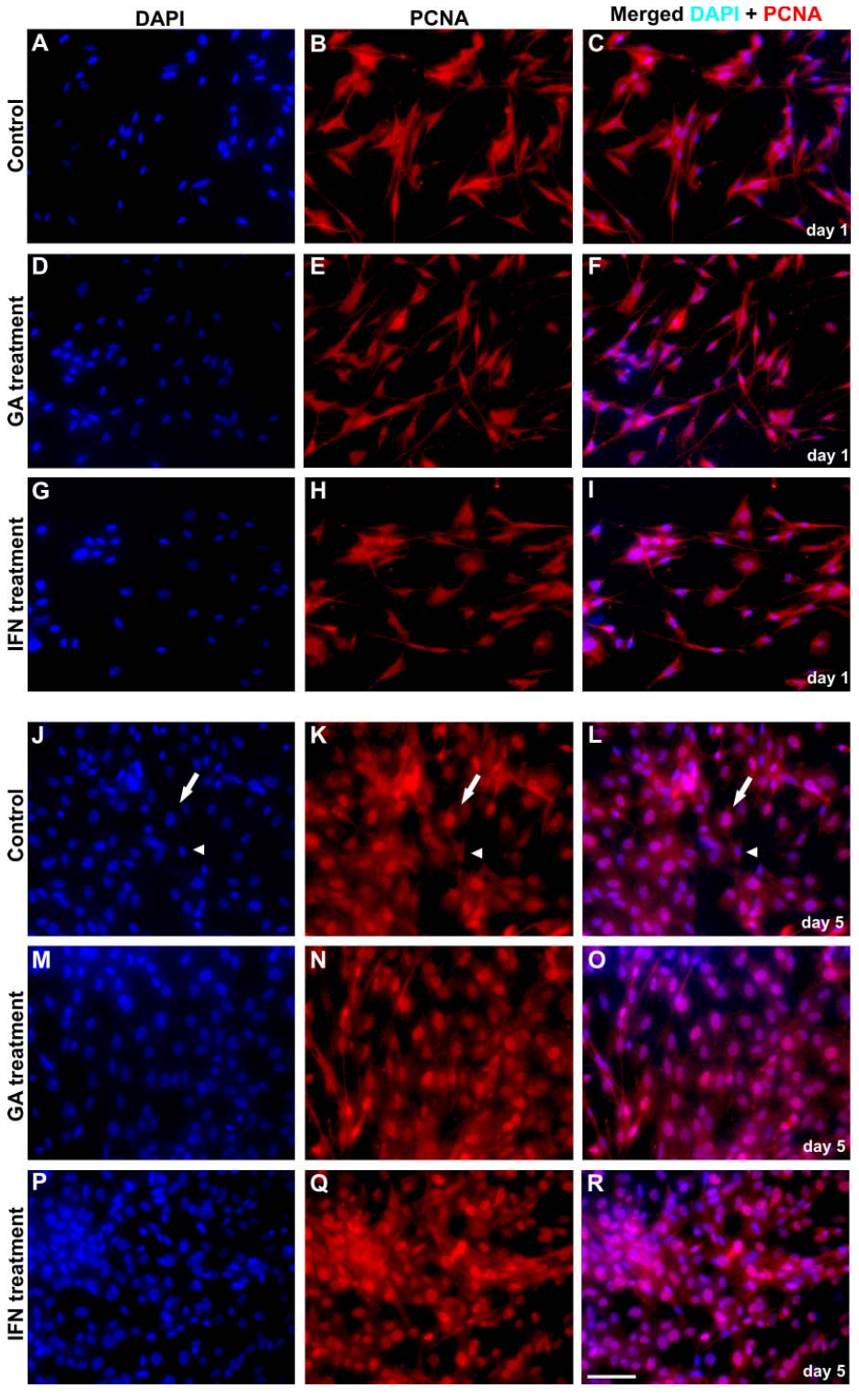

Fig. 2. (A–I) control (untreated), GA and IFN beta treated cultures (first to third line). (J–R) SCs at the fifth day of treatment. DAPI nuclei staining (blue) is presented on the first row, followed by PCNA positive cells (red). Merged images are shown sequentially. Note that at the beginning of the treatment, only a small number of SCs are PCNA positive (nuclear staining). Such cell proliferation marker is clearly more expressed into nuclei of SCs at the end of the treatment, especially in the IFN treated cultures (M–O). Arrows indicate double labeled cells, while arrow heads indicate cell nuclei only positive to DAPI staining. Scale is 100 μm.

An amount of 10^4 cells/well were seeded in culture plates with 24 wells (Corning/Costar Corporation, Cambridge, MA, USA) and the treatment with different doses of IFN beta (100, 500 and 1 000 IU/ml) and of GA (1.2, 2.5 and 5.0 μg/ml) started after 24 h and was continued for five consecutive days. The cultures were kept at a temperature of 37°C with an atmosphere of 5% CO_2, and all experiments were performed in triplicate. The cell cultures were examined and documented every day, and for each culture well, 10 random areas were captured with an inverted microscope (Nikon eclipse TS100) connected to a Nikon camera (DXM1200F). The number of cells was quantified with the Image Tool software (version 3.0, UTHSCSA, USA) by counting of DAPI stained nuclei.

After treatment, the cells were fixed in 4% paraformaldehyde for 10 minutes (Reagen, Brazil), and washed in 0.1 M phosphate buffer (PB), pH 7.4, at 37°C. To avoid nonspecific staining, the specimens were incubated for 45 minutes with 1% BSA in PB containing 0.25% Triton-X. The purity of the cultures was evaluated with the S100β marker and the proliferation study with PCNA (proliferating cell nuclear antigen) nuclei labeling (24 h after plating, third and fifth day of treatment). The samples were incubated for 2 hours with a rabbit anti-S100β (dilution 1:1 000) or with rabbit anti-PCNA antibody (dilution 1:200). In sequence, the cells were rinsed in PB at 37°C and incubated with Cy3 conjugated anti-rabbit secondary antibody (dilution 1:500) for 45 minutes at room temperature. Following immunostaining, cytochemistry with DAPI was performed for nuclei labeling. After several washes in PB, cell cultures were observed under a microscope equipped for fluorescence acquisition.

After 24 h of culturing in 24 well plates, the total number of cells was obtained at each day of treatment, for the construction of the growth curve. Figure 1 shows the control, GA and IFN beta treated cultures and depict that both drugs presented a considerable mitogenic effect following five days of treatment. The purity of the cultures was confirmed with S100β immunolabeling, an antibody commonly used for SC identification.

Figure 2 contains representative images of cultures treated with GA and IFN highest doses. Under such conditions the mitotic rate was calculated by the ratio of PCNA/DAPI labeling. Following 24 h of the first administration of GA (5.0 μg/ml) or IFNbeta (1 000 IU/ml) no statistical differences were obtained among the experimental groups. Nevertheless, mitotic effects could be identified after 5 days with IFN beta treatment. GA treatment did not result in statistical differences in comparison to control cultures (Fig. 3C).

Considering the growth curves of the treated cultures and the final number of cells (DAPI stained nuclei), a statistical difference after both treatments could be detected in all doses of IFN beta and in the two highest doses of GA (Fig. 3A–B). Five days after treatment with both drugs, the cells were quantified and compared to control cultures. Control cultures showed an increment of 199.9% ± 36 (Mean ± SE) while the IFN beta treated cells demonstrated the following growth percentages: 100 IU/ml – 402.4% ± 43, $P<0.01$; 500 IU/ml – 351.3% ± 27, $P<0.01$; 1 000 IU/ml – 384.3% ± 13, $P<0.01$ (Mann Whitney *U* test). GA treated cultures resulted in the following growth rates: 1.2 μg/ml – 308.2% ± 61, $P>0.05$; 2.5 μg/ml – 352.4% ± 39, $P<0.05$; 5.0 μg/ml – 420.5% ± 47, $P<0.05$ (Mann Whitney *U* test).

Figure 3C indicates the quantification and the statistical analysis of the mitotic rate. Such rate was calculated by the PCNA/DAPI ratio in purified cultures, previously tested with anti-S100 immunolabeling. Although not statistically significant, GA treatment showed an increase of 5% in cell growth in comparison to control cultures (day 5: 0.78 ± 0.04 – GA and 0.74 ± 0.01 – control, *t* test). Regarding the IFN beta treatment, an increase 10% in the third day and 15% in the fifth day of treatment was obtained (3^{rd} day: 0.70 ± 0.03, $P>0.05$; 5^{th} day: 0.85 ± 0.03, $P<0.05$, *t* test).

Injury to a peripheral nerve induces the so-called Wallerian degeneration process, which is more prominent in the distal stump (Chen et al. 2007). A hallmark of this process is that SCs shift to a more active state, detaching from axons and discarding the myelin sheaths that are broken down and phagocyzed by the SCs themselves and by resident and recruited macrophages (Ide 1996, Chen et al. 2007). Once free from contact with the axons, SCs proliferate and form Büngner bands that are columns of cells lined up inside the endoneural tubes. Such an arrangement is of crucial importance to the establishment of effective pathways for the guided growth of regenerating axons (Ide 1996, Chen et al. 2007). Since SCs play such a critical role in the regeneration process, their use has become central in tissue engineering approaches for nerve repair (Armstrong et al. 2007).

In this context, the use of artificial nerve conduits containing viable SCs is one of the most promising

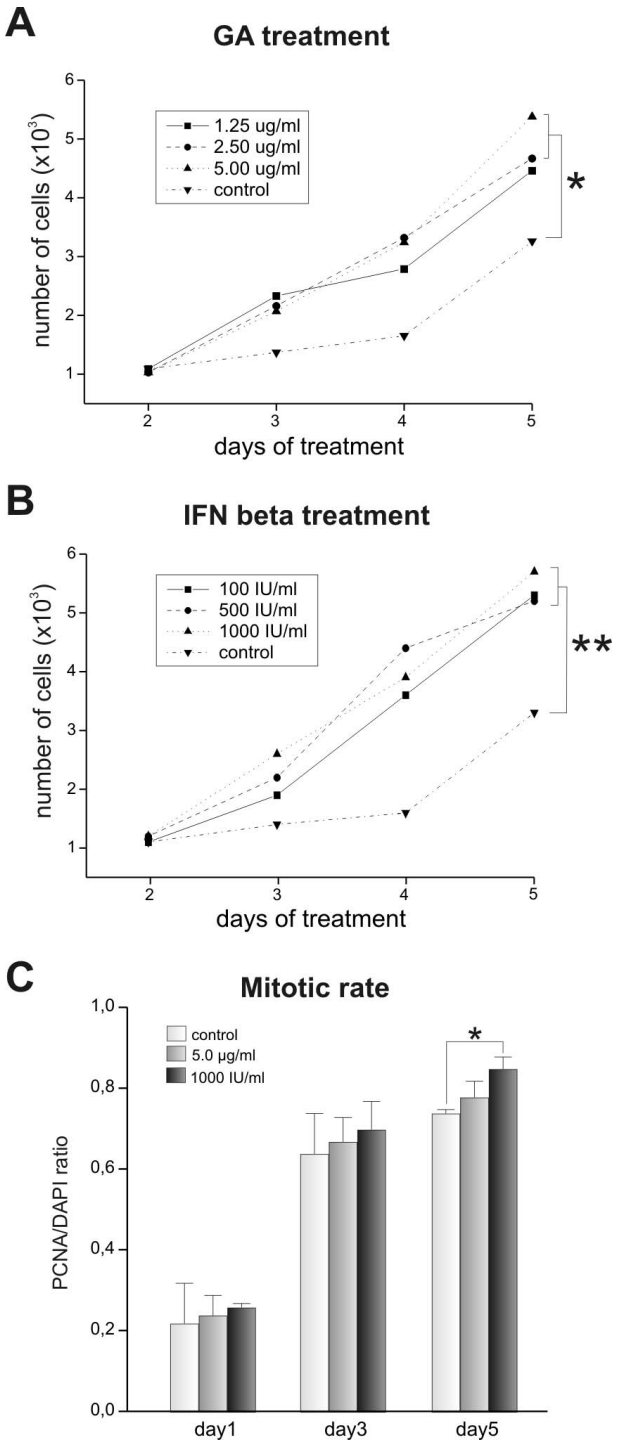

Fig. 3. (A), (B) Growth curves of control and treated cultures during whole period of treatment. Observe that both drugs stimulated cell proliferation at the fifth day. (C) PCNA/DAPI ratio at three time points of treatment (1st, 3rd and 5th day of culture) for control, GA (5.0 µg/ml) and IFN beta (100 µl/ml) treated cultures. Only IFN beta induced a statistically significant increase of PCNA nuclear labeling at the end of the treatment (5 days).

strategies to repair peripheral nerve injury (Kim et al. 2007). However, it is important to emphasize that the therapeutic effectiveness of autologous SC transplantation may be affected by *in vitro* proliferation issues, which have to be addressed. Proliferation and differentiation of SCs are regulated by various extracellular signals, such as humoral cytokines, membrane bound molecules (especially those on the surface of axons), and extracellular matrix molecules (Ogata et al. 2006). In this way, strategies to induce stronger proliferative responses by the SCs when cultured *in vitro* may be useful in order to increase the cell harvest and reduce the need for nerve fragments from the patient (Kim et al. 2007, Lavdas et al. 2008). The results presented herein address such an issue and demonstrate that the use of both interferon beta and glatiramer acetate resulted in SC proliferative stimulation.

Interferon beta is a pro-inflammatory cytokine which has significant impact on the treatment of the relapsing-remitting form of multiple sclerosis (Arnason 1996). It was used previously in the authors' laboratory to investigate peripheral nerve regeneration, demonstrating the ability to positively act in the spinal cord microenvironment, stimulating synaptic plasticity (Zanon and Oliveira 2006). In the present study, we demonstrated the positive impact of INF beta treatment on SC culture proliferation, which may persist during the regeneration process *in vivo*. In this sense, the use of IFN gamma, which displays similar functions than IFN beta, showed a direct influence on SCs proliferation, increasing the number of BrdU (mitosis marker) positive nuclei (Conti et al. 2002). Besides the possible direct effect of IFN beta, this cytokine may induce growth factors expression, such as BDNF (Hamamcioglu and Reder 2007) that could also positively interfere on SC proliferation process. It is well known that neutrophic factors are proteins which regulate growth and differentiation, and also programmed cell death in developing and mature neural crest derived cells (Blanchette and Neuhaus 2008). Regarding Schwann cells, neurotrophins are key mediators for myelination. Also, BDNF and NT3 are essential components for SC-neuronal interactions (Chan et al. 2001). In this sense, BDNF treatment increases the proliferation of SCs, the number of myelinating axons and the thickness of the myelin sheath *in vivo* and augment the expression of proteins of the extracellular matrix *in vitro* (Chan et al. 2001, Groot et al. 2006).

Regarding glatiramer acetate, it is known that it induces an elevation of BDNF, GDNF, NT-3/4, TGF-β, IL-4 and 10 by T cells within the Nervous System (Aharoni et al. 2005, Liu et al. 2007, Blanchette and Neuhaus 2008). It is possible that the positive effects of GA on SC proliferation are related to a stimulation of neurotrophic factor or cytokine synthesis. This would, in turn, induce an autocrine effect, accelerating the cell cycle and increasing the culture growth rate.

An important point to be emphasize is that, since SCs play a crucial role in the endogenous repair of peripheral nerves, due to their ability to dedifferentiate, migrate, proliferate, express growth promoting factors and myelinate regenerating axons (Oudega and Xu 2006), *in vitro* pre-treatment with growth and proliferative substances may increase their *in vivo* performance after grafting. Such a strategy would also be useful for certain CNS lesions, where SCs grafting is one of the most promising approaches for achieving functional repair. In addition to transplantation into the spinal cord, large numbers of SC would be necessary to fill, for example, injury-induced cystic cavities and to have a strong neurotrophic and neuroprotective effect when transplanted into lesioned areas of the brain (Zujovic et al. 2007, Lavdas et al. 2008). In this way, both drugs tested herein may be candidates to induce SC proliferation and neurotrophic factor expression. With this propose, studies are underway in our laboratory to address possible functional improvements after INF and GA treatment following peripheral lesions, as well as after *in vitro* SC pre-treatment followed by grafting in to CNS and PNS.

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