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Expression of basal lamina components by Schwann cells cultured on poly(lactic acid) (PLLA) and poly(caprolactone) (PCL) membranes

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Abstract The present in vitro study investigated the expression of basal lamina components by Schwann cells (SCs) cultivated on PCL and PLLA membranes prepared by solvent evaporation. Cultures of SCs were obtained from sciatic nerves from neonatal Sprague Dawley rats and seeded on 24 well culture plates containing the polymer membranes. The purity of the cultures was evaluated with a Schwann cell marker antibody (anti-S-100). After one week, the cultures were fixed and processed for immunocytochemistry by using antibodies against type IV collagen, laminin I and II. Positive labeling against the studied molecules was observed, indicating that such biomaterials positively stimulate Schwann cell adhesion and proliferation. Overall, the present results provide evidence that membrane-derived biodegradable polymers, particularly those derived from PLLA, are able to provide adequate substrate and stimulate SCs to produce ECM molecules, what may have in turn positive effects in vivo, influencing the peripheral nerve regeneration process.

1 Introduction

Peripheral nervous system regeneration is considered a highly complex phenomenon, which results in a chain of events expressed in a chronological and synchronized fashion, aiming at the readjustment of the lesioned nerve microenvironment [1, 2]. Following a peripheral nerve lesion, a series of changes occur in the distal stump, namely the Wallerian degeneration. The main events at this stage are degeneration of myelin sheath and axons that were disconnected from the neuronal cell body [3, 4].

Schwann cells (SCs) are the most important element during the events after peripheral nerve lesion. Soon after lesion there is an intense proliferation of such cells which together with macrophages, phagosize myelin and axon debris in the degenerating axotomized microenvironment. Of pivotal importance is that SCs organize themselves longitudinally forming the so called bands of Büngner (BB), that guide the growing axons to the target denervated organ [2, 5, 6].

Additionally to the BB formation, SCs actively modify and reorganize the extracellular microenvironment of the lesioned nerve, as well as synthesize different neurotrophic factors, such as nerve growth factor (NGF), brain derived neurotrophic factor (BDNF), cilliary neurotrophic factor (CNTF), contributing to the recovery of the nerve homeostasis and providing support to the growing axons [4, 7].

Among the factors described above, certain elements of the extracellular matrix (ECM, e.g., collagen and laminins) effectively contribute to the regenerative process, since the nerve sprouts from the proximal stump express receptors which recognize these molecules that serve as substrate to the guided axonal elongation [8–12].

Up to date, several biodegradable synthetic materials have been investigated and implanted in different tissues. Among the studied polymers, the poly (-hydroxy acid) constitutes one of the most up-and-coming group of biomaterials. The important advantage of such biopolymers is the degradation process, based on de-esterification. In this

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sense, once degraded, the monomeric components of each polymer are removed from the body by natural pathways [13]. The most often utilized synthetic polymers are the poly(glycolic acid) (PGA), the poly(lactic acid) (PLA), the poly(lactic-co-glycolide) (PLGA) and the poly(caprolactone) (PCL) [6, 14, 15]. These biomaterials display important properties regarding degradation rates, porosity and resistance to stress. Also, a critical characteristic is that they can be molded in different sizes and shapes [6, 14].

Taking into account the different properties of the biomaterials, the importance of the SCs, as well as the components of the ECM to the success of the PNS regeneration, the present study investigated the expression of type I and II laminins and type IV collagen by SCs cultured on membranes of PLLA and PCL. Such membranes were produced by solvent evaporation, providing a homogeneous and yet thin and resistant substrate for cell culturing. The results were analyzed by immunocytochemistry and scanning electron microscopy (SEM).

2 Material and methods

2.1 Biopolymer membrane preparation

The membranes used in the present study were produced by the technique of solvent evaporation, as described by Aldini et al. [16]. Briefly, 0.85 g of PLLA (molecular weight = 300 kDa) and 1.65 g of PCL (molecular weight = 100 kDa) (PURAC—Gerinchem, Holland). The biopolymers were dissolved separately in 33 ml of solvent (dichloroethane, Merck, Darmstadt, Germany), so that the final concentrations were 2.5 and 5%, respectively (weight/volume). The solutions were left at room temperature for 12 h and then poured into a flat glass chamber so that, after evaporation of the dichloroethane, a thin and uniform membrane was obtained (200 μm). Eventual solvent residues were removed by placing the membranes in a vacuum chamber for 5 min. The obtained PLLA and PCL membranes were then cut to fit the diameter of the culture wells and were disinfected in 70% alcohol.

2.2 Culture of SCs

The SC cultures were prepared following the method described by Brockes et al. [17] and Assouline et al. [18], with slight modifications. Briefly, sciatic nerves from neonatal Sprague Dawley rats ($n = 10$) were dissected out and freed from the epineurium and contaminating tissue. The nerves were then reduced into small fragments and incubated in collagenase and trypsin for 30 min at 37°C. The enzymes were inhibited with fetal calf serum and the tissue was triturated, filtrated, centrifuged and resuspended in Dubelcco's modified Eagle's medium (DMEM) with 10% fetal calf serum. The medium was enriched with forskolin and pituitary extract (10 μg/ml, Sigma). The obtained cells were then seeded on 24 well culture plates containing the polymer membranes. After 24 h of culturing, a further cell purification was performed by adding 10 nM of cytosine arabinoside for 48 h. The purity of the cultures was evaluated with the anti-S100 antibody (Dako, Glostrup, Denmark). The cultures, once purified, were maintained for one week on the biopolymers and the medium was changed every second day. All experiments were performed in triplicate.

2.3 Imunocytochemistry for basal lamina components

Following one week of culturing, the cells were fixed in 4% paraformaldehyde for 10 min (Merk) and washed twice in 0.1 M phosphate buffer (PB), pH 7.4, for 5 min. Preincubation with 1% bovine albumin for 45 min was followed by overnight incubation at 4°C with primary antisera (anti-S-100, anti-laminin I—double labeling against alpha 1 and beta 1 chains, anti-laminin II—double labeling against alpha 2 and beta 1 chains and anti-type IV collagen). After three rinses in PB, the cultures were incubated for 45 min at room temperature with secondary antibodies Cy-2 or

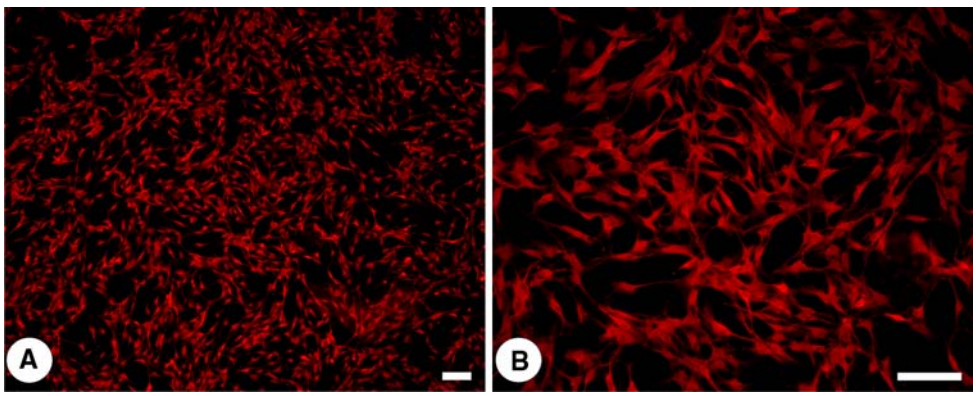

Fig. 1 **a** and **b** Representative examples of purified Schwann cell cultures stained with anti-S100 antibody. Scale = 100 μm

Cy-3 conjugated donkey anti-goat or anti-rabbit according to the primary antibodies. After rinsing in PB, the preparations were mounted in a mixture of glycerol/PB (3:1) and examined in a Nikon inverted microscope equipped with epifluorescence and appropriate filter combinations for the fluorophores used.

For quantitative measurements, five representative images of each culture (4,350 μm^2 each) made on PCL and PLLA membranes were captured at a final magnification of $\times 40$. Quantification was performed with the enhance contrast and density slicing feature of IMAGEJ software (version 1.33u, National Institutes of Health, USA). The integrated density of pixels was systematically measured and the mean value for each group was calculated. Eventual statistical differences were accessed as described below. Data is represented as mean ± standard deviation (SD).

2.4 Preparation for scanning electron microscopy

The cultures were fixed in Karnovisky (2% glutaraldehyde and 1% paraformaldehyde in 0.1 M cacodylate buffer, pH 7.4) for 15 min. A secondary fixation with 1% osmium tetroxide was performed, followed by buffer washing and dehydration in ethanol (30, 50, 70, 90 and 100%, 15 min each step). The specimens were then taken at the critical point (Balzers, CTD030) and coated with gold for 120 s at 20 mA using a sputter coater, resulting in an approximate coating thickness of 40 nm. The specimens were then viewed under a scanning electron microscope (Jeol, JXA 840A) operated at 10 kV. The area of 20 representative cells from each experimental group was obtained using the measurement tool of the Image Tool software (Version 3.0, The University of Texas Health Center in Santo Antonio, USA).

2.5 Statistical analysis

The parametric and non-parametric results were analyzed by the Student's *t*-test and the Mann–Whitney *U* test, respectively. The *P*-value considered as significant was $P < 0.05$ (*).

3 Results

Under phase contrast it was possible to observe that SCs displayed the characteristic bipolar morphology with thin cell processes. The immunolabeling against S-100 revealed that the cultures were highly pure, as seen in Fig. 1.

An important observation was that the number of SCs adhered to the PLLA was evidently greater when compared to the PCL biomembranes. Such a fact was also reinforced by the immunostaining against S-100, indicating that the tested biomaterials provided different adhesion conditions to the SC. The quantification of the immunostaining indicated a statistically significant greater S-100 expression on the PLLA membranes (57,465.33 ± 8,907.06—PCL;

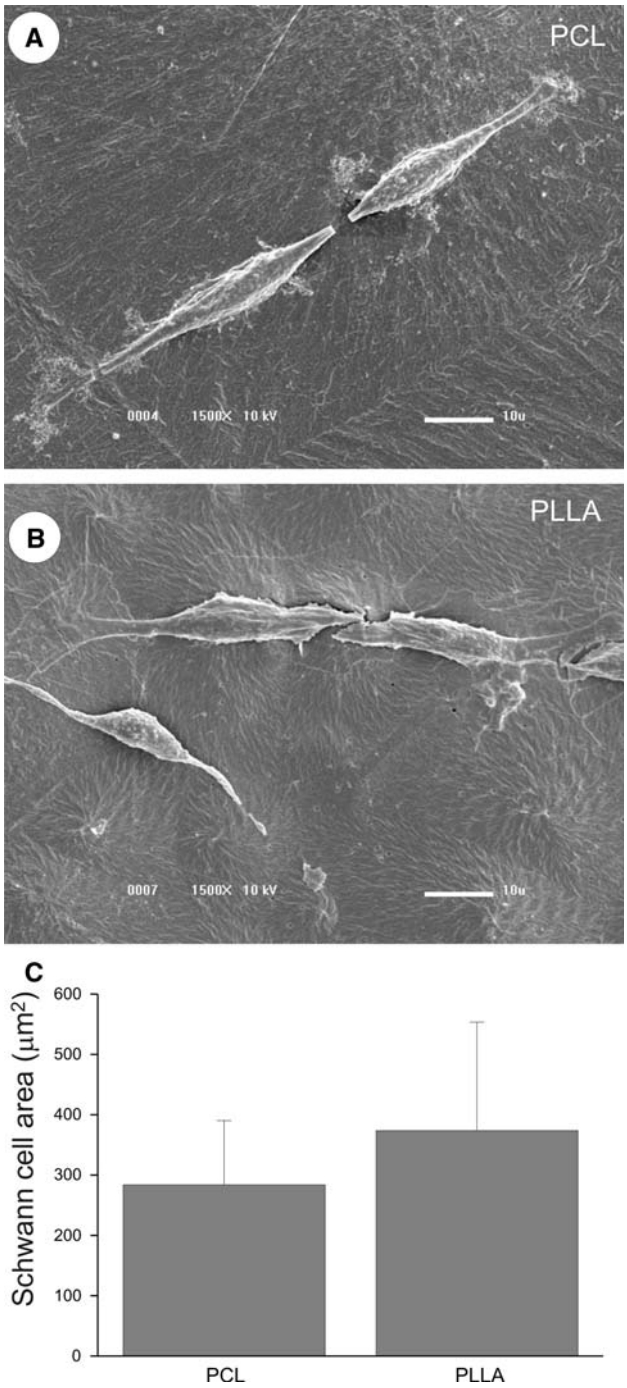

Fig. 2 **a** Schwann cells cultured on PCL membranes viewed by scanning electron microscopy. **b** Schwann cells cultured on PLLA membranes. **c** Although PLLA substrate provided a better adhesion surface to the cultures, no difference in cell area could be observed. Scale = 10 μm

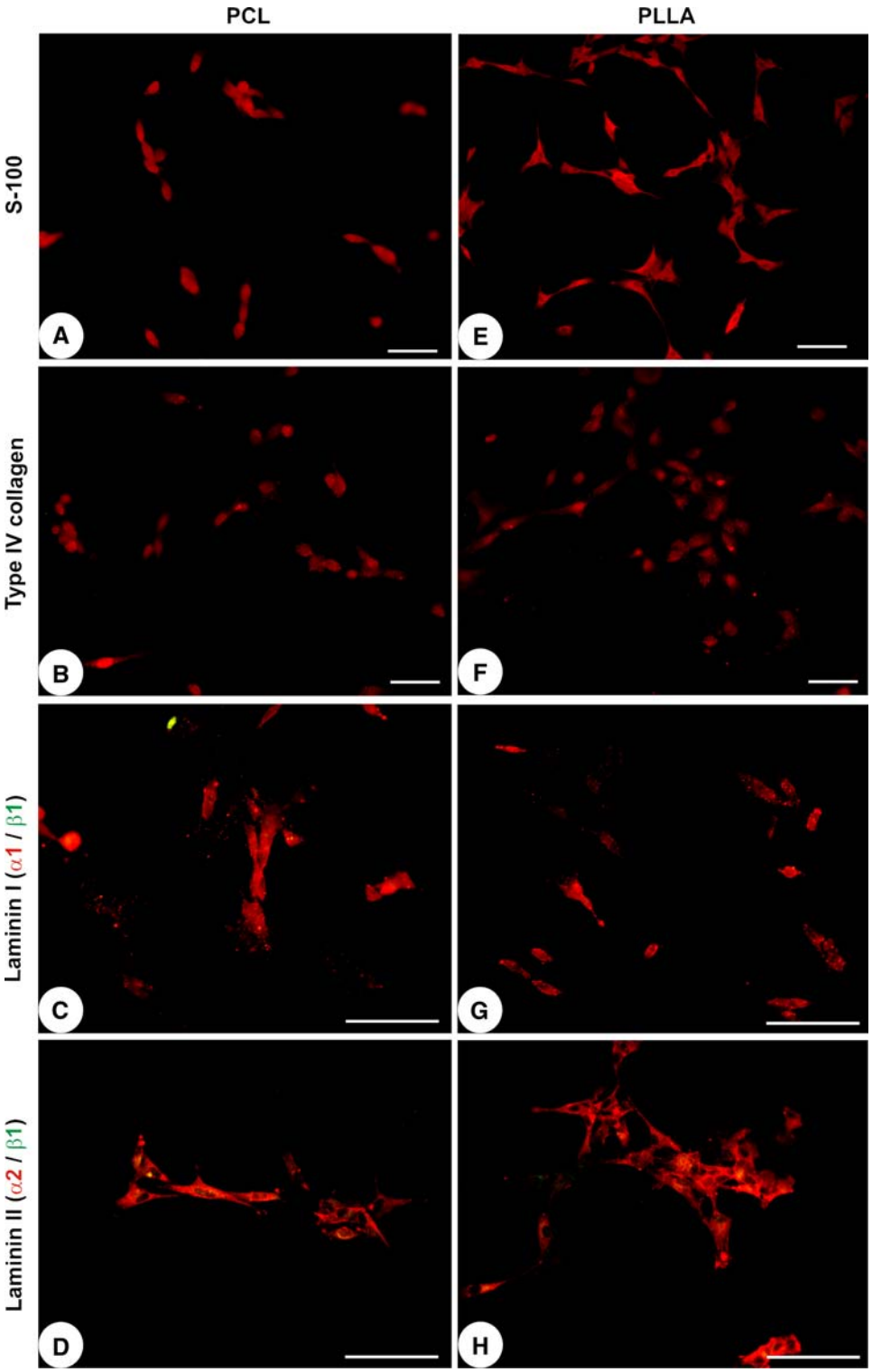

Fig. 3 Schwann cell culture immunolabeling on PCL and PLLA membranes. Observe the greater cell density of the cultures developed on PLLA substrate. Scale = 100 μm

69,517.60 ± 3,449.56—PLLA; integrated density of pixels/4,350 μm^2; $P < 0.05$; Fig. 4a).

The scanning electron microscopy further revealed that the SCs cultured on PCL presented less cytoplasmic branches over the substrate so that the cell body was less attached to the membrane. On the contrary, cells cultured on PLLA displayed several fine cytoplasmic projections and were visibly more attached to the membrane (Fig. 2). Nevertheless, the total cell area measured in both experimental conditions revealed no

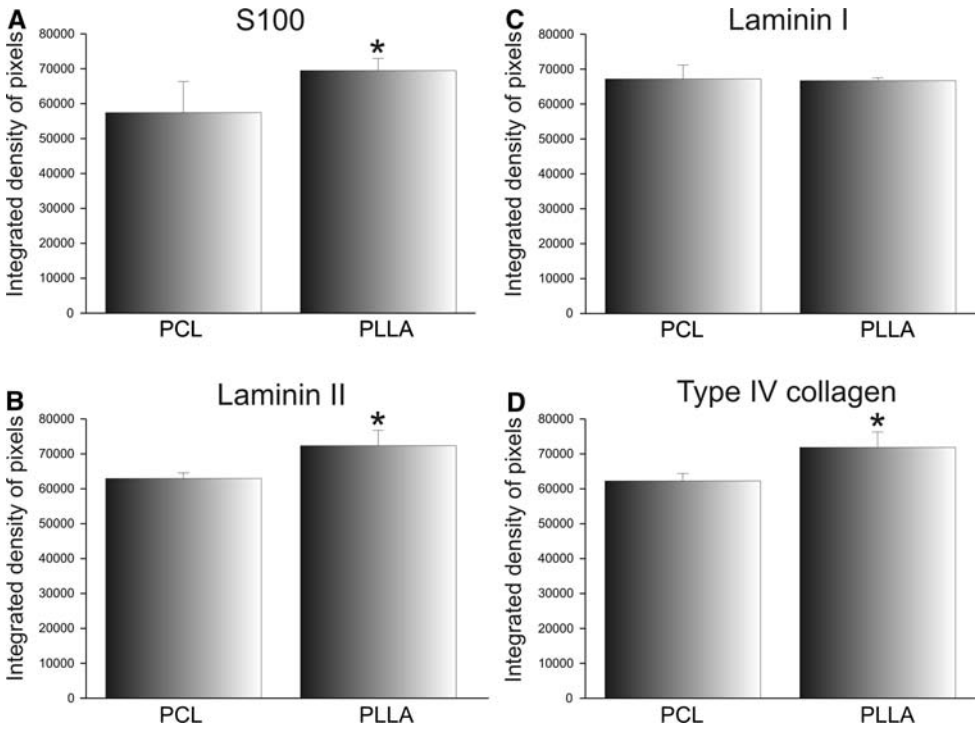

Fig. 4 Quantification of the immunostaining (integrated density of pixels/area) against S100, laminin I, laminin II and type IV collagen in SC cultures on PCL and PLLA membranes prepared by solvent evaporation. Observe that PLLA provided a better substrate to the cultured cells, which expressed increased levels of S100, Laminin I and type IV collagen. (*) = $P < 0.05$

statistical differences regarding SC area of contact with the substrate (284.42 ± 106.03 μm^2—PCL; 373.96 ± 179.79 μm^2—PLLA).

Laminin II expression was greater in SCs cultured on PLLA (Figs. 3 and 4b; 63,023.67 ± 1,527.52—PCL; 72,375.25 ± 4,369.34—PLLA; integrated density of pixels/4,350 μm^2; $P < 0.05$). On the other hand, the quantification of the immunolabeling against laminin I revealed no statistical difference among the experimental groups (67,256.00 ± 3,906.50—PCL; 66,779.00 ± 731.12—PLLA; integrated density of pixels/4,350 μm^2—Fig. 4c).

A strong immunoreactivity was obtained with the anti-collagen antiserum in the SC cultures on both PCL and PLLA membranes. Nonetheless, cultures on PLLA membranes resulted in a statistically significant increase of type IV collagen expression (62,283.94 ± 2,117.46—PCL; 71,891.20 ± 4,403.59—PLLA; integrated density of pixels/4,350 μm^2; $P < 0.05$—Fig. 4d).

4 Discussion

The use of biomaterials has been regarded as an efficient alternative to the allograft, since it significantly reduces the risk of rejection and local inflammation [19–22]. Also, the method of preparation of the biopolymer together with its intrinsic properties may influence its degradation rate, flexibility and cell adhesion, which may in turn positively stimulate the nerve regeneration process. [13, 16, 23].

Sangsanoh et al. (2007) [24] have studied the in vitro responses of a schwannoma cell line derived from a chemically induced rat peripheral neurotumor. They have analyzed different fibrous scaffolds of biodegradable polymers, including PLLA and PCL, obtaining similar results to those reported herein, regarding cell adhesion and proliferation. An important difference, however, is that in the present study, primary SC cultures have been used instead of a cell line, what may provide more accurate cell behavior results. Also, the culturing period of 7 days was 48 h longer than the investigated in the work of Sangsanog et al. (2007) [24].

The present work also analyzed the expression of basal lamina components, namely laminin I and II and type IV collagen. In this sense, Letourneau [25] described that the interaction between axonal sprouts and such ECM components, as well as neurotrophic factors, positively influence the nerve regeneration process [26].

In line with this concept, the SCs cultured on PLLA presented better adhesion, which provided a greater density of cells producing basal lamina components that are fundamental to the axonal regeneration. Such a fact is reinforced by the quantitative analysis of the immunostaining that revealed a greater expression of Laminin I and type IV collagen.

An important fact to be highlighted is that the better adhesion to the substrate results in synthesis of neurotrophic factors. In fact, Hurtado et al. [27] demonstrated in vitro that SCs cultured on porous PDLLA synthesized greater amounts of neurotrophins.

Miller et al. [28] observed that addition of laminin to the suface of PDLLA resulted in an increased cell adhesion as well as orientation of SCs, similarly to that observed during the BB formation in vivo. Our results are in accordance to that, since SCs cultured on PLLA proliferated more and displayed a general parallel organization, compatible to what happens in vivo during the initial steps of the nerve regeneration process.

Another important aspect observed herein and by other authors [24] is that the SC morphology may vary depending on the polymeric surface. As observed in our results, SCs cultivated on a more hydrophobic substrate, namely the PCL membranes, displayed different morphologies than the ordinary bipolar profile. This may negatively reflect in the synthesis of neurotrophic factors and cell proliferation. Nevertheless, no statistical differences were observed regarding the overall cell area in contact to both polymers.

In summary, the present work shows that SCs cultured on PCL and PLLA membranes display different levels of laminin I and type IV collagen, but not laminin I. Also, the better cell adhesion on PLLA membranes, associated with the differential expression of certain ECM molecules suggest that it is an adequate substrate for construction of prosthesis aiming at peripheral nerve repair.

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