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Adhesion and proliferation of human Schwann cells on adhesive coatings

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Abstract

Attachment to and proliferation on the substrate are deemed important considerations when Schwann cells (SCs) are to be seeded in synthetic nerve grafts. Attachment is a prerequisite for the SCs to survive and fast proliferation will yield large numbers of SCs in a short time, which appears promising for stimulation of peripheral nerve regeneration. The aim of the present study was to compare the adhesion and proliferation of human Schwann cells (HSCs) on different substrates. The following were selected for their suitability as an internal coating of synthetic nerve grafts; the extracellular matrix proteins fibronectin, laminin and collagen type I and the poly-electrolytes poly(D-lysine) (PDL) and poly(ethylene-imine) (PEI). On all coatings, attachment of HSCs was satisfactory and comparable, indicating that this factor is not a major consideration in choosing a suitable coating.

Proliferation was best on fibronectin, laminin and PDL, and worst on collagen type I and PEI. Since nerve regeneration is enhanced by laminin and/or fibronectin, these are preferred as coatings for synthetic nerve grafts seeded with SCs.

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1. Introduction

Schwann cells (SCs) play an important role in mediating peripheral nerve regeneration [1,2]. After nerve transection SCs in the distal nerve stump align to form the so-called 'bands of Bungner', which conduct the outgrowing regenerating axons to the target organ [3]. In the presence of axons, SCs assemble a complete extracellular matrix (ECM) [4,5], and express cellular adhesion molecules (CAMs; N-CAM, MAG, L1/Ng-CAM and N-cadherin) on their surface. These CAMs interact with the CAMs, integrins and integrin-related ECM receptors on the neuronal growth cones [2].

Introduction of cultured SCs into the lumen of a synthetic nerve graft enhances peripheral nerve regeneration [6–11]. For SCs to survive in the graft, attachment is mandatory, since attachment is a prerequisite for survival and proliferation of SCs [12,13]. In the absence of axons, it seems necessary to supply the SCs with additives, i.e. an adhesive coating, to allow them to assemble basal-lamina-like structures, and thus to adhere to the grafts' interior. Preferably such an adhesive coating should also stimulate proliferation, because the few studies that evaluated the influence of the number of SCs in artificial nerve grafts on nerve regeneration, advocate the addition of a large number of cells [6,9,11]. The success of predegenerated autologous nerve grafts seems to be at least partially based on SC proliferation subsequent to Wallerian degeneration [14,15].

Fibronectin, laminin and collagen type I [16–18], interact with the integrins on the SC surface [13,19], and support SC attachment and proliferation. The positively charged poly-electrolytes poly(D-lysine) (PDL) [20,21] and poly(ethylene-imine) (PEI) [22] allow attachment of negatively charged SCs.

Although the adhesive and proliferation stimulating properties of these coatings are important in deciding what coating to apply to the synthetic nerve guide,

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no previous study, to the best of our knowledge, systemically quantified and compared attachment and proliferation on these coatings before. In the present study we compared both attachment and proliferation of human Schwann cells (HSCs) on fibronectin, laminin, collagen, PDL, and PEI amongst each other and against customary coatings in SC culturing, like gelatin, gelatin crosslinked with glutaraldehyde [23] and uncoated glass.

2. Materials and methods

2.1. Isolation and culture of human Schwann cells

Small pieces of human sural nerve that remained after nerve transplantation were used. All material was obtained from the Department of Neurosurgery at the Leiden University Medical Center. Patients agreed by informed consent to the use of this material.

After stripping the epineurium and connective tissue, the sural nerve was cut into pieces of ca. 1 mm^3. These were placed in gelatin-coated (see below) culture flasks (Greiner, The Netherlands) and covered with a thin layer of LAK culture medium [24], consisting of Dulbecco's modified Eagle's medium (DMEM; BioWhittaker Europe, Belgium), 10% lymphokine activated killer cells conditioned medium (LAK) [25], 5% fetal calf serum (FCS; Gibco BRL, Life Technologies, Germany), 0.25 μl/ml phytohaem-agglutinin (PHA; Difco Laboratories, USA), 100 IU/ml penicillin (Gist-Brocades, The Netherlands), and 50 μg/ml streptomycin (Gist-Brocades).

To obtain highly purified HSC cultures, a sequential explantation technique was used [26]. Fibroblasts migrate faster out of the nerve pieces and after three to five explantation cycles, only SCs emerge. Cultures were regularly sampled, and the presence of fibroblasts in these samples was assessed with immunostainings. If more than 2% of the sampled cells (rough estimate) were fibroblasts, 10 ml of 1×10^{-3} M arabinoside-C was added to the culture medium [26]. After 2 days, arabinoside-C was removed and cells were thoroughly rinsed with fresh LAK culture medium. In the subsequent weeks, SCs were allowed to recover.

2.2. Immunocytochemistry of human Schwann cells

Standard immunocytochemical stainings were used to determine the identity of the cultured cells. Cell cultures were made on crosslinked gelatin-coated coverslips (see below), fixed with Cryofix® (Merck, Darmstadt, Germany), rinsed three times with phosphate buffered saline (PBS; 0.1 M, pH 7.2) and incubated with antibodies appropriately diluted in PBS containing 0.1% bovine serum albumin (BSA; Sigma, St. Louis, Missouri) and 1% normal goat serum (NGS; CLB, Amsterdam, The Netherlands). To identify SCs, antibodies directed against S100 (1:1000; Sigma) and GFAP (1:500; Boehringer Mannheim Biochemica, Germany) were added. For fibroblast identification, antibodies directed against fibroblasts (1:100; S5 clone, Sigma) and Thy 1.1 (1:10,000; Serotec, Oxford, United Kingdom) were used. After overnight incubation at 4°C in a moist chamber, cultures were again rinsed three times with PBS and subsequently stained with GAM/FITC (Molecular Probes, The Netherlands) (anti-S100, anti-fibroblast, and anti-Thy 1.1.) and GAR/FITC (GFAP) appropriately diluted in PBS containing 0.1% BSA and 1% NGS. After extensive rinsing with PBS, sections were mounted, dehydrated, and coverslipped with Fluoromount® (Merck) and viewed with a fluorescence microscope.

SC identity was further confirmed with a reverse transcriptase polymeric chain reaction (RT-PCR) protocol [27]. In short, cDNA was generated from total RNA isolated from trypsinized SC cultures. As a control, cDNA was also generated from total RNA isolated from fibroblasts. PCR reactions were allowed to take place between the generated cDNA and primers for 2′,3′-cyclic nucleotide-3′-phosphatase (CNPase), S100β and GFAP. After amplification, the PCR products were applied to an agarose gel and visualized under UV.

As a final control, HSCs were collected from the gelatin-coated culture flasks by incubation with a solution of 0.25% trypsin (Difco Laboratories, USA) and 10 mM ethylenediamine tetra-acetic acid (EDTA) in PBS [24] for approximately 2 min at room temperature, followed by the addition of an equal amount of culture medium. The cell number was estimated using a Bürker-Türk chamber. The cell suspension was centrifuged for 5 min at 1600 rpm and subsequently the cells were incubated in 2 ml 40 μM DiI in culture medium for 30 min. After a second identical centrifugation step, the cells were resuspended in fresh culture medium to a final concentration of ca. 2×10^3 cells/ml and seeded on cultures of DRG neurons [28]. After 7 days, the ability of the HSCs cells to align along neurites was evaluated with a fluorescence microscope.

2.3. Coatings

Round glass coverslips with a diameter of 15 mm (176.7 mm^2) were washed in a 10% g/v potassium dichromate in 10% H_2SO_4 solution, rinsed and sterilized. All glass coverslips were first placed in a 24-well plate and subsequently coated. For each time point three glass coverslips were prepared with each coating, cf. [18]. Glass coverslips coated with gelatin and gelatin crosslinked with glutaraldehyde, and uncoated glass coverslips were used as controls.

2.3.1. *Fibronectin and laminin*

A 50 μg/ml solution of either fibronectin or laminin (Boehringer Mannheim, Almere, The Netherlands) in PBS (200 μl) was applied to glass coverslips. After 45 min of incubation (37°C and humidified air/5% CO_2), the surplus of fluid was removed. In order to prevent the surface from drying out, HSCs were seeded on the surface immediately.

2.3.2. *Collagen type I*

Collagen type I from bovine dermis (Vitrogen 100, Collagen Corporation, Fremont, CA) was applied (500 μl) to glass coverslips. After overnight incubation at 37°C and humidified air/5% CO_2, the surface was rinsed thoroughly with PBS. In order to prevent the surface from drying out, HSCs were seeded on the surface immediately.

2.3.3. *Poly(D-lysine) and poly(ethylene-imine)*

A 0.1% solution (w/v) of either PDL or PEI (Sigma) was applied to glass coverslips. After 2 h, the coverslips were rinsed thoroughly with PBS. The surfaces were dried overnight in a flow chamber.

2.3.4. *Gelatin*

A 0.5% solution (w/v) of gelatin (400 μl; Difco Laboratories, USA) in PBS was applied to glass coverslips. After 45 min, the surplus of gelatin was removed and the surface was rinsed with PBS. The surfaces were dried overnight in a flow chamber.

2.3.5. *Crosslinked gelatin*

A 0.5% solution (w/v) of gelatin (400 μl) in PBS was applied to glass coverslips. After 45 min, the surplus of gelatin was removed and the surface was treated for 15 min with a 0.5% solution (w/v) of glutaraldehyde (400 μl). This was done to prevent the gelatin from dissolving in the culture medium [23]. Then the surface was thoroughly rinsed with PBS. The surfaces were dried overnight in a flow chamber.

2.4. *Human Schwann cell seeding*

HSCs were collected from the gelatin-coated culture flasks, by incubation for approximately 2 min at room temperature with a solution of 0.25% trypsin and 10 mM EDTA in PBS, followed by the addition of an equal amount of culture medium. A sample of the suspension was stained with True Blue (Janssen Chemica, Belgium) to evaluate cell viability. The cell suspension was centrifuged for 5 min at 1600 rpm, and subsequently the cell pellet was washed with LAK culture medium. The cell number was estimated using a Bürker-Türk chamber. After a second identical centrifugation step, the cells were resuspended in fresh culture medium to a final concentration of 2×10^3 cells/ml for the attachment study and to a final concentration of 3.8×10^3 cells/ml for the proliferation study. The concentration of HSCs was lower in the attachment study to reduce attachment of HSCs to each other. Aliquots of the final cell suspension (500 μl) were added to the (coated) glass slides in the wells and incubated at 37°C in humidified air/5% CO_2. Medium was refreshed three times a week.

2.5. *Human Schwann cell adhesion and proliferation*

For evaluation at the indicated times, the (coated) glass coverslips in the 24-well plate were gently rinsed with PBS to remove the non-attached cells. Cryofix® was added for fixation and gently rinsed away after 20 min. Coverslips were taken out of the 24-well plate and stained with 0.25 wt/vol% Coommassie blue solution in methanol:water:acetic acid (5:5:1). Subsequently (coated) glass coverslips with adhering stained HSCs were coverslipped with Aquamount® (Merck) and examined under a light microscope (Olympus) at 100 × or 200 × magnification. The morphology of the cells was described and pictures of representative cells on all slides were made. All cells present on the coatings and glass were counted and the logarithms of the number of cells were compared. When a confluent cell layer was present, the total number of cells was calculated from representative samples.

Attachment of HSCs was evaluated 1, 3, 6 and 24 h after seeding [29]. The attachment ratio was calculated as the lognumber of cells counted after 24 h, divided by the lognumber of cells seeded.

Proliferation of HSCs was evaluated 3, 6, 9, 12 and 15 days after seeding. The proliferation rate was calculated as the slope of the line resulting from applying a linear regression analysis to the logarithms of the cell numbers on each coating or glass at the studied time points. First, this was done for the overall proliferation (time interval days 3–12), and subsequently for the time intervals days 0–3, days 3–6, days 6–9 and days 9–12, separately.

2.6. *Statistical data analysis*

The lognumber of cells in the attachment and proliferation studies and the attachment ratios are presented as mean ± standard deviation (SD) and were analyzed using a one-way ANOVA, followed by a Tukey's least significant differences multiple comparisons test if there was a difference between the groups beyond a significance level of $p = 0.05$. Proliferation rates were presented as mean ± standard deviation (SD) and compared using a univariate general linear model with a difference contrast for time. The SPSS statistical program, version 11.0, was used to perform all calculations. p-values of less than 0.05 were regarded as significant.

3. Results

3.1. Purification and characterization of Schwann cell cultures

Purification by the sequential explantation method resulted in cultures that stained almost exclusively with S100 and GFAP antibodies, and only sporadically with fibroblast and Thy1.1 antibodies, indicating that highly purified HSC cultures were obtained. RT-PCR demonstrated that HSC cultures expressed the SC markers CNPase, S-100β and GFAP. Control fibroblast cultures were negative in RT-PCR for all SC markers. Virtually, all cells in the samples stained with True Blue turned blue, indicating high HSC viability. DiI labeled HSCs, cultured on rat DRG neurons, were observed to align along the neurites, thus expressing a specific SC property (Fig. 1).

3.2. Human Schwann cell adhesion on adhesive coatings

At 1 h after seeding, most HSCs adhering to the (coated) coverslips demonstrated small lamellipodial extensions and resembled 'fried eggs' (Fig. 2). After 3 h, most cells stretched out, although there was also a considerable number of cells that appeared partially folded, indicating that the cells were not yet fully attached. Except on PEI, cells were stretched out or transformed to a spindle shape and formed clusters after 6 h. On PEI, after 24 h a lot of cells were still not fully stretched. Mitotic figures were absent throughout the cell cultures on all coatings at 24 h, indicating that multiplication of cells did not influence attachment ratios.

Initially, the lognumber of adhering cells differed among the coatings, but these differences disappeared progressively during the first 6 h of culturing (Fig. 3A). At 1 h after seeding the lognumbers of cells adhering to collagen, PDL, gelatin and glass were lower compared to crosslinked gelatin, and the lognumbers of cells on collagen and glass were also lower compared to fibronectin and PEI. After 3 h, the lognumber of cells on collagen was lower compared to gelatin and crosslinked gelatin, and the lognumber of adhering cells to glass was lower compared to all other coatings, except collagen. At 6 and 24 h after seeding, no significant differences could be detected in the lognumbers of adhering cells to the coatings and glass. Moreover, the attachment ratio was equal ($p = 0.09$; Fig. 3B).

3.2.1. Human Schwann cell proliferation on adhesive coatings

HSCs proliferated on all coatings and on uncoated glass, between 3 and 15 days of culturing (Fig. 4). Except on PEI HSCs appeared stretched out and demonstrated a typical bi- or tripolar morphology with oval nuclei [30]. After 6 days of culturing, cell layers reached confluency. On PEI it took the cells more than 6 days to fully recover from the seeding procedure; it was not until the ninth day of culturing that HSCs on PEI were fully stretched out and reached confluency. The increase in number of cells on the coatings and on glass was accompanied by a decrease in cell size. This is clearly illustrated by comparing the cells on days 12 and 15 (enlargement 200 ×) to the cells on days 3, 6 and 9 (enlargement 100 ×). On day 15, the number of cells increased to such an extent that the cell layers started to detach from the coated surfaces and from the glass. Mitotic figures were present throughout the cell cultures on all coatings.

The proliferation rates on all coatings and glass in the time interval 3–12 days were in the same range, but the

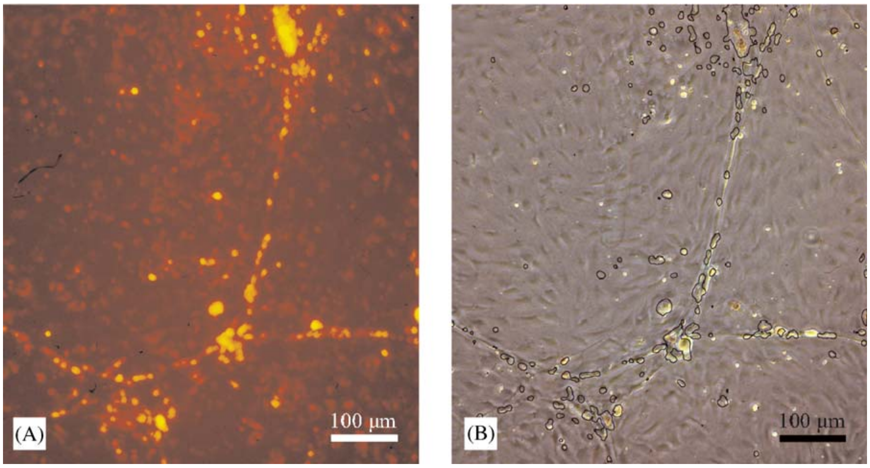

Fig. 1. HSCs stained with DiI seeded on DRG neurites. The left figure is a phase contrast micrograph of HSCs aligning along DRG neurites. The neurites are easily recognizable. The fluorescence micrograph (right figure) clearly demonstrates the alignment of the fluorescent HSCs along the DRG neurites.

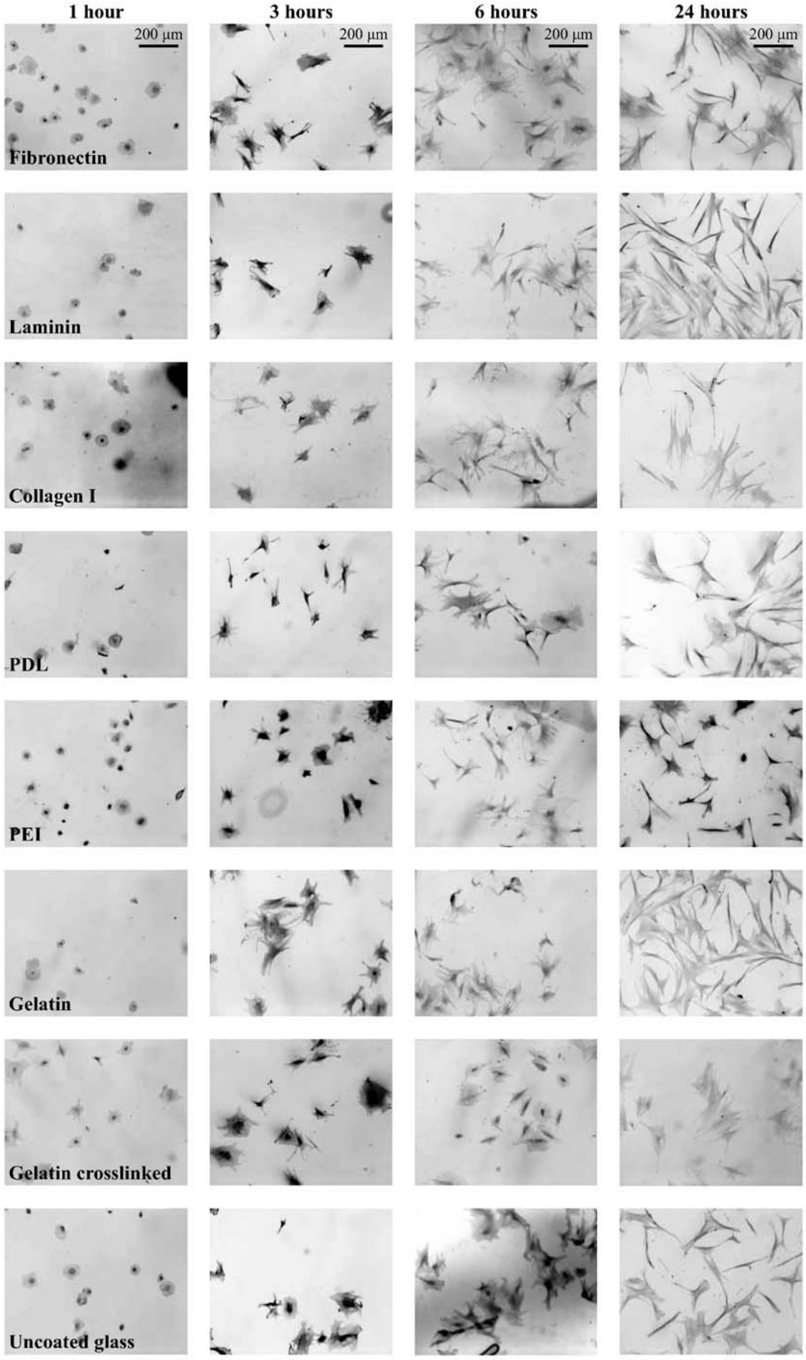

Fig. 2. Attachment of HSCs on coatings—qualitative. Morphological appearances of HSCs on fibronectin, laminin, collagen type I, PDL, PEI, gelatin, crosslinked gelatin and glass, 1, 3, 6 and 24 h after seeding. Historically, SCs were described to be spindle shaped [52,53]. However, in later years it was demonstrated that, although many of cultured SCs have this characteristic spindle-shaped morphology, some have a more flattened (fibroblast-like) morphology [32,54–58].

accompanying correlation coefficients were relatively low (Table 1). For this reason we wanted to gain insight in the trends of proliferation within the 0–12 days period. Figs. 5A and B demonstrate that the proliferation rate during the first 3 days after seeding was highest on laminin and crosslinked gelatin, and also high on fibronectin and PDL. As a result, the lognumber of cells at day 3 on collagen, PEI, gelatin and glass was lower compared to laminin and crosslinked gelatin. Moreover, the logarithm of cell numbers on gelatin and collagen was also lower compared to PDL.

From 3 to 6 days of culturing, proliferation rates changed considerably. On PEI, the proliferation rate increased more than 20 times, on fibronectin, collagen,

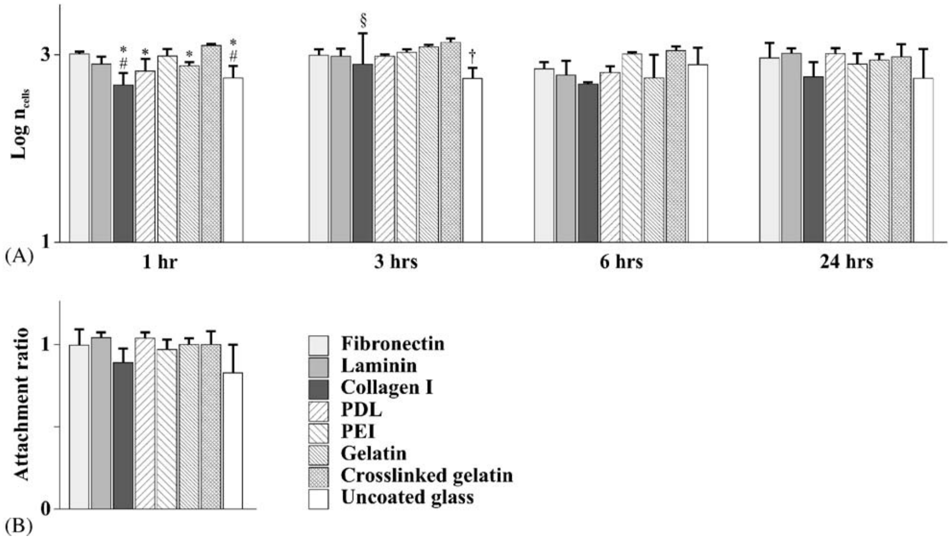

Fig. 3. Attachment of HSCs on coatings—quantitative. (A) The lognumber of HSCs adhering to different coatings and glass at 1, 3, 6 and 24 h after seeding. The number of cells seeded was 1000 cells/well (log 1000 = 3). Data are represented as mean ± SD. The seven coatings and glass were compared among each other: * lower compared to crosslinked gelatin at the corresponding time point; # lower compared to fibronectin and PEI at the corresponding time point; § lower compared to gelatin and crosslinked gelatin at the corresponding time point; † lower compared to all surfaces, except collagen at the corresponding time point. (B) Attachment ratios calculated as the lognumber of cells present after 24 h divided by the lognumber of cells seeded. Data are represented as mean ± SD.

gelatin and glass, the proliferation rate increased 6–10 times. The proliferation rate on laminin increased only a little, in contrast to a three-fold increase in the cell number during the first 3 days. On crosslinked gelatin the proliferation rate even decreased. Consequently, the lognumber of cells was approximately the same at 6 days after seeding, except on crosslinked gelatin and PEI, which displayed a lower lognumber of cells.

From 6 to 9 days, the lognumber of cells on the coatings and glass remained constant, with the exception of PEI, that demonstrated a considerable proliferation rate, though also lower in comparison with the previous interval (Fig. 5B). After 9 days, no significant differences could be detected in the lognumber of cells on the adhesive coatings or on uncoated glass (Fig. 5A).

Between 9 and 12 days, proliferation rates again increased on all coatings and glass, except on collagen and PEI. On collagen, the number of cells remained approximately the same, and on PEI the number of cells even decreased. Consequently, after 12 days the lognumber of cells on collagen became smaller compared to laminin and the lognumber of cells on PEI became smaller compared to all other coatings, except collagen (Fig. 5A).

After 15 days of culturing, the number of cells on all coatings and on glass increased to such an extent that the cell layer detached from the underlying surface. It was therefore not possible to count and to compare the cells quantitatively. Qualitatively, however, the space between the cells was larger on collagen and PEI (Fig. 4).

4. Discussion

Using a relatively simple explantation technique, we were able to obtain confluent HSC cultures from which fibroblasts were virtually absent. Purity was assessed by positive staining of S100- and GFAP-antibodies, and negative staining with fibroblast- and Thy 1.1-antibodies. The positive staining results were confirmed with RT-PCR SC identification. We demonstrated that the cultured cells aligned along neurites, a highly specific SC property. Therefore, we qualified this explantation technique as adequate to obtain sufficient numbers of HSCs for subsequent seeding on the coatings and glass.

4.1. *Attachment of human Schwann cells*

The attachment study demonstrated that within 6 h after seeding, HSCs attached as well to ECM proteins, poly-electrolytes, gelatin and crosslinked gelatin as to glass. On PEI, however, complete stretching of HSCs took more than 24 h.

It was demonstrated in several studies that SCs could satisfactorily attach to ECM proteins and poly-electrolytes [16,18,20,21,31]. However, attachment to these substrata was never studied comparatively. The present results demonstrate that the number of cells adhering to these coatings is comparable. Moreover, no differences could be detected in SC morphology, with the exception of HSCs on PEI which still demonstrated partial folding after 24 h in culture. Fibronectin was previously demonstrated to encourage cell spreading [32], and

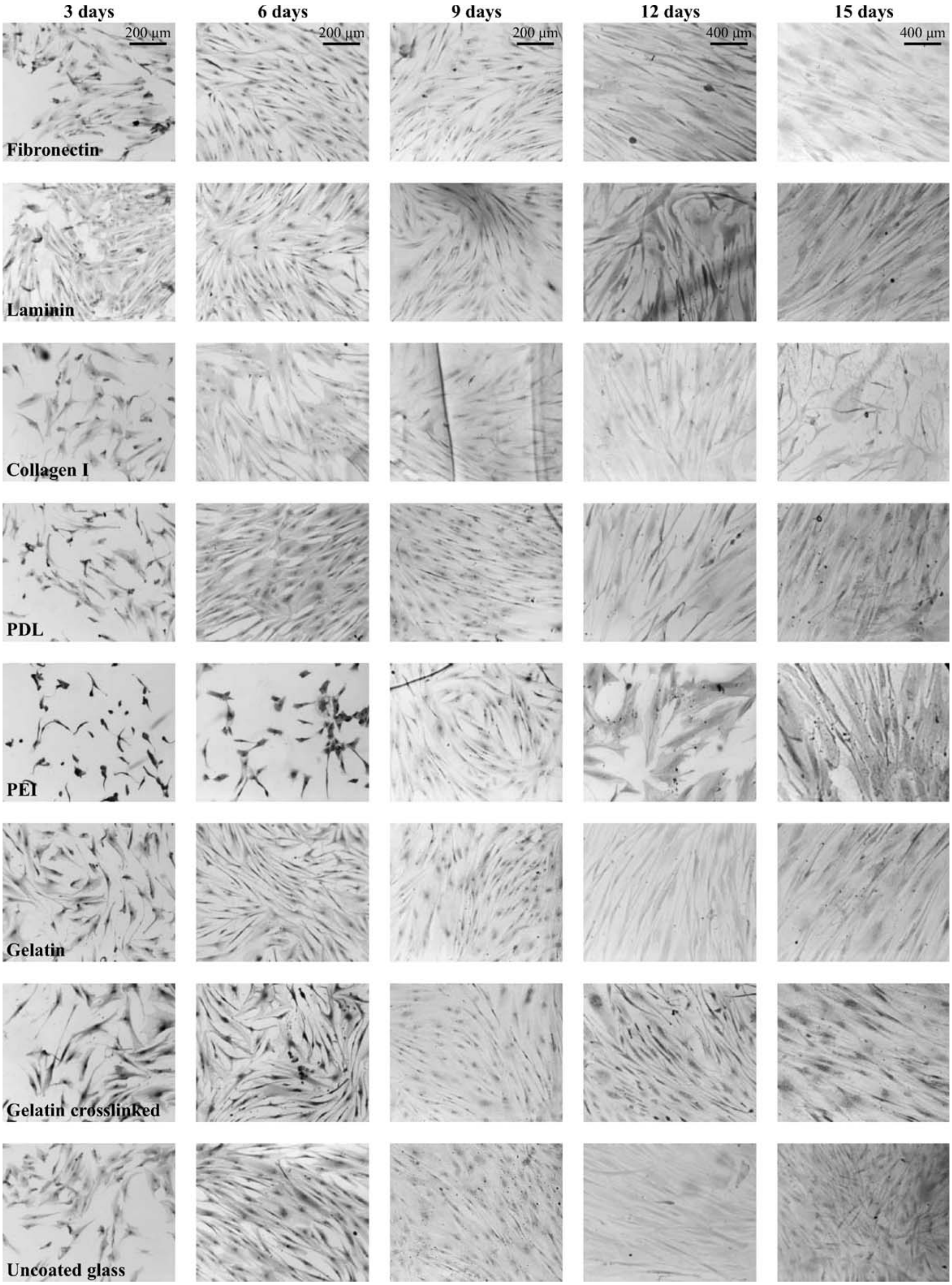

Fig. 4. Proliferation of HSCs on coatings—qualitative. Morphological appearances of HSCs on coatings, 3, 6, 9, 12 and 15 days after seeding. SCs can appear either as spindle-shaped or as flat cells.

laminin to stimulate SCs to elongate [17], but we did not observe more spreading or elongation of the HSCs on fibronectin or laminin compared to other coatings (Fig. 2).

In a previous paper we described SC attachment to different biomaterials, and likewise demonstrated that within 6 h after seeding, attachment was the same [33]. Apparently SCs easily attach to almost any culture

Table 1
Proliferation rate of human Schwann cells on coatings—time interval 3–12 days

	Correlation coefficient of linear fit (*R*)	Proliferation rate (3–12 days)
Fibronectin	0.820	0.10±0.02
Laminin	0.950	0.09±0.01
Collagen	0.799	0.13±0.03
PDL	0.900	0.09±0.02
PEI	0.798	0.09±0.02
Crosslinked gelatin	0.901	0.07±0.01
Gelatin	0.859	0.14±0.03
Glass	0.853	0.12±0.02

Note: The proliferation rate of the cells on the coatings for the whole culturing period (day 3–12) was calculated as the slope of the line resulting from applying a linear regression analysis to the lognumbers of cells on each surface on all time points.

substratum. Thus, contrary to the prevailing opinion, attachment is not an important consideration in choosing a suitable material for coating the interior of a synthetic nerve guide.

4.2. *Proliferation of human Schwann cells*

Proliferation rates of HSCs varied largely during the first 6 days of culture. The first increase in proliferation (3–6 days of culture) was most profound on fibronectin, laminin, PDL and crosslinked gelatin, and less on collagen type I, PEI, gelatin and glass. Proliferation diminished whenever a density of ca. 10 cells/mm^2 (1850 cells/176.7 mm^2) was reached, but after 9 days, a second increase in cell proliferation was observed.

The initial increase in proliferation was least prominent on collagen type I, PEI, gelatin and glass. The attachment ratios were also low on these surfaces. It seems plausible that the proliferation rate is partially dependent on the attachment ratio [17]. Newly emerged cells must attach prior to the next proliferation cycle, and delayed attachment (low attachment ratio) may thus slow down the proliferation rate. Insignificant differences in attachment may thus be magnified to significant differences in proliferation.

We observed a decrease in proliferation rate upon reaching a density of approximately 10 cells/mm^2. Cessation of proliferation upon increase of HSC density has been observed before, and was recently ascribed to a contact-mediated mechanism of growth regulation [34].

Contrary to the other coatings and glass, the second increase in proliferation rate after 9 days was not observed on collagen type I and PEI. The number of HSCs on collagen remained equal, while the number of HSCs on PEI even declined.

In conclusion, the proliferation rate is lowest on collagen type I and on PEI because (i) proliferation during the first 9 days of culturing is slower; (ii) the second burst in proliferation does not occur. Moreover, on PEI cells eventually die. Thus, when a coating is needed to encourage HSC proliferation, we do not recommend choosing collagen type I or PEI from our panel. The results on collagen are remarkable, since it is frequently used as a matrix or coating to culture SCs [20]. However, systematic comparison of HSC proliferation on different coatings has not been performed before, and it was therefore not known that other coatings enhance HSC proliferation more.

Previous studies in which the SC number seeded in an artificial nerve graft was varied, demonstrated that the total number of axons and the number of myelinated axons were larger in those groups in which a large number of SCs were seeded [6,9]. It is to be expected that SC proliferation ceases upon arrival of axons, since axonal signals control SC differentiation and myelination by regulation of the Oct-6 SC-specific enhancer [35], and cause SCs to transform into a non-proliferating cell state [19]. Regenerating axons will start ingrowth into an artificial nerve guide approximately 2–3 weeks after implantation [36]. It is therefore necessary that the coating chosen enhances SC proliferation especially during this period. The coating on which SCs proliferate the most is therefore preferable. Fibronectin, laminin and PDL show maximal proliferation of HSCs during the first 15 days of culturing, and are therefore the most suitable candidates from our panel for coating of a synthetic nerve graft.

The extent to which the coating enhances neurite extension is also of great importance. Poly-electrolytes allow neurite extension from peripheral and central nervous system neurons in vitro [37–39], but, to the best of our knowledge, PDL was never used to coat a synthetic nerve graft in vivo. Fibronectin and laminin, to the contrary, were repeatedly demonstrated not only to enhance neurite extension in vitro [38,40,41] but also to stimulate nerve regeneration in vivo in a synthetic nerve graft [3,42–44]. It was even demonstrated that laminin and fibronectin act synergistically with respect to the enhancement of neurite outgrowth, both in vitro [16] and in vivo [45–47].

The enhancement of neurite regeneration by laminin and fibronectin can be explained by the observation that regenerating axons upregulate receptors for ECM molecules in order to establish an integrin mediated interaction with these proteins [48,49]. Recently, the $\alpha 7\beta 1$ integrin, a specific receptor for the laminin 1, 2 and 4 isoforms, was demonstrated to be strongly upregulated after axotomy, indicating an important role for this integrin in axonal regeneration [50].

Combining the HSC proliferation promotive capacities that we demonstrated in this study, and the neurite outgrowth promotive capacities described in literature, we would choose to coat a synthetic nerve guide with a combination of laminin and fibronectin. It would even

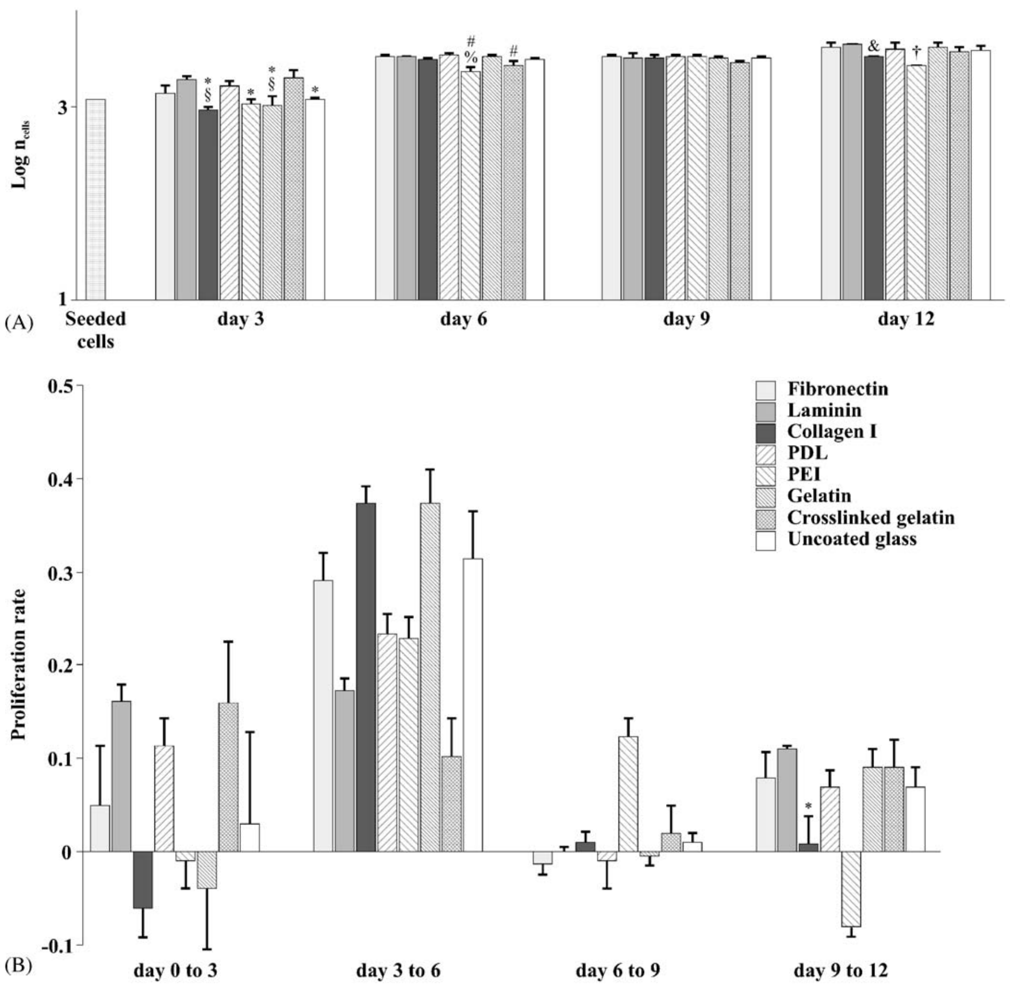

Fig. 5. Proliferation of HSCs on coatings—quantitative. (A) The lognumber of HSCs adhering to different coatings and glass at 3, 6, 9 and 12 days after seeding. The initial amount of cells was 1900 cells/well (log 1900 = 3.3). Data are represented a mean ± SD. The seven coatings and glass were compared among each other: $*$ lower compared to laminin and crosslinked gelatin at the corresponding time point; § lower compared to PDL at the corresponding time point; % lower compared to collagen and glass at the corresponding time point; # lower compared to fibronectin, laminin, PDL and gelatin at the corresponding time point; & lower compared to laminin at the corresponding time point; † lower compared to all surfaces, but collagen at the corresponding time point. (B) Proliferation rates calculated as the slope of the linear fit calculated from the logarithm of the number of cells over the time intervals: 0–3, 3–6, 6–9 and 9–12 days. For all coatings, the comparison of proliferation rates differed significantly when two time intervals were compared, except for collagen when the proliferation rates of the time interval 6–9 days were compared with the time interval 9–12 days (indicated with an $*$).

be better to disperse these ECMs in a three-dimensional matrix as to allow the outgrowing neurites to exploit the entire volume of the synthetic nerve graft. Results obtained with Matrigel®, a matrix consisting of 80% laminin, were contradictory [6,42–44,51], while results obtained with a lower concentration of laminin (50 μg/ml; 5% m/v) were uniformly positive [3,38,40,45–47].

At such low concentrations, however, laminin does not aggregate into a gel. Thus, we need another matrix protein to constitute the bulk of the gel. Although we demonstrated that collagen type I did not promote the proliferation of HSCs, it did not inhibit proliferation either. Therefore, collagen gel is suitable to serve as a matrix into which low concentrations of laminin and fibronectin (50 μg/ml; 5% m/v) can be dispersed. In a follow up study we will evaluate whether the introduction of this gel into a synthetic nerve graft, indeed improves peripheral nerve regeneration.

5. Conclusion

Attachment and proliferation of human Schwann cells on various culture substrata were compared, with the aim to select among these substrata the most suitable ones for coating the interior of synthetic nerve guides. The attachment study demonstrated that the number of Schwann cells adhering to the coatings was comparable, as was their morphology. We concluded that human Schwann cells easily attach to almost any culture substratum, and that attachment is not an important consideration in the selection of suitable materials for coating the interior of synthetic nerve guides.

In the first 6 days after seeding the proliferation rate was high on fibronectin, laminin, PDL and crosslinked gelatin, but only moderate on collagen type I, PEI, gelatin and glass. On all coatings and on glass the proliferation rate diminished whenever a density of ca. 10 cells/mm^2 was reached. After 9 days, on all coatings, except on collagen type I and on PEI, Schwann cell proliferation increased again, which was ascribed to a contact-mediated mechanism of growth regulation. The number of Schwann cells on collagen, however, remained equal, while the number of Schwann cells on PEI even declined.

Considering the relative poor performance of collagen type I or PEI with respect to Schwann cell proliferation, we do not recommend the application of these materials to synthetic nerve guides. Fibronectin, laminin and PDL demonstrated the best proliferation rates during the 15-day culture period. Of these fibronectin and laminin have a proven ability to enhance neurite extension. Fibronectin and laminin are therefore the most suitable candidates from our panel for coating the interior of a synthetic nerve guide.

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