In vivo bioluminescent imaging of Schwann cells in a P(DLLA-ε-CL) nerve guide

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INTRODUCTION

Artificial nerve guides for supporting axonal regeneration in peripheral nerve defects are widely used alternatives for traditional autologous nerve grafts (Chalfoun, et al., 2006; Ikema-Paassen, et al., 2004; Schlosshauer, et al., 2006; Meek and Coert, 2002; Navarro, et al., 2003). Since regenerating nerve stumps can only bridge gaps up to 30 mm across a synthetic nerve guide in humans, several studies have used nerve guides seeded with Schwann cells (SCs) in order to optimize the micro-environment for nerve regeneration (Evans, et al., 2002; Fansa and Keilhoff, 2004; Mackinnon and Dellon, 1990; Schlosshauer, et al., 2003; Zhang, et al., 2002). SCs play a crucial role in the intrinsic regenerative response after peripheral nerve injury and stimulate neurite outgrowth by producing a variety of factors, such as cytokines MCP-1 and LIF, neurotrophic factors NGF, BDNF, CNTF and NT3, extracellular matrix proteins laminin-2 and fibronectin, and adhesion molecules L1 and N-CAM (Frostick, et al., 1998; Nadim, et al., 1990; Terenghi, 1999; Tofaris, et al., 2002). Various recent studies have put forward promising autologous sources for SCs, such as bone marrow and adipose stem cells, neural crest derived hair follicle stem cells, skin stem cells and, more recently induced pluripotent stem cells (IPS) (Amoh, et al., 2005; Caddick, et al., 2006; Cuevas, et al., 2002; Jiang, et al., 2008; Keilhoff, et al., 2006; Marchesi, et al., 2007; Takahashi, et al., 2007). However, before SC implantation can be safely applied clinically, fate and biological behavior of these cells, such as survival, migration, proliferative activity and functionality have yet to be elucidated in detail. In this study we investigated the applicability of bioluminescent imaging (BLI) as a noninvasive longitudinal imaging technique for monitoring the fate of SCs seeded in a commercially used nerve guide.

MATERIAL AND METHODS

Isolation and transfection of SCs

Sciatic nerves were excised from the hindlimbs of newborn (P4) Wistar rats. The nerves were cleaned from adherent tissue and collected in Hanks buffered saline solution (HBSS) with 0.6% glucose, 1% penicillin/streptomycin (Invitrogen, Breda, The Netherlands) and 13 mM HEPES. SCs were purified and cultured as described previously (Evans, et al., 2002). Arabinoside-cytosine (Ara-C) was added to the medium at a concentration of 1%, 24 hours after isolation. The culture medium was switched 48 hours after isolation to SC medium (SCM) containing 200 μg/ml Bovine Pituitary Extract (BPE, Sigma-Aldrich), penicillin/streptomycin (Invitrogen, Breda, The Netherlands) and 1% glutaraldehyde in 0.1M sodium cacodylate buffer for 3 hours at pH 7.4. Further processing and SEM was carried out according to standard procedures. Pre-implantation and post-explantation, nerve guides seeded with SCs were cut longitudinally into two halves using a scalpel blade. They were fixed with 2% glutaraldehyde in 0.1M sodium cacodylate buffer for 4 hours and post-fixed in 1% osmium tetroxide for 1 hour. Specimens were dehydrated in an ascending ethanol series, critical point-dried, sputter-coated, and observed under Scanning electron microscopy (SEM).

Preparation of nerve guides and seeding of Fluc-SCs

The fabrication of P(DLLA-ε-CL) nerve guides has been described previously (Meek and Coert, 2008). The nerve guides were manufactured by Polyganics. One hour prior to SC seeding, the inner lining of the nerve guides was coated with a solution of 1 μg/ml fibronectin and 5 μg/ml laminin in DMEM (Invitrogen). The coating mixture was injected into the lumina of the tubes. The supernatant was removed after 1 hour and immediately prior to cell seeding to prevent desiccation of the coating. Transfected SCs (Fluc-SCs) were harvested using a cell scraper 48 hours after the start of transfection, and about 3 x 10^6 cells in 45 μl SCM were injected into the nerve guides. The nerve guides were immersed in SCM enriched with 200 μg/ml BPE and incubated at standard culture conditions.

Scanning electron microscopy (SEM)

Pre-implantation and post-explantation, nerve guides seeded with SCs were cut longitudinally into two halves using a scalpel blade. They were fixed with 2% glutaraldehyde in 0.1M sodium cacodylate buffer for 3 hours at pH 7.4. Further processing and SEM was carried out according to standard procedures.

Implantation of nerve guides

Implantation experiments were performed in 8 adult female Wistar rats 48 hours after seeding of Fluc-SCs. Animals were anesthetized (2.5% isoflurane in oxygen 1.5 L/min), and the relevant skin area was shaved. A 3 cm incision was made in the flank, and the nerve guides were carefully placed in the groin inside an intermuscular pocket (at an estimated depth of 5 mm). The skin was sutured using separate nylon sutures.

Bioluminescent imaging (BLI)

For in vitro imaging of Fluc-SCs in the nerve guides, fresh SCM with beetle D-luciferin (150 μg/ml) was allowed to diffuse into the nerve guides. Luciferase activity was assessed using the In Vivo Imaging System IVIS®100 (Xenogen, Alameda, CA, now Caliper Life Sciences, Hopkinton, USA). For in vivo imaging of the implanted Fluc-SCs, a bolus of beetle D-luciferin solution (150mg/kg; Xenogen) in 0.9% NaCl was injected intraperitoneally just prior to imaging. Grey-scale images were taken as reference. Animals were imaged from the lateral side for 40 minutes. To reduce background signals, the skin was shaved locally. Sequential images were taken with an integration time of 5 minutes and a delay time of 1 minute (binning factor 16, field of view 15, f/stop 1, open filter). Data were analyzed using Living Image®2.50 (Xenogen) and Igor Pro® (WaveMetrics, Lake Oswego, USA) software.

Immunocytochemistry (ICC)

Pre-implantation and post-explantation, Fluc-SCs were fixed with 4% paraformaldehyde and washed with PBS. Standard ICC was performed using the following antibodies: anti-Oct6 (1:100, kindly provided by Dr. D. Meijer, #1909), anti-p75 (MAB375, Chemicon, Hampshire, UK, 1:300), and anti-luciferase...
RESULTS

We were able to obtain SC cultures with a purity >90% and stable Fluc-SC cultures with a purity of 70.3% ± 3.1%, as established with SC specific (double) immunostaining for Oct6, p75 and luciferase (Figure 1A and B). Treatment with Ara-C and BPE appeared to be essential for inhibiting fibroblast overgrowth and maximizing SC purity. To induce luciferase expression in the SCs, the transient non-viral transfection procedure JetPEI was used. Expression of the transfected gene is known to be detectable for a period of 12-14 days with this method. Double immunostaining for luciferase and p75 revealed a transfection efficiency of 77.4% ± 2.5%. The inner linings of the nerve guides were coated with extracellular matrix proteins fibronectin and laminin to promote optimal SC spreading and proliferation (Vleggeert-Lankamp, et al., 2004). Phase-contrast microscopy (PCM) and scanning electron microscopy (SEM) revealed efficient adhesion and spreading of SCs across the coated surface within 2 days after seeding (Figure 1D and E). Fluc-SCs were tested for bioluminescence both in vitro (before implantation and after implantation) and in vivo. In vitro imaging showed a strong signal from the SC seeded nerve guide (Figure 1F). Nerve guides were implanted subcutaneously 4 days after transfection. In vivo signal intensity as detected with IVIS was on average 21.8% of in vitro intensity (Figure 1G and H). Peak luminescence was measured at 35 minutes after D-luciferin injection. Due to the transient nature of luciferase expression, a gradual decrease of the signal over time was observed, as expected. Transcutaneous signals could be detected up to 7 days after implantation. In one tube, no signal could be detected 1 day after implantation. After uncovering and visualizing the tube, it was observed that a fibrin capsule had formed around the nerve guide, and blood clots obstructed the tube’s openings. Removal of the capsule and blood clots restored free diffusion of luciferin into the tube lumen. After re-implantation a signal could again be detected for the following 5 days. Nerve guides were explanted on day 8. PCM, SEM and ICC showed SCs with a healthy appearance throughout the nerve guides (Figure 1I-K). The total number of viable cells was approximately 80% of the amount of cells seeded (240,000 cells). Most cells were arranged longitudinally, parallel to the long axis of the tube as typical for mature functional SCs (Bozkurt, et al., 2009). Like the original SC suspension, 65.9 ± 4.87% of the cells were positive for both p75 and luciferase (Figure 1L). This indicates that the majority of implanted Fluc-SCs cells survived and still expressed luciferase after 7 days of implantation.
DISCUSSION

We demonstrate that SCs can effectively be transplanted to express luciferase. They can be detected transcutaneously after subcutaneous implantation in rats by means of bioluminescent imaging (BLI), after seeding in commercially available CE/FDA approved poly(DL-lactic acid-co-glycolic acid) (P(DLLA-e-CL)) nerve guides (Meek and Coert, 2008). Since the nerve guides are not permeable until onset of hydrolysis, supply of luciferin to the SCs depends on unimpeded diffusion through the open tube endings (Meek, et al., 2004). Our findings in one animal stress the importance for a careful and bloodless technique to prevent blood clot formation (e.g., diathermic surgery, tube implantation remote from the incision). In clinical usage, the viability of implanted cells might be endangered if diffusion of nutrients to the tube lumen is obstructed. In the present report, we used as a proof of concept a non-viral gene transfection method, which was chosen for its high efficiency and low toxicity. Stable long-term expression of luciferase would require either the use of a lentiviral vector for luciferase gene transfection or isolation of SCs from luciferase knock-in animals.

BLI provides a powerful tool for molecular imaging of ongoing in vivo biological processes (Contag, 2007; Sadikot and Blackwell, 2005). We have demonstrated that it also can be applied to study the fate and functionality of SCs in an implanted nerve guide, in case free diffusion of luciferin is guaranteed in an in vivo setting. By using gene constructs in which the luciferase gene is coupled to, and its expression is under the control of, promoters for the transcription of functional proteins, BLI might offer a significant contribution to evaluate the role of Schwann cells in the axonal bridging of large peripheral nerve gaps. This way, a functional molecular imaging model of nerve regeneration can be established.

P(DLLA-e-CL) nerve guides have proven their regenerative capacity in preclinical randomized controlled trials, and have been used with similar outcomes compared to autologous nerve grafting in a clinical study (as Neurolac®). Neurolac® nerve guides are among the best-documented nerve guides regarding functional outcome as well as biocompatibility. The transparency of Neurolac® offers an advantage for pre-clinical studies, especially in combination with reporter technologies such as bioluminescence. Neurolac® is the only transparent FDA-approved nerve guide currently available. Disadvantages described in animal studies include foreign body reactions, swelling with lumen blockage, incomplete degradation, and premature fragmentation and collapse leading to neurroma formation (Kehoe, et al., 2012). These effects will be relevant when choosing the appropriate material for preclinical long-term studies (as well as for clinical use).

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