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Collagen (NeuraGen®) nerve conduits and stem cells for peripheral nerve gap repair

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HIGHLIGHTS

- FDA-approved collagen nerve guides are efficient scaffold for cell delivery.
- Schwann cells show affinity for collagen, influencing distal cell infiltration.
- Proximal regeneration at 2-weeks was not influenced by regenerative cells.
- Endogenous Schwann cells influence sprouting dynamics of growth fronts.
- Cell neurotrophic potential may be improved by material functionalization.

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ABSTRACT

Collagen nerve guides are used clinically for peripheral nerve defects, but their use is generally limited to lesions up to 3 cm. In this study we combined collagen conduits with cells as an alternative strategy to support nerve regeneration over longer gaps. *In vitro* cell adherence to collagen conduits (NeuraGen® nerve guides) was assessed by scanning electron microscopy. For *in vivo* experiments, conduits were seeded with either Schwann cells (SC), SC-like differentiated bone marrow-derived mesenchymal stem cells (dMSC), SC-like differentiated adipose-derived stem cells (dASC) or left empty (control group), conduits were used to bridge a 1 cm gap in the rat sciatic nerve and after 2-weeks immunohistochemical analysis was performed to assess axonal regeneration and SC infiltration. The regenerative cells showed good adherence to the collagen walls. Primary SC showed significant improvement in distal stump sprouting. No significant differences in proximal regeneration distances were noticed among experimental groups. dMSC and dASC-loaded conduits showed a diffuse sprouting pattern, while SC-loaded showed an enhanced cone pattern and a typical sprouting along the conduits walls, suggesting an increased affinity for the collagen type I fibrillar structure. NeuraGen® guides showed high affinity of regenerative cells and could be used as efficient vehicle for cell delivery. However, surface modifications (*e.g.* with extracellular matrix molecule peptides) of NeuraGen® guides could be used in future tissue-engineering applications to better exploit the cell potential.

1. Introduction

Autografts are currently the gold standard in nerve repair, but have the drawback requiring sacrifice of a functional nerve. A few nerve conduits (NC), tubular structures designed to bridge the nerve gap created after nerve injury, have received approval for mass production from the US Food and Drug Administration (FDA) and Conformit Europe (CE) reviewed in [2,17]. These include synthetic non-resorbable polyvinyl alcohol hydrogels (SaluBridge™)

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and polymer tubes with various degradation times (Neurotube®, made of polyglycolide acid and Neurolac®, made of poly D,L-lactide-ε-caprolactone). Two of the NC are made from collagen, NeuraGen® nerve guide and NeuroMatrix, Neuroflex®. While commercial nerve guides represent a promising alternative to autografts when direct tensionless repair is not possible, their use is limited to lesions up to 3 cm [8]. For this reason, researchers have focused on different biomaterials and the inclusion of coatings, growth factors, intraluminal structures as well as the incorporation of supportive cells. The rationale of cell transplantation into neural conduits is that the presence of a cellular supportive platform within the NC can secrete a constant flow of neurotrophic factors and matrix proteins to assist nerve repair. The beneficial effects of *in vivo* transplantation of Schwann cells (SC) are widely reported [2]. Problems do however exist in clinical applications of autologous SC, with limited tissue availability, time consuming derivation and expansion *ex vivo* and donor site morbidity [20]. Adult stem cells such as bone marrow-derived mesenchymal stem cells (MSC) and adipose-derived stem cells (ASC) have shown profound plasticity, showing *in vitro* differentiation into non-mesenchymal fates, including SC-like phenotypes [14,24]. Our group firstly described the *in vivo* transplantation and effects of dASC using biodegradable fibrin nerve conduits [11], showing significant improvements in regeneration rates, similar to the autografts [9]. To our knowledge, besides their growing applicability in tissue engineering and nerve regeneration, dASC have never been tested with a commercially available conduit. In this study we evaluated the interactions of both dASC and SC-like differentiated bone marrow derived MSC (dMSC) with the Neuragen® conduit *in vitro*. The *in vivo* early nerve regeneration across empty Neuragen® conduits and Neuragen® conduits coupled with dMSC, dASC and SC was assessed using a 1-cm rat sciatic nerve gap in a two-weeks experiment.

2. Methods

2.1. Experimental animals

All animal protocols were approved by the local veterinary commission, in accordance with the European Community Council directive 86/609/ECC for the care and use of laboratory animals. Male Sprague–Dawley rats (Janvier, France) weighing 250 g were used for this study.

2.2. Cell harvest and adult stem cells mesodermal differentiation

Adult Schwann cells (SC) were isolated from rat sciatic nerves as previously described [11] and maintained in Dulbecco's Modified Eagle's Medium plus Glutamax (DMEM, Invitrogen, UK) containing 10% fetal bovine serum (FBS), 1% penicillin–streptomycin (P/S) and supplemented with 14 μM forskolin (Sigma, UK) and 100 ng/ml neuregulin-1 β1 (R&D Systems, UK). Adipose-derived stem cells (ASC) were harvested as described previously [14] and maintained in Modified Eagle Medium (α-MEM; Invitrogen, UK) containing 10% (v/v) FBS, and 1% (v/v) P/S solution. Bone marrow-derived mesenchymal stem cells (MSC) were harvested from adult Sprague-Dawley rat femoral bones as previously described [3] and maintained in the same growth medium as for ASC. In order to confirm stem cell multi-potency, bone marrow MSC and ASC were incubated with specific media to induce differentiation into the three mesodermal-derived lineages as in previous literature, using adipogenic, osteogenic and chondrogenic induction media for three weeks [21].

2.3. Adult stem cells differentiation to a Schwann cell phenotype

At early passages (P2–P3), when cells were sub-confluent, bone marrow-derived MSC and ASC were treated with differentiation medium (DM) containing 5 ng/mL platelet-derived growth factor, 10 ng/mL basic fibroblast growth factor (both from PeproTech Ltd.), 14 mM forskolin (Sigma) and 200 ng/ml of neuregulin-1 β1 (R&D Systems, UK) as previously described [11,14]. Cultures were incubated under these conditions for at least 2 weeks with DM changes every 48 h. Stem cell differentiation into SC-like cells was assessed by immunocytochemistry for typical SC markers S100 and GFAP in parallel to SC as positive controls [3,14]. Negative controls with lack of primary antibodies were also included.

2.4. Scanning electron microscope in vitro imaging

To study cell-conduit interactions *in vitro* 1×10^6 cells (either Schwann cells or dASC) were suspended in 50 μl of respective medium and seeded into longitudinally cut NeuraGen® conduits. Top up of media was performed after 2 h. After 48 h incubation, conduits were washed with PBS and fixed in 2.5% glutaraldehyde in PBS for 1 h at 4 °C. After fixation, further washing in PBS and dehydration was performed using increasing concentrations of ethanol, followed by washes in hexamethylsidilazane (HMDS). After dehydration, samples were mounted on stubs and gold sputtered for scanning electron microscopy qualitative analysis (VPSEM, Zeiss EVO60, Zeiss, Germany, up to 1000×; Phenom, G2 pro desktop, Lambda Photometrics, UK, up to 5000×).

2.5. Cell seeding for in vivo experiments

Prior to implantations in rats, the different regenerative cells were detached from the flask by trypsinization. After centrifugation and aspiration of the supernatant, 2×10^6 cells were resuspended in 50 μl of DM (or SCGM for the SC group) and carefully pipetted into the NeuraGen® conduits. The conduits without cells contained just 50 μl of DM. To avoid cell or medium dispersion during this procedure, one side of the collagen tube was temporarily sealed using a vascular clip. Conduits with cells were kept at 37 °C with 5% CO_2 until surgical implantation (for a maximum of 3 h). Before implantation the vascular clips were cut away, leaving a conduit of 14 mm length.

2.6. Microsurgical technique

Five conduits were implanted for each different group involved in the study (empty nerve guide, guide seeded with SC, dASC, dMSC) for a total $n = 20$. The operation was performed on the left sciatic nerve under aseptic conditions as previously described [9]. The sciatic nerve was transected 1 cm proximal to its distal branches. Proximal and distal nerve stumps were inserted 2 mm into the NeuraGen® conduit, thus leaving a 10 mm gap, and fixed to it by a single epineural suture at each end (9/0 Prolene, Ethicon). Muscles and fascia layers were closed with single resorbable stitches (4/0 Softcat, Braun, Germany) and the skin by a continuous running suture (4/0 Prolene, Ethicon, Germany).

2.7. Conduits harvesting and immunohistochemistry

Animals were sacrificed by CO_2 euthanasia followed by cervical dislocation. Harvested conduits where fixed according to a previously published protocol [11] and embedded in OCT freezing media (Tissue-tek, Sakura, Japan), frozen with dry ice and stored at −80 °C. Longitudinal cryo-sections (14 μm) were prepared onto slides (Superfrost plus, Menzel-Gläser, Germany). A total of 60 sections were taken for each conduit explanted and stored at −20 °C.

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