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Promoting engraftment of transplanted neural stem cells/progenitors using biofunctionalised electrospun scaffolds

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ABSTRACT

With the brain's limited capacity for repair, new and innovative approaches are required to promote regeneration. While neural transplantation for a number of neural disease/injuries have been demonstrated, major limitations in the field include poor cell survival and integration. This, in part, is due to the non-conducive environment of the adult brain, failing to provide adequate chemical and physical support for new neurons. Here we examine the capacity of fibrous poly ε-caprolactone (PCL) scaffolds, biofunctionalised with immobilised glial cell-derived neurotrophic factor (GDNF), to influence primary cortical neural stem cells/progenitors *in vitro* and enhance integration of these cells following transplantation into the brain parenchyma. Immobilisation of GDNF was confirmed prior to *in vitro* culturing and at 28 days after implantation into the brain, demonstrating long-term delivery of the protein. *In vitro*, we demonstrate that PCL with immobilised GDNF (iGDNF) significantly enhances cell viability and neural stem cell/progenitor proliferation compared to conventional 2-dimensional cultureware. Upon implantation, PCL scaffolds including iGDNF enhanced the survival, proliferation, migration, and neurite growth of transplanted cortical cells, whilst suppressing inflammatory reactive astroglia.

1. Introduction

Development of the central nervous system (CNS) is dependent on a tightly orchestrated sequence of events involving the appropriate temporal and spatial presentation of chemical cues and physical support. These same sequences of events are required to repair the injured CNS, however they are either inhibited or significantly attenuated to an extent that repair is extremely limited. Furthermore, current therapies for the treatment of CNS disease or trauma are non-existent, minimally effective and/or associated with unwanted side effects, thereby highlighting the need for new innovative therapies. In this regard, stem cells, due to their self-renewing and differentiation capacity, have received significant attention for their potential in cell-based therapies. While cell transplantation using stem cells/progenitors has shown promise for a number of neurological conditions, and in some clinical trials (see reviews [1–3]), extensive variability, poor cell survival and insufficient integration/reinnervation remain common limitations impeding their further development. Combined, this highlights the need for the development of technologies to improve the microenvironment for transplanted stem cells and residual endogenous cells in an effort to promote neural repair. In this regard the engineering and functionalisation of biomaterials are of increasing interest.

While numerous biomaterials are available, electrospinning of polymers has drawn attention for neural repair due to the ability to recapitulate the local tissue environment through the manipulation of fiber alignment, diameter and inter-fibre distance. These scaffolds provide physical support for new and residual cells, while also maintaining the architecture at the injury site [4–6]. In particular, a number of studies to date have demonstrated the ability of poly (ε-caprolactone) (PCL) to support neural cells *in vitro* and *in vivo* (see reviews [7–9]). Previously we showed the ability of PCL to support neural stem cells (NSC) *in vitro*, resulting in altered proliferation, differentiation and enhanced neurite growth [10–12].

Abbreviations: CNS, central nervous system; CRT, cell replacement therapy; E, embryonic day; ED, ethylene diamine; ELISA, enzyme-linked immunosorbent assay; GDNF, glial-cell derived neurotrophic factor; GFP, green fluorescent protein; iGDNF, immobilised glial derived neurotrophic factor; NSC, neural stem cell; PCL, poly ε-caprolactone; sGDNF, soluble glial derived neurotrophic factor.

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Additionally we have demonstrated that, following implantation into the brain, host derived neurites surrounding the injury site were capable of penetrating the PCL scaffolds, thereby demonstrating biocompatibility and integration [13]. It now remains to be determined to what extent these scaffolds are able to support transplanted NSC/progenitors *in vivo*.

In addition to providing physical support, scaffolds can also be utilised to present chemical cues. A number of studies to date have demonstrated the benefits of administering proteins to influence cell transplantation. Proteins have been administered intracerebrally to promote cell survival or suppress cell death cascades, enhance differentiation of transplanted cell and encourage axonal growth and connectivity on implanted cells [14–17]. However, delivery of these proteins is commonly dependent on the co-transplantation of overexpressing cells, implantation of cannulas and infusion pumps or viral infection of host tissue. Each of these approaches are hindered by problems, including inability to accurately control the site of protein expression, mis-expression in neighbouring nuclei resulting in inappropriate targeting of axons, compromised translation of genes to proteins in cases of severe trauma as well as the inability to down regulate proteins after new cells have appropriately integrated. This highlights the need for improved methods of *in vivo* protein delivery. Several studies now have demonstrated the ability to tether proteins onto scaffolds. In many instances the presentation of immobilised proteins has been shown to be superior to soluble proteins, as endocytosis is prevented, thereby prolonging the period of cellular stimulation [18–20]. We recently immobilised brain derived neurotrophic factor onto PCL scaffolds where it was shown to influence cellular proliferation as well as promote the differentiation of neurons and oligodendrocytes from cortical neural stem cells in culture [12]. Whilst immobilised proteins have been examined *in vitro*, their ability to induce long-term functional outcomes *in vivo*, particularly in the context of supporting neural transplants, requires further investigation.

As two of the major stumbling blocks for the integration of neural transplants into the injured brain are poor cell survival and inadequate reinnervation of the host tissue, in the present study we chose to examine the effects of tethered glial-cell derived neurotrophic factor (GDNF) on cortical neural stem/progenitor cells *in vitro* and *in vivo*. GDNF has been shown to regulate neural cell behaviour including survival, proliferation, differentiation, and neurite outgrowth *in vitro* [21–24]. Additionally, *in vivo* delivery of GDNF using conventional methods has improved the survival rate and/or neurite growth of both endogenous and transplanted neurons in a number of models of neural injuries [25–30].

The aim of this study was to investigate the potential for electrospun PCL fibrous scaffolds to present tethered GDNF to support cortical neural stem/progenitor cell *in vitro* and upon transplantation. We investigated their ability to support cell survival, proliferation, differentiation and enhance neurite growth, thereby enhancing graft integration.

2. Materials and methods

2.1. Preparation of poly (ε-caprolactone) scaffolds and aminolysation

Poly(ε-caprolactone) (PCL) was obtained from Sigma Aldrich (St Louis, MO, USA, molecular weight = 70000–90000). Polymer solutions of 10% (w/v) were prepared for electrospinning by dissolving the PCL in 5 ml of chloroform and methanol (Merck Pty Ltd, Australia) at a ratio of 3:1 (v/v). The solution was placed in a glass syringe with a 18 - gauge needle for electrospinning at +20 kV to −5 kV with a 0.394 mL/h flow rate and a working distance of 10 cm from the plate. The collected PCL scaffolds were dried in a vacuum oven overnight at 30 °C. Scaffolds were then cut into squares (0.5 cm^2) and aminolysed by immersion in 0.05 M ethylene diamine (ED, Sigma Aldrich, USA) diluted with 2-propanol (Merck Pty, Australia) for 15 min at room temperature. The samples were subsequently washed in milliQ water three times for 10 min and stored in a desiccator under vacuum. The samples were sterilised in 70% ethanol for 15 min and washed with sterilised PBS prior to *in vitro* and *in vivo* testing.

2.2. Scanning electron microscopy (SEM)

The scaffolds were coated with 2 nm of platinum using a Cressington sputter coater. PCL samples were tilted at 45° and splutter coated at approximately 50–100 turns/per minute for 30 s. A scanning electron microscope was then used for examination of the scaffold architecture (JEOL JSM-840A SEM W filament). The SEM variables were set as: 20.0 kV (accelerating voltage), 8 mm (working distance), 3000X (magnification) and 1×10^{-9} A (probe current). Fibre diameters were measured using Image J software.

2.3. Biofunctionalisation of PCL scaffolds with glial-cell line derived neurotrophic factor

For immobilisation of GDNF onto the PCL scaffolds, 4-(*N*-maleimidomethyl) cyclohexane-1-carboxylic acid 3-sulfo-*N*- hydroxysuccinimide ester sodium salt (sulfo-SMCC) was used as a cross-linker, as previously described by Horne et al., 2010. In brief, a 2.5 mg/mL sulfo-SMCC solution (Sigma Aldrich, USA) was prepared in PBS with 1 h agitation at room temperature prior to filtration (0.22 μm filter). The PCL scaffolds, treated with ED, were immersed in sulfo-SMCC for 2 h at room temperature, prior to being transferred to a solution containing recombinant human GDNF (0.5 or 4 μg/ml; R & D Systems, USA) overnight at 4 °C.

2.4. Confirmation of GDNF attachment by enzyme-linked immunosorbent assay and immunhistochemistry

An enzyme-linked immunosorbent assay (ELISA) was used to verify the attachment of GDNF onto the scaffolds in samples prepared in parallel to those scaffolds used *in vitro* and *in vivo*. Scaffolds (PCL, PCL with soluble GDNF or PCL with immobilised GDNF) were washed 3 times in PBST (PBS containing 0.05% Tween-20) prior to blocking in 5% normal donkey serum. The scaffolds were then immersed in 1 μg/mL of goat anti-GDNF antibody (R & D Systems, USA) prepared in PBST for 2 h at 37 °C. The scaffolds were then washed three times in PBST before being incubated in anti-goat horseradish peroxidase (HRP, 1:2000 in PBST solution containing 2% donkey serum). Scaffolds were again washed (3 × 10 min in PBST) and placed in a 96-well plate where the bound HRP activity was assayed by color development using TMB microwell peroxidase system (R & D Systems, USA). The reaction was stopped by addition of 1M HCl, and finally the absorbance (450 nm) was measured with a microtitre plate reader (SpectraMax). ELISA was performed on triplicate scaffolds for each treatment group and repeated for the 3 independent *in vitro* cultures as well as the *in vivo* implantation of scaffolds.

The attachment of GDNF onto the PCL-ED scaffolds was additionally confirmed by immunohistochemistry at 28-days after implantation. Brain sections containing the scaffolds were mounted onto slides for immunostaining against GDNF. All slides were washed (3 × 10 min in PBS) and quenched in endogenous peroxidase (10%, methanol, 10%, hydrogen peroxide and 80% PBS) for 20 min before additional washes. Subsequently the primary antibody, goat anti-GDNF (2 μg/mL) prepared in PBS containing 0.3% triton-X, was applied overnight at room temperature. The next day the slides were washed prior to blocking in 10% donkey serum in PBS for 30 min. The secondary antibody, biotinylated anti-goat (1:500, DAKO), was added for 1.5 h at room temperature. The sections were then incubated in avidin peroxidase (Vectastain® ABC system kit) and then reacted with diaminobenzidine (DAB, Sigma). The slides were washed in PBS before dehydrating in ethanol, delipiding in X3B and coverslipping with DPX mounting medium.

2.5. Animals

All procedures were conducted in accordance with the Australian National Health and Medical Research Council's published Code of Practice for the Use of Animals in Research, and experiments were approved by the Florey Neuroscience Institute animal ethics committee. Mice and rats were housed on a 12 h light/dark cycle with ad libitum access to food and water. Cells used for *in vitro* culturing and transplantation were obtained from mice that were time mated overnight, with visualisation of a vaginal plug on the following morning taken as embryonic day (E) 0.5. All tissue was isolated at mouse embryonic day 14.5 (E14.5). *In vitro* culturing of primary cortical cells was performed using tissue obtained C57BL/6 time mated mice while donor tissue for transplantation was obtained from C57BL/6 time-mated mice expressing green fluorescent protein (GFP) under the β-actin promoter. The ubiquitous expression of GFP within the donor tissue enabled distinction of the grafted cells within the host brain.

2.6. Preparation of primary cortical cell suspensions for in vitro and in vivo application

Pregnant mice (E14.5) were anesthetised with isoflurane prior to cervical dislocation. The collected embryos were immersed in chilled L15 medium (invitrogen), the brains removed and cortices microdissected. Subsequently the tissue