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The reduction in immunogenicity of neurotrophin overexpressing stem cells after intra-striatal transplantation by encapsulation in an *in situ* gelling collagen hydrogel

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ABSTRACT

Delivery of neurotrophic factors to the brain via genetically modified bone marrow-derived mesenchymal stem cells (MSCs) offers a promising neuroprotective strategy for neurodegenerative diseases. However, MSCs delivered to the CNS typically show poor survival post-transplantation, which is accompanied by microglial activation and astrocyte recruitment at the graft site. Recent studies have shown the potential of biomaterials to provide a supportive matrix for transplanted cells which may assist in the grafting process. In this study, an *in situ* gelling type I collagen hydrogel was evaluated as an intracerebral transplantation matrix for delivery of glial cell line-derived neurotrophic factor (GDNF)-overexpressing MSCs to the rat brain (GDNF-MSCs). *In vitro* analyses demonstrated that this collagen hydrogel did not affect the viability of the GDNF-MSCs nor did it prevent GDNF secretion into the surrounding medium. *In vivo* analyses also confirmed that the collagen hydrogel did not negatively impact on the survival of the cells and permitted GDNF secretion into the striatal parenchyma. Importantly, this study also revealed that transplanting GDNF-MSCs in a collagen hydrogel significantly diminished the host brain's response to the cells by reducing the recruitment of both microglia and astrocytes at the site of delivery. In conclusion, this hydrogel, which is composed of the natural extracellular matrix, collagen, was shown to be a well-tolerated cell delivery platform technology which could be functionalised to further aid cell support and graft integration.

1. Introduction

Neurotrophic factors show promise for neuroprotection in disorders such as Alzheimer's disease (AD) [1], Parkinson's disease (PD) [2,3] and Huntington's disease [4,5], however their clinical development is limited by issues related to their delivery to the brain. *Ex vivo* gene therapy, where cells are genetically engineered *ex vivo* to over-express and secrete growth factors, may circumvent some of the problems associated with direct delivery of neurotrophic factors to the brain. However, a major limitation associated with this approach is that cells survive poorly after transplantation into the brain [6–10] and are associated with activation of the host brain's neuroimmune microglia and astrocytes [8,10]. Biomaterial systems, such as preformed scaffolds or *in situ* forming hydrogels, show potential to improve the grafting procedure by acting as supportive and protective matrices for the cells following intracerebral transplantation [11–18] (reviewed elsewhere [19]).

Whilst scaffolds for applications in traumatic brain injury [20,21] or spinal cord injury [22,23] may be implanted by invasive surgical techniques, intervention for neurodegenerative diseases of the brain must be injectable. Materials with the ability to form hydrogels *in situ* [24] or in response to stimuli such as body temperature [25] offer an injectable biomaterial platform to aid cell transplantation. As cell survival post-transplantation is poor [6–9], a predominant goal of using hydrogels to deliver cells to the brain is to 1) improve the cell engraftment by providing an adherent

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substrate and 2) to provide a physical barrier to protect the transplanted cells against the host response [11–18]. The commercially available gel formulation, Matrigel™, composed of extracellular matrix proteins derived from mouse sarcomas, has been used to enhance stem cell transplantation [15,18]. Co-injection of embryonic stem (ES) cell-derived neural precursor cells (NPCs) with the Matrigel™ into the mouse striatum resulted in greater cell survival, as determined by the graft volume, than cells transplanted in medium [15]. Additionally, pan-leukocytic CD45-positive cells (microglia and lymphocytes) were reduced at the early time point (24 h), while another study showed less graft infiltration of GFAP positive cells (astrocytes) when the Matrigel™ was used [18]. Whilst these studies provide proof-of-concept for inclusion of a supportive and protective matrix in cell transplantation studies, the murine derivation of Matrigel™ combined with its multitude of growth factors (even when used as a factor poor version), negates its clinical translatability. Degradable biomaterial systems that have been granted approval for clinical use offer greater translatable potential, warrant pre-clinical evaluation as biomaterial matrices in cell transplantation studies.

The natural extracellular matrix, collagen, is a clinically accepted biomaterial which has received comparatively less attention for cell transplantation in the brain [16]. Unlike synthetic hydrogels such as those composed of poly *N*-(2-hydroxypropyl)-methacrylamide (HPMA) [11] or poly(ethylene glycol)–poly(propylene sulfide) (PEG–PPS) [17] which require laborious addition of RGD peptide sequences to facilitate cellular attachment, collagen has the advantage having an endogenous RGD sequence allowing cell adhesion without requiring further functionalization chemistries [26]. In order for a collagen hydrogel to prove useful in the assistance of *ex vivo* therapies, it is important that its composition does not hinder therapeutic molecule delivery and diffusion at the target parenchymal tissue. Moreover, the ability of the collagen hydrogel to modulate the host response to transplanted cells could play an important role in cell transplantation procedures in the CNS, potentially allowing better graft integration or survival.

To this end, the aim of this study was to assess the potential use of a collagen hydrogel for cell delivery applications in the brain. It was hypothesised that the hydrogel, consisting of type I atelocollagen cross-linked with four arm Star-PEG, would reduce the host response to grafted rat mesenchymal stem cells transplanted into the striatum, without hindering the release and diffusion of the over-expressed GDNF to the surrounding brain tissue.

2. Materials and methods

2.1. Animals

All procedures were carried out in accordance European Union Directive 2010/63/EU and S.I. No. 543 of 2012, and were approved by the Animal Ethics Committee of the National University of Ireland, Galway. Bone marrow-derived MSCs were extracted from the femora and tibiae of green fluorescent protein (GFP) transgenic Sprague–Dawley rats, and were characterised as MSCs as described previously [27]. MSCs were then transduced to over-express human GDNF using a murine leukaemia virus as previously described [28]. Type I collagen was extracted from bovine Achilles tendon and held as an acidified solution at 5 mg/ml in ethanoic acid as previously described [29].

2.2. Fabrication of cell-seeded cross-linked collagen type I hydrogels

Under standard sterile cell culture conditions MSCs were cultured in a 1:1 DMEM:Alpha-MEM mix that contained 10% FBS and 1% penicillin/streptomycin in T75 flasks in humid conditions at 37 °C with 5% CO_2. MSCs were trypsinised and resuspended in cell transplantation medium (phenol-red free DMEM with 10% FBS and 1% penicillin/streptomycin) at a concentration of 25,000 cells/μl so that a final concentration of 10,000 cells/μl could be obtained in the hydrogel. For the preparation of the collagen hydrogel, all components were placed on ice to prevent premature gelation. For a final hydrogel volume of 100 μl, 50 μl of collagen, neutralised with 1 M NaOH, was added to 10 μl of 4× phosphate buffered saline. 40 μl of the cell suspension was added to an eppendorf containing 40 mg of poly(ethylene glycol) ether tetrasuccinimidyl glutarate (4S-StarPEG) to dissolve this cross-linker. The cell/cross linker solution was then added to the collagen/PBS solution and mixed thoroughly. For *in vitro* experiments, 4 μl samples were transferred onto a previously sterilised (UV radiation) super hydrophobic surface (Teflon® tape) and placed at 37 °C to gel (~10–15 min). Images of the gels either immediately after formation or after one day in culture were obtained using a fluorescent microscope (Olympus). Image overlays were produced from the intrinsic GFP expression, DAPI nuclear counterstain and bright field images. For *in vivo* experiments, the cell seeded collagen hydrogel was held on ice prior to transplantation to prevent gelation. The overall design of the study is shown in Fig. 1.

2.3. In vitro assessment of the impact of the hydrogel on cell viability and GDNF release

2.3.1. Astrocyte viability assay

Before embarking on *in vivo* studies, confirmation that the collagen hydrogel itself was non-toxic to neural cells was needed. To do so, primary astrocytes were extracted from new-born rat pups, as described previously [30], using a combination of two protocols [31,32]. The astrocytes were cultured in a 1:1 DMEM:F12 mixture supplemented with 10% FBS and 1% penicillin/streptomycin and seeded at a density of 20,000 cells per well of a 24 well-plate and left overnight to attach. The astrocytes then received either no treatment, or they were incubated with transplantation medium samples (three samples of 4 μl each) or unseeded collagen hydrogels (three gels of 4 μl each) for 48 h. As an indicative measure of cell viability, metabolic activity of the cells was measured using the alamarBlue® assay as previously described [33]. Briefly, 100 μl of a 10% solution of alamarBlue® (Invitrogen) solution in HBSS was added to each well and incubated for 3 h. Absorbance at 550 nm and 595 nm was analysed using a Varioskan Flash plate reader (Thermo Scientific) with SkanIt® software. Viability was calculated by normalisation of all results to control wells.

2.3.2. MSC viability assay

To determine the effect of the collagen hydrogel on the viability of MSCs seeded within it, 120,000 MSCs in either cell transplantation medium or seeded into the collagen hydrogel (three gels of 4 μl each containing 10,000 cells/μl) were added to 24 well-plates containing 1 ml of MSC growth medium. Once again, as a measure of cell viability, metabolic activity of the cells was measured using the alamarBlue® assay as described above (MSCs were incubated with the alamarBlue® solution for 6 h due to 3D hydrogel culture hindering alamarBlue® diffusion time).

2.3.3. GDNF assay

Analysis of the impact of the collagen hydrogel on GDNF release into the surrounding medium was performed by seeding 120,000 GDNF-MSCs per well of a 24 well-plate, either within (three gels of 4 μl each containing 10,000 cells/μl) or without a collagen hydrogel. 50 μl of the total 1.5 ml cell supernatant was removed one, three and five days post seeding for analysis by the human GDNF enzyme-linked immunosorbent assay (ELISA, human GDNF DuoSet, R&D Systems) according to the manufacturer's protocol.

2.4. In vivo assessment of the impact of the hydrogel on cell viability, GDNF release and host response

After the initial *in vitro* analyses, two *in vivo* studies using male Sprague Dawley rats (Charles River, UK) were conducted. The first was a pilot study designed to assess the host response to the collagen hydrogel in the rat brain, while the second was the main *in vivo* study which was designed to assess the impact of the collagen hydrogel on the survival of, GDNF release from, and host response to, GDNF-MSCs transplanted into the rat striatum.

2.4.1. Stereotactic surgery

All surgeries were conducted under isoflurane gaseous anaesthesia (5% in O_2 for induction and 2% in O_2 for maintenance) in a stereotaxic frame with the nose bar set at −2.3 mm. The striatum was infused bilaterally at coordinates AP = 0.0, ML ± 3.7 (from bregma) and DV −5.0 below dura. All infusions were completed in a total volume of 3 μl at a rate a 1 μl/min with a further 2 min allowed for diffusion. For the pilot *in vivo* study, rats ($n = 4$) received either 3 μl of collagen hydrogel or 3 μl of transplantation medium at the above coordinates. For the main *in vivo* study, rats ($n = 23$) received a bilateral injection of 30,000 GDNF-MSCs in 3 μl of either collagen hydrogel or transplantation medium.

2.4.2. Immunohistochemistry

At day 4 (pilot study) or days 1, 4, 7, and 14 (main study) post-transplantation, rats were sacrificed by terminal anaesthesia (50 mg/kg pentobarbital i.p.) and transcardially perfused with 100 ml heparinised saline followed by 150 ml 4% paraformaldehyde. Brains were rapidly removed, post-fixed in 4% paraformaldehyde overnight and cryoprotected in 25% sucrose solution. Serial coronal sections (30 μm) were cut using a freezing stage sledge microtome (Bright, Cambridgeshire, UK.) and free-floating immunohistochemistry for GDNF, microglial activation and astrocyte recruitment was performed as previously described [27]. In brief, endogenous