Optimizing a multifunctional microsphere scaffold to improve neural precursor cell transplantation for traumatic brain injury repair

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Abstract

Tissue engineering using stem cells is widely used to repair damaged tissues in diverse biological systems; however, this approach has met with less success in regenerating the central nervous system (CNS). In this study we optimized and characterized the surface chemistry of chitosan-based scaffolds for CNS repair. To maintain radial glial cell (RGC) character of primitive neural precursors, fibronectin was adsorbed to chitosan. The chitosan was further modified by covalently linking heparin using genipin, which then served as a linker to immobilize fibroblast growth factor-2 (FGF-2), creating a multifunctional film. Fetal rat neural precursors plated onto this multifunctional film proliferated and remained multipotent for at least 3 days without providing soluble FGF-2. Moreover, they remained less mature and more highly proliferative than cells maintained on fibronectin-coated substrates in culture medium supplemented with soluble FGF-2. To create a vehicle for cell transplantation, a 3% chitosan solution was electrosprayed into a coagulation bath to generate microspheres (range 30–100 µm, mean 64 µm) that were subsequently modified. Radial glial cells seeded onto these multifunctional microspheres proliferated for at least 7 days in culture and the microspheres containing cells were small enough to be injected, using 23 Gauge Hamilton syringes, into the brains of adult rats that had previously sustained cortical contusion injuries. When analysed 3 days later, the transplanted RGCs were positive for the stem cell/progenitor marker Nestin. These results demonstrate that this multifunctional scaffold can be used as a cellular and growth factor delivery vehicle for the use in developing cell transplantation therapies for traumatic brain injuries. Copyright © 2013 John Wiley & Sons, Ltd.

Keywords chitosan; fibroblast growth factors; fibronectin; immobilized growth factors; multifunctional scaffold; neurotrauma; radial glia; regenerative medicine

1. Introduction

Stem cell therapeutics is a promising field for tissue regeneration but it has shown limited success in repairing the brain after severe injury. Brain injuries often cause extensive tissue damage characterized by neuronal and glial cell death where there is virtually no functional replacement of cells from the endogenous neural stem cells (NSCs). In an animal model of stroke, Arvidsson et al. (2002) reported that less than 1% of the destroyed neurons are replaced from the endogenous neural precursors of the subventricular zone (SVZ) (Arvidsson et al., 2002). Similar results have been obtained in animal models of traumatic brain injury (TBI) (Salman et al., 2004). Salman et al. (2004) observed that neural precursors (NPs) from the SVZ repopulated a mechanically injured cortex. The SVZ cells proximal to the injured area produced a very small percentage of new neurons (not quantified), with the majority of the transplanted cells becoming...
astrocytes. Direct transplantation of NPs into the penumbra of brain lesions has yielded minor advancements (Richardson et al., 2010; Sanberg et al., 2012). Most of the transplanted cells either do not survive (Shindo et al., 2006; Harting et al., 2009; Wallenquist et al., 2009) or differentiate into glial cells instead of neurons (Shear et al., 2004; Boockvar et al., 2005; Ma et al., 2011; Sun et al., 2011). Shear et al. (2004) and Boockvar et al. (2005) found that NG2 positive glial cells were produced upon transplanting NPs and Sun et al. (2011) observed that the majority of the precursors they transplanted became Olig2 positive cells (presumably glia). Ma et al. (2011) reported that only 4% of NPs that they transplanted were NSCs, whereby only 11% differentiated into cells expressing a neuronal marker. Transplanting stem cells attached to a supportive matrix directly into the lesion site may be more effective in promoting regeneration. Tate et al. (2009) showed improvement in the long-term survival of NPs that were transplanted within a supportive fibronectin and laminin matrix after TBI. Animals receiving these transplants also showed improved performance in spatial learning tasks compared with injured mice that did not receive NPs (Tate et al., 2002, 2009).

Using principles from material engineering and molecular biology, tissue engineers are developing organic substitutes to support or replace portions of malfunctioning tissues or organs to create substitutes (Langer and Vacanti, 1993). The common approach to create these substitutes is to use living cells, scaffolding and signalling molecules (Kim et al., 2008). Evans (2000) identified four components necessary for nervous tissue scaffolds: growth factors, extracellular matrix (ECM), support cells and molecules that will promote axonal regeneration (Evans, 2000). Traumatic brain injuries are appropriate for the application of biomaterial scaffolds because there is extensive and localized loss of cells and ECM. A scaffold can serve as an artificial matrix and support network for engrafted cells as well as for the host tissue. Furthermore, it serves as both a physical and chemical barrier against glial scarring, which is well known to inhibit axonal regeneration (Hou et al., 2005; Cui et al., 2006; Li et al., 2009; Bozkurt et al., 2010; Jurga et al., 2011; Martinez-Ramos et al., 2012). The ECM is also an important regulator of cell function. Interactions of ECM and integrin govern cellular processes such as proliferation, survival, migration and differentiation (Ingber, 1992; Mooney et al., 1992; Meredith et al., 1993; Aplin et al., 1999; Aplin and Juliano, 1999). Accordingly, biomaterial systems that mimic the native ECM should be considered when designing regenerative therapies for neural tissue.

Chitosan is a naturally occurring biodegradable polysaccharide formed from the deacetylation of chitin, which is the major structural component of the exoskeletons of animals such as crabs and insects. Chitosan has been shown to be a suitable biomaterial for neural tissue engineering applications (Haipeng et al., 2000), eliciting high cellular compatibility with low toxicity. The abundance of side-chains in chitosan allows for easy modification and addition of other peptides or molecules. Hydrophilic polymers, such as polylysine, allow for a more polar and wettable material that increases the affinity of cells when added to chitosan (Khor and Lim, 2003). Peptide sequences such as arginine–glycine–aspartic acid 'RGD' (found in fibronectin, collagen or gelatin) or isoleucine-lysine-valine-alanine-valine 'IKVAV' (found in laminin) can be immobilized easily to chitosan to aid in cell adhesion, migration, proliferation and differentiation (Muzzarelli, 1977; Gupta and Ravi Kumar, 2000; Kumar, 2000). The RGD peptide sequence can bind to integrin receptors found on stem cells, activating integrin-signalling pathways to prevent differentiation and increase proliferation (Comisar et al., 2007; Dellatore et al., 2008; Martino et al., 2009). Heparin is a highly sulphated glycosaminoglycan with high binding affinity for growth factors such as fibroblast growth factor (FGF), platelet-derived growth factor (PDGF), and vascular endothelial growth factor (VEGF) (Ashikari-Hada et al., 2004). The binding of heparin to these growth factors protects them from proteolytic degradation (Sommer and Rifkin, 1989).

In this study, a chitosan-based biomaterial scaffold was engineered to promote CNS regeneration from primitive neural precursors. A method to manufacture an injectable multifunctional microsphere scaffold was optimized and the surface chemistry of the biomaterial was subsequently modified in order to achieve a scaffold that was highly suitable as a vehicle for cell transplantation to repair traumatic brain injuries.

2. Methods

2.1. Reagents

Chitosan (low molecular mass, ~ 50 kDa), heparin sodium salt from bovine intestinal mucosa, MITT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium], 4,6-diamidino-2-phenylindole (DAPI) and phalloidin-conjugated rhodamine were purchased from Sigma (St Louis, MO, USA). Genipin was purchased from Wako Pure Chemical Industries, Ltd (Osaka, Japan). Recombinant human-fibroblast growth factor-2 (FGF-2) was purchased from Peprotech (Rocky Hill, NJ, USA). Nitrocellulose and NuPAGE 4–12% Bis-Tris Gels were purchased from Life Technologies (Carlsbad, CA, USA). Fibronectin, laminin and collagen were purchased from BD BioSciences (Franklin Lakes, NJ, USA). Bovine serum albumin, gelatin, poly-L-lysine (PLL), acetic acid, sodium hydroxide and methanol were purchased from Fisher Scientific (Pittsburgh, PA, USA). Common laboratory chemicals were purchased from either Sigma or VWR International (Radnor, PA, USA).

2.2. Chitosan scaffold

Chitosan powder (1.5 g) was dispersed in 50 ml of water containing 2.0% v/v acetic acid to create a 3% chitosan solution. The chitosan solution was mechanically stirred at 700 rpm until completely dissolved. The resulting solution was collected and centrifuged at 193 × g for
10 min. Subsequently, the supernatant was collected and the remaining impurities at the bottom were discarded.

### 2.2.1. Chitosan films

A 3% acid chitosan solution was pipetted into two-well glass chamberslides (NUNC, Rochester, NY, USA) to coat the bottom of chamber. The remaining solution was removed and the slides were set to dry for 2–3 h at room temperature. Chitosan coatings were neutralized in 0.5 M NaOH for 10 min and then rinsed three times in sterile distilled water for 5 min each. For substrate cell adhesion, chitosan was subsequently adsorbed with solutions of fibronectin 10 μg/ml, 20 μg/ml laminin, 0.1% gelatin, 0.1 mg/ml collagen type I, or 0.05 mg/ml PLL, all prepared in distilled water. For two-dimensional multifunctional scaffolds, chitosan films were prepared and chemically modified as described below.

### 2.2.2. Chitosan microspheres

Chitosan microspheres were formed by extruding 3% acid chitosan solution through a syringe with a 30-gauge needle (30-gauge needle method) into a basic coagulation bath, consisting of 2.5 M sodium hydroxide–methanol–water (20:30:50 v/v). To create smaller spheres, two modifications were devised: the coaxial airflow method, which consisted of adding a coaxial air pressure around the 30-gauge needle to reduce surface tension on the end of the needle as described previously (Skop et al., 2013) and the electrospray method. The electrospray method reduces needle surface tension greater than the coaxial airflow technique, thus further reducing the size of the microspheres. A 25 kV electric current was applied to the tip of a 23-gauge needle while the chitosan solution was extruded through a syringe. This method is adapted from the electrospraying technique, which is commonly used to form material fibre meshes. The electric current applies a high voltage to the chitosan solution droplet contained within the syringe. The charge on the liquid solution is enough to overcome the surface tension at the opening of the needle where the droplet is released more quickly. By reducing the surface tension, the droplet is also expelled at a smaller size, producing microspheres of reduced diameter. Next, the spheres were removed from the ionic solution and rinsed four times in distilled water to eliminate any residual sodium hydroxide and methanol. Microspheres were then sterilized in 70% ethanol for 30 min. Microspheres were photographed using phase contrast in an inverted microscope (Nikon Ti-S) and camera (Nikon DS-RiI). The microsphere size was determined by measuring the microsphere diameter using Sigma SCAN PRO 5 software.

### 2.2.3. Chitosan surface chemistry modification

Chitosan films or microspheres were rinsed in distilled water. To crosslink the heparin to the scaffolds, chitosan was incubated overnight with 0.5 mg/ml heparin and 0.45 mM genipin in 50 mM 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid, N-(2-Hydroxyethyl)piperazine-N'- (2-ethanesulfonic acid) (HEPES) buffered solution containing 0.9% NaCl pH 7.4 (HBS), as previously described (Skop et al., 2013). The following day the heparin crosslinked chitosan films or spheres were rinsed three times for 10 min each in HBS and incubated overnight with 10 μg/ml fibronectin in distilled water and for 2 h in HBS containing 1 μg/ml FGF-2 in 1 mg/ml bovine serum albumin (BSA) solution. Spheres were then centrifuged at 730 g and the supernatant discarded. The spheres were rinsed in HBS to remove any unbound FGF-2 and then resuspended in radial glia media (RGM) containing Dulbecco’s modified Eagle’s Medium (DMEM)/F12 media supplemented with B27, 50 μg/ml gentamicin and 50 μg/ml apotransferrin. Chitosan films were rinsed once in HBS to remove unbound FGF-2. For the MTT reduction assay, chitosan films were modified with only one or more of the surface ingredients, as indicated in the Results section.

### 2.3. Cell culture

#### 2.3.1. RG3.6 cell line

The RG3.6 cell line is an immortalized cell line obtained by introducing v-myc to radial glial cells from embryonic cortex day 13.5 of green fluorescent protein positive (GFP⁺) rats (donated by Dr Martin Grumet, Rutgers, New Brunswick, USA). The RG3.6 cells were grown as neurospheres for culture propagation or as adherent monolayers on coated Petri dishes in RGM media containing 10 ng/ml FGF-2 prepared with heparan sulphate at 1 ng/ml final concentration (RGM-FGF). Ten per cent of the medium was changed every day and replaced with equal volume of 10× FGF-2-containing media (100 ng/ml). When growing RG3.6 cells on modified chitosan microspheres, cells were seeded at a 20:1 ratio cells over spheres (1 000 000 cells for 50 000 spheres) and incubated at 37°C overnight. The RG3.6 cell line was used instead of primary cells for specific experiments to eliminate several variables seen with heterogeneous primary cell cultures and to achieve greater consistency during substrate optimization.

#### 2.3.2. Primary radial glial cells

Primary radial glial cells were harvested from embryonic day 13.5 EGFP [Sprague–Dawley–Tg(GFP)Bal/2Rrrc (RRRC:0065)] rat neocortex, provided by the Missouri Research Animal Diagnostics Laboratory (RADIL, Columbia, MO). Cells were grown as neurospheres for culture propagation or as adherent monolayers on coated Petri dishes in RGM-FGF. Ten per cent of the medium was changed every day and replaced with equal volume of 10× FGF-2-containing media (100 ng/ml). To obtain secondary RGCs, neurospheres were collected by centrifugation at 200 g. The pellet was then resuspended in 70% accutase (Millipore, Billerica, MA, USA) for 5 minutes at 37°C to dissociate the cells. An equal volume of conditioned RGM was then added and
cells were centrifuged at 200 g. The supernatant was aspirated and cells were resuspended in fresh RGM. The suspension was centrifuged for a final time at 240 g and the pellet was resuspended in RGM-FGF. The RGCs were triturated gently to ensure cell dissociation. Cells were then counted and seeded into Petri dishes in RGM-FGF.

2.3.3. Cell differentiation

The RG3.6 cells or RGCs were seeded onto PLL and laminin-coated dishes, and maintained for 24 h in RGM-FGF. Cells were differentiated by removing the mitogen FGF-2 from the media. Seven days later cells were fixed with 3% paraformaldehyde and stained with a mouse monoclonal antibody against class III beta-tubulin (βIII TUB) 1:500 (Covance, Princeton, NJ, USA), rabbit polyclonal anti-glial fibrillary acidic protein (GFAP) antiserum 1:500 (Sigma) and mouse monoclonal antibody supernatant O4 (1:4) (produced in-house) to identify neurons, astrocytes and oligodendrocytes, respectively.

2.3.4. Process length and number

Cells were fixed in 3% paraformaldehyde and stained for F-actin with phalloidin conjugated to rhodamine, at 0.1 mg/ml (Sigma). Fluorescence photomicrographs were taken at ×20 magnification in four fields per well in triplicates. Cell process lengths were measured using Sigma Scan PRO 5 software and the number of processes extending from each cell was calculated manually. A total of 400–500 cells per condition were evaluated. Statistical analyses were performed using ANOVA with Tukey post hoc. Data are expressed as the mean ± standard error of mean (SEM).

2.3.5. Ki67 Staining

Cells were grown in chamber slides and stained using a rabbit polyclonal anti-Ki67 antibody at 1:1000 (Vector Laboratories, Burlingame, CA, USA) and counterstained with 1 μg/ml DAPI. Positive cells were counted from photomicrographs taken at ×20 magnification in four fields/well in triplicate. A total of 200–300 cells per condition were analysed. The percentage of proliferative cells was calculated as the number of Ki67+ cells over the total number of cells stained with DAPI.

2.4. MTT reduction assay

The MTT assay is a colorimetric assay that measures the reduction of a yellow substrate (MTT) in the cell into an insoluble purple formazan product (Mosmann, 1983). The assay was performed in 96-well plates previously coated with 50 μl of 3% chitosan. Cells were seeded at 5 × 10^4 cells/well in 100 μl of media. Two days later, 10 μl of a 5 mg/ml MTT solution in PBS was added to each well and incubated on the cells for 2–4 h at 37°C. The reaction was stopped by adding 100 μl of a solution containing 50% (w/v) N,N-dimethylformamide and 20% sodium dodecyl sulphate (SDS) (pH 4.8). The plates were maintained overnight in the incubator at 37°C and absorption at 560–690 nm was determined using a microtitre plate reader (PowerWave 200; Bio-tek Instruments, Winooski, VT).

2.5. Western blot analyses

Cells were scraped from six-well plates in lysis buffer containing PBS, 1% Triton-X 100, 0.1% SDS, 1% 0.1 M sodium orthovanadate and 1% protease inhibitor cocktail (Roche Diagnostics, Indianapolis, IN, USA). Protein concentrations were determined using the BCA assay (ThermoScientific, Rockford, IL, USA). Ten micrograms of protein per well was loaded onto NuPAGE 4–12% Bis-Tris gels and electrophoresed. Approximately, 2 μl of MagicMark XP (Invitrogen, Carlsbad, CA) was loaded for standard molecular weight markers. Gels were transferred to nitrocellulose. The blots were blocked with 5% milk in PBS-Tween followed by incubation with primary antibody in 5% BSA in PSB-Tween overnight at 4°C with gentle rocking. The primary antibodies used were: rabbit polyclonal anti-brain lipid binding protein (BLBP) antiserum at 1:1000 (Abcam, Cambridge MA, USA); rabbit polyclonal anti-sex determining region Y-box 2 (Sox2) at 1:200 (Chemicon, Temecula, CA, USA); mouse monoclonal βIII TUB at 1:1000 (Covance); rabbit polyclonal anti-microtubule associated protein-2 (MAP2) at 1:200 (Sigma); rabbit polyclonal anti-GFAP at 1:500 (DAKO, Carpinteria, CA, USA); and mouse monoclonal anti-proliferating cells nuclear antigen (PCNA) at 1:1000 (Cell Signaling, Beverly, MA, USA). The Ki67 antibody was inadequate for Western blot. Following three rinses with PBS-Tween the following day, the blots were incubated with corresponding secondary antibodies such as donkey anti-rabbit horseradish peroxidase (DAR-HRP)-conjugated (1:10000; Jackson ImmunoResearch, West Grove, PA, USA) or donkey anti-mouse horseradish peroxidase (DAM-HRP)-conjugated (1:5000; Jackson ImmunoResearch) for 2 h at room temperature. The blots were washed and signal developed with Western Lightning chemiluminescence reagent (PerkinElmer, Wellesley, MA, USA) as per manufacturer’s guidelines. The bands were visualized using a UVP EpiChem3 and processed with LABWORKS 4.0 digital quantification software (UVP, Upland, CA, USA).

2.6. Controlled cortical impact

Two-month-old adult Sprague–Dawley male rats were anaesthetized by intraperitoneal injection (i.p.) of a ketamine and xylazine mixture (90 mg/kg and 10 mg/kg, respectively). The fur covering the head was removed using an electric razor and a midline incision made through the scalp using a scalpel. The skin was deflected and a craniectomy was made using a drill with a 5-mm diameter trephine. The trephine was placed midway between Bregma and Lambda, with the edge of the trephine adjacent to midline. Cold PBS was suffused onto the surface of the skull during the craniotomy to reduce the generation of heat that could cause damage to the
underlying dura mater and neocortex. The skull flap was removed and the animal placed into a stereotactic apparatus under the controlled cortical impactor device (eCCI 6.3 device built by Custom Design and Fabrication, Richmond, VA, USA). The anvil tip of 3.5 mm diameter was zeroed by bringing it into contact with the exposed Dura mater. The velocity of the impactor was set at 4.0 ± 0.2 m/s, depth of penetration was 1.5 mm and the duration of deformation was 150 ms. After impact, the integrity of the Dura mater was confirmed and the scalp incision sutured with 3–0 nylon thread. Buprenorphine (0.05 mg/kg, subcutaneously) was administered postoperatively and the rats were placed on heating pads at 37°C and monitored continuously for 2 h after surgery. In addition, immediately after surgery, all subjects received 3% body weight of 0.9% saline subcutaneously to prevent dehydration.

### 2.7. Cell transplantation

Subacute transplantations were performed 7 days after controlled cortical impact injury. The animals were anesthetized by i.p. injection of a ketamine and xylazine mixture and the sutures were removed to expose the skull. Cell–microsphere complexes were collected from culture dishes and resuspended in phenol-free media without supplements. A 23-gauge Hamilton syringe (inner diameter of 260 μm) was used to inject the scaffold at three different depths: 1.5, 1.0 and 0.5 mm below the dura mater. One microlitre was injected at each depth over 5 min, with 5-min intervals between each injection. The needle was withdrawn 10 min after the last injection. The scalp incision was sutured with 3–0 nylon thread and the animals placed onto a 37°C heating pad until they were fully awake. All animal procedures performed in this report were approved by the New Jersey Medical School IACUC under animal protocol #08056.

### 2.8. Immunofluorescence of brain sections

Rats were deeply anesthetized with the ketamine and xylazine mixture perfused 3 days post-transplantation using 4% paraformaldehyde (PFA). The brains were collected and kept in 4% PFA overnight. Next day, the brains were rinsed with PBS and cryoprotected by immersion in 30% sucrose in distilled water. After one change of sucrose solution, the brains were placed into plastic cryomoulds and frozen in optical curing temperature (OCT) embedding media (Sakura Finetek, Torrance, CA, USA) on a dry ice–ethanol slush. The brains were cryosectioned at 40 or 15 μm thickness and stained with primary antibodies overnight at 4°C using mouse monoclonal anti-Nestin antibody 1:5 (Developmental Studies Hybridoma Bank, Iowa City, IA, USA) and chicken polyclonal anti-green fluorescent protein (GFP) antibody 1:2500 (AVES, Tigard, OR, USA). Sections were incubated in secondary antibodies 1:200 (Jackson ImmunoResearch) for 2 h at room temperature. All secondary antibody combinations were carefully examined to ensure that there was no bleed-through between fluorescent dyes or cross-reactivity between secondary antibodies. No signal above background was obtained when the primary antibodies were replaced with pre-immune sera. After secondary antibody incubation the sections were washed, counterstained with 1 μg/ml DAPI for 5–10 min and coverslipped with GelMount (Biomed, Foster City, CA, USA).

### 3. Results

#### 3.1. Effect of chitosan films adsorbed with adhesion proteins on morphology and proliferation of RGCs

To establish which surface modifiers best maintained the morphology and proliferation of RGCs, chitosan films were adsorbed with different ECM proteins that mediate cell adhesion, and used as substrate for the adhesion and growth of RGCs. RG3.6 cells, a cell line derived from embryonic RGC, were seeded onto chitosan films adsorbed with fibronectin, laminin, gelatin, type 1 collagen or the polymer poly-L-lysine and maintained in growth media for 4 days. Cell morphology was visualized by staining of F-actin, which clearly revealed cell processes. RGCs are morphologically distinct cells that project a few long processes, typically non-branching and in opposing directions. The RG3.6 cells adopted strikingly different morphologies (especially the length and number of processes) when grown on chitosan films absorbed with varying adhesive proteins. Chitosan films coated with fibronectin best preserved the RGC morphology (Figure 1 a–c). They had a small cell body and one to three straight processes that lacked branches and that on occasion were very long (49 ± 5 μm). RG3.6 cells grown on chitosan films coated with laminin also possessed few processes but they were shorter (35 ± 3 μm) than the processes of cells grown on fibronectin–chitosan films. Cells grown on gelatin had processes with significantly fewer branches than the control condition (unmodified chitosan films) but they were much shorter than the fibronectin condition, with an average of 31 ± 2 μm. Cells grown on PLL had numerous, very short processes (17 ± 1 μm) that branched frequently, very similar to the control condition. Based on these observations, fibronectin was the most appropriate ECM protein to use as surface modifier to maintain the RGC morphology.

These substrates did not affect the proliferation of RGCs, as determined by immunostaining for Ki67. Ki67 is a marker of cells in all active phases of the cell cycle G1, S, G2 and mitosis but not resting phase G0; therefore, Ki67 is an indicator of proliferating cells. RG3.6 cells were grown in log phase on chitosan films adsorbed with different ECM proteins for 5 days and stained for Ki67. Figure 1d shows that the rate of RG3.6 cell proliferation was high in all conditions. Fibronectin- and gelatin-chitosan substrates produced a slightly
higher proliferation rate than the other conditions (92 ± 1% and 93 ± 2%, respectively; Figure 1d). The Ki67 indices of cells grown on the other substrates were: laminin, 89 ± 2%; collagen, 87 ± 4%; PLL 77 ± 5%; and unmodified chitosan 82 ± 1%. However, the differences were not statistically significant using ANOVA.

3.2. Multifunctional scaffold effect on cell proliferation and maintenance of stemness

Each component of the modified chitosan scaffold has specific functions. Heparin was chosen because of its high...
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affinity to bind to FGF-2. Genipin, as a natural crosslinker, keeps heparin in place and firmly attached to chitosan. As a result, FGF-2 is stably tethered to the scaffold (Skop et al., 2013). FGF-2 is an essential growth factor for RGC cells to sustain their survival, proliferation and stemness (Lewis, 1996; Yoon et al., 2004). In culture, FGF-2 loses over 80% of its biological activity within 24 h, making daily addition of FGF-2 an absolute requirement to maintain stemness of RGCs (Rifkin and Moscatelli, 1989a, 1989b; Caldwell et al., 2004). In contrast, removing FGF-2 from the medium induces RGC differentiation. This study evaluated whether the CHG-FN-FGF was capable of sustaining survival and proliferation in the absence of soluble FGF-2. Control cells were grown on chitosan films coated with fibronectin, and soluble FGF-2 was added daily to the medium (C-FN + sFGF). As negative control for growth, cells were seeded on chitosan films modified with genipin–heparin and fibronectin (CHG-FN) lacking FGF-2, and they did not receive FGF-2 in the medium. RGC were seeded on these substrates in media lacking FGF-2 for 3 days, whereupon the cells were photographed and assayed for MTT reduction. As expected, cells grown in the absence of FGF-2 did not proliferate and cell death was observed (Figure 2, CHG-FN) because of the absence of FGF-2 at all times during culture, including seeding. In contrast, the control condition (Figure 2, C-FN + sFGF) promoted survival and proliferation. Surprisingly, the multifunctional scaffold was even more efficient at promoting survival and proliferation (Figure 2, CHG-FN-FGF). In this condition, cells had higher levels of MTT reduction than the control condition where cells received FGF-2 daily in the media (Figure 2b). The higher percentage of MTT reduction could result from greater proliferation or decreased cell death in the CHG-FN-FGF condition. In either case, FGF-2 was more efficient in sustaining survival/proliferation of RGCs when immobilized to the scaffold than when soluble in the media. The same effect of immobilized FGF-2 was obtained using the immortalized RGCs (Skop et al., 2013).

Another desired feature of the multifunctional scaffold is the capacity to maintain neural stem cells in undifferentiated state or stemness. Radial glia are multipotent progenitors that generate neurons, astrocytes and oligodendrocytes. In vitro, RGCs begin to differentiate 24 h after FGF-2 is removed to generate neurons, astrocytes and oligodendrocytes (Figure 3b). Therefore, we aimed to establish whether the multifunctional scaffold maintains the stemness of RGCs. Secondary RGCs were grown on CHG-FN-FGF multifunctional scaffolds for 4 days (Figure 3a) and the expression of stem cell and differentiation markers was compared with differentiated RGCs and proliferative RGCs. Differentiated RGCs were produced by seeding secondary RGCs on PLL–laminin-coated substrate (Diff condition) and withdrawing FGF-2 (Figure 3 A). Proliferative cells were produced by growing RGCs in standard culture conditions namely polyornithine–fibronectin-coated substrates that received soluble FGF-2 daily (Non-Diff condition) (Figure 3a). As expected, cells survived well in all conditions. The expression of stem cell and differentiation markers was evaluated by Western blot analyses (Figure 3c,d). RGCs grown on the CHG-FN-FGF scaffold expressed high levels of the stem cell and progenitor markers BLBP and SOX2 (Figure 3c,d) comparable to RGCs maintained under standard culture conditions (Figure 3c,d), revealing that the

Figure 2. Effect of immobilized fibroblast growth factor-2 (FGF-2) on survival/proliferation of radial glial cells (RGCs). RGCs were passaged twice, then seeded and grown for 4 days in 96-well plates coated with chitosan–fibronectin receiving daily soluble FGF-2 in the media (C-FN + sFGF), chitosan with heparin–genipin, fibronectin and immobilized FGF-2 (CHG-FN-FGF multifunctional scaffold) or chitosan with heparin–genipin and fibronectin lacking FGF-2 (CHG-FN) during the culture. (a) Phase contrast images of RGCs grown for 4 days on the substrates indicated. Scale bar = 100; C, chitosan; H, heparin; G, genipin; FN, fibronectin; FGF, FGF-2. (b) Percentage 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction of RGCs cultured on different substrates. Assay was performed in triplicate in three independent experiments. Values represent mean ± SEM. Statistical significance was determined by ANOVA with Tukey’s post hoc (**p < 0.001 compared with CHG-FN and ***p < 0.001 compared with C-FN + sFGF)
multifunctional scaffold maintained their stemness. Furthermore, the astrocytic marker GFAP was barely expressed by cells grown on the multifunctional scaffold condition compared with the standard condition (Figure 3d). In contrast, differentiated RGCs showed an increase in neuronal markers, βIIIITub and MAP-2 and an increase in GFAP. RGCs grown on the multifunctional scaffold had similar levels of the proliferation marker PCNA (Figure 3c,d) compared with the standard condition confirming the previous result that the multifunctional scaffold maintains the proliferative status. Oligodendrocyte markers were not evaluated as RGC rarely produce oligodendrocytes within 4 days. These data support the conclusion that the multifunctional scaffold promotes the stemness and proliferation of RGCs. In particular, the results indicate that the immobilized FGF-2 was superior to soluble FGF-2 in maintaining their stemness, limiting their differentiation and even delaying the formation of astrocytes.

3.3. Optimization of chitosan microsphere size and cell attachment

The goal of this study has been to manufacture a vehicle to facilitate the delivery of neural precursors into brain injuries with the cells adhered to the surface of the microspheres. A schematic for the vehicle design is presented in Figure 4a. The authors have previously generated microspheres that ranged between 200 μm and 500 μm in diameter using a coaxial airflow method (Skop et al., 2013). Such spheres are too large to pass through the needles used for transplantation (23-gauge).s.
As the optimal sphere size to pass through a 23-gauge Hamilton syringe is less than 100 μm, the size of the microspheres was reduced using the electrospray method, which was designed for this purpose. Using this method, microspheres were formed that ranged between 30 μm and 100 μm with an average diameter of 64 μm (Figure 4b,c). The majority of the spheres fell within the 40–50 μm range as seen in the frequency distribution graph in Figure 4d.

The multifunctionality of the chitosan microspheres was enhanced when modified with genipin–heparin, fibronectin and fibroblast growth factor-2 (FGF-2). Staining the microspheres using Toluidine Blue revealed the presence of bound heparin (Figure 4e). To establish whether RGCs would adhere and grow on these microspheres, a single-cell suspension of RG3.6 cells was mixed with microspheres and incubated for 18 h or up to 10 days. Cells were well attached to the spheres after 18 h (not shown), proliferated and sustained excellent survival/proliferation after 10 days in culture, as observed by phase-contrast microscopy (Figure 4f).

### 3.4. Transplantation

Numerous studies have encapsulated cells inside spheres or other delivery vehicles to enable the cells to produce soluble growth and trophic factors (Maysinger et al., 1996; Zielinski and Aebischer, 1994; Skardelly et al., 2011; Nicodemus and Bryant, 2008). However, the goal of this research has been to manufacture a delivery vehicle with the cells adhered to the surface of the microspheres. This configuration will enable the progeny of the stem cells to migrate off the scaffold into the adjacent tissue, which will be crucial to reconstruct damaged brain tissue. To test our approach, multifunctional microspheres containing RG3.6 cells attached to the surface were transplanted into a neocortical lesion cavity 7 days after a CCI. As the RG3.6 cells express GFP, they could be distinguished from the host cells using fluorescence microscopy. RG3.6 cells are pluripotential and can generate neurons, astrocytes...
and oligodendrocytes when they were differentiated \textit{in vitro} (Figure 5a). At 3 days after transplantation, microspheres could be recognized within the wound cavity adjacent to the host tissue (Figure 5b–d). The RG3.6 cells, were largely still adhered to the microspheres (Figure 5b–d) and many of them were positive for the stem cell/progenitor marker Nestin (Figure 5e–g: magnified boxed insert in b and arrows). Some of the transplanted cells, or their progeny, were seen far from the beads in the adjacent tissue. These data support the conclusion that the cells thrive on the spheres and withstand the mechanical forces of the syringe, while surviving 3 days post-transplantation.

4. Discussion

The brain is arguably the most difficult organ to repair after an injury because of the complexity of the CNS and its limited capacity to regenerate on its own. Neurons do not undergo mitosis and endogenous NSCs are unable to replace the quantity of neurons lost after a typical injury. The transplantation of NSCs has become an effective tool for studying the mechanisms of CNS regeneration. Primary fetal neural cells have shown success upon transplantation in Parkinson disease and Huntington disease, with notable symptomatic relief (Dunnett et al., 2000; Kendall et al., 2000; Bjorklund et al., 2003; Lindvall and Bjorklund, 2004). Their application to spinal cord injury shows their ability to survive, integrate and improve functional outcome while also reducing lesion volume (Hasegawa et al., 2005; Bonner et al., 2011). Published data have suggested that engrafting rat or mouse stem/progenitor cells improves outcome following experimental TBI. Cognitive and sensorimotor recovery has been observed, and in some cases neuronal differentiation has been obtained (Hoane et al., 2004; Shear et al., 2004; Boockvar et al., 2005; Bakshi et al., 2006; Gao et al., 2006). Many studies have shown that transplanted cells can survive in the host brain for up to a year; however, only a small percentage, typically less than 2% of the donor cells, engraft (Harting et al., 2009; Wallenquist et al., 2009). Furthermore, most of the cells that do engraft differentiate into glia, not neurons (Shear et al., 2004; Boockvar et al., 2005; Ma et al., 2011; Sun et al., 2011) The brain also has endogenous NSCs located in distinct regions such as in the dentate gyrus and subventricular zone (Snyder, 1994; Shear et al., 2004; Kulbatski et al., 2005; Longhi et al., 2005). However, these cells are limited in the types of neurons they can produce. More primitive cells, the RGCs that are found in the fetal

Figure 5. Multifunctional microsphere scaffolds for cell replacement therapies after central nervous system injury. (a) Multipotentiality of RG3.6 cells differentiated \textit{in vitro} for 7 days and stained for βIII-tub (beta III-tubulin; red), GFAP (glial fibrillary acidic protein; blue) and GFP (green fluorescent protein). (b–d) Immunofluorescence of brain sections from animals that received transplants of multifunctional microsphere scaffolds with adhered RG3.6 cells 7 days after traumatic brain injury. Sections were stained for GFP (c), nestin (red) (d) and DAPI (4,6-diamidino-2-phenylindole; blue). White arrows illustrate co-localization. Inset was magnified to show co-localization of GFP and nestin (red) in panels e–g. (a) was taken at × 20, (b–d) at × 10 magnifications. Spheres were injected at three depths: 1.5 mm, 1.0 mm and 0.5 m at 1 μl each.

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J Tiss Eng Regen Med (2013)

DOI: 10.1002/term
ventricular zone, have the potential to differentiate into a wider variety of neurons and glia (Reid and Walsh, 2002; Merkle et al., 2007)

One possible reason that the survival rate of transplanted cells is low is that the cystic cavity formed by the injury creates a harsh, non-permissive environment that lacks nutrients, survival factors and, most importantly, a habitable substrate (Tate et al., 2002; Crompton et al., 2007; Tate et al., 2009; Mo et al., 2010). A scaffold would serve as a structural and functional support for the cells. Stem cells, when transplanted within a biomaterial matrix, have shown promise in addressing this issue for various brain injuries. Park et al. (2002) demonstrated greater engraftment using a polymer scaffold in a stroke model (Park et al., 2002). They used a fibrous poly(glycolic acid) scaffold seeded with the C17.2 NSC cell line and showed that these cells could differentiate and reduce the extent of inflammation and glial scarring. Tate et al. (2009) used a collagen gel containing laminin and fibronectin to improve neural precursor cell engraftment and survival after TBI. The study extended up to 8 weeks in which there was a significant difference in outcome when the NPs were seeded within a matrix versus control. Many cells were positive for NG2 and the animals showed improvements in spatial memory tasks (Tate et al., 2002, 2009).

Brain injuries are not uniform in shape or size; therefore a scaffold that is injectable and will mould to the injured tissue will be necessary. Chitosan was selected as the bulk material for scaffolds in this study. The chemical structure of chitosan allows it to be easily modified, making it a very attractive and versatile material. Chitosan has been previously demonstrated as a suitable material for nerve cell affinity (Haipeng et al., 2000). Chitosan’s biocompatibility was also evaluated, as shown in Figure 1. Although the chitosan was not toxic (data not shown), RGCs require adhesive peptides to grow, therefore ECM proteins were incorporated into the scaffold to enhance the efficiency of growth on the scaffold. Proliferative rates were high on all chitosan substrates, but they were noticeably higher when the chitosan was coated with fibronectin or gelatin.

Microspheres comprised of poly(lactic-co-glycolide) (PLGA) have been successfully transplanted into the brain (Nicholas et al., 2002). However, synthetic polymers can degrade into caustic by-products. For example, Kou et al. (1997) showed that PLGA degraded into acidic by-products when transplanted into the brain, exacerbating the inflammation and damage produced by TBI. In contrast, chitosan degrades into non-toxic metabolites, making it appealing for CNS applications. The utility of chitosan has been demonstrated in studies using microcapsules containing the neurotrophic factor NT-3. These neurotrophin-containing microcapsules increased neuronal regeneration of the rat hippocampus, demonstrating that chitosan is safe for in vivo applications (Mo et al., 2010).

In the authors’ studies the chitosan microspheres were modified by the addition of fibronectin to aid cell adherence, proliferation and stemness. Arginine-glycine-aspartic acid (RGD), first identified in fibronectin, is contained within other ECM proteins such as collagen, vitronectin, thrombospondin, von Willebrand factor, fibrinogen, gelatin and some laminins (Ruoslathi and Pierschbacher, 1986; Ruoslathi, 1996). Consistent with a role for the RGD peptide in enhancing the proliferation of the RGCs, RG3.6 cells grown on fibronectin-coated substrates exhibited a higher Ki67 index (Figure 1). Collagen also possesses an RGD (Ruoslathi, 1996) sequence, but the RG3.6 cells did not proliferate as significantly when attached to collagen. This may be because of collagen’s negative charge and the resulting gel-like coating formed when bound to chitosan. Importantly, the RGD sequence binds to the α5β1 integrin receptor whose intracellular amino-terminus influences cellular migration (Hocking et al., 1998), proliferation, self-renewal and differentiation (Campos et al., 2004; Leone et al., 2005). Yoshida et al. (2003) observed that reducing α5β1 expression in cortical progenitors increased their differentiation (Kimura et al., 2003). Cui et al., 2006 applied this concept to an in vivo application using a hyaluronic acid-based hydrogel with immobilized RGD for brain tissue engineering. Transplantations after cortical damage using hyaluronic acid–RGD scaffolds enhanced cell infiltration and angiogenesis into the matrix, while simultaneously inhibiting glial scar formation. An increase in neurite extension was also observed.

The modified chitosan microspheres used in this study were designed to allow FGF-2 to be tethered to the surface of the scaffold, which differs from many studies that have used microspheres to encapsulate growth factors. FGF-2 is a known survival factor for RGCs and maintains them in a primitive state. It has also been shown to increase the numbers of stem/progenitor cells in the SVZ following TBI (Yoshimura et al., 2003; Sun et al., 2009). Soluble FGF-2 has been reported to have a half-life of 24 h at 32°C (Rifkin and Moscatelli, 1989a,1989b) and less than 5 h at 37°C (Shiba et al., 2003), whereas its stability increases when affixed to heparin or heparan sulphate proteoglycans (Sommer and Rifkin, 1989). Caldwell et al. (2004) reported over an 80% reduction in FGF-2 stability when incubated at 37°C for 24 h, whereas less than 20% was degraded with the addition of heparin under the same conditions. There was also an observed increase in striatal precursors grown in culture with the addition of heparin. Interestingly, neither BSA, fetal calf serum, nor other proteoglycans, such as chondroitin sulphate, dermatan sulphate, keratin sulphate and hyaluronic acid, prevented FGF-2 denaturation like heparin at 37°C (Caldwell et al., 2004). By immobilizing FGF-2 to the surface of the chitosan, it is presented to the cells in a more biologically active form (owing to heparin binding).

A key finding of this study, which has implications for the broader field of stem cell research is that cells maintained on the multifunctional film did not need to be fed for at least 3 days after plating, and yet the proportion of proliferating and undifferentiated cells was significantly greater than cells propagated under standard growth conditions. Stem cells normally require feeding on a daily basis; however, with this matrix, the cells can clearly be left untended for at least 3 days. We believe that
stem cell researchers studying multiple types of stem and progenitor cells would benefit from using this platform. Kang et al. (2012) obtained similar results in studies on the effects of a recombinant FGF-2 protein on human adipose stem cells. In their study, FGF-2 was linked to a maltose-binding protein, which was then immobilized to polystyrene dishes. Interestingly, the bound FGF-2 promoted the specification of adipocytes while inhibiting the production of osteoblasts. They also noted that heparin inhibited the cells from binding to the immobilized FGF-2, thus demonstrating the strong interaction that heparin has for FGF-2.

Ideally, while the FGF-2 is present, the cells will begin to proliferate and form processes that extend to the pial surface, mimicking embryonic neurogenesis. Neuroblasts and other progeny would migrate along their processes, ultimately generating neurons appropriate to each cortical layer and supportive glia as the FGF-2 dissipates. Concurrently, the scaffold will degrade over time revealing a regenerated, and ordered cortex. A schematic depicting a method for CNS repair using stem cells delivered upon a multifunctional scaffold after TBI is provided in Figure 6.

5. Conclusion

In this study, multifunctional microspheres using natural biopolymers were optimized for cell replacement therapies for CNS injury. The combination of fibronectin and heparin-immobilized FGF-2 provides neural stem cells with a niche that resembles in many ways the region that they normally inhabit. It has been shown that fetal rat RGCs plated onto this multifunctional film remain in a primitive, multipotent and proliferative state for at least 3 days. The electrospray method can be used to produce microspheres that are small enough to be conveniently injected and that RGCs attached to these multifunctional microspheres can be delivered into the core lesion produced by a focal TBI. When analysed 4 days later,
the transplanted RGCs were positive for the stem cell/progenitor marker Nestin. Together, these results demonstrate that this approach can be used as a cellular and growth factor delivery vehicle to promote the regeneration of nervous tissue after brain injuries. While this application of brain tissue engineering shows promise, more detailed in vivo studies are required to assess survival and differentiation of transplanted neural precursors as well as detailing the extent of anatomical and functional recovery from TBI.

Conflict of interest

The authors have declared that there is no conflict of interest.

Acknowledgements

This study was partly supported by the New Jersey Commission on Brain Injury Research grant number 08.001.BIR2 awarded to CDG and SWL and grant number CBIR12FEL025 awarded to NBS.

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