Injectable alginate hydrogel loaded with GDNF promotes functional recovery in a hemisection model of spinal cord injury

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Abstract

We hypothesized that local delivery of GDNF in spinal cord lesion via an injectable alginate hydrogel gelifying *in situ* would support spinal cord plasticity and functional recovery. The GDNF release from the hydrogel was slowed by GDNF encapsulation in microspheres compared to non-formulated GDNF (free GDNF). When injected in a rat spinal cord hemisection model, more neurofilaments were observed in the lesion when the rats were treated with free GDNF-loaded hydrogels. More growing neurites were detected in the tissues surrounding the lesion when the animals were treated with GDNF microsphere-loaded hydrogels. Intense GFAP (astrocytes), low βIII tubulin (neural cells) and RECA-1 (endothelial cells) stainings were observed for non-treated lesions while GDNF-treated spinal cords presented less GFAP staining and more endothelial and nerve fiber infiltration in the lesion site. The animals treated with free GDNF-loaded hydrogel presented superior functional recovery compared with the animals treated with the GDNF microsphere-loaded hydrogels and non-treated animals.

Keywords: GDNF, microspheres, spinal cord injury, injectable hydrogel, alginate

1 Introduction

Neuroprotection and regeneration facilitated by administration of exogenous neurotrophic growth factors has been considered as a potential treatment for spinal cord injury (SCI) (Wang et al., 2008). Brain-derived neurotrophic factor (BDNF), glial-derived neurotrophic factor (GDNF), neurotrophin 3 and 4/5 (NT3 and NT-4/5), and fibroblast growth factor (FGF) have already been tested for spinal cord regeneration (Awad et al., 2013). GDNF belongs to the transforming growth factor-β superfamily and promotes survival and neurite outgrowth of dopaminergic, motor, peripheral sensory, and sympathetic neurons (Chou et al., 2005). In addition, the upregulation of GFRα1 and c-Ret gene expression, two GDNF receptors, following SCI may further increase the responsiveness of injured neurons and axons to GDNF (Widenfalk et al., 2001). Iannotti et al. demonstrated that long-term GDNF intrathecal infusion elicited neuroprotective effects on contused adult rat spinal cords, resulting in a substantial degree of tissue and axonal sparing (Iannotti et al., 2004). GDNF mRNA expression is highly upregulated 8 hours post-SCI and downregulated from 12 hours to 12 weeks post-SCI (Gerin et al., 2011). Therefore, treatments that promote GDNF-mediated survival should be administered by 12 hours post-SCI and should last up to 12 weeks (Hill et al., 2008). These data suggest that GDNF treatments during the chronic phases, when endogenous GDNF is no longer provided, might be an effective strategy for promoting cell survival (Gerin et al., 2011).

Several strategies have been used to deliver GDNF to the central nervous system, e.g., delivery by an infusion pump (Behrstock et al., 2006), gene therapy (Chou et al., 2005), and genetic engineering of cells so that they release GDNF (Lu et al., 2003). However, these techniques present several disadvantages, such as pump refilling necessity, lack of control of

the duration of the transgene expression and the viral spread outside the target area, reduced rate of cellular survival within the implanted graft, and immune rejection of the grafted cells by the host tissue (Garbayo et al., 2009). An alternative approach is the encapsulation of GDNF in biodegradable and biocompatible polylactide-co-glycolide (PLGA) microspheres that allow controlled, sustained, and localized delivery in addition to protect the GDNF from enzymatic degradation (Garbayo et al., 2009; Wang et al., 2008).

To combine sustained release and GDNF localization at the lesion site, a drug-delivery system composed of GDNF-loaded microspheres dispersed within an alginate hydrogel was selected. Injectable matrices require less invasive surgeries, shortening the surgical operation time and reducing the post-operative loss of function, pain, and scar size (Zhao et al., 2010). Several hydrogels have been investigated for use in spinal cord regeneration (Slaughter et al., 2009), including alginate. Alginate has been widely used for drug delivery and cell encapsulation and as an injectable cell transplantation vehicle due to its biocompatibility, low toxicity, and relatively low cost (Tan et al., 2009). Its solid, non-injectable form has also been used for spinal cord regeneration (Erdogan et al., 2010; Kataoka et al., 2004; Prang et al., 2006; Tobias et al., 2005). When co-injected with calcium into the lesion, alginate can gelify *in situ* and fill the spinal cord lesion (des Rieux, unpublished data).

We hypothesized that the delivery of GDNF from a hydrogel directly injected into a spinal cord lesion could stimulate spinal cord plasticity and functional recovery. The objectives were then to: i) evaluate the influence of GDNF encapsulation on its release from alginate hydrogels; ii) study the influence of GDNF formulation on neurite growth; and iii) evaluate functional recovery in a rat hemisection model of SCI.

2 Materials and methods

2.1 Materials

Human recombinant GDNF was obtained from Invitrogen (Carlsbad, CA, USA). Alginate was purchased from Pronova (Ultrapure MVG alginate, Mw > 200 kDa, produced under GMP guidelines by fermentation from non-animal origin material, FMC BioPolymers, NovaMatrix, Philadelphia, PA). Calcium chloride ($CaCl_2$) was purchased from Sigma-Aldrich (Saint Louis, MO, USA). The used fibrinogen was a component from a Tisseel™ fibrin sealant kit, kindly provided by Baxter Innovations GmbH Inc. (Wien, Austria). A GDNF enzyme-linked immunosorbent assay kit (GDNF Emax® ImmunoAssay System, ELISA) was purchased from Promega (Madison, WI, USA). Polyclonal anti-GDNF antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Polylactic-co-glycolic acid (PLGA) RG 503H (MW 34 kDa) was provided by Boehringer-Ingelheim (Ingelheim, Germany). Dichloromethane, acetone, dimethylsulphoxide, and glycerine were obtained from Panreac Quimica S.A. (Barcelona, Spain). Polyvinyl alcohol (88% hydrolyzed, MW: ~ 125,000) was obtained from Polysciences, Inc. (Warington, USA). Rat pheochromocytoma PC-12 cells were purchased from the American Type Culture Collection (ATCC) (Rockville, MD, USA). The Transwell® inserts were provided by Corning (NY, USA). General laboratory reagents were purchased from Sigma–Aldrich unless otherwise specified.

2.2 GDNF encapsulation in PLGA microspheres by TROMS

Among the methods used to encapsulate GDNF, the Total Recirculation One-Machine System (TROMS) method is a very gentle technique that avoids shear stress, allowing high

encapsulation efficiency and producing very homogeneous batches on a semi-industrial scale (Garbayo et al., 2009).

GDNF-loaded microparticles were prepared with a solvent extraction/evaporation method using the TROMS technology (Garbayo et al., 2009). Briefly, the organic solution containing the polymer was injected through a needle with an inner gauge diameter of 0.25 mm at 38 ml/min into the inner aqueous phase to form the first emulsion. The inner aqueous phase contained 100 µg of GDNF in 10 mM phosphate, 50 mM sodium chloride (PBS), pH 7.9, 5 mg HSA, and 5 µl PEG 200. The primary emulsion (W_1/O) was recirculated through the system and was injected into 20 ml of the external aqueous phase (W_2) composed of 0.5% PVA. The turbulent injection through the needle with an inner gauge diameter of 0.25 mm resulted in the formation of a multiple emulsion ($W_1/O/W_2$), which was further homogenized by circulation through the system for another 30 seconds. The $W_1/O/W_2$ emulsion was stirred at 1000 rpm at room temperature for 4 h to allow solvent evaporation and microsphere formation. Finally, the GDNF-loaded microspheres were washed with ultrapure water by centrifugation and then freeze-dried.

The mean particle size and size distribution of the microspheres were determined with laser diffractometry using a Mastersizer-S® (Malvern Instruments, Malvern, UK). The microspheres were dispersed in ultrapure water and analyzed under continuous stirring. The average particle size was expressed as the volume mean diameter in micrometers (n=3). To determine microparticle protein content 0.5 mg of loaded microparticles were dissolved in 20 µl DMSO (n=3). PBS was added to dilute the sample for western blot analysis. SDS-PAGE was performed onto 12% polyacrylamide gels. Proteins were then transferred onto nitrocellulose membranes. Membrane was blocked during 2h at room temperature with Tris-Buffered Saline 0.05% Tween (TBST) pH 7.4 containing 5% (w/v) of nonfat dried milk. Incubation with 1:2000 rabbit antihuman GDNF antibody (SantaCruz Biotechnology, Santa

Cruz, USA) diluted in blockade solution lasted 2 h at RT. Horseradish peroxidase (HRP)-conjugated donkey anti-rabbit IgG antibody (GE Healthcare-Amersham) diluted 1:2000 in blockade solution,1 h, RT, was performed to detect bounded antibody. Chemiluminescence detection was performed using LumiLight Plus western blotting substrate (Roche Diagnostics, Mannheim, Germany). The GDNF signal was quantified by densitometry using the Quantity One software (Bio-Rad Laboratories, Inc., Munich, Germany). Samples containing defined quantities of GDNF were prepared under the same conditions (PBS and DMSO) and used as standard curve.

2.3 GDNF-loaded hydrogel preparation

A 0.5% (w/v) alginate solution (Pronova UPMVG (medium viscosity, 60% guluronate), FMC BioPolymers, NovaMatrix, Philadelphia, PA) was prepared in MilliQ water, incubated with charcoal for 30 min, and filter sterilized (MillexTM, MA). The endotoxin level of NovaMatrix alginates is guaranteed to be below 100 EU/g.

A 50 mM calcium chloride ($CaCl_2$) solution was prepared in MilliQ water and sterilized by filtration. Alginate hydrogels were formed by the co-injection of 0.5% alginate and 50 mM $CaCl_2$ solutions. Fibrinogen was added to the alginate (5 mg/ml) to improve its biocompatibility. The fibrinogen used was a component of the Tisseel™ fibrin sealant kit, kindly provided by Baxter Innovations GmbH Inc., and was reconstituted with the supplied aprotinin solution at 100 mg/ml. Free GDNF (0.5 µg/µl) or GDNF-loaded microspheres were mixed with the 0.5 % alginate:fibrinogen solution prior to gelification.

2.4 *In vitro* GDNF release profiles

In addition, 2 µg of GDNF, either in solution (free) or encapsulated (microspheres), was incorporated into 300 µl of the alginate:fibrinogen solution, and hydrogels were formed by

the addition of $CaCl_2$ (n=3). The hydrogels were incubated in PBS + 0.01% sodium azide and 0.5% BSA at 37°C for 1 month. The amount of released GDNF was measured using a sandwich ELISA (GDNF Emax® ImmunoAssay System, Promega) and was expressed as a percentage of the incorporated GDNF.

2.5 *In vitro* bioactivity assay of released GDNF

The bioactivity of the GDNF released from the alginate:fibrinogen hydrogel was assessed using a PC-12 neurite outgrowth bioassay (Garbayo et al., 2007). PC-12 cells were plated onto a 12-well culture plate at a low density (2×10^3 cells/cm^2). Then, 24 hours later, free GDNF-loaded alginate:fibrinogen hydrogels (0.5 µg GDNF in 300 µl of hydrogel) were formed in the apical compartment of the 0.45-µm Transwell® inserts that were placed in the 12-well plate containing the cells (n=6), allowing the GDNF to diffuse through the gel and to reach the PC12 cells. Neurite outgrowth was visualized 7 days later under phase-contrast illumination with an Accu-Scope inverted microscope connected to a Micrometrics digital camera. PC-12 cells incubated with 50 ng/ml of recombinant NGF were used as a positive control.

2.6 GDNF-loaded hydrogel injection into a rat spinal cord hemisection model

The animal experiments were approved by the ethical committee for animal care of the health science sector of Université catholique de Louvain. Female Long Evans rats (Janvier, Saint Berthevin, France) were anesthetized using a rodent anesthesia system (Equipement Veterinaire Minerve, Esternay, FR) with vaporized isoflurane (Isoba, Schering-Plough Animal Health, Merck Animal Health, Boxmeer, NL) prior to a laminectomy at T9-10 and exposure of the spinal cord. A lateral hemisection resulting in a gap of 4 mm along the rostro-

caudal axis and extending up to the midline was created with a microscalpel in the left side of the spinal cord (De Laporte et al., 2009a; De Laporte et al., 2009b). Hydrogel (10 µl) was formed by the co-injection in the injury of an alginate solution and a $CaCl_2$ solution with a double syringe system (30G insulin syringes), and gelation occurred within 5 min. No gel was injected into the untreated operated animals (negative control). Then, paravertebral muscles were sutured on the midline, and the skin was stapled. Post-operative care included the subcutaneous administration of Baytril (enrofloxacin, 2.5 mg/kg s.c., once per day for 2 weeks), buprenorphine (0.01 mg/kg s.c., twice per day for 3 days), and lactated Ringer's solution (5 mL/100 g, once per day for 5 days). In addition, the bladder was expressed twice per day until bladder function recovered. To study the influence of GDNF formulation, and thus GDNF release kinetics, on the damaged spinal tissues, free GDNF and GDNF-loaded microspheres were incorporated in alginate:fibrinogen hydrogels and injected into the rat spinal cord hemisection model (n=12). Non-treated operated animals were used as negative controls. For the experiments, 10 µl of the alginate:fibrinogen hydrogel was loaded with 2 µg of GDNF and injected.

2.7 Immunohistochemistry and immunofluorescence analyses of spinal cords

At 6 weeks and 3 months post-implantation, the rats were transcardially perfused with 4% phosphate-buffered formaldehyde to fix the tissues.

Immunohistochemistry

The injury site was embedded in paraffin and sliced transversally into 12-µm-thick sections. Every section was collected and processed for immunohistochemical analysis. Primary antibodies against neurofilaments (mouse anti-pan-neurofilament, 1/1000, Covance, Emeryville, CA) and growth-associated protein (mouse anti-GAP43, 1/10000, Millipore, Temecula, CA) were used in combination with a secondary immunoperoxidase stain

(biotinylated anti-mouse IgG (Vector Laboratories, Burlingame, CA, 1:200). The staining was performed with an ABC Elite kit (Vector Laboratories) and DAB (Sigma). Hematoxylin was used as a counterstain. Negative controls were generated by omitting the primary antibodies. Image acquisition was performed using a MIRAX (Zeiss, Zaventem, BE) slide scanner, allowing the acquisition of entire sections. The level of staining (4-6 sections/rat, n=4 per group) was quantified using Frida software (The Johns Hopkins University). Neurofilament staining was quantified within the lesion (zone delimited on morphological observations), while GAP43 (growing neurites) was quantified in a spinal cord portion centered on the lesion by an investigator blinded to the treatment. This latter spinal cord portion extended from 3250 μm rostral to 3250 μm caudal of the lesion and for the entire width of the section.

Immunofluorescence

The hemisected spinal cord was stained using the whole mount staining method (WMS) as previously described (Kardon, 1998). WMS involves the staining of small pieces of tissue without sectioning onto slides first. The spinal cords were cut transversally in two equal parts and were stained for endothelial cells (mouse anti-RECA IgG1, 1/75, AbD Serotech, Oxford, UK), neuronal cells (mouse anti-βIII tubulin IgG2a, 1/500, Covance), and astrocytes (rabbit anti-glial fibrillary acidic protein [GFAP], 1/200, Abcam, Cambridge, UK). Alexa® Fluor 647 goat anti-mouse IgG1 (γ1), Alexa® Fluor 488 goat anti-mouse IgG2a (γ2a), and Alexa® Fluor 594 goat anti-rabbit IgG (H+L) (Life Technologies, Eugene, OR, USA) secondary antibodies were used for the detection of endothelial cells, βIII tubulin, and GFAP, respectively. Once the immunofluorescence was completed, the tissues were vitrified. Each half was imaged (300-500 μm stacks) with a Cell Observer Spinning Disk (Zeiss). The image stacks were merged using AxioVision (Zeiss), and the adjacent zones were assembled using Adobe Photoshop.

2.8 Assessment of the functional recovery of the rats using Catwalk™

The functional recovery of the rats was evaluated using the Catwalk™ test (Catwalk 7, Noldus, Wageningen, The Netherlands). The paw angle and base of support (BOS) of the hind paws and the intensity of left hind paw (injured side) were studied. Paw print recording allowed analysis of various aspects of walking steps, such as the BOS, the intensity of the paw prints, the duration of contact with the floor (stance phase duration), and the sequence regularity of steps. Paw angle is an estimate of the angle in degrees of the paw axis relative to the horizontal plane. The BOS is the average width between the hind or front paws. The 'intensity' parameter refers to the intensity of the paw prints that is detected by the system during gait and is related to weight support by the paws. Up to 5 runs were recorded per animal, and analysis was performed on the fastest uninterrupted run (<2s) by blinded experimenters (Mignion et al., 2013). The rats were trained 2 weeks before surgery, and the runs were recorded just before the surgery; 9 and 14 days; and 1, 2, and 3 months following surgery. The BOS, paw angle of hind paws, and intensity of the left hind paw (the side affected by the surgery) were considered. Five rats per group were used in these behavioral experiments.

2.9 Statistical analysis

Statistical analyses were performed using PRISM (GraphPad Software, CA, USA). One-way or two-way ANOVA with post hoc Bonferroni multiple comparison tests were performed; p-values between 0.05 and 0.001 were considered significant (*** $p<0.001$, ** $p<0.01$, * $p<0.05$). Error bars represent the standard error of the mean in all figures except for Figure 1, where they represent the standard deviation.

3 Results

3.1 Formulation of alginate hydrogel and GDNF-loaded PLGA microspheres

For this study, 0.5% MVG alginate, supplemented with fibrinogen (5 mg/ml v/v), was selected based on a previous study (des Rieux, unpublished data). It was well tolerated by cells and spinal cord tissues and allowed the sustained release of VEGF.

GDNF was encapsulated in PLGA-based microspheres using the TROMS technique with a 88% encapsulation efficiency and a loading of 1.75 µg/mg of microspheres. The mean microsphere diameter was 12.3 ± 2.78 µm.

3.2 Influence of GDNF formulation on its release from alginate:fibrinogen hydrogel

The *in vitro* release of GDNF, either in its free form or encapsulated in PLGA microspheres, was measured. Different release profiles were observed for the free GDNF and GDNF-loaded microspheres incorporated into the alginate:fibrinogen hydrogel (Figure 1). GDNF release from microspheres was 2.5-fold slower than free GDNF release (28% versus 70%, respectively). A total of 25% of the GDNF encapsulated in microspheres was released within 14 days, and the release then reached a plateau (less than 0.3%/day). Free GDNF was rapidly released the first 4 days (30%, 7.5% per day), and the release then slowed down progressively to 2.3%/day over the 12 following days and to 0.93%/days over the remaining 2 weeks. A small burst release was observed for the two formulations (6.7 % and 4.3 % after 4 hours for free GDNF- and GDNF microsphere-loaded hydrogels, respectively). Release of free GDNF from the hydrogel showed a good fit to the Higuchi model (R^2=0.9674) (Figure 1, insert) (Gunduz et al., 2013). This indicates that free GDNF release from the hydrogel occurred mainly by diffusion. GDNF release from microspheres incorporated in the hydrogel was first

fitting the Higuchi model, followed by a very slow release. GDNF was probably first released by desorption from microsphere surface and then by microsphere degradation.

3.3 Bioactivity of GDNF released from the alginate:fibrinogen hydrogel

The bioactivity of the GDNF released from the alginate:fibrinogen hydrogel was assessed by assaying its capacity to induce PC12 cell differentiation into a neuronal phenotype characterized by the presence of neurites. The bioactivity of the GDNF released from microspheres has been previously demonstrated (Garbayo et al., 2009). Here, the objective was to study the influence of GDNF incorporation in alginate:fibrinogen hydrogel on its bioactivity once released from the hydrogel.

Whereas no neurites were observed for the cells cultured with the alginate:fibrinogen hydrogel alone, PC12 cells cultured with DMEM supplemented with NGF or with GDNF-loaded hydrogels developed neurites (Figure 2). This assay demonstrated the bioactivity of GDNF after its release into the culture medium.

3.4 Influence of GDNF release profiles on neurite outgrowth

The hemisected spinal cords of rats were injected with either free GDNF-loaded hydrogels or GDNF microsphere-loaded hydrogels, and non-treated operated animals were used as controls. The presence of neurites in the lesion and of neurite growth around the lesion site was evaluated at 6 weeks and 3 months post-injection. Neurofilament staining highlighted neurites, whereas GAP43 staining revealed growing neurites.

At 6 weeks and 3 months post-injection, residual hydrogel could be observed in the lesion site (Figure 3). For non-treated operated animals, the tissue filling the defect was largely composed of fibrous scarring, whereas the tissue that was formed in the lesion site treated with GDNF-loaded hydrogels was denser and more homogenous.

Hemisections filled with free GDNF-loaded hydrogel displayed more neurofilament staining at 6 weeks (2-fold more compared with non-treated ($p<0.01$) and GDNF microsphere-treated animals ($p<0.05$)) (Figure 3A and 4a). After 3 months, more neurofilaments were observed for rats injected with free GDNF (2.2-fold, $p<0.01$) and GDNF microspheres (1.8-fold more, $p<0.1$) compared with controls (Figure 3A and 4b). Neurofilament staining decreased between 6 weeks and 3 months for the non-treated and free GDNF-treated rats (1.6-fold less) but did not decrease for GDNF microsphere-treated rats (0.56% at 6 weeks and 0.6% at 3 months).

At 6 weeks, 3 to 4-times more GAP43 immunoreactivity was detected in GDNF microsphere-loaded hydrogels compared with free GDNF-treated ($p<0.05$) and non-treated rats ($p<0.01$) (Figure 3C and 4c). The percentage of GAP43 positive staining decreased over time, slightly more for the non-treated (1.85-fold less) than for the GDNF-treated animals (1.3-folds less). At 3 months, the GAP43 staining was still more important for GDNF microsphere-loaded hydrogels than for free GDNF-treated and non-treated rats ($p<0.05$) (Figure 3D and 4d). Growing neurites were mainly observed at the lesion periphery and surrounding tissue but not directly within the lesion (Figure 4D).

WMS was applied for the first time to adult spinal cord tissues. To a certain extent, visualization was allowed to assess the influence of GDNF treatment on tissue organization. By performing immunofluorescence staining of entire tissues, this technique provides a more accurate idea of the tissue organization. Intense GFAP (astrocytes, red), low βIII tubulin (neuronal cells, green), and RECA-1 (endothelial cells, white) stainings were observed for non-treated lesions (Figure 5a). Cavities, bordered by astrocytes, could also be seen. The GDNF-treated spinal cords presented less GFAP staining and more endothelial and nerve fiber infiltration in the lesion site (Figure 5b and 5c). Some hydrogel remains were detectable, filling part of the hemisection in which neurites, endothelial cells and blood vessels were

detected. Some level of organization was also visible, particularly in the spinal cords injected with GDNF microsphere-loaded hydrogels (Figure 5c).

3.5 Influence of GDNF delivery on the gait of spinal cord injured rats

The gait of each rat was followed over time using the Catwalk™ system to assess the effect of GDNF delivery on functional recovery.

The hind paw angle of both the non-treated and GDNF microsphere-treated rats progressively increased from 12° to 50-70° at 3 months, whereas the free GDNF-treated rat value remained close to the pre-op value (19° after 3 months versus 14°) ($p<0.05$) (Figure 6a). Positive angle values indicate an outward position of the paw, relative to the horizontal plane, during stance. Previously, it was reported that thoracic spinal cord injury does not affect the BOS of the front paws but leads to an increase in the BOS of the hind paws (Deumens et al., 2013; Hamers et al., 2001). In our study, we observed the opposite: lower BOS values and higher paw angles reflected the position of the hind paws of injured rats with knees close to each other and paws facing outwards. The hind paw BOS of all groups decreased post-surgery. After one month, the BOS values of the hind paws of the free GDNF-treated animals were closer to pre-op values, whereas the BOS of non-treated and GDNF microsphere-treated rats were 2-fold lower ($p<0.05$) (Figure 6b). At two months post-surgery, there was no significant difference between the values according to GDNF formulation, and both BOS values were significantly higher than the values observed in non-treated rats (3.6-fold higher, $p<0.05$). At three months, even though the same general tendencies were observed, the differences were no longer significant.

At nine days post-surgery, a decrease of the ipsilateral hind paw (left side) intensity, reflecting loss of weight support, was observed for all the conditions (Figure 6c). However, the left paw of the free GDNF-treated rats displayed a 3.6-fold higher intensity than the GDNF

microsphere-treated and the non-treated operated animals ($p<0.05$). Over time, a steady increase of the ipsilateral hind paw intensity for free GDNF-treated rats was recorded, whereas the same parameter was more than 3-fold lower for the GDNF microsphere-loaded hydrogel treated rats and non-treated operated animals ($p<0.001$).

4 Discussion

The expression levels of some growth factors, such as GDNF, are highly upregulated during the initial hours post-SCI and then quickly downregulated during the following days (Gerin et al., 2011). In this report, we incorporated GDNF, either free or encapsulated in microspheres, in an injectable alginate:fibrinogen hydrogel, and we evaluated the influence of GDNF-controlled delivery on hemisected spinal cord neurite outgrowth and functional recovery.

GDNF controlled release was achieved both when the GDNF was incorporated free and when it was incorporated in microspheres in the hydrogel. As expected, faster release was observed when the free GDNF was incorporated into hydrogels as compared with the microspheres. The GDNF release kinetics and the burst release were much slower when the microspheres were incorporated into the alginate:fibrinogen hydrogel than when they were directly incubated in PBS (Garbayo et al., 2009). This difference can be explained by the hindered diffusion of GDNF across the PLGA-hydrogel boundary (Stanwick et al., 2012), by interactions between the alginate and GDNF, or by slower microsphere degradation. Most likely, a combination of these factors was involved. An assay performed on PC-12 cells demonstrated the preserved activity of released GDNF. Given that the GDNF incorporation in the alginate:fibrinogen hydrogel occurred during a very mild gelation process, the preservation of GDNF activity was expected.

When injected into a rat spinal cord hemisection model, the GDNF-loaded hydrogels elicited a different tissue response than observed for the non-treated operated animals. More homogenous and denser tissues were observed in the hemisections. The tendency to see differences in tissue organization at the lesion site in treated animals may be due to the hydrogel itself or could be simply a result of filling the initial tissue void (Cholas et al., 2012).

Hemisections filled with free GDNF-loaded hydrogel displayed more neurofilament staining than GDNF microsphere-filled lesions or non-treated operated animals. Neurofilament staining decreased over time for the non-treated and free GDNF-treated rats but did not decrease for the GDNF microsphere-treated rats. GDNF is one of several potent neurotrophic factors that regulate many critical behaviors of neurons, including neurite branching and neuroprotection (Cholas et al., 2012) (des Rieux et al., unpublished data). The greater neurofilament staining observed at 6 weeks in the hydrogel-filled lesions of free GDNF-treated rats compared with the GDNF microsphere-treated animals might have been due to a rapid release of enough GDNF to stimulate neurite growth. GDNF mRNA expression is highly upregulated by 8 hours post-injury (a 6-fold increase compared with control) and is 4-fold downregulated compared with the control from 12 hours to 12 weeks post-injury (Gerin et al., 2011). In addition, when exogenous GDNF is applied immediately or within 30 minutes over the traumatized area, it significantly enhances cell viability and survival (Sharma, 2006). These results suggest that the injured spinal cord needs to be stimulated by an exogenous supply of GDNF to compensate for the loss of endogenous expression. Therefore, early GDNF delivery followed by a sustained release lasting up to 12 weeks or until behavioral changes are observed should promote cell survival (Hill et al., 2008). The GDNF fast release kinetics could explain why neurofilament staining observed 3 months post-injury in the free GDNF treated rats was lower than at 6 weeks. However, neurofilament staining in the GDNF microsphere-treated rats did not diminish and was close to being

significantly higher than the staining in the controls (significant for α=0.1). This may be related to the low but sustained GDNF release from microsphere-loaded hydrogel (approximately 6 ng/day).

Growing neurites were mainly observed at the lesion periphery and in the surrounding tissue but not directly within the lesion. The highest GAP43 immunoreactivity was observed for rats treated with GDNF microsphere-loaded hydrogels. Several studies have shown that GDNF administration following SCI significantly increases both the number of myelinated axons and the total number of regenerating axons (Iwase et al., 2005; Zhang et al., 2009). Another study reported an increased number of axons regrowing into Schwann cell grafts when GDNF treatment was provided and attributed this response either to a higher number of neurons that regrew their axons or to branching of existing axons (Zhang et al., 2009). The greater number of growing neurites detected in the periphery of the lesion in GDNF microsphere-treated rats in our study could be due to a prolonged action of GDNF over time, even after hydrogel degradation. Indeed, GDNF encapsulation in microspheres confers protection and sustained release of GDNF, whether microspheres are incorporated into a hydrogel or released in the surrounding tissues. As the hydrogel slowly degrades, the microspheres detach and migrate/diffuse through the tissue from their injection site and continue to deliver GDNF, whereas free GDNF is released and quickly degraded. Thus, we hypothesize that free GDNF is released within and close to the lesion site and has to act quickly to be efficient in the immediate vicinity and provide a local effect. However, GDNF microspheres should act for longer periods, increasing the GDNF half-life and stimulating neurite growth around the lesion by diffusion of GDNF into surrounding tissues. In addition, GDNF-treated spinal cords displayed apparently less GFAP staining and more endothelial and nerve fiber infiltration in the lesion site. Some level of organization was also visible, particularly in the spinal cords injected with GDNF microsphere-loaded hydrogels. Deng et

al. reported that GDNF treatment significantly reduced the production of GFAP and chondroitin sulfate proteoglycans (CSPGs) in both *in vitro* and *in vivo* models (Deng et al., 2011). Regarding tissue organization, the hydrogel itself would most likely provide a mechanical support for axonal and blood vessel growth in addition to delivering growth factors. The main drawback of the hydrogel is the lack of directionality.

The last part of our study was dedicated to the evaluation of the impact of GDNF delivery on functional recovery of treated animals. Free GDNF-loaded hydrogel-treated animals showed improved functional outcome after SCI compared to non-operated animals and even compared to GDNF microsphere-loaded hydrogels. Significant improvements were observed for paw angle of hind paws and left hind paws intensity parameters. These data suggest that an early sustained GDNF delivery from an alginate hydrogel, as obtained by treatment with free-GDNF hydrogels, is associated with better functional outcome than a slower GDNF release that is more sustained. As free-GDNF was also associated with the highest degree of neurofilament staining, functional outcomes in our investigation were better with early increases in neurofilament staining within the lesion site. Despite a higher degree of GAP43 staining and an increase of neurofilament staining over the course of three months, GDNF microsphere-treated rats did not show significant functional improvements. As such, it may be speculated that an improved functional outcome required early sustained GDNF delivery from an alginate hydrogel that may have induced an important degree of neurofilament survival or early growth in and around the lesion site. However, it would be over-simplistic to draw a direct line between neuroprotection and functional recovery, particularly as neurofilament immunoreactivity in the lesion site significantly decreased 3 months post-surgery, whereas the Catwalk™ parameters were still significantly superior for the free GDNF treatment compared with the GDNF microspheres. In addition, the neurite growth that was observed within the lesions is encouraging but represents only the very first

step in the spinal cord healing process. Additional processes that limit spinal cord regeneration, and ultimately functional recovery, need to be addressed. These include the reestablishment of functional connections between the rostral and caudal sides of the lesion. Other parameters might also influence the functional outcome, such as neuropathic pain. Indeed, the intensity parameter of the Catwalk™ has previously been correlated with neuropathic pain behavior such as mechanical allodynia (Vrinten and Hamers, 2003). GDNF has potent analgesic effects (Boucher et al., 2000) and it might, therefore, be possible that early delivery of a high enough dose of GDNF would also reduce neuropathic pain in our investigation, thereby improving the rat walking pattern. The pain-reducing effect of GDNF has also been reported in an animal model of spinal cord injury. A mitigation of allodynia was noted when spinally injured mice received a transplant of GDNF producing-embryonic neural stem cell (NSC) as compared to NSC alone; this difference correlated with the level of CGRP/GAP43 immuno-reactivity and sprouting observed in the cervicothoracic dorsal horns (Macias et al., 2006). Both allodynia- and CGRP/GAP43-positive afferent sprouting were lower in the GDNF-transfected group compared with the NSC alone. Non-directed axonal sprouting might also cause (Berger et al., 2011). Kalous et al. provided evidence that the anatomical reorganization of sensory and nociceptive dorsal horn circuits rostral to an injury could factor in the development or maintenance of SCI pain (Kalous et al., 2009). The effect of GDNF on CGRP/GAP43 immunoreactivity might, thus, be both protective and analgesic on SCI-evoked neuropathic pain (Macias et al., 2006).

5 Conclusion

Our hypothesis was that GDNF-loaded injectable hydrogels would stimulate neurite growth and functional recovery after SCI. We demonstrated that local GDNF delivery from alginate:fibrinogen hydrogel gelifying *in situ* significantly increased the ingrowth of neurites

in and around the site of spinal cord hemisection. Free GDNF delivery elicited more neurofilament staining at the lesion, whereas GDNF microspheres induced more neurite growth around the lesion. The animals treated with free GDNF-loaded hydrogel experienced superior functional recovery compared with the animals treated with GDNF microsphere-loaded hydrogels and non-treated animals. We hypothesize that these results could be linked to different GDNF release profiles that influence GDNF actions on damaged spinal cord.

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