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Research paper

Effect of GDNF-releasing biodegradable microspheres on the function and the survival of intrastriatal fetal ventral mesencephalic cell grafts

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Abstract

The transplantation of fetal ventral mesencephalic (FVM) cell suspensions into the brain striatal system is an alternative approach for the treatment of Parkinson's disease (PD). However, one objection to this procedure is the relatively poor survival of implanted cells. Attempts have been made to improve the survival of grafted dopaminergic neurons using glial cell line-derived neurotrophic factor (GDNF). Nevertheless, the clinical application of GDNF is limited, due to the difficulties in administering a protein to the brain tissue and due to the ubiquity of its receptor, thus leading to neurological side effects. A strategy to deliver GDNF in the brain based on the intracerebral implantation of biodegradable poly(D,L-lactic acid-co-glycolic acid) sustained release microspheres has been developed. Such microparticles can be easily implanted by sterotaxy in precise and functional areas of the brain without causing damage to the surrounding tissue. Moreover, the release profile of the GDNF-loaded microspheres showed a sustained release over 56 days of biologically active GDNF at clinically relevant doses. The present study shows that the implantation of GDNF-loaded microspheres at a distance to the site of FVM cells in the 6-hydroxydopamine-lesioned rat model of PD improves dopaminergic graft survival and function. Furthermore, the unloaded and the GDNF-loaded microspheres, when they are mixed with FVM cells, may provide a mechanical support and a 3D environment inducing differentiation and increased function of dopaminergic neurons. Taken together, these results show that GDNF microspheres represent an efficient delivery system for cell transplantation studies.

Keywords: Parkinson's disease; Cell therapy; GDNF; Transplantation; Microspheres; Poly(lactic acid-co-glycolic acid)

1. Introduction

Parkinson's disease (PD) is a progressive neurodegenerative disorder, characterized by the loss of nigrostriatal dopaminergic neurons [1]. Transplantation of fetal ventral mesencephalic (FVM) tissue has been explored as a therapeutic approach for PD patients [2,3]. However, cell survival in brain tissue grafts is poor, with survival rates of only 5–15% of grafted dopaminergic neurons two weeks after grafting [4–7]. Attempts have been made to improve the survival of grafted dopaminergic neurons using neurotrophic factors, antioxidants, and antiapoptotic agents [8].

Among the different neurotrophic factors, glial cell line-derived neurotrophic factor (GDNF), a member of the transforming growth factor-β superfamily, exerts a potent and specific trophic effect on dopaminergic neurons in vitro [9] and in vivo [10–16]. As GDNF does not readily cross

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the blood–brain barrier and as its diffusion from the cerebrospinal fluid into the brain parenchyma is limited, GDNF must be delivered within the central nervous system itself, close to its target [17]. Several studies have shown that GDNF, delivered in the vicinity of FVM grafts, improves survival, growth, and function of fetal dopaminergic cells grafted in the 6-hydroxydopamine (6-OHDA)-lesioned rat model of PD [18–26].

To date, in clinical studies, direct intraparenchymal delivery of GDNF is achieved by pumps [27–29]. This approach is well tolerated and has a good safety profile. However, it is hampered by problems such as requirement of catheter repositioning, poor protein stability in the delivery reservoir fluid, and accumulation of GDNF near the site of delivery limiting its diffusion in the parenchyma [27]. Recent efforts have concentrated on the transplantation of cells genetically modified in vitro to express GDNF and the direct delivery of the GDNF gene using recombinant viral vectors [17,30]. These strategies allow a prolonged and intraparenchymal delivery of the protein, but the safety of these methods and the long-term expression of the transgene still need to be addressed. Moreover, the delivered doses, that depend on cell survival and the stability of the transgene, could be difficult to control in a precise manner. These limitations have prompted to develop another GDNF delivery approach, based on the intracerebral implantation of poly(D,L-lactic acid-co-glycolic acid) (PLGA) microspheres [31,32]. PLGA microspheres can be implanted by stereotaxy, are biocompatible and totally biodegradable in the brain [33,34]. Moreover, they can release bioactive neurotrophic factors in a controlled and sustained manner [35–38]. Recently, GDNF-loaded microspheres have been characterized [39,40] and their striatal implantation promotes recovery of motor function in a partial model of PD [12,13,41].

The aim of this work was to investigate whether GDNF-loaded microspheres can improve the survival and function of FVM cells in the 6-OHDA-lesioned striatum. In order to study the effect of a 3D environment and to further simplify the implantation procedure for a future clinical application, microspheres were implanted together with FVM cells at a single site in the lesioned striatum. Furthermore, with this approach, GDNF is more accessible to grafted cells. The effectiveness of this implantation protocol was compared to microspheres implanted at a distance to the site of the FVM cells.

2. Materials and methods

2.1. Materials

Uncapped (free carboxylic acid group at the terminal end) PLGA, 37.5/25, was obtained from Phusis (Saint Ismier, France). The composition of the chains was 37.5% D-lactic units, 37.5% L-lactic units, and 25% glycolic units. PLGA molecular weight was 30,000 Da ($I = 2$) as determined by size exclusion chromatography. Recombinant human GDNF and enzyme-linked immunosorbent assay (ELISA) kits were purchased from R&D Systems (Abingdon, UK). ^{125}I-labeled GDNF was produced by a chemical method using Bolton Hunter reagent (Perkin Elmer, Paris, France) as described in a previous work [39]. Polyethylene glycol (PEG 400) was purchased from Cooper (Melun, France) and poly(vinyl alcohol) (PVA; 88% hydrolyzed, Rhodoviol® 4/125) was from Merck Eurolab (Paris, France). Human serum albumin (HSA), bovine serum albumin (BSA), D-amphetamine, polyornithine, insulin, transferrin, sodium selenite, putrescin, progesterone, 6-OHDA, paraformaldehyde, and diaminobenzidine (DAB) were supplied from Sigma–Aldrich (Saint Quentin Fallavier, France). Dulbecco's modified Eagle's medium (DMEM), Ham's F-12 medium, and fetal calf serum (FCS) were purchased from Cambrex (Verviers, Belgium). Antibodies to tyrosine hydroxylase (TH; dopaminergic neuron marker), glial fibrillary acidic protein (GFAP; astrocyte marker), and cluster of differentiation 11b (CD11b; clone OX-42; microglial cell/macrophage marker) were obtained from Jacques Boy (Reims, France), DakoCytomation (Trappes, France), and Serotec (Cergy Saint-Christophe, France), respectively.

2.2. Microsphere formulation and characterization

PLGA microspheres were prepared by a w/o/w emulsion solvent extraction–evaporation process as previously described [39,42]. Briefly, a 60 μl internal aqueous phase (16 mM citrate buffer, pH 5.0, containing 5% w/w HSA with respect to the amount of PLGA), 75 μg rhGDNF (0.14% w/w with respect to the amount of PLGA), and a fraction of ^{125}I-labeled GDNF were mixed with 90 μl PEG 400 and then emulsified in 2 ml of an organic phase (3:1 methylene chloride/acetone) containing 50 mg PLGA. This w/o emulsion was subsequently poured into an external aqueous solution of PVA (30 ml, 5% w/v) containing NaCl (10% w/v) and mechanically stirred at 500 rpm for 1 min (Heidolph RGH 500, Merck Euroloab, Paris, France). The resulting w/o/w emulsion was added to deionized water (400 ml) containing NaCl (10% w/v) and magnetically stirred for a further 25 min to extract the organic solvent. Finally, the formed microparticles were filtered, washed, and then freeze-dried. The resulting microspheres were characterized, notably by the volume average diameter and size distribution, using a Coulter® Multisizer (Coultronics, Margency, France). Unloaded microspheres were prepared in the same way, except that GDNF was omitted from the procedure.

The protein encapsulation yield was quantified by measuring the radioactivity (cpm) of microspheres with a gamma counter Cobra® II Packard Instrument (Paris, France) as published elsewhere [39]. In vitro release of GDNF from 20 mg [^{125}I]GDNF PLGA microspheres ($n = 3$) was determined using a continuous flow system. For the duration of the study, eluent (citrate buffer 10 mM + 0.1% BSA + 150 mM NaCl + 0.02% sodium azide, pH 5.0) was

supplied to the chamber inlet at 5 μl/min. Eluent containing the released protein was collected in fractions over 24 and 48 h intervals, and subsequently frozen (−20 °C) until further required. The released GDNF was quantified by measuring the radioactivity and by ELISA.

To confirm the biological activity of the released GDNF, a bioassay was also performed. Cultures of dopaminergic neurons were prepared from the ventral mesencephalon of E14 rat embryos. After dissection, the tissue was mechanically dissociated in culture medium. The cell suspension was plated at a density of 2×10^5 cells onto 14 mm diameter dishes, precoated with polyornithine (50 μg/ml) in DMEM/Ham's F-12 medium supplemented with 10% FCS. Cultures were maintained in a humidified 5% CO_2/95% air atmosphere at 37 °C. After 24 h, the medium was replaced with a serum-free supplemented medium consisting of DMEM/Ham's F-12 with 25 μg/ml insulin, 100 μg/ml transferrin, 5.2 pg/ml sodium selenite, 9.6 μg/ml putrescin, and 6.29 pg/ml progesterone. Collected fractions containing the released GDNF at different timepoints and diluted to 1 ng/ml based on the ELISA results, or a standard GDNF solution at 1 ng/ml (positive control), were added to this medium. After 11 days in vitro, cells were fixed in 4% paraformaldehyde and then dopaminergic cells were identified with antibodies anti-TH. The number of surviving dopaminergic cells in a culture dish was counted by microscopic analysis, and the size and the number of neurites of each neuron was calculated using an image analysis software (ImageJ, NIH).

2.3. 6-OHDA lesions

Sprague–Dawley rats, 220–250 g in weight, were anesthetized and positioned in a Kopf stereotaxic instrument. A unilateral lesion was performed with two stereotactic injections of 6-OHDA hydrochloride (4.0 mg/ml dissolved in 0.9% saline with 1 mg/ml ascorbic acid, pH 4.3) into the right medial forebrain bundle [Coordinates: posterior to bregma (AP), −4.4 mm; lateral (L), −1.3 mm; ventral from dura (V), −7.8 mm; toothbar (TB) set at −2.3 mm (2.5 μl), and AP, −4.0 mm; L, −1 mm; V, −8.0 mm; TB set at +3.4 mm (2.0 μl)].

2.4. Behavioral assay

In order to assess the extent of dopamine depletion in the striatum ipsilateral to the lesion, the rats were submitted to amphetamine-induced rotations in an automated rotometer bowl. D-Amphetamine (5 mg/kg) was administered intraperitoneally. Left and right full-body turns were monitored over a 90-min period. Only rats with a rotational asymmetry of >6 net ipsilateral turns per minute 2 and 3 weeks after the lesion were used for the subsequent experiments. Amphetamine-induced rotations were quantified in the same manner, 2 and 3 weeks after intracranial grafting, to evaluate the functional recovery.

2.5. Grafting and injection of microspheres

Tissue pieces of FVM were dissected as described above and the cell suspension was prepared in the same manner. The microspheres were suspended in a dispersing medium (DMEM, Tween 80 0.2% w/w, CMC 0.25% w/w, and mannitol 0.8% w/w). Three weeks after the lesion, rats were divided into two groups:

- group I (FVM cells separated from microspheres) first received 0.5×10^6 FVM cells/5 μl in DMEM (AP, +1.0 mm; L, −2.5 mm; V, −5.0 mm; TB set at −3.3 mm) and subsequently 0.4 mg/5 μl unloaded or GDNF-loaded microspheres in dispersing medium 1.3 mm posterior and 0.5 mm laterally to the FVM cells (AP, −0.3 mm; L, −3.0 mm; V, −5.0 mm; TB set at −3.3 mm);
- group II (FVM cells together with microspheres) received 0.4 mg/5 μl unloaded or GDNF-loaded microspheres in dispersing medium mixed just before injection with 0.5×10^6 FVM cells/5 μl in DMEM at the following coordinates (AP, +0.5 mm; L, −2.8 mm; V, −5.0 mm; TB set at −3.3 mm).

For each group, control animals received sham injections of the appropriate solution, either DMEM or dispersing medium at the same coordinates.

2.6. Immunohistochemistry

Three weeks after grafting, rats were anesthetized and perfused through the heart with 150 ml of ice-cold 0.9% saline, followed by 300 ml of ice-cold 4% paraformaldehyde in phosphate-buffered saline (PBS), pH 7.4. Coronal sections were cut at 30 μm and collected in PBS–sodium azide 0.01% for free-floating immunochemistry. Sections were prepared for free-floating TH, GFAP, CD11b, and GDNF immunohistochemistry and revealed by a peroxidase-DAB reaction.

2.7. Cell counts and fiber density

TH-immunoreactive (IR) cell bodies were counted and measured every third section throughout the rostro-caudal extent of the lesioned/transplanted striatum.

To quantify fiber outgrowth from grafts, low power (2.5×) images of brain sections containing TH-IR grafts were analyzed using a computerized image analysis system (Meta View, Roper Scientific, France). The background was substracted from an area of the cortex. The area extending 0.5 mm from the graft-host border around the entire graft was measured. The striatal TH-IR was measured on three sections at the level of the larger portion of the graft.

2.8. Statistical analysis

The data were analyzed by the non-parametric Mann–Whitney *U* test and the one-way ANOVA. Data are

reported as means ± standard error of the mean (SEM) and were considered significantly different when $P < 0.05$.

3. Results and discussion

The use of GDNF is a promising strategy to improve the post-transplantation survival of fetal dopaminergic grafts in PD [43]. However, intracerebral delivery devices, suitable for clinical applications, which allow localized, regulated delivery of GDNF must be developed. In this regard, biocompatible and biodegradable PLGA sustained release microspheres have been produced for intraparenchymal delivery of GDNF. These microspheres can be easily implanted by stereotaxy, admixed or at a certain distance from the grafted cells. As published elsewhere [39], microspheres containing GDNF were smooth and spherical with a mean diameter of 27 ± 10 μm. The encapsulation yield determined by dosing radioactivity was 92.0 ± 5.6%. Thus, total GDNF was successfully encapsulated with a loading of 0.13% w/w (with respect to the amount of PLGA). Released GDNF from microspheres was assessed in a low continuous flow system and was quantified by both radioactivity using ^{125}I-labeled GDNF (data not shown) and ELISA. During the first 24 h, 10 μg GDNF was released for 20 mg microspheres and approximately 10 ng per day was then released during the next 8 weeks (Fig. 1). The bioassay confirmed the bioactivity of the released protein. Table 1 shows that TH-IR cell body areas and neurite outgrowth were significantly increased by the released GDNF collected at Day 1, Day 2, Day 4, and Day 16. This effect was in the same order of magnitude as the positive control (1 ng/ml), showing that the protein was released in biologically active amounts. However, the positive control as well as the released GDNF, at these different time-points, did not have an important effect on dopaminergic cell survival (Table 1). The absence of this survival effect by GDNF in vitro has been already observed and may depend on the culture conditions [24]. The fractions collected following Day 16 could not be tested due to the high dilution of the released GDNF with the continuous flow system.

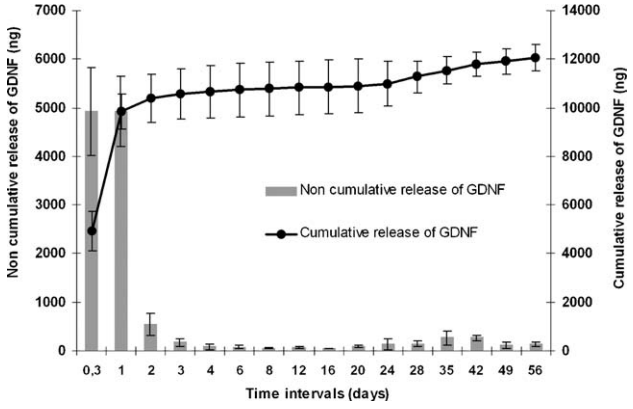

Fig. 1. In vitro cumulative and non-cumulative release of GDNF (ng) for 20 mg PLGA microspheres in eluent buffer (citrate buffer 10 mM + 0.1% BSA + 150 mM NaCl + 0.02% sodium azide, pH 5.0) as determined by ELISA. During the first 24 h, 10 μg GDNF was released for 20 mg MS (corresponding to about 35% of the total GDNF encapsulated) and approximately 10 ng/day was released thereafter for the next 8 weeks.

The goals of this study were to determine if GDNF-loaded microspheres could improve the survival and function of FVM cells in the 6-OHDA-lesioned animals. Two protocols for microsphere implantation were evaluated. In the first one, microspheres were implanted at a distance from the site of FVM cells. Indeed, several studies have shown that devices releasing GDNF separated by a very small distance from FVM transplants improved the survival and/or fiber outgrowth of transplanted dopaminergic neurons [18–20,22–24,26]. In the second protocol, the microspheres were mixed with FVM cells and implanted at a single site in the lesioned striatum (0.4 mg microspheres for 0.5×10^6 FVM cells). This ratio was chosen after testing different proportions of microspheres and FVM cells in culture and evaluating the survival and differentiation of TH-IR neurons (data not shown). The second protocol reduced the length of the implantation procedure, the risk of infections encountered with several injections and provided a 3D microenvironment for the cells.

Assessment of graft function was monitored by amphetamine-induced rotating behavior. Table 2 shows that three weeks after implantation of FVM cells and microspheres, an improvement of behavior was observed for animals receiving GDNF microspheres at a distance from the site of FVM cells and animals receiving the mixture of microspheres and FVM cells. On the contrary, lesioned animals receiving sham injections only or unloaded microspheres at

Table 1
Trophic effects of the defined medium alone (negative control), the rhGDNF standard solution (1 ng/ml; positive control) and the released GDNF from microspheres at Day 1, Day 2, Day 4, and Day 16 (diluted at 1 ng/ml) on primary embryonic TH-IR neurons

	Number of TH-IR cells per cm^2	TH-IR cell body areas (μm^2)	Relative density of TH-IR neurites per mm^2
Defined medium	116 ± 29	165.64 ± 4.51	1034.09 ± 59.35
rhGDNF (1 ng/ml)	107 ± 16	292.37 ± 11.48**	1316.33 ± 54.45**
Day 1	85 ± 7	326.88 ± 10.64**	1389.53 ± 85.78**
Day 2	171 ± 10	296.10 ± 11.66**	1494.79 ± 101.10**
Day 4	222 ± 7	268.08 ± 7.32**	1415.49 ± 78.49**
Day 16	124 ± 27	193.37 ± 5.32**	1230.39 ± 172.29*

* $P < 0.05$.
** $P < 0.001$ (one-way ANOVA), significantly different from defined medium cultures.

Table 2
Rotational behavior of 6-OHDA-lesioned rats after amphetamine challenge at pre-grafting and at two and three weeks after grafting FVM cells and application of unloaded or GDNF-loaded microspheres

Treatments	Pre-graft	Post-graft	
		2 weeks	3 weeks
Microspheres at a distance from FVM cells			
Controls ($n = 7$)	11.56 ± 1.15	13.66 ± 1.1	15.57 ± 1.91
Unloaded microspheres ($n = 5$)	15.78 ± 3.3	16.24 ± 3.51	12.48 ± 3.3
GDNF-loaded microspheres ($n = 5$)	10.98 ± 1.62	9.42 ± 2.01	$6.32 \pm 2.65^{*}$
Microspheres together with FVM cells			
Controls ($n = 5$)	15.58 ± 2.48	13.84 ± 2.93	17.02 ± 4.01
Unloaded microspheres ($n = 6$)	12.7 ± 1.4	12.67 ± 1.33	$5.67 \pm 2.29^{*}$
GDNF-loaded microspheres ($n = 5$)	9.48 ± 1.3	13.28 ± 3.1	$6.68 \pm 1.61^{*}$

* $P < 0.05$ Mann–Whitney U test, significantly different from control groups three weeks post-graft.

a distance from the site of FVM cells failed to show any behavioral recovery. All control and treatment groups presented a virtually complete loss of TH-IR neurons in the ipsilateral substantia nigra (data not shown). Improved functional recovery is thus believed to be a direct consequence of grafted dopaminergic cell survival and function in the host tissue. Accordingly, quantitative analysis of TH-IR cells within the graft showed that implantation of GDNF-loaded microspheres at a distance to the site of the FVM cells induced a 3-fold increase in TH-IR neuron survival (Fig. 2A). This result is consistent with previous transplant studies where recombinant GDNF protein was delivered near the FVM grafts by repeated injections [18,19], continuous infusions [23] or ex vivo gene transfer techniques [20,24,26]. Furthermore, an increase in the size of the surviving TH-IR neurons was observed, suggesting a differentiation of these cells (Fig. 2B). Indeed, the average cell size in the GDNF group was 164 ± 3 μm^2 while in the control group it was 142 ± 3 μm^2.

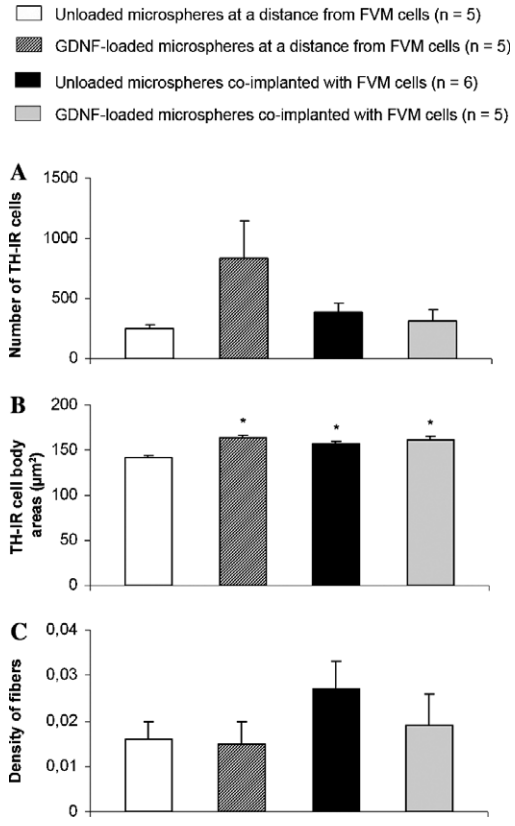

Fig. 2. Quantification of the number of TH-IR neurons/graft (A), cell body areas of TH-IR neurons (B), and density of TH-IR staining in a 0.5 mm zone surrounding the graft (C) $^{*}P < 0.05$ (one-way ANOVA), significantly different from group treated with unloaded microspheres implanted at a distance from FVM cells.

On the contrary, the implantation of GDNF microspheres mixed with FVM cells did not increase the survival of TH-IR neurons (Fig. 2A). The lack of this effect could be due to the observation that within the graft, the microspheres were not well dispersed and formed aggregates probably reducing FVM cell survival (Fig. 3). Nevertheless, an increase in the size of surviving TH-IR neurons was observed in the presence of unloaded or GDNF-loaded microspheres (Fig. 2B), suggesting that when mixed with the cells, the microspheres provided a mechanical support and a 3D environment for the cells, inducing their differentiation. This hypothesis is in accordance with previous studies showing that artificial scaffolds providing a 3D structure induced differentiation of bovine adrenal chromaffin cells [44] and PC12 cells into neuron-like cells [45]. No difference in the size of TH-IR cells was observed between unloaded or GDNF-loaded microspheres as the mechanical support provided by the microspheres probably masked the effect of GDNF.

In the two protocols for microsphere implantation, GDNF-loaded microspheres were still immunoreactive for GDNF antibodies three weeks after their implantation (data not shown). Furthermore, the unloaded and GDNF-loaded microspheres were surrounded by a weak and non-specific astrocytic reaction and some macrophagic cells as previously described [12]. This confirms the biocompatibility of microspheres in brain tissue.

The evaluation of the overall density of TH-IR fibers around the grafts showed that GDNF microspheres had no effect on TH fiber outgrowth whether they are separated or mixed with FVM cells (Fig. 2C). Moreover, the growth of TH-IR fibers toward the GDNF microspheres injected at a distance was variable (data not shown). Thus, while

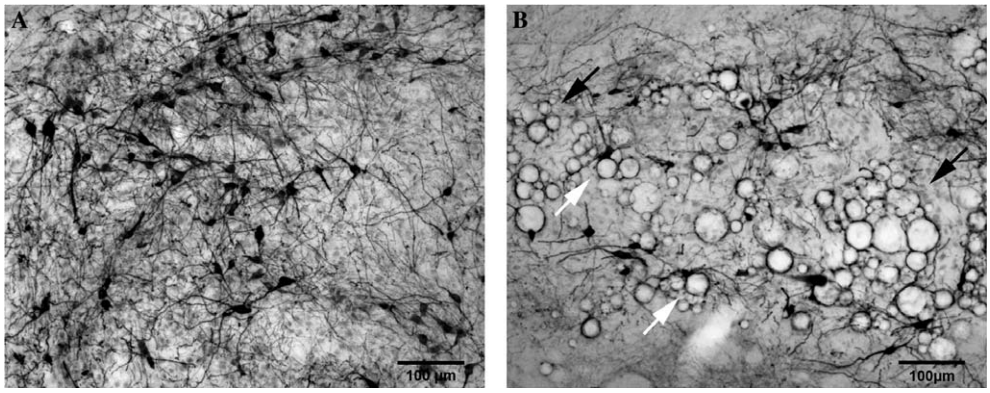

Fig. 3. Photomicrographs of sections throughout the graft stained with antibodies against TH showing FVM cells implanted at a distance from GDNF-loaded microspheres (A) or mixed with GDNF microspheres (B). Black arrows in (B) point to microsphere aggregates and white arrows show dopaminergic cells in contact with microspheres.

GDNF microspheres implanted at a distance from FVM cells increased the number of TH-IR neurons within the graft, no increased fiber outgrowth was observed. This result is consistent with some studies where GDNF was delivered near the FVM graft [23–25]. Nevertheless, other studies described an increase of TH-IR fibers around the graft and their preferential growth toward the source of GDNF [18,20–22,46]. The absence of a correlation between cell survival and fiber outgrowth could be due to the GDNF release profile previously described in vitro. The burst corresponding to a total dose of 0.2 μg GDNF per rat might be sufficient to increase the TH-IR cell survival. Accordingly, previous studies have shown that similar doses of GDNF protected the grafted dopaminergic neurons from cell death [19,20,25]. In contrast, the low amount of GDNF released after 24 h (0.2 ng GDNF per rat) together with the reduced diffusion distance of proteins in the brain ended up providing very low levels of GDNF to the grafted cells which were probably not enough for a long-term effect on subsequent fiber outgrowth. An improvement in the GDNF microsphere formulation by modifying the physical properties of the polymer or by including an additive in the microspheres should increase the quantity of GDNF released after 24 h.

4. Summary

This work demonstrates that GDNF microspheres improve dopaminergic graft function and survival when they are implanted at a distance from the grafted cells. This delivery strategy is safe, localized, sustained for at least two months in vitro but is also transient. This time period of delivery is important since a recent study showed that overexpression of GDNF in the striatum for 6 months could impair dopamine release or the establishment of synaptic connections between the graft-derived dopaminergic fibers and the surrounding host tissue, thus compromising the function of the FVM grafts [25]. This present study also shows that microspheres mixed with FVM cells may provide a mechanical support inducing differentiation of TH-IR neurons, which was accompanied by an ameliorated behavior of the animals. This effect is in accordance with the increased functional outcome obtained with the dopamine secreting human retinal pigment epithelial cells conveyed on microcarriers (Spheramine®) and grafted in parkinsonian paradigms [47]. In this regard, the use of microspheres as microcarriers that may convey the cells on their surface while delivering an active molecule is probably a more suitable approach. For this purpose, our laboratory has developed a new tool, the pharmacologically active microcarriers (PAM) [48]. PAM are biocompatible and biodegradable microparticles coated with cell adhesion or extracellular matrix molecules, conveying cells on their surface, and presenting a controlled delivery of a growth factor. Recently, NGF-releasing PAM, conveying PC12 cells were produced and characterized [45]. These NGF-releasing PAM induced PC12 cell differentiation and functional restoration in 6-OHDA-lesioned rat model of PD [49]. The production and the characterization of GDNF-releasing PAM conveying FVM cells are currently underway.

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