LONG-TERM NEUROPROTECTION AND NEURORESTORATION BY GLIAL CELL-DERIVED NEUROTROPHIC FACTOR MICROSPHERES FOR THE TREATMENT OF PARKINSON`S DISEASE

Elisa Garbayo[1], Eduardo Ansorena[1], Jose Luis Lanciego[2], María Jose Blanco-Prieto[1*] and María S. Aymerich[2,3*]

[1]Departamento de Farmacia y Tecnología Farmacéutica, Facultad de Farmacia, Universidad de Navarra, E-31008 Pamplona, Spain

[2]Área de Neurociencias, CIMA, Universidad de Navarra, E-31008 Pamplona, Spain

[3]Departamento de Bioquímica y Biología Molecular, Universidad de Navarra, E-31008 Pamplona, Spain

*Corresponding authors:

Dr. María S. Aymerich

Avda. Pío XII 55, E-31008 Pamplona, Spain

Phone: + 34 948 194700 ext. 2003

Fax: + 34 948 194715

e-mail: maymerich@unav.es

Dr. María J. Blanco-Prieto

C/Irunlarrea 1, E-31080 Pamplona, Spain

Phone: + 34 948 425600 ext. 6519

Fax: + 34 425649

e-mail: mjblanco@unav.es

Word count: 1558

Running title: GDNF microspheres for PD treatment

Financial Disclosure:

This work was supported by the UTE-project/Foundation for Applied Medical Research (FIMA), by the Department of Health (63/04) and Education (17/04) of the Government of Navarra, the Mapfre Medicine Foundation and Caja Navarra Foundation.

ABSTRACT

Background: Glial cell-derived neurotrophic factor (GDNF) is a survival factor for dopaminergic neurons and a promising candidate for the treatment of Parkinson´s disease (PD). However, the delivery issue of the protein to the brain still remains unsolved.

Purpose: The aim of the study was to investigate the effect of long-term delivery of encapsulated GDNF within microspheres.

Methods: A single dose of microspheres containing 2.5 μg of GDNF was implanted intrastriatally in animals 2 weeks after a 6-hydroxydopamine lesion.

Results: The amphetamine test showed a complete behavioral recovery after 16 weeks of treatment which was maintained until the end of the study (week 30). This effect was accompanied by an increase in dopaminergic striatal terminals and neuroprotection of dopaminergic neurons.

Conclusions: The main achievement was the long-term neurorestoration in parkinsonian animals induced by encapsulated GDNF, suggesting that microspheres may be considered as a means to deliver GDNF for PD treatment.

KEYWORDS

Parkinson´s disease

GDNF

Microspheres

Neurodegeneration

6-OHDA rat

Tyrosine hydroxylase.

ABREVIATIONS

GDNF: glial cell-derived neurotrophic factor

PD: Parkinson´s disease

SNpc: substantia nigra pars compacta

VTA: ventral tegmental area

PLGA: poly-lactic-co-glycolic acid

TH: tyrosine hydroxylase

pTH: phosphorylated tyrosine hydroxylase

6-OHDA: 6-hydroxydopamine

BDA: biotinylated dextran amine

INTRODUCTION

Glial cell derived neurotrophic factor (GDNF) was initially described as a potent neurotrophic factor for dopaminergic neurons, regarded as a promising therapeutic agent for PD [1]. Several animal studies and a few clinical trials have set the requirements for proper GDNF treatment [2-4]. Intracerebroventricular injection of GDNF in PD patients caused severe side effects, without improving parkinsonian symptoms [5, 6], probably because GDNF did not reach the target tissues [7]. For these reasons, GDNF should be delivered locally to the caudate-putamen in humans or striatum in rodents [8]. The results from two independent open-label trials showed that the sustained delivery of GDNF within the putamen using a mechanical pump reduced the motor symptoms and achieved a partial restoration of the nigrostriatal pathway [9, 10]. These promising results were in contrast to the findings in the first double-blind placebo-controlled study in which GDNF infusion, at a lower dose and using a wider catheter, failed to improve motor symptoms in patients [11]. The lack of efficacy in this trial, the appearance of antibodies against GDNF and cerebellar lesions in monkeys receiving very high doses of GDNF, resulted in the withdrawal of GDNF from all clinical tests. These studies concluded that a new delivery system should be developed before considering GDNF infusion in human patients. This has prompted researchers to seek alternative methods for long-term protein delivery.

In the present study we propose the use of biodegradable and biocompatible non-toxic poly-lactic-co-glycolic acid (PLGA) microspheres for sustained GDNF delivery into the brain. The goal of this work was to asses whether a single low dose of microencapsulated GDNF would be sufficient to maintain long-term beneficial neuroestorative and/or neuroprotective effect in the partial 6-hydroxydopamine (6-OHDA) rat model of PD. We provide evidence that encapsulated GDNF may be

delivered to the striatum, inducing a long-lasting recovery of lesion-induced motor impairment. Restoration of functional behavior is accompanied by striatal dopaminergic fiber sprouting, neuroprotection of dopaminergic cells at the SNpc level and an increase in tyrosine hydroxylase phosphorylation.

MATERIAL AND METHODS

Microsphere preparation.

Rat recombinant glycosylated GDNF was expressed and purified as previously described [12]. GDNF-loaded microspheres were prepared using the solvent extraction/evaporation method utilizing TROMS technology [13]. Fluorescent microspheres were prepared similarly but encapsulating rhodamine B isothiocyanate (0.1 mg) in the inner aqueous phase instead of GDNF.

Animal procedures.

Animals. Female Sprague-Dawley rats of 220-240 g were obtained from Harlan (Barcelona, Spain). Animals were kept in standard animal facilities with free access to food and water, in a temperature and humidity-controlled room with 12 h on–off light cycle. Procedures involving animals were in accordance with the guidelines established by the normative of the European Council (86/609/EEC).

Experimental parkinsonism. Animals were anesthetized via an intraperitoneal injection of a 1:1 mixture of ketamine (75 mg/kg) and xylazine (10 mg/kg). Rats received two 6-OHDA (Sigma) injections, each one of 10 µg in 5 µL of saline containing 0.1% ascorbic acid, into the striatum at the following coordinates relative to bregma [14]: AP: +0.5 mm, ML: 2.5 mm DV: 5 mm and AP: -0.5 mm, ML: 4.2 mm, DV: 5 mm.

Microsphere implantation. Stereotaxic surgery was performed 15 days after the 6-OHDA lesion using the same coordinates. Each rat received a dose of 2.5 µg of GDNF in two implantations, each one comprising 1.5 mg of microspheres (1.25 µg of GDNF).

Amphetamine test. The rotational behavior of the animals induced after an intraperitoneal injection of 5 mg/kg D-amphetamine (Sigma) was assessed on a computerized rotometer (Panlab).

Histological techniques

Animals were perfused transcardially with a fixing solution containing 4% paraformaldehyde in 0.125 M phosphate buffer, pH 7.4. Coronal sections (40 µm thick) were obtained. Coronal sections (40mm thick) were use for immunohistochemistry. Histological techniques and optic density measurements were performed as described previously[15].

Statistical analysis

Those data following a normal distribution were analyzed using independent Student´s t test and non-parametric data using a Wilcoxon test for independent samples. A *p* value < 0.05 was considered significant.

RESULTS

Assessment of biodegradability of PLGA microspheres in vivo

We first evaluated whether GDNF-microspheres were present 28 weeks after their implantation. Microspheres loaded with rhodamine were prepared and injected within the rat striatal parenchyma. Animals were sacrificed at different times and rhodamine-microspheres were visualized by confocal microscopy (Fig. 1A). Between the first and second week there was a large decrease in the microsphere injection size that continued to decrease, at a lower rate, over time. Importantly, a significant amount of microspheres were still detectable at week 28th post-injection.

Microencapsulated GDNF reverses altered motor behavior in a PD animal model

To evaluate the long-term effect of encapsulated GDNF the partial 6-OHDA animal model was used. The extent of the lesion was assessed 13 days after the toxic administration by the amphetamine-induced rotational behavior test. Two days later, animals received an injection of either empty or GDNF-loaded microspheres. Striatal implantation of GDNF-microspheres significantly reduced amphetamine-induced ipsilateral rotational behavior (Fig. 1B) after 8 weeks of treatment. Furthermore, the imbalance in rotational behavior completely disappeared at week 16 and the recovery was maintained up to week 30.

Effect of GDNF-microsphere treatment on TH-positive cells

The effect of GDNF-microspheres at the striatal level was evaluated by TH immunohistochemistry. Intrastriatal injections of GDNF-microspheres induced profuse TH-immunoreactive (TH-ir) fiber sprouting surrounding the injection areas (Fig. 1C). In contrast, only the injection tract was visible around the injection sites of empty microspheres. Aberrant sprouting in the globus pallidus was not observed [16]. Densitometric analysis at three different striatal rostrocaudal levels (Fig. 1D) showed a

22% increase in the area of TH immunoreactivity at the most caudal striatal levels. Our results suggest that these fibers are likely to arise from sprouting by the remaining terminals located within the ventral striatum and the nucleus accumbens.

TH-ir neurons were counted in three sections throughout the central portion of the SNpc, at the level of the medial terminal nucleus of the accessory optic tract (Fig. 2A). A statistically significant 1.7-fold increase in the number of TH-ir cells was observed within the SNpc of animals treated with GDNF-microspheres (Fig. 2B). The degree of TH activity within these cells was studied by double immunofluorescence to detect the form of the enzyme TH phosphorylated at ser31 [17, 18] (Fig. 2C). All cells detected were double labeled and fluorescence signal corresponding to pTH with respect to TH-positive cells was quantified. A significant increase in this ratio at the SNpc level was found in GDNF-treated animals (Fig. 2D). This was not observed at the VTA level (Fig. 2E). These results suggest that the improvement in behavior induced by GDNF-microspheres could be due to both a neuroprotective effect on dopaminergic cells and an increase in TH activity.

DISCUSSION

The results of the present study demonstrate that PLGA microspheres are an efficient vehicle for sustained delivery of neurotrophic factors to the brain. Biodegradable and biocompatible PLGA microspheres offer an interesting alternative and have already been used in clinical practice for the treatment of human gliomas [19, 20]. Microspheres release drugs locally for an extended period of time, have a low risk of side effects and protect the encapsulated protein from degradation by tissue proteases.

Several studies to determine neuroprotection have been performed by administrating GDNF prior to performing the 6-OHDA lesion [16, 21-24]. While these studies are interesting for testing the efficacy of GDNF, the administration of the trophic factor after a lesion would have greater clinical relevance. For this reason, GDNF-microspheres were implanted intrastriatally 14 days after the 6-OHDA lesion. At 28 weeks post-implantation, microspheres were still present and the GDNF beneficial effect was preserved. GDNF was clearly detected by immunohistochemistry 5 weeks after microparticles implantation [15]. In the present study, the immunohistochemistry procedure failed to detect the presence of GDNF, probably because the remaining protein was under the detection threshold of the technique. It may be assumed that very low GDNF doses are enough to maintain the beneficial effect as previous studies have demonstrated that protein withdrawal in PD patients [25] or GDNF viral expression switch off, reversed GDNF effect [26]. The distribution of the trophic factor is another important issue. In this study, two intrastriatal microparticle injections were sufficient to promote a stable functional recovery of the 6-OHDA animal model. The number of microparticle injections may be increased for non-human primates or human brains with a larger putaminal volume.

An additional issue with GDNF-based therapy is the production of neutralizing antibodies against GDNF [11]. An immune reaction against the *E. coli* unglycosylated recombinant protein used in the clinical trial could be responsible for the production of autoantibodies [27]. To overcome this potential problem, glycosylated recombinant rat GDNF was used in our study [12] and the absence of anti-GDNF antibodies was confirmed [15].

The low dose required in the present approach to achieve a functional effect was also remarkable. A total amount of 2.5 µg of GDNF was sufficient for the long-term effect of GDNF treatment. Considering that the human putamen volume is 171 times larger than the rat striatum [28 29], an estimation of the dose necessary for patients for a 28 week treatment would be 427 µg.

The main achievement of this study was the stable long-term functional recovery in parkinsonian animals induced by GDNF treatment initiated after the 6-OHDA lesion. The results of this work indicate that microspheres should be considered as a method to deliver GDNF directly to the striatum for the treatment of PD.

ACKNOWLEGMENTS

We acknowledge Carmen Molina for technical support and Dr. Rafael Franco for critical review of the manuscript.

AUTHOR ROLES

Elisa Garbayo: Research project, execution; Manuscript, review and critique

Eduardo Ansorena: Research project, execution; Manuscript, review and critique

Jose Luis Lanciego: Research project, conception

María Jose Blanco-Prieto: Research project, conception; Manuscript, review and critique

María S. Aymerich: Research project, conception and execution; Manuscript, writing of the first draft.

REFERENCES

1. Lin LF, Doherty DH, Lile JD, Bektesh S, Collins F. GDNF: a glial cell line-derived neurotrophic factor for midbrain dopaminergic neurons. Science 1993;260(5111):1130-1132.

2. Tomac A, Lindqvist E, Lin LF, et al. Protection and repair of the nigrostriatal dopaminergic system by GDNF in vivo. Nature 1995;373(6512):335-339.

3. Gash DM, Zhang Z, Ovadia A, et al. Functional recovery in parkinsonian monkeys treated with GDNF. Nature 1996;380(6571):252-255.

4. Björklund A KD, Rosenblad C, Georgievska B, Lundberg C, Mandel RJ. Towards a neuroprotective gene therapy for Parkinson's disease: use of adenovirus, AAV and lentivirus vectors for gene transfer of GDNF to the nigrostriatal system in the rat Parkinson model. Brain Res 2000;886

(1-2):82-98.

5. Kordower JH, Palfi S, Chen EY, et al. Clinicopathological findings following intraventricular glial-derived neurotrophic factor treatment in a patient with Parkinson's disease. Ann Neurol 1999;46(3):419-424.

6. Nutt JG, Burchiel KJ, Comella CL, et al. Randomized, double-blind trial of glial cell line-derived neurotrophic factor (GDNF) in PD. Neurology 2003;60(1):69-73.

7. Sariola H, Saarma M. Novel functions and signalling pathways for GDNF. J Cell Sci 2003;116(Pt 19):3855-3862.

8. Kirik D, Georgievska B, Bjorklund A. Localized striatal delivery of GDNF as a treatment for Parkinson disease. Nat Neurosci 2004;7(2):105-110.

9. Gill SS, Patel NK, Hotton GR, et al. Direct brain infusion of glial cell line-derived neurotrophic factor in Parkinson disease. Nat Med 2003;9(5):589-595.

10. Slevin JT, Gerhardt GA, Smith CD, Gash DM, Kryscio R, Young B. Improvement of bilateral motor functions in patients with Parkinson disease through the unilateral intraputaminal infusion of glial cell line-derived neurotrophic factor. J Neurosurg 2005;102(2):216-222.

11. Lang AE, Gill S, Patel NK, et al. Randomized controlled trial of intraputamenal glial cell line-derived neurotrophic factor infusion in Parkinson disease. Ann Neurol 2006;59(3):459-466.

12. Garbayo E, Ansorena E, Lanciego JL, Aymerich MS, Blanco-Prieto MJ. Purification of bioactive glycosylated recombinant glial cell line-derived neurotrophic factor. Int J Pharm 2007;344(1-2):9-15.

13. Garbayo E, Ansorena E, Lanciego JL, Aymerich MS, Blanco-Prieto MJ. Sustained release of bioactive glycosylated glial cell-line derived neurotrophic factor from biodegradable polymeric microspheres. Eur J Pharm Biopharm 2008;69(3):844-851.

14. Paxinos G, Watson C. The main achievement of this study is the long-term stable functional recovery in parkinsonian animals induced by GDNF treatment initiated after the 6-OHDA lesion. The results of this work indicate that microspheres should be taken into consideration as a method to deliver GDNF directly to the striatum for the treatment of PD. 6th ed. New York: Academic Press, 2007.

15. Garbayo E, Montero-Menei CN, Ansorena E, Lanciego JL, Aymerich MS, Blanco-Prieto MJ. Effective GDNF brain delivery using microspheres--a promising strategy for Parkinson's disease. J Control Release 2009;135(2):119-126.

16. Georgievska B, Kirik D, Bjorklund A. Aberrant sprouting and downregulation of tyrosine hydroxylase in lesioned nigrostriatal dopamine neurons induced by long-lasting

overexpression of glial cell line derived neurotrophic factor in the striatum by lentiviral gene transfer. Exp Neurol 2002;177(2):461-474.

17. Salvatore MF, Zhang JL, Large DM, et al. Striatal GDNF administration increases tyrosine hydroxylase phosphorylation in the rat striatum and substantia nigra. J Neurochem 2004;90(1):245-254.

18. Salvatore MF, Gerhardt GA, Dayton RD, Klein RL, Stanford JA. Bilateral effects of unilateral GDNF administration on dopamine- and GABA-regulating proteins in the rat nigrostriatal system. Exp Neurol 2009;219(1):197-207.

19. Menei P, Capelle L, Guyotat J, et al. Local and sustained delivery of 5-fluorouracil from biodegradable microspheres for the radiosensitization of malignant glioma: a randomized phase II trial. Neurosurgery 2005;56(2):242-248; discussion 242-248.

20. Menei P, Montero-Menei C, Venier MC, Benoit JP. Drug delivery into the brain using poly(lactide-co-glycolide) microspheres. Expert Opin Drug Deliv 2005;2(2):363-376.

21. Sauer H, Rosenblad C, Bjorklund A. Glial cell line-derived neurotrophic factor but not transforming growth factor beta 3 prevents delayed degeneration of nigral dopaminergic neurons following striatal 6-hydroxydopamine lesion. Proc Natl Acad Sci U S A 1995;92(19):8935-8939.

22. Kearns CM, Cass WA, Smoot K, Kryscio R, Gash DM. GDNF protection against 6-OHDA: time dependence and requirement for protein synthesis. J Neurosci 1997;17(18):7111-7118.

23. Sullivan AM, Opacka-Juffry J, Blunt SB. Long-term protection of the rat nigrostriatal dopaminergic system by glial cell line-derived neurotrophic factor against 6-hydroxydopamine in vivo. Eur J Neurosci 1998;10(1):57-63.

24. Kirik D, Rosenblad C, Bjorklund A. Preservation of a functional nigrostriatal dopamine pathway by GDNF in the intrastriatal 6-OHDA lesion model depends on the site of administration of the trophic factor. Eur J Neurosci 2000;12(11):3871-3882.

25. Slevin JT, Gash DM, Smith CD, et al. Unilateral intraputamenal glial cell line-derived neurotrophic factor in patients with Parkinson disease: response to 1 year of treatment and 1 year of withdrawal. J Neurosurg 2007;106(4):614-620.

26. Yang X, Mertens B, Lehtonen E, et al. Reversible neurochemical changes mediated by delayed intrastriatal glial cell line-derived neurotrophic factor gene delivery in a partial Parkinson's disease rat model. J Gene Med 2009;11(10):899-912.

27. Tatarewicz SM, Wei X, Gupta S, Masterman D, Swanson SJ, Moxness MS. Development of a maturing T-cell-mediated immune response in patients with idiopathic Parkinson's disease receiving r-metHuGDNF via continuous intraputaminal infusion. J Clin Immunol 2007;27(6):620-627.

28. Yin D, Valles FE, Fiandaca MS, et al. Striatal volume differences between non-human and human primates. J Neurosci Methods 2009;176(2):200-205.

29. Bemelmans AP, Horellou P, Pradier L, Brunet I, Colin P, Mallet J. Brain-derived neurotrophic factor-mediated protection of striatal neurons in an excitotoxic rat model of Huntington's disease, as demonstrated by adenoviral gene transfer. Hum Gene Ther 1999;10(18):2987-2997.

FINANCIAL DISCLOSURE FOR THE PAST YEAR

UTE-project/Foundation for Applied Medical Research (FIMA)

LEGENDS

Figure 1: Neurorestorative effect of GDNF-microspheres. (A) Degradation of rhodamine microspheres implanted intrastriatally. The same formulation of microspheres used for GDNF treatment was prepared and loaded with rhodamine. Rhodamine-microspheres were injected intrastriatally and the extent of the deposit was followed at different time intervals. Microspheres are still present at week 28. Scale bar: 100 µm. (B) Amphetamine-induced rotation was performed 13 days after the 6-OHDA lesion. All animals that fulfilled the inclusion criteria (>9 full body turns per minute) received the injection of either empty or GDNF-loaded microspheres 48 h later. At 10 weeks post-6-OHDA and 8 weeks after beginning the GDNF treatment, animals receiving GDNF-microspheres showed a significant improvement in their rotational behavior when compared to animals that received empty microspheres. The rotational response was completely reversed at week 16 and the recovery maintained until the end of the study (week 30). Control animals treated with empty microspheres (open circles), n = 6; animals treated with GDNF microspheres (filled circles), n = 8. *p<0.05. (C) TH immunohistochemistry was performed in animals sacrificed 30 weeks after 6-OHDA lesion. Images at the striatal level of the two injection sites show an increase in TH-positive fiber density surrounding the GDNF-microspheres deposits that is not detectable around the empty-microspheres deposits in lesioned animals. Scale bar: 1 mm. (D) Quantification of the TH-positive fiber density at three different rostrocauldal levels. *p<0.05.

Figure 2: GDNF effect in the substantia nigra *pars compacta*. (A) Immunohistochemistry for TH revealed an increase in the number of surviving cells in the SNpc of GDNF-treated compared to empty-microspheres-treated animals 30 weeks

after the 6-OHDA lesion. (B) Quantification of TH-positive neurons from the SNpc showed a statistically significant increase in the number of cells present in animals treated with GDNF microspheres compared to animals that received empty microspheres. Scale bar: 1 mm. Wilcoxon test *p<0.05. (C) Double immunohistochemistry for TH and pTH showed that all cells were double labeled and presented an increase in pTH level. Quantification of fluorescence intensity of pTH with respect to the total amount of TH at the level of SN (D) and VTA (E) showed a significant increase of pTH in TH-ir neurons from the SNpc of GDNF-treated animals compared to the contralateral side. Scale bar: 20 μm. t-Student *p<0.05.