Journal of Controlled Release 135 (2009) 119–126

Contents lists available at ScienceDirect

Journal of Controlled Release

journal homepage: www.elsevier.com/locate/jconrel

Effective GDNF brain delivery using microspheres—A promising strategy for Parkinson's disease

E. Garbayo [a], C.N. Montero-Menei [b], E. Ansorena [a], J.L. Lanciego [c], M.S. Aymerich [c], M.J. Blanco-Prieto [a,*]

[a] Department of Pharmacy and Pharmaceutical Technology, University of Navarra, Spain
[b] Inserm U646, Laboratoire d'Ingénierie de la Vectorisation Particulaire; Université d'Angers, France
[c] Department of Neurosciences, Center for Applied Medical Research (CIMA and CIBERNED) University of Navarra Medical College, Spain

ARTICLE INFO

Article history:
Received 18 September 2008
Accepted 14 December 2008
Available online 25 December 2008

Keywords:
GDNF
PLGA microparticles
TROMS
Serum IgG antibodies against GDNF
Parkinson's disease

ABSTRACT

Glial cell line-derived neurotrophic factor (GDNF) has shown promise in the treatment of neurodegenerative disorders of basal ganglia origin such us Parkinson's disease (PD). In this study, we investigated the neurorestorative effect of controlled GDNF delivery using biodegradable microspheres in an animal model with partial dopaminergic lesion. Microspheres were loaded with N-glycosylated recombinant GDNF and prepared using the Total Recirculation One-Machine System (TROMS). GDNF-loaded microparticles were unilaterally injected into the rat striatum by stereotaxic surgery two weeks after a unilateral partial 6-OHDA nigrostriatal lesion. Animals were tested for amphetamine-induced rotational asymmetry at different times and were sacrificed two months after microsphere implantation for immunohistochemical analysis. The putative presence of serum IgG antibodies against rat glycosylated GDNF was analyzed for addressing safety issues. The results demonstrated that GDNF-loaded microspheres, improved the rotational behavior induced by amphetamine of the GDNF-treated animals together with an increase in the density of TH positive fibers at the striatal level. The developed GDNF-loaded microparticles proved to be suitable to release biologically active GDNF over up to 5 weeks *in vivo*. Furthermore, none of the animals developed antibodies against GDNF demonstrating the safety of glycosylated GDNF use.

1. Introduction

Neurotrophic factors have emerged as promising tools for the treatment of a wide variety of neurodegenerative diseases. Among them, the glial cell-line derived neurotrophic factor (GDNF) was selected as the most suitable candidate for the treatment of Parkinson's disease (PD) due to its strong trophic effect on the dopaminergic system [1,2]. The initial successful results obtained in relevant animal models of the disease led to different clinical trials in PD patients. The outcome obtained in two independent Phase I clinical trials known as the "Bristol" and "Kentucky" studies [3–5], using direct intraputaminal infusion of naked nonglycosylated GDNF through mini-pumps, was not further confirmed by a double-blind Phase II study using a similar strategy [6]. Several safety concerns were reported, such as the appearance of blocking antibodies against GDNF, together with the presence of unexpected cerebellar damage in a toxicology study carried out in parallel in primates treated with high doses of GDNF. However, the main reason for the discontinuation of the study was the failure of reaching the primary endpoint [6]. Differences in GDNF doses, catheter size and infusion methods may have resulted in different GDNF spread and bioavailability. Recently, the statistical design of this Phase II study was also questioned [7].

Several strategies have been used for GDNF release in the central nervous system (CNS). A catheter connected to an infusion pump has already been used in PD patients [3–6]. This method has some disadvantages, such as the need of high concentrations of the neurotrophic factor as well as pump refilling, together with limited tissue diffusion of the delivered protein [8]. Another feasible option is the use of gene therapy using different strains of modified viruses carrying the GDNF gene [9–11]. This approach also presents some disadvantages such as the lack of control of the duration of the transgene expression, the viral spread outside the target area, and the difficulties in calculating the exact amount of GDNF produced from the viral-infected neurons. Finally, a different alternative would be the use of cell therapy strategies using cells genetically engineered to release GDNF [12]. However, several concerns have been raised, related to the reduced rate of cellular survival within the implanted graft, as well as the presence of immune rejection of the grafted cells by the host tissue.

When compared to the existing strategies, the use of biodegradable and biocompatible microspheres for the controlled brain release of GDNF could represent an attractive alternative for several reasons. First of all, microparticles are prepared with biodegradable polymers

* Corresponding author. Department of Pharmacy and Pharmaceutical Technology, School of Pharmacy, University of Navarra, C/Irunlarrea 1, E-31080 Pamplona, Spain. Tel.: +34 948 425 600x6519; fax: +34 948 425 649.
E-mail address: mjblanco@unav.es (M.J. Blanco-Prieto).

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doi:10.1016/j.jconrel.2008.12.010

that do not require removal once the treatment is finished. Secondly, brain biocompatibility of particles prepared with poly (lactic-co-glycolic) acid (PLGA) polymers has already been well established [13–16] and therefore the appearance of host immune reaction against injected microparticles is very unlikely. Finally, the drug release profile of PLGA microspheres brings another important advantage. Therefore, GDNF dosage could be diminished, leading to a reduction of the possible side effects. However, protein encapsulation is not an easy task due to the labile nature of these macromolecules. Among the methods described, multiple emulsion solvent evaporation technique (W/O/W) is generally accepted as the most suitable to encapsulate proteins and peptides [17]. Nevertheless, proteins may lose their biological activity during the manufacturing process. Since shear stress and vortexing are avoided, multiple emulsion prepared by Total Recirculation One-Machine System (TROMS) may be a feasible way of overcoming protein denaturation during microparticle preparation [18]. TROMS technology also produces very homogeneous batches on a semi-industrial scale, which is of great interest considering future scaling-up and industrial issues.

Different formulations loaded with glycosylated rat recombinant GDNF were previously analyzed to optimize the neurotrophic factor microencapsulation by TROMS technology, the stability of the protein during the manufacturing process and the drug release profile [19]. In the present work we move one step forward by testing the *in vivo* efficacy and safety of GDNF-loaded microspheres in a rodent model of PD. We are particularly interested in evaluating their ability to restore the dopaminergic innervation in a model of partial dopaminergic fiber depletion that mimics the situation encountered in PD patients. Rotational testing, histological assessment as well as antibody response to glycosylated GDNF were performed to analyze the effects of GDNF-loaded microparticles after implantation in Parkinsonian rats.

2. Materials and methods

2.1. Materials

Rat recombinant glycosylated GDNF was expressed and purified as previously described [20]. Recombinant insect cell-derived rat GDNF was purchased from SIGMA (Steinheim, Germany). GDNF enzyme linked immunosorbent assay kit (ELISA) was purchased from Promega (Madison, USA). Poly (lactic-co-glycolic) acid (PLGA) with a lactic: glycolic ratio of 50:50 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). Poly (vinyl alcohol) 88% hydrolyzed (MW: ~125,000) was obtained from Polyscences, Inc (Warington, USA). The rat pheochromocytome PC-12 cells were purchased from American Type Culture Collection (ATCC) (Rockville, MD, USA). Normal goat serum, normal rabbit serum, biotinylated rabbit anti goat IgG and the Vectastain ABC kit were provided by Vector Laboratories (Burlingame, CA, USA). Triton X-100, ExtrAvidin®-Peroxidase, mouse anti TH monoclonal antibody, 6-hydroxydopamine hydrochloride, 3, 3′-diaminobenzidine, D-amphetamine sulphate, and rhodamin B isothiocyanate were from Sigma-Aldrich (Barcelona, Spain). DPX was obtained from BDH Chemicals (Poole, UK). H_2O_2 and paraformaldehyde were purchased from Merck (Barcelona, Spain). Carboxymethylcellulose and mannitol were obtained from Cooper Pharmaceutique (Melun, France). Polysorbate 80 was provided by Prolabo (Paris, France). Biotinylated goat anti mouse IgG was obtained from Jackson ImmunoResearch (West Grove, PA, USA). Goat anti GDNF antibody was purchased from R&D systems (Minneapolis, MN, USA), rabbit anti GFAP antibody was obtained from DAKO (Trappes, France), mouse anti CD11b antibody was purchased from AbD Serotec (Oxford, England) and rat anti dopamine transporter monoclonal antibody were obtained from Chemicon International (Temecula, CA, USA). Donkey anti rabbit and donkey anti mouse coupled to Alexa®488 were from Molecular Probes (Eugene, OR, USA). Horseradish-peroxidase-conjugated goat anti-rat IgG, horseradish-peroxidase-conjugated donkey anti rabbit IgG and horseradish-peroxidase-conjugated sheep anti mouse IgG were from Amersham GE Healthcare (Buckinghamshire, UK). o-Phenylenediamine dihydrochloride was obtained from SIGMA (Saint Louis, MO, USA). The rabbit anti GDNF polyclonal antibody and the mouse anti GDNF monoclonal IgG1 antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

2.2. Preparation of GDNF-loaded microspheres

GDNF-loaded microparticles were prepared by solvent extraction/evaporation method using TROMS as previously described [19]. Briefly, the organic solution composed of 2 ml of dichlorometane: acetone (3:1) containing 100 mg of Resomer RG 503H was injected through a needle with an inner gauge diameter of 0.17 mm at a ratio of 30 ml/min into the inner aqueous phase (200 µl). The inner aqueous phase contained 135 µg of GDNF in 10 mM phosphate, 50 mM sodium chloride (PBS), pH 7.9, 5 mg of HSA and 5 µl of PEG 400. Next, the primary emulsion (W_1/O) was recirculated through the system for 3 min under a turbulent regime at a flow rate of 30 ml/min. The first emulsion was then injected into 30 ml of the external aqueous phase (W_2) composed of 1.5% PVA. The turbulent injection through the needle with an inner gauge diameter of 0.50 mm resulted in the formation of a multiple emulsion ($W_1/O/W_2$), which was further homogenized by circulation through the system for 4 min. The $W_1/O/W_2$ emulsion was stirred at 1000 rpm at room temperature for at least 3 h to allow solvent evaporation and microspheres formation. Finally, particles were washed with ultrapure water and freeze-dried. For fluorescence-labelled microparticles, rhodamin B isothiocyanate (0.5 mg/ml) was added to inner aqueous phase and microspheres were prepared as described above.

2.3. Characterization of microspheres

2.3.1. Particle size analysis

The mean particle size and size distribution of the microspheres were examined by laser diffractometry using a Mastersizer-S® (Malvern Instruments, Malvern, UK). Microspheres were dispersed in ultrapure water and analyzed under continuous stirring. The average particle size was expressed as the volume mean diameter in micrometers. Samples were read in triplicate.

2.3.2. Particle morphology

Both the microsphere shape and surface structure were evaluated by SEM using Zeiss DSM 940A microscope with a digital imaging capture system (DISS of Point Electronic GmbH).

2.3.3. Drug content

The amount of GDNF encapsulated in the microspheres was determined by dissolving 5 mg of freeze-dried loaded particles in 1 ml of dimethyl sulfoxide (DMSO). Previously, it was verified that DMSO did not affect GDNF stability. The quantity of GDNF was measured in triplicates by ELISA using the GDNF Emax® ImmunoAssay System according to the manufacturer's instructions.

2.3.4. In vitro release of GDNF from PLGA microspheres

GDNF-loaded microspheres (1 mg, $n=3$) were resuspended by vortexing in 0.5 ml of PBS, pH 7.4 containing 0.1% BSA and 0.02% w/w sodium azide. Incubation took place in rotating vials at 37 °C. At defined times ranging from 30 min to 40 days, samples were centrifuged at 25,000 ×g, for 15 min. Due to the instability of the protein in the release medium, the amount of drug released was indirectly determined by measuring the quantity of GDNF remaining