Gene therapy using lactoferrin-modified nanoparticles in a rotenone-induced chronic Parkinson model

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1. Introduction

Neurodegenerative disorders are a class of chronic and progressive brain diseases such as Parkinson’s disease (PD). These disorders have a long course and may last for decades. A long-term therapy is required to maintain the healthy neurons and repair degenerated ones, hoping to slow or reverse the progressive course of these diseases. Moreover, the therapeutic method must overcome the blood–brain barrier (BBB) efficiently, so that the active molecules could reach the diseased regions when given by intravenous administration.

Gene therapy has been implicated as a feasible approach starting from the etiology for neurodegenerative diseases. However, most current gene delivery methods to the brain were achieved via direct intracerebral injection of viral vectors such as adeno-associated virus (AAV), because these gene vectors themselves could not cross the BBB through an intravenous injection [1]. This administration method is considered highly invasive, and impractical for repetitive or continuous treatments [2]. Therefore, long-term and effective gene therapy for neurodegenerative disorders requires an efficient gene delivery vector to cross the BBB and an acceptable administration way for long-lasting treatments.

Non-viral vectors were reported to be able to cross the BBB via transvascular administration [3]. A potential non-viral vector was constructed in our previous studies, using polyamidoamine (PAMAM) conjugated with lactoferrin (Lf) through bifunctional polyethylene glycol (PEG) [4]. Lf was used as a brain-targeting ligand here to bind specifically with Lf receptors on brain cells through a receptor-mediated pathway. Lf-modified nanoparticles (NPs) could significantly enhance the extravasation of foreign gene expression in the brain via a receptor-mediated BBB-crossing mechanism, about 5.2-fold of that of unmodified ones [4]. However, the gene expression of non-viral vectors could only last for several days, not a desired time for long-term gene therapy. Multi-dose administration is one of the conventional means to maintain the effective concentration of therapeutic drugs. In this study, multiple dosing intravenous administration was attempted to enhance and maintain therapeutic gene products within the brain.

In the present study, PD was chosen as a representative for neurodegenerative disorders to evaluate the pharmacodynamic effect of Lf-modified NPs and multiple dosing administration. Over the years, the unilateral 6-hydroxydopamine (6-OHDA) rat model has emerged as one of the most popular tool for PD studies [5]. Following stereotaxic injection of 6-OHDA into the striatum or substantia nigra (SN), this model produced symptoms of PD in the short term [6]. However, the strong unilateral lesion in rats is not consistent with the real progress of PD and cannot show direct relevance to human PD [7]. It has been reported that rotenone, a commonly used pesticide, is one important environmental factor in the etiology of PD [8,9]. In recent years, many

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studies showed that the chronic exposure of rotenone could cause highly selective lesions of dopaminergic neurons in the whole nigrostratal system [10]. On the basis of these facts, Betarbet and colleagues developed a new experimental model of PD via chronic, continuous exposure of rats to rotenone [11]. The rotenone model could produce most of the features of PD including Lewy body formation in the nigral neurons, unlike other existing models [12]. Consequently, the rotenone-induced PD model was considered to more adequately mimic the pathogenesis and progress of PD than previously used models [13].

Till now, gene therapy of PD using therapeutic genes encoding glial cell line-derived neurotrophic factor (GDNF) family has been extensively studied in animals, even advanced to clinical testing [14,15]. GDNF is generally believed to possess most potent trophic effects on dopaminergic neurons [16]. In the present study, human GDNF gene (hGDNF) was chosen as the therapeutic gene to be encapsulated in Lf-modified brain-targeting NPs. A regimen of multiple dosing intravenous administration of Lf-modified NPs every other day was designed; then the neuroprotective effects were evaluated in the rotenone-induced chronic model of PD.

2. Materials and methods

2.1. Materials

PAMAM dendrimer [generation = 5, 21.43% w/w solution in methanol, containing 128 surface primary amino groups (MW 28,826)], was purchased from Dendritech (Midland, MI, U.S.A.), α-Maleimidyl-ω-N-hydroxysuccinimidyl polyethylene glycol (NHS-PEG-MAL, MW 3400) was obtained from Nektar Therapeutics (Huntsville, AL, U.S.A.). 5,5′-Dithiobis(2-nitrobenzoic acid) (Ellmann’s reagent) was purchased from Acros (Belgium). GDNF enzyme-linked immunosorbent assay (ELISA) kits were obtained from Promega (GDNF Emax® ImmunoAssay System). Anti-tyrosine hydroxylase (TH), clone LNC1, was purchased from Chemicon (Temecula, CA, U.S.A.). Secondary antibody labeled with horseradish peroxidase (the ABC kit) and 3,3′-diaminobenzidine (DAB) were from Zymed Laboratories (CA, U.S.A.). The therapeutic plasmid DNA, pEF-Bos-peroxidase (the ABC kit) and 3,3′-tyrosine hydroxylase (TH), clone LNC1, was purchased from Chemicon (University of Helsinki). The plasmid pEGFP-N2 coding green protein (GFP) (Clontech, U.S.A) and pEF-Bos-hGDNF were purified by using Qiagen plasmid Mega Kit (Qiagen GmbH, Germany). Lf from bovine colostrum, human holo-transferrin (Tf), rotenone, and other reagents, if not specified, were purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.).

2.2. Animals

Male Sprague–Dawley rats weighing 250–300 g of 2 months of age (purchased from Shanghai Experimental Animal Center, Chinese Academy of Sciences) were housed under standard laboratory conditions. They were allowed free access to food and water through the experiments. The rats were kept in a 12-h dark/light environment. All animal experiments were carried out in accordance with the guidelines evaluated and approved by the ethics committee of Fudan University.

2.3. Synthesis of PAMAM derivatives and preparation of various NPs

A 1:2 (mol/mol) conjugate of PAMAM and PEG (PEGylated vector), a 1:2:1 (mol/mol/mol) conjugate of PAMAM, PEG and Tf (TF-modified vector), and a 1:2:1 (mol/mol/mol) conjugate of PAMAM, PEG and Lf (Lf-modified vector) were synthesized successfully, as described previously [4,17].

PAMAM and its derivatives were freshly prepared and diluted to appropriate concentrations in distilled water. In 50 mM sodium sulfate solution, DNA solution was added to obtain specified weight ratios (10:1, PAMAM to DNA, w/w) and immediately vortexed for 30 s at room temperature. Reporter gene (GFP) and the therapeutic gene (hGDNF) were used to prepare different NPs, according to different purposes. Agarose gel electrophoresis was carried out to verify the complete complexation of dendrimers with DNA. Freshly prepared NPs were used in the following experiments.

2.4. GDNF quantification by ELISA

Normal rats were injected via the tail vein with saline or different NPs loading hGDNF at a dose of 200 μg DNA/rat. On the specified day, animals were sacrificed and their brains were rapidly removed. Then the brains were carefully washed with distilled water, and homogenized in 4 ml lysis reagent (Promega, Madison, WI, U.S.A.) using a Y92-ll tissue homogenizer. The homogenate was incubated for 30 min on ice and subsequently centrifuged at 15,000 × g for 15 min at 4 °C. GDNF expression in rat brains was measured using ELISA according to the supplier’s recommendations. The tissue protein was determined by a Bio-Rad Protein Assay Kit. The results were expressed as pg GDNF/mg protein.

2.5. Treatments and experimental groups

Rotenone emulsified in corn oil at 1.25 mg/ml was given intra-peritonely once a day at 2.5 mg/kg for 45 days. Rotenone might have peripheral toxicity to some extent. The therapeutic effect of NPs loading hGDNF was evaluated here, focusing on the lesion of rotenone on nigrostratal system. Throughout the study, animals were monitored for weight loss and signs of motor impairments, such as hypokinesia and rigidity. As a result, 4 groups of animals (n = 15 in each group) were categorized as follows: group 1, rats injected with corn oil only (without rotenone) every day without any therapy, used as the blank control; group 2, rats injected with rotenone every day and treated with five injections of Lf-modified NPs loading GFP every other day from 35th day, considered as the negative control group; group 3, rats injected with rotenone every day and treated with triple injections of Lf-modified NPs loading hGDNF every other day from 39th day, and group 4, rats injected with rotenone every day and treated with five injections of Lf-modified NPs loading hGDNF every other day from 35th day. The detailed design is shown in Table 1.

Table 1

<table>
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<tr>
<th>Group</th>
<th>Days</th>
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<td>Group 3 (Lf-modified NPs loading hGDNF)</td>
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<td>Group 4 (Lf-modified NPs loading hGDNF)</td>
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<td>BT, IH</td>
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*represents one injection of corresponding NPs; BT, behavioral test; IH, immunohistochemistry.
2.6. Behavioral tests (open-field test)

Each animal was placed in an open field of 80 cm × 48 cm, with a 50 cm wall around. The floor of the field was divided into 15 small equal-sized squares (16 cm × 16 cm). The movement and behavior of the rats were observed for 5 min. Five parameters were measured in this experiment: line crossing (number of lines crossed), rearing (number of rears), active sitting (time in seconds), head dipping (number of dips), and inactive sitting (time in seconds). Locomotor activity was measured on 15th, 25th, 35th and 45th day from the beginning of rotenone injection. Evaluation was done by an investigator unaware of the treatments.

2.7. TH immunohistochemistry

Rats were anaesthetized by 10% chloral hydrate on specified days and perfused transcardially with saline followed by 4% paraformaldehyde in phosphate buffer solution (PBS). The brains were rapidly removed and postfixed for 24 h, then transferred to PBS containing 30% sucrose at 4 °C until subsidence. Coronal brain sections were made at a thickness of 30 μm and processed for TH immunohistochemical staining to examine the extent of dopaminergic neuronal loss. Sections were incubated in 0.25% Triton X-100 for 30 min followed by 0.3% hydrogen peroxide for 15 min, then blocked with 5% BSA for 2 h. After that, the sections were incubated with anti-TH monoclonal antibody (1:200) overnight at 4 °C. Subsequently, the sections were incubated with secondary biotinylated antibody for 1 h at room temperature, followed by incubation with a streptavidin-biotinylated-horseradish peroxidase complex, following the instructions of the ABC kit. The staining was developed with DAB as the chromogen. Digital photomicrographs were captured with a Canon IXUS 950IS camera (striatum) and Olympus IX51 microscope (SN). For quantitative analysis of the numbers of TH-positive neurons, 10 coronal sections (30 μm) across the center of the SN were counted bilaterally using software Image-Pro Plus Version 6.0, in a blind fashion. TH immunoreactivity was also examined in the striatum using the same software package, expressed as the mean optical density of TH-positive striatal fibers.

2.8. HPLC analysis of monoamine neurotransmitter levels

At the completion of the study, rats were deeply anesthetized by 10% chloral hydrate and rapidly decapitated using a guillotine. Striata were dissected and homogenized by sonication in ice-cold processing solution (0.2 M perchloric acid, 0.2 mM sodium pyrosulfite, and 0.01% EDTA-2Na), and then centrifuged at 14,000 rpm for 15 min at 4 °C. The supernatants were measured for the contents of dopamine (DA) and its metabolites, dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA). Briefly, samples were quantified by reverse-phase HPLC coupled to an electrochemical detector (EC). The mobile phase consisted of 40 mM sodium acetate, 15 mM citric acid, 0.25 mM sodium octanesulfonate, 0.2 mM EDTA-2Na, and 16% methanol (pH 4.3). The filtered supernatants of samples were introduced into the autosampler carousel (Angilent 1100) with flow rate 1 ml/min using a C18 column (DIAKMA). The amount of DA and its metabolites were quantified using a standard curve generated by determining the ratio between the known amounts of amine and a constant amount of an internal standard (DHBA) and represented as ng/g wt.

2.9. Statistical analysis

All data were expressed as mean ± standard error of the mean (S.E.M). Statistical analysis was performed by one-way ANOVA followed by Student–Newman–Keuls post hoc test, using GraphPad InStat software. Statistical significance was defined as p < 0.05.

3. Results

3.1. GDNF quantification by ELISA in brain

Rats were sacrificed 2 days post a single injection of different NPs loading hGDNF. As shown in Fig. 1A, GDNF expression of PEGylated NPs, TF-modified NPs and LF-modified NPs was significantly higher than that of the saline group. A single injection of LF-modified NPs had the highest GDNF expression (653.03 ± 88.77 pg GDNF/mg protein), about 3.67-fold of that of the saline group (177.84 ± 72.44 pg GDNF/mg protein) and 1.76-fold of that of TF-modified NPs (371.13 ± 196.87 pg GDNF/mg protein). Gene expression of LF-modified NPs with single or multiple dosing intravenous injections was evaluated next. The results showed that GDNF expression with a single injection of LF-modified NPs decreased markedly in terms of time. Gene expression examined 2 days post injection was highest (653.03 ± 88.77 pg GDNF/mg protein), about 2.82-fold of that measured 10 days later (231.22 ± 29.45 pg GDNF/mg protein) (Fig. 1B). Furthermore, multiple injections of LF-modified NPs had higher GDNF expression than that of a single injection. GDNF expression with five injections of LF-modified NPs (1446.15 ± 160.62 pg GDNF/mg protein) was about 6.25-fold of that with a single injection (231.22 ± 29.45 pg GDNF/mg protein) (Fig. 1B).

3.2. Construction of rotenone-induced rat model of PD

TH immunohistochemistry results showed that dopaminergic neurons and terminals were not obviously decreased in the SN and striatum of the rotenone model animals (Fig. 2A–C and E–G) till 35th day. On the 45th day, rotenone caused a significant TH neurons and fibers loss in both the striatum and SN (Fig. 2D and H), indicating the successful development of PD rat rotenone model. These results were verified by the quantitative analysis. The number of TH-positive neurons in the SN and the mean optical density of striatal fibers were decreased on 2 days post injection of different NPs loading hGDNF. The results showed that GDNF expression with multiple injections of LF-modified NPs decreased markedly in terms of time. Gene expression examined 2 days post injection was highest (653.03 ± 88.77 pg GDNF/mg protein), about 2.82-fold of that measured 10 days later (231.22 ± 29.45 pg GDNF/mg protein) (Fig. 1B). Furthermore, multiple injections of LF-modified NPs had higher GDNF expression than that of a single injection. GDNF expression with five injections of LF-modified NPs (1446.15 ± 160.62 pg GDNF/mg protein) was about 6.25-fold of that with a single injection (231.22 ± 29.45 pg GDNF/mg protein) (Fig. 1B).

Fig. 1. Detection of GDNF content in rat brains by ELISA. A: Rats were treated with a single injection of different NPs loading hGDNF, using saline as controls. GDNF expression was examined 2 days after treatments. B: Rats were treated with a regimen of LF-modified NPs loading hGDNF, using saline as controls. GDNF expression was examined 2 days after treatments.
significantly reduced on the 45th day, about 39.1% and 14.2%, relative to those on the 35th day, respectively (Fig. 2I and J).

3.3. Behavioral observation

No rat died in the blank group without rotenone treatment. The percent mortality was 20% in the negative group (group 2), and 6.7% in the therapeutic groups (group 3 and 4), respectively.

Five parameters of behavioral observation were measured to evaluate the neuroprotective effects. Better locomotor activity was defined when parameters were observed closer to normal rats. Regarding the line-crossing parameter, there was no significant difference in the former 35 days. On the 45th day, multiple injections of Lf-modified NPs loading hGDNF (group 3 and 4) could markedly increase the number of line crossings compared to the negative group (group 2), indicating the improved locomotor activity of rats (Fig. 3A, Table 1). The result of

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**Fig. 2.** TH-immunoreactivity in the striatum and SN. Representative immuno-staining sections of model rats were shown on the 15th (A, E), 25th (B, F), 35th (C, G) and 45th (D, H) day from the beginning of continuous exposure to rotenone. Panel A–D show the striatal dopaminergic terminals, while panel E–H show the dopaminergic soma in the SN. Original magnification: 100× (panel E–H). For quantitative analysis, the number of TH-positive neurons (I) and the mean optical density of striatal fibers (J) were determined using Image-Pro Plus software. Data are expressed as mean±S.E.M (n = 3). Significance: ***p<0.001.
3.4. TH immunohistochemical staining

TH immunohistochemistry revealed severe loss of dopaminergic cells in SN and fibers in striata in the negative group treated with Lf-modified NPs loading hGDNF compared to the negative group treated with Lf-modified NPs loading reporter gene encoding green fluorescent protein (GFP), also suggesting the improved locomotor activity of rats (Fig. 3B). Furthermore, no significant difference was seen in the evaluation of rearing, head dipping and active sitting between different groups (data not shown).

3.5. Monoamine neurotransmitter analysis

Treatment with Lf-modified NPs loading GFP (group 2) could not prevent the significant decrease in the levels of DA, DOPAC and HVA in the striatum (Fig. 5). The amount of DA in group 3 treated with triple injections of Lf-modified NPs loading hGDNF (4868.95 ± 434.88 ng/g wt), about 4.8-fold of that in the negative group treated with five injections of LF-modified NP loading GFP (group 2) (1012.19 ± 56.93 ng/g wt). Furthermore, group 4 with five injections of Lf-modified NP loading hGDNF had the highest DA level (8188.77 ± 637.37 ng/g wt). In terms of DOPAC and HVA, group 4 with five injections of Lf-modified NPs loading hGDNF still had the highest level (Fig. 5B and C).

4. Discussion

In the present study, the neuroprotection in the rotenone-induced chronic PD model using a non-viral gene therapy via multiple dosing intravenous administration was successfully demonstrated.

The rotenone-induced parkinsonian rat model was reported to be more adequately mimicking the progressive features of PD than previously used models, such as gradual loss of dopaminergic neurons and development of Lewy bodies [13], besides causing highly selective nigrostriatal dopaminergic lesions [10]. The results of TH immunohistology (Fig. 2) and behavioral test (Fig. 3) verified the lesion in dopaminergic neurons of rotenone in the rat model, which presented more sustained progress without sudden high-dose external lesion. The TH immunohistological results showed severe loss of dopaminergic neurons in the bilateral striatum and SN 45 days after continuous exposure to rotenone, demonstrating the successful development of the chronic rotenone model in this study (Fig. 2D, H and J). This lesion was gradual and uniform in a global area in the nigrostriatal system, unlike 6-OHDA-induced model which caused local degeneration of neurons. However, the severe lesion of the nigrostriatal system led to significantly reduced locomotor activity and body weight in rotenone-treated rats, some of which died during the experiment (especially after 35 days exposure to rotenone). This condition can be improved by intensive care of the lesioned rats. The phenomenon of significantly reduced locomotor activity of model rats is consistent with a recent report in which Cormier and colleagues demonstrated that rats treated with rotenone continuously through osmotic mini-pumps developed severe motor and postural deficits characteristic of PD [18]. In addition, Huang and co-workers reported the selective degeneration of the nigrostriatal dopaminergic system in rotenone microspheres-treated rats [7]. In short, the rotenone model constructed in our lab produced the tendency of motor and postural deficits and significant features of PD.

The regimen was designed according to the fact that typical symptoms of PD were observed from the 35th day with continuous exposure to rotenone. Prompt therapy from the point when decreased locomotor activity was observed might rescue the dopaminergic neurons of PD rats to a maximum extent. In addition, multiple injections of DNA-loaded nanoparticles could maintain the protein expression in a higher level (Fig. 1B). Therefore, the first administration of the multiple dosages was given on the 35th day.

Till date, studies of the therapy in rotenone-induced PD animal model have been focused on small molecular drugs, such as nicotine and melatonin [18, 19]. Most recently, Marella and colleagues revealed the neuroprotective effect via a single, unilateral injection of recombinant AAV carrying the NADH dehydrogenase gene into the SN of rotenone-treated PD rats [20]. In this study, the neuroproductive effects of Lf-modified NPs encapsulating hGDNF were examined in the rotenone-induced PD rat model.

Lf-modified NPs were demonstrated to target LF receptors on brain capillary endothelial cells (BCECs) and brain parenchyma cells [14]. The characteristics of NPs were carefully investigated in previous studies [14]. Compared to non-modified NPs, Lf-modified NPs could enhance the expression of reporter genes in the brain by about 4-fold [14]. Till now, non-viral gene therapy has been applied in treating brain diseases [21, 22]. It has been reported that a single administration of OX26 (anti-transferrin receptor antibody)-modified liposomes targeting to LF receptors significantly improved the behavioral symptoms of 6-OHDA-lesioned rats [22]. Our previous studies demonstrated that exogenous gene expression of Lf-modified NPs in brains was about 2.3-fold of that...
of Tf-modified NPs [14]. And, GDNF expression of Lf-modified NPs was significantly higher than that of Tf-modified NPs after a single intravenous injection (Fig. 1A). In addition, Faucheux and coworkers reported an overexpression of Lf receptors in both microvessels and neurons of the SN in PD patients [23]. The relatively concentrated expression of exogenous genes encapsulated in Lf-modified NPs in neurons of the SN has also been demonstrated [14]. All the results showed great potential of Lf-modified NPs in treating PD.

Actually, Lf-modified NPs exhibited significantly higher neuroprotective effects in 6-OHDA-lesioned PD model in our previous work [24]. To fully verify the potential of Lf-modified NPs in gene therapy of PD, the pharmacodynamic study was further evaluated in the chronic

Fig. 4. TH-immunoreactivity in the striatum and SN. Representative immuno-staining sections of rats selected from group 1 (A, E), group 2 (B, F), group 3 (C, G), and group 4 (D, H) were shown on the 45th day from the beginning of continuous exposure to rotenone. Panel A–D show the striatal dopaminergic terminals, while panel E–H show the dopaminergic soma in the SN. Original magnification in panel E–H: 100×. For quantitative analysis, the number of TH-positive neurons (I) and the mean optical density of striatal fibers (J) were determined using Image-Pro Plus software. Data are expressed as mean±S.E.M (n=3). Significance: *p<0.05; **p<0.01; ***p<0.001.
viral gene delivery systems could obtain significant effects. Co-workers demonstrated that multiple intravenous dosing of non-viral gene delivery systems could obtain significantly enhanced neuroprotective effects than a single intravenous dose in 6-OHDA-induced PD rats [26]. Otherwise, the acute toxicity of different NPs was further investigated through immunohistochemical analysis of specific proteins on monocytes/macrophage. The results indicated that five injections of the Lf-modified NPs in the dosage used in this study did not mediate apparent toxicity in main organs [24], showing its potential in non-viral gene therapy of chronic brain diseases. However, a very recent study showed that high spinal gene expression of GDNF might associate with cord long-term potentiation which should be kept in mind [27].

Given that human and rat GDNF has very similar sequences of amino acids, and the final goal of our study is to push the potential Lf-modified NPs for the clinical application, the therapeutic gene encoding human GDNF was used for gene therapy of PD rats in this study. This can further close to the clinical application without affecting the pharmacodynamic evaluation of Lf-modified NPs in PD models.

Current results clearly demonstrate that Lf-modified NPs could successfully deliver therapeutic genes to the brain of rotenone-induced PD rats via intravenous administrations. In addition, multi-dose intravenous administration of Lf-modified NPs could achieve higher and longer expression of therapeutic proteins than a single-dose injection, and then efficiently improve the symptoms of PD rats. Therefore, this study provides a practical idea to enhance and maintain the transfection efficiency of non-viral gene vectors which are increasingly attractive in gene therapy for chronic brain disorders.

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**References**


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*Fig. 5.* Levels of DA (A), DOPAC (B) and HVA (C) in the striatum of rats in different groups. Data are expressed as mean ± S.E.M (n = 6). Significance: ***p < 0.001; **p < 0.01; *p < 0.05.*