GDNF-Transfected Macrophages Produce Potent Neuroprotective Effects in Parkinson’s Disease Mouse Model

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Abstract

The pathobiology of Parkinson’s disease (PD) is associated with the loss of dopaminergic neurons in the substantia nigra pars compacta (SNpc) projecting to the striatum. Currently, there are no treatments that can halt or reverse the course of PD; only palliative therapies, such as replacement strategies for missing neurotransmitters, exist. Thus, the successful brain delivery of neurotrophic factors that promote neuronal survival and reverse the disease progression is crucial. We demonstrated earlier systemically administered autologous macrophages can deliver nanoformulated antioxidant, catalase, to the SNpc providing potent anti-inflammatory effects in PD mouse models. Here we evaluated genetically-modified macrophages for active targeted brain delivery of glial cell-line derived neurotropic factor (GDNF). To capitalize on the beneficial properties afforded by alternatively activated macrophages, transfected with GDNF-encoded pDNA cells were further differentiated toward regenerative M2 phenotype. A systemic administration of GDNF-expressing macrophages significantly ameliorated neurodegeneration and neuroinflammation in PD mice. Behavioral studies confirmed neuroprotective effects of the macrophage-based drug delivery system. One of the suggested mechanisms of therapeutic effects is the release of exosomes containing the expressed neurotrophic factor followed by the efficient GDNF transfer to target neurons. Such formulations can serve as a new technology based on cell-mediated active delivery of therapeutic proteins that attenuate and reverse progression of PD, and ultimately provide hope for those patients who are already significantly disabled by the disease.

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Introduction

An extraordinary goal, such as brain delivery of therapeutic proteins, requires exceptional measures. The blood brain barrier (BBB) is a major obstacle to the successful treatment of many devastating diseases of central nervous system (CNS). Among them are neurodegenerative disorders (PD, Alzheimer’s disease, and amyotrophic lateral sclerosis [1–3]), infectious diseases (meningitis, encephalitis, prion disease, and HIV-related dementia [4,5]), stroke [6,7], and lysosomal storage diseases [8,9]. PD is the second most common neurodegenerative disorder of people over 65 year age; 70,000 new cases are registered in US every year. Regrettably, no therapies are currently available that can attenuate disease progression. The use of existing drugs provides only symptomatic relief to patients that are hindered by the development of drug resistance and progressive adverse side effects such as motor complications and dyskinesia [10]. In addition, most current therapies are based on pharmacological replacement of lost striatal dopamine, which only masks or reduces the declining dopaminergic activity in PD patients. Thus, novel therapies are urgently required.

Disease symptoms are characterized by lack of neurotransmitter dopamine due to the loss of dopaminergic neurons located in the nigrostriatal system. A growing body of evidence indicates the usefulness and efficacy of neurotrophic factors for the treatment of PD [11–17]. It has been confirmed that BDNF expression levels are decreased in the SNpc of PD patients [18]. Thus, the neurotrophic factors, and in particular GDNF, can promote regeneration of DA neurons and protect these cells from toxic insults [19]. Furthermore, intracranial infusions of GDNF [20,21] and BDNF [22] have been shown to provide protection and restoration of DA neurons in rodent models of PD, and in 1-methyl-4-phenyl-1,2,3,6-tetrahydro-pyridine (MPTP)- or 6-hydroxydopamine (6-OHDA)-lesioned and intact aged primates...
Unfortunately, intracranial infusion is an invasive procedure that carries a high risk of adverse effects. Thus, the development of new drug delivery systems for neurotrophic factors that can be administered systemically is crucial.

The majority of CNS disorders have in common an inflammatory component [26] resulting in the excessive production of reactive oxygen species (ROS) and subsequent neurodegeneration. Immunoocytes that include mononuclear phagocytes (dendritic cells, monocytes and macrophages) exhibit an intrinsic homing property of migrating toward the inflammation site via the processes known as diapedesis and chemotaxis [27]. Thus, immunoocytes are reported to cause BBB breakdown following brain inflammation [28–31] trafficking primarily between adjacent endothelial cells, i.e., paracellulary through the junctional complexes [32,33]. Considering that immunoocytes readily home to the sites with inflammation, we propose harnessing this natural mechanism, and use macrophages for the active targeted delivery of therapeutic proteins to the brain.

Two approaches are utilized in cell-mediated drug delivery. First, host cells are loaded with a drug, usually incorporated into a protective container, and then carry the drug to the disease side. One of the main obstacles of this approach is efficient disintegration of the entrapped foreign particles by monocytes. Second, cell-carriers are genetically modified to produce therapeutically active molecules. For example, neurotropic factors, such as glial cell-line derived neurotropic factor (GDNF), neurturin (NTN), or vascular endothelial growth factor (VEGF) produced by transfected neural stem cells (NSC) [34–36] and bone marrow-derived macrophages and microglia [37,38] were used in PD mouse models. In particular, bone marrow stem cells were transduced ex vivo with lentivirus expressing a GDNF gene driven by a synthetic macrophage-specific promoter and then transplanted into recipient mice. Macrophage-mediated GDNF delivery dramatically ameliorated PD-related inflammation and neuronal degeneration in SNpc [37] resulting in axon regeneration and reversed hypactivity in mice. Furthermore, transfected NSC were used for delivery of neurotrophic factors [39–43] in Alzheimer’s disease mouse model.

Our earlier studies suggest cell-carriers offer distinct advantages over standard drug administration regimens by providing disease-specific homing, sustained on-site drug delivery, and improved therapeutic efficacy [44–52]. Based on our previously developed cell-mediated drug delivery system, present work utilized genetically-modified autologous macrophages for active targeted delivery of GDNF to the inflamed brain. The overall scheme of these investigations is depicted in Figure 1. Macrophages were transfected ex vivo to produce GDNF, and administered intravenously in mice with PD model. This resulted in significant increases in dopaminergic neurons survival and decreases in inflammation in SNpc. Of note, genetically-modified macrophages released exosomes with incorporated GDNF that may facilitate GDNF transport into the target cells and preserve it against degradation.

Exosomes are nanosized vesicles secreted by a variety of cells, in particular, cells of the immune system: dendritic cells [53], macrophages [54], B cells [55], and T cells [56]. These extracellular vesicles were initially thought to be a mechanism for removing unneeded proteins. Recent studies revealed, they are actually specialized in long-distance intercellular communications facilitating transfer of proteins [57], functional mRNAs and microRNAs for subsequent protein expression in target cells [58,59]. To shuttle their cargo, exosomes can attach by a range of surface adhesion proteins and specific vector ligands (tetraspanins, integrins, CD11b and CD18 receptors), and fuse with target cellular membranes delivering their payload [53,60,61]. Indeed, exosomes, comprised of cellular membranes, have an exceptional ability to interact with target cells. Thus, we demonstrated here that exosomes showed an extraordinary ability to abundantly adhere andoverflow neuronal cells as was visualized by confocal microscopy. This mechanism may play a significant role in GDNF-mediated protection effects, increasing the blood circulation time, reducing immunogenicity, and facilitation of the protein transfer across the BBB and into target neurons. In addition, the membranotropic properties of GDNF-carrying exosomes may facilitate GDNF binding to GFRα-1 receptors expressed on DA neurons [62]. The present data indicate, intrinsic properties of macrophages can overcome the limitations of current common therapies, alleviate and reverse the symptoms, and may ultimately improve the quality of life of patients with various neurodegenerative disorders.

Materials and Methods

Plasmids

The gW1 high expression vectors encoding the reporter gene green fluorescent protein (GFP) (gWIGFP) under control of an optimized human cytomegalovirus (CMV) promoter followed by intron A from the CMV immediate-early (IE) gene were used throughout the study (Gene Therapy Systems, San Diego, CA). Human GDNF cDNA (NM_199234) was provided by OriGene (Rockville, MD). All plasmids are expanded in DH5α E. coli and isolated using Qiagen endotoxin-free plasmid Giga-prep kits (Qiagen, Valencia, CA) according to the supplier’s protocol.

Reagents

GenePORTER 3000 transfection agent was purchased from AMS Biotechnology, England. Lipopolysaccharides (LPS), 6-hydroxydopamine (6-OHDA), and Triton X-100 were purchased from Sigma-Aldrich (St. Louis, MO, USA). A lipophilic fluorescent dye, 1,1'-dioctadecyl-3,3,3',3'-tetramethylindo-carbocyanine perchlorate (DIL), was purchased from Invitrogen (Carlsbad, CA). Interferon gamma (IFN-γ), and murine macrophage colony-stimulating factor (MCSF) were purchased from Peprotech Inc (Rocky Hill, NJ).

Cells

Raw 264.7, a mouse macrophage cell line, was purchased from ATCC (cat # TIB-71), and cultured in Dulbecco’s Modified Eagle’s Media (DMEM) (Invitrogen, Carlsbad, CA, USA) supplemented with 2.5% horse serum and 15% FBS. Neuronal PC12 rat adrenal pheochromocytoma cell line was obtained from ATCC, and cultured in Dulbecco’s modified Eagle medium (HyClone, Utah, USA) supplemented with 10% FBS, and 1% (v/v) of both penicillin and streptomycin. The cells were grown in an incubator with optimal culture conditions of 37°C and 5% CO₂, and the medium was routinely replaced every 2–3 days.

Animals

BALB/C female mice (Charles River Laboratories, USA) eight weeks of age were treated in accordance to the Principles of Animal Care outlined by National Institutes of Health and approved by the Institutional Animal Care and Use Committee of the University of North Carolina at Chapel Hill.
Isolation of Exosomes
Condensed media from genetically-modified Raw 264.7 macrophages grown on T-75 flasks (2 x 10^6 cells/flask) was collected, and exosomes were isolated using ultracentrifugation [53]. In brief, the culture supernatants were cleared of cell debris and large vesicles by sequential centrifugation at 300 x g for 10 min, 1000 x g for 20 min, and 10,000 x g for 30 min, followed by filtration using 0.2-μm syringe filters. Then, the cleared sample was spun at 100,000 x g for 1 hour to pellet the exosomes, and supernatant was collected. The collected exosomes (10^{11}-10^{12} exosomes/flask) were washed twice with PBS. To avoid contamination of the FBS-derived exosomes, FBS was spun at 100,000 x g for 2 hours to remove exosomes before use. The recovery of exosomes was estimated by measuring the protein concentration using the Bradford assay and by Nanoparticle Tracking Analysis (NTA). The obtained exosomal fraction was re-suspended in PBS (500 μl, 1 mg/mL total protein), and characterized for size and polydispersity by Dynamic Light Scattering (DLS) and Atomic Force Microscopy (AFM). The obtained exosomal fraction was evaluated for protein content.

Western Blot Analysis
Western blots were utilized to evaluate the presence of GDNF in transfected macrophages as well as in exosomes secreted by GDNF- or empty vector-transfected macrophages. Genetically-modified macrophages and exosomes isolated from the conditioned media were treated with lysis buffer (1% Triton X-100) and protease inhibitors (Sigma) and protein concentration was determined by BCA assay. Samples were mixed with 2 x SDS sample buffer, boiled for 5 min and then separated on a 4-20% SDS-PAGE gel (BioRad, USA). Proteins were transferred to nitrocellulose membranes and GDNF was visualized with sheep polyclonal antibodies to GDNF (Millipore, AB5252, 1:2000 dilution) and secondary antibodies donkey anti-sheep IgG-HRP (Jackson ImmuneResearch; 1:5000 dilution). To correct for loading differences in cellular lysates and exosomal fractions, the levels of proteins were normalized to constitutively expressed β-actin in cells with goat polyclonal antibodies to β-actin (Abcam, ab8229; 1:500 dilution); and TSG101 in exosomes with goat polyclonal antibodies to TSG101 (Santa Cruz, SC6037; 1:200 dilution). Furthermore, for the characterization of different subtypes of polarized macrophages and released from them exosomes, membranes with corresponding transferred protein bands from macrophages or exosomes were blotted with rabbit polyclonal antibodies to CD63 (Santa Cruz, SC15363; 1:200 dilution), iNOS (Santa Cruz, SC650; 1:200 dilution), Arg1 (Santa Cruz, SC18351; 1:200 dilution) and CD206 (Santa Cruz, SC6037; 1:200 dilution) overnight at 4°C, and incubated with appropriate HRP-conjugated secondary antibodies: goat anti-rabbit IgG-HRP (Santa Cruz, SC2004; 1:2500 dilution), or donkey anti-goat IgG-HRP (Santa Cruz, SC2020; 1:5000 dilution). Membranes were washed and the expression levels were visualized with chemiluminescent substrate (Thermo Scientific) and a FluorChem E imaging system.
Figure 2. Expression of GDNF by genetically-modified macrophages. Raw 264.7 macrophages were pre-transfected with GDNF encoding pDNA and GenePorter 3000 reagent for 4 hours. Then, exosomes were collected from concomitant macrophages media for 24 hours, and GDNF levels in cellular lysates (lines 2–3) and in exosomes (lines 5–6) were examined by western blot. Commercially available GDNF (line 1) served as a positive control. Significant amount of GDNF was detected in the cells (line 3) and exosomes released from GDNF-transfected macrophages (line 5), but not in macrophages transfected with empty vector (line 2). Expressed GDNF was protected in exosomes against degradation by pronase (line 5), while control GDNF was degraded at these conditions (line 4). Destruction of exosomes by sonication eliminated their protective effect (line 6). β-actin and TSG101 served as controls for cell lysates and exosomes, respectively.

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RT-PCR analysis

Total RNA of macrophages as well as exosomal RNA was extracted using RNeasy mini kit (Qiagen, CA, USA) according to manufacturer’s instructions. Residual genomic DNA of macrophages and exosomes was removed by incubating with Rnase-free DNase set (Qiagen). RNA was analyzed and quantified using nanodrop 2000c (Thermo Scientific, USA). As quality controls of RNA samples purity from contaminating DNA and chaotropic salts was obtained by absorbance Ratio A260/A280 and A260/A230, respectively. RNA (1 μg) isolated from resting and polarized macrophages cells and their respective exosomes was reverse transcribed with Superscript III First-Strand synthesis system for RT-PCR (Invitrogen, CA, USA) according to manufacturer’s protocol. To quantify mRNA levels, quantitative reverse transcription PCR was performed using an ABI StepOne Plus Detection System (Applied Biosystems, MA, USA). TaqMan PCR Universal Master Mix and Expression Assays were from Applied Biosystems. Assay IDs: iNOS Mm00440502_m1, CD206 Mm00485148_m1 and CD63 Mm01966817_g1. CD63 was used as exosomal marker and CD11b as macrophage cells marker.

Confocal Microscopy Studies

To visualize exosomal accumulation in target neurons, Raw 264.6 macrophages were transfected with GDNF-encoding DNA, and cultured in exosome-free media for two days. Exosomes were collected from macrophage conditioned media by ultracentrifugation, and stained with the lipophilic fluorescent dye, DIL (red). PC12 neurons were cultured with DIL-labeled exosomes or PLGA nanoparticles (as a control) for three days, washed with PBS, and stained with phalldin for actin microfilaments (green). The accumulation levels were examined by confocal microscopy.

To track systemically injected macrophages in the inflamed brain, macrophages were transfected with GFP-encoding DNA as described above. For 6-OHDA intoxications, mice were stereotactically injected with 6-OHDA solution (10 μg 6-OHDA in 0.9% NaCL with 0.02% ascorbic acid), flow rate of 0.1 μL/min into the striatum (AP: +0.5; L: –2.0 and DV: –3.0 mm) [52]. Animals with brain inflammation induced were injected with GFP-expressing Raw 264.7 macrophages on day twenty one after intoxication with 6-OHDA into the intrajugular vein (i.j.). Two days later, animals were sacrificed and perfused as described [52], brains were removed, washed, post-fixed in 10% phosphate-buffered paraformaldehyde, and evaluated by confocal microscopy.

Immunohistochemical and Stereological Analyses

6-OHDA-intoxicated mice were i.j. injected with PBS, or GDNF-transfected macrophages, or macrophages transfected with empty vector 48 hours after intoxication. Twenty four days later, animals were sacrificed, perfused; brains were removed, washed, post-fixed, and immunohistochemical analysis was performed in 30 μm thick consecutive coronal brain sections [46]. For detection of microglia activation, tissue sections were incubated with primary monoclonal rat anti mouse anti-CD11b antibodies (AbD Serotec, Raleigh, NC) 1:500 dilution), and secondary biotinylated goat anti-rat antibodies (Vector Laboratories, Burlingame, CA, 1:200 dilution). For the assessment of neuroprotection effect, tyrosine hydroxylase (TH) staining was used to quantitate numbers of dopaminergic neurons [63]. The total number of TH-positive SN neurons and CD11b-positive microglia cells were counted by using the optical fractionator module in StereoInvestigator software (MicroBrightField, Inc., Williston, VT) [46].

Figure 3. Effect of exosomes released from GDNF-transfected macrophages on the axonal growth in PC12 neurons. PC12 neurons were cultured for 3 days in: (A) control media without GDNF; (B) in the presence of 100 ng/ml GDNF; or suplemented with (C) conditioned media collected from GDNF-transfected macrophages; or (D) exosomes isolated from conditioned media released from GDNF-transfected macrophages. Exosomes were fluorescently labeled with lipophilic dye, DIL (red) before the addition to the neurons (D). Following incubation, the cells were washed with PBS, and stained with phalldin for actin microfilaments (green). Confocal images revealed the pronounced development of axons upon treatment with media (C) and especially exosomes (D) released from GDNF-transfected macrophages. The bar: 20 μm.

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Behavioral Tests

For the traditional constant speed rotarod test, mice were trained and tested as previously described with slight modifications [64]. 6-OHDA-intoxicated mice were i.v. injected with PBS, or GDNF-transfected macrophages 48 hours after intoxication and the latency to fall from the rotarod was determined at three speeds (4, 5, and 7 rpm). Healthy mice i.c. injected with PBS were used as a control [65]. For apomorphine test, the animals were injected with apomorphine (0.05 mg / kg, s.c.) and rotations were scored every 10 min for 90 min [66].

Statistical Analysis

For the all experiments, data are presented as the mean ± SEM. Tests for significant differences between the groups in in vitro experiments investigating transfection of macrophages, as well as in in vivo evaluations of therapeutic effects of different drug formulations were performed using a one-way ANOVA with multiple comparisons (Fisher’s pairwise comparisons) using GraphPad Prism 5.0 (GraphPad software, San Diego, CA, USA). A standard T-test was performed when only two groups (for example, for the evaluation of expression levels of specific proteins by western blot) were compared. A minimum \( p \) value of 0.05 was chosen as the significance level for all tests.

Results

Exosomes secreted from genetically-modified macrophages contain GDNF

The optimal conditions that provide for high levels and duration of therapeutic proteins expression in macrophages identified previously [48] were used to transfect Raw 264.7 macrophages. Next, exosomes were collected from conditioned macrophages...
macrophages did not cross the BBB in the absence of brain inflammation (Fig. 4 A–C). Slides were stained for expression of mannose receptor (CD206 antibodies). Co-localization of GFP-expressing macrophages and CD206 antibodies to mannose receptor manifested in yellow staining (arrows) confirmed presence of significant amounts of M2 macrophages (Fig. 4 A, B). Healthy mice without brain inflammation (with PBS i.c. injections) were used as a control group (C). Slides were stained with for expression of mannose receptor (CD206 antibodies). Twenty four hours later mice were sacrificed, and perfused with PBS and 4% PFA. Brains were frozen, sectioned with a cryostat (10 µm thick), and examined by confocal microscopy (60 x magnification) (A, B). Healthy mice without brain inflammation (with PBS i.c. injections) were used as a control group (C). Slides were stained with for expression of mannose receptor (CD206 antibodies). Co-localization of GFP-expressing macrophages and CD206 antibodies to mannose receptor manifested in yellow staining (arrows) confirmed presence of significant amounts of the M2 genetically-modified cells in the intoxicated brain endothelial microvessles (A), and parenchyma (B). No fluorescence in the healthy brain was found (C) indicating that systemically administered Raw 264.7 macrophages did not cross the BBB in the absence of brain inflammation. The bar: 20 µm.

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Effects of GDNF-transfected Macrophages on axonal growth in vitro

A profound therapeutic effect of GDNF released from pre-loaded M2 macrophages in vitro was demonstrated in PC12 neurons that are known to express GDNF receptor (Fig. 3). Neurons were cultured for 72 hours in: (A) control media without GDNF; (B) media supplemented with high concentration of commercially available GDNF (100 ng/ml); (C) conditioned media collected from GDNF-transfected macrophages, or (D) exosomes isolated from GDNF-transfected macrophages. Following the incubation, the cells were stained with phallolin for actin microfilaments (green) and DAPI for cell nucleus (blue). In parallel, exosomes were stained with a lipophilic fluorescent dye, DID (red). Confocal images revealed the significant axonal growth in neurons cultured with conditioned media from GDNF-preloaded macrophages (Fig. 3 C) suggesting GDNF was released from the cell-carriers in a functional state. Noteworthy, treatment of PC12 neurons with exosomes released from pre-transfected macrophages resulted in the drastic increase of neuronal maturation with a pronounced outgrowth of axons and dendrites (Fig. 3 D). The effects were greater than those caused by a high dose of commercially-available GDNF (Fig. 3 B). It is likely, similar to catalase-transfected macrophages [48], exosomes with incorporated overexpressed neurotropic factor promote GDNF transfer, and secure its efficient accumulation and favorable intracellular localization in target neurons.

Differentiation of Macrophages and Using an Alternatively Activated Neuroregenerative M2 Subtype

To enhance the therapeutic effects of GDNF and avoid the pro-inflammatory neurotoxic effects of classically-activated M1 macrophages [67], Raw 264.7 macrophages were differentiated to M2 regenerative subtype. For this purpose, macrophages were cultured in the presence of Interleukin 4 (IL 4) for M2 subtype; or Interferon gamma (IFN-γ) and lipopolysaccharides (LPS), as a negative control for M1 pro-inflammatory subtype. The obtained subsets of macrophages were characterized by confocal microscopy (Fig. 4 A–C) and RT-PCR (Fig. 4 D). Mannose receptor (CD206), and Arginase 1 (Arg1) were chosen as principal markers for identifying M2 macrophages subtype. Levels of inducible Nitric Oxide Synthases (iNOS, CD86) were examined as marker of M1 macrophages subtype. Noteworthy, Arg1 and iNOS [68] were demonstrated to have anti-inflammatory and pro-inflammator properties, respectively.

Macrophages differentiated in the presence of INF-γ/LPS demonstrated considerable expression of CD 86, and low, if any, levels of mannose receptor (Fig. 4 A) that is indicative for classically activated pro-inflammatory M1 subtype. In contrast, Raw 264.7 macrophages differentiated in the presence of IL-4 showed high expression levels of mannose receptor, and low, if any, expression of CD 86 (Fig 4B) that indicative for “alternately activated” anti-inflammatory macrophages. Non-activated Mo macrophages showed low, if any expression of both CD86 and CD206 receptors (Fig 4 C). Noteworthy, the polarization to different macrophages subtypes altered the cells morphology. RT-PCR studies confirmed elevated levels of iNOS mRNA in M1 cells, and high levels of CD206 and Arginase 1 (Arg1) mRNA in M2 macrophages (Fig. 4 D).

Next, we hypothesized that exosomal content, at least in part, should reflect the content of parental macrophages. Thus, exosomes released from M2 macrophages may exhibit neuroprotective and regenerative properties by themselves. A presence of Arg1 mRNA and Arg1 protein that indicative for M2 macrophages subtype was evaluated by RT-PCR (Fig. 5 A) and western blot analyses (Fig. 5 B). The obtained data confirmed exosomes secreted by M2 regenerative macrophages, but not M1 pro-inflammatory macrophages showed high levels of Arg1 and CD206 mRNA and Arg1 protein. In contrast, expression of iNOS mRNA and protein (marker for M1 macrophages) was detected in exosomes released from M1 macrophages, but not in those secreted by M2 macrophages. Noteworthy, although the overall trend of the expression of specific markers in different subtypes macrophages and exosomes is analogous, these levels might not be completely the same, as exosomes should reflect, but not copy the content of parental macrophages they were released from. This approach was utilized further to differentiate the cell-carriers towards the M2 phenotype prior to the infusion to capitalize on the beneficial properties afforded by alternatively activated M2 macrophages in the context of PD, and minimize the potential of the cells converting to the pro-inflammatory M1 subtype.

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GDNF-Transfected Macrophages for Parkinson’s Disease Treatment
Systemically-administered M2 Macrophages Home Inflamed Brain Tissues and Sustain their Phenotype in Brain Tissues

Raw 264.6 macrophages were transfected with GFP-encoding pDNA, and then differentiated to M2 regenerative subtype as described above. BALB/c mice were i.c. intoxicated with 6-OHDA into SNpc. Twenty one days later (at the pick of inflammation), mice were systemically injected with GFP-expressing macrophages (5×10⁶ cells/ mouse in 100 μl). Twenty four hours later, the mice were sacrificed, and perfused with PBS and 4% PFA. Healthy mice without brain inflammation were used as a control group. Brain slides were stained with primary antibodies to CD 206, a marker for mannose receptor attributed to M2 subtype of macrophages. Confocal images of brains sections indicate systemically infused macrophages home the inflamed brain and localize around brain endothelial microvessels (Fig. 6 A), and parenchyma (Fig. 6 B). Co-localization of GFP-expressing macrophages (green) and CD206 staining (red) manifested in yellow confirmed, the macrophages sustain their M2 phenotype in the intoxicated brain. No macrophages were found in healthy brain (Fig. 6 C) indicating the cells do not cross the BBB in the absence of inflammation.

Macrophage-mediated Neuroprotection in PD mice

A potent neuroprotective effect of GDNF-transfected macrophages was demonstrated in the 6-OHDA-intoxicated mice (Fig. 7). GDNF-overexpressing Raw 264.7 macrophages were systemically administered to BALB/c mice with brain inflammation (1×10⁶ cells/100 μl) 48 hours after i.c. intoxication with 6-OHDA (PD mouse model). Non-intoxicated mice were used as healthy controls. 21 day later, mice were sacrificed, perfused, and the brain slides were stained for tyrosine hydroxylase (TH)-expressing DA neurons. I.c. intoxications of 6-OHDA caused substantial neurodegeneration in the SNpc (Fig. 7 C, Table 1) compared to healthy mice (Fig. 7 A, Table 1). Systemic administration of GDNF-transfected macrophages protected DA neurons against 6-OHDA intoxications (Fig. 7 E, Table 1). The numbers of TH+ neurons in SNpc of 6-OHDA animals treated with GDNF-transfected macrophages were significantly (p<0.05) greater than those 6-OHDA intoxicated, and then PBS-injected animals (Table 1). Noteworthy, empty-transfected macrophages slightly improved neuronal survival in PD animals, probably due to their regenerative M2 subtype, although this effect was not statistically significant (Fig. 7D, Table 1). Indeed, empty-transfected macrophages did not affect neuronal survival in healthy mice (Fig. 7 B, Table 1). This signifies that GDNF-transfected macrophages can efficiently protect DA neurons against 6-OHDA-induced intoxication. The therapeutic effect of GDNF-transfected macrophages was also manifested in significant decreases in inflammation and levels of activated microglia in SNpc (Figure 8, Table 1).

Finally, behavioral tests demonstrated statistically significant improvements in motor functions upon treatment with GDNF-transfected macrophages (Fig. 9). Specifically, the loss of dopaminergic input due to the lesion of the left nigro-striatal pathway resulted in number of full-body contralateral rotations induced by a dopaminergic agent, apomorphine (Fig. 9 A). In contrast, systemic administration of genetically-modified macrophages to 6-OHDA intoxicated mice drastically (p<0.005) reduced number of...
these rotations on the seventh week following the intoxication in apomorphine test to the levels of the non-intoxicated animals. Furthermore, the motor functions were preserved by systemic administration of GDNF-transfected macrophages in 6-OHDA intoxicated animals at the levels similar to those of control non-intoxicated mice, as demonstrated in rotarod test (Fig. 9 B).

**Discussion**

A long-term objective of these investigations is to develop an active targeted cell-mediated delivery of therapeutic proteins to the brain. We demonstrated here that genetically-modified macrophages can produce therapeutically-active neurotrophic factor, GDNF, and release it in extracellular vesicles, exosomes. The most important finding of this work is that GDNF-transfected macrophages provide significant neuronal protection in a PD mouse model. Our in vivo experiments demonstrated that systemically administered GDNF-expressing macrophages migrate across the BBB towards the inflammation site in large numbers, and provide efficient sustained delivery of their drug payload at the disease site that is consistent with previously reported findings [45–49]. To enforce outcomes of the new formulation, a specific subset of “alternatively activated” (M2) macrophages with regenerative functions was used. Thus, macrophages, polarized to M2 regenerative subtype, represent biologically-active carriers that can promote neuronal regeneration enhancing the therapeutic efficacy of drug formulations.

**Table 1.** Effect of GDNF-transfected macrophages on inflammation and neurodegeneration in mice with PD modela.

| Treatment                        | CD11b+ (cells per mm²) | Total N of neurons × 10⁴ |
|----------------------------------|------------------------|--------------------------|
|                                  | PBS                    | 6-OHDA                   | PBS          | 6-OHDA                 |
| PBS                              | 10.1±1.2               | 90.0±11 (**)             | 5.9±1.4     | 2.15±0.3 (**/##)       |
| GDNF-transfected macrophages     | n/a                    | 63.5±5.2 (*)             | n/a         | 3.3±0.6 (##)           |
| Empty-transfected macrophages    | 9.8±1.0                | 87.0±11.1                | 5.7±1.5     | 2.5±0.7                |

aBALB/c mice were i.c. injected with 6-OHDA. Forty eight hours later, the animals were i.v. injected with various macrophage-based formulations or PBS. A control group was i.c. injected with PBS, and then 48 hours later i.v. injected with PBS.

bTotal number of neurons was calculated in ipsilateral hemisphere.

cStatistical significance is shown by asterisk: *p<0.05 (*), and **p<0.005 (**) compared to mice with i.c. PBS injections followed by i.v. PBS injections (healthy controls); or #p<0.05 (###), compared to mice with i.c. 6-OHDA injections followed by i.v. PBS injections (PD controls). Errors are mean ±SEM; N=7.

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*Figure 8.** GDNF-transfected macrophages reduce neuro-inflammation in PD mice. BALB/c mice were i.c. injected with 6-OHDA. Forty eight hours later, animals were i.v. injected with GDNF-transfected or empty-transfected macrophages, or PBS, and 21 days later they were sacrificed, and mid-brain slides were stained for expression of CD11b, a marker for activated microglia. A 6-OHDA-mediated intoxication up-regulated expression of CD11b by microglia within the SNpc as exhibited a more amoeboid morphology in 6-OHDA-treated mice compared to ramified microglia in PBS-treated mice. In contrast, treatment of 6-OHDA-intoxicated mice with GDNF-transfected macrophages resulted in the decreased levels of CD11b compared with 6-OHDA-intoxicated control animals. Administration of empty-vector transfected macrophages did not affect the number of dopaminergic neurons in PD or healthy mice.

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The mechanism of the macrophage-mediated drug delivery is not fully understood. Thus, several independent processes may serve to improve GDNF-expressing macrophage therapeutics. In addition to the targeted tissue-specific delivery of therapeutics in macrophages [46,52], drug-carrying macrophages were shown to increase time circulation in the blood, and therefore permit sustained release of the therapeutic protein allowing the drug to enter the brain, independent of carrier cells ("depot effect") [46,52]. Furthermore, we reported earlier that significant catalase transport across the BBB can be attributed to the efficient interaction of exosomes with brain microvessel endothelial cells followed by facilitated protein transfer to the brain [47]. Similar mechanism may be employed in the GDNF transfer from carrier macrophages across the BBB. We demonstrated that exosomes secreted from preloaded with nanoformulated catalase macrophages attach to the plasma membranes, and discharge their cargo into target cells [47]. As a result, the drug-incorporated nanoparticles were transferred from macrophages to adjacent cells, and diffused broadly throughout the recipient cells avoiding degradation in lysosomes. This mechanism enabled the drug to reach different intracellular compartments such as mitochondria, cytoplasm, and endoplasmic reticulum, and produce a powerful therapeutic effects [47]. Indeed, the mechanism of GDNF action differs from catalase neuroprotective effects, as specific binding to GFRα-1 receptors is required in order to accomplish GDNF-mediated neuroprotection. Nevertheless, we hypothesized that the efficient interactions of exosomal carriers with plasma membranes of DA neurons may facilitate GDNF binding to their receptors, and result in the profound therapeutic effect. Unfortunately, at this stage of understanding, we cannot discriminate, which mechanism is the most important for the therapeutic effects of cell-based formulations. A comprehensive examination and detailed understanding of these mechanisms would be of great value in designing strategies for cell-mediated drug delivery.

Several trials human and nonhuman (primate) were initiated to evaluate efficiency of GDNF for PD treatment as well as its off-side effects. Thus, GDNF showed therapeutic promise when injected intracranially through a surgically implanted shunt [69–71]. Although there were significant improvements followed GDNF infusions, some primate subjects revealed damage in the cerebellum found in brain autopsies. This was attributed to the rapid withdrawal from large amounts of GDNF and damaging tissues by the catheter, rather than to the direct GDNF toxic effects [71]. To this end, we believe that cell-mediated GDNF delivery can eliminate these issues, providing continuous targeted GDNF delivery specifically to the inflamed brain areas. Using genetically-modified macrophages as a cell-mediated drug delivery system will target the therapeutic proteins to the brain, prolong drug half-live, and diminish drug immunogenicity. In addition, properly differentiated immune cells accumulating in traumatic, degenerative, ischemic, infectious, and autoimmune lesions of the nervous system might provide a neuroprotective effect, which may further boost the therapeutic effect of cell-mediated drug delivery systems. It is anticipated that these studies will lead to the developing a new technology based on active targeted cell-mediated delivery of therapeutic polypeptides that produce neuroprotection and neuroregeneration in patients with PD.

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Author Contributions

Conceived and designed the experiments: MH AVK EVB. Performed the experiments: YZ MH RG JB ZH. Analyzed the data: EVB. Contributed to the writing of the manuscript: EVB.
57. Johnstone RM (1992) The Jeanne Manery-Fisher Memorial Lecture 1991. Maturation of reticulocytes: formation of exosomes as a mechanism for shedding membrane proteins. Biochem Cell Biol 70: 179–190.

58. Zomer A, Vendreg T, Hopmans ES, van Eijndhoven M, Middeldorp JM, et al. (2010) Exosomes: Fit to deliver small RNA. Commun Integr Biol 3: 447–450.

59. Valadi H, Ekstrom K, Bossios A, Sjostrand M, Lee JJ, et al. (2007) Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells. Nat Cell Biol 9: 654–659.

60. Thery C, Ostroski M, Segura E (2009) Membrane vesicles as conveyors of immune responses. Nat Rev Immunol 9: 581–593.

61. Rana S, Yue S, Stadel D, Zoller M (2012) Toward tailored exosomes: the exosomal tetraspanin web contributes to target cell selection. Int J Biochem Cell Biol 44: 1574–1584.

62. Eketjall S, Fainzilber M, Murray-Rust J, Ibanez CF (1999) Distinct structural elements in GDNF mediate binding to GFRalpha1 and activation of the GFRalpha1-c-Ret receptor complex. EMBO J 18: 5901–5910.

63. Tieu K, Perier C, Caspersen C, Trismann P, Wu DC, et al. (2003) D-beta-hydroxybutyrate rescues mitochondrial respiration and mitigates features of Parkinson disease. J Clin Invest 112: 892–901.

64. Rozas G, Guerra MJ, Labandeira-Garcia JL (1997) An automated rotarod method for quantitative drug-free evaluation of overall motor deficits in rat models of parkinsonism. Brain Res Brain Res Protoc 2: 75–84.

65. Keshet GL, Tolswani RJ, Trejo A, Kraft P, Doyonnas R, et al. (2007) Increased host neuronal survival and motor function in BMTC Parkinsonian mice: involvement of immunosuppression. J Comp Neurol 504: 690–701.

66. Papatheou M, Sweeney A, McCraey A, Jenner P (2011) Induction and expression of abnormal involuntary movements is related to the duration of dopaminergic stimulation in 6-OHDA-lesioned rats. Eur J Neurosci 33: 2247–2254.

67. Kigerl KA, Gesnel JC, Ankeny DP, Alexander JK, Donnelly DJ, et al. (2009) Identification of two distinct macrophage subsets with divergent effects causing either neurotoxicity or regeneration in the injured mouse spinal cord. J Neurosci 29: 13435–13444.

68. Suschek CV, Schnoor O, Kolb-Bachofen V (2004) The role of iNOS in chronic inflammatory processes in vivo: is it damage-promoting, protective, or active at all? Curr Mol Med 4: 763–775.

69. McLeod M, Hong M, Mukhida K, Sadi D, Uhalia R, et al. (2006) Erythropoietin and GDNF enhance ventral mesencephalic fiber outgrowth and capillary proliferation following neural transplantation in a rodent model of Parkinson’s disease. Eur J Neurosci 24: 363–370.

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

71. Nutt JG, Burchiel KJ, Comella CL, Jankovic J, Lang AE, et al. (2003) Randomized, double-blind trial of glial cell line-derived neurotrophic factor (GDNF) in PD. Neurology 60: 69–73.