Journal of Controlled Release 172 (2013) 841–851

Contents lists available at ScienceDirect

Journal of Controlled Release

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

GENE DELIVERY

Doxycycline-regulated GDNF expression promotes axonal regeneration and functional recovery in transected peripheral nerve

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ARTICLE INFO

Article history:
Received 9 July 2013
Accepted 4 October 2013
Available online 17 October 2013

Chemical compounds studied in this article:
Doxycycline (PubChem CID 54686183)
DiI (PubChem CID 2762626)
Forskolin (PubChem CID 47936)
MTT (PubChem CID 64965)

Keywords:
GDNF
Conditional expression
Doxycycline
Peripheral nerve
Regeneration
Lentivirus

ABSTRACT

Increased production of neurotrophic factors (NTFs) is one of the key responses seen following peripheral nerve injury, making them an attractive choice for pro-regenerative gene therapies. However, the downside of over-expression of certain NTFs, including glial cell line-derived neurotrophic factor (GDNF), was earlier found to be the trapping and misdirection of regenerating axons, the so-called 'candy-store' effect. We report a proof-of-principle study on the application of conditional GDNF expression system in injured peripheral nerve. We engineered Schwann cells (SCs) using dendrimers or lentiviral transduction with the vector providing doxycycline-regulated GDNF expression. Injection of GDNF-modified cells into the injured peripheral nerve followed by time-restricted administration of doxycycline demonstrated that GDNF expression in SCs can also be controlled locally in the peripheral nerves of the experimental animals. Cell-based GDNF therapy was shown to increase the extent of axonal regeneration, while controlled deactivation of GDNF effectively prevented trapping of regenerating axons in GDNF-enriched areas, and was associated with improved functional recovery.

1. Introduction

Neurotrophic factors (NTFs) comprise several families of proteins essential for survival, maintenance and regeneration of neurons [1–3]. Many of them are dramatically up-regulated after peripheral nerve injury, suggesting their role in the regenerative response [4,5]. GDNF belongs to the TGF-β family of neurotrophic factors and performs versatile and distinct roles in neuronal signaling pathways. Elevation of GDNF in the sciatic nerve was reported in different injury models [4–7], implying the importance of the observed molecular changes in the successful axonal regeneration program. This led to the idea of supplementation of this and other NTFs to facilitate regeneration in the injured peripheral nerve.

NTF delivery in peripheral nerve regeneration models has been performed in many diverse ways, including direct protein application via osmotic mini-pump [8], fibrin sealants [9], injection of lentiviral [10,11] or adenoviral [12] particles, microspheres [13,14], modified scaffolds and conduits [15–18] and other methods, including gene therapy [19–23]. However, unlike some other NTFs, GDNF over-expression was reported to cause excessive trapping of regenerating axons in NTF-enriched area, resulting in impaired regeneration [10,24–26].

In search for strategies to avoid or reduce adverse side effects of prolonged GDNF exposure, we tested two approaches for time-restricted GDNF expression in Schwann cells as a physiologically relevant carrier platform. SCs are myelinating cells in the peripheral nervous system and are therefore preferred candidates for cell-based gene therapy in PN repair. NTF over-expressing SCs have been previously shown by us and others to stimulate regeneration both in the peripheral and central nervous system [27–29]. The first approach we utilized involved non-viral GDNF expression using two types of cationic dendrimers, proven earlier by us to confer plasmid-based and therefore inherently transient NTF release [30,31]. Our second approach applied a lentiviral (LV) doxycycline-regulated GDNF expression system [32]. Our results show that controlled time-restricted GDNF over-expression is capable of inducing axonal outgrowth and functional

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http://dx.doi.org/10.1016/j.jconrel.2013.10.004

recovery, while timely deactivation of GDNF synthesis will prevent regenerating axons from being misdirected or trapped locally by transgenic cells.

2. Materials and methods

2.1. Study outline

We have first modified Schwann cells (SCs) for lentivirus- or dendrimer-driven GDNF expression under a doxycycline-controlled promoter. Transgenic cells were next injected into transected and repaired sciatic nerves of isogenic Lewis rats to assess GDNF expression at 1 and 3 weeks, and axonal outgrowth and trapping at week 3. Finally, an 11 week experiment was made to analyze behavioral and functional outcomes of controlled GDNF gene therapy after sciatic nerve injury. Detailed outline is also available in Suppl. Fig. 1.

2.2. Cell culture

Schwann cells (SCs) were isolated from sciatic nerves of P2 Lewis rats according to established protocols [33,34] with minor modifications. Briefly, sciatic nerves were excised, stripped of the epineurium, and cut into 1 mm^2 pieces. Nerve segments were placed on poly-D-lysine coated 35 mm culture dishes in DMEM/F12 medium supplemented with 10% FBS, 1% penicillin/streptomycin and 0,25 μg/ml Fungizon for 3 days, allowing fibroblasts migration out from the nerve. Media were then changed to serum-free DMEM containing 1% N2, 10 ng/ml heregulin-1β, 4 μM forskolin (SC-medium) for another 3 days to stimulate Schwann cell outgrowth. At day 6, fragments were removed from the dish and explanted onto another 35-mm dish in SC-medium with 2.5% FBS. The explant procedure was repeated until little outgrowth of fibroblasts was observed, then the explants were discarded and all dishes containing SCs were purified in serum-free SC-medium. Purity of SC cultures was assessed by p75 and GFAP immunolabeling and a heregulin exclusion test [35]. Human embryonic kidney 293 T cells (HEK 293 T) were grown in DMEM-Glutamax (Gibco) with 10% heat-inactivated FBS (HyClone). Cells were routinely maintained on plastic tissue culture flasks and plates (Falcon) at 37 °C in a humidified atmosphere containing 5% CO_2/95% air.

2.3. Plasmids

Plasmids for this experiment were generated by Drs Patrick Aebischer and Didier Trono [32] and obtained from Addgene (Cambridge, USA). Transfection efficiency and promoter strength were assessed using vectors pLVTHM (Addgene plasmid 12247, constitutive GFP expression under EF1α promoter), pLVPT-tTR-KRAB (Addgene plasmid 11642, doxycycline-inducible GFP expression under PGK promoter), and pLVPT-rtTR-KRAB-2SM2 (Addgene plasmid 11652, doxycycline-repressible GFP expression under PGK promoter). Experiments involving controlled GDNF expression were made using vectors pLVPT-GDNF-tTR-KRAB (Addgene plasmid 11646, doxycycline-inducible GDNF expression) or pLVPT-GDNF-rtTR-KRAB-2SM2 (Addgene plasmid 11 647, doxycycline-repressible GDNF expression). Helper plasmids for lentivirus production were pMD2.G (Addgene plasmid 12259) and psPAX2 (Addgene plasmid 12260). Plasmids were propagated in the provided *Escherichia coli* strains DH5α or Stbl3 and isolated by Plasmid Maxi kit (Qiagen) according to the manufacturer's instructions. Purified plasmids DNA with an A260/A280 ratio of 1.8 were used for transfection.

2.4. Gene delivery vehicles and procedures

GFP or GDNF-expressing cassettes were delivered to SCs using either transduction via HIV-based lentiviruses or transfection using cationic dendrimers. Viral particles were produced by co-transfection of plasmids pMD2.G, psPAX2, and a transfer vector into HEK293 cell line as described [32]. Virus titer was determined by HEK transduction in consecutive dilutions. SC transductions were performed in 96-well polylysine-coated plates. When SCs reached 70–80% confluence, lentiviral particles were added to the well at a multiplicity of infection (MOI) of 20 for 24 h.

The dendrimers were represented by two types — polyamidoamine (PAMAM) and cationic phosphorus (CPD) dendrimers, each of fourth generation (G4). Biophysical characterization of dendrimer/vector binding properties is detailed below. PAMAM-NH_2 and CPD dendrimers were diluted to a concentration of 10 mM in terms of nitrogen residues, to allow uniform conditions for comparative analysis of their DNA condensing and gene-delivering properties [36,37]. For SC transfections, complexes of plasmid DNA (2 μg) and each of dendrimers at a charge ratio of 1:1–1:1,2 were prepared in 50 μl of 150 mM NaCl solution and the mixtures were vortexed and incubated for 15 min at room temperature. Transfection was carried out in 96-well or 6-well plates in SC culture medium, respectively, at ~65–70% confluence by 4 h or overnight exposure to a dendriplex. Doxycycline (0.5 μg/ml) was added with the media change next day post-transduction or immediately post-transfection. Expression of the reporter gene GFP was visible directly under a fluorescence microscope, and GDNF ELISA (Promega, USA) was carried out according to the manufacturer's instructions to assess GDNF production with or without doxycycline.

2.5. Biophysics of plasmid/dendrimer complexation

In ethidium bromide (EB) intercalation assay, EB in the final concentration of 7 μmol/l was added to the vectors solution (0.6 nmol/l of each vector in PBS) and its fluorescence was monitored using an LS-55B spectrofluorimeter (PerkinElmer, USA). The excitation wavelength was 484 nm; the excitation and emission slits were 10 nm. The emission spectra were recorded between 500 and 700 nm and the position of emission maximum was determined. The 'dye-vector' complex was then titrated with a dendrimer and changes in the fluorescence parameters (intensity and λ_{max}^{em}) were recorded. The data graphs were modified so that the changes in fluorescence intensity of the EB–plasmid complex when dendrimers were added were presented as

$$F^{rev} = \frac{F^{complex} - F^{pureEB}}{F_0^{complex} - F^{pureEB}} \quad (1)$$

where $F^{complex}$ is the fluorescence of EB–plasmid in the presence of dendrimer, F^{pureEB} is the fluorescence of pure (free) EB, and $F_0^{complex}$ is the fluorescence of the EB–plasmid complex in the absence of dendrimer when EB is fully bound by the plasmid.

The particle size and zeta-potential of plasmid/dendrimer complexes were measured using a Zetasizer Nano ZS (Malvern Instruments Ltd., UK) at 25 °C in a disposable Malvern plastic cuvette. Charge ratios for all dendriplexes studied were 5:1 (P:N) for both PAMAM G4 and CPD G4 dendrimers.

2.6. MTT assay

Target cells were seeded in 96-well plates allowed to grow to 60–70% confluence before transfection and subjected to the exposure of dendrimer–plasmid complex, as described above. 24 h later, 100 μl MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium-bromide) substrate solution (5 mg/ml) was added to the cells to measure mitochondrial activity. After 3 h, the supernatant was removed and the formed formazan crystals were dissolved in isopropanol/HCl, and absorbance was measured at 570 nm with a reference wavelength of 690 nm. All points were performed in triplicate. All results were calculated as a percentage related to control cells.