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Direct hippocampal injection of pseudo lentivirus-delivered nerve growth factor gene rescues the damaged cognitive function after traumatic brain injury in the rat

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ABSTRACT

Traumatic brain injury (TBI) treatment is a long-term process and requires repeated medicine administration, which, however, can cause high expense, infection, and hemorrhage to patients. To investigate how a long-term expression of nerve growth factor (*Ngf*) gene affects the injured hippocampus function post-TBI, in this study, a pseudo lentivirus carrying the *β-Ngf* fusion gene, with green fluorescence protein (GFP) gene, was constructed to show the gene expression and its ability of protecting cells from oxidative damage *in vitro*. Then, the pseudo lentivirus-carried *β-Ngf* fusion gene was directly injected into the injured brain to evaluate its influence on the injured hippocampus function post-TBI *in vivo*. We found that the expression of the pseudo lentivirus-delivered *β-Ngf* fusion gene lasted more than four-week after the cell transduction and the encoded β-NGF fusion protein could induce the neuron-like PC12 cell differentiation. Moreover, the hippocampal injection of the pseudo lentivirus-carried *β-Ngf* fusion gene sped the injured cognitive function recovery of the rat subjected to TBI. Together, our findings indicate that the long-term expression of the *β-Ngf* fusion gene, delivered by the pseudo lentivirus, can promote the neurite outgrowth of the neuron-like cells and protect the cells from the oxidative damage *in vitro*, and that the direct and single dose hippocampal injection of the pseudo lentivirus-carried *β-Ngf* fusion gene is able to rescue the hippocampus function after the TBI in the rat.

1. Introduction

Since the secondary injury of the traumatic brain injury (TBI) is a preventable process, various therapeutic approaches, including medicine administration, have been explored to treat the TBI regardless of the long-term process of the treatment. The administration of the neurotrophic factors, such as nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF), has been proved to be efficacious in repairing the injuries in both the central nervous system (CNS) and the peripheral nerve system (PNS) [21,39,40]. For example, in the CNS, there is evidence that continuous NGF infusion into brain lateral ventricle can prevent cholinergic neuronal injury post-trauma in the rat [21]. Likewise, the BDNF delivery has been reported to reduce cortical infarct volume and improve neurological deficits via the brain ventricle after brain ischemia [33] as well as to promote the connections of corticospinal neurons in a rat dorsal over-hemisection model of spinal cord injury [40]. Meanwhile, in the PNS, the BNDF infusion to the injured site of the transected sciatic nerve of the rat increases mean axonal diameter [39]. Nonetheless, such invasive and long-lasting therapeutic approaches can potentially cause hemorrhage and infection [3,29]. On the other hand, the intravenous administration of the molecules with large molecular weights, such as the NGF, is easily hampered by the blood–brain barrier (BBB).

Gene therapy is a therapeutic approach to transfer exogenous genes into target cells as a drug to treat or prevent diseases. The vectors delivering a therapeutic gene into the cells *in vitro* and *in vivo* include viral and nonviral vectors. The viral vectors comprise retroviruses, adenoviruses, adeno-associated viruses, and herpes simplex viruses, and so on, while the nonviral vectors consist of DNA plasmids, liposome, and particles, etc. The nonviral vectors show their advantages such as the capacity to allow the insert of the gene sequence with a larger size and no/very low toxicity, yet its

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disadvantages, including low transfection ability, short period of gene expression, and limited diffusion, hamper its application [9]. In contrast, the viral vectors can break down these obstacles [42].

Studies have indicated that the gene transfer via the viral vectors can improve the CNS conditions and treat diseases. For instance, there is evidence that the injection of an adenoviral vector encoding the BDNF in the lateral ventricle of the rat brain can increase the number of the neurons in the CNS [4]. Meanwhile, adeno-associated virus (AAV)-mediated *Bdnf* gene has been documented to promote the locomotor recovery of the spinal cord-injured rat [6].

Lentivirus is a subtype of retrovirus, some characteristics of which make it different from other viruses, e.g., its capability of infecting only non-dividing cells may make it suitable for neurons. It has been reported that the lentiviral vector is able to extend the exogenous gene expression to at least 12 weeks, yet the expression of the gene carried by the adenoviral vectors lasts only 2–3 weeks duo to the immunogenic response of the host [42]. To our knowledge, there has been no report showing the lentiviral vector carrying the *Ngf* gene is directly applied in the brain for the TBI treatment so far, although a study indicates an NGF-expressing graft contributes to the function recovery of the injured hippocampus in the immunosuppressed mice [23].

Therefore, to explore how the lentiviral vector-mediated *Ngf* gene directly influences the TBI, we hypothesized the expression of the *Ngf* gene delivered by the lentiviral vector could improve the damaged hippocampus function of the rat subjected to the TBI. We first cloned the gene of *β-Ngf*, which encodes one of three NGF subunits exhibiting all biological activities of the NGF, into a lentiviral vector to produce the pseudo lentivirus containing the *β-Ngf* gene, fused with a green fluorescent protein (GFP) gene. Then, we evaluated the expression of the *β-Ngf* fusion gene in neuron-like PC12 cells and its ability of protecting the cells from oxidative injury *in vitro*. Next, an *in vivo* study was used to test whether a single dose injection of the pseudo lentivirus-mediated *β-Ngf* fusion gene in the injured brain could ameliorate the lesioned hippocampus function after the TBI. We found that the *β-Ngf* fusion gene expression was not only able to last more than four weeks after the transduction, but also able to show the bioactivity to induce the differentiation of the PC12 cells. Moreover, the direct injection of the pseudo lentivirus-delivered *β-Ngf* fusion gene into the rat brain could contribute to the recovery of the impaired cognitive function following the TBI.

2. Materials and methods

2.1. Production of the pseudo lentivirus carrying the β-Ngf fusion gene

Rat total RNA was extracted and used as templates to generate cDNA library with the PrimeScript™ RT reagent Kit (Cat. #RR037A, Takara, Dalian, China). Polymerase chain reaction (PCR) was performed to amplify *β-Ngf* gene from the cDNA library by using forward primer 5′-CCGCTCGAGGCCACCATGTCCATGTTGTTCTACACTCTGA-3′ and reverse primer 5′-CGGGATCCGCCTCTTCTTGCAGCCTTC-3′. The *β-Ngf* gene was then cloned into the modified lentiviral vector of the pLVX-IRES-ZsGreen1 (Clontech, Mountain View, CA, USA) to generate a *β-Ngf* fusion gene with GFP gene.

Human embryonic kidney 293 T cells (HEK293T, ACTT, Manassas, VA, USA) were used as packaging cells. The lentiviral vector containing the *β-Ngf* fusion gene and the packaging plasmid mixes (Clontech, Mountain View, CA, USA) were co-transfected into the 293 T packaging cells. 48 hours (h) later, the pseudo lentivirus containing the *β-Ngf* fusion gene (Lv-*Ngf*-GFP) was harvested, and the titers were determined. The lentiviral vector without the *β-Ngf* gene (Lv-GFP) was used as a control.

2.2. Transduction of neuron-like PC12 cells with the pseudo lentivirus

PC12 cells were cultured in the Nutrient Mixture F12 Ham Kaighn's Modification (F12K) medium (N3520, Sigma–Aldrich, St. Louis, MO, USA) with 2.5% fetal bovine serum (Gibco, Invitrogen, Carlsbad, CA, USA) and 15% horse serum (Gibco, Invitrogen, Carlsbad, CA, USA) on a 24-well plate, which was pre-coated with 100 ug/mL poly-L-lysine (PLL, P-1399, Sigma–Aldrich, St. Louis, MO, USA) to enhance cell attachment. The procedure of the PC12 cell transduction with the pseudo lentivirus carrying the *β-Ngf* fusion gene is described as follows: first, 6×10^4 of the PC12 cells were plated in each well of the 24-well plate and incubated at 37 °C with 5% CO_2 for 24 h; second, 24 h later, the cells were divided into three groups, which were respectively transduced with no virus (PC12 only), the Lv-GFP, and the Lv-*Ngf*-GFP in the complete cell culture medium; third, the cell culture medium was replaced with the fresh medium 24 h post-transduction and changed every 3 days (d); fourth, the cells were harvested for proteins in 3, 7, 14, and 21 d after the transduction.

2.3. Expression of the pseudo lentivirus-mediated β-Ngf fusion gene in the PC12 cells

At above time points, the cells in each well were harvested and lyzed with lysis buffer (50 mM Tris–HCl, pH 8.0, 150 mM NaCl, 1% NP-40, 0.5% deoxycholate, and 0.1% SDS) for 40 minutes (min). After three freeze–thaw cycles, the cell lysate was centrifuged at 12,000 × *g* for 10 min, and the supernatant was collected. The protein concentration was determined by BCA protein assay kit (Pierce, Rockford, IL, USA). The expression of the *β-Ngf* fusion gene was analyzed by western blotting as follows: 50 micrograms (μg) proteins of each sample were loaded and separated on a 10% Tris-glycine gel, which were then transferred onto a PVDF membrane with a 0.45 micrometers (μm) pore size. The membrane was blocked with 5% nonfat dry milk in PBS-0.05% Tween-20 (PBS-T) at 4 °C, overnight, then sequentially incubated with goat anti-β-NGF primary antibody (Ab) (1:250, Sigma–Aldrich, St. Louis, MO, USA), diluted in the blocking buffer, at room temperature (RT) for 1 h. After being washed in PBS-T for 10 min, 3 times, the blot was incubated with Peroxidase AffiniPure Donkey anti-Goat IgG (1:5000, Jackson ImmunoResearch, West Grove, PA, USA) in the blocking buffer at RT for 1 h. After another round of PBS-T washing, the β-NGF antigen was detected with Immobilon™ Western Chemiluminescent HRP substrate (EMD Millipore, Billerica, MA, USA). An internal control of each sample was determined with HRP-conjugated anti-β-Actin IgG (1:5000, Proteintech Group, Chicago, USA).

Densitometric analysis was performed using the NIH Image Program (ImageJ 1.43u). The relative density of the β-NGF band, representing the expression level of the *β-Ngf* fusion gene, was determined using a ratio of the β-NGF optical density to that of the internal control β-Actin of the same sample.

2.4. Neurite outgrowth of the neuron-like PC12 cells post-transduction

3, 7, 14, 21, and 28 d after the cell transduction with the pseudo lentivirus containing the *β-Ngf* fusion gene, the neurite outgrowth in the PC12 cells was observed and photographed. The cells treated with the commercially available β-NGF protein (15 ng/mL, Sigma–Aldrich, St. Louis, MO, USA) were used as a positive control.