Intrathecal injection: An effective technique to implement gene transfection inside central nervous system
Research Article


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Summary
Spinal cord injury (SCI) will lead to functional deficit of limbs and sometimes even affect the patient’s life. Now, little measures are effective except administration of great dosage of methylprednisolone and decompression surgery in early stage. But general application of the drug may cause side effects and operation can only remove the mass that compress the dura or spinal cord. More safe and effective methods will be needed during the operation. We use intrathecal injection to implement gene transfection inside central nervous system (CNS). Through gene delivery technique, continuous expression of functional protein, such as neuro-protective factor at local site may be possible. In mRNA level, expression of reporter gene was demonstrated at the 2-week point by display of Lac Z-mRNA via RT-PCR. However, no bands of control group can be detected by this way except β-actin. Experiment group represents blue dyed cell (neuron or glial cell) in spinal cord, but control group doesn’t. The tissue proximal to injection point represents more positive cells than that of distal to the point (according to quantity analysis). The intrathecal injection technique applied in the current study make regional gene transfection within CNS system possible.

I. Introduction
Applying neurotrophic factor can be one of the possible strategies of gene therapy for spinal cord injury (SCI). Although administration of exogenous neurotrophic proteins has been used widely as a therapeutic strategy, the clinical use of this approach is limited because of short serum half life period, large molecular weight, high cost, and the blood brain barrier (BBB). During the past few years, significant progress has been made in the development of techniques for transfecting genes into spinal cord and exploring their potential to treat SCI(Wu et al. 2013). Immune responses to viral structures, and the potential for transactivation of oncogenes, may, however, limit the utility of virus-mediated gene transfer (Yang et al. 1994). Some physical methods may be efficient but rarely used in vivo (Li 2004). Within the CNS, a few attempts have been made, and they have yielded mixed results (Roessler et al. 1994; Thorsell et al. 1996; Kato et al. 2005; Pardridge et al. 2005; da Cruz et al. 2005; Lu et al. 2005; Metz et al. 2000; Tanaka et al. 2004; Zhang et al. 2004). Here we reported an expression construct encoding LacZ cDNA (Zhu et al. 2004) which can be delivered into the spinal cord of adult rats by cation transfection agent (Okumura et al. 2005) using the intrathecal injection method. In vivo studies demonstrated the cellular uptake of the complexes, and presence of transgene mRNA for up to 2 weeks.

II. Materials and methods
A. Materials
A total of 24 Sprague-Dawley (SD) (Hutson et al. and Wang et al. 2012) rats weighing between 340 to 360 g (12 per group) received either recombinant plasmid containing report gene (LacZ) or original plasmid pcDNA3.1. Animals were living in Specific pathogen Free (SPF) environment.
Room temperature was from 20 to 26°C. Light/dark cycle was 12/12h. Access to food and water was in a regular way. Plasmid pcDNA3.1-Lac Z was provided by Institute of Biochemistry and Cell Biology, Shanghai Institute for Biological Science, Chinese Academy of Science (Shanghai, China). The transfection agent DOTAP was obtained from Roche (Shanghai, China). *E. coli* DH5α, HindIII and XbaI were purchased from Takara Co. (Shanghai, China).

### B. DNA isolation and general molecular manipulations

pcDNA3.1-LacZ was transformed into *E. coli* DH5α competent cell according to the standard procedures (reference such as Molecular Cloning: A Laboratory Manual). The candidate clones were selected on LB agar plates supplemented with ampicilin (1mg/L). The plasmid PcDNA3.1-Lac Z was verified by digestion with restriction enzyme HindIII and XbaI, and the sequence was subsequently confirmed by DNA sequencing Otogenetic Co. (Shanghai, China). Large scale preparation of plasmid DNA was done using alkaline lysis method.

### C. Enzyme digestion and gene sequencing

After the recombinant eukaryotic expression vector for LacZ-pcDNA3.1 was digested with HindIII and XbaI, electrophoresis revealed 300bp fragment for Lac Z gene and 5.4kb fragment for pcDNA3.1 vector. (Fig.2) After that, gene sequencing was conducted with universal primer T7. The result was confirmed by gene bank / Fig.3.

### D. Preparation of plasmid-DOTAP mixture

The pcDNA3.1-LacZ plasmid DNA (dsDNA) for transfection was purified by column chromatography and gradient centrifugation of cesium chloride to avoid possible cytotoxic effects. 5 µg DNA (RNA) was diluted to a concentration of 0.1 µg/µl in HBS (20 mM HEPES, cell culture grade, 150 mM NaCl, pH 7.4) buffer (final volume 50 µl) in a sterile reaction tube. In a separate sterile reaction tube, 30 µl DOTAP was mixed with HBS buffer to a final volume of 100 µl. The nucleic acid solution (50 µl) was transferred to thereaction tube containing the DOTAP in HBS buffer (100 µl). The resulting transfection mixture was mixed by gently pipetting several times. Subsequently, the transfection mixture was incubated at 15-25°C for 10-15 min.

### E. Intrathecal injection of mixture

Adult SD rats were anesthetized by injection of sodium pentobarbital (Nembutal; 45mg/kg, i.p.). A T11 laminectomy was performed to expose the underlying thoracic spinal cord segment(s). To create an entry point for intrathecal injection, an incision of the ligamentum flavum between T11 and T12 was made by using a pair of micro scissors. A sterile micro syringe was then used to puncture a small hole in the dura/arachnoid mater. Penetration into the spinal subarachnoid space was confirmed by cerebrospinal fluid (CSF) leakage out from the puncture site.

The micro syringe was then inserted into the space and the previously prepared mixture was then administrated slowly (no less than 1 min) into it. To avoid the rising of intracranial pressure (ICP), CSF of same volume was drawn from subarachnoid space pre-injection. The muscle and skin were finally closed in layers using a 5-0 suture. The experimental

![Gene sequence of pcDNA3-Lac Z with universal primer T7](image-url)
protocol was previously approved by the Animal Research Ethical Committee of the University of Shanghai Jiao Tong University.

**F. Histochemistry**

Following protocols (Bianchi et al. 2002) were approved by the Shanghai Jiao Tong University Animal Care and Use Committee. Fourteen days after injection, animals were given an overdose of sodium pentobarbital (Nembutal; 60mg/kg, i.p.). Related spinal cord was then harvested, and 1 ml of 4% paraformaldehyde was infused transcardially. The spinal cord was then immersion-fixed for an additional 30–60 min. After fixation, tissues were rinsed in PBS. Spinal cord was decalcified before or after lac z staining (0.12 M EDTA; 24–48 hr). Both methods produced similar results. To detect [5-bromo-4-chloro-3-indolyl]-β-D-galactopyranoside (X-gal) activity, spinal cord was washed in lac Z wash buffer (2 mM MgCl2, 0.01% deoxycholate, 0.02% NP-40) for 30min. The spinal cord was then incubated overnight (16–24hr) at 37°C in staining buffer (wash buffer with 0.2% X-gal, 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide). Decalcified tissues were then dehydrated through a graded series of alcohols, cleared in xylene, and embedded in low melting temperature paraffin. Tissues were sectioned at 4µm and collected on glass slides. Estimates of X-gal activity were made from hematoxylin and eosinstained serial sections. The number of pixels with blue dyed tissue was counted in every fourth section (16µm intervals) under microscope by certain image processing software. Statistical analysis was completed using Student’s t-test.

**G. RT-PCR**

Total RNA was isolated by an RNeasy Mini kit (Qiagen®) according to the manufacturer’s instructions. Total tissue RNA was quantified spectrophotometrically. First-strand cDNA was reverse-transcribed from 2 µg of total RNA using random hexamer primers in a 25 µl reaction volume. After soaking gels into 0.5 µg/ml ethidium bromide solutions in 1x TAE for 20 min, reaction (final) products were visualized by UV transilluminator (Renner GmbH). Images were captured by a computer-assisted gel documentation system (multigenius®).

**III. Results**

**A. Toxicity and side effects evaluation**

There was no mortality or apparent morbidity after intrathecal administration. A lack of toxicity was also indicated by normal weight gain of treated animals (Fig. 1). The mean initial weights of control and experiment group were 270±5.0 and 250±8.0 respectively. 14 days after operation the weights of two groups were 361±4.0 and 345±6.0. The mean weight gain between two groups showed no significant difference (P>0.05). (SAS, version 8.10)

![Figure 1. Post-surgical weight development of lipid: DNA-complex-injected animals and lipid-empty vehicle complex treated controls. No difference in weight development was seen. The mean initial weight of control and experiment group was 270±5.0 and 250±8.0 respectively. 14 days after operation the weights of two groups were 361±4.0 and 345±6.0. The mean weight gain between two groups was of no significant difference (P>0.05). (Statistic software: SAS, version 8.10)](image1)

**B. PCR detection**

In agreement with the fact that Lac Z is not endogenously expressed in rat spinal cord, Lac Z activity was consistently absent in spinal cord tissue of empty vehicle (pcDNA3) treated controls. The primers of Lac Z and β-actin were designed and synthesized by Institute of Biochemistry and Cell Biology, Chinese Academy of Science. In mRNA level, expression of reporter gene was demonstrated at the 2-week point by display of Lac Z mRNA via RT-PCR (Fig 4). However, no bands of control group can be detected by this way except β-actin. (The annealing temperature was initially defined by corresponding prime’s structure and practical PCR effect.)

![Figure 4: RT-PCR result. Reporter gene expression was demonstrated at the 2-week point by demonstration of Lac Z mRNA (in Lane 6), no bands of control group can be detected (in Lane 2) except β-actin (in Lane 4). Lane 1 : Marker ; Lane 2 : Rat spinal cord that received empty vector-liposome complexes , LacZ (−) ; Lane 3 : Rat spinal cord that received plasmid-liposome complexes, β-actin (+) ; Lane 4 : Rat spinal cord that received empty vector-liposome complexes, β-actin (+) . Lane 5 : PcdNA3-LacZ( + ) ; Lane 6 : Rat spinal cord that received plasmid-liposome complexes injection , LacZ (+) ;](image2)
C. Histochemistry

While in protein level, its expression is confirmed by histochemistry (Fig.5). Transfection efficiency was monitored by histochemical staining of tissues with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside(X-gal). Experiment group (Fig.5b and 5c) represents blue dyed cell (neuron or glial cell) in spinal cord, but control group (Fig.5a) doesn’t. The tissue proximal to injection point (Fig.5b) represents more positive cells than that (Fig.5c) of distal to the point (according to quantity analysis in Tab 1), which suggests cerebrospinal fluid of subarachnoid space may affect the transfection efficiency of exogenous gene to central nervous system CNS.

A. Non-viral gene delivery

Great prospects have been demonstrated from basic study and clinic test. However, the gene therapy in CNS is still in stage of laboratory.etc. With gene delivery technique, the function recovery after SCI has achieved great success. Cografting of gel and Lingo-1 RNAi significantly promoted functional recovery and nerve regeneration, enhanced neurite outgrowth and synapses formation, preserved myelinated axons, and induced the proliferation of glial cells. In addition, the combined implantation also improved neuronal survival and inhibited cell apoptosis, which may be associated with the attenuation of endoplasmic reticulum (ER) stress after SCI. Together, it is indicated that delivering Lingo-1 shRNA by gel scaffold was a valuable treatment approach to SCI and PF-127 delivery of viral vectors to the spinal cord may provide strategy to study and develop therapies for SCI(Wu et al. 2013).

Nowadays, gene therapy in SCI is studied in many laboratories and the problems include: 1. The ideal components of transfer gene. 2. The choice of carrier. 3. Immune reaction, and prolonged survival and persistent expression of the receptor cells in the spinal cord. In the current study, we investigated whether a liposome mediated gene intrathecal administration can be expressed in spinal cord tissue of SD rats. The potential risks of using viruses to deliver and integrate DNA into host cells in gene therapy have been poignantly highlighted in recent clinical trials. Safer, non-viral gene delivery approaches have been largely ignored in the past because of their inefficient delivery and the following transient transgenic expression (Dominic et al. 2005). Some various new advances make it possible in the first time for us to achieve long-term therapeutic gene expression in humans through non-viral approaches. Thorsell et al. (1996) using commercially available lipids and a CMV-promoter driven prepro-NPY expression construct, demonstrated the feasibility of lipid mediated gene delivery and its expression in the adult rat brain, and the presence of vector derived transcript in brain tissue extract could be detected for up to 4 weeks after delivery. Okumura et al. (2005) evaluated the anti-tumor effects of osteosarcoma(HOSM-1) cells via transfer of the Bax gene, which inducing an apoptosis and caspase-independent cell death by using a cationic liposome. A supramolecular structure of liposome forming Surface-functionalized complexes is potentially suitable for transfection In vivo(Ewert et al.2005). It is demonstrated that the complexes were made by complicated structures in which the liposomes tend to aggregate and the DNA is surrounded by lipid material(Ruozi et al.2003).

**In vivo transfection inside CNS**

Gene transfer to spinal cord cells may be crucial for therapy in spinal cord injury. After intraparenchymal injection In vivo, transduction is mainly neuron. Vectors are efficient for transduction of a variety of cells like...
neurons, interneuron, dorsal root ganglia neurons and astroglia (Peluffo et al. 2012). Gene transduction therapy by vector containing NT-3 can achieve improvement in locomotor’s recovery and decrease in scar size by BBB scale and immunohistochemistry examination (Donnelly et al. 2012).

In order to directly address the issue whether expression of functional protein can be obtained in the CNS using lipid mediated gene transfer, we therefore turned to an established functional reporter system, in which activity of an enzyme protein, β-galactosidase (Lac Z), is assayed (Chunni et al. 2004). Following lipid mediated CNS gene transfer of a CCK expression unit, the functional abnormality was corrected in these animals over the period of 21 days (Zhang et al. 2004). In agreement with above report, our current data supports the feasibility of obtaining expression of functional protein in the adult rats’ spinal cord tissue following lipid mediated gene transfer. As expected, endogenous Lac Z activity in rat spinal cord tissue was absent, allowing a sensitive detection of gene transfer efficiency. This was obtained in apparent absence of adverse effects. There is the possibility that Lac Z activity will be detected even when mRNA expression no longer occurs. To control this possibility, we ensured that Lac Z mRNA (as well as plasmid DNA) was present 2 weeks after delivery of lipid: DNA complexes using RT-PCR. After that, histochemistry was done to ensure the identity of the amplified sequence. It is possible that this is in relation with advantageous properties of lipid complexes used in the current study and/or careful optimization of lipid: DNA ratios (Thorsell et al. 1996). The intrathecal injection technique applied in the current study make regional gene transfection possible, which has already been succeeded in delivery of siRNA into spinal cord and peripheral neurons (Luo et al. 2005).

B. Transfection efficiency and persistence

Adeno-associated virus (AAV) mediated gene delivery had achieved higher transduction efficiency in non-human primates (NHP). The identification of AAV1 (certain AAV vector serotype) as the optimal serotype for transducing corticospinal neurons CSNs should facilitate the design of future gene therapy strategies targeting the corticospinal tract CST for the treatment of SCI (Hutson et al. 2012). So it is encourage using the approach in human to treat motor neuron disease. In addition, the vector can enter into everywhere throughout the whole CNS (Gray et al. 2013). But there’s still some side effect like potential possibility of virus infection.

The goal of present study was therefore to optimize our delivery and expression strategies, and examine whether detectable amounts of functional protein can be expressed following lipid mediated gene transfer by intrathecal injection in the CNS.

Our data here supports the feasibility of functional transgene expression in the adult rat CNS using intrathecal delivery of lipid: DNA complexes. The most likely structure to take up and express delivered constructs appears to be the neuron and glia cell. This suggests the possibility that gene products delivered and expressed in this manner maybe secreted into the cerebrospinal fluid. And by diffusion through spinal parenchyma, can they act on other spinal cord structures. Though expression quantity was comparatively less, positive cells can still be seen in spinal segments distal to injection point. It confirmed the high transfection efficiency of our expression system. With careful optimization, expression may be lasted for more than 2 weeks or even longer.

C. Potential application of our technique

It was found (Tian W et al. 2013) that intrathecal injection of epigallocatechin-3-gallate (EGCG) can significantly improve locomotor’s recovery after spinal cord injury (SCI) by up-regulation of brain-derived neurotrophic factor (BDNF) and glial cell line-derived neurotrophic factor (GDNF). Neurotrophin like brain-derived neurotrophic factor (BDNF) has been identified as a candidate for neuroprotective strategies which can promote regeneration and re-myelination (Weishaupt et al. 2012). But those methods applied by administration protein directly won’t guarantee a long term gene level in site as gene transfection.

In vivo study in CNS (spinal cord) establishes a platform for gene therapy. In that case certain functional protein remains in low expression level after injury, which impedes the regrowth of axon and the following functional rehabilitation. And application of neurotrophic factors is one of the potent means (Blesch et al. 2012) to guide regeneration of axons across a lesion site and augment its response to injury. By these techniques, useful facts can enter into spinal cord during decompression operation by means of intrathecal injection. And 2 weeks’ continuous expression is long enough to protect the spinal cord from further attack. Therefore, we postulate intrathecal injection of certain carrier with target gene, which can reduce secondary damage and prevent spinal cord from further attack.

Recent study (Kramer et al. 2012) found induced pluripotent stem cell (IPSC) become a new cell transplantation candidate for potential clinical application in therapies designed to promote regeneration of CNS, whose effect was equivalent to embryonic stem cells (ESCS), So we think the combination of above cell transplantation with our gene transfection technique will be a new hot spot in relevant research.

Conclusion

An expression construct encoding Lac Z cDNA combined with commercially available lipids will led to cellular uptake and presence of transgene mRNA for at least 2 weeks. The current study therefore can optimize our delivery and expression strategies. Besides, detectable
amounts of functional protein can be expressed by lipid mediated gene transfer in the CNS, which probably establish a new prospect in field of gene therapy of acute spinal cord injury.

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Reference


