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A novel system for *in vivo* neprilysin gene delivery using a syringe electrode

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ABSTRACT

Existing methods for *in vivo* gene transfer are generally inefficient and have several technical problems. In the present study we aimed to develop a safe, simple and efficient gene-delivery system for *in vivo* therapeutic applications. We focused on delivery of a plasmid encoding human neprilysin (hNEP), an enzyme implicated in the degradation of toxic amyloid-beta (Abeta) peptide, with potential application in Alzheimer's disease therapy. We report the development of a syringe electrode device in which DNA is injected via one of the two syringes and DNA uptake is stimulated by application of a brief pulsed square-wave electrical field between the two syringe needles. Using this device, purified plasmid DNA encoding hNEP was injected into hindlimb skeletal muscle of 6-week-old KunMing mice and electrostimulation (50 V/cm, 6 pulses, 20 ms per pulse) was applied to the syringe needles. hNEP protein was detected in muscle, serum and brain of treated mice by western blotting and ELISA at 7, 14 and 30 days post-transfer. Importantly, hNEP levels following DNA injection alone, but without electrostimulation, were barely above background. Only low levels of muscle damage were detected following DNA injection and electrotransfer. These results demonstrate that DNA delivery by the syringe electrode technique can give rise to efficient long-term expression of the encoded polypeptide, and that the electrotransfer protocol is essential for effective plasmid DNA uptake and expression. This technique provides a safe and efficient non-viral method for *in vivo* gene delivery with potential applications in both basic research and in gene therapy of neuronal disease.

1. Introduction

The development of a simple, safe and effective gene-delivery system is needed for gene therapy of neurodegenerative conditions including Alzheimer disease (AD). AD is predominantly diagnosed in people over 65 years of age, with increases in life expectancy the prevalence of AD has increased markedly worldwide. AD is characterized by extracellular deposits of beta-amyloid (A beta), an abnormal processing product of the amyloid precursor protein (APP), and A beta toxicity has been causally implicated in neuronal loss and disease progression. Evidence is emerging that neprilysin (NEP, including MME/CD10 and MMEL1), a protein about 97 kDa molecule weight, can degrade A beta *in vivo* and slow the progression of AD. Increasing age is associated with partial NEP deficiency, and this is thought to exacerbate A beta pathology and impair cognitive function; conversely, elevation of NEP levels can delay the deposition of Abeta plaques in a mouse model of AD (Marr et al., 2003). For this reason much attention has focused on the possibility of increasing NEP levels as a therapeutic approach to AD (Mohajeri and Wolfer, 2009). To date, several studies have reported that increasing NEP levels by gene transfer into animal models of AD can decrease A beta plaques and preserve brain function (Hong et al., 2006; Iwata et al., 2004). Direct delivery into the brain of lentivirus vectors expressing human (h) NEP has been attempted (El-Amouri et al., 2008). Although this approach could have short-term benefits, the development of an immune response to the vector is ported that lentivirus-mediated hNEP gene transfer into bone marrow cells, likely to restrict clinical utility. In a parallel approach it was followed by intravenous transplantation of gene-modified cells, gave results similar to those obtained with direct gene delivery (Guan et al., 2009). More recently a novel method of lentivirus-mediated mouse NEP gene delivery into skeletal muscle was proposed, and there was no evidence of adverse effects via interference with the processing of other physiological peptide

Abbreviations: hNEP, human neprilysin; AD, Alzheimer disease; A beta, beta-amyloid; ELISA, enzyme-linked immunosorbent assay; mAb, monoclonal antibody; i.p., intraperitoneal injection; i.v., caudal vein injection; EB, Evans Blue; EBD, Evans Blue dye; PBS, Phosphate Buffer Solution; CNS, central nervous system.

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targets for NEP (Liu et al., 2009). These studies aimed to increase systemic NEP levels with a view to decreasing toxic A beta levels *in vivo*. Despite these encouraging results, there is a clear need for a gene-delivery system for the treatment of neurological diseases that would achieve long-lasting expression while avoiding immune responses against vector components and simple to manipulate. Conventional methods above for gene delivery to brain tissue generally rely on virus vectors, but the drawbacks of these approaches, in addition to anti-vector immunity, include secondary toxicity and low gene-transfer efficiency (Bergen et al., 2008; Boulaiz et al., 2005). For this reason in recent years, considerable attention has focused on the possibility of using electric pulses to delivery therapeutic molecules directly to tissues and organs, and this non-viral approach has been demonstrated to be a safe, efficient, simple and inexpensive method of gene transfer *in vivo* (Isaka and Imai, 2007). Intramuscular injection and electrotransfer of naked DNA has yielded particularly promising results, and in many instances stable and long-lasting gene expression has been reported (Quigley et al., 2006). Furthermore, gene expression in skeletal muscle can give rise sustained secretion of therapeutic proteins into the blood, as skeletal muscle has a long lifespan, and such proteins can therefore access peripheral tissues including the brain via the circulation (Trollet et al., 2006).

In this study, we aimed to develop a novel and simple approach to enhance hNEP gene delivery and protein expression levels *in vivo* using the syringe electrode of our design, that involving the injection of naked plasmids encoding hNEP into skeletal muscle combined with electroporation to facilitate DNA uptake. The method described would provide a simple, safe and effective gene-delivery system with potential therapeutic applications in AD and other diseases.

2. Materials and methods

2.1. Materials and apparatus

hNEP plasmid (PCSC-SP-PW-hNEP), in which hNEP gene expression is under the control of the cytomegalovirus (CMV) promoter, was a kind gift from Dr. Louis B Hersh and Dr. Yinxing Liu, Department of Molecular and Cellular Biochemistry, University of Kentucky. Purified monoclonal antibody (mAb) against hNEP (anti-human CD10 mAb clone MEM-78) was from Biolegend (CA, USA) and the hNEP enzyme-linked immunosorbent assay (ELISA) kit was from R&D Systems (Minneapolis, MN). Goat anti-mouse β-actin antibody was from sigma corp (MO, USA). Cell lysis buffer for Western and IP and BCA protein assay kit were all from Beyotime corp (Shanghai, China). A square-wave electroporater MPS-3002L-3 was purchased from MATRIX (ShenZhen, China). The syringe electrode (Fig. 1c) consists of two U-100 syringes bonded together with a distance of 0.6 cm between the two needles (28G1/2). The needles themselves serve as electrodes and are connected to the square-wave generator by insulated wires and clamps. DNA solution can be injected through one or both of the syringes. The square-wave output device is of our design (Fig. 1a). Six-week-old KunMing male mice (~25 g) were obtained from the Experimental Animal Centre, The Fourth Military Medical University (Xi'an, China). All experimental procedures were conducted under protocols approved by the Institutional Animal Care and Use Committee.

2.2. Electrotransfer of hNEP plasmid

In order to determine the efficiency of gene delivery, all animals were divided into five groups in random ($n = 6$ per group), including four experimental groups and one blank control group. Experimental groups were anesthetized by intraperitoneal injection (i.p.) of amobarbital (concentration of 0.5%, 0.01 ml/g). Hindlimb muscles were surgically exposed and one of the needles of the syringe electrode was inserted into the quadriceps muscle parallel to the long axis of the muscle fibers. The other needle loaded with plasmid solution (1 mg/ml in 0.9% saline) was inserted into the gastrocnemius. Unless otherwise stated, 10 μg of DNA in 20 μl of 0.9% saline was injected into the right gastrocnemius; the contralateral (left) muscle provided the control. Electric field strength is reported in terms of the ratio of the applied voltage to the distance between the electrodes (V/cm). Electric pulses were immediately applied by the syringe electrode except one experimental group with injected plasmid DNA only. The polarity of the two electrodes had no effect on the efficiency of gene transfer; the optimal electroporation protocol waves were 50 V/cm, 6 pulses, 20 ms per pulse (Fig. 1b).

2.3. Western blotting of hNEP protein

Mice were killed at 7, 14 or 30 days after gene delivery. Hindlimb muscles and brain tissues were taken out simultaneously, then homogenized in 1 ml of cell lysis buffer (20 mM Tris, pH7.5, 150 mM NaCl, 1% Triton X-100, sodium pyrophosphate, β-glycerophosphate, EDTA, Na_3VO_4, leupeptin) and added a protease inhibitor (PMSF, 1 μl) using an Ultra-Turrax T-25 basic homogenizer (IKA, Wilmington, NC, USA). Homogenates were centrifuged at 13,000 × *g* for 40 min and supernatants were collected. Protein concentrations were determined using the BCA protein assay kit. Muscle and brain homogenates (10 μg) were subjected to SDS-PAGE and western blot analysis. Bolts were incubated for an hour at room temperature with mouse anti-hNEP mAb (1:500 dilution), β-actin using goat anti-mouse IgG (1:10,000 dilution) couple with horseradish peroxidase provided the internal control.

2.4. hNEP protein levels in blood plasma

Whole blood at 7, 14 or 30 days following gene delivery was obtained via orbital sinus venipuncture and collected in tubes with added EDTA, then centrifuged at 1500 × *g* for 10 min. Levels of secreted hNEP proteins in plasma were determined by ELISA.

2.5. Muscle damage assessment

To assess damage induced by the gene-delivery method we used a dye that is selectively taken up by damaged myocytes. Evans Blue (EB) was dissolved in Phosphate Buffer Solution (PBS) (10 mg/ml) and sterilized by filtration through 0.2 μm filters. 24 h following gene delivery, mice were intravenously injected with 0.5 mg dye per 10 g body weight ($n = 6$ per group). One day after dye injection muscle tissues were examined under a fluorescence microscope.

2.6. Statistical analysis

Data were presented as means ± SEM and were analyzed by Student's *t*-test using SPSS 16.0 software (SPSS, Chicago, IL, USA). $P \leq 0.05$ was taken to indicate statistical significance.

3. Results

3.1. hNEP expression following syringe electrode delivery into skeletal muscle

We investigated whether the syringe electrode (Fig. 1c) would facilitate hNEP gene transfer. After 7, 14 and 30 days, the homogenates of skeletal muscle tissue were examined for hNEP expression by western blotting. As shown in Fig. 2, a band corresponding to hNEP was clearly visible in the treated (right, R) muscle with electrotransfer at 7, 14 and 30 days following gene delivery (R_7, R_{14} and R_{30}). A weaker signal was also seen in the contralateral