Journal of Controlled Release 212 (2015) 1–9

Contents lists available at ScienceDirect

Journal of Controlled Release

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

Focused ultrasound-induced blood-brain barrier opening for non-viral, non-invasive, and targeted gene delivery

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ARTICLE INFO

Article history:
Received 20 February 2015
Received in revised form 28 May 2015
Accepted 9 June 2015
Available online 11 June 2015

Keywords:
Focused ultrasound
Gene delivery
Blood-brain barrier opening
Liposomes
Luciferase
GDNF

ABSTRACT

Focused ultrasound (FUS) exposure in the presence of microbubbles can temporally open the blood-brain barrier (BBB) and is an emerging technique for non-invasive brain therapeutic agent delivery. Given the potential to deliver large molecules into the CNS *via* this technique, we propose a reliable strategy to synergistically apply FUS-BBB opening for the non-invasive and targeted delivery of non-viral genes into the CNS for therapeutic purpose. In this study, we developed a gene-liposome system, in which the liposomes are designed to carry plasmid DNA (pDNA, containing luciferase reporter gene) to form a liposomal-plasmid DNA (LpDNA) complex. Pulsed FUS exposure was delivered to induce BBB opening (500-kHz, burst length = 10 ms, 1% duty cycle, PRF = 1 Hz). The longitudinal expression of luciferase was quantitated *via* an *in vivo* imaging system (IVIS). The reporter gene expression level was confirmed *via* immunoblotting, and histological staining was used to identify transfected cells *via* fluorescent microscopy. In a comparison of gene transduction efficiency, the LpDNA system showed better cell transduction than the pDNA system. With longitudinal observation of IVIS monitoring, animals with FUS treatment showed significant promotion of LpDNA release into the CNS and demonstrated enhanced expression of genes upon sonication with FUS-BBB opening, while both the luciferase and GDNF protein expression were successfully measured *via* Western blotting. The gene expression peak was observed at day 2, and the gene expression level was up to 5-fold higher than that in the untreated hemisphere (compared to a 1-fold increase in the direct-inject positive-control group). The transfection efficiency was also found to be LpDNA dose-dependent, where higher payloads of pDNA resulted in a higher transfection rate. Immunoblotting and histological staining confirmed the expression of reporter genes in glial cells as well as astrocytes. This study suggests that IV administration of LpDNA in combination with FUS-BBB opening can provide effective gene delivery and expression in the CNS, demonstrating the potential to achieve non-invasive and targeted gene delivery for treatment of CNS diseases.

1. Introduction

Neurodegenerative diseases, such as ALS, Parkinson's, Alzheimer's, and Huntington's, involve the progressive, loss of neuronal structure or function, resulting in progressive degeneration and/or neuronal cell death. So far, there is no definite treatment approach to prevent or slow neurodegenerative disease progression. For example, in Parkinson's, patients continue to worsen in terms of controlling their movement and other such symptoms, despite progressive therapeutic intervention [1]. Recently, numerous preclinical and clinical studies have demonstrated the potential use of gene therapy for neurodegenerative diseases [2,3]. With the delivery of genes into the CNS, it may be possible to genetically modify neuronal cells that are directly functionally impaired, potentially relieving symptoms or even reversing the progression of the neurodegenerative diseases [4,5].

To deliver therapeutic genes into the CNS, the first obstacle to overcome is the blood-brain barrier (BBB), which effectively blocks delivery to the brain [6,7]. The BBB is formed by the tight junctions between the endothelial cells responsibly for the barrier function, preventing uptake of most therapeutic agents into the brain. To overcome this, current

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http://dx.doi.org/10.1016/j.jconrel.2015.06.010
0168-3659/

clinical trials have applied local gene delivery through direct injection [5,8], however, the craniotomy/burr-hole installation process prior to gene injection is highly invasive. When attempting non-invasive gene delivery through the circulation, there is the challenge of preventing the rapid degradation of naked DNA by nucleases in circulation and rapid clearance of DNA by the RES systems [9]. Currently to overcome these limitations, numerous nanocarrier-mediated delivery technologies, including viral- and non-viral gene delivery systems, are being developed to more effectively perform gene delivery and gene transfection [10]. Nevertheless, even novel vehicle technology that has been developed to permeate the BBB will encounter the challenge of how to locally express the therapeutic genes. Continued development of novel strategies is necessary to enhance gene transfection efficiency and improve therapeutic treatments of neurodegenerative diseases. Therefore, the two above-mentioned obstacles greatly limit the success of non-invasive CNS gene delivery for brain disease treatment.

To solve the first obstacle, a technology based on targeted focused ultrasound (FUS) exposure in the presence of microbubbles (MBs) has been developed to temporally and locally open the BBB. It has also become one of the most promising strategies to achieve non-invasive and targeted CNS gene delivery [6,7,11–13]. Ultrasound-mediated MB destruction has the potential to open the BBB tight junctions and trigger therapeutic agent deposition at specific sites with non-invasive sonication [14,15]. The interaction of ultrasonic waves with the MBs enhance acoustic cavitation-related microstreaming, sheer stress, and radiation forces directly on capillary endothelial cells, the temporal destruction of tight junctions. The subsequent leaks temporarily increase the endothelial porosity and vascular permeability [16]. Then, to solve the second hurdle, the therapeutic gene can be carried by a plasmid that is incorporated into vehicles to protect it from degradation, such as encapsulation in liposomes, creating a construct called liposomal plasmid DNA (LpDNA). We therefore hypothesized that the combination of LpDNA and FUS-induced BBB opening can be an effective gene delivery system for the brain that could provide a substitute for currently used invasive viral-vector based approaches [17,18].

The aim of this study was to develop a non-viral gene delivery system based on liposomes containing plasmid DNA (LpDNA) and concurrent use of FUS-BBB opening to induce stable CNS transgenic expression. A functionalized liposome containing a plasmid with the luciferase pLuc-N3 and glial cell line-derived neurotrophic factor (GDNF) genes was used to determine whether the developed nanocarrier was effectively delivered to the brain through FUS-induced BBB opening. The approach of using LpDNA in combination with an *in vivo* imaging system (IVIS) and nano-carriers enables semi-quantitative bioluminescent imaging assessment of transgene expression of luciferase in brains. We used IVIS to semi-quantitatively evaluate LpDNA expression in the brains, and immunoblotting analysis was employed to identify luciferase and GDNF expression. The transduced cell types were also confirmed *via* immunohistochemistry staining.

2. Material and methods

2.1. Materials and reagents

Phospholipid dipalmitoylphosphatidylcholine (DPPC), cholesterol (Chol), and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] (DSPE-PEG(2000)-amine) were purchased from Avanti Polar Lipids Inc. (Alabaster, AL). Antibodies of goat anti-luciferase, rabbit anti-IBA1, rat anti-GDNF, and mouse anti-GFAP were obtained from Promega Corp. (Madison, WI), Wako Pure Chem. Ind. (Osaka, Japan), R&D Sys. (Minneapolis, MN), and Dako Inc. (Carpinteria, CA), respectively. Goat anti-rabbit antibody conjugated with fluorescence 594 and donkey anti-mouse/anti-goat antibody conjugated with fluorescence 488 were purchased from Molecular Probes, Inc. (Grand Island, NY). Anti-fade reagent with the nuclear marker DAPI was ordered from Calbiochem. (San Diego, CA). Other chemicals, if not specified, were reagent grade from Aldrich-Sigma (St, Louis, MO).

2.2. Plasmid preparation

A single bacterial colony containing a plasmid encoding both the luciferase pLuc-N3 gene (marker gene) and the GDNF gene (therapeutic gene) was selected and inoculated in 500 mL LB medium. The mixture was then incubated for about 24 h at 37 °C with shaking at about 300 rpm. The bacterial cells were harvested by centrifugation at 3000 ×*g* for 30 min at 4 °C. Plasmid DNA (pDNA) was separated by maxiprep according to the manufacturer's instructions and concentrated using ethanol precipitation. The samples were centrifuged at 15,000 ×*g* for 10 min and the supernatant was decanted. Then, 200 μL double-distilled autoclaved water (DDAC) was added to the pellet, followed by 20 μL NaOAc along with 550 μL cold ethanol. The mixture was centrifuged at 4 °C for about 15 min. Finally the supernatant was gently removed and 100 μL of DDAC water was added to the plasmid. A spectrophotometer was used to measure the plasmid concentration. The absorbance at 260- and 280-nm was measured using a Nanodrop (ND-1000, Thermo Fisher Scientific Inc. Waltham, MA) and a ratio greater than 1.8, indicated that the purified pDNA was free of contaminants.

2.3. Liposomal plasmid DNA (LpDNA) formation and characteristics

Liposomes containing DPPC, Chol, DSPE-PEG (2000)-amine, and α-tocopherol in a 3:1:1:0.004 molar ratio were made by the film hydration method [19,20]. Briefly, the lipid mixture dissolved in chloroform was dried onto a flask to produce a homogeneous lipid film. The film was hydrated with a suspension of condensed pDNA at 42 °C until the film dispersed from the bottom of the flask. The suspension was extruded 10 times through 200-nm polycarbonate filters and 10 times through 100-nm filters using an Avanti Mini Extruder (Alabaster, AL), and then passed through a spin column to remove the unencapsulated pDNA. After centrifugation at 12,500 rpm for 15 min and collecting the supernatant, the concentration of LpDNA in the liposomes was determined spectrophotometrically by measuring the absorbance at 260 nm (Hitachi F-7000, Tokyo, Japan). Encapsulation efficiency was calculated as the fraction of original pDNA that was incorporated into the LpDNA vesicles. The size of LpDNA was measured by dynamic light-scattering (DLS) on a Nano-ZS90 particle analyzer (Malvern Instruments, Malvern, Worcestershire, UK). Each run required about 3 min, and 10 runs were averaged. The sample was prepared and imaged by cryogenic-transmission electron microscopy (cryo-TEM).

2.4. In vivo animal model

This study used a total of eighty 8-week-old Balb/c male mice, each weighing about 25 g. The mice were anesthetized with vaporized isoflurane (2%). The experimental procedure met the criteria outlined by the Institutional Animal Care and Use Committee of Chang Gung University (CGU-IACUC), and mice were handled according to the guidelines in *The Handbook of the Laboratory Animal Center*, Chang Gung University.

2.5. Focused ultrasound system

A single-element FUS transducer (Imasonics SAS, France; center frequency = 500 kHz, diameter = 60 mm, radius of curvature = 80 mm, electric-to-acoustic efficiency of 70%) was placed in an acrylic water tank filled with distilled and degassed water. The focus of the ultrasonic field was positioned to the desired region. The signals from the function generator (33120A, Agilent, Palo Alto, CA) were boosted with a power amplifier (150A100B, Amplifier Research, Souderton, PA), measured with an inline power meter (Model 4421, Bird Electronics Corp., Cleveland, OH), and used to drive the FUS transducer as shown in