Biochemical and Biophysical Research Communications xxx (2018) 1–10

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

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

Noninvasive, targeted gene therapy for acute spinal cord injury using LIFU-mediated BDNF-loaded cationic nanobubble destruction

Zhaojun Song [a], Yongjie Ye [a], Zhi Zhang [a, *], Jieliang Shen [b], Zhenming Hu [b], Zhigang Wang [c], Jiazhuang Zheng [a]

[a] Department of Orthopaedics, Suining Central Hospital, Sichuan, People's Republic of China
[b] Department of Orthopaedics, The First Affiliated Hospital of Chongqing Medical University, Chongqing, People's Republic of China
[c] Institution of Ultrasound Imaging, The Second Affiliated Hospital, Chongqing Medical University, Chongqing, People's Republic of China

ARTICLE INFO

Article history:
Received 17 January 2018
Accepted 19 January 2018
Available online xxx

Keywords:
BDNF
Cationic nanobubble
LIFU
Spinal cord injury
Gene therapy

ABSTRACT

Various gene delivery systems have been widely studied for the acute spinal cord injury (SCI) treatment. In the present study, a novel type of brain-derived neurotrophic factor (BDNF)-loaded cationic nanobubbles (CNBs) conjugated with MAP-2 antibody (mAb_{MAP-2}/BDNF/CNBs) was prepared to provide low-intensity focused ultrasound (LIFU)-targeted gene therapy. In vitro experiments, the ultrasound-targeted tranfection to BDNF overexpressioin in neurons and efficiently inhibition neuronal apoptosis have been demonstrated, and the elaborately designed mAb_{MAP-2}/BDNF/CNBs can specifically target to the neurons. Furthermore, in a acute SCI rat model, LIFU-mediated mAb_{MAP-2}/BDNF/CNBs transfection significantly increased BDNF expression, attenuated histological injury, decreased neurons loss, inhibited neuronal apoptosis in injured spinal cords, and increased BBB scores in SCI rats. LIFU-mediated mAb_{MAP-2}/BDNF/CNBs destruction significantly increase transfection efficiency of BDNF gene both *in vitro* and in vivo, and has a significant neuroprotective effect on the injured spinal cord. Therefore, the combination of LIFU irradiation and gene therapy through mAb_{MAP-2}/BDNF/CNBs can be considered as a novel non-invasive and targeted treatment for gene therapy of SCI.

1. Introduction

In recent years, gene therapy for spinal cord injury (SCI) has emerged as an exciting new direction for research, and substantial progress has been made with improved gene delivery systems. Microbubbles (MBs) have been approved for clinical application because of their good biological tolerance, and ultrasound (US)-targeted microbubble destruction (UTMD)-mediated gene delivery has demonstrated the ability to enhance the transfection efficiency of the gene into the spinal cord in vivo [1]. Common MBs with a neutral or slightly negative surface charge are often called neutral MBs. Such MBs have limited capacities for carrying plasmids because nucleic acids and the MB surfaces repulse each other [2]. Furthermore, traditional MBs are usually too large to pass through the intervals of vascular endothelial cells and reach the target cells. The recently developed cationic microbubbles (CMBs) carry a positive surface charge, which increases their gene loading capacity [3].

Special antibodies or ligands that bind to disease-associated molecular markers are often linked to microbubbles to endow them with good targeting properties. Several neuronal markers have been reported, among which microtubule-associated protein 2 (MAP-2) is considered a neuron-specific molecular marker and is best known for its microtubule-stabilizing activity and proposed roles in regulating microtubule networks in the axons and dendrites of neurons [4,5]. Brain-derived neurotrophic factor (BDNF) is one of the best-studied neurotrophic factors and has been shown to promote the growth, differentiation, survival, and synaptogenesis of central and peripheral neurons [6,7], also can protect neurons against apoptosis [8].

Herein, we designed nanoscale and targeted BDNF-loaded CNBs conjugated with MAP-2 antibody, implemented low-intensity focused ultrasound (LIFU)-targeted destruction of these CNBs and investigated the ability of this strategy to treat rats after SCI.

* Corresponding author.
E-mail address: zhangzhispine@163.net (Z. Zhang).

https://doi.org/10.1016/j.bbrc.2018.01.123
0006-291X/

2. Materials and methods

2.1. Preparation of the cationic nanobubbles

A plasmid for expression of rat BDNF was constructed by OriGene Technologies, Inc. (Beijing, China). This recombinant plasmid was designed to encode green fluorescent protein (GFP) as the reporter gene.

CMBs comprised a perfluoropropane gas (C_3F_8) core encapsulated by a PEGylated lipid shell and were prepared as previously reported [9].

Briefly, dipalmitoylphosphatidylcholine (DPPC), 2-distearoyl-sn-glycero-3-phosphoethanolamine-N-carboxy polyethylene glycol 2000 (DSPE-PEG2000-COOH) and 1,2-dipalmitoyl-3-trimethylammonium-propane (DPTAP) (Daigang Biomaterial Co., Ltd, China) were dissolved in chloroform at a molar ratio of 9: 2: 1 and dried via a rotary evaporator to form a film. The film was then hydrated with 5 μL/mL glycerol-PBS, and the CMBs were fabricated by agitating at 120 rpm and room temperature for 3 h. Then, C_3F_8 gas was injected to replace the air, and the CMBs were subjected to additional sonication (100 W, 30 s).

Sonication, which was used to reduce the size of the initial CMBs, was carried out with a 100-kMz probe at 120 W power for 10 min. To further reduce the size, low-speed centrifugation at 800 rpm was performed for 5 min to separate the small bubbles from the large ones in a thin upper layer in the suspension. Finally, the bottom layer containing phospholipid fragments and liposomes was discarded after the suspension was centrifuged at 5000 rpm for 5 min, and the precipitated CNBs were washed with deionized water.

2.2. Preparation of $mAb_{MAP\text{-}2}$-targeted BDNF-loaded cationic nanobubbles ($mAb_{MAP\text{-}2}$/BDNF/CNBs)

A carbodiimide method was used to link the free amino groups of anti-MAP-2 antibody ($mAb_{MAP\text{-}2}$) to the carboxyl groups of DSPE-PEG2000 on the CNBs. Briefly, 1×10^6 prepared CNBs were dissolved in 1 mL of MES buffer (0.1 mol/L, pH = 6.0), and then EDC/NHS (Sigma-Aldrich, USA) at a molar ratio of 1:1 were added to the CNB solution. This mixture was oscillated and incubated for 2 h at room temperature. The remaining EDC/NHS was completely removed by centrifuging in MES buffer at 5000 rpm. Both the precipitate and 1 mg/mL monoclonal $mAb_{MAP\text{-}2}$ (ABclonal Biotechnology Co., Ltd., USA) were dispersed in MES buffer (0.1 mol/L, pH = 8.0) and incubated at 4 °C for 8 h. Finally, the upper layer of the suspension was collected and washed (1000 rpm, 5 min) three times to remove the excess free antibodies and obtain the $mAb_{MAP\text{-}2}$ CNBs. These CNBs were freeze-dried and stored at −20 °C until use.

Deionized water was used to adjust the concentration of the $mAb_{MAP\text{-}2}$ CNBs to 1×10^8/mL. Next, 1×10^6 prepared $mAb_{MAP\text{-}2}$ CNBs and 20 μg of synthesized GFP-labelled BDNF plasmid were mixed well in 1 mL of deionized water and incubated at 4 °C for 1 h. Then, the suspension was centrifuged at 800 rpm for 5 min and washed with deionized water. The suspension was subsequently stored at 4 °C for another 30 min for stratification. The upper layer with the $mAb_{MAP\text{-}2}$-targeted BDNF-loaded CNBs ($mAb_{MAP\text{-}2}$/BDNF/CNBs) were collected and stored at −20 °C until use.

To assess whether the antibody had combined with the CNBs successfully, a secondary antibody labelled with FITC was incubated with the $mAb_{MAP\text{-}2}$/BDNF/CNBs at room temperature for 2 h. After a wash with deionized water, the binding between the antibody and the CNBs was observed using laser confocal scanning microscopy (LCSM, Nikon Corporation, Tokyo, Japan).

2.3. Characterization of $mAb_{MAP\text{-}2}$- targeted BDNF-loaded cationic nanobubbles

The morphological characterization of the $mAb_{MAP\text{-}2}$/BDNF/CNBs was performed using light microscopy (Leica DM500 ICC50, Germany) and transmission electron microscopy (TEM, Hitachi H-760 0, Japan). The mean diameter, polydispersity index (PDI), and zeta potential of the samples were analysed by a laser particle size analyser system (Zeta SIZER, Malvern, USA). Evaluation of the amount of plasmid DNA that can bind to the CNBs was performed as described by Panje et al. [10]. Each experiment was performed in triplicate.

2.4. Cell culture

This study was conducted in strict accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and all the experimental protocols were approved by the Animal Ethics Committee of the Sichuan Health and Family Planning Commission.

Spinal cord primary cultures were prepared from embryonic day 14 Sprague-Dawley rats as described previously [11]. Cell cultures were maintained by changing one-half of the medium once a week and used for experiments after 12–28 days *in vitro* as indicated.

2.5. In vitro assessment of the target binding

The spinal neurons were seeded into a 6-well plate with the number of 1×10^5 cells per well. After 24 h culture, 5 μg of 1,1′-dioctadecyl-3,3,3′,3′-tetramethyl-indocarbocyanine perchlorate (DiI)-labelled $mAb_{MAP\text{-}2}$/BDNF/CNBs or DiI-labelled BDNF-loaded CNBs was added into each well and incubated for 2 h at 37 °C. Then, the cells were washed with PBS buffer, fixed with 4% paraformaldehyde for 15 min, and stained with 4′,6-diamidino-2-phenylindole (DAPI) for visualization of the nuclei. The target binding of CNBs to the neurons was observed using LCSM (Nikon Corporation, Tokyo, Japan).

2.6. In vitro UTMD-mediated gene therapy

Firstly, the spinal neurons were maintained in culture media for 48 h and the culture dishes were sealed with parafilm and immersed for 2 h in a circulating water bath thermo-regulated at 43 °C for the heat stress treatments to induce apoptosis as described previously [12]. Then the treated cells were randomly divided into four groups: BDNF group (0.5 mL, 0.1 mg/mL), BDNF + LIFU group (0.5 mL, 0.1 mg/mL), BDNF/CNBs + LIFU group (0.5 mL, 0.1 mg/mL), and $mAb_{MAP\text{-}2}$/BDNF/CNBs + LIFU group (0.5 mL, 0.1 mg/mL). An LIFU gene transfection instrument (UTG 1025, Institution of Ultrasound Imaging, Chongqing Medical University) was utilized for irradiation at a radiation frequency of 1.5 W/cm^2 for 60 s. BDNF protein level was evaluated by Western blot, GFP was detected by LCSM (Nikon Corporation, Tokyo, Japan), and apoptotic incidence was detected by flow cytometry (FCM) with an Annexin V-PE/PI Apoptosis Detection Kit (BD Biosciences, USA) 48 h after LIFU-mediated gene therapy.

2.7. In vivo UTMD-mediated BDNF gene therapy

In the present study, 92 male Sprague-Dawley rats (weight, 220–250 g) were randomly divided into 4 groups (n = 23) and administered the following treatments:

1. BDNF group, 0.5 mL of BDNF plasmid (50 μg) solution was injected via the tail vein.

Please cite this article in press as: Z. Song, et al., Noninvasive, targeted gene therapy for acute spinal cord injury using LIFU-mediated BDNF-loaded cationic nanobubble destruction, Biochemical and Biophysical Research Communications (2018), https://doi.org/10.1016/j.bbrc.2018.01.123