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Enhanced blood–brain barrier penetration and glioma therapy mediated by a new peptide modified gene delivery system

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ABSTRACT

Successful glioma gene therapy lays on two important factors, the therapeutic genes and efficient delivery vehicles to cross the blood–brain barrier (BBB) and reach gliomas. In this work, a new gene vector was constructed based on dendrigraft poly-L-lysines (DGL) and polyethyleneglycol (PEG), conjugated with a cell-penetrating peptide, the nucleolar translocation signal (NoLS) sequence of the LIM Kinase 2 (LIMK2) protein (LIMK2 NoLS peptide, LNP), yielding DGL-PEG-LNP. Plasmid DNA encoding inhibitor of growth 4 (ING4) was applied as the therapeutic gene. DGL-PEG-LNP/DNA nanoparticles (NPs) were monodispersed, with a mean diameter of 90.6 ± 8.9 nm. The conjugation of LNP significantly enhanced the BBB-crossing efficiency, cellular uptake and gene expression within tumor cells. Mechanism studies suggested the involvement of energy, caveolae-mediated endocytosis and macropinocytosis in cellular uptake of LNP-modified NPs. MTT results showed that no apparent cytotoxicity was observed when cells were treated with synthesized vectors. Furthermore, LNP-modified NPs mediated strongest and most intensive apoptosis on the tumor site, and the longest median survival time of glioma-bearing mice. All the results demonstrated that LNP is a kind of efficient CPPs especially for BBB-crossing application, and DGL-PEG-LNP/DNA is a potential non-viral platform for glioma gene therapy via intravenous administration.

1. Introduction

Glioma is considered one of the most aggressive tumors, whose therapy is extensively limited by the existence of blood–brain barrier (BBB) at the early stage [1]. And also, lack of sharp border of glioma makes conventional therapies (surgery, chemotherapy and radiotherapy) less efficient [2]. Gene therapy has been considered one of the most promising approaches for glioma therapy. Successful glioma gene therapy lays on two important factors, the therapeutic genes and efficient delivery vehicles to cross the BBB and reach gliomas.

The therapeutic genes are very important for successful gene therapy. Several gene medicines, especially plasmid DNA encoding human tumor necrosis factor-related apoptosis-inducing ligand (hTRAIL) [2–4], have been exploited for glioma gene therapy. Recently, inhibitor of growth 4 (ING4), which was dramatically downregulated in a variety of tumors such as glioma [5], lung cancer [6] and melanoma [7], has attracted much attention as a strong tumor suppressor. ING4 can exert its tumor-suppressive effect via multiple pathways, for example, to have important impacts in oncogenesis, DNA repair, tumor growth, angiogenesis migration and gene transcription regulation [8]. Most interestingly, ING4 has a potential role on the growth suppression and apoptosis enhancement in glioma via the activation of mitochondrial-induced apoptotic pathway and the hindrance of the cell cycle progression [9]. And also, autophagy contributes to ING4-induced glioma cell death [10]. The low expression and dysfunction of ING4 might be correlated with the tumorigenesis and progression of gliomas [5,9]. Thus, upregulation and supplement of ING4 is considered a probable way to treat gliomas. However, current therapy based on ING4 gene focuses on adenovirus-mediated gene

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transfer [11,12]. Usage of viral vectors might cause dangerous side effects such as immunological responses [13]. Successful construction of plasmid DNA encoding ING4 (ING4) has been reported [14]. To create more safe and efficient gene delivery vehicles is the key to the success of non-viral gene therapy.

Non-viral gene therapy attracts lots of interest due to its relative biosafety [15]. However, the therapeutic efficiency is of high demand to be enhanced. Till now, variety of non-viral gene delivery systems including polyplexes [2,4,16], liposomes [3,17], micelles [18], and nanobubbles [19], have been reported for glioma gene therapy. Among these systems, dendrigraft poly-L-lysines (DGL) was proved to be a potential gene delivery vector due to its cationic, monodispersed, well-defined, biodegradable and biocompatible properties [20] and easy modification of targeting ligands such as a choline derivate [4].

Peptide-mediated drug delivery has recently been widely explored, especially cell-penetrating peptides (CPPs) [21,22]. CPPs are small peptides containing 10-30 amino acids, which have the ability to cross the cell membranes [23]. CPPs including HIV-1 transactivating protein (Tat) [24,25] and Antennapedia (Antp) [26] have been reported to successfully assist drug delivery systems from extracellular to intracellular to play their functions. Also, some CPPs could mediate therapeutic molecules across the BBB and subsequent delivery into central nervous systems (CNS) [27], showing promising potential in developing drug delivery technologies for brain disorders. It has been reported that the nucleolar translocation signal (NoLS) sequence of the LIM Kinase 2 (LIMK2) protein (LIMK2 NoLS peptide, LNP) could not only transport cargoes to nucleus but also have the characteristic cell-penetrating function [28]. Furthermore, an in vivo assay confirmed that LNP has the potential for transporting biomolecules across the BBB [28]. The BBB-crossing, cell-penetrating and nucleolar localizing characteristics make LNP extremely suitable for drug delivery to CNS, especially for glioma drug delivery.

In this study, a new peptide LNP-modified gene delivery system was constructed based on DGL dendrimer and polyethyleneglycol (PEG). ING4 was constructed and exploited as the therapeutic gene for glioma therapy. Complex of the cationic DGL-based vector with the negative gene via electrostatic interaction was achieved as DGL-PEG-LNP/ING4 nanoparticles (NPs). The physicochemical characterizations, BBB-crossing efficiency, cellular uptake, gene expression and the therapeutic effect to glioma-bearing mice were systematically investigated in this work.

2. Materials and methods

2.1. Materials

LNP with the sequence "KKRT LRKN DRKK RC" was synthesized and characterized by Chinese Peptide Company (Hangzhou, China). Dendrigraft poly-L-lysines (DGL) containing 123 primary amino groups for generation 3 was purchased from COLCOM (Montpellier Cedex, France). α-Malemidyl-ω-N-hydroxysuccinimidyl polyethyleneglycol (NHS-PEG-MAL, MW 3500) was obtained from Jenkem Technology Co., Ltd (Beijing, China). The plasmid pEGFP-N_2 (GFP, Clontech, Palo Alto, CA, USA), plasmid DNA encoding luciferase pGL2-control vector (Promega, Madison, WI, USA) and pcDNA3.1-ING4 (ING4, Boshang Biotech, Shanghai, China) were purified using QIAGEN Plasmid Mega Kit (Qiagen GmbH, Hilden, Germany). Fluorescent dyes, ethidium monoazide bromide (EMA), YOYO®-1 iodide (YOYO-1) and 4′,6′-diamidino-2-phenylindole (DAPI) were purchased from Molecular Probes (Eugene, OR, USA). Temozolomide capsules were kindly provided by Prof. Wei Shi in Affiliated Hospital of Nantong University. TdT-mediated dUTP nick end labeling (TUNEL) apoptosis detection kit (FITC-labeled) was purchased from KeyGEN Biotech (Nanjing, China). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), phenylarsine oxide (PhAsO), filipin complex (from Streptomyces filipinesis), colchicine, dimethylsulfoxide (DMSO) and other reagents, if not specified, were purchased from Sigma–Aldrich (St. Louis, MO, U.S.A.). All the chemicals were used without further purification.

2.2. Synthesis and characterization of different vectors

The synthesis of different vectors was performed according to previous work with proper modification [29]. Briefly, DGL-PEG was obtained via the reaction of NH_2- in DGL and NHS- in PEG in phosphate-buffered solution (PBS, pH 8.0), at room temperature for 2 h with a DGL to PEG molar ratio at 1:2, purified by ultrafiltration using a 5-kDa molecular weight cutoff membrane. Then DGL-PEG was resolved in PBS (pH 7.0) and further conjugated with LNP via the reaction of MAL-in PEG and SH- in LNP, at room temperature for 24 h with a DGL to LNP molar ratio at 1:1. The vector DGL-PEG-LNP was obtained after ultrafiltration through a membrane (cutoff = 3 kDa). To characterize whether DGL-PEG-LNP was successfully synthesized, the vector was dissolved in D_2O and analyzed in a 400 MHz nuclear magnetic resonance (NMR) spectrometer (Varian, USA).

2.3. Preparation of different NPs

The preparation of different NPs was performed according to previous work [20]. DGL-PEG and DGL-PEG-LNP were freshly prepared and diluted to appropriate concentrations with PBS (pH 7.4). DNA solution (100 μg/ml in 50 mM sodium sulfate) was added to obtain specified weight ratios and immediately vortexed for 30 s at room temperature. Freshly prepared NPs were used in all experiments.

DNA was labeled with two different dyes, red EMA [20] and green YOYO-1 (according to the manufacturer's instruction). Fluorescent NPs were prepared using different dye-labeled plasmid DNA.

2.4. Agarose gel electrophoresis

The DNA-binding efficiency was determined via agarose gel electrophoresis. DGL-PEG-LNP/DNA at various DGL to DNA ratios (w/w) were mixed with appropriate amounts of 6× loading buffer and then electrophoresed on 0.7% (w/v) agarose gel containing ethidium bromide (0.25 μg/ml). The location of DNA in the gel was analyzed on an electrophoresis apparatus and photographed.

2.5. Physicochemical characterization

The mean diameter and zeta potential of DGL-PEG-LNP/DNA with a DGL to DNA weight ratio at 3:1 were measured by dynamic light scattering using a Nano-ZS instrument (Malvern, UK). To observe the morphology of DGL-PEG-LNP/DNA, transmission electron microscopy (TEM) was performed on a JEM-2010 instrument with an acceleration voltage of 200 kV (JEOL, Japan).

2.6. Cell culture

Brain capillary endothelial cells (BCEC) were kindly provided by Prof. J. N. Lou (Clinical Medicine Research Institute of the Chinese-Japanese Friendship Hospital). BCEC were expanded and maintained in special Dulbecco's modified Eagle medium (DMEM) supplemented with 20% heat-inactivated fetal bovine serum (FBS), 100 μg/ml epidermal cell growth factor (ECGF), 2 mM L-glutamine, 40 U/ml heparin, 100 U/ml penicillin, 100 μg/ml streptomycin, and cultured at 37 °C under a humidified atmosphere containing 5% CO_2.

Human glioma cells (U87), human hepatocellular carcinoma cells (Bel-7402) and human neuroblastoma cells (SH-SY5Y) were maintained in DMEM, RMPI-1640 and DMEM, respectively, with the addition of 10% heat-inactivated FBS, 1% L-glutamine, 1% penicillin and 1% streptomycin, at 37 °C and in 5% CO_2. All the cells were obtained from Shanghai Cell Bank, Chinese Academy of Sciences.

2.7. Transport of NPs across the BBB monolayer

BBB monolayers were established according to our previous work [30]. Briefly, BCEC were seeded at a density of 5×10^4 cells per cm^2 in 24-well transwell filters (Falcon Cell Culture Insert, Becton Dickinson Labware, NJ, USA). After about a week, BCEC were examined for complete confluency under the microscope. The monolayer integrity was monitored using an epithelial volt-ohmmeter (Millicell ERS®, Millipore, MA, USA) to measure the transendothelial electrical resistance (TEER). BBB monolayers with TEER above 200 Ω cm^2 were used for transport studies.

DGL was radiolabeled with ^{125}I as described previously with radioactivity of 1 μCi/μg DGL [30,31]. ^{125}I-DGL was used to prepare DGL-PEG/DNA and DGL-PEG-LNP/DNA with a DGL to DNA weight ratio at 3:1. While BBB monolayers were examined for complete confluency, 0.6 μCi ^{14}C-sucrose in 250 μl FBS-free medium was added to the donor chamber at time 0 to monitor the integrity of BBB monolayers during the whole experiment. Simultaneously, BBB monolayers were treated with radiolabeled NPs with a final amount of 20 μg DGL per well. The incubation was performed at 37 °C on a shaking platform. An aliquot of 20 μl sample was removed from each acceptor chamber and replaced with the same volume of fresh FBS-free medium, at specified time point. The radioactivity of ^{125}I was assessed using a γ-counter (SN-695, China). Half of the 20 μl aliquot was dissolved in 1 ml scintillation cocktail, and analyzed in a liquid scintillation counter (Beckman, USA). The correction factor of ^{14}C-sucrose was 25% for the equipment. The apparent permeability (P_{app}) was calculated according to Irvine et al. as follows [32]:

$$P_{app} = \frac{\partial Q}{\partial t \cdot C_0 \cdot A}$$

where $\partial Q/\partial t$ is the permeability rate (nmol/s), C_0 is the initial concentration (nmol/ml) in the donor chamber, and A is the surface area (cm^2) of the filter membrane. The data of cumulative transport amount of NPs were also presented.