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Angiogenesis-targeting microbubbles combined with ultrasound-mediated gene therapy in brain tumors

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A B S T R A C T

The major challenges in gene therapy for brain cancer are poor transgene expression due to the blood-brain barrier (BBB) and neurologic damage caused by conventional intracerebral injection. Non-viral gene delivery using ultrasound-targeted microbubbles (MBs) oscillation *via* the systematic transvascular route is attractive, but there is currently no high-yielding and targeted gene expression method. In this study, we developed a non-viral and angiogenesis-targeting gene delivery approach for efficient brain tumor gene therapy without brain damage. We developed a VEGFR2-targeted and cationic microbubbles (VCMBs) gene vector for use with transcranial focused ultrasound (FUS) exposure to allow transient gene delivery. The system was tested in a brain tumor model using the firefly luciferase gene and herpes simplex virus type 1 thymidine kinase/ganciclovir (pHSV-TK/GCV) with VCMBs under FUS exposure for transgene expression and anti-tumor effect. *In vitro* data showed that VCMBs have a high DNA-loading efficiency and high affinity for cancer cells. *In vivo* data confirmed that this technique enhanced gene delivery into tumor tissues without affecting normal brain tissues. The VCMBs group resulted in higher luciferase expression (3.8 fold) relative to the CMBs group (1.9 fold), and the direct injection group. The tumor volume on day 25 was significantly smaller in rats treated with the pHSV-TK/GCV system using VCMBs under FUS ($9.7 \pm 5.2\,mm^3$) than in the direct injection group ($40.1 \pm 4.3\,mm^3$). We demonstrated the successful use of DNA-loaded VCMBs and FUS for non-viral, non-invasive and targeted gene delivery to brain tumors.

1. Introduction

Glioblastoma multiforme (GBM) is the most common malignant brain cancer. Despite the available standard treatments (surgery, radiotherapy and chemotherapy), patients have a poor outcome and high recurrence rate [1]. Moreover, available therapies have several side effects, including brain tissue damage and systemic drug toxicity [2]. Compared with traditional treatments, gene therapy provides targeted and selective killing of tumor cells *via* the insertion of genes that alter cellular sensitivity to drugs. Antitumor suicide gene therapy is a new strategy that involves tumor-targeted transfection of a suicide gene that encodes an enzyme for converting non-toxic prodrugs into toxic products to kill tumor cells. The most widely investigated genetic prodrug activation system is the herpes simplex virus thymidine kinase (pHSV-TK)/ganciclovir (GCV) paradigm, which has shown good success in many tumor models, including GBM [3–6]. The HSV-TK gene encodes thymidine kinase, which phosphorylates GCV to monophosphorylate GCV, which is then metabolized to its triphosphate form by cellular kinases. Triphosphorylated GCV can incorporate into nascent DNA and terminate DNA replication, resulting in death of tumor cells

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and surrounding non-transfected tumor cells (*i.e.*, bystander effect) [7]. However, gene transfection methods such as viral vector with transcranial direct injection or systemic administration of non-viral gene carrier often induce significant inflammatory responses, brain damage and off-target effects [8–11]. Hence, it is important to develop a non-viral, non-invasive, and target-specific gene delivery platform for GBM treatment.

Currently, cationic microbubbles (CMBs), which consist of positively charged lipid shells, provide a simple strategy to carry nucleic acids *via* charge interactions, due to the anionic phosphate backbone of DNA [12–14]. The lipid shell of CMBs can also protect DNA from DNase degradation in serum before the DNA arrives at the lesion site [12–14]. With focused ultrasound (FUS) sonication, the cavitation effects from circulating MBs can produce local intracellular gap junctions of the cerebral vesicular endothelial cells, endocytotic cellular uptake and vessel disruption, increasing the blood-brain barrier/blood-tumor barrier (BBB/BTB) permeability of surrounding cerebral vasculature, allowing trans-vascular gene delivery [15,16]. In addition, MBs disintegrate into several nanometer-sized gene-loaded fragments under FUS exposure, promoting the introduction of DNA into cells in the immediate perivascular region [17,18]. However, gene delivery by DNA-loaded MBs is limited by an insufficient gene transfection rate, which can be attributed to (1) the presence of only a few MBs in the FUS field, and (2) the need of MBs to be close to, or in direct contact with the cell membrane. Further, because glioma cells are typically surrounded by normal neural cells, a tumor cell-specific gene delivery platform needs to be developed to reduce off-target effects. To solve these issues, a more efficient design that involves modifying special antibodies or ligands that bind to disease-associated molecular markers expressed on vascular endothelial cells of the tumor are needed to promote local aggregations of MBs to increase the gene concentration. Few studies have focused on the antitumor effects of DNA-loaded CMBs in the central nervous system (CNS) system, none of which used MBs capable of active targeting.

Angiogenesis involves the recruitment of new blood vessels to provide adequate blood supply during solid tumor growth [19]. Vascular endothelial growth factor (VEGF) receptor 2 (VEGFR2) is a key regulator of angiogenesis, and activation of the VEGF/VEGFR2 axis triggers multiple signaling networks that result in endothelial cell survival, differentiation, and vascular permeability. Also, VEGFR2 is a specific endothelial molecular marker of angiogenesis, and is overexpressed in tumor endothelial cells during tumor angiogenesis occurring in GBM [20]. The properties of VEGFR2 make it amenable for use in VEGFR2-targeted strategies for tumor theranostics [21]. It has been reported that inhibition of the VEGF/VEGFR2 pathway with humanized antibody bevacizumab results in prolonged survival in cancer patients [22]. Also, several VEGFR2-targeted imaging strategies have been designed for detecting tumor location, evaluating treatment outcome and assessing tumor malignancy [23].

In this study, we developed a non-viral and angiogenesis-targeting gene delivery platform for increasing gene delivery efficiency by taking advantage of the active targeting capability. The suicide gene (pHSV-TK) or reporter gene (red firefly luciferase gene, pLUC) were utilized to form DNA-loaded and VEGFR2-targeted CMBs (DNA-VCMBs). First, the DNA payload and tumor-binding capability of VCMBs were estimated. Then, the gene delivery efficiency of DNA-VCMBs and FUS was assessed by live-cell imaging and bioluminescence imaging *in vitro*. The *in vivo* transgene expression and distribution were assessed by enzyme-linked immunosorbent assays and bioluminescence imaging. Finally, the antitumor ability of FUS-mediated pHSV-TK gene transfection and GCV was validated in a rat GBM tumor model by estimating tumor volume and animal survival rate. The results demonstrated that DNA-VCMBs are useful non-viral gene carriers for brain tumor-specific gene delivery.

2. Materials and methods

2.1. Plasmid preparation

Expression vectors for pLUC (6.6 kb, Origene Technologies) and pHSV-TK (7.2 kb were prepared as described in a previous study) [24], and both of them driven by the cytomegalovirus promoter. All plasmid DNA was purified from a culture of *Escherichia coli* DH5α with a Plasmid Maxi Kit (NucleoBond Xtra Maxi EF, Macherey-Nagel, Düiren, Germany). The DNA concentration was determined by measuring the absorbance at 260 nm (A260) by NanoDrop (NanoDrop 2000, Thermo Fisher Scientific, IL, USA). The A260/A280 ratio of all plasmid DNA was between 1.8 and 1.9, which indicated that the purified DNA was free of proteins and RNA.

2.2. Preparation and property of DNA-loaded VEGFR2-targeted CMBs

2.2.1. Preparation of DNA-loaded VEGFR2-targeted CMBs

The lipids 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC, Avanti Polar Lipids, AL, USA), 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[carboxy(polyethylene glycol)-2000] (DSPE-PEG-2000, Avanti Polar Lipids), 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[biotinyl (polyethylene glycol)-2000] (DSPE-PEG2000-Biotin, Avanti Polar Lipids) and 1,2-dipalmitoyl-3-trimethylammonium-propane (DPTAP, Avanti Polar Lipids) were dissolved in chloroform at a weight ratio of 9:1:1:1, and drained *via* rotary evaporator to form a dried film. The film was then hydrated with glycerol-PBS buffer (5%) in an airtight vial. The gas in the vial was removed and refilled with perfluoropropane (C_3F_8) gas. The vial was produced by intense shaking with an agitator [25]. During the agitation process, part of the lipid structures disrupted and further encapsulated C_3F_8 gas to re-form monolayer air bubbles with C_3F_8 gas core. Mechanical shaking by agitator for 45 s gave rise to MBs with a C_3F_8 gas core and lipid shell. Biotinylated MBs were centrifuged for 2 min at 6000 rpm to remove unincorporated free lipids and resuspended with glycerol-PBS buffer. Then, 1 mg FITC-labeled avidin (10 mg/mL) (Sigma Aldrich, MO, USA) was added to the biotinylated MBs and incubated for 10 min at room temperature (RT). The MBs were centrifuged again for 2 min at 6000 rpm to remove excess avidin and resuspended with glycerol-PBS buffer. Finally, 75 μg biotin-conjugated rat anti-mouse CD309 (VEGFR2) monoclonal antibody (0.5 mg/mL) (Biolegend, CA, USA) was added to the avidin-biotinylated MB suspension and incubated for 10 min [26–28]. The VCMBs were centrifuged again for 2 min at 6000 rpm and re-suspended to remove excess biotinylated antibody. For comparison, the control MBs and CMBs were fabricated by the same method as that used for VCMBs, but without the addition of DPTAP and anti-VEGFR2 antibody. Details on control MBs and CMBs preparation, and antibody binding efficiency are described in Supplementary material.

The pLUC or pHSV-TK was mixed with VCMBs by gentle inversion for 30 min to promote DNA attachment to VCMBs. The DNA-loaded VCMB sample was centrifuged at 6000 rpm for 1 min to separate unbound pLUC/pHSV-TK from well-conjugated pLUC-VCMBs/pHSV-TK-VCMBs. For comparison, the pLUC-CMBs/pHSV-TK-CMBs were fabricated by the same method used for VCMBs.

2.2.2. Characteristics of DNA-loaded VCMBs

The size and concentration of pLUC-VCMBs, pLUC-CMBs, and control MBs were measured by coulter counter (Multisizer 3, Beckman Coulter Inc., CA, USA). The zeta-potential of the MBs was analyzed by a dynamic light-scattering system (Nanosizer-S, Malvern, London, UK). The morphology and dispersion of the MBs were observed under bright-field microscopy (Eclipse Ti, Nikon, Tokyo, Japan). The green fluorescence image of pLUC-VCMBs showed avidin-biotin interaction by adding FITC-avidin to the shell of MBs. The DNA loaded onto the shell of MBs was visualized by propidium iodide staining (PI,

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