Biomaterials 34 (2013) 2142–2155

Contents lists available at SciVerse ScienceDirect

Biomaterials

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

Antiangiogenic-targeting drug-loaded microbubbles combined with focused ultrasound for glioma treatment

Ching-Hsiang Fan [a,1], Chien-Yu Ting [a,1], Hao-Li Liu [b,**], Chiung-Yin Huang [c], Han-Yi Hsieh [b], Tzu-Chen Yen [d], Kuo-Chen Wei [c], Chih-Kuang Yeh [a,*]

[a] Department of Biomedical Engineering and Environmental Sciences, National Tsing Hua University, No. 101, Section 2, Kuang-Fu Road, Hsinchu 30013, Taiwan, ROC
[b] Department of Electrical Engineering, Chang-Gung University, No. 259, Wen-Hwa 1st Road, Kuei-Shan, Tao-Yuan 33302, Taiwan, ROC
[c] Department of Neurosurgery, Chang Gung University and Memorial Hospital, No. 5, Fu-Shing Road, Kuei-Shan, Tao-Yuan 33305, Taiwan, ROC
[d] Department of Nuclear Medicine and Molecular Imaging Center, Chang Gung University and Memorial Hospital, No. 5, Fu-Shing Road, Kuei-Shan, Tao-Yuan 33305, Taiwan, ROC

ARTICLE INFO

Article history:
Received 28 September 2012
Accepted 27 November 2012
Available online 14 December 2012

Keywords:
Targeting microbubble
Focused ultrasound (FUS)
Glioblastoma multiforme (GBM)
Chemotherapy
Blood–brain barrier (BBB)
Antiangiogenic therapy

ABSTRACT

Current chemotherapeutic agents do not only kill tumor cells but also induce systemic toxicity that significantly limits their dosage. Focused ultrasound (FUS) in the presence of microbubbles (MBs) is capable of transient and local opening of the blood–brain barrier (BBB) that enhances chemotherapeutic drug delivery into the brain parenchyma for glioma treatment. Our previous results demonstrated the success of combining the use of drug (1,3-bis(2-chloroethyl)-1-nitrosourea, BCNU)-loaded MBs with FUS-induced BBB opening to improve local drug delivery and reduce systemic toxicity. Here we introduce novel VEGF-targeting, drug-loaded MBs that significantly further enhance targeted drug release and reduce tumor progression in a rat model, using the FUS-BBB opening strategy. This study suggests a promising direction for future MB design aimed at targeted brain tumor therapy, and the possible future extension of MB application towards theragnostic use.

1. Introduction

Glioblastoma multiforme (GBM) is the most aggressive and common glioma in humans and is classified as tumor grade 4 by the World Health Organization (WHO) grading scheme [1–3]. The conventional treatment of GBM with a combination of surgical resection, radiotherapy and chemotherapy results in a median patient survival of only 1–2 years after diagnosis and no significant differences in overall survival [4–6]. 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU, Carmustine) is one of the most widely used antineoplastic agents against malignant glioma [7] and acts as an alkylating agent to induce interstrand cross-links in DNA, RNA and proteins [8]. The clinical effectiveness of BCNU, however, is limited by its short half-life (about 20–50 min *in vitro* and less than 15 min *in vivo* [7,9]), by the small fraction of the systemically administered dose reaching the tumor mass at an effective concentration, and by its accompanying systemic toxic side-effects including myelosuppression, hepatic and renal toxicity, and pulmonary fibrosis [10]. The blood–brain barrier (BBB) provides another significant barrier to effective chemotherapy by preventing all large molecules and more than 98% of small-molecule drugs from entering the brain parenchyma [11].

Angiogenesis is an essential process to provide adequate blood supply for sustaining the growth of solid tumors with diameters greater than 1–2 mm [12,13]. GBM and other malignant brain tumors are highly vascularized and rich in vascular endothelial growth factor (VEGF) that promotes angiogenesis [14,15]. VEGF-A plays a key role in paracrine regulation of glioma angiogenesis by binding to and activating VEGF receptor tyrosine kinase type R2 (VEGF-R2; also called Flk-1 or KDR) on endothelial cells. Endothelial cells in gliomas are known to overexpress VEGF-R2 [16], and mis-regulation of VEGF-A/VEGF-R2 interactions induce excessive angiogenesis in tumor regions that cause endothelial cell proliferation and migration, increasing the risk of tumor growth, recurrence, and metastasis [17,18]. The antiangiogenic drug bevacizumab is a humanized monoclonal antibody that targets VEGF-A and prevents it from activating its receptors, especially VEGF-R2 [19,20]. It was approved by the FDA in 2009 to be combined with chemotherapeutic agent delivery (Temozolomide) in GBM patients, with the assumption that targeting chemotherapeutics to regions of

* Corresponding author. Tel.: +886 3 571 5131x34240; fax: +886 3 571 8649.
** Corresponding author. Tel.: +886 3 211 8800x5677; fax: +886 3 211 8026.
E-mail addresses: haoliliu@mail.cgu.edu.tw (H.-L. Liu), ckyeh@mx.nthu.edu.tw (C.-K. Yeh).
[1] These authors contributed equally to this work.

0142-9612/$ – see front matter © 2012 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.biomaterials.2012.11.048

VEGF-A overexpression could suppress the microvascular proliferation of GBM [21].

A new technique using microbubbles (MBs) with transcranial focused ultrasound (FUS) exposure to transiently and locally open the BBB has been developed to allow better delivery and penetration of chemotherapeutic agents into brain tumors [22,23]. This approach can facilitate the delivery of therapeutic agents, genes, and antibodies from vasculature into the brain parenchyma that is normally restricted because of the BBB [24]. Targeted drug delivery is achieved by encapsulating a chemotherapeutic agent in MBs and then inducing its local release at BBB-opened regions. This concept has been successfully validated by our previous development of high-payload drug (BCNU)-carrying MBs (BCNU-MBs) which improved BBB permeability and enhanced local drug concentration upon FUS exposure [25].

The aim of this study was to propose a design involving conjugation of VEGF-A ligand to BCNU-encapsulating MBs to produce antiangiogenic-targeting drug-loaded MBs (VEGF-BCNU-MBs). We hypothesized that these VEGF-BCNU-MBs would be directed to regions of tumor vasculature where overactive angiogenesis is marked by overexpression of VEGF-R2 receptor.

2. Materials and methods

2.1. Preparation of drug-loaded and antiangiogenic-targeting microbubbles

2.1.1. Preparation of biotinylated drug-loaded MBs

Biotinylated lipid-formed BCNU-MBs were prepared by the thin-film hydration method as previously described [25]. The lipid shell composition of the MBs was 66 mol% 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), 17 mol% 1,2-distearoyl-sn-glycero-3-phosphoethanolamine (DSPE)–polyethylene glycol (PEG) 2000, and 17 mol% DSPE–PEG2000–Biotin (Avanti Polar Lipids, AL, USA). Briefly, the lipids were mixed in chloroform with 2.8 mg BCNU solution (100 mg BCNU dissolved in 3 mL purified ethanol) (Bristol-Myers Squibb, NY, USA) in a vial and dried at 10 °C under reduced pressure over 24 h with a rotary evaporator (Rotavapor R-210, Büchi Labortechnik AG, Flawil, Switzerland). The dried lipid-film was then hydrated with 5 μL/mL glycerol phosphate-buffered saline (PBS), and the gas in the vial was replaced with perfluoropropane (C_3F_8). Biotinylated BCNU-MBs were formed by shaking method with an agitator for 45 s. The hydrophobicity of the BCNU drug molecules (molecular weight: 214.05) allowed them to be embedded in the MBs where they were attached to the phospholipid shell by both electrostatic and hydrophobic interactions.

2.1.2. Preparation of antiangiogenic-targeting drug-loaded MBs

The concept of targeting and drug-loaded MBs is demonstrated in Fig. 1. Biotinylated BCNU-MBs were centrifuged for 3 min at 6000 rpm (mini-micro centrifuge, Bertec Enterprise Co., Ltd., Taiwan) to remove unencapsulated drugs and resuspended with fresh glycerol PBS buffer. The biotinylated BCNU-MBs were then incubated with fluorescein amidite (FAM)-labeled avidin (10 mg/mL) (Sigma Aldrich, MO, USA) for 10 min at room temperature (RT). The MBs were subsequently centrifuged and resuspended with fresh glycerol PBS buffer to remove excess avidin. Finally, 75 μg biotin-conjugated rat anti-mouse CD309 (VEGF-R2, Flk-1) monoclonal antibody (0.5 mg/mL) (Biolegend, CA, USA) was added to the MB suspension and incubated for 10 min. The VEGF-BCNU-MBs were again centrifuged and resuspended to remove excess biotinylated antibody. Control pure lipid MBs, with and without drug encapsulation, and with and without anti-VEGF-R2 antibody were also prepared.

2.2. Characterization of antiangiogenic-targeting drug-loaded MBs

2.2.1. Size distribution, concentration and drug loading efficiency

The size distribution and concentration of VEGF-BCNU-MBs were determined by a Coulter counter equipped with a 30 μm sensor orifice (Multisizer 3, Beckman Coulter, FL, USA) and a 0.7–20 μm range. Smaller particles (<1 μm) were measured by dynamic light scattering (Nanosizer-S, Malvern, London, UK). The drug loading efficiency was determined by a reverse method using high performance liquid chromatography (HPLC) with a UV detector (L-2400, Hitachi, Tokyo, Japan) as previously described [25].

2.2.2. Estimation of conjugation efficiency

The antibody labeling efficiency was determined by measuring the absorbance of the standard samples and VEGF-BCNU-MBs with a fluorescence plate reader system (Tecan Infinite M200, Tecan Trading AG, Switzerland) at an emission wavelength range of 490–600 nm and an excitation wavelength of 450 nm. The amounts of antibody on the MBs (binding efficiency (%)) were calculated as the ratio of intensity of VEGF-BCNU-MBs to the intensity of free avidin.

2.3. In vitro affinity and cytotoxicity test of VEGF-BCNU-MBs

2.3.1. Cell culture and in vitro binding test

C-6 glioma cells were cultured in Dulbecco's modified Eagle's medium containing growth factor F12 (DMEM/F12) (Gibco, NY, USA) supplemented with 10% Fetal Bovine Serum (FBS, Gibco), 1% penicillin–streptomycin (Gibco) and 1.2 g $NaHCO_3$.

Cells were grown on 6-cm-diameter dishes at 2×10^5 cells/dish. BCNU-MBs and VEGF-BCNU-MBs (both labeled with FAM) were adjusted to the same concentration with Dulbecco's phosphate-buffered saline (DPBS, Biological Industries, Kibbutz Beit Ha'emek, Israel). Because of the buoyancy of the MBs, the dishes were inverted to make the cells in contact with the bubbles. After 10 min of targeting at RT, the dishes were washed twice with DPBS and examined under an inverted optical microscope system (IX-71, Olympus, NY, USA) using a 10× objective. Images were acquired using a CCD camera (Olympus DP72, Olympus). Pictures were taken from 5 randomly selected fields of view per dish, and the attachment of MBs to cell surface in each field was counted from 5 randomly selected cells. Five dishes were used for each experiment group. The adhesion of MBs to the cells was presented as the mean and standard deviation of the mean. Blocking tests were performed by pre-incubating C-6 cells with anti-VEGF-R2 antibody for 10 min followed by washing and VEGF-BCNU-MBs interaction. The attachment of VEGF-BCNU-MBs to the cells was calculated as mentioned above.

2.3.2. Flow cytometry

To avoid damage to the surface receptors, cells were washed once with DPBS, and then gently harvested with 0.02% PBS/Ethylenediaminetetraacetic Acid (EDTA) (containing 4.5 g/L glucose and 5 mM $MgCl_2$). The cell suspensions (10^6 cells/mL as determined by a hemocytometer) were then incubated with either BCNU-MBs or VEGF-BCNU-MBs (both labeled with FAM) for 10 min at 4 °C and washed twice with DPBS. Cell targeting of different MB samples was analyzed by flow cytometry (Cytomics FC 500, Beckman-Coulter, CA, USA). Analysis was done with the CXP software (Beckman-Coulter). Each group contained 3 samples and the measurements were performed in triplicate. To further evaluate the effects of amounts of antibody conjugated to MBs on binding of VEGF-BCNU-MBs to C-6 cells, we also prepared VEGF-BCNU-MBs conjugated with 2-fold more antibody.

2.3.3. In vitro ultrasound-triggered drug release experiment setup

A single-element 1-MHz FUS transducer (V302, Panametrics, MA, USA; diameter = 38 mm, focus length = 60 mm) was used to trigger the release of BCNU from VEGF-BCNU-MBs. A function generator (WW2571, Tabor electronics, Haifa, Israel) created sonication pulses which were amplified with a RF power amplifier (150A100B, AR, PA, USA) to drive the 1-MHz FUS transducer. A self-assembled external impedance matching circuit was used to match the electric impedance of the transducer with the output impedance of the amplifier. A polyvinylidene-difluoride type hydrophone (HNP-0400, Onda, CA, USA) was used to measure and calibrate the axial and lateral acoustic fields generated by the 1-MHz FUS transducer in a tank filled with degassed, distilled water. The half-maximum pressure amplitude at the focal zone had a diameter of 3 mm and length of 26 mm.

A removable water cone mounted on the 1-MHz FUS transducer was used to direct the 1-MHz FUS beam into the dish. In order to avoid any undesired cell contamination, an ultrasound transmittable polyurethane membrane was placed between the water cone and the cell dish. A thin layer of water and ultrasound coupling gel (Aquasonic 100, Parker laboratories, NJ, USA) was placed between the membrane and the water cone to maximize the transmission of ultrasound between the transducer and the cell. FUS sonication was applied with peak-rarefactional pressure of 0.5 MPa, 10,000 cycles, and 5 Hz of PRF, sonication duration of 1 min and a total of 2 sonication sites.

2.3.4. In vitro cytotoxicity measurement

The oxidation-reduction indicator Alamar Blue (AbD Serotec, Oxford, UK) was used for colorimetric determination of cell proliferation and viability [26,27]. After collection, cells were grown in a 96-well tissue culture-treated plate at a density of 1×10^4 cells/well in 100 μL of culture medium. Each experiment group contained at least 9 wells of the cells. All cells were incubated overnight and maintained in a humidified atmosphere at 37 °C and 5% CO_2. Cells were treated the following day. MB samples were adjusted to the same concentration and contained the same amounts of drug. Wells without drug served as controls. To eliminate the influence of drug leakage on cell viability, each group of drugs (at the same given dose) was administrated to the cells followed by a 10 min targeting period. The cells were then washed three times with DPBS before finally applying FUS.

After 2 h of incubation, the medium was removed and cells were washed twice with DPBS and resuspended in culture medium. Following a 24 h incubation time, 10 μL Alamar Blue with 90 μL FBS and penicillin–streptomycin-free DMEM/F12 medium was added to the cells and cells were further incubated for 2 h. The absorbance of each plate was measured with a fluorescence plate reader system at