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Inhibition of human brain malignant glioblastoma cells using carmustine-loaded catanionic solid lipid nanoparticles with surface anti-epithelial growth factor receptor

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ABSTRACT

Innovated catanionic solid lipid nanoparticles (CASLNs) carrying carmustine (BCNU) (BCNU-CASLNs) were grafted with anti-epithelial growth factor receptor (EGFR) (anti-EGFR/BCNU-CASLNs) and applied to inhibiting the propagation of human brain malignant glioblastomas cells. U87MG cells were treated with anti-EGFR/BCNU-CASLNs and stained for the expression of EGFR. The minimal average diameter of BCNU-CASLNs and maximal entrapment efficiency of BCNU emerged when the concentration of catanionic surfactants was 1 mM. An increase in the weight percentage of cacao butter (CB) reduced the zeta potential, enhanced the viability of human brain microvasscular endothelial cells (HBMECs), and decreased the expression of tumor necrosis factor-α by HBMECs. The dissolution rate of BCNU and inhibition against the multiplication of U87MG cells using anti-EGFR/BCNU-CASLNs followed the order: 100% CB > 0% CB > 50% CB. Anti-EGFR/BCNU-CASLNs demonstrated the properties including an effective delivery to U87MG cells and antiproliferative efficacy against the growth of malignant brain tumors.

1. Introduction

Glioblastoma multiforme (GBM) is the most commonly encountered and the highest grade (grade 4) glioma in the central nervous system [1]. GBM is also regarded as the most aggressive brain carcinoma and the most malignant form of astrocytoma. Gliomas normally express certain types of growth factor receptor, which is a transmembrane protein modulating cell proliferation, viability, and differentiation [2]. Therefore, labeling a monoclonal antibody against specific growth factor receptor can target a brain carcinoma and becomes a focused therapeutic strategy [3]. In addition, the tumor vessels are leakier than the normal tissue vessels, in general. In fact, the interstitial gap junction among vascular endothelia of tumor is about 100–600 nm [4]. Thus, the design of a drug delivery system for anticancer therapeutics should include an optimal size to extend the residence of carriers in neoplasm.

The catanionic microemulsion is a recently developed colloidal system containing a mixture of oppositely charged surfactants to form vesicles or micelles [5]. The elimination of charge on the head groups and the different embedded hydrophobic chains could reduce the energy barrier between catanionic micelles and yield unordered isometric clusters [6]. The application of supermolecular catanionic aggregates to 3T6 and HeLa cells impaired the cell membrane via fusion or endocytosis and induced the subsequent apoptotic processes [7]. In a study on human mononuclear U-937 macrophages, positively charged catanionic vesicles could alter the mitochondrial membrane potential and cell size [8]. In addition, the lipid core of solid lipid nanoparticles (SLNs) exhibited a very high affinity to hydrophobic drugs [9]. SLNs were also efficacious in carrying anticancer compounds to shun high tissue toxicity, poor specificity and stability, and high incidence of drug resistance in tumor cells [10]. In a pharmacokinetic study, it was confirmed that doxorubicin-loaded SLNs prolonged the circulation period as compared with free drug solution [11].

1,3-bis(2-chloroethyl)-1-nitrosurea (carmustine, BCNU) is an important chemotherapeutic drug for treating solid brain tumors in human. BCNU could deactivate the molecular pathways related to p53 in GBM U87MG cells [12]. A dose-escalation approach was concluded to be a reliable method in analyzing the sustained delivery of BCNU from polymers in rats [13]. In addition, the release of BCNU from electrospun fibers could mediate the antitumor activity against the propagation of rat glioma C6 cells [14]. The

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application of polymer matrix containing BCNU substantially suppressed the growth of tumors in nude mice over 2 months [15]. The side effects of BCNU often observed in clinical practice were the interstitial pneumonitis and pulmonary fibrosis [16]. This was due to an injury to DNA in the alveolar lining cells and the suppression of hematopoiesis by high alkylating activity of BCNU [17]. The efficacy of BCNU was also limited by myelosuppression and hepatic toxicity [18].

This study aims to investigate the possibility of BCNU-encapsulated catanionic SLNs (BCNU-CASLNs) with surface-grafting anti-epithelial growth factor (EGFR) (anti-EGFR/BCNU-CASLNs) to suppress the growth of GBM. In addition, this study attempts to combine catanionic system with SLNs. We demonstrated the particle size, zeta potential, release of BCNU, expression of tumor necrosis factor-α (TNF-α) by human brain-microvascular endothelial cells (HBMECs), inhibition against the growth of human GBM U87MG cells, and expression of EGFR on U87MG cells.

2. Materials and methods

2.1. Fabrication of anti-EGFR/BCNU-CASLNs

BCNU-CASLNs were prepared by microemulsion of hexadecyltrimethylammonium bromide (HTMAB, Sigma) and sodium dodecylsulfate (SDS, Sigma, St. Louis, MO). Cacao butter (CB, OCG Cacao Inc., Whitinsville, MA), stearic acid (SA, Sigma), and BCNU (Sigma) were mixed at 75 °C and 400 rpm. The weight ratios of CB in CB and SA were 0, 0.5, and 1. BCNU was 7.5% (w/w) the lipid phase. The lipid phase was mixed with an equimolar HTMAB and SDS with 0.25, 0.5, 1, 1.5, or 2 mM, 5% (w/w) 1-butanol (Riedel-de Haën, Seelze, Germany), and 0.1% (w/w) 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[carboxy(polyethylene glycol)-2000] (DSPE-PEG (2000)-carboxylic acid, Avanti Polar Lipid, Alabaster, AL) at 75 °C and 400 rpm for 30 min. The lipid phase was 4% (w/w) the emulsified fluid. Fluorescent BCNU-CASLNs were prepared by incorporating 7.5% (w/w) fluorescein isothiocyante-conjugated dextran 70,000 (Sigma) in the lipid phase. One aliquot of the microemulsion was added into ten aliquots of ultrapure water (Barnstead, Dubuque, IA) at 400 rpm and 3 °C for 20 min. The suspension was filtrated with a filtration paper containing pores of 1 μm. The filtrate including BCNU-CASLNs was centrifuged by a superspeed refrigerated centrifuge (AVANTij-25, Beckman Coulter, Palo Alto, CA) at 159000 × g and 4 °C for 10 min. The pellet was resuspended in ultrapure water containing 2% (w/v) D-mannitol (Sigma), refrigerated in an ultralow temperature freezer (Sanyo, Osaka, Japan) at −80 °C for 30 min, and lyophilized by a freeze dryer (Eyela, Tokyo, Japan) at 2–4 torr and −80 °C over 24 h. BCNU in the supernatant was analyzed by a high performance liquid chromatograph (HPLC, Jasco, Tokyo, Japan) with a reverse phase BDS Hypersil C-18 column (Thermo Hypersil-Keystone, Bellefonte, PA) and detected by an ultraviolet (UV) detector (Jasco, Tokyo, Japan) at 230 nm. The entrapment efficiency of BCNU, E_e, was calculated by Ref. [19].

$$E_e = [(W_t - W_s)/W_t] \times 100\%$$

where W_t and W_s were the total weight of BCNU used in the fabrication and the weight of BCNU in the supernatant, respectively.

To graft anti-EGFR (Millipore, Bedford, MA), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (Sigma) with 2 m and N-hydroxysuccinimide (Acros, Morris, NJ) with 5 mM were added into the resuspended BCNU-CASLNs at 80 rpm and 25 °C for 1 h. After centrifugation at 159,000 × g and 4 °C for 30 min, the pellet was resuspended in a solution containing 0.1% (w/v) anti-EGFR at 80 rpm and 25 °C for 2 h. The suspension containing anti-EGFR/BCNU-CASLNs was purified using a dialysis tube of 100 kDa, frozen, and lyophilized in the presence of D-mannitol. The powder products were stored in the refrigerator at 4 °C.

2.2. Characterization of particulate formulation

The cumulant Z-average diameter, D, and zeta potential, ζ, of BCNU-CASLNs were obtained by a zetasizer 3000 HS_A with a photon correlation spectroscope and a laser Doppler velocimeter (Malvern, Worcestershire, UK) at 25 °C. The concentration of BCNU-CASLNs in 0.1 M tris buffer (Riedel-de Haën) was 2 mg/mL for this analysis.

A field emission scanning electron microscope (FE-SEM, JSM-6330 TF, Jeol, Tokyo, Japan) was used to examine the surface morphology of BCNU-CASLNs. The samples were vacuum-dried and sputter-coated with platinum with accelerating voltage of 8 kV for 90 s.

0.2% (w/v) BCNU-CASLNs in tris buffer was loaded on a carbon-coated 200-mesh copper grid for 2 min. Then, the samples were pretreated with 2% (w/v) phosphotungstic acid (Sigma) solution to stain the highly electron-transmissible atoms for 24 h. The images were obtained by a transmission electron microscope (TEM, JEM-1400, Jeol, Tokyo, Japan).

An X-ray photoelectron spectroscope (XPS, Kratos, Kanagawa, Japan) with a vacuum grade of 2×10^{-7} Pa and 300 W was used to evaluate the surface atoms on BCNU-CASLNs. The samples were vacuum-dried on a cover slide of 5 × 5 mm for 15 min before test.

2.3. Dissolution of BCNU

0.2% (w/v) anti-EGFR/BCNU-CASLNs were resuspended in Dulbecco's phosphate buffered saline (DPBS, Sigma) containing 0.05% sodium azide (Sigma) as a biocide at pH 7.4. A dialysis bag was pretreated with ethylene diamine tetra acetic acid (Riedel-de Haën), loaded with 5 mL of the sample, placed in a flask of 100 mL with 20 mL of DPBS, and swayed in a bath-reciprocal shaker at 60 rpm and 37 °C over 48 h. 100 μL of the fluid containing released BCNU was analyzed by the HPLC-UV system at specific time points. The liquid volume in the flask was immediately compensated with 100 μL of fresh DPBS. The cumulative percentage of BCNU released, P_{BCNU}, was calculated by

$$P_{BCNU}(\%) = Q_m/Q_t \times 100\%$$

where Q_m and Q_t were the cumulative quantity of BCNU in the dissolution medium and the total quantity of BCNU in anti-EGFR/BCNU-CASLNs, respectively.

2.4. Cytotoxicity and inflammation assay

HBMECs (Biocompare, South San Francisco, CA) with a density of 6×10^4 cells/well were seeded into a 96-well MicroWell™ polystyrene plate (Nalge Nunc, Rochester, NY) and each well contained 150 μL of endothelial cell medium (Biocompare). These HBMECs were incubated with 0.02% (w/v) anti-EGFR/BCNU-CASLNs in a humidified CO_2 incubator (NuAire, Plymouth, MN) at 37 °C for 4 h. The toxicity of anti-EGFR/BCNU-CASLNs to HBMECs was assayed with 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT, Biological Industries, Beit Haemek, Israel) using an enzyme-linked immunosorbent assay (ELISA) spectrophotometer (Bio-tek, Winooski, VT) at 450 nm. The incubated HBMECs per well was reacted with 75 μL XTT mixture containing 2% (v/v) activation solution in the CO_2 incubator at 37 °C for 4 h. The viability of HBMECs, P_{CV}, was calculated by

$$P_{CV} = [(OD_{H,BCNU} - OD_{XTT})/(OD_{HBMECs} - OD_{XTT})]$$

where $OD_{H,BCNU}$, OD_{HBMECs}, and OD_{XTT} were the optical density of HBMECs incubated with anti-EGFR/BCNU-CASLNs, the optical density of HBMECs, and the optical density of XTT, respectively.

HBMECs with a density of 1×10^5 cells/well were seeded on a 24-well tissue culture plate (Corning, Horseheads, NY) and incubated with 100 μL of culture medium containing 0.15% (w/v) anti-EGFR/BCNU-CASLNs in the CO_2 incubator for 2 h. 50 μL of culture medium after incubation and 100 μL of ultrapure water were reacted in a plate pre-coated with anti-human TNF-α instant ELISA (Bender Med, Vienna, Austria) at room temperature for 3 h. The plate was rinsed with 400 μL of wash buffer for 6 times, stained with 100 μL of tetramethylbenzidine (Bender Med) solution in darkness for 10 min, and treated with 100 μL of stop solution. The sample was analyzed by the ELISA spectrophotometer at primary wavelength of 450 nm and reference wavelength of 620 nm. The total protein of HBMECs in the culture was determined with a bicinchoninic acid protein assay kit (Sigma). HBMECs on a 24-well tissue culture plate after incubation were treated with protein extraction buffer, comprising 100 μL of mammalian protein extraction reagent and 1 μL of protease inhibitor, and centrifuged by the superspeed refrigerated centrifuge at 25,000 × g and 4 °C for 10 min. 10 μL of sample from the supernatant were reacted with 100 μL of standard working reagent at 37 °C for 30 min. The total quantity of cell protein was determined by the ELISA spectrophotometer at 562 nm.

2.5. Inhibition against U87MG cells and expressed EGFR

The human GBM U87MG cells (Bioresource Collection and Research Center, Hsin-Chu, Taiwan) were obtained from a female GBM patient of 44 years old. U87MG cells with a density of 1×10^5 cells/well were seeded on a 96-well MicroWell™ polystyrene plate and each well contained 150 μL of α-minimum essential medium (Gibco, Carlsbad, CA) supplemented with 10% fetal bovine serum (Hyclone, Logan, UT) and 1% penicillin–streptomycin–glutamate solution (Gibco, Carlsbad, CA). U87MG cells were cultured with 0.02% (w/v) BCNU-CASLNs or anti-EGFR/BCNU-CASLNs in the humidified CO_2 incubator (NuAire, Plymouth, MN) at 37 °C for 6, 12, 24, and 48 h. Based on the entrapment efficiency, the same quantity of BCNU was applied to all formulations for the growth inhibition study. The viability of U87MG cells, P_{U87MG}, was assayed with XTT, analyzed by the ELISA spectrophotometer at 450 nm, and calculated by

$$P_{U87MG} = [(OD_{U,BCNU} - OD_{XTT})/(OD_{U87MG} - OD_{XTT})]$$

where $OD_{U,BCNU}$ and OD_{U87MG} were, respectively, the optical density of U87MG cells incubated with the BCNU formulations and the optical density of U87MG.