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Intracellular drug delivery by sulfatide-mediated liposomes to gliomas

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Abstract

We described here a liposomal carrier system in which the targeting ligand was sulfatide, a glycosphingolipid known to bind several extracellular matrix (ECM) glycoproteins whose expression was highly up-regulated in many tumors. In vitro experiments with human glioma cell lines demonstrated that robust intracellular uptake of the liposomes depended specifically on the presence of sulfatide as the key liposomal component. Significant amount of the liposomes remained largely intact in the cytoplasm for hours following their internalization. When anticancer drug doxorubicin (DOX) was encapsulated in such liposomes, most of the drug was preferably delivered into the cell nuclei to exert its cytotoxicity. Use of this drug delivery system to deliver DOX for treatment of tumor-bearing nude mice displayed much improved therapeutic effects over the free drug or the drug carried by polyethylene glycol (PEG)-grafted liposomes. Our results demonstrate a close link between effective intracellular uptake of the drug delivery system and its therapeutic outcome. Moreover, the sulfatide-containing liposomes (SCL) may represent an interesting ligand-targeted drug carrier for a wide spectrum of cancers in which sulfatide-binding ECM glycoproteins are expressed.

Keywords: Intracellular drug delivery; Liposomes; Sulfatide; Gliomas; Doxorubicin

1. Introduction

Liposomes have attracted considerable attention as one of the most promising drug delivery systems [1]. Despite many existing pharmaceutical agents and newly synthesized biological macromolecules such as nucleic acids, proteins and peptides have the potential of being encapsulated in liposomes to serve as liposomal drugs, their in vivo efficacy depends largely on how they are delivered to the right sites of action, which are often located inside the cells. Efficient intracellular drug delivery relies on specific interactions between the drug carriers and the diseased cells. For example, ligand-targeted liposomes may specifically bind to their receptors and be internalized by receptor-mediated endocytosis [2], whereas sterically stabilized long-circulating liposomes may not directly interact, at least in vitro, with certain tumor cells such as ovarian carcinoma cells [3] and gliomas [4]. In the latter case, the therapeutic effect of the liposomal drug appears to depend on the perivascular accumulation of the liposomes, in addition to the cytotoxic effect of the drug carried in them [5].

An obvious strategy to enhance cellular binding and uptake of liposomal drugs is to incorporate in the drug carrier a ligand that would bind to its receptor on the cell surface or in the extracellular matrix (ECM) of the cells [2,6–8]. Since the ECM composition surrounding the tumor cells is very different from that of their normal counterparts and certain ECM glycoproteins such as tenascin-C (TN-C) are highly up-regulated in many different cancers including breast cancer, ovarian cancer, prostate cancer

Abbreviations: ECM, extracellular matrix; TN-C, tenascin-C; DOX, doxorubicin; DOPE, 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine; GalCer, galactosylceramide; Rh-PE, Lissamine™ rhodamine B 1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine; FITC, fluorescein isothiocyanate; PFA, paraformaldehyde; SCL, sulfatide-containing liposomes; SCL-DOX, DOX encapsulated in sulfatide/DOPE (30:70, mol/mol) liposomes; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide; EMEM, Eagle's minimum essential medium; FCS, fetal calf serum; PEG, polyethylene glycol; PEGL-DOX, DOX encapsulated in PEG-grafted liposomes; DOPC, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine; DOPG, 1,2-dioleoyl-*sn*-glycero-3-phosphoglycerol; GM1, gangliosides GM1; TEA, triethanolamine.

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and gliomas [9], it is therefore possible to formulate a liposome containing a ligand specifically interacts with the ECM of the tumor cells. To this end, we have focused on a sulfatide-containing liposomal system as the potential carrier for anticancer drugs. Sulfatide has been found in a number of mammalian tissues and is involved in a variety of biological processes such as cell adhesion, platelet aggregation, cell growth, protein trafficking, signal transduction, neuronal plasticity, cell morphogenesis and disease pathogenesis [10–12]. It has been found that the incorporation of sulfatide into phospholipids such as 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE) vesicles greatly enhances the stability of the liposomes formed, even in the presence of plasma, presumably due to the hydration of the negatively charged sulfate headgroup of the glycosphingolipid [13]. More interestingly, sulfatide is known to bind several ECM glycoproteins including particularly TN-C [14]. Based on this information, sulfatide-containing liposomes (SCL) are expected to interact directly with the targeting tumor cells that express sulfatide-binding ECM glycoproteins. It is hypothesized that such liposome-cell interactions would facilitate the intracellular drug delivery and thus improve the therapeutic outcome of the liposomal drugs.

In this study, we examined the internalization of SCL by the human glioma cells in vitro, the intracellular drug distribution following uptake of the liposomal doxorubicin (DOX) and the consequent cytotoxicity of the drug. The in vivo therapeutic properties of SCL with encapsulated DOX were evaluated in nude mice xenografts of the human glioma cells. Our results show that the sulfatide-containing liposomal drug was more potent than free DOX and DOX encapsulated in nontargeted liposomes in terms of inhibition of tumor growth and extension of the survival time of the tumor-bearing animals.

2. Materials and methods

2.1. Chemicals

Sulfatide (3′-sulfogalactosylceramide), galactosylceramide (GalCer), ganglioside GM1 (GM1), 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[methoxy (poly-ethylene glycol)-2000] (PEG-DSPE), DOPE and other phospholipids were obtained from Avanti Polar Lipids, Inc. (Alabaster, AL). Lissamine™ rhodamine B 1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine (Rh-PE) was from Molecular Probes (Eugene, OR). All other chemicals were from Sigma-Aldrich (St. Louis, MO).

2.2. Human glioma cell lines and culture conditions

Human U-87MG and CCF-STTG1 glioblastoma cell lines were obtained from American Type Culture Collection (Rockville, MD). U-87MG cells were grown in EMEM containing 2 mM L-glutamine, 2.2 g/L $NaHCO_3$, 110 mg/L sodium pyruvate, and 10% fetal calf serum (FCS). CCF-STTG1 cells were maintained in RPMI 1640 medium supplemented with 10% FCS. Cells were cultured in a humidified atmosphere containing 5% CO_2 at 37 °C and were passaged following treatment with trypsin (0.05%)/EDTA (0.02%).

2.3. Liposome preparation

All lipids were dissolved in chloroform:methanol (2:1, v/v) except GalCer that was dissolved in hot ethanol. The lipid thin films of various formulations were prepared as described by Wu and Li [13]. For fluorescence microscopy studies, 0.5 mol% of Rh-PE was included in the lipid mixture that was hydrated with PBS. For liposomes used for encapsulation of DOX, each batch consisting of 10 μmol total lipids was hydrated in 250 mM ammonium sulfate (pH 8.5), followed by vigorous vortex and brief sonication (1 min). The liposomes were formed by extrusion 19 times through two stacked polycarbonate membranes with a defined pore size of 100 nm at 25 °C (Avestin, Inc., Canada) [15]. The extruded liposomes were then dialyzed extensively against a 100-fold volume of 10% sucrose (in 25 mM Trizma at pH 8.5), four changes over 24 h at 4 °C. DOX dissolved in the above buffer was actively loaded into the liposomes through establishment of ammonium sulfate gradient [16]. Nonentrapped DOX was removed by passing the liposomes through a Sephadex G-50 column equilibrated with degassed PBS. DOX concentrations were determined by measurement of the fluorescence intensity of the drug (λex = 480 nm, λem = 550 nm) against a standard curve and the encapsulation efficiency was generally greater than 90%, with a drug to phospholipid ratio of approximately 100 μg/μmol.

2.4. Fluorescence microscopy

Cells were examined by using an inverted microscope (Olympus IX71) and a Zeiss laser scanning confocal microscope system (LSM 510). Cells were seeded in 24-well plates containing glass coverslips for 24 h before co-incubated with the liposomes (total lipid concentration: 80 μM) for up to 1 h. The cells were then washed three times with ice-cold PBS and fixed with 3.7% paraformaldehyde (PFA) (1 h at 25 °C). The coverslips were thoroughly rinsed with PBS and mounted on slides with anti-fade mounting media (Invitrogen, CA) before viewing. For experiments on uptake of liposomes, Rh-PE was excited at 543 nm and the emitted fluorescence was collected using a 560 nm long-pass filter. For quantitative analysis, the images were processed with the Image-Pro Plus software (Media Cybernetics, Inc., USA), where cell contours for each set of fields were traced out manually in the corresponding phase-contrast images and then used to mask the fluorescence images. The fluorescence intensities of 7–10 fields of ~10 cells/field/condition were analyzed.

2.5. Colocalization studies

To investigate whether the integrity of SCL was maintained after internalization, fluorescein isothiocyanate (FITC)-dextran loaded liposomes were prepared by adding 1 ml FITC-dextran in PBS (45 mg/ml) to the Rh-PE-labeled lipid films [17]. The lipid dispersion was briefly sonicated and then extruded through the polycarbonate membranes as described above. A Sephadex G-50 (Sigma) gel filtration column (15 cm × 1.5 cm) was used to remove the un-encapsulated FITC-dextran. Lipid concentration of the eluted liposomes was estimated with the colorimetric method by Stewart [18]. To the cultured U-87MG cells,