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Research Article

Sulfatide-tenascin interaction mediates binding to the extracellular matrix and endocytic uptake of liposomes in glioma cells

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Abstract. Tenascin-C is an extracellular matrix glycoprotein, whose expression is highly restricted in normal adult tissues, but markedly up-regulated in a range of tumors, and therefore serves as a potential receptor for targeted anticancer drug or gene delivery. We describe here a liposomal carrier system in which the targeting ligand is sulfatide. Experiments with tenascin-C-expressing glioma cells demonstrated that binding of liposomes to the extracellular matrix relied essentially on the sulfatide-tenascin-C interaction. Following binding to the extracellular matrix, the sulfatide-containing liposomes were internalized via both caveolae/lipid raft- and clathrin-dependent pathways, which would ensure direct cytoplasmic release of the cargoes carried in the liposomes. Such natural lipid-guided intracellular delivery targeting at the extracellular matrix glycoproteins of tumor cells thus opens a new direction for development of more effective anticancer chemotherapeutics in future.

Keywords. Ligand-targeted drug delivery, liposomes, sulfatide, tenascin-C, extracellular matrix.

Introduction

Tumor-targeted drug delivery relies on specific interactions between the ligands associated with the drug carriers and the antigens or receptors either uniquely expressed or overexpressed on the tumor cells [1]. The most frequently used ligands for the purpose of targeted drug delivery to tumor cells are antibodies or their fragments that recognize the tumor-associated antigens ([1, 2] and references therein). Although this approach possesses a high level of specificity to the targeted tissues, its applications might be limited by problems such as immunogenicity and low percentage of tumors that express any given antigen. Therefore, design of new drug delivery systems based on alternative and yet highly specific ligand-receptor interactions could have important implications for the development of novel anticancer therapeutics.

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The extracellular matrix (ECM) surrounding tumor cells is very different from that surrounding normal cells. In particular, tenascin-C (TN-C), a multifunctional ECM glycoprotein, is highly up-regulated in many different cancers such as glioma, breast cancer, ovarian cancer and prostate cancer ([3] and references therein). Under normal circumstance, TN-C is only expressed during the early stages of development and is absent or much reduced in developed tissues [4]. Since the reappearance of TN-C is closely associated with pathological conditions like carcinogenesis and usually indicative of poor prognosis [3], it makes an attractive target for ligand-targeted therapeutic strategies, similar to other ECM-associated targets such as integrins and ED-B fibronectin [5–7]. In principle, any molecule that specifically binds this tumor-specific ECM glycoprotein has the potential to be exploited in the development of novel drug carrier systems to improve the therapeutic outcomes and/or reduce the systemic toxicity of 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 [8–10]. More interestingly, sulfatide binds several ECM glycoproteins including specially TN-C [11–14]. In other words, sulfatide, a natural acidic glycosphingolipid consisting of a hydrophobic ceramide and a hydratable galactose residue sulfated at the C3 position, is a promising candidate as the ligand of targeted carriers to deliver anticancer drugs to tumor cells via binding to TN-C. In fact, sulfatide had been chosen previously as a minor lipid component in liposomes preparation [15–17]. It was found that incorporation of sulfatide into phospholipids such as 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE) vesicles greatly enhanced the stability of the liposomes formed, even in the presence of plasma, presumably due to hydration of the negatively charged sulfate headgroup of the glycosphingolipid [17].

In this study, we examined the molecular mechanisms involved in binding and subsequent internalization of sulfatide-containing liposomes (SCL) by human glioma cells. The results demonstrate a ligand-targeted intracellular delivery system based on interaction between a glycosphingolipid and a tumor-specific ECM glycoprotein, which may lead the way for formulation and development of novel chemotherapeutics to treat a wide spectrum of cancers.

Materials and methods

Chemicals. Sulfatide (3'-sulfogalactosylceramide), galactosylceramide (GalCer), ganglioside GM1, 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE), 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), cholera toxin B subunit (CTxB) and bodipy-lactosylceramide (Bodipy-LacCer) 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). Mouse anti-human TN-C monoclonal antibody, anti-O4 monoclonal antibody and the IgM negative control were from Chemicon (Temecula, CA). T7●Tag® monoclonal antibody was from Merck (Whitehouse Station, NJ). All other chemicals were from Sigma-Aldrich (St. Louis, MO).

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 Eagle's minimum essential medium (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 Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FCS. Cells were cultured in a humidified atmosphere containing 5% CO_2 at 37°C.

Liposome preparation. All lipids were dissolved in chloroform:-methanol (2:1, v/v) except GalCer, which was dissolved in hot ethanol. Appropriate amounts of lipids were transferred from their respective stock solutions into glass tubes and dried by evaporation under a nitrogen stream, as described by Wu and Li [17]. The ratio of sulfatide to phospholipid was chosen such that liposomes would be formed with the optimal stability [16, 17]. For fluorescence microscopy studies, 0.5 mol% of Rh-PE was included. The samples were stored under vacuum for 24 h at 4°C and the thin lipid film formed on the wall of the glass tubes was hydrated with phosphate-buffered saline (PBS), briefly sonicated (1 min), and extruded repeatedly through two layers of polycarbonate membranes with a pore size of 100 nm at room temperature (Avestin, Inc., Canada) [18].

ECM binding and intracellular uptake of SCL. ECM binding and internalization of Rh-PE-labeled SCL were examined using an inverted microscope (Olympus IX71) or a Zeiss laser-scanning confocal microscope system (LSM 510), respectively. Cells were seeded into 24-well plates containing glass coverslips for 24 h before co-incubation with the liposomes (final total lipid concentration: 80 μM) for 1 h. The cells were then washed three times with ice-cold PBS and immediately fixed with 3.7% paraformaldehyde (PFA). The coverslips were thoroughly rinsed with PBS and mounted on slides with anti-fade mounting media (Invitrogen, CA) before viewing. 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 (version 4.5.1, Media Cybernetics, Inc., USA), where cell contours or the ECM areas surrounding the cells for each set of the fields were traced out manually in the corresponding phase-contrast images and then used to mask the fluorescence images. The fluorescence intensities of seven to ten fields of ~10 cells/field/condition or five fields of ~10 ECM areas/field/condition were analyzed to quantitate the cellular uptake or the ECM binding, respectively, of the liposomes.

Antibody perturbation. Sulfatide/DOPE/Rh-PE (30:69.5:0.5; mol/mol/mol) liposomes were incubated with anti-O4 monoclonal antibody (100 μg/ml) or mouse IgM (100 μg/ml) (Chemicon, CA) for 30 min at 37°C. U-87MG cells were seeded into 4-well chambered coverglass system for 24 h before being incubated with either the anti-O4-pretreated, the IgM-pretreated or the untreated liposomes for 1 h, followed by quantitative analysis of the ECM binding of SCL as described above.

1,25-Dihydroxyvitamin D_3 treatment. U-87MG cells were treated with up to 1 μM 1,25-dihydroxyvitamin D_3 (VD_3), which is known to inhibit TN-C expression in various types of cells [19, 20], for 24 h at 37°C and the expression level of TN-C protein was estimated by Western blotting (see below). After washing with PBS, the cells

were incubated with Rh-PE-labeled SCL for 1 h at 37°C and quantitative analysis of the binding level of the liposomes was conducted as described above.

Small interfering RNA preparation and transfection. A TN-C-specific small interfering RNA (siRNA) duplex corresponding to bases 5209–5227 from the open reading frame of the human TN-C mRNA was designed and synthesized by Proligo (Singapore): 5'-GUGGAGAGCUUCCGGAUUA-dTdT- 3'. The RNA sequence without known homology to mammalian genes, which was used as a negative control, was: 5'-UUCUCCGAACGUGUCACGU-dTdT-3'. Knockdown of TN-C expression by siRNA was carried out according to the manufacturer's instructions. Briefly, cells were transfected at 60–80% confluence using Lipofectamine^{TM}2000. The final concentration of TN-C siRNA and the negative control siRNA used was 0.2 μM. The medium was replaced with fresh complete medium 4 h after transfection, and analyses on TN-C knockdown efficiency and SCL binding to the ECM of the cells were conducted at 24 and 72 h after transfection, respectively.

Western blotting. Cells were lysed in the lysis buffer containing 10 mM Tris-HCl, 150 mM NaCl, 1% NP-40, 1 mM EDTA (pH 7.4) and various protease inhibitors (Roche, Basal, Switzerland). Aliquots of the cell lysate (20 μg protein) were then resolved by 5% SDS-PAGE and electroblotted onto polyvinylidene difluoride (PVDF) membranes (Pierce, Rockford, IL). After blocking with 5% skim milk for 1 h at room temperature, TN-C and the clathrin hub were detected by Western blot analysis using the monoclonal antibody against TN-C (1 : 1000 dilution) and clathrin hub (1:10 000 dilution), respectively. Immunoreactive bands were visualized using a secondary antibody conjugated to horseradish peroxidase (Bio-Rad, CA) and the SuperSignal enhanced chemiluminescence reagent (Pierce, Rockford, IL).

Effects of pharmacological inhibitors/phospholipase on liposome uptake. Cultured cells were seeded on glass coverslips for 24 h before treatment with various pharmacological inhibitors for 1 h to differentiate clathrin-dependent from clathrin-independent internalization of SCL. The concentration of each reagent used (all from Sigma) was 10 mg/ml (2-hydroxypropyl)-β-cyclodextrin (2-HCD) [21], 10 μg/ml cytochalasin D [22], 5 μM sphingosine [23], 0.5 M hyperosmolar sucrose [24] and 0.3 IU/ml phosphatidylinositol-specific phospholipase C (PI-PLC) [25, 26]. The treated U-87MG cells were then incubated with the Rh-PE-labeled SCL for 1 h for quantitative analysis of the rate of liposome uptake. Cell viability was >90% for all treatments as judged by trypan blue staining.

Transfection of U-87MG cells with clathrin hub. Expression of a fragment of clathrin heavy chain, clathrin hub, as a dominant-negative inhibitor of clathrin-mediated endocytosis was performed by following the method of Liu et al. [27], with slight modifications. In brief, the cDNA encoding bovine clathrin heavy chain residues 1073–1675 was cloned into the *Bam*HI and *Hin*dIII sites of the vector pET23d (Novagen, Inc.) after the T7 gene 10-leader peptide sequence. The PCR-amplified T7Hub introduced a Kozak sequence as well as a *Not*I restriction site at the end of the T7Hub. The PCR products digested with *Eco*R V were ligated with *Eco*R V-cleaved pIRES-EGFP (where EGFP is enhanced green fluorescent protein) vectors (BD bioscience, NJ) followed by transformation of DH-5α competent cells. The positive clones were inoculated to LB medium and incubated overnight. The T7Hub-pIRES-EGFP plasmid was extracted, analyzed by enzymatic cleavage and amplified in *E. coli* and finally purified with the DNA Purification Systems (Promega). The U-87MG cells were seeded in plates for 24 h before transfection with Lipofectamine-2000 (Invitrogen)/T7Hub-pIRES-EGFP complex according to the manufacturer's protocol. The EGFP expression was detected with the 488-nm laser, which was used to indicate the expression of the plasmid. For liposome internalization experiments, U-87MG cells were incubated, 48 h after transfection, with Rh-PE-labeled SCL for 1 h and then viewed and quantitated with confocal fluorescence microscopy after washing.

Statistical analysis. Data were evaluated by either unpaired Student's *t*-test (two-sided) or one-way ANOVA using SPSS 12.0 software (SPSS Inc., Chicago) and presented as means ± SD. All results were derived from at least three independent experiments.

Results

Sulfatide was required for binding of liposomes to the ECM of human glioma cells. Liposomes consisting of GM1, GalCer or sulfatide as the minor component (30 mol%) and either DOPC or DOPE as the major component (70 mol%) were prepared by extrusion through polycarbonate membranes of average pore size 100 nm (see Materials and methods for details). The mean diameter of such liposomes formed was ~82 nm with a polydispersity of <0.5, as determined by dynamic light scattering (data not shown). It was found that presence of sulfatide was essential for effective binding of the Rh-PE (0.5mol%)-labeled liposomes to the ECM of U-87MG, a typical human glioblastoma cell line (Fig. 1Aa–d).

Evidently, presence of a galactose group, such as that of GalCer in GalCer/DOPC liposomes, or a complex oligosaccharide headgroup, such as that of gangliosides GM1 in GM1/DOPC liposomes, was insufficient for effective ECM binding to occur. In addition, DOPE significantly enhanced the ECM binding of SCL by approximately twofold compared with that of sulfatide/DOPC liposomes (Fig. 1B). Similar effects of sulfatide on binding of SCL to the ECM of another human glioblastoma cell line, CCF-STTG1 cells, were also observed (data not shown).

To further confirm that sulfatide is required for effective binding by the glioblastoma cells, SCL were pretreated with monoclonal anti-sulfatide (anti-O4) antibody (100 μg/ml) or mouse IgM (100 μg/ml). Figure 2 shows that treatment with IgM led only to a slight decrease (~25%) in ECM binding of the liposomes, whereas treatment with anti-O4 under identical conditions resulted in a near complete inhibition of ECM binding of the liposomes, indicating that binding of antibody to sulfatide on the outer surface of liposomes had prevented it from interacting with its receptor(s) in the ECM of the cells. These data suggest that sulfatide plays an essential role in mediating the binding of liposomes to the ECM of the glioma cells.

TN-C was involved in binding of SCL to the ECM of human glioma cells. It has been reported that heparan sulfate proteoglycan (HSPG) plays an important role in cellular uptake of liposomes [28, 29]. To rule out the possibility that binding of SCL to the ECM of the glioma cells was mediated by HSPG, the cells were pretreated with heparinase I (3.0 U/ml, 30 min at 37°C) or the liposomes were incubated with the cells in the presence of heparin (100 μg/ml), a HSPG competitor. Such treatments affected neither the binding of SCL to the ECM of the glioma cells nor the internalization rate of the liposomes, suggesting that HSPG was unlikely to be involved in interactions between SCL and the glioma cells. On the other hand,

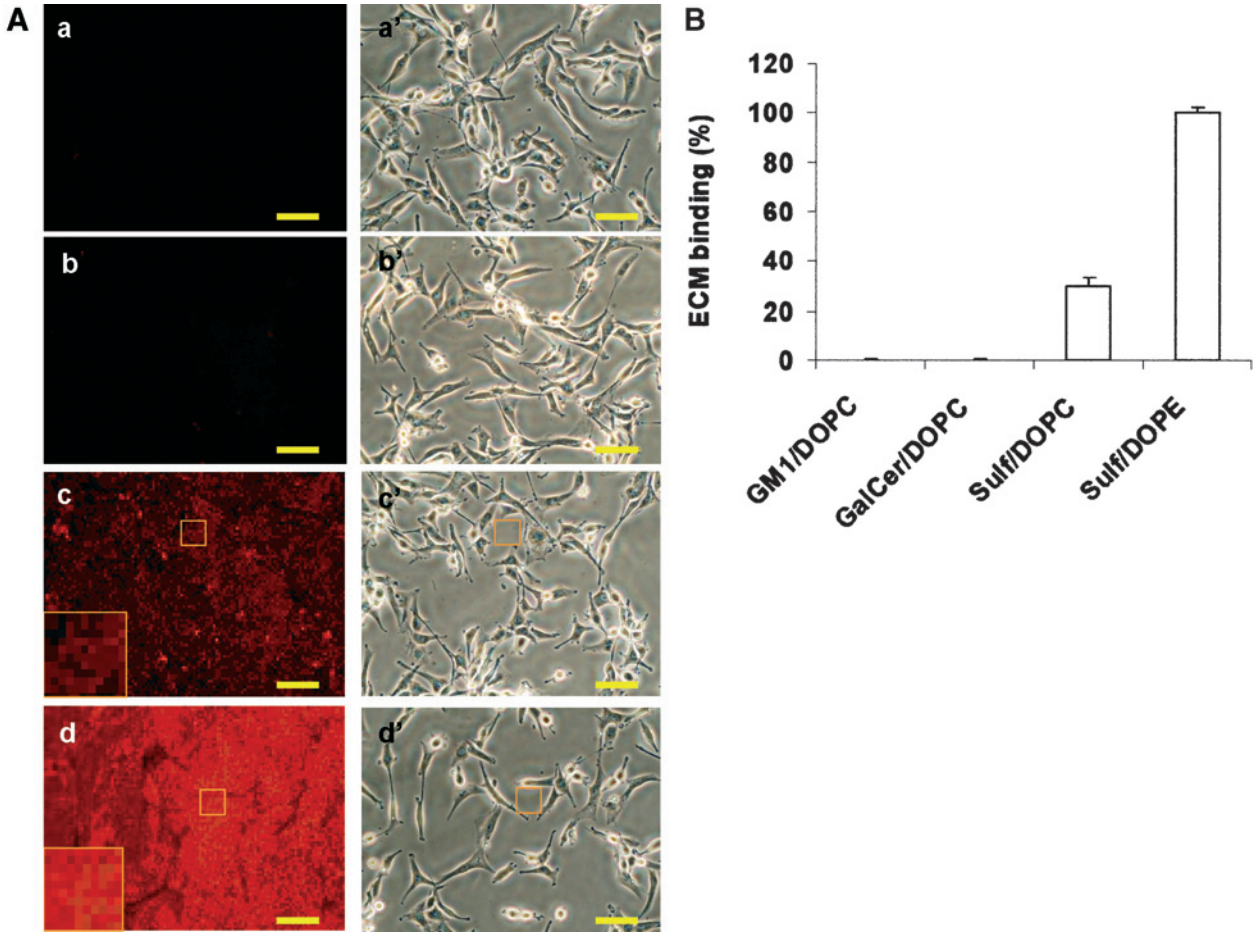

Figure 1. Sulfatide is specifically required for binding of phospholipid liposomes to the ECM of U-87MG cells. (A) Fluorescence and corresponding phase-contrast micrographs of U-87MG cells after incubation with Rh-PE-labeled liposomes for 1 h at 37°C. Liposomes (total lipid concentration: 80 μM) used: a and a', GM1/DOPC (30:70, mol/mol); b and b', GalCer/DOPC (30:70, mol/mol); c and c', sulfatide/DOPC (30:70, mol/mol) and d and d', sulfatide/DOPE (30:70, mol/mol). The scale bar represents 50 μm. The boxed regions in c' and d' and their corresponding regions in c and d are enlarged by approximately threefold in the insets to show typical ECM binding of the liposomes. (B) Quantitative analysis of the binding level of liposomes by the ECM of U-87MG cells. The average binding level represented by the fluorescence intensity of Rh-PE-labeled sulfatide/DOPE (30:70, mol/mol) liposomes was set as 100%. Values are mean ± SD of at least three independent experiments.

sulfatide is known to be able to bind tenascins, in particular, TN-C [11], which is abundantly expressed in human glioma cell lines [30]. It was therefore of interest to ascertain if this glycoprotein is responsible for the ECM binding of SCL. Therefore, VD_3 was used to treat the cells for 24 h to inhibit the expression of TN-C before the liposomes were introduced. Figure 3A shows that TN-C expression was significantly attenuated by VD_3 treatment, as detected by Western blot with TN-C-specific antibody. Correspondingly, binding of SCL to the ECM of U-87MG cells treated with 1 μM VD_3 for 24 h reduced by ~35% compared with that in the control cells (Fig. 3B). We further confirmed the specific interaction between SCL and TN-C by transfection of the U-87MG cells with a siRNA duplex targeted to the TN-C mRNA. Figure 4A shows the immunoblots probed for TN-C protein in U-87MG cells 24 or 72 h after transfection. TN-C expression was knocked-down by ~80% 72 h after transfection with TN-C-specific siRNA compared with that in untreated cells and cells transfected with a control siRNA of random sequence. We next examined whether the knockdown of TN-C would affect the ECM binding of SCL. Figure 4B shows that binding of SCL to the ECM of TN-C siRNA transfected U-87MG cells was reduced by ~50% 72 h after cell transfection compared with that in the untreated cells. Not surprisingly, transfection of cells with the control siRNA did not affect the binding of SCL to the ECM of U-87MG cells. Taken together, these results suggest that TN-C, the ECM glycoprotein known to be able to bind sulfatide, was indeed involved in binding of SCL to the ECM of the human glioma cells.

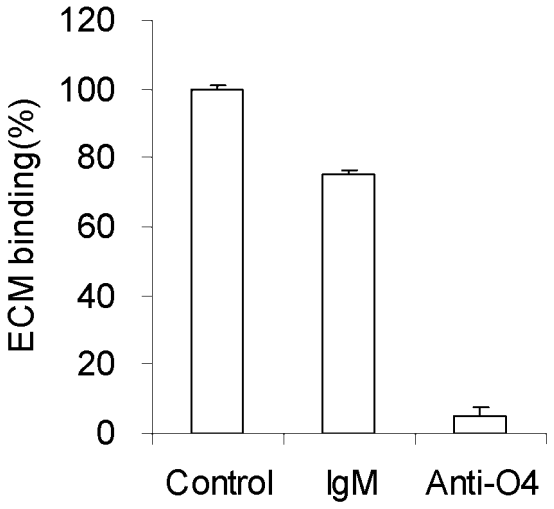

Figure 2. Effect of pretreatment of sulfatide/DOPE (30:70, mol/mol) liposomes with anti-O4 antibody or IgM on their binding to the ECM of U-87MG cells. The average ECM binding level in the control experiments, where untreated Rh-PE-labeled SCL were used, was set as 100%. Values are mean ± SD of at least three independent experiments.

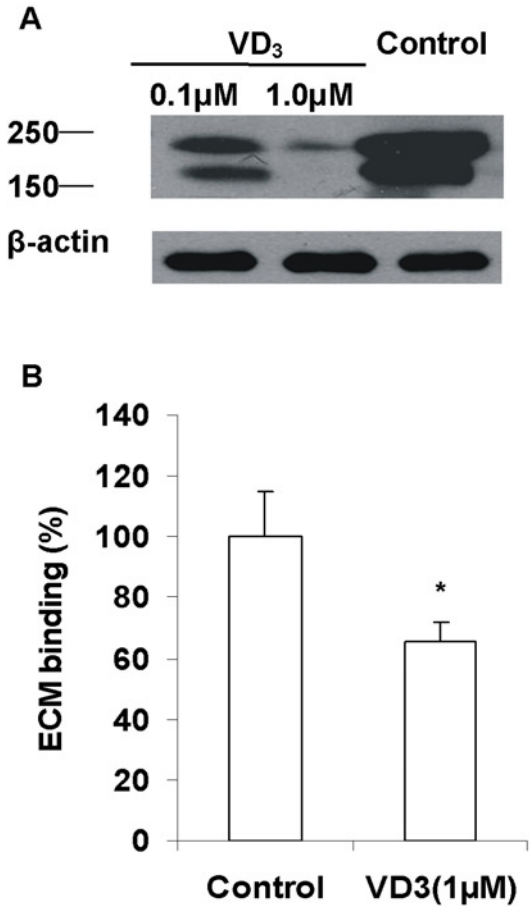

Figure 3. Effect of VD_3 on binding of sulfatide/DOPE (30:70, mol/mol) liposomes to the ECM of U-87MG cells. (A) Effect of VD_3 on TN-C expression in U-87MG cells as examined by immunoblotting with anti-TN-C antibody. β-Actin was used as a loading control. The blots are representatives of three independent experiments. Treatment of the cells with VD_3 attenuated TN-C expression in a dose-dependent manner. (B) Binding of Rh-PE-labeled sulfatide/DOPE (30:70, mol/mol) liposomes to the ECM of U-87MG cells was significantly inhibited by VD_3 treatment. The concentration of VD_3 used here is 1 μM and higher concentration was tested to be toxic to the cells. Values are mean ± SD of at least three independent experiments. *$p<0.005$ *versus* the non-treated control.

SCL were internalized via both clathrin-dependent and -independent endocytosis. After binding of the liposomes to the target cells, the route of their internalization becomes important for optimal therapeutic effects. For example, strategies may need to be developed for endosomal escape of the drugs to avoid actions by lysosomal enzymes if the drug delivery system enters the cell via the clathrin-dependent pathway. To identify the pathway(s) responsible for uptake of SCL, we first tried to exclude the possibility of macropinocytosis as the major pathway for liposomal internalization by pre-treatment of the cells with cytochalasin D. It is known that this mode of internalization requires a functional actin cytoskeleton while cytochalasin D destabilizes the actin filaments [22, 31]. Pre-treatment of the cells with cytochalasin D (10 μg/ml) had no effect on the internalization of SCL (data not shown), suggesting that macropinocytosis is unlikely to be involved in uptake of the liposomes. It has been well-established that various types of endocytosis may co-exist and some, such as caveolae/lipid raft- and clathrin-mediated endocytosis, are affected by depletion of cholesterol of the cell plasma membranes [32, 33]. We then examined whether the internalization of SCL by the human glioma cells was susceptible to cholesterol depletion. When the cells are pre-treated with 2-HCD, an approximately ~70% decrease in internalization of the fluorescence-labeled SCL was observed (Fig. 5A), suggesting that the endocytic pathways of SCL were cholesterol dependent. However, since both clathrin-dependent and -independent endocytosis may be affected by cholesterol depletion, further experiments were needed to ascertain that either or both of these pathways had contributed to the uptake of SCL. In these experiments, markers internalized almost exclusively via caveolae-dependent pathway (CTxB and Bodipy-LacCer) or clathrin-dependent pathway (fluorescein-conjugated transferrin) were used as controls [33–35].

We first examined whether caveolae/lipid raft-mediated endocytosis was responsible for uptake of the liposomes. The cells were pre-treated with PI-PLC, a well-established procedure to remove glycosylphosphatidylinositol(GPI)-anchored proteins from the cell surface [25, 26]. Figure 5B shows that PI-PLC treat-

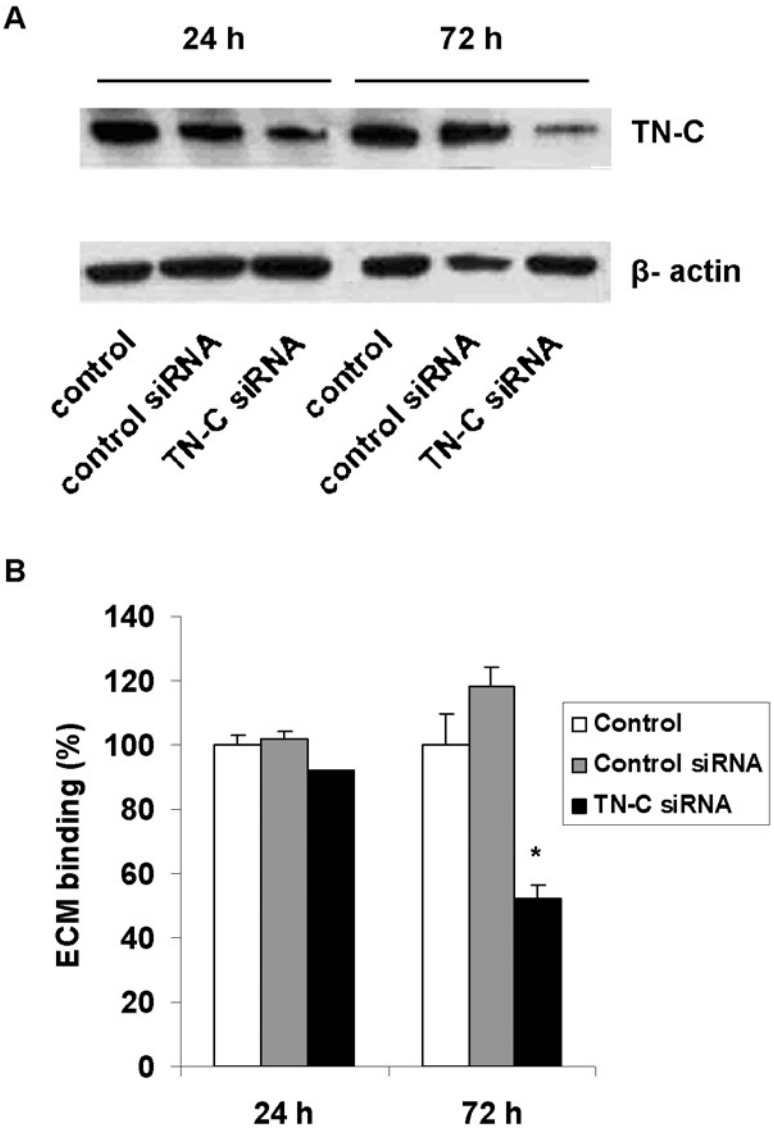

Figure 4. Effects of TN-C siRNA on TN-C expression in U-87MG cells and ECM binding of sulfatide/DOPE (30:70, mol/mol) liposomes. (A) TN-C expression in U-87MG cells following treatment with TN-C siRNA and control siRNA for up to 72 h as detected by Western blot analysis. The blots are representatives of three independent experiments. β-Actin was used as the internal control. (B) Quantitative analysis of TN-C siRNA treatment on ECM binding of Rh-PE-labeled sulfatide/DOPE (30:70, mol/mol) liposomes by U-87MG cells. The average level of ECM binding of untreated liposomes was set as 100%. Values are mean ± SD of at least three independent experiments. *$p<0.001$ *versus* both the non-treated control and the control siRNA-treated sample.

ment caused a ~55% reduction in the uptake of the fluorescence-labeled liposomes compared with the untreated control cells, a clear indication that perturbation to caveolae/lipid rafts did affect the internalization of SCL. However, it is likely that clathrin-dependent endocytosis was also involved, as the internalization rate of SCL was significantly higher than that of CTxB. The situation became more complicated as treatment by PI-PLC also disturbed somehow the uptake of transferrin, a typical marker for clathrin-mediated endocytosis.

We next investigated whether SCL could be internalized by the clathrin-mediated pathway. It is well-known that hypertonic sucrose inhibits clathrin-mediated endocytosis by disrupting formation of clathrin-coated pits [24]. The human glioma cells were treated with 0.5 M sucrose before uptake of liposomes was allowed to take place. It was found that such treatment decreased the internalization of SCL by as much as 70% compared with that in the control cells (Fig. 5C). This implies that uptake of SCL was indeed also mediated by clathrin. This conclusion was verified by examining the uptake of SCL following treatment of the cells with 5 μM sphingosine, a cationic amphiphilic drug known to interrupt assembly-disassembly of clathrin from coated pits and endosomes [23]. Figure 5D shows that sphingosine had similar effects to hypertonic sucrose and internalization of SCL was reduced by ~60%, suggesting that SCL were internalized, at least partly, by the clathrin-mediated pathways.

To obtain further support for a mechanism that would involve clathrin-mediated endocytosis, we then expressed a dominant-negative hub fragment of clathrin by transfection of U-87MG cells, which has been known to perturb clathrin-mediated endocytosis [27,

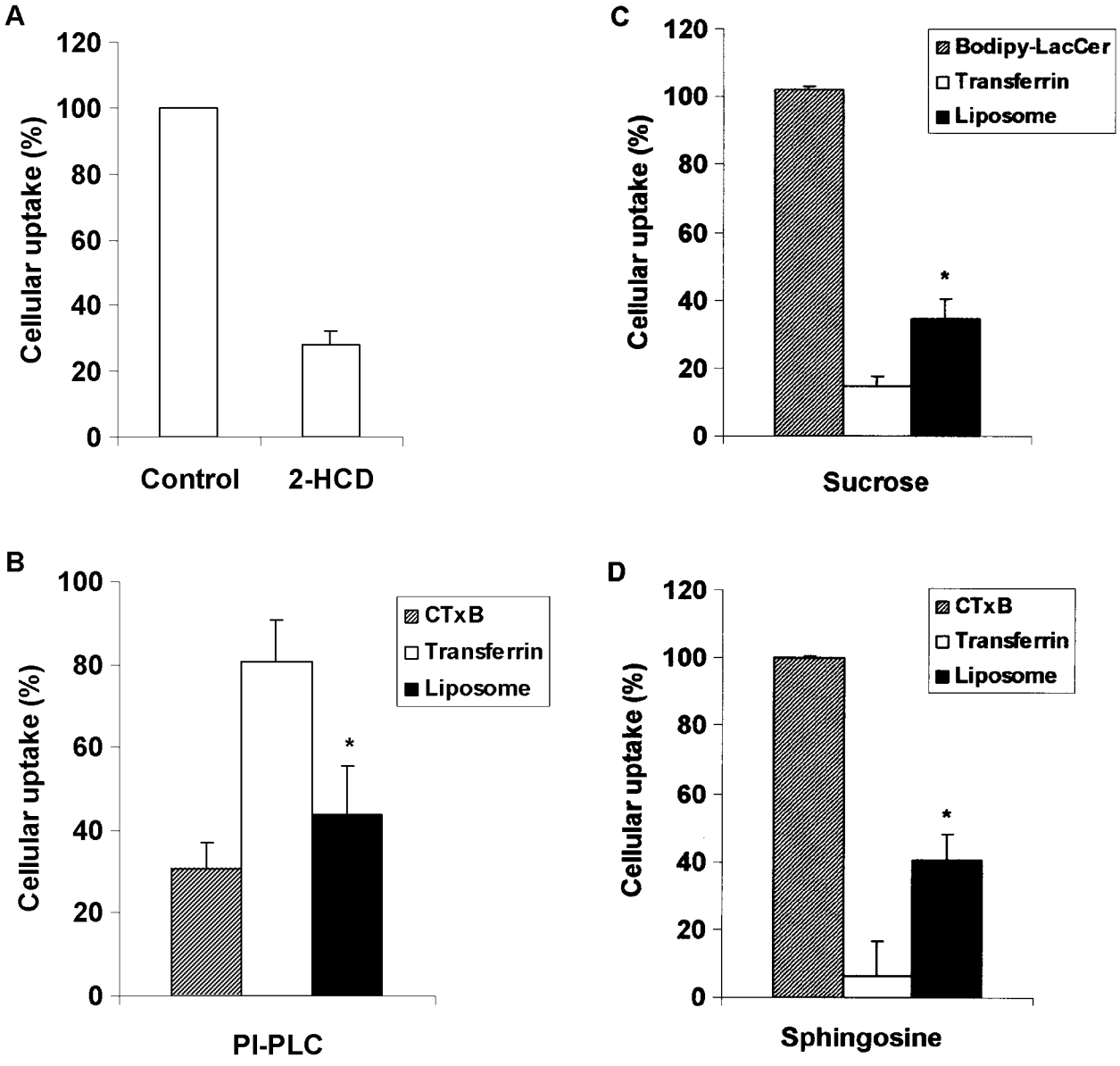

Figure 5. Effects of pretreatment of U-87MG cells with 2-HCD, PI-PLC, sucrose and sphingosine on the internalization of sulfatide/DOPE (30:70, mol/mol) liposomes. The cells were pretreated with 1% (w/v) 2-HCD (A), 0.3 IU/ml PI-PLC (B), 0.5 M sucrose (C) or 5 μM sphingosine (D), respectively, for 1 h at 37°C before the liposomes were introduced. Control cells were treated with vehicle and the uptake of Rh-PE-labeled liposomes by control cells was set as 100% (not shown in the figure). Values are mean ± SD of at least three independent experiments. *$p<0.01$ *versus* the uptake levels of both CTxB (or Bodipy-LacCer) and transferrin.

36]. Clathrin hub expression was detected, using anti-T7 epitope antibody, by 24 h and reached its maximum 48 h after transfection (Fig. 6A), at which time uptake of SCL by the transfected cells was examined. Figure 6B shows that clathrin hub expression inhibited ~50% liposomal endocytosis. Taken together, these data are consistent with the notion that both clathrin-dependent and -independent (or caveolae/lipid raft-dependent) pathways are involved in the internalization of SCL by the glioblastoma cells.

Discussion

In this study, we have shown that sulfatide was specifically required for effective binding of phospholipid liposomes to the ECM of human glioblastoma cells such as U-87MG and CCF-STTG1 cells. In contrast, when ganglioside GM1 and GalCer were used to formulate the liposomes, no detectable binding of such liposomes were observed (Fig. 1A, B). The specific requirement for sulfatide has been further confirmed by examining the effect of anti-O4 antibody on binding of SCL to the ECM of the glioblas-

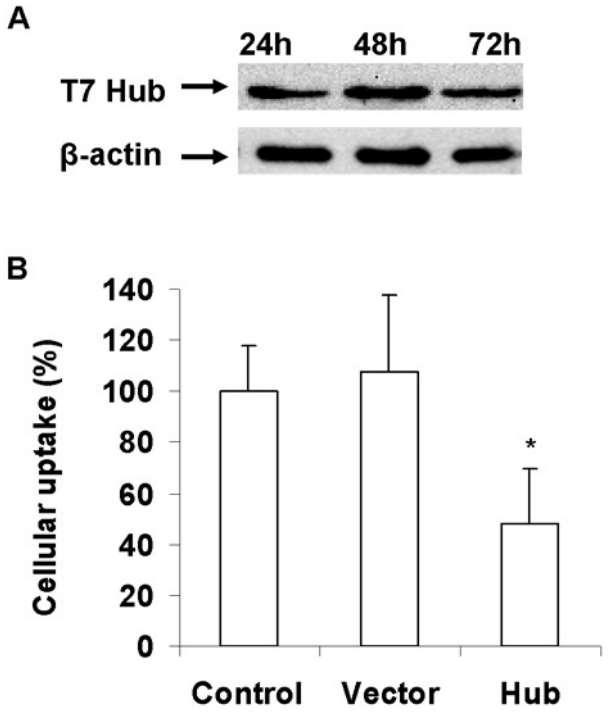

Figure 6. Effect of expression of clathrin hub on internalization of sulfatide/DOPE (30:70, mol/mol) liposomes by U-87MG cells. (A) Expression of clathrin hub in U-87MG cells as detected by Western blot with anti-hub antibody**.** The blots are representatives of three independent experiments performed. β-Actin was used as a loading control. (B) Internalization of Rh-PE–labeled sulfatide/DOPE (30:70, mol/mol) liposomes was studied in U-87MG cells 48 h after clathrin hub transfection. Values are mean ± SD of at least three independent experiments. *$p<0.01$ *versus* the non-transfected control and the vector-transfected cells.

toma cells (Fig. 2). The small reduction in ECM binding caused by IgM could be due to sterical hindrance resulting from nonspecific binding of IgM at the liposomal surface, which, due to its large molecular weight, would affect the interactions between sulfatide and its receptor(s) at the cell surface. This postulation was supported by the observation that albumin, at a similar concentration, did not affect the binding of the liposomes (data not shown). We hypothesize that TN-C, the ECM glycoprotein, plays an essential role in mediating the binding of SCL. Firstly, it is well known that TN-C binds specifically to sulfatide but not to other gangliosides or GalCer [11], which is consistent with our observations in this work (Fig. 1). Secondly, inhibition to TN-C expression by VD_3 caused significant reduction in binding of SCL to the ECM of the glioblastoma cells (Fig. 3B). Thirdly, knockdown of TN-C by specific TN-C siRNA provided supporting evidence for a link between this matrix protein and SCL binding (Fig. 4B). Finally, the expression level of tenascins is known to be much higher than that of other sulfatide-binding ECM proteins like laminin in human glioblastoma cells [37]. In addition, expression of TN-C is highly up-regulated in many different cancers (reviewed in [3]). Hence, a targeted liposomal drug delivery system mediated by specific sulfatide-TN-C interactions may hold the promise of development of new therapeutics for treatment of a wide spectrum of cancers.

The binding and internalization of SCL by U-87MG cells are well-defined sequential events. Fluorescence real-time images showed that liposomes bound to the ECM were detachable from the cell bodies after the cells were treated with trypsin, while those inside the cells were not affected (data not shown). This observation that binding of SCL to the ECM was sensitive to trypsin digestion also supports the involvement of ECM protein components in binding of the liposomes. It is still unclear what the internalization machinery is at this stage. Nevertheless, it has been demonstrated that both clathrin-dependent and –independent endocytosis contributed to the uptake of SCL (Figs 5 and 6). Since the particle size affects the route of its internalization [33], a simple explanation for such a dual entry mechanism could be the intrinsic heterogeneous size distribution of the liposomes. However, this does not rule out the possibility that distinguishable internalization machineries exist for clathrin-dependent and -independent endocytosis of the liposomes. For effective intracellular drug and/or gene delivery, early release of the cargo is always desirable to avoid lysosomal degradation in clathrin-mediated endocytosis. Presence of DOPE definitely reduces the chance of lysosomal degradation as the liposomes would become unstable in endosomes with an acidic pH [16, 17]. Moreover, it is advantageous to have a significant portion of SCL internalized via the clathrin-independent pathway, *e.g.*, internalization mediated by caveolae/lipid rafts in which the caveosomes could deliver their contents into other non-lysosomal subcellular compartments. Bypassing the acidic and harmful milieu is always considered a major advantage, among others, for drug delivery via the clathrin-independent pathway over the clathrin-dependent pathway [38, 39].

In conclusion, we have demonstrated, at least theoretically, a potential targeted drug delivery system utilizing sulfatide as the binding or targeting motif, which should markedly improve the targeting efficiency of drug delivery, and thus treatment effect for cancers that involve overexpression of sulfatide-binding proteins like TN-C. Because expression of TN-C is known to be highly up-regulated in many different types of cancers, use of this natural lipid-guided liposomal formulation of anticancer drugs thus possesses promising clinical potential, and preliminary therapeutic effects of SCL with encapsulated doxorubicin have recently been evaluated *in vitro* in nude

mice xenografts of human glioma cells [40]. Furthermore, as the stability of this drug delivery system is pH sensitive [16, 17] and its internalization by cells involves both clathrin-dependent and –independent pathways, it may have broad applications for delivery of substances such as imaging agents, therapeutic proteins and peptides directly into the cytoplasm of the target tumor cells. All these warrant further exploration.

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