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Cadherin-integrated liposomes with potential application in a drug delivery system

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ABSTRACT

N-cadherin (CDH2) proteins were reconstituted with liposomes using a baculovirus expression-liposome fusion method. CDH2 budded viruses were fused with giant liposomes containing dioleoylphophogycerol/dioleoylphosphatidylcholine (DOPG/DOPC) at pH 4.5 and the localization of CDH2 on the liposome membrane was observed by confocal laser scanning microscopy. CDH2 liposomes showed Ca^{2+}-dependent association. CDH2-mediated association/dissociation in CDH2 liposomes was specific to Ca^{2+} and reversible. CDH2-expressing LN-229 cells (human glioblastoma cell) adhered to CDH2 liposomes and small CDH2 liposomes (diameter approximately 150 nm), in particular, were internalized by endocytosis and partly escaped endosomes. Cadherin-containing liposomes show high potential as a new cell-specific proteoliposome. The baculovirus expression-liposome fusion method is useful as a new enabling technology for biomedical applications of functional proteoliposomes.

1. Introduction

Cadherins are single-pass transmembrane receptors that mediate calcium-dependent cell–cell adhesion at adherens junctions and play an essential role in regulating major cellular behaviors, including cell growth, motility, and differentiation [1,2]. Type I classical cadherins, which include epithelial (E)-cadherin, neuronal (N)-cadherin and placental (P)-cadherin are homophilic, calcium-dependent glycoproteins. The changes in cadherin expression or "cadherin switching" plays a critical role during tumor progression [3,4]. A wide variety of tumor cells change from E-cadherin expression to N-cadherin expression [5,6]. N-cadherin plays a key role in the processes that are essential for tumor growth. The inhibiting activity of N-cadherin in cells that unnecessarily express this protein has an impact on tumor therapy. The synthetic cyclic peptide "N-Ac-CHAVC-NH2" (ADH-1) was discovered to be an N-cadherin antagonist [7]. The effects on tumor growth by which contains the sequence "His-Ala-Val" (HAV), are found in the first extracellular (EC1) domains of type I classical cadherins, and metastasis are likely to be multifaceted and complex by virtue of the wide variety of normal and tumor cells that express N-cadherin. ADH-1 has been shown to inhibit cell growth and motility *in vitro*, and tumor growth and invasion in vivo [8]. It has been shown at the histological level that ADH-1 disrupts tumor angiogenesis in several mouse solid tumor models [9].

We report here proteoliposomes containing adhesion proteins (cadherins) for the potential application to drug delivery systems. Recently, we developed new methods for the preparation of proteoliposomes without using detergents. The expressed membrane protein was incorporated directly into the liposome membrane upon *in vitro* synthesis, leading to pure membrane protein-containing liposomes [10–12]. An alternative method uses membrane fusion between liposomes and glycoprotein 64 (gp64) displayed on recombinant budded viruses (BVs) of baculovirus (*Autographa californica* nuclear polyhedrosis viruses (AcNPV)) under acidic conditions [13–17]. The original orientation and conformation of the membrane proteins are maintained by the fusion method. In this study N-cadherin (CDH2)-containing giant liposomes (GLs; ~5 μm in diameter) or small liposomes (about 150 nm in diameter) were prepared by using the baculovirus-liposome fusion method. Calcium ion dependent association of the CDH2 liposomes and interaction with cultured cells were investigated.

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2. Materials and methods

2.1. Materials

1,2-Dioleoyl-*sn*-3-phospho-*rac*-(1-glycerol) sodium salt (DOPG), 1,2-dioleoyl-*sn*-glycero-3- phosphocholine (dioleoylphosphatidylcholine; DOPC) and 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl) (Rh-DOPE) were purchased from Avanti Polar Lipids (Alabaster, AL). pBluescriptR/CDH2 (Accession No. BC036470) was purchased from Open Biosystems (Huntsville, AL). Rabbit anti-N cadherin IgG antibody (H-63) and mouse anti-baculovirus glycoprotein gp64 IgG antibody (AcV5) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Horseradish peroxidase conjugated anti-rabbit IgG was purchased from GE Healthcare (Piscataway, NJ). Alexa Fluor 546 $(ab')_2$ fragment of goat anti-rabbit IgG (H + L), Lysotracker Green, Sf-900 III SFM medium, Dulbecco's Modified Eagle Medium (DMEM) and fetal bovine serum (FBS) were purchased from Invitrogen (Carlsbad, CA). Human glioblastoma LN-229 cells were purchased from ATCC (Manassas, VA).

2.2. Construction of recombinant baculovirus and Sf9 cell culture

The recombinant baculovirus was constructed using a BaculoGold kit (BD Biosience, San Jose, CA). The pBluescriptR/CDH2 cDNA was designated with *Not*I and *BamH*I and these fragments were treated with cohesive ends to blunt ends. The blunt end fragment inserted into pVL1393 vector at *Sma*I. Sf9 cells (Invitrogen, Carlsbad, CA) were co-transfected with the vector for CDH2 and linearized baculovirus genome DNA. Sf9 cells were cultured in Sf-900 III SFM (Invitrogen) at 27 °C. The supernatant containing recombinant BVs was collected at 72 h after the co-transfection. The viral concentration was amplified three times.

2.3. BV envelopes preparation

An insect cell line Sf9 derived from the fall armyworm *Spodoptera frugiperda* was cultured in ten culture flasks (75 cm^2) containing 12 mL Sf-900 III medium each until they covered ~80% of the bottom of the flask. A suspension of CDH2 AcNPV BVs was spread in each flask at a multiplicity of infection (MOI) of 1. Flasks were incubated at 27 °C for 94 h and then placed at 4 °C. The culture medium was then centrifuged at 1000×*g* for 5 min at 4 °C to precipitate and remove cells. The supernatant was ultracentrifuged at 100,000×*g* for 1 h at 15 °C and the resultant pellet was resuspended in phosphate buffered saline (PBS, 1 mM Na_2HPO_4, 10.5 mM KH_2PO_4, 140 mM NaCl, 40 mM KCl, pH 6.2). That suspension was then centrifuged at 40000×*g* for 30 min at 15 °C in a stepwise sucrose density gradient (10, 15, 20, 25 and 30% sucrose (w/v) in PBS). Two bands resulted: the lower one contained intact BVs and the upper contained BV envelopes without nucleocapsids. Bands were recovered separately, diluted in PBS, and ultracentrifuged again at 100,000×*g* for 1 h at 15 °C. The pellet was dissolved in a Tris buffer (0.5 mL) and the amount of BV proteins was estimated using Protein Assay Kit (BIO-RAD, Hercules, CA).

2.4. Western blot analysis of recombinant budded viruses

One volume of the sample buffer (50 mM Tris-HCl (pH 6.8), 2% SDS, 0.1% bromophenol blue, 10% Glycerol, 100 mM DTT) was added to the samples. Proteins expressed CDH2 on Sf9 cells and BVs were separated by SDS-PAGE using 12.5% SDS-polyacrylamide gel and transferred to PVDF membranes. The membranes were treated with 0.5% skim milk in Tris buffered saline (TBS, 20 mM Tris, 500 mM NaCl pH7.4)-0.05% Tween 20 buffer (TBST) for blocking and were reacted with primary antibody in TBST. The following antibodies were used: polyclonal rabbit anti-N cadherin IgG antibody (H-63) and monoclonal mouse anti-baculovirus glycoprotein gp64 IgG (AcV5). The membranes linked with the primary antibody were then reacted with horseradish peroxidase conjugated anti-rabbit IgG and horseradish peroxidase conjugated anti-mouse IgG. The membrane was reacted with ECL Western Blotting Detection Reagents (GE Healthcare) and bands were visualized using LAS-4000 EPUV mini (FUJIFILM, Tokyo, Japan).

2.5. Giant liposome preparation

Giant liposomes (GLs) were prepared through gentle hydration of dry lipid films. A phospholipid solution of DOPG and DOPC (1:1 molar ratio) were dissolved in chloroform and put into a glass micro test tube and the solution evaporated under flowing argon gas until lipid films formed at the bottom of the tube. Films were then hydrated by adding buffers at various pHs [20 mM CH_3COOH/CH_3COONa (pH 4.5) or 20 mM Na_2HPO_4/NaH_2PO_4 (pH 7.0)] and incubated for more than 12 h at 27 °C to spontaneously form GLs.

2.6. CDH2 liposomes preparation

The giant liposomes [DOPG/DOPC (1:1)] hydrated with the sodium acetate buffer (pH 4.5) and the phosphate buffer (pH 7.0) were mixed with a suspension of recombinant CDH2 BVs or wild type BVs (about 5 μg), and kept for 30 min at 27 °C while giant CDH2 liposomes were prepared. To obtain a homogeneous particle diameter, the suspension was filtered using an Avanti Mini-extruder with 0.2 μm pore polycarbonate membranes. The liposome suspension was centrifuged at 40,000 × g for 30 min at 15 °C on a stepwise sucrose density gradient [10, 15, 20, 25 and 30% sucrose (w/v) in 10 mM Tris-HCl (pH 7.5)], and the liposome fraction was collected.

2.7. Microscopic observation of giant CDH2 liposomes

One mM of giant CDH2 liposomes (200 μL) or 1 mM giant liposomes fused with wild type BV (200 μL) was subjected to centrifugation (10,000×*g*, 10 min), and the resulting pellet was then resuspended with 200 μL of 10 mM Tris-HCl (pH 7.5). The suspension was treated with rabbit anti-N cadherin IgG antibody (H-63) as the primary antibody and incubated for 1 h at 37 °C. Next, the suspension was treated with the Alexa Fluor 546-conjugated $(ab')_2$ fragment of goat anti-rabbit IgG (H + L) as a secondary antibody and incubated for 30 min at 37 °C. The samples were observed using a confocal laser scanning microscope (LSM 510 META, Carl Zeiss, Oberkochen, Germany) with an oil-immersion objective lens (×63) at 560–615 nm (for Alexa Fluor 546) using a helium-neon laser (543 nm).

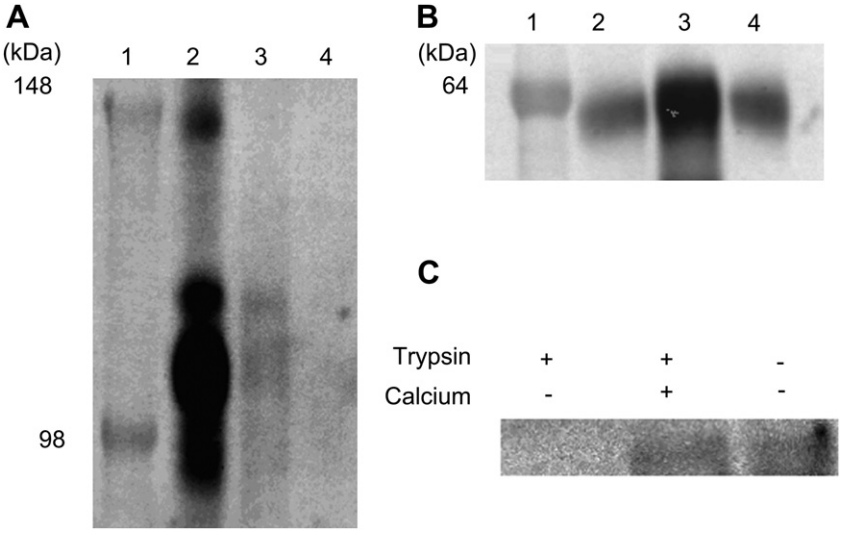

Fig. 1. Western blot analyses of recombinant CDH2 budded viruses (BVs). The blotted membrane was immuno-stained with either anti-CDH2 (A) or anti-gp64 (B). Lane 1, marker; lane 2, Sf9 cell infected with recombinant CDH2 BV; lane 3, recombinant CDH2 BV (5 μg of total protein); lane 4, wild type BV. Trypsinization experiment of CDH2 performed on recombinant CDH2 BV (C). Cadherins displayed on BVs are stable to proteolysis in the presence of Ca^{2+} but labile in its absence.