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Rapid communication

Modification of the C16Y peptide on nanoparticles is an effective approach to target endothelial and cancer cells via the integrin receptor

Nobuhito Hamano[a,1], Yoichi Negishi[a,*,1], Azusa Fujisawa[a], Maya Manandhar[a], Hinako Sato[a], Fumihiko Katagiri[b], Motoyoshi Nomizu[b], Yukihiko Aramaki[a]

[a] Department of Drug Delivery and Molecular Biopharmaceutics, School of Pharmacy, Tokyo University of Pharmacy and Life Sciences, Hachioji, Tokyo, Japan
[b] Department of Clinical Biochemistry, School of Pharmacy, Tokyo University of Pharmacy and Life Sciences, Hachioji, Tokyo, Japan

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ABSTRACT

Liposomes have been explored as potential drug and gene-delivery particles. In recent years, tumor-targeted liposomes have been developed to improve the efficacy of antitumor treatment. The C16Y peptide is a modified C16 peptide, which is derived from the laminin γ1 chain, and binds to integrins $\alpha_v\beta3$ and $\alpha5\beta1$ on endothelial cells. In this study, we prepared integrin-targeted C16Y peptide-modified liposomes (C16Y-L) to enhance the intracellular uptake of drugs and genes specifically into tumor tissues. The selectivity of C16Y-L for endothelial cells and cancer cells, which strongly express integrins $\alpha_v\beta3$ and $\alpha5\beta1$, was assessed by flow cytometry and confocal microscopy. The cellular uptake of C16Y-L by both cell types was higher than uptake of the un-labeled and scramble peptide-modified liposomes. Next, to ascertain the involvement of receptor-mediated endocytosis in the process, these cells were treated with C16Y-L for 1 h at 37 °C or at 4 °C. We found that uptake was also dependent on the temperature. Moreover, to evaluate whether the uptake depended on an integrin–ligand interaction, we examined the inhibition of C16Y-L uptake using recombinant integrin αVβ3 and found that the cellular uptake of C16Y-L treated with αVβ3 integrin decreased. This result suggests that C16Y-L can selectively target cells that highly express integrin αVβ3. Thus, the modification of the C16Y peptide on a Drug Delivery System (DDS) carrier may be a feasible approach for drug or gene delivery into tumors.

In tumor treatment, liposomes have been utilized as potential drug and gene-delivery particles. Compared with free drugs and genes, liposomal formulations typically exhibit prolonged systemic circulation times and increased tumor localization through their enhanced permeability and retention (EPR) effects (Maeda et al., 2000). Recently, tumor-targeted liposomes were used to improve the efficacy of antitumor treatment (Hatakeyama et al., 2007) and resulted in a significant suppression of tumor growth compared with non-targeted formulations. Active targeting of liposomes has been proposed to aid liposomes in reaching the target site effectively and to improve the cellular uptake of liposomes; for active targeting, the development of a carrier that can selectively deliver drugs or genes to specific receptors, such as transferrin or folate, is required and should be studied in detail (Suzuki et al., 2008; Xiang et al., 2008). In this study, we focused on the C16Y peptide, a 12-amino acid synthetic peptide, which is a modified C16 peptide, derived from the globular domain of the laminin γ1 chain that binds to the endothelial cell integrins, $\alpha_v\beta3$ and $\alpha5\beta1$ (Ponce et al., 2001, 2003). Integrin $\alpha_v\beta3$ has been reported to be strongly expressed on activated endothelial cells and cancer cells. In this study, we developed C16Y peptide-modified liposomes (C16Y-L) and examined whether C16Y-L can target endothelial and cancer cells via integrins $\alpha_v\beta3$ and $\alpha5\beta1$.

The C16Y peptide (DFKLFAVYIKYR-GGC) and a scramble peptide (IKDYLYFARVKF-GGC) as a control were synthesized manually using a 9-fluorenylmethoxycarbonyl (Fmoc)-based solid-phase strategy, prepared in the COOH terminal amide form and purified by reverse-phase high-performance liquid chromatography. The purity and identity of the peptides were confirmed by analytical HPLC and electrospray ionization mass spectrometry at the Central Analysis Center, Tokyo University of Pharmacy and Life Sciences. We first prepared C16Y peptide-modified liposomes (C16Y-L) that were composed of 1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC), 1,2-distearoylphosphatidylethanolamine-methoxy-polyethyleneglycol (DSPE-PEG_{2000}-OMe) and 1,2-distearoyl-*sn*-glycero-3-phosphatidylethanolamine-polyethyleneglycol-maleimide (DSPE-PEG_{2000}-Mal) at a molar ratio of 94:4:2 by a reverse-phase

* Corresponding author at: Department of Drug Delivery and Molecular Biopharmaceutics, School of Pharmacy, Tokyo University of Pharmacy and Life Sciences, 1432-1 Horinouchi, Hachioji, Tokyo 192-0392, Japan. Tel.: +81 42 676 3183, fax: +81 42 676 3183.
E-mail address: negishi@toyaku.ac.jp (Y. Negishi).
[1] The first two authors contributed equally to this work.

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evaporation method. The un-labeled (control) liposomes were also composed of DSPC and DSPE-PEG$_{2000}$-OMe (DSPC:DSPE-PEG$_{2000}$-OMe = 94:6). In brief, all of the reagents were dissolved in 1:1 (v/v) chloroform/diisopropylether. 2-(4-[2-Hydroxyethyl]-1-piperazinyl) ethanesulfonicacid (HEPES) buffer (10 mM, pH 6.0) was then added to the lipid solution, and the mixture was sonicated and then evaporated at 65 °C. The organic solvent was completely removed, and the size of the liposomes was adjusted to approximately 150 nm using extruding equipment and a sizing filter (Nuclepore Track-Etch Membrane; Whatman plc, UK). For the fluorescent labeling of the lipid membrane, 1,1-dioctadecyl-3,3,3,3-tetramethyl-indocarbocyanine perchlorate (DiI: 0.1 mol% of total lipids) was added. For coupling, C16Y or the scramble peptide was added to the liposomes at a molar ratio of 1.5-fold DSPE-PEG$_{2000}$-Mal in the presence of TCEP (final concentration: 20 mM). The mixture was incubated for 6 h at room temperature to conjugate the cysteine of the peptide with the maleimide of the liposomes using a thioether bond. The resulting peptide-modified liposomes (C16Y-L or Scramble-L) were passed through a Sephadex G-50 spin column to remove any excess peptide. The liposomal size and ζ potential value were measured by dynamic light scattering (Nicomp 380ZLS-S; Particle Sizing Systems, Santa Barbara, CA, USA). As shown in Table 1, the mean diameter of C16Y-L was estimated to be 199 nm, and the ζ potential of C16Y-L was estimated to be −17.70 mV. The ζ potential value of C16Y-L showed that C16Y-L was more negative than the un-labeled liposomes (PEG-L).

Table 1
Size and ζ potential of peptide-modified liposomes.

Liposomes	Mean diameter (nm) ± S.D	ζ, potential (mV) ± S.E
PEG-L (um-labeled)	165.5 ± 9.5	−6.60 ± 3.94
C16Y-L	199.2 ± 4.7	−17.70 ± 0.73
Scramble-L	160.3 ± 1.9	−18.16 ± 0.35

We next examined the selective cellular uptake of C16Y-L via the integrin receptor on human umbilical vein endothelial cells (HUVECs) and a murine melanoma cell line (B16) using flow cytometry analysis. As shown in Fig. 1, the cells treated with C16Y-L showed enhanced fluorescence intensities compared with PEG-L and scramble-L. In addition, to elucidate the intracellular uptake of C16Y-L, the intracellular localization of C16Y-L was examined after liposome treatment using confocal microscopy (Fig. 1C and D). The intracellular localization of the liposomes in the cells treated with C16Y-L was observed on the surface of the cell membrane and in the cytoplasm. The cellular uptake of C16Y-L on both of the cells was higher than that of PEG-L and scramble-L. Next, to

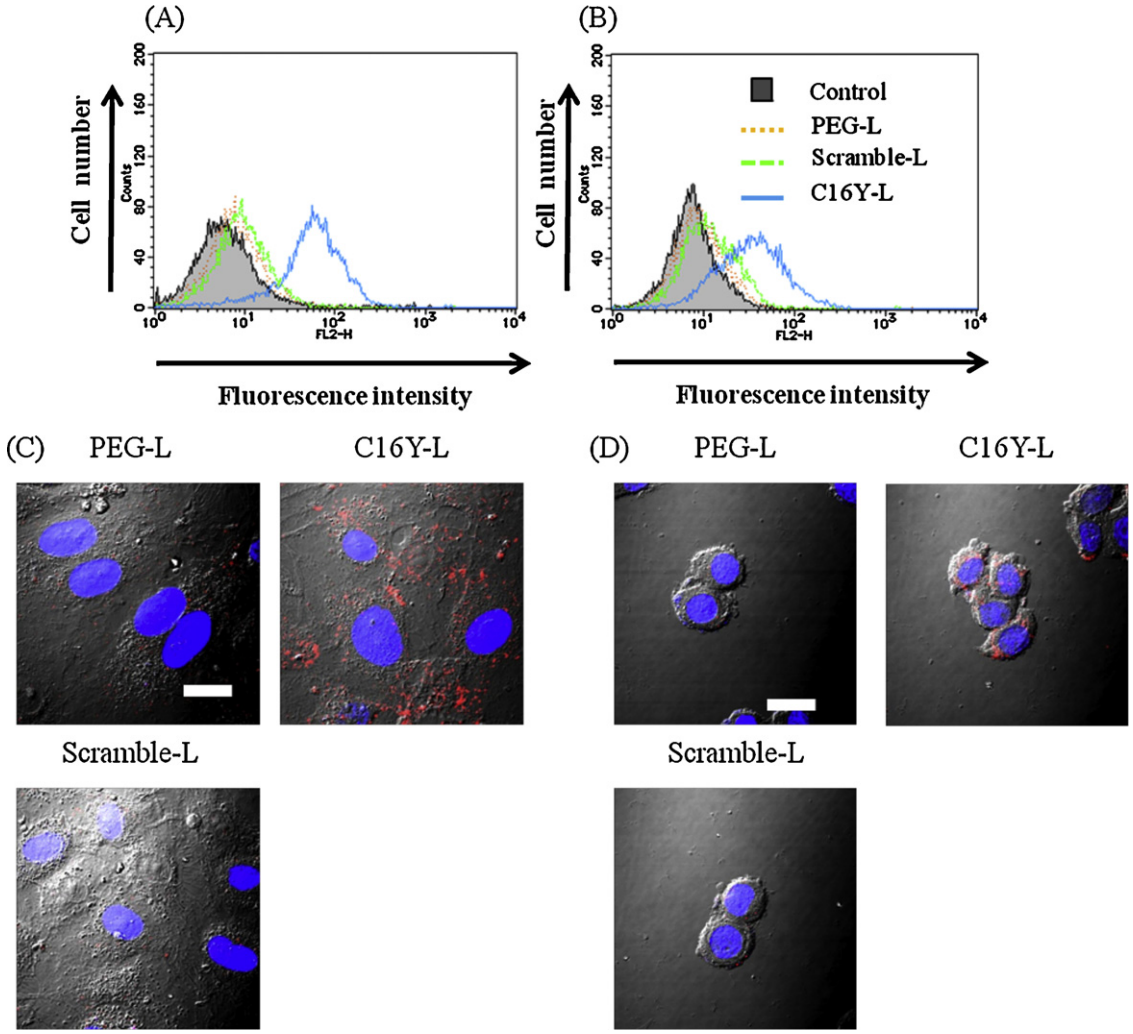

Fig. 1. Cellular uptake of peptide-modified liposomes by HUVECs (A) or B16 cells (B) and confocal microscopy images of HUVECs (C) or B16 cells (D). Cells were treated with DiI-labeled liposomes for 1 h at 37 °C. After incubation, the cells were washed, and the fluorescence intensities were measured by flow cytometry (A) or (B). The cells were then treated with DAPI (blue) for nuclear staining (C) and (D). Blue: DAPI fluorescence. Red: DiI. Scale bars represent 20 μm.

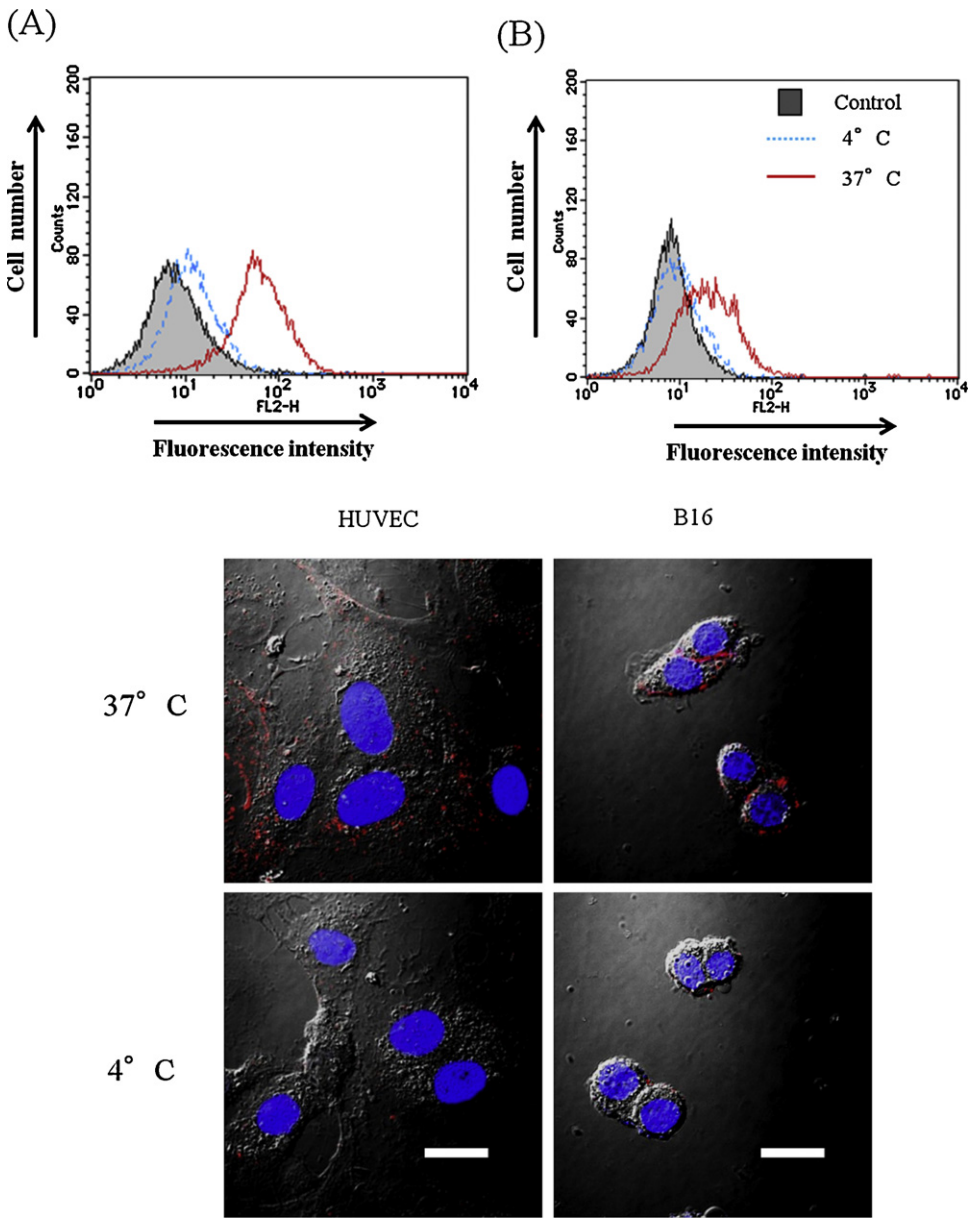

Fig. 2. Cellular uptake of C16Y peptide-modified liposomes into cells. HUVECs (A) and B16 cells (B) were treated with C16Y-liposomes for 1 h at 37 °C or 4 °C. After incubation, the cells were washed, and the fluorescence intensities were measured by flow cytometry. Confocal microscopy images of HUVECs or B16 cells. Cells were treated with DiI-labeled C16Y-liposomes for 1 h at 37 °C or 4 °C (C). After incubation, the cells were washed, fixed and treated with DAPI (blue) for nuclear staining. Blue: DAPI fluorescence. Red: DiI. Scale bars represent 20 μm.

examine the involvement of receptor-mediated endocytosis in the process, the cells were treated with C16Y-L for 1 h at 37 °C or at 4 °C. As shown in Fig. 2, the fluorescence intensity of the cells treated with C16Y-L at 4 °C was decreased compared to the treatment with C16Y-L at 37 °C. By blocking the cellular uptake at a low temperature, we found that C16Y-L could enhance the intracellular uptake via energy-dependent endocytosis.

Moreover, to verify that C16Y-L binds integrin αVβ3, we examined the inhibition of the cellular uptake of C16Y-L due to blockage by the recombinant αVβ3 protein. As shown in Fig. 3, the uptake of C16Y-L in the HUVECs was inhibited by the treatment with the recombinant αVβ3 protein. The cellular uptake of scramble-L also was not observed (Fig. 1). These results suggest that C16Y-L can selectively target integrin αVβ3.

In addition, C16Y-L did not show significant cytotoxicity in the HUVECs and B16 cells (data not shown), suggesting that C16Y-L could be useful as a drug or gene-delivery particle. The C16Y peptide itself has been reported to also inhibit angiogenesis and tumor growth (Ponce et al., 2003; Kim and Csaky, 2010); however, the present study indicated that there was no cytotoxicity of C16Y-L in the HUVECs and B16 cells as long as a small amount of the peptide modification was employed. Therefore, we are attempting to develop a C16Y-L coating that contains a higher amount of the peptide as a dual-function particle with effective targetability and cytotoxicity for cancer therapy.

In summary, we developed C16Y peptide-modified liposomes to enhance the specific intracellular uptake of drugs or genes by tumor tissues. The C16Y peptide is known as a ligand for integrins, which are expressed in tumor endothelial cells and various cancer cells. The intracellular uptake of C16Y-L was higher than that of PEG-L and scramble-L in the HUVECs and B16 cells. Moreover, the cellular uptake of C16Y-L was inhibited by low temperature and the recombinant αVβ3 protein, suggesting that

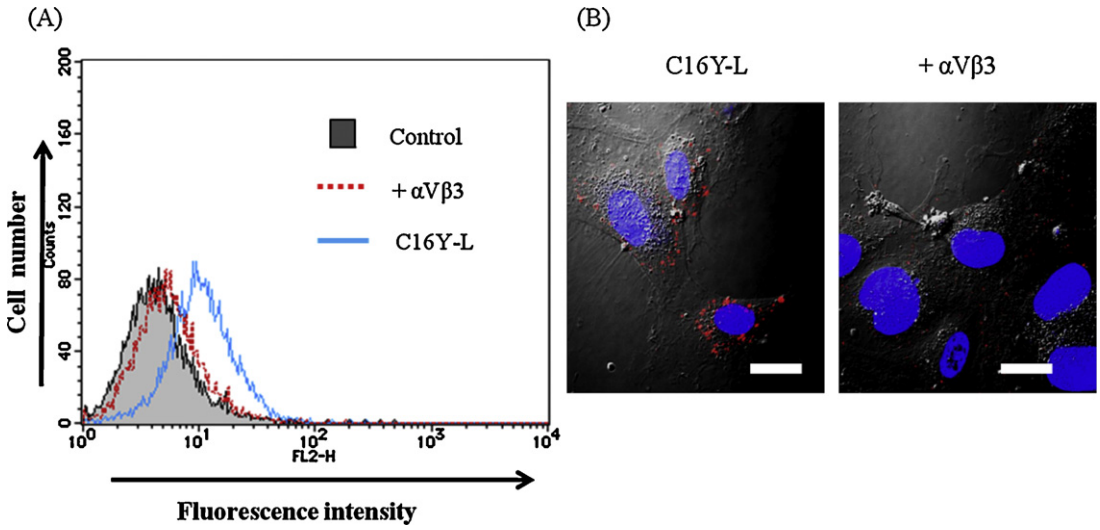

Fig. 3. Inhibition of the cellular uptake of C16Y-L owing to treatment with recombinant αVβ3 protein. The uptake of C16Y-L by HUVECs (A) and confocal microscopy images of HUVECs (B). C16Y-L was first incubated with recombinant αVβ3 protein for 30 min at 37 °C. Subsequently, the cells were treated with the mixture for 30 min at 37 °C. After incubation, the cells were washed and treated with DAPI (blue) for nuclear staining. Blue: DAPI fluorescence. Red: DiI. Scale bars represent 20 μm.

C16Y-L can selectively target cells that highly express integrin αVβ3. Thus, the modification of the C16Y peptide on a DDS carrier may be a feasible approach for drug or gene delivery into tumors.

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