Preparation and Characterization of Laminin-Derived Peptide AG73-Coated Liposomes as a Selective Gene Delivery Tool

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Targeted gene delivery to cancer cells is considered as a promising strategy for cancer therapy. Since, several targeting ligands have been studied for cancer gene therapy, such as transferrin, folate, anisamide, RGD-peptide, and antibodies. We have focused on AG73 peptide, which is derived from the globular domain of the laminin a1 chain. AG73 peptide is known as a ligand for syndecans, one of the major heparin sulfate-containing transmembrane proteoglycans. Syndecan-2 is highly expressed in various cancer cells and plays a role in angiogenesis. In this study, we prepared AG73-labeled polyethylene glycol-modified liposomes (AG73-PEG liposomes) for gene delivery to syndecan-2 overexpressing cancer cells, and assessed the characterization of AG73-PEG liposomes. We confirmed the conjugation of AG73 peptide to PEG liposomes by reverse-phase high-performance liquid chromatography analysis. Electron microscopy analysis showed that monodisperse AG73-labeled liposomes were prepared. We also assessed the gene transfection efficiency of AG73-PEG liposomes in syndecan-2 overexpressing cancer cells or syndecan-2 less expressing cancer cells. As a result, AG73-mediated liposomal gene transfection efficiency was increased by 100-fold in syndecan-2 overexpressing cancer cells compared to syndecan-2 less expressing cancer cells. These results suggested that AG73-PEG liposomes were successfully prepared from a point of view of the modification of AG73 peptide to PEG-liposomes and the particle size of liposomes, which presented nano size. Furthermore, our results suggest that AG73-PEG liposomes can be a useful targeted gene delivery vehicle for syndecan-2 overexpressing cancer cells.

Key words AG73 peptide; gene delivery; liposome; syndecan

In gene therapy, it is necessary that the development of delivery vehicles or vectors can selectively deliver therapeutic genes to target cells safely and with high efficiency. There are two main approaches in gene delivery, viral gene delivery and non-viral gene delivery. Although viral vector have high transfection efficiencies over a wide range of cell targets, they have major limitations, including virally-induced inflammatory responses and oncogenic effects.12) Non-viral vectors, which are generally delivered as a complex with chemical and/or biochemical vectors such as cationic lipids or polymers, continue to be an attractive alternative to viral vectors due to their safety, versatility, and ease of preparation and scale-up. Non-viral vectors, however, generally suffer from relatively low transfection efficiencies.3,4)

Selective gene therapy approaches have been considered as a promising cure for various cancers. Therefore, it is necessary to develop delivery vehicles that can selectively deliver therapeutic genes to target cancer cells safely and with high efficiency. Several different targeting moieties have been used in studies on cancer gene therapy, such as transferrin, folate, anisamide, RGD-peptide, and antibodies.5–10)

In this context, the present study focused on AG73 peptide, which is 12 amino acid synthetic peptide derived from the globular domain of the laminin a1 chain. AG73 peptide is as a ligand for syndecans, one of the major heparin sulfate-containing transmembrane protein.11–13) It has been reported that syndecan-2 is highly expressed in various cancer cells and play a role in angiogenesis.14–18)

Therefore, AG73-labeled polyethylene glycol-modified liposomes (AG73-PEG liposomes) were prepared, which were capable of encapsulating a gene condensed by poly-l-lysine.

In this study, we assessed the characterization and transfection efficiency of prepared AG73-PEG liposomes. To confirm the conjugation of AG73 peptide to liposomes and the morphology of AG73-PEG liposomes, we performed high-performance liquid chromatography (HPLC) analysis and electron microscopy observation. Furthermore, we examined whether AG73-PEG liposomes can deliver genes to cells selectively via syndecan-2.

MATERIALS AND METHODS

Materials The plasmid pCytomegalovirus (CMV)-Luc is an expression vector encoding the firefly luciferase gene under the control of a cytomegalovirus promoter.

Cell Lines and Cultures A 293T human embryonic kidney carcinoma cell line, stably overexpressing syndecan-2 (293T-Syn2), was cultured in Dulbecco’s modified Eagle’s medium (DMEM; Kohjin Bio Co., Ltd., Tokyo, Japan), supplemented with 10% fetal bovine serum (FBS; Equitech Bio Inc., Kerrville, TX, U.S.A.), penicillin (100 U/ml), streptomycin (100 μg/ml), and puromycin (0.4 μg/ml), at 37°C in an humidified 5% CO2 atmosphere.

Preparation of AG73-PEG Liposomes The Cys-AG73 peptide (CGG-RKRLQVQLSIRT) and scrambled Cys-AG737 control peptide (CGG-LQQRRSVLRTKI) were synthesized manually using the 9-fluorenylmethoxycarbonyl (Fmoc)-based solid-phase strategy, prepared in the COOH-terminal amide form. 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. AG73-labeled...
PEG liposomes were prepared by the hydration method. pDNA (plasmid DNA) diluted in 10 mM HEPES (N-(2-hydroxyethyl)piperazine-N'-2-ethanesulfonic acid) buffer (pH 7.4) (0.1 mg/ml) was condensed using poly-L-lysine (PLL) (0.1 mg/ml) (SIGMA-Aldrich Co., St. Louis, MO, U.S.A.). The pDNA/PLL complex was prepared at nitrogen/phosphate (N/P) ratio of 2.4. The complex of pDNA and PLL was added to a lipid film composed of 1,2-dioleoyl-sn-glycerol-3-phospho-rac-1-glycerol (DOPG) (AVANTI Polar Lipids Inc., Alabaster, AL, U.S.A.), 1,2-dioleoyl-sn-glycerol-3-phosphoethanolamine (DOPE) (AVANTI Polar Lipids Inc., Alabaster, AL, U.S.A.), 1,2-distearoyl-sn-glycerol-3-phospho(1-yl)-ethanolamine-polyethylene glycol-maleimide (DSPE-PEG2000-Mal) in a molar ratio of 2:9:0.57, followed by incubation for 10 min at room temperature to hydrate the lipids. The solution was sonicated for 5 min in a bath-type sonicator (42 kHz, 100 W) (BRANSONIC 2510J-DTH, Branson Ultrasonic Co., Danbury, CT, U.S.A.). For coupling, AG73 peptide, at a molar ration of five-fold DSPE-PEG2000-Mal, was added to the PEG liposomes, and the mixture was incubated for 6 h at room temperature to conjugate cysteine of Cys-AG73 peptide with the maleimide of the PEG liposomes using a thioether bond. The resulting AG73-peptide-conjugated PEG liposomes (AG73-PEG liposomes) were dialyzed to remove any excess peptide. AG73-PEG liposomes using a thioether bond. The resulting AG73-peptide-conjugated PEG liposomes (AG73-PEG liposomes) were dialyzed to remove any excess peptide. AG73-PEG liposomes were modified with 5 mol% PEG and 5 mol% peptides. The particle size and ζ-potential of prepared liposomes were measured by NICOMP 380 ZLS (Particle Sizing Systems, Santa Barbara, CA, U.S.A.).

High Performance Liquid Chromatography (HPLC) Reverse-phase HPLC was performed with a C18 column (US 120 250×4.6 mm, Shiseido Co., Tokyo, Japan) was used with a gradient of water and acetonitrile with 0.1% (v/v) trifluoroacetic acid (20—40% acetonitrile over 20 min) with detection by UV absorption at 215 nm and flow rate was 1 ml/min.

Electron Microscopy Analysis Prepared AG73-PEG liposomes were visualized under transmission electron microscope by negative staining technique at ×50000 or ×20000 magnification.

Flow Cytometry Analysis 293T and 293T-Syn2 cells (6×10⁵) were treated with anti-syndecan-2 antibody (R&D Systems Inc., Minneapolis, U.S.A.) or isotype control antibody (Bechman Coulter, Inc., Brea, CA, U.S.A.) for 30 min at 4 °C. Cells were subsequently treated with Alexa Fluor 488-conjugated secondary antibody (Invitrogen Japan K.K., Tokyo, Japan) for 30 min at 4 °C, and then the fluorescence intensities were measured by flow cytometry.

Transfection of pDNA into Cells Using AG73-PEG Liposomes The 2 d before the experiments, 293T and 293T-Syn2 cells (1×10⁵) were seeded in a 48-well plate. The cells were treated with AG73-PEG liposomes (pDNA: 0.9 μg/well) in serum-free medium for 4 h at 37 °C. pDNA was complexed with Lipofectamine2000 (LF2000) (Invitrogen Japan K.K., Tokyo, Japan) according to the manufacturer’s instructions. After replacement with fresh medium, the cells were cultured for 20 h and then luciferase activity was measured. Cell lysate were prepared with a lysis buffer (0.1 M Tris–HCl (pH 7.8), 0.1% Triton X-100, and 2 mM EDTA (ethylenediaminetetraacetic acid). Luciferase activity was measured using a luciferase assay system (Promega, Madison, WI, U.S.A.) and a luminometer (LB96V, Belthold Japan Co., Ltd., Tokyo, Japan). The activity is indicated as relative light units (RLU) per mg protein.

RESULTS AND DISCUSSION

For selective gene delivery, we prepared AG73-PEG liposomes, which encapsulating pDNA condensed by poly-L-lysine. AG73 peptide is considered as a ligand for syndecans, and it has been reported that syndecan-2 is highly expressed in various cancer cells. In this study, we assessed the characterization and transfection efficiency of prepared AG73-PEG liposomes.

Some methods for modification of ligands to liposomes were reported.¹⁹) One of the most useful and efficient coupling procedure is conjugation of thiolated ligands to liposomes containing maleimide group. The reaction of maleimide and thiol groups is rapid and proceeds close to completion.²⁰) Furthermore, the reaction is proceeds at close to neutral pH, at ambient temperature, and even when relatively low concentrations of the reactants are present. So, it is useful methods for conjugation of ligands with liposomes. Therefore, we modified peptide to liposomes by thioether bond by PEG liposomes and peptide mixture.

To confirm the conjugation of AG73 to PEG liposomes, reverse-phase HPLC analysis was performed. As a result, free AG73 dissolved in the mobile phase eluted at a retention time of around 10 min (Fig. 1A). Furthermore, when the mixture of AG73 peptide and PEG liposomes was injected, the peak for the free AG73 peptide was obtained (Fig. 1B). In contrast, when AG73-PEG liposomes following the coupling step were injected, AG73-PEG liposomes dissolved in the mobile phase eluted at a retention time of around 13 min (Fig. 1C). The mixture of AG73 peptide and AG73-PEG liposomes were also injected, and two peaks of the corresponding free AG73 peptide and AG73-PEG liposomes were obtained. These result suggested that Cys-AG73 peptide conjugated to DSPE-PEG2000-Mal could give a different retention time than the free AG73 peptide, and AG73 peptide could conjugate with DSPE-PEG2000-Mal.

We evaluated the average size and zeta potential of non-labeled, AG73, or AG73T-PEG liposomes with 5 mol% DSPE-PEG2000 and modified 5 mol% peptides. The size and zeta po-

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Fig. 1.  HPLC Confirmation of AG73 Conjugation to Liposomes

(A) Free AG73 eluted with a retention time of around 10 min. (B) The mixture of AG73 peptide and PEG liposomes was injected. (C) AG73-PEG liposomes eluted with a retention time of around 13 min. (D) The mixture of AG73 peptide and AG73-PEG liposomes was injected.
The potential of the liposomes were determined as about 200 nm with a slight negative charge (Table 1).

Next, the morphology of AG73-PEG liposomes was confirmed by electron microscopy. As shown in Fig. 2, the transmission electron microscopic (TEM) observations revealed that monodisperse liposomes had been successfully produced.

It was examined whether AG73-PEG liposomes could selectively deliver genes to syndecan-2 overexpressing cells. We confirmed that the expression of syndecan-2 in a 293T human embryonic kidney carcinoma cell line and a stable syndecan-2 overexpressing 293T cell line (293T-Syn2 cell) by flow cytometry analysis. As a result, 293T-Syn2 cells treated with anti-syndecan-2 antibody had significantly higher fluorescence intensity compared with 293T cells (Fig. 3). This result suggested that 293T-Syn2 cells expressed syndecan-2 highly.

It was determined whether AG73-mediated liposomal gene transfection could be achieved selectively. Two days before the experiments, 293T-Syn2 cells (1×10⁵) were seeded in a 48-well plate. The cells were treated with AG73-PEG liposomes encapsulating pCMV-Luc, which encodes the firefly luciferase gene under the control of a cytomegalovirus enhancer/promoter (pDNA: 0.9 μg/well), in serum-free medium for 4 h at 37°C. After replacement with fresh medium, the cells were cultured for 20 h and then luciferase activity was measured. As shown in Fig. 4, the luciferase activity was increased by the treatment of AG73-PEG liposomes in 293T-Syn2 cells compared to 293T cells. However, the luciferase activity was not significantly different between 293T-Syn2 cells and 293T cells by the treatment of Non-labeled, AG73T-PEG liposomes, and Lipofectamine2000 (LF2000). These results suggested that AG73-PEG liposomes could selectively deliver genes into syndecan-2 overexpressing cells.

We also confirmed that AG73-PEG liposomes could selectively associated with syndecan-2 overexpressing cells by flow cytometry analysis (data not shown), and the significant cytotoxicity was not observed by the treatment of AG73-PEG liposomes (data not shown). It has been reported that the transfection efficiency was enhanced up to about 100-fold by transferrin coated lipoplexes compared with non-coated lipoplexes in HeLa cells (human epithelial carcinoma cell line). On the other hands, our results indicated that AG73-PEG liposomes had about 100-fold higher gene transfection efficiency than that of non-labeled PEG liposomes in 293T-Syn2 cells (Fig. 4). Therefore, AG73-PEG liposomes may

### Table 1. Particle Size and ζ-Potential of Prepared Liposomes

<table>
<thead>
<tr>
<th>pDNA/PLL complexes</th>
<th>Non-labeled PEG liposomes</th>
<th>AG73-PEG liposomes</th>
<th>AG73T-PEG liposomes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diameter (nm)</td>
<td>155.5±21.3</td>
<td>215.4±29.6</td>
<td>240.7±31.7</td>
</tr>
<tr>
<td>ζ-Potential (mV)</td>
<td>19.4±0.5</td>
<td>-5.0±1.0</td>
<td>-1.8±3.4</td>
</tr>
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</table>

a) Data represent means and S.D.
have an equal utility of transferrin coated liposomes for selective gene delivery in vitro. However, it is necessary that the transfection efficiency and selectivity of AG73-PEG liposomes are evaluated in vivo.

In summary, it was shown that AG73-PEG liposomes were successfully prepared from a point of view of the modification of AG73 peptide to PEG-liposomes and the particle size of liposomes, which presented nano size. Our result suggested that PEG liposomes modified with the AG73 peptide, which is considered as a ligand for syndecans, could be a useful targeted gene delivery vehicle for syndecan-2 overexpressing cancer cells. Therefore, AG73-PEG liposomes may be a promising method to achieve selective gene delivery for cancer gene therapy.

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