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Cancer cell specific gene delivery by laminin-derived peptide AG73-labeled liposomes

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ABSTRACT

We developed laminin-derived AG73 peptide labeled liposomes for cancer specific gene therapy. AG73 peptide is well known as a ligand for syndecan-2 which is highly expressed in various cancer cells. Liposomes labeled with AG73 showed high efficient transfection efficiency in syndecan-2 overexpressing cells, and found that AG73 could be a superior molecule in the development of non-viral vector using liposomes for the gene delivery to syndecan-2 overexpressing cancer cells.

Approaches of cancer specific gene therapy have been considered as a promising cure. Therefore, the development of delivery vehicle that can selectively deliver therapeutic genes to target cancer cells safely and with high efficiency would be required, and the design of highly efficient carriers for gene delivery has been studied by many research group.[1–3] Some targeting molecules have been reported in studies for cancer gene therapy, such as transferrin,[4] folate,[5] RGD-peptide,[6] and antibodies.[7] In this Letter, we focused on AG73, which is 12 amino acid synthetic peptide and is located in the carboxyl end of the laminin α1 chain. AG73 peptide is known a ligand for syndecans, one of the major heparan sulfate-containing transmembrane proteoglycans.[8] Syndecan-2 is highly expressed in various cancer cell lines and plays a role in angiogenesis.[9,10] We thus developed AG73-labeled liposomes (AG73-liposomes), which were capable of encapsulating a gene condensed by poly-L-lysine. In this Letter, we examined whether AG73-liposomes can deliver genes to cancer cells selectively via syndecan-2.

The stearyl-AG73 peptide (stearyl-GGG-RKRLQVQLSIRT) and scrambled stearyl-AG73 peptide (stearyl-GGG-LQQRRSVLRTKI), control peptide, were prepared according to Futaki et al.[11] The Gly-AG73 peptide and scrambled Gly-AG73T control peptide were synthesized manually using the 9-fluorenylmethoxycarbonyl (Fmoc)-based solid-phase strategy with the COOH-terminal amide form and purified by reverse-phase HPLC.[12] Structures of peptides used in this study were shown in Figure 1. AG73-labeled liposomes were prepared by the hydration method.[13] To condense plasmid DNA encoding luciferase (pCMV-Luc), the DNA (0.1 mg/mL) dissolved in 10 mM HEPES buffer (pH 7.4) was mixed with poly-L-lysine (PLL, mw. 27,400; Invitrogen) solution (0.1 mg/mL) under vortexing at room temperature. The nitrogen/phosphate (N/P) ratio of condensed PLL/DNA was 2.4. A condensed DNA solution (0.25 mL) was added to the lipid film, formed by the evaporation of a chloroform solution of 137.5 nmol lipids composed of 1,2-dioleoyl-*sn*-glycero-3-[phospho-*rac*-(1-glycerol)] (DOPG; Avanti Polar Lipids, Alabaster, AL) and 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE; Avanti Polar Lipids, Alabaster, AL) (DOPG/DOPE = 9:2 (molar ratio)) on the bottom of a glass tube, followed by incubation for 10 min to hydrate lipids. The glass tube was then sonicated for 5 min in the bath type sonicator (Bransonic 2510, Branson Ultrasonics, Danbury, CT). After sonication, the liposomes were incubated with stearyl-AG73 or stearyl-AG73T (5% of total lipids) for 30 min at room temperature to anchor these peptide at liposomal surface. In the case of flow cytometry and confocal microscopic experiments, 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-*N*-(lissamine rhodamine B sulfonyl) (rhodamine-DOPE, Avanti Polar Lipids, Alabaster, AL) was incorporated at 0.5% of the total lipid. Liposomes were extruded through Mini-Extruder with polycarbonate membrane filter (Nucleopore, Pleasanton, CA) of 100 nm. The liposomal size was measured by a dynamic light scattering (Nicomp 380ZLS-S, Internationa Businessl., Ltd, Tokyo), and mean diameter was estimated as 197 nm.

We examined whether laminin-derived peptide AG73 could be candidate for a selective gene delivery to cells overexpressing syndecan-2. The expression of syndecan-2 in a 293T human embryonic kidney carcinoma cell line (293T cells) and stably

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Stearyl-AG73: $CH_3(CH_2)_{16}$-CONH-Gly-Gly-Gly-Arg-Lys-Arg-Leu-Gln-Val-Gln-Leu-Ser-Ile-Arg-Thr-$CONH_2$
Stearyl-AG73T: $CH_3(CH_2)_{16}$-CONH-Gly-Gly-Gly-Leu-Gln-Gln-Arg-Arg-Ser-Val-Leu-Arg-Thr-Lys-Ile-$CONH_2$

Figure 1. Structures of peptides used in this study.

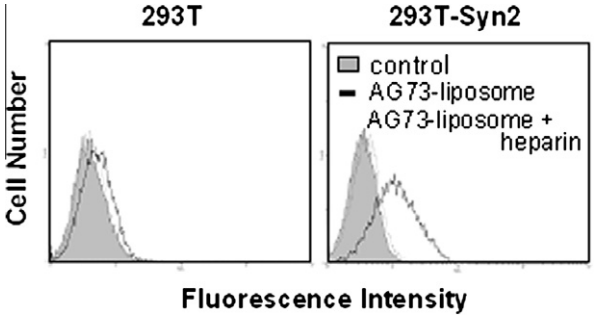

Figure 2. Association of AG73-liposomes to cells. 293T and 293T-Syn2 cells were treated with rhodamine-labeled AG73-liposomes (20 nmol/well) in the presence or absence of heparin (1 μg/mL) for 1 h at 37 °C, and the association of liposomes to the cells was evaluated with flow cytometry.

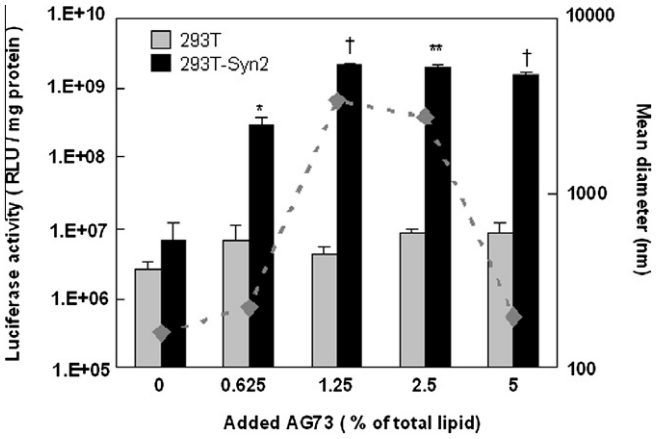

Figure 4. Effect of AG73 coating on luciferase activity by AG73-liposomes. 293T cells and 293T-Syn2 cells were treated with liposomes (10 nmol/well) labeled with different amount of AG73 in the absence of FBS at 37 °C for 4 h, and the luciferase activity was measured using Luciferase Assay System (Promega). Dashed line indicates the mean diameter of AG73-liposomes. *; p <0.05, **; p <0.05, †; p <0.005 versus 293T (n = 3).

overexpressing syndecan-2 393T cell line (293T-Syn2 cell) was evaluated by flow cytometry, and was confirmed that 293T-Syn2 cells are over expressing syndecan-2 on the cell surface (data not shown). We then examined the association of AG73-labeled liposomes to 293T and 293T-Syn2 cells using flow cytometry. As shown in Figure 2, fluorescence intensity derived from rhodamin, a component of liposomes, increased when 293T-Syn2 cells were treated with AG73-liposomes, but no increase was observed in 293T cells. It is reported that AG73 site functions as a heparin/heparan sulfate binding and cell attachment site in laminin-1, and the site is important for the interaction between laminin-1 and syndecans, cell surface receptors.[8] The attachment of fibroblast to AG73 coating plate was inhibited completely by the addition of heparin.[8] Thus the effects of heparin (1 μg/mL) on the association of AG73-liposomes to cells were examined, and heparin drastically inhibited the binding of liposomes to the 293T-Syn2 cells. These findings suggest that AG73-liposomes associate with 293T-Syn2 cells via the syndecan-2.

Cells were incubated with naked liposomes, AG73-liposomes, and AG73T-liposomes at 37 °C for 1 h, and the association of liposomes to 293T and 293T-Syn2 cells was also evaluated by confocal microscopy (Fig. 3). Although fluorescence derived from rhodamin was faintly observed in cells treated with naked liposomes, the highest fluorescence was observed in 293T-Syn2 cells treated with AG73-liposomes. No fluorescence was observed in cells treated with liposomes labeled with scrambled AG73 peptide (AG73T-liposomes).

The cells (1×10^5/48-well plate) were treated with AG73-liposomes encapsulating pCMV-Luc encoded the firefly luciferase gene under the control of a cytomegalovirus enhancer/promoter (encapsulated pDNA: 3 μg/mL) 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. Figure 4 shows the expression of luciferase activity following the transfection with liposomes labeled with different amount of AG73 peptide. Although luciferase activity increased with an increase in amount of surface AG73 peptide, the saturation was observed at 1.25% and almost the same luciferase expression was observed when the cells were transfected with 5% AG73. Mean diameter of various liposomes were measured by Nicomp 380ZSL, and evaluated as

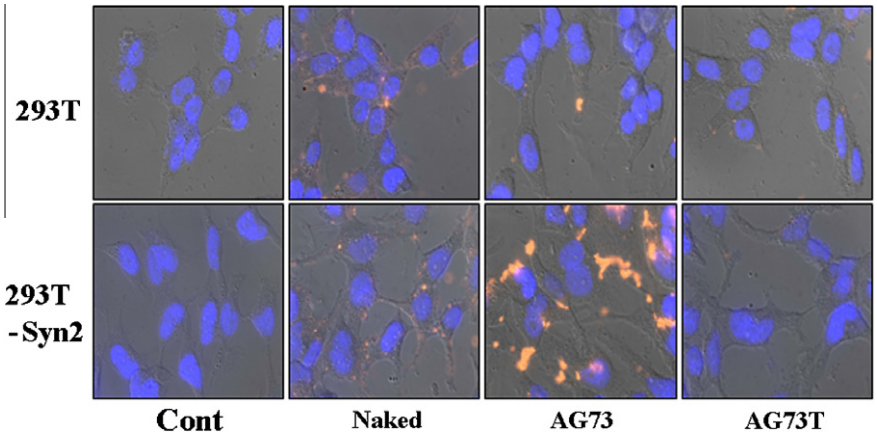

Figure 3. 293T and 293T-Syn2 cells were incubated with non-labeled (naked) liposomes, AG73-liposomes and AG73T-liposomes at 37 °C for 1 h, and localization of liposomes labeled with rhodamine-DOPE was evaluated by confocal microscopy. Red; rhodamine-DOPE (liposome), Blue: 4′,6-diamidino-2-phenylindole (DAPI) (nucleus).

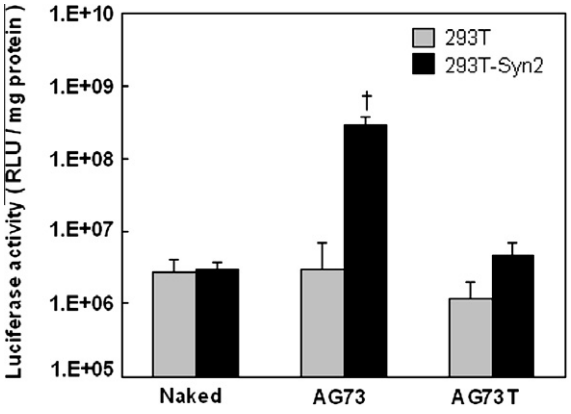

Figure 5. Luciferase activity following the transfection with AG73-liposomes. 293T cells and 293T-Syn2 cells were transfected with AG73-liposomes (10 nmol/well) in the absence of FBS at 4 °C for 4 h. After replacement with fresh medium containing FBS, the cells were cultured 37 °C for 20 h, and then luciferase activity was measured using Luciferase Assay System (Promega). †; p <0.005 versus 293T (n = 3).

159.6, 222.8, 3391.3, 2707.8, and 197.1 nm for naked liposomes, 0.625%, 1.25%, 2.5%, and 5% AG73-liposomes, respectively. Large size of liposomes is restricted for in vivo use, thus AG73-liposomes labeled with 5% AG73 with a size of about 200 nm were used following experiments.

In order to confirm the selective gene delivery of laminin-derived peptide AG73-labeled liposomes, transgene expression was evaluated using liposomes labeled with AG73 peptide and scramble peptide AG73T. As shown Figure 5, the highest luciferase expression was observed when 293T-Syn2 cells were transfected with AG73-liposomes, but not with AG73T-liposomes. In 293T cells with low level of syndecan-2 expression, luciferase activity was low even when the cells were transfected with AG73-liposomes. Cytotoxicity of AG73-liposomes was also evaluated by Cell Counting Kit-8 (Dojindo, Kumamoto, Japan). Cell viability was over 85% of control cells, indicating no significant cytotoxicity of AG73-liposomes. AG73-liposomes, but not AG73T-liposomes, also showed cancer cell specific gene expression in HepG2 cells, human hepatoblastoma cells, overexpressing syndecan-2 (data not shown). These findings suggested that liposomes labeled with laminin-derived peptide AG73 could be candidate for a selective gene delivery to cells overexpressing syndecan-2.

In summary, many research groups have reported that some vehicles labeled with targeting molecules, such as transferrin,[4] folate,[5] RGD-peptide,[6] and antibodies[7] are candidate for non-viral vectors of cancer gene therapy. We prepared liposomes labeled with AG73 peptide, which is considered as a ligand for syndecans, and examined the transfection efficacy in syndecan-2 overexpressing cancer cells. Extensively high transgene expression was observed in 293T-Syn2 and HepG2 cells, overexpressing syndecan-2, suggesting that AG73 could be found as a superior molecule for the development of cancer cell specific non-viral vector of liposomes.

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