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Enhanced Laminin-Derived Peptide AG73-Mediated Liposomal Gene Transfer by Bubble Liposomes and Ultrasound

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Abstract: A promising strategy as a cancer therapeutic is tumor-targeted gene delivery. The AG73 peptide derived from the laminin α1 chain is a ligand for syndecans, and syndecan-2 is highly expressed in some cancer cells. In this study, AG73-PEG liposomes were developed for selective gene delivery to syndecan-2 overexpressing cancer cells. AG73-PEG liposomes were used in combination with Bubble liposomes and ultrasound exposure to enhance transfection efficiency by promoting the escape of the liposomes from the endosome to the cytosol. AG73-PEG liposomes showed selective gene delivery to syndecan-2 overexpressing cancer cells. Furthermore, AG73-mediated liposomal gene transfection efficiency was enhanced by 60-fold when Bubble liposomes and ultrasound exposure were used, despite the absence of an increase in the uptake of AG73-PEG liposomes into the cells. Confocal microscope analysis revealed that the Bubble liposomes and ultrasound promoted intracellular trafficking of the AG73-PEG liposomes during gene transfection. Thus, the combination of AG73-PEG liposomes with Bubble liposomes and ultrasound exposure may be a promising method to achieve selective and efficient gene delivery for cancer therapy.

Keywords: AG73 peptide; Bubble liposomes; gene delivery; syndecan-2; ultrasound

Introduction

The success of human gene therapy depends upon the development of delivery vehicles or vectors that can selectively deliver therapeutic genes to target cells safely and with high efficiency. There are two main approaches in gene delivery, namely, viral gene delivery and nonviral gene delivery. Although viral vectors have high transfection efficiencies over a wide range of cell targets, they have major limitations, including virally induced inflammatory responses and oncogenic effects.[1,2] Nonviral vectors, which are generally delivered as a complex with chemical and/or biochemical

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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. Nonviral vectors, however, generally suffer from relatively low transfection efficiencies.[3,4]

A promising strategy for enhancing cancer gene therapy is tumor-targeted gene delivery. Some targeting moieties have been used in studies for cancer gene therapy, such as transferrin, folate, anisamide, RGD-peptides, and antibodies.[5−10]

The present study focused on AG73, which is 12 amino acid synthetic peptide derived from the globular domain of the laminin α1 chain. AG73 peptide is a ligand for syndecans, one of the major heparan sulfate-containing transmembrane proteoglycans.[11−13] Syndecan-2 is highly expressed in various cancer cell lines and plays a role in angiogenesis.[14−18] Therefore, AG73-labeled polyethyleneglycol-modified liposomes (AG73-PEG liposomes) were developed, which are capable of encapsulating a gene condensed by poly-L-lysine. However, PEG modification of liposomes can enhance the stability of pDNA in serum and also suppress the association of liposomes with cells or cause endosomal escape of liposomes, leading to a decrease in transfection efficiency.[19−23]

A novel approach to the administration of a drug or gene is the use of ultrasound (US)-enhanced delivery. US-enhanced delivery exploits the cavitation bubbles produced by the pressure oscillations of US. Furthermore, US waves above a certain threshold can cause oscillating bubbles to undergo a violent collapse known as inertial cavitation. Inertial cavitation is believed to enhance the permeability of a tissue or a cell membrane transiently.[24−28] Microbubbles, which are contrast agents for medical US imaging, improve

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permeability after US-induced cavitation.[29−34] However, microbubbles have problems associated with their size, stability, and targeting function. Therefore, the present study developed echo-contrast gas entrapping liposomes, also known as Bubble liposomes (BLs). We found that BLs and US exposure could enhance the permeability of a tissue or the cell membrane transiently.[35−38] We hypothesized that BLs and US may affect not only the cell membrane but also intracellular vesicles and could enhance the escape of pDNA from endosomes to the cytoplasm.

This study assessed the selectivity of AG73-PEG liposomes for syndecan-2 overexpressing cells and examined whether AG73-mediated liposomal gene transfection could be enhanced by BLs and US exposure to achieve highly efficient transfection.

Experimental Section

Materials. The plasmid pCMV-Luc is an expression vector encoding the firefly luciferase gene under the control of a cytomegalovirus promoter. Chloroquine was purchased from Sigma (St. Louis, MO). Cy3-labeled pDNA was purchased from Mirus Bio LLC, Madison, WI. Alexa Fluor 488-conjugated transferrin was purchased from Molecular Probes, Inc. (Eugene, OR).

Cell Lines and Cultures. A 293T human embryonic kidney carcinoma cell line, stably overexpressing syndecan-2 (293T-Syn2 cell), 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), penicillin (100 U/mL), streptomycin (100 μg/mL), and puromycin (0.4 μg/mL), at 37 °C in a humidified 5% CO_2 atmosphere.

Preparation of AG73-PEG Liposomes. The Cys-AG73 peptide (CGG-RKRLQVQLSIRT) and scrambled Cys-AG73T control peptide (CGG-LQQRRSVLRTKI) were synthesized manually using the 9-fluorenylmethoxycarbonyl (Fmoc)-based solid-phase strategy, prepared in the COOH-terminal amide form and purified by reverse-phase high-performance liquid chromatography. AG73-labeled PEG liposomes were prepared by the hydration method. pDNA diluted in 10 mM HEPES 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). The complex of pDNA and PLL was added to a lipid film composed of 1,2-dioleoyl-*sn*-glycero-3-phospho-*rac*-1-glycerol (DOPG) (AVANTI Polar Lipids Inc., Alabaster, AL), 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE) (AVANTI Polar Lipids Inc., Alabaster, AL), and 1,2-distearoyl-*sn*-glycero-3-phosphatidylethanolamine-polyethyleneglycol-maleimide (DSPE-PEG_{2000}-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, Bransoni Ultrasonic Co., Danbury, CT). For coupling, AG73 peptide, at a molar ratio of 5-fold DSPE-PEG_{2000}-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 were modified with 5 mol % PEG and 3 mol % peptides. The peptide conjugates were confirmed by protein assay (Thermo Scientific Inc., MA).

Preparation of Bubble Liposomes. PEG liposomes composed of 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) (NOF Corporation, Tokyo, Japan) and 1,2-distearoyl-*sn*-glycero-3-phosphatidylethanolamine-polyethyleneglycol (DSPE-PEG_{2000}-OMe) (NOF corporation, Tokyo, Japan) in a molar ratio of 94:6 were prepared by a reverse-phase evaporation method. In brief, all reagents were dissolved in 1:1 (v/v) chloroform/diisopropyl ether. Phosphate buffered saline was added to the lipid solution, and the mixture was sonicated and then evaporated at 47 °C. The organic solvent was completely removed, and the size of the liposomes was adjusted to less than 200 nm using extruding equipment and

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a sizing filter (pore size: 200 nm) (Nuclepore Track-Etch Membrane, Whatman plc, U.K.). The lipid concentration was measured using a Phospholipid C test Wako (Wako Pure Chemical Industries, Ltd., Osaka, Japan). BLs were prepared from liposomes and perfluoropropane gas (Takachio Chemical Ind. Co. Ltd., Tokyo, Japan). First, 2 mL sterilized vials containing 0.8 mL of liposome suspension (lipid concentration: 1 mg/mL) were filled with perfluoropropane gas, capped, and then pressurized with a further 3 mL of perfluoropropane gas. The vial was placed in a bath-type sonicator (42 kHz, 100 W) (BRANSONIC 2510j-DTH, Branson Ultrasonics Co., Danbury, CT) for 5 min to form BLs.

Transfection of pDNA into Cells Using AG73-PEG Liposomes. The two days before the experiments, 293T-Syn2 cells (1×10^5) were seeded in a 48-well plate. The cells were treated with AG73-PEG liposomes (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.

Transfection of pDNA into Cells by Combination of AG73-PEG Liposomes with BLs and US Exposure. The two days before the experiments, 293T-Syn2 cells (1×10^5) were seeded in a 48-well plate. The cells were treated with AG73-PEG liposomes (encapsulated pDNA: 3 μg/mL) in serum-free medium for 4 h at 37 °C. After incubation, the cells were washed twice to remove any excess AG73-PEG liposomes that were not associated with the cells and BLs (120 μg/mL) were added. Then, US exposure was applied through a 6 mm diameter probe placed in the well (frequency, 2 MHz; duty, 50%; burst rate, 2 Hz; intensity, 1.0 W/cm^2; time, 10 s). A Sonopore 3000 (NEPA GENE, CO., Ltd., Chiba, Japan) was used to generate the US exposure. The cells were cultured for 20 h, and then luciferase activity was determined and cell viability was measured using an MTT assay.

Measurement of Luciferase Expression. Cell lysate was prepared with a lysis buffer (0.1 M Tris-HCl (pH 7.8), 0.1% Triton X-100, and 2 mM EDTA). Luciferase activity was measured using a luciferase assay system (Promega, Madison, WI) and a luminometer (LB96 V, Belthold Japan Co. Ltd., Tokyo, Japan). The activity is indicated as relative light units (RLU) per mg protein.

Flow Cytometry Analysis. The two days before the experiments, 293T-Syn2 cells (2×10^5) were seeded in a 24-well plate. 0.2 mol % Rhodamine-labeled AG73-PEG liposomes (pDNA: 3 μg/mL) were added to the cells and incubated for 1 h at 37 °C. The cells were then collected, and the fluorescence intensities were measured by flow cytometry to evaluate the cellular association of liposomes.

To examine the effect of BLs and US exposure on cellular uptake of pDNA, AG73-PEG liposomes (encapsulated Cy3-labeled pDNA: 3 μg/mL) were added to cells and incubated for 4 h at 37 °C. After incubation, the cells were washed twice and BLs (120 μg/mL) were added. Then, US exposure was applied (frequency, 2028 kHz; duty, 50%; burst rate, 2.0 Hz; intensity, 1.0 W/cm^2; time, 10 s). Subsequently, the cells were incubated for 10 or 180 min, and then the cells were collected by trypsinization and washed with PBS supplemented with heparin (50 μg/mL) three times to remove AG73-PEG liposomes and pDNA bound to the cell surface. The fluorescence intensities were measured by flow cytometry.

Table 1. Characteristics of Prepared Liposomes[a]

	diameter (nm)	ζ-potential (mV)
nonlabeled PEG liposomes	155.6 ± 37.2	−4.77 ± 2.28
AG73-PEG liposomes	152.0 ± 17.2	−1.54 ± 0.25
AG73T-PEG liposomes	156.7 ± 38.5	−1.38 ± 0.33

[a] Data represent means and SD of three different determinations.

Confocal Laser Scanning Microscopy (CLSM). 293T-Syn2 cells (7×10^4) were seeded two days before the experiments. The cells were treated with AG73-PEG liposomes (Cy3-labeled pDNA: 3 μg/mL) and Alexa Fluor 488-conjugated transferrin (50 μg/mL) for 4 h at 37 °C. After incubation, the cells were washed and BLs (120 μg/mL) were added. Then, US exposure was applied (frequency, 2028 kHz; duty, 50%; burst rate, 2.0 Hz; intensity, 1.0 W/cm^2; time, 10 s). Subsequently, the cells were incubated for 10, 60, or 180 min and then fixed with 4% paraformaldehyde for 1 h at 4 °C. Then, CLSM was performed. To differentiate the AG73-PEG liposomes internalized into the cytoplasm following attachment to the surface of the cell membrane, the cytoplasm was distinguished from the cell membrane as shown in a previous paper.[39,40] The rate of colocalization of Cy3-labeled pDNA with Alexa Fluor 488-conjugated transferrin was quantified as follows: amount of colocalization (%) = Cy3 pixels$_{colocalization}$/Cy3 pixels$_{total}$ × 100, where Cy3 pixels$_{colocalization}$ represents the number of Cy3 pixels colocalizing with Alexa Fluor 488-conjugated transferrin and Cy3 pixels$_{total}$ represents the number of all Cy3 pixels.

Results

Characteristics of AG73-PEG Liposomes. We evaluated the average size and zeta potential of nonlabeled, AG73, or AG73T-PEG liposomes with 5 mol % DSPE-PEG$_{2000}$ and modified with 3 mol % peptides. The size and zeta potential of the liposomes were determined as about 150 nm with a slight negative charge (Table 1).

Receptor-Mediated Gene Delivery by AG73-PEG Liposomes. We evaluated the selective association of AG73-PEG liposomes with 293T-Syn2 cells (Syndecan-2 overexpressing cell) via the syndecan-2 receptor. The cells were incubated with either nonlabeled, AG73, or AG73T-PEG liposomes containing Rhodamine-DOPE for 1 h at 37 °C, and fluorescence intensities were examined by flow cytom-

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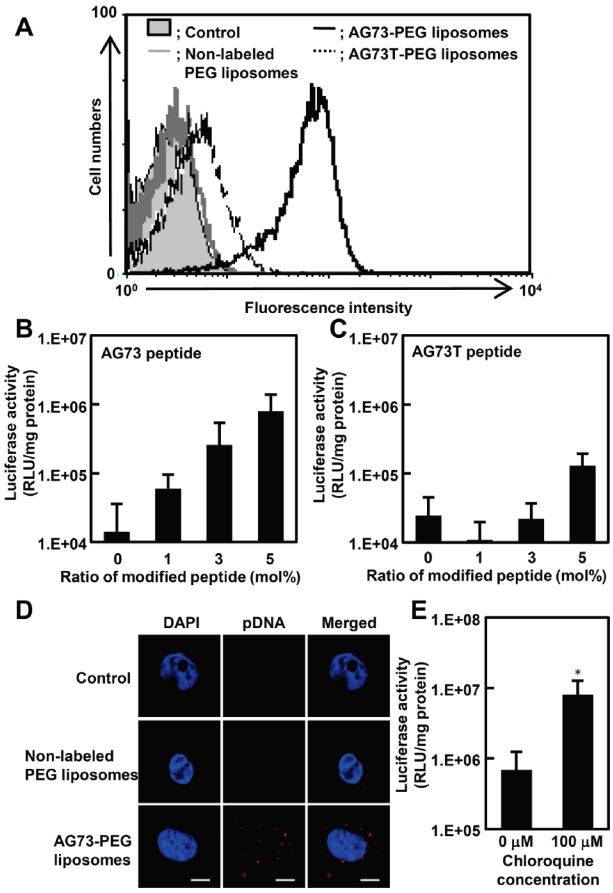

Figure 1. Effect of AG73 coating on liposome cell binding and gene transfer. (A) 293T-Syn2 cells were treated with either Rhodamine-labeled nonlabeled, AG73- or AG73T-PEG liposomes for 1 h at 37 °C. The fluorescence intensities were measured by flow cytometry. (B, C) The cells were incubated with the PEG liposomes modified with AG73 or AG73T in various ratios for 4 h at 37 °C. After replacement with fresh medium, the cells were cultured for 20 h and then luciferase activity was determined. Data are shown as means ± SD ($n = 4$). (D) The cells were treated with nonlabeled or AG73-PEG liposomes encapsulating Cy3-labeled pDNA (red) for 1 h at 37 °C. The nucleus was stained with DAPI (blue), and then the cells were observed by CLSM. The scale bars represent 10 μm. (E) The cells were preincubated with or without chloroquine for 30 min before transfection and then treated with AG73-PEG liposomes in the presence or absence of chloroquine for a further 4 h at 37 °C. After replacement with fresh medium, the cells were cultured for 20 h and luciferase activity was determined. Data are shown as means ± SD ($n = 4$). *$p < 0.05$ compared with treatment in the absence of chloroquine.

etry. The cells treated with AG73-PEG liposomes showed an enhancement of fluorescence intensities compared with nonlabeled and AG73T-PEG liposomes (Figure 1A). The delivery efficiency of AG73-PEG liposomes was higher than that of the nonlabeled liposomes. Next, the effects of AG73 coating of PEG liposomes on gene transfection were examined. The cells were treated with AG73-PEG liposomes that were modified with AG73 at various ratios for 4 h at 37 °C, and then luciferase activity was measured after an additional incubation for 20 h. The increase in luciferase activity depended upon the ratio of AG73 modified with PEG liposomes, while the luciferase activity was not enhanced by AG73T-PEG liposomes (Figure 1B,C). Furthermore, to elucidate the subcellular localization of pDNA after uptake by syndecan-2 receptors, AG73-PEG liposomes containing Cy3-labeled pDNA were monitored in the cells by confocal laser scanning microscopy. In the cells treated with AG73-PEG liposomes, the fluorescence of pDNA was observed on the surface of the cell membrane and in the cytoplasm after incubation for 1 h. In contrast, the fluorescence of pDNA was weak in the cytoplasm of cells treated with nonlabeled PEG liposomes after a 1 h incubation (Figure 1D).

Although AG73-PEG liposomes could introduce genes into the cells via syndecan-2, it is believed that PEG-modification of liposomes affects the endosomal escape of liposomes, leading to a decrease in gene expression after transfection.[19−23] Therefore, to assess the ability for endosomal escape of AG73-PEG liposomes, cells were transfected with AG73-PEG liposomes in the presence of chloroquine, which is recognized as an endosomolytic agent.[41−43] The resulting luciferase activity was 10-fold higher than that following treatment with AG73-PEG liposomes in the absence of chloroquine (Figure 1E). We conclude that AG73-PEG liposomes can selectively deliver genes to the cells via syndecan-2, but they may not release genes into cytoplasm and nucleus efficiently.

Enhancement of AG73-Mediated Liposomal Gene Transfection by BLs and US. To investigate the effect of BLs and US exposure on the transfection efficiency of AG73-PEG liposomes, 293T-Syn2 cells were treated with either nonlabeled or AG73-PEG liposomes for 4 h at 37 °C in serum-free medium, and then the cells were treated with BLs and US exposure. After treatment with AG73-PEG liposomes, the luciferase activity was enhanced up to 60-fold by BLs and US exposure when compared with that of AG73-PEG liposomes alone. Furthermore, the combination of AG73-PEG liposomes with BLs and US exposure had about 60-fold higher luciferase activity than that of nonlabeled liposomes with BLs and US exposure (Figure 2A). We also examined the transfection efficiency by treatment of AG73-PEG liposomes with US in the absence of BLs. As a result,

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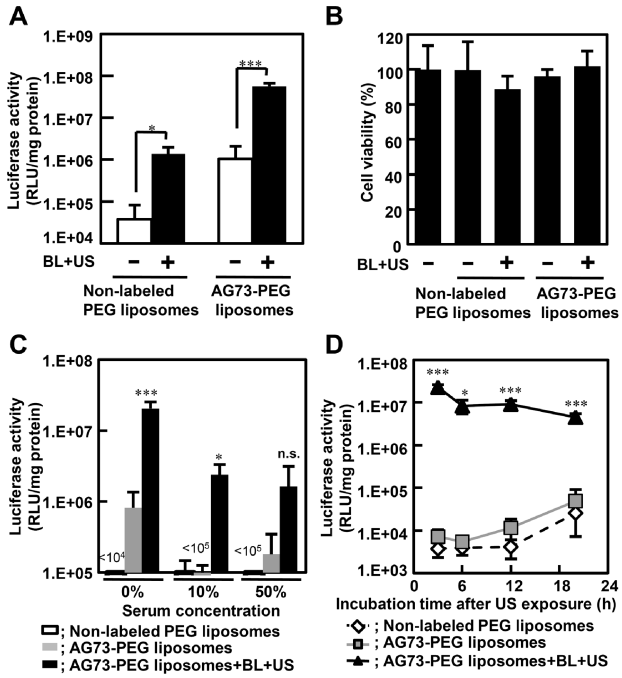

Figure 2. Effect of BLs and US exposure on AG73-mediated liposome-mediated gene transfection. (A, B) Nonlabeled or AG73-PEG liposomes were added to 293T-Syn2 cells. After 4 h incubation, the cells were washed and BLs were added. Then, the cells were exposed to US and cultured for 20 h. Then luciferase activity was determined and cell viability was measured using a MTT assay. Data are shown as means ± SD ($n = 4$). *$p < 0.05$, ***$p < 0.005$. (C) The cells were treated with either nonlabeled or AG73-PEG liposomes in the various serum concentrations. After the incubation for 4 h, the cells were washed and BLs were added. Then, the cells were exposed to US and cultured for 20 h. Then, luciferase activity was determined. Data are shown as means ± SD ($n = 4$). *$p < 0.05$, ***$p < 0.005$ compared with AG73-PEG liposomes. (D) Cells were treated with either nonlabeled or AG73-PEG liposomes. After incubation for 4 h, the cells were washed and BLs were added. Then, the cells were exposed to US and incubated for 3, 6, 12, or 20 h. Then, luciferase activity was determined. Data are shown as means ± SD ($n = 4$). *$p < 0.05$, ***$p < 0.005$ compared with AG73-PEG liposomes.

the transfection efficiency was barely enhanced by treatment with AG73-PEG liposomes with US compared with that of AG73-PEG liposomes alone (data not shown). The cytotoxicity of the combination of AG73-PEG liposomes with BLs and US exposure was determined using an MTT assay. Cell viability was more than 90% even after each transfection (Figure 2B). Since cancer gene therapy in the clinic needs to be effective in the presence of serum, we examined the effects of serum on gene transfection by AG73-PEG liposomes with BLs and US exposure. As shown in Figure 2C, the luciferase activity after treatment with AG73-PEG liposomes was increased by BLs and US exposure even in the presence of serum.

The mechanism of transfection involving the combination of AG73-PEG liposomes with BLs and US exposure could be different from that of AG73-PEG liposomes alone. Therefore, to investigate the kinetics of gene expression, cells were treated with either nonlabeled or AG73-PEG liposomes for 4 h, and then the cells were treated with BLs and US exposure. Luciferase activity was measured sequentially after US exposure. As shown in Figure 2D, the luciferase activity after the treatment with AG73-PEG liposomes was enhanced by BLs and US exposure within 3 h after US exposure compared with that of nonlabeled or AG73-PEG liposomes alone. However, the luciferase activity after the treatment with either nonlabeled or AG73-PEG liposomes alone increased in a time-dependent manner; and the luciferase activity with the combination of AG73-PEG liposomes with BLs and US exposure was maintained at a higher level at all time points. We conclude that BLs and US exposure can enhance AG73 mediated liposomal gene transfection.

The Mechanism of Transfection by AG73-PEG Liposomes with BLs and US Exposure. To evaluate the effects of BLs and US exposure on the cellular uptake of pDNA, flow cytometry analysis was performed to measure the fluorescence intensities of Cy3-labeled pDNA in 293T-Syn2 cells transfected with AG73-PEG liposomes with or without BLs and US exposure. Cellular uptake of pDNA showed almost no difference in the presence of AG73-PEG liposomes with or without BLs and US exposure (Figure 3A). To examine the involvement of endocytosis in the process, the cells were first treated with AG73-PEG liposomes for 1 h at 37 °C or at 4 °C, and then the fluorescence intensity was measured by flow cytometry. As shown in Figure 3B, the fluorescence intensities of the cells treated with AG73-PEG liposomes for 1 h at 4 °C were decreased compared with the treatment of AG73-PEG liposomes for 1 h at 37 °C. Next, the involvement of endocytosis in the transfection with AG73-PEG liposomes was examined. The cells were transfected by AG73-PEG liposomes with or without BLs and US exposure at 37 or 4 °C. Twenty-three hours after transfection, the luciferase activity was measured. When the cells were transfected by AG73-PEG liposomes with BLs and US exposure at 37 °C, the luciferase activity was increased compared with that of the cells treated with AG73-PEG liposomes alone. In contrast, the luciferase activity did not change in the cells treated with AG73-PEG liposomes with BLs and US exposure at 4 °C compared with the treatment of AG73-PEG liposomes alone (Figure 3C).

We further established whether BLs and US exposure could enhance gene expression with AG73-PEG liposomes. The cells were treated with AG73-PEG liposomes for 4 h at 37 °C, and then AG73-PEG liposomes attached to the surface of the cell membrane were removed with trypsin and heparin, followed by BLs and US exposure treatment. As a result, the luciferase activity was increased by BLs and US exposure even when AG73-PEG liposomes attached to the surface of the cell membrane were removed (Figure 3D). The effects of chloroquine on gene transfection by AG73-PEG liposomes with BLs and US exposure were also examined. The cells

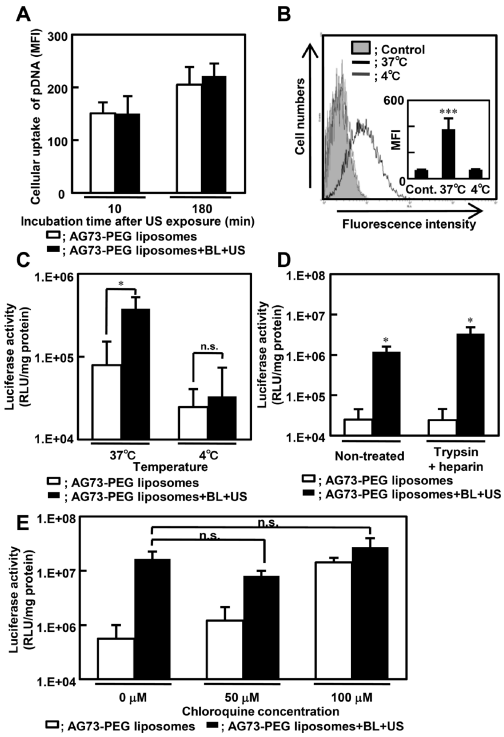

Figure 3. Mechanism of accelerated AG73-mediated liposomal gene transfer by BLs and US exposure. (A) 293T-Syn2 cells were incubated with AG73-PEG liposomes encapsulating Cy3-labeled pDNA for 4 h at 37 °C. After the incubation, the cells were washed and BLs were added. Then the cells were exposed to US and incubated for 10 or 180 min. Then, the cells were collected and washed with heparin-containing PBS three times. The fluorescence intensities were measured by flow cytometry. Data are shown as means ± SD ($n = 3$). (B) The cells were preincubated for 30 min at 37 or 4 °C before transfection and then treated with Rhodamine-labeled AG73-PEG liposomes for a further 1 h at 37 or 4 °C. The cells were then collected and washed with heparin-containing PBS three times. The fluorescence intensities were measured by flow cytometry. (C) The cells were preincubated for 30 min at either 37 or 4 °C before transfection and then treated with AG73-PEG liposomes for a further 1 h at 37 or 4 °C. After incubation, the cells were washed and BLs were added. Then, the cells were exposed to US and cultured for 23 h. Luciferase activity was determined. Data are shown as means ± SD ($n = 4$). *$p < 0.05$. (D) The cells were treated with AG73-PEG liposomes for 4 h at 37 °C and then washed with heparin-containing PBS following BLs and US exposure. The cells were cultured for 20 h and luciferase activity was determined. Data are shown as means ± SD ($n = 4$). *$p <$ 0.05 compared with AG73-PEG liposomes. (E) The cells were preincubated with or without chloroquine for 30 min before transfection and then treated with AG73-PEG liposomes in the presence or absence of chloroquine for a further 4 h. After incubation, the cells were washed and BLs were added. Then, the cells were exposed to US and cultured for 20 h. Then luciferase activity was determined. Data are shown as means ± SD ($n = 4$).

were pretreated for 30 min with chloroquine and transfected with pDNA using AG73-PEG liposomes with or without BLs and US exposure in the presence of chloroquine. As shown in Figure 3E, the cells treated with AG73-PEG liposomes alone showed the enhancement of luciferase activity in the presence of chloroquine (0−100 μM). In contrast, for cells treated using AG73-PEG liposomes with BLs and US exposure, the luciferase activity was not affected significantly even at the high dose of chloroquine. We conclude that BLs and US exposure can affect AG73-PEG liposomes internalized into the cells.

Intracellular Distribution of pDNA. To evaluate the intracellular distribution of pDNA, 293T-Syn2 cells were treated with AG73-PEG liposomes containing Cy3-labeled pDNA in the presence of Alexa Fluor 488-conjugated transferrin for 4 h, and then the cells were treated with BLs and US exposure. After the US exposure, cells were incubated for 10, 60, or 180 min and observed by confocal laser scanning microscopy. In the cells treated with AG73-PEG liposomes alone, the fluorescence of pDNA colocalized with the fluorescence of transferrin (Figure 4A). In contrast, the fluorescence of pDNA colocalized with transferrin was reduced. The ratio of Cy3-labeled pDNA with Alexa Fluor 488-conjugated transferrin was also quantified. As shown in Figure 4B, the ratio of colocalization of Cy-3 labeled pDNA with Alexa Fluor 488-conjugated transferrin was decreased by treatment with AG73-PEG liposomes with BLs and US exposure compared with the treatment of AG73-PEG liposomes alone. We conclude that BLs and US exposure can affect the intracellular trafficking of pDNA and enhance the transfection efficacy of AG73-PEG liposomes.

Discussion

The selective delivery of pDNA into tumors could enhance cancer gene therapy, and some targeting of ligands, such as transferrin, folate, anisamide, RGD-peptides, and antibodies, has been used.[5−10] For selective gene delivery into tumors, AG73-PEG liposomes encapsulating pDNA were developed. The laminin-derived AG73 peptide is a known ligand for syndecans, and it has been reported that syndecan-2 is highly expressed in various cancer cells.[11−13,15,17]

First, it was assessed whether the AG73-PEG liposomes could deliver genes to syndecan-2-overexpressing cancer cells (293T-Syn2 cells) selectively. The cellular association (Figure 1A), transfection efficacy (Figure 1B,C), and cellular uptake (Figure 1D) were examined. AG73-PEG liposomes could strongly associate with 293T-Syn2 cells, deliver pDNA effectively into the cells, and increase luciferase gene expression depending upon the ratio of AG73 peptide modified PEG liposomes used. These results suggested that AG73 is involved in the association of the liposomes with the cells via Syndecan2, and that pDNA can be internalized efficiently into cells, leading to the increase in gene expression.

PEG-modification is believed to suppress cellular association and/or endosomal escape of liposomes, and it decreased

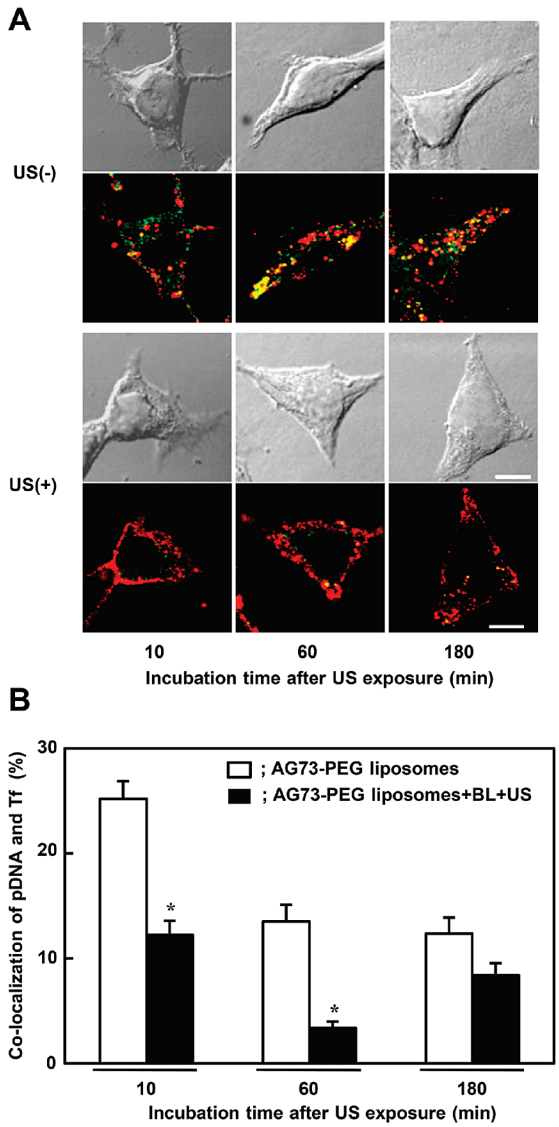

Figure 4. Effect of BLs and US exposure on the colocalization of pDNA and transferrin. (A, B) 293T-Syn2 cells were treated with AG73-PEG liposomes encapsulating Cy3-labeled pDNA (red) and Alexa Fluor 488-conjugated transferrin (green) for 4 h at 37 °C. After incubation, the cells were washed and BLs were added. Then, the cells were exposed to US and incubated for 10, 60, or 180 min. Then the cells were fixed with 4% paraformaldehyde for 1 h at 4 °C and observed by CLSM. The scale bars represent 10 μm. The ratio of colocalization of Cy3-labeled pDNA with Alexa Fluor 488-conjugated transferrin was quantified. Data are shown as means ± SE ($n = 100$). *$p < 0.05$ compared with AG73-PEG liposomes (Mann−Whitney's U test).

gene expression.[19−23] Therefore, it was assessed whether AG73-PEG liposomes could transport pDNA into the cytoplasm and nucleus by escaping from endosomes efficiently. In the presence of chloroquine, the luciferase gene expression by AG73-PEG liposomes was apparently increased when compared with incubation without chloroquine (Figure 1D). This result suggested that AG73-PEG liposomes may not deliver the encapsulated pDNA into the cytoplasm and nucleus efficiently. It was also confirmed that AG73-liposomes without PEG modification generated higher gene expression when compared with AG73-PEG liposomes (data not shown). However, it was necessary to stabilize liposomes in the blood to obtain therapeutic efficacy in systemic cancer gene therapy. Therefore, it is necessary to further enhance the transfection efficacy by PEG-modified liposomes for cancer gene therapy *in vivo*.

The present study developed echo-contrast gas entrapping liposomes (BLs) and found that BLs and US exposure could enhance the permeability of tissue cell membranes transiently.[35−38] It was also hypothesized that BLs and US might affect not only the cell membrane but also intracellular vesicles and enhance the escape of pDNA from endosomes into the cytoplasm. We assessed whether AG73-mediated liposomal gene transfection could be enhanced by BLs and US exposure. To determine the effects of BLs and US exposure on AG73-PEG liposomes either within the cells or attached to the surface of the cell membrane, excess AG73-PEG liposomes in the medium were removed after a 4 h incubation. Gene transfection efficiency with AG73-PEG liposomes was enhanced by BLs and US exposure even in the presence of serum (Figure 2A,C), and higher luciferase gene expression was observed at 3 h after US exposure compared with treatment with AG73-PEG liposomes alone (Figure 2D). It was shown that luciferase expression remained at a plateau, rather than decreasing, following treatment using AG73-PEG liposomes with BLs and US exposure (Figure 2D). For this reason, it may be considered that pDNA/poly-L-lysine complexes may be first decondensed and the naked pDNA may be degraded gradually after treatment with BLs and US exposure, which could affect the intracellular trafficking of pDNA leading to enhancement of the release of pDNA from endosomes into the cytosol and the transfer of pDNA to the nucleus. Therefore, luciferase expression could increase at an early time point and then decrease in a time-dependent manner. In contrast, in treatments with AG73-PEG liposomes or nonlabeled PEG liposomes alone, luciferase expression increased (Figure 2D). For this reason, it may be considered that AG73-PEG liposomes attached to the cell membrane via syndecan-2 or nonlabeled PEG liposomes attached to the cell membrane nonspecifically, which could not be removed completely and might be internalized in a time-dependent manner, leading to a gradual increase in luciferase expression subsequently. We also confirmed an enhancement of the transfection efficiency of AG73-PEG liposomes by BLs and US exposure in B16 melanoma cells expressing syndecan-2 (data not shown). These results suggest that BLs and US exposure can lead to enhanced gene expression.

We determined the mechanism of the enhancement of AG73-mediated liposomal gene transfection by BLs and US exposure. It had been speculated that BLs and US exposure could affect cell membranes and/or intracellular vesicles and enhance the intracellular transport of AG73-PEG liposomes

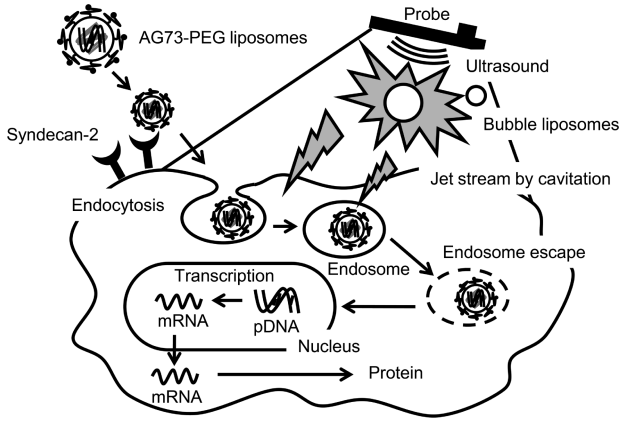

Figure 5. Diagram of gene transfer by AG73-PEG liposomes with Bubble liposomes and ultrasound exposure.

and the trafficking of AG73-PEG liposomes. Therefore, the effect of BLs and US exposure on cellular internalization of pDNA was investigated by flow cytometry (Figure 3A). However, the fluorescence intensities of pDNA in the cells were not affected remarkably by BLs and US exposure. Next, it was examined whether BLs and US exposure could affect AG73-PEG liposomes either attached to the surface of the cell membrane or internalized into cells. The uptake of AG73-PEG liposomes into the cells was diminished at 4 °C. The transfection efficiency by itself was not enhanced significantly by BLs and US exposure at 4 °C. In contrast, luciferase activity was increased when BLs and US exposure were applied to the cells internalizing AG73-PEG liposomes (Figure 3B−D). In addition, although the transfection efficiency of AG73-PEG liposomes alone was enhanced in the presence of chloroquine, the transfection efficiency of AG73-PEG liposomes with BLs and US exposure was not increased significantly (Figure 3E). These results suggested that BLs and US exposure could affect AG73-PEG liposomes internalized into the cells but did not affect AG73-PEG liposomes attached to the surface of the cell membrane and could present effects such as chloroquine (Figure 5). It may be possible that BLs and US exposure affect RNA transcription, but this seems unlikely because further increases in gene expression were not observed with the enhanced endosomal escape by chloroquine (Figure 3D). Furthermore, the intracellular distribution of pDNA was observed. The ratio of colocalization of pDNA and transferrin was decreased by treatment with BLs and US exposure (Figure 4). This result suggested that BLs and US exposure could affect the intracellular trafficking of pDNA and increase the transfection efficacy of AG73-PEG liposomes (Figure 5). It has been reported that microbubble and US exposure could directly deliver lipoplex into the cytoplasm of the cell.[44] However, we suggested that AG73-PEG liposomes could not significantly enter into cytoplasm directly by the treatment of BLs and US exposure (Figure 3C). Therefore, there is a possibility that AG73-PEG liposomes associated with syndecan-2 receptor of the cells might not directly enter into cytoplasm by the treatment of BLs and US exposure.

However, we should elucidate a more particular mechanism by which BLs and US exposure may affect directly intracellular vesicle morphology or induce several biological effects such as influx of calcium ions or generation of reactive oxygen species,[45−48] but that has not yet been determined.

In conclusion, it was shown that PEG liposomes modified with AG73 peptide, which is a ligand for syndecans, could be a useful vector for syndecan-2 overexpressing cancer cells. In addition, the combination of BLs and US exposure could enhance AG73-mediated liposomal gene transfection. BLs and US exposure could not promote the transportation of AG73-PEG liposomes into cells but did affect the intracellular trafficking of AG73-PEG liposomes, leading to an increase in gene expression. BLs and US exposure may overcome the disadvantages of PEG-modified liposomes and enhance the delivery efficiency of genes into the cytoplasm and nucleus. Thus, the combination of AG73-PEG liposomes with BLs and US exposure may be a promising method to achieve selective and efficient gene delivery for cancer gene therapy via systemic administration.

Abbreviations Used

PEG, polyethyleneglycol; BLs, Bubble liposomes; US, ultrasound; pDNA, plasmid DNA; DOPE, 1;2-dioleoyl-*sn*-glycero-3-phosphoethanolamine; DOPG, 1,2-dioleoyl-*sn*-glycero-3-phospho-*rac*-1-glycerol; DSPE, 1,2-distearoyl-*sn*-glycero-3-phosphatidylethanolamine; Mal, maleimide; Fmoc, fluorenylmethoxycarbonyl; FBS, fetal bovine serum; PLL, poly-L-lysine.

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