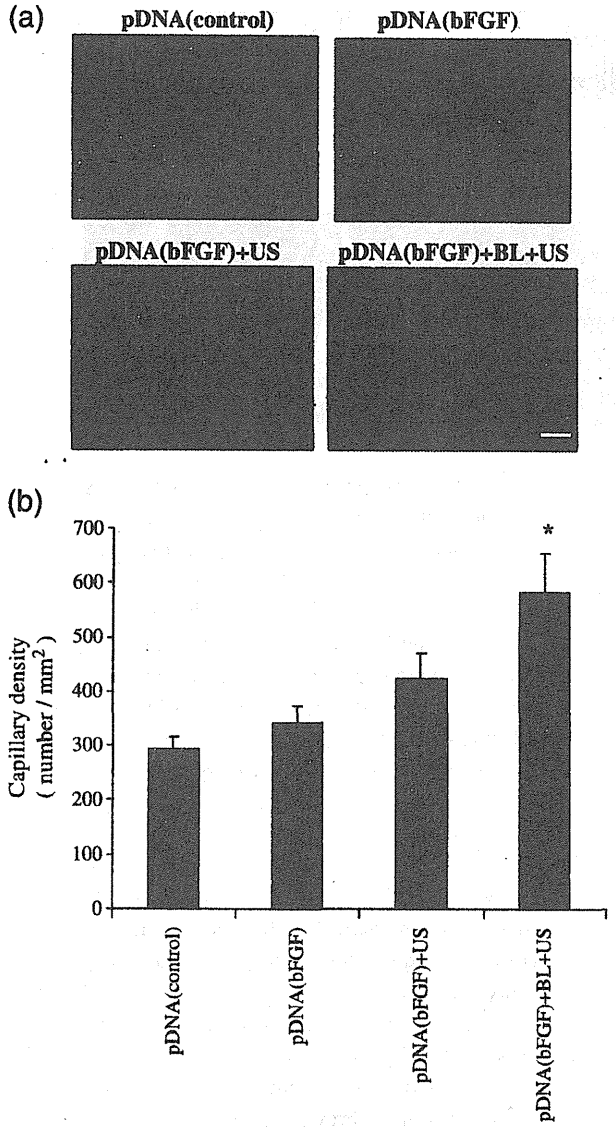

Fig. 4 Effect of BL and US-mediated bFGF gene transfer into hindlimb ischemia on capillary density. (a) CD31 staining of hindlimb muscle sections 14 days after BL and US-mediated bFGF gene transfer. The stained sections were analyzed by fluorescent microscopy. (b) CD31 positive vessels were measured. Green dots indicate CD31 positive vessels stained with an FITC-labeled anti-CD31 antibody. Scale bar represented 50 μm. *$P < 0.05$ *vs.* other treatment groups.

exposure; in contrast, without BL, only a few fibers could be observed in a treatment of US exposure without BL. Consequently, we found that a gene delivery method using BL and US exposure helped to both improve the transfection efficiency in the US focused site with a minimally invasive transfection procedure.

It is unclear whether BL with US exposure could improve transgene expression. Previously, we reported that BL could induce cavitation by a short duration (1–10 s) of US exposure and lead to efficient gene transfer into various types of cells (19,20). Therefore, the major biological effect of BL for gene delivery into the muscle may be through a cavitation induction, as was shown in previous reports (19). In contrast, in the case of Lipofectamine 2000, a commercial cationic lipid that is widely used in gene delivery, the transfection efficiency in the muscle was markedly lowered (Fig. 1a). This result is consistent with reports that serum proteins interact with and disturb cationic liposomes (29). It is expected that more time is required for this transfection, because cationic lipid/pDNA complexes (lipoplex) are entered into the cytoplasm by an endosomal pathway. Therefore, when the lipoplex with Lipofectamine 2000 was directly injected into the muscle, before it could enter into the cytoplasm by an endosomal pathway, it is possible that the degradation of pDNA or the aggregation of lipoplexes easily occurred. In contrast, once a solution of both BL and pDNA is administered into the muscle, US exposure is immediately applied at the injection site, leading to efficient gene expression, as shown in Fig. 1. In this way, unlike with lipoplexes, this simple method with BL and US exposure does not require a long time to achieve an efficient gene transfection. Our previous report has demonstrated that, by BL and US exposure, siRNA could directly enter into the cytoplasm without an endosomal pathway (22). In this report, because the level of gene expression corresponding

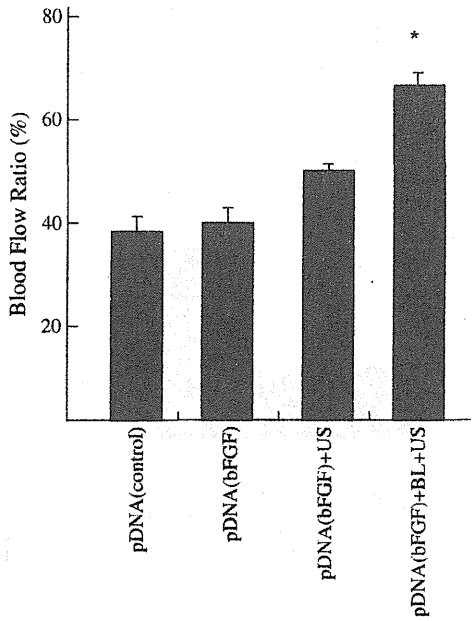

Fig. 5 Effect of BL and US-mediated bFGF gene transfer on the recovery of blood flow in ischemic limbs. After femoral artery ligation, mice were treated with BL and US-mediated intramuscular bFGF gene transfer. After the transfection, blood flow was measured at 14 days using a laser Doppler blood flow meter. *$P < 0.05$ *vs.* other treatment groups. Blood Flow Ratio (%): ischemic / normal blood flow ratio. pDNA (pBLAST-bFGF): 10 μg, BL: 30 μg, US exposure (Frequency: 1 MHz, Duty: 50%, Intensity: 2 W/cm^2, Time: 60 s).

to half of the expression in BL with a 1-minute US exposure could also be observed by BL with an only 10-second US exposure (data not shown), it may be thought that this transfection method by BL with US exposure enables immediate and direct pDNA delivery into the cytoplasm of muscle cells. The transfection efficiency might increase due to the appearance of transient holes in the cell membrane caused by the spreading of the BL, followed by their eruption with US exposure, which is consistent with previous reports using Optison (9).

Recently, a therapeutic strategy delivering angiogenic gene factors has been widely studied for clinical use in ischemic diseases (30). The delivery of naked plasmid DNA encoding an angiogenic gene into the ischemia has also been reported in clinical trials. However, the transfection efficiency is still insufficient for effective angiogenesis without side effects (30). Therefore, we assessed the feasibility and the effectiveness of BL for a gene therapy by trying to deliver a plasmid expressing bFGF, a key angiogenic factor, into the skeletal muscle of hindlimb ischemia model mice by the combination of BL and US exposure. As expected, with the gene delivery of the bFGF plasmid into an intramuscular injection with the combination of BL and US exposure, the capillary density and the blood flow ratio of the ischemic to non-ischemic hindlimb were markedly increased in the hindlimb transfected with the bFGF plasmid DNA through the combination of BL and a low intensity of US exposure compared to the plasmid DNA injection alone (Figs. 4 and 5). In addition, it has been reported that low-intensity US exposure can induce angiogenesis (31,32). However, no significant recovery in ischemic hindlimbs was observed with the combination of BL and US exposure without bFGF plasmid or with US exposure alone without the bFGF plasmid (data not shown). These results apparently indicate that therapeutic angiogenesis using naked plasmid DNA transfer that is enhanced by BL and US exposure could be a potential method in a clinical setting. We believe that there are several possibilities for BL usage in therapeutic angiogenesis with naked plasmid DNA in clinical use. The novel method using the combination of BL and US exposure may possibly reduce the amount of naked plasmid DNA, administration times, and the achievement of efficient gene transfer non-invasively without a viral vector, thereby enabling the decrease of the potential cost in clinical settings.

CONCLUSION

The present studies demonstrated a novel gene delivery method into skeletal muscle by the combination of BL and US exposure. Applied as gene therapy in a mouse model of ischemic limb muscle, intramuscular injection of bFGF as an angiogenic gene with BL followed by US exposure enabled improvement of an angiogenesis followed by apparent increased blood flow in the ischemic muscle. Because intramuscular injection of naked plasmid DNA alone may be inefficient and restrict its clinical use, this US-mediated BL technique may provide an effective non-invasive and non-viral method for angiogenic gene therapy for limb ischemia as well as for wound healing, ischemic heart disease, myocardial infarction, peripheral arterial diseases, and other various diseases.

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molecular pharmaceutics

Brief Article

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Involvement of Ca^{2+} and ATP in Enhanced Gene Delivery by Bubble Liposomes and Ultrasound Exposure

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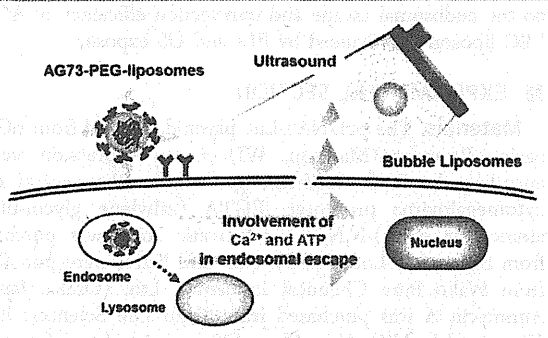
ABSTRACT: Recently, we reported the accelerated gene transfection efficiency of laminin-derived AG73-peptide-labeled polyethylene glycol-modified liposomes (AG73-PEG liposomes) and cell penetrating TAT-peptide labeled PEG liposomes using PEG-modified liposomes, which trap echo-contrast gas, "Bubble liposomes" (BLs), and ultrasound (US) exposure. BLs and US exposure were reported to enhance the endosomal escape of AG73-PEG liposomes, thereby leading to increased gene expression. However, the mechanism behind the effect of BLs and US exposure on endosomes is not well understood. US exposure was reported to induce an influx of calcium ions (Ca^{2+}) by enhancing permeability of the cell membrane. Therefore, we examined the effect of Ca^{2+} on the endosomal escape and transfection efficiency of AG73-PEG liposomes, which were previously enhanced by BLs and US exposure. For cells treated with EGTA, the endosomal escape and gene expression of AG73-PEG liposomes were not enhanced by BLs and US exposure. Similarly, transfection efficiency of the AG73-PEG liposomes in ATP-depleted cells was not enhanced. Our results suggest that Ca^{2+} and ATP are necessary for the enhanced endosomal escape and gene expression of AG73-PEG liposomes by BLs and US exposure. These findings may contribute to the development of useful techniques to improve endosomal escape and achieve efficient gene transfection.



KEYWORDS: *AG73 peptide, atp, Bubble liposomes, calcium ions, gene delivery, endosomal escape, ultrasound*

INTRODUCTION

For successful gene therapy, various nonviral vectors such as lipid- and polymer-based carriers have been developed. However, they generally have relatively low transfection efficiencies, which need to be overcome.[1] Recent reports have emphasized the importance of subcellular and intracellular trafficking of gene delivery carriers. To achieve efficient gene transfection, carriers must overcome several steps including cellular internalization, endosomal escape, nuclear transfer and intracellular transcription.[2,3] Of these steps, endosomal escape is considered one of the most important, because most carriers are internalized into cells via an endocytic pathway. When escape from endosomes is impossible, the genes are degraded in lysosomes. Indeed, some groups have developed carriers and protocols that involve monitoring functions, such as pH sensitivity, temperature dependence, or photosensitivity, to deliver genes to the cytosol from endosomes.[4−7]

Previously, we developed laminin-derived AG73 peptide-labeled polyethylene glycol (PEG)-modified liposomes (AG73-PEG liposomes) as tumor targeted gene delivery carriers.[8] We also reported that the transfection efficiency of AG73-PEG liposomes and TAT-PEG liposomes, which were labeled with a TAT peptide (a cell penetrating peptide derived from human immunodeficiency virus trans-acting transcriptional activator), could be accelerated by PEG-modified liposomes, which trap echo-contrast gas, "Bubble liposomes" (BLs), and ultrasound (US) exposure.[9,10] BLs and US exposure enhanced the endosomal escape of AG73-PEG liposomes and TAT-PEG liposomes, leading to increased gene expression. However, the mechanism behind the effect of BLs and US exposure on endosomes and the resulting enhanced endosomal escape of carriers is not well understood. To promote this method as a more useful gene delivery tool, it is necessary to understand the detailed interactions at a fundamental level.

US pressure above a certain threshold can cause oscillating bubbles to undergo a violent collapse known as inertial cavitation. Microbubbles can be the nuclei of cavitation, and

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subsequent US exposure can induce more efficient cavitation. Inertial cavitation is thought to cause transient disruptions in cell membranes, which enable the transport of extracellular molecules into cells.[11−16] However, US exposure has also induced several biological effects, such as bone fracture healing, wound healing, and induction of apoptosis.[17−19] Moreover, the induced influx of calcium ions, the generation of reactive oxygen species, or the activation of some signals at a cellular level can be attributed to US exposure.[20−23]

Calcium ions (Ca^{2+}) have important roles in cells and are involved in various events such as cell proliferation and cell death.[24,25] US exposure induces the influx of Ca^{2+} by enhancing permeability of the cell membrane. Ca^{2+} also adjusts endosomal acidification and vesicle fusion.[26−29] Therefore, we focused on Ca^{2+} and hypothesized that BLs and US enhance the endosomal escape of gene delivery carriers via Ca^{2+} influx. We also investigated the involvement of ATP in enhanced gene delivery. In this study, we examined the effect of Ca^{2+} and ATP on the endosomal escape and transfection efficiency of AG73-PEG liposomes enhanced by BLs and US exposure.

EXPERIMENTAL SECTION

Materials. The pcDNA3-Luc plasmid, derived from pGL3-basic (Promega, Madison, WI), is an expression vector encoding the firefly luciferase gene under the control of a cytomegalovirus promoter. EGTA (ethylene glycol-bis(2-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid) was purchased from Sigma (St. Louis, MO). NaF and NaN_3 were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Antimycin A was purchased from Enzo Life Sciences, Inc. (Farmingdale, NY). 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 humidified 5% CO_2 atmosphere.

Preparation of AG73-PEG Liposomes. The Cys-AG73 peptide (CGG-RKRLQVQLSIRT) was synthesized manually using the 9-fluorenylmethoxycarbony (Fmoc)-based solid-phase strategy. The peptide was 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. The pDNA was diluted to a concentration of 0.1 mg/mL in 10 mM HEPES buffer (pH 7.4) and was condensed using 0.1 mg/mL poly-L-lysine (PLL); (SIGMA-Aldrich Co., St. Louis, MO). The complex of pDNA-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-polyethylene glycol-maleimide (DSPE-PEG_{2000}-Mal) (NOF Corporation, Tokyo, Japan) 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 the cysteine of Cys-AG73 peptide to 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.

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-polyethylene glycol (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 a sizing filter (pore size: 200 nm) (Nuclepore Track-Etched Membrane, GE Healthcare, 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 using perfluoropropane gas (Takachio Chemical Ind. Co. Ltd., Tokyo, Japan). First, 2 mL sterilized vials containing 0.8 mL of the 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 vials were placed in a bath-type sonicator (42 kHz, 100 W) (BRANSONIC 2510j-DTH, Branson Ultrasonics Co., Danbury, CT) for 5 min to form BLs.

Gene Transfection by AG73-PEG Liposomes with BLs and US Exposure. 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 (encapsulating pDNA: 3 μg/mL) in serum-free medium for 4 h at 37 °C. The cells were washed twice with Ca^{2+}-free DMEM containing 10 mM EGTA. To deplete ATP, the cells were treated with NaN_3 (0.1%), NaF (10 mM), and antimycin A (1 μg/mL) for 30 min, and then the BLs were added. Within 2 min, 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. The cells were transferred to fresh medium and cultured for 20 h, and then luciferase activity was determined.

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

Assessment of Localization of pDNA and Transferrin. The 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 the 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). To assess the involvement of Ca^{2+} and ATP, the cells were treated as described in the above section. Subsequently, the cells were incubated for 10 min and then fixed with 4%

paraformaldehyde for 1 h at 4 °C followed by visualization using confocal laser scanning microscopy (CLSM). 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 previously.[9,10,30,31] 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 total number of Cy3 pixels.

Assessment of Localization of pDNA and lamp-2. The 293T-Syn2 cells were first treated with AG73-PEG liposomes (Cy3-lableled pDNA: 3 μg/mL) for 4 h at 37 °C and then with BLs and US exposure. To assess the involvement of Ca^{2+} and ATP, cells were treated as described in the above section. Subsequently, the cells were incubated for 1 h and then fixed with 4% paraformaldehyde for 1 h at 4 °C. The cells were washed with PBS and permeabilized for 5 min in 0.2% saponin, followed by treatment with 10% goat serum in PBS. Finally, the cells were incubated with anti-lamp2 Ab (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) overnight at 4 °C and treated with Alexa Fluor 488-conjugated secondary Ab (Invitrogen Co., Carlsbad, CA) for 1 h at room temperature in the dark. Then, CLSM and analysis was performed as described in the above section.

RESULTS AND DISCUSSION

In previous reports, we have showed that BLs and US exposure could enhance endosomal escape and gene transfection of AG73-PEG liposomes. We have proposed the mechanism that the cavitation induced in the outside of cells by US exposure and BLs could affect endosomes, and then AG73-PEG liposomes internalized by endocytosis escaped from endosomes, leading to enhanced gene expression. It have been also confirmed that AG73-PEG liposomes could not be introduced into cytoplasm directly through the cell membrane after the US-mediated disruption of BLs. However, the exact mechanism of accelerated endosomal escape of carriers was not clear. US exposure induces the influx of Ca^{2+} by enhancing permeability of the cell membrane.[21] In addition, Ca^{2+} adjusts endosomal acidification and vesicle fusion.[26−29] Therefore, to evaluate the mechanism by which BLs and US exposure could promote the endosomal escape of AG73-PEG liposomes, we examined the effect of Ca^{2+} on the endosomal escape and transfection efficiency of AG73-PEG liposomes enhanced by BLs and US exposure. ATP is involved in various reactions, such as acidification of endosomes, intracellular trafficking of vesicles and fusion of vesicles.[26] We also investigated the involvement of ATP-dependent processes in enhanced gene delivery.

First, to evaluate the involvement of Ca^{2+} and ATP in gene expression enhanced by BLs and US exposure, we examined the effect of Ca^{2+} and ATP on gene expression efficiency of AG73-PEG liposomes using 293T-Syn2 cells. The cells were incubated with AG73-PEG liposomes containing pcDNA3-Luc, and then treated with BLs and US exposure. After 20 h incubation, luciferase activity was assayed. BLs and US exposure enhanced the luciferase activity of AG73-PEG liposomes by approximately 60-fold compared to that of AG73-PEG liposomes alone.[9] By contrast, when the cells were treated with 10 mM EGTA before the treatment of BLs and US exposure, the enhancement ratio of luciferase activity by BLs and US exposure was decreased. To examine the effect of ATP on gene transfection efficiency, the cells were treated with NaN_3, NaF, and antimycin A to deplete ATP. The subsequent luciferase assay showed insignificant enhancement by BLs and US exposure. Conversely, when cells were treated with AG73-PEG liposomes alone, luciferase activity was not affected by Ca^{2+} and ATP depletion (Figure 1). These results suggest that

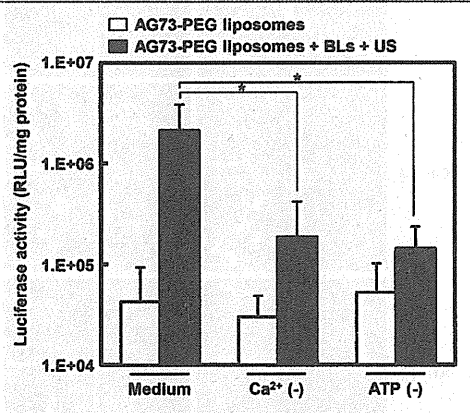

Figure 1. Effects of Ca^{2+} and ATP on gene expression by AG73-PEG liposomes with BLs and US exposure. 293T-Syn2 cells were treated with AG73-PEG liposomes for 4 h at 37 °C, and then cells were washed twice with Ca^{2+}-free DMEM containing 10 mM EGTA for a depleted Ca^{2+} condition. ATP was depleted by pretreating cells for 30 min before US exposure with 1 μg/mL antimycin A, 10 mM NaF, and 0.1% NaN_3. BLs (120 μg/mL) were added to cells followed by immediate US exposure. After replacement with fresh medium, the cells were cultured for 20 h and luciferase activity was determined. The data are shown as the means ± SD (n = 4). *p < 0.05.

Ca^{2+} and ATP may be necessary to enhance gene transfection efficiency of AG73-PEG liposomes by BLs and US exposure. On the other hand, it is reported that extracellular Ca^{2+} plays important roles to repair the cell membrane disruption and maintain cell survival.[32] Therefore, we examined the cell viability in Ca^{2+}-depleted condition. As a result, in this condition, the cell viability had almost no difference in the treatment with or without BLs and US exposure (data not shown). This result suggested that the decreased enhancement ratio of luciferase activity by the treatment of BLs and US exposure in Ca^{2+}-depleted condition was not due to a change of cell viability.

Recent reports have emphasized the importance of subcellular and intracellular trafficking of gene delivery carriers.[2,3] Among the several steps involeved, endosomal escape is considered one of the most important. In previous study, we have reported that enhanced endosomal escape of AG73-PEG liposomes by BLs and US exposure could increase gene expression.[9] Therefore, we evaluated the involvement of Ca^{2+} and ATP on the endosomal escape of gene delivery carriers. We examined the effects of Ca^{2+} and ATP on localization of pDNA encapsulated in AG73-PEG liposomes and transferrin, as an endosome marker,[33] by confocal microscopy. BLs and US exposure enhanced the endosomal escape of AG73-PEG liposomes and decreased the ratio of colocalization of pDNA and transferrin.[9] The 293T-Syn2 cells were first incubated with AG73-PEG liposomes containing Cy3-labeled pDNA and Alexa Fluor 488-conjugated transferrin and then treated with BLs and US exposure. The cells were observed by confocal microscopy to assess the colocalization of

Cy3-labeled pDNA and Alexa Fluor 488-conjugated transferrin. As shown in Figure 2a, the pDNA internalized into cells were

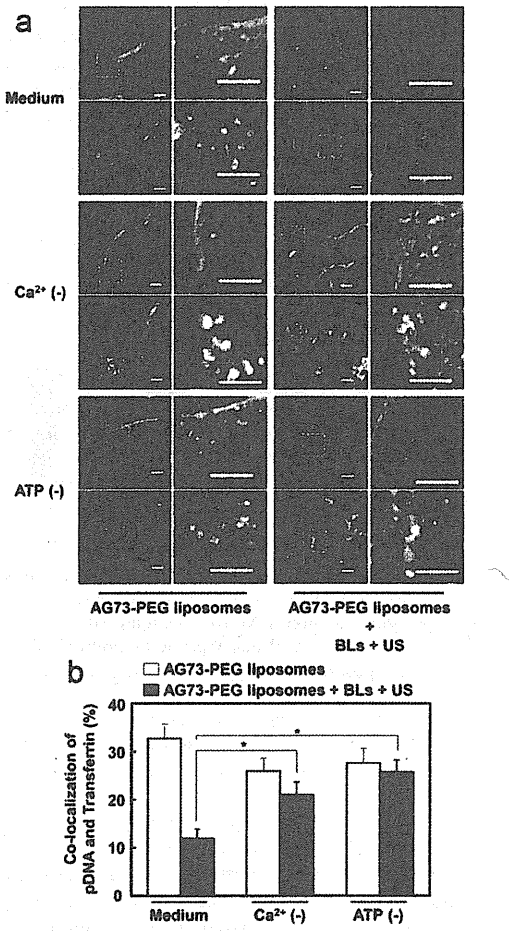

Figure 2. Effects of Ca^{2+} and ATP on intracellular localization of pDNA and endosomes. (a, b) The 293T-Syn2 cells were treated with AG73-PEG liposomes encapsulating Cy3-labeled pDNA (red) and Alexa Fluor 488-conjugated transferrin (green) for 4 h at 37 °C and then washed twice with Ca^{2+}-free DMEM containing 10 mM EGTA to create Ca^{2+}-depleted conditions. ATP was depleted by pretreating cells for 30 min before US exposure with 1 μg/mL antimycin A, 10 mM NaF, and 0.1% NaN_3. BLs (120 μg/mL) were added to cells followed by immediate US exposure. The cells were incubated for 10 min, fixed with 4% paraformaldehyde for 1 h at 4 °C and observed by CLSM. The areas within the dotted square are shown as enlarged images. The scale bars represent 5 μm. The ratio of colocalization of Cy3-labeled pDNA with Alexa Fluor 488-conjugated transferrin was quantified. The data are shown as means ± SE (n = 50). *p < 0.05 compared with AG73-PEG liposomes alone (Mann–Whitney's U test).

colocalized with transferrin, whereas BLs and US exposure decreased the colocalization of the pDNA and transferrin. However, when cells were treated with 10 mM EGTA, BLs and US exposure did not affect the intracellular localization of the pDNA and transferrin. In the ATP-depleted state, BLs and US exposure had no effect on the intracellular localization of the pDNA and transferrin. Furthermore, we calculated the ratio of colocalization of the pDNA and transferrin and found that BLs and US exposure decreased the ratio of colocalization. By contrast, when cells were treated with 10 mM EGTA or were exposed in an ATP-depleted state, BLs and US exposure did not affect the ratio of colocalization of pDNA and transferrin (Figure 2b). These results suggest that Ca^{2+} and ATP may be required for endosomal escape of AG73-PEG liposomes after the addition of BLs and US exposure.

Efficient gene transfection requires sufficient delivery of genes from the endosomes to the cytosol, to avoid the degradation of genes in lysosomes. Therefore, we assessed the intracellular localization of pDNA and lysosomes and the effects of Ca^{2+} and ATP on localization of pDNA and lysosomes. The 293T-Syn2 cells were treated with AG73-PEG liposomes containing Cy3-labeled pDNA, followed by the addition of BLs and application of US. The cells were fixed and stained with antibodies for lamp-2, a lysosome marker.[34] As a result, the pDNA internalized into cells was colocalized with lamp-2 at 10 or 60 min, whereas BLs and US exposure decreased the colocalization of pDNA and lamp-2 at 60 min after US exposure (Figure3). Moreover, when cells were treated with 10 mM EGTA and depleted of ATP, BLs and US exposure did not decrease the localization of pDNA and lamp-2 (Figure

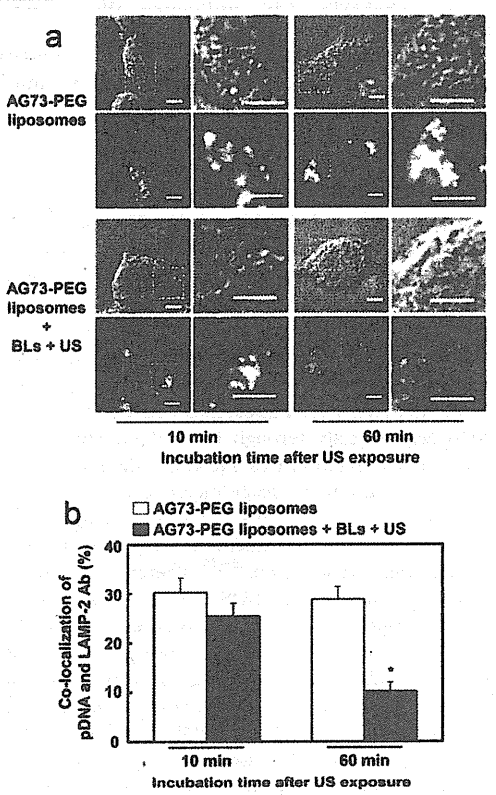

Figure 3. Effect of BLs and US exposure on intracellular localization of pDNA and lysosomes. The 293T-Syn2 cells were treated with AG73-PEG liposomes encapsulating Cy3-labeled pDNA (red) for 4 h at 37 °C. BLs (120 μg/mL) were added to cells followed by immediate US exposure. The cells were incubated for 10 or 60 min and then fixed with 4% paraformaldehyde for 1 h at 4 °C followed by staining with antibodies for lamp-2 (green), a marker for lysosomes. The cells were observed by CLSM. The areas within the dotted square are shown as enlarged images. The scale bars represent 5 μm. The ratio of colocalization of Cy3-labeled pDNA with lamp-2 was quantified. The data are shown as means ± SE (n = 50). *p < 0.05 (Mann–Whitney's U test).

4a). We also evaluated the ratio of colocalization of pDNA and lamp-2. In normal medium, the ratio of colocalization of pDNA and lamp-2 was decreased by the application of BLs and US. By contrast, the decrease in the ratio of colocalization of pDNA and lamp-2 could be abrogated by 10 mM EGTA and ATP depletion (Figure 4b). These results suggest that BLs and US exposure could decrease the ratio of colocalization of pDNA and lysosomes. Furthermore, Ca^{2+} and ATP may be involved in the escape of AG73-PEG liposomes from lysosomes. We also confirmed the change of localization of pDNA with endosomes or lysosomes. When 293T-Syn2 cells were treated by AG73-PEG liposomes with BLs and US exposure, a decrease in colocalization of pDNA and endosomes was observed at 10 min after US exposure,[9] whereas a decrease in colocalization of pDNA and lysosomes was observed at 60 min after US exposure (Figure 3). These results suggest that BLs and US exposure might significantly affect endosomes, leading to the decrease in colocalization of pDNA and endosomes. In addition, the increase in the release of genes to the cytosol from endosomes might decrease gene delivery from endosomes to lysosomes.

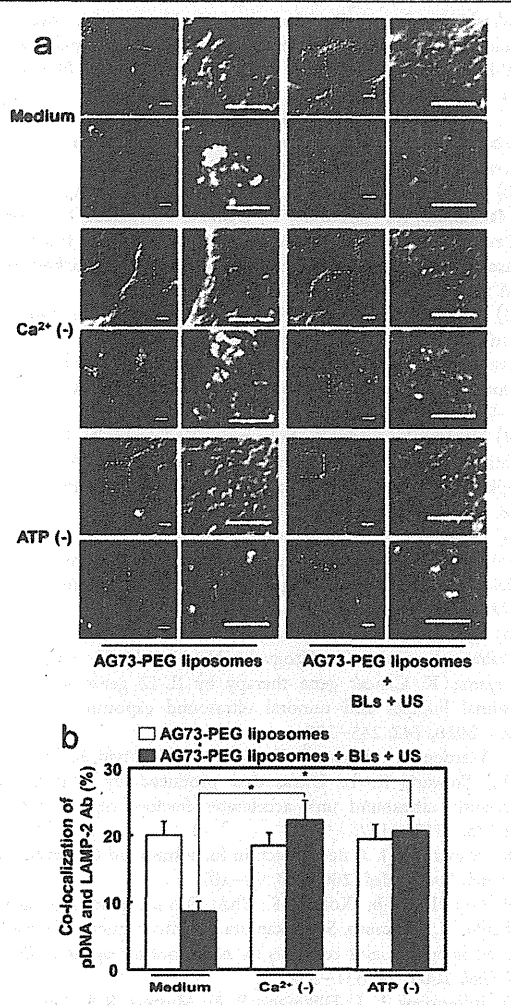

Figure 4. Effects of Ca^{2+} and ATP on intracellular localization of pDNA and lysosome. (a, b) The 293T-Syn2 cells were treated with AG73-PEG liposomes encapsulating Cy3-labeled pDNA (red) for 4 h at 37 °C and then washed twice with Ca^{2+}-free DMEM containing 10 mM EGTA to create Ca^{2+}-depleted conditions. ATP was depleted by pretreating cells for 30 min before US exposure with 1 μg/mL antimycin A, 10 mM NaF, and 0.1% NaN_3. BLs (120 μg/mL) were added to cells followed by immediate US exposure. The cells were incubated for 1 h, fixed with 4% paraformaldehyde for 1 h at 4 °C and stained with antibodies for lamp-2 (green), a marker for lysosomes. The cells were observed by CLSM. The areas within the dotted square are shown as enlarged images. The scale bars represent 5 μm. The ratio of colocalization of Cy3-labeled pDNA with lamp-2 was quantified. The data are shown as means ± SE (n = 50). *p < 0.05 (Mann–Whitney's U test).

On the other hand, it has been also reported that US exposure could affect the transcription by oxidative stress or activation of NFκB.[35,36] It may be possible that an activated transcription is involved in enhanced gene transfection. We need more study to clarify the detailed mechanism concerning transcription in the enhanced gene delivery by BLs and US exposure. However, the endosomal escape of AG73-PEG liposomes induced by BLs and US exposure was significantly suppressed in Ca^{2+} or ATP-depleted condition (Figure 3). Therefore, our results suggest that BLs and US exposure can enhance at least the endosomal escape followed by gene expression via Ca^{2+} and ATP.

Although Ca^{2+} and ATP were involved in enhanced endosomal escape and gene expression efficiency of AG73-PEG liposomes by BLs and US exposure, how Ca^{2+} and ATP enhance the endosomal escape of carriers is still unclear. More investigations into the detailed mechanism of enhanced endosomal escape of AG73-PEG liposomes by BLs and US exposure are required. Moreover, endosomal acidification is adjusted by Ca^{2+}, suggesting that the influx of Ca^{2+} by BL and US exposure may affect endosomal acidification.[26] This could lead to the destabilization of endosomes and hydrogen pumps, such as H^+/K^+-ATPase. However, Ca^{2+} and ATP are involved in endosomal membrane fusion.[27,28] Therefore, an influx of Ca^{2+} by BLs and US exposure and ATP may affect endosomal membrane fusion. Our study demonstrated the involvement of Ca^{2+} and ATP in enhanced endosomal escape and gene expression efficiency of AG73-PEG liposomes by BLs and US exposure. Significantly, BLs and US exposure enhanced endosomal escape through biological effects rather than physical effects. In fact, our results suggest that BLs and US exposure could affect more endosomes than lysosomes. It is expected that BLs and US exposure could be safer tools for the enhancement of endosomal escape by setting the appropriate US exposure conditions.

In conclusion, our study focused on Ca^{2+} and ATP and investigated the particular mechanism of enhanced endosomal escape and gene expression of AG73-PEG liposomes by BLs and US exposure. When cells were treated in Ca^{2+}- and ATP-depleted conditions, endosomal escape and gene expression of AG73-PEG liposomes were not enhanced by BLs and US exposure. These results suggest that both Ca^{2+} and ATP are necessary for enhanced endosomal escape and gene expression of AG73-PEG liposomes by BLs and US exposure. These findings may contribute to the development of useful gene transfection methods to achieve efficient gene transfection by improving endosomal escape.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS USED

BLs, Bubble liposomes; CLSM, confocal laser scanning microscopy; 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; FBS, fetal bovine serum; Fmoc, fluorenylmethoxycarbonyl; Mal, maleimide; pDNA, plasmid DNA; PEG, polyethylene glycol; US, ultrasound

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RESEARCH ARTICLE

Enhanced gene delivery using Bubble liposomes and ultrasound for folate-PEG liposomes

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Abstract

We have previously reported that the transfection efficiency of laminin-derived AG73-peptide labeled polyethyleneglycol-modified liposomes (AG73-PEG liposomes) was enhanced by echo-contrast gas entrapping PEG liposomes (Bubble liposomes, BLs) and ultrasound (US) exposure by improving endosomal escape. However, it has not been well understood whether BLs and US exposure can enhance the transfection efficiency of other carriers except AG73-PEG liposomes. In this study, to evaluate whether BLs and US exposure can be generally applied to gene delivery carriers, we focused on folate as a model ligand and examined whether BLs and US exposure could enhance the transfection efficiency of folate-PEG liposomes. Folate-PEG liposomes could internalize into cells efficiently, whereas they could not deliver genes into cytosol from endosomes sufficiently. BLs and US exposure could enhance the transfection efficiency of folate-PEG liposomes compared with folate-PEG liposomes alone without their direct induction into cells. These results suggested that BLs and US exposure could enhance the transfection efficiency of folate-PEG liposomes in the same manner as AG73-PEG liposomes. Thus, BLs and US exposure may be a promising tool to achieve efficient gene transfection into various gene carriers in general.

Keywords: Bubble liposomes, endosomal escape, folate, targeted gene delivery, ultrasound

Introduction

For successful gene therapy, it is necessary to develop vectors or carriers that can deliver therapeutic genes to targeted tissues or cells efficiently, selectively, and safely. Although virus vectors have high transfection efficiency in various tissues and cells, they have limitations, including tumorigenic and immunogenic effects (Dewey et al., 1999; Sun et al., 2003). Non-viral vectors, such as lipid-based and polymer-based carriers, have advantages in their safety, versatility, and ease of preparation; however, their insufficient transfection efficiency is generally a major limitation (Hirko et al., 2003; Zhang et al., 2004; Mae et al., 2009; Yamano et al., 2011).

Recently, the importance of subcellular and intracellular trafficking of gene delivery carriers has been reported. Several steps, such as cellular internalization, endosomal escape, nuclear transfer and intracellular transcription, should be rationally regulated to achieve efficient gene transfection (Varga et al., 2005; Hama et al., 2006). In these steps, endosomal escape is considered one of the most important step, because most carriers internalize into cells via the endocytic pathway, and when they cannot escape from endosomes, the genes degrade in lysosomes. Indeed, some studies have developed carriers and methods equipped with functions such as pH sensitivity, temperature dependence, or photo sensitivity to achieve efficient endosomal escape (Subbarao et al., 1987; Høgset et al, 2004; Lee et al., 2008; Hatakeyama et al., 2009).

We have previously developed laminin-derived AG73-peptide labeled polyethyleneglycol (PEG)-modified

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liposomes (AG73-PEG liposomes) as tumor-targeted gene delivery carriers (Negishi et al., 2010a). We also reported that PEG-modified liposomes entrapping echo-contrast gas, "Bubble liposomes" (BLs), and ultrasound (US) exposure could enhance endosomal escape of AG73-PEG liposomes, leading to increased transfection efficiency (Negishi et al., 2010b). Although the detailed mechanism of the endosomal escape enhanced by BLs and US exposure was not well understood, we found that cavitation induced by BLs and US exposure was involved in the enhancement of endosomal escape. The cavitation may affect directly intracellular vesicle morphology or induce several biological effects to enhance endosomal escape. In this method, BLs and US exposure were applied after the treatment of AG73-PEG liposomes; therefore, it is expected that BLs and US exposure may be applied to other carriers and enhance their transfection efficiency. However it has not been well understood whether BLs and US exposure can enhance the transfection efficiency of carriers other than AG73-PEG liposomes, which are modified by several ligands and internalize into cells via several receptors and endocytic pathways.

Selective gene delivery has been considered a promising method. In this purpose, folate, RGD peptide, transferrin, and anisamide have been used as ligands to develop selective gene delivery carriers (Li et al., 2008; Ng et al., 2009; Koppu et al., 2010; Kibria et al., 2011; Morris & Sharma, 2011). Folate is a high-affinity ligand for the folate receptor, which is overexpressed in ovarian carcinomas as well as numerous other cancers, including endometrial, kidney, lung mesothelioma, breast, brain, and myeloid leukemia, but the expression is low in normal tissues (Garin-Chesa et al., 1993; Leamon et al., 2003; Yoshida et al., 2006; Kawano et al., 2009); therefore, folate receptor is expected as a specific receptor of cancer cells, and folate-modified carriers have been developed to achieve selective gene delivery. The majority of these carriers was internalized via endocytosis and is required to deliver genes from endosomes to cytosol for high gene expression. For efficient endosomal escape, some studies have developed folate-modified carriers, which have functions to enhance endosomal escape (Shi et al., 2002); therefore, we chose folate-modified liposomes as a model to assess the utility of BLs and US exposure in general.

In this study, to evaluate whether BLs and US exposure can be generally applied to gene delivery carriers to improve transfection efficiency, we examined whether BLs and US exposure can enhance the transfection efficiency of folate-PEG liposomes.

Methods

Materials

The plasmid pCMV-Luc is an expression vector encoding the firefly luciferase gene under the control of cytomegalovirus promoter. Chloroquine, chlorpromazine, and protamine were purchased from Sigma (St. Louis, MO). Genistein was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Amiloride was purchased from Calbiochem (San Diego, CA).

Cell lines and cultures

KB cells (human nasopharyngeal epidermoid carcinoma cells) were cultured in folate-free Dulbecco's modified Eagle's medium supplemented with 4 mM glutamine and 0.15% $NaHCO_3$ (DMEM; Sigma) at 37°C in a humidified 5% CO_2 atmosphere. This medium were supplemented with 10% fetal bovine serum (FBS: Equitech Bio Inc., Kerrville, TX), penicillin (100 U/mL), and streptomycin (100 μg/mL).

Preparation of folate-PEG liposomes

Folate-PEG liposomes were prepared by the hydration method. pDNA diluted in 10 mM HEPES buffer (pH 7.4) was condensed using protamine (N/P = 5.0). The complex of pDNA and protamine 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.), and 1,2-distearoyl-sn-glycero-3-phophatidylethanolamine-polyethyleneglycol-folate (DSPE-PEG_{2000}-Folate) (AVANTI Polar Lipids Inc.) in a molar ratio of 2: 9: 0.34, 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; Bransonic Ultrasonic Co., Danbury, CT). Folate-PEG liposomes were modified with 3 mol% PEG and 3 mol% folate of total lipid. The particle size and ζ-potential of prepared liposomes were measured by NICOMP 380 ZLS (Particle Sizing Systems, Santa Barbara, CA). Three independent determinations were performed.

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-PEG2000-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 a sizing filter (pore size: 200 nm) (Nuclepore Track-Etch Membrane; Whatman plc, UK). The lipid concentration was measured using a phopholipid C test Wako (Wako Pure Chemical Industries, Ltd., Osaka, Japan). BLs were prepared from liposomes and perfluoropropane gas (Takachio Chemical Ind. Col. Ltd., Tokyo, Japan). First, 2 mL sterilized vials containing 0.8 mL liposome suspension (lipid concentration: 1 mg/mL) were filled with perfluoropropane gas, capped and then

pressurized with a further 3 mL perfluoropropane gas. The vial was placed in a bath-type sonicator (42 kHz, 100 W) (BRANSONIC 2510j-DTH; Branson Ultrasonics Co.) for 5 min to form BLs.

Flow cytometry analysis

To evaluate the cellular association of liposomes, the day before experiments, KB cells (1×10^5) were seeded in a 12-well plate. 0.2 mol% DiI-labeled folate-PEG liposomes (pDNA: 3 μg/mL) were added to the cells and incubated for 1 h at 37°C. To examine the cellular uptake pathway of folate-PEG liposomes, the cells were incubated with chlorpromazine (10 μg/mL), genistein (400 μM) or amiloride (1 mM) for 30 min and then treated with DiI-labeled folate-PEG liposomes in the presence of endocytic inhibitor for a further 1 h at 37°C. The cells were then collected and the fluorescence intensities were measured by flow cytometry (BD FACSCanto, Franklin Lakes, NJ). The mean fluorescence intensity of cells was quantified. The results are presented as a mean ± SD obtained from three sample ($n = 3$).

Confocal laser scanning microscopy

KB cells were seeded the day before the experiments. 0.2 mol% DiI-labeled folate-PEG liposomes (pDNA: 3 μg/mL) were added to the cells and incubated for 1 h at 37°C. After incubation, the cells were fixed with 4% paraformaldehyde for 1 h at 4°C, followed by CLSM (FV1000D; Olympus Corporation, Tokyo, Japan).

Transfection of pDNA into cells using folate-PEG liposomes

The day before the experiments, KB cells (3×10^4) were seeded in a 48-well plate. The cells were treated with folate-PEG liposomes (encapsulated pDNA: 3 μg/mL) in serum-free medium for 4 h at 37°C. The cells was also treated with pDNA and Lipofectamine$_{2000}$ (Invitrogen Japan K.K., Tokyo, Japan) complexes prepared according to the manufacturer's instructions for 4 h. 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 folate-PEG liposomes with BLs and US exposure

The day before the experiments, KB cells (3×10^4) were seeded in a 48-well plate. The cells were treated with folate-PEG liposomes (encapsulated pDNA: 3 μg/mL) in serum-free medium for 4 h at 37°C. After incubation, the cells were washed twice within 10 min to remove any excess folate-PEG liposomes that were not associated with the cells and BLs (60 μg/well) were added. Within 2 min, 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 a WST-8 assay (Cell Counting Kit-8; Dojindo Laboratories, Kumamoto, Japan).

Measurement of luciferase expression

Cell lysate was prepared with 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). Activity is indicated as relative light units (RLU) per mg protein ± SD, four independent determinations were performed for each transfection experiment ($n = 4$).

Statistical analysis

Statistical analyses were performed to establish the significance of variation using Student's t-test. Differences with a value of $p < 0.05$ were considered statistically different.

Results

Physical properties of prepared liposomes

We prepared non-labeled PEG liposomes and folate-PEG liposomes encapsulating pDNA condensed by protamine as a model to evaluate the utility of BLs and US exposure in general. We first examined the physical properties of prepared liposomes. The average size and zeta potential of prepared liposomes were about 150 nm and with a positive charge (Table 1).

Cellular association and internalization of folate-PEG liposomes

We evaluated the effect of folate labeling on the cellular association and internalization of liposomes. KB cells were incubated with either non-labeled PEG liposomes or folate-PEG liposomes containing DiI for 1 h at 37°C, and fluorescence intensity was examined by flow cytometry. The cells treated with folate-PEG liposomes showed enhanced fluorescence intensity compared with non-labeled PEG liposomes (Figure 1a). The mean fluorescence intensity of cells treated with folate-PEG liposomes was significantly increased compared with that of cells treated with non-labeled PEG liposomes ($p < 0.05$) (Figure 1b). To elucidate the subcellular localization of liposomes after uptake by folate receptor, folate-PEG liposomes containing DiI were monitored in the cells by confocal laser scanning microscopy (CLSM). In cells treated with folate-PEG liposomes, the fluorescence of liposomes was observed in the cytoplasm after incubation for 1 h. In contrast, the fluorescence of liposomes was weak in the cytoplasm of cells treated with non-labeled

Table 1. Physical properties of prepared liposomes.

Prepared liposomes	PEG liposomes	Folate-PEG liposomes
Particle size(nm)	148.9 ± 20.7	146.6 ± 5.9
z potential (mV)	13.37 ± 0.6	18.46 ± 2.3

Data represent means and SD of three different determinations.

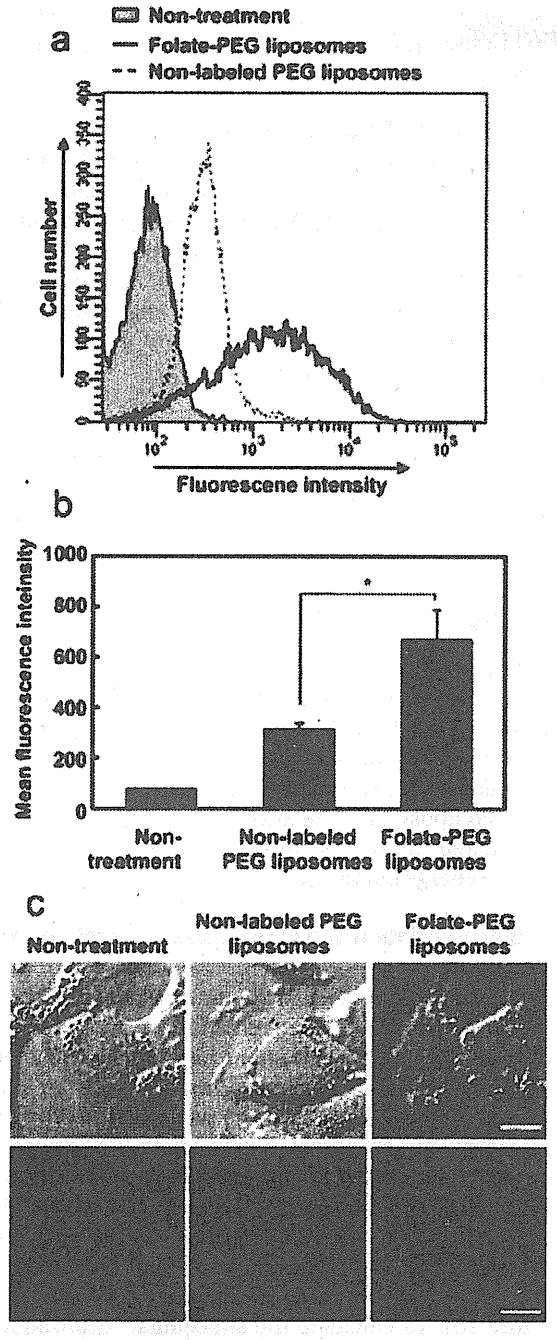

Figure 1. Cellular association and internalization of folate-PEG liposomes. KB cells were treated with DiI-labeled, non-labeled or folate-PEG liposomes for 1 h at 37°C. (a) Fluorescence intensity was measured by flow cytometry. (b) The mean fluorescence intensity of cells was quantified. (c) Cells were observed by CLSM. Scale bars represent 10 μm. Data are the means ± SD ($n = 3$). *$p < 0.05$.

PEG liposomes (Figure 1c). These results suggested that folate-PEG liposomes could efficiently associate with and internalize into cells.

Endocytic pathway of folate-PEG liposomes

It is possible that the difference in the cellular uptake pathway of carriers may affect the enhancement of transfection efficiency by BLs and US exposure. We assessed the endocytic pathway of folate-PEG liposomes using several inhibitors. We evaluated the involvement of clathrin-mediated endocytosis, raft-dependent endocytosis, and macropinocytosis in the cellular uptake of folate-PEG liposomes. To inhibit clathrin-mediated endocytosis, KB cells were treated with chlorpromazine, which blocks the assembly of coated pits at the plasma membrane (Wang et al., 1993). Raft-dependent endocytosis was inhibited by genistein, which is a thyrosin kinase inhibitor (Pelkmans et al., 2002). We also used amiloride, a specific inhibitor of the Na^+/H^+ exchange required for macropinocytosis (Wadia et al., 2004). The cells were treated with DiI-labeled folate-PEG liposomes in the presence or absence of several inhibitors, and fluorescence intensity of cells was measured by flow cytometry. As shown in Figure 2, when the cells were treated with genistein, fluorescence intensity was significantly decreased compared with that of folate-PEG liposomes in the absence of genistein. The fluorescence intensity of folate-PEG liposomes was slightly decreased by treatment of chlorpromazine and amirolide. The mean fluorescence intensity of cells treated with genistein or chlorpromazine was decreased compared with that of folate-PEG liposomes in the absence of endocytic inhibitors ($p < 0.05$) (Figure 2b). These results suggested that raft-dependent endocytosis was the major cellular uptake pathway of folate-PEG liposomes, and they could partially internalize into cells via clathrin-mediated endocytosis and macropinocytosis.

Endosomal escape of folate-PEG liposomes

Folate-PEG liposomes were efficiently associated with cells and internalized into cells via raft-dependent endocytosis, whereas it is needed to deliver genes to cytosol efficiently from endosomes to obtain high gene expression. We next examined the efficacy of endosomal escape in folate-PEG liposomes. KB cells were treated with non-labeled or folate-PEG liposomes in the presence or absence of chloroquine, which is considered an endosomolytic agent (Sonawane et al., 2003). KB cells were transfected by non-labeled or folate-PEG liposomes with or without chloroquine. The resulting luciferase activity of folate-PEG liposomes with chloroquine was sevenfold higher than that of folate-PEG liposomes without chloroquine ($p < 0.05$) (Figure 3). It was suggested that folate-PEG liposomes could not deliver genes into cytosol from endosomes sufficiently, although they could internalize into cells.

Enhanced gene transfection of folate-PEG liposomes by BLs and US exposure

Our previous study showed that BLs and US exposure could improve endosomal escape, leading to enhancement of the gene transfection efficiency of AG73-PEG liposomes. Folate-PEG liposomes could not deliver genes into cytosol from endosomes sufficiently and there was

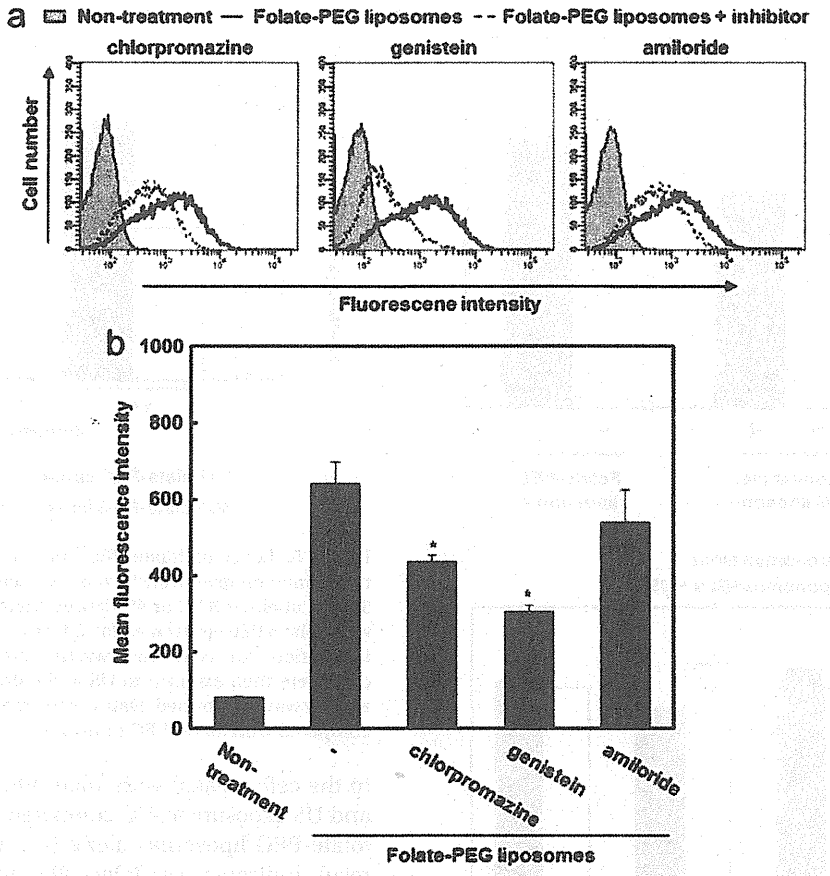

Figure 2. Endocytic pathway of folate-PEG liposomes. KB cells were incubated with chlorpromazine (10 μg/mL), genistein (400 μM) or amiloride (1 mM) for 30 min and then treated with DiI-labeled folate-PEG liposomes in the presence of endocytic inhibitors for a further 1 h at 37°C. (a) Fluorescence intensity was measured by flow cytometry. (b) The mean fluorescence intensity of cells was quantified. Data are the means ± SD ($n = 3$). *$p < 0.05$ compared with folate-PEG liposomes without endocytic inhibitor.

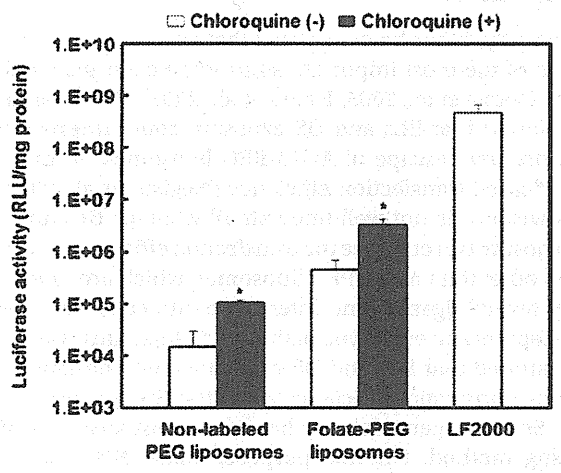

Figure 3. Endosomal escape of folate-PEG liposomes. KB cells were preincubated with chloroquine (200 μM) for 30 min before transfection and then treated with non-labeled or folate-PEG liposomes in the presence of chloroquine for a further 4 h at 37°C. The cells were also transfected by Lipofectamine$_{2000}$ (LF2000) for 4 h. After replacement with fresh medium, the cells were cultured for 20 h and then luciferase activity was determined. Data are the means ± SD ($n = 4$). *$p < 0.05$ compared with liposomes without chloroquine.

the potential to improve transfection efficiency by BLs and US exposure. To investigate the effect of BLs and US exposure on gene transfection of folate-PEG liposomes, KB cells were incubated with folate-PEG liposomes for 4 h at 37°C, and then the cells were treated with BLs and US exposure. As a result, luciferase activity was enhanced up to about five-fold by treatment with BLs and US exposure compared with folate-PEG liposomes alone. Furthermore, the luciferase activity of folate-PEG liposomes with BLs and US exposure was five-fold higher than that of non-labeled PEG liposomes with BLs and US exposure ($p < 0.05$) (Figure 4a). We also examined the cytotoxicity of the treatment of folate-PEG liposomes with BLs and US exposure. Significant cytotoxicity was not observed, and cell viability was more than 80% even after each transfection (Figure 4b). These results suggested that BLs and US exposure could enhance the transfection efficiency of folate-PEG liposomes without significant cytotoxicity.

Effect of folate-PEG liposomes attached to the cell membrane on gene transfection

It is reported that BLs and US exposure could increase the permeability of the cell membrane. It is possible that

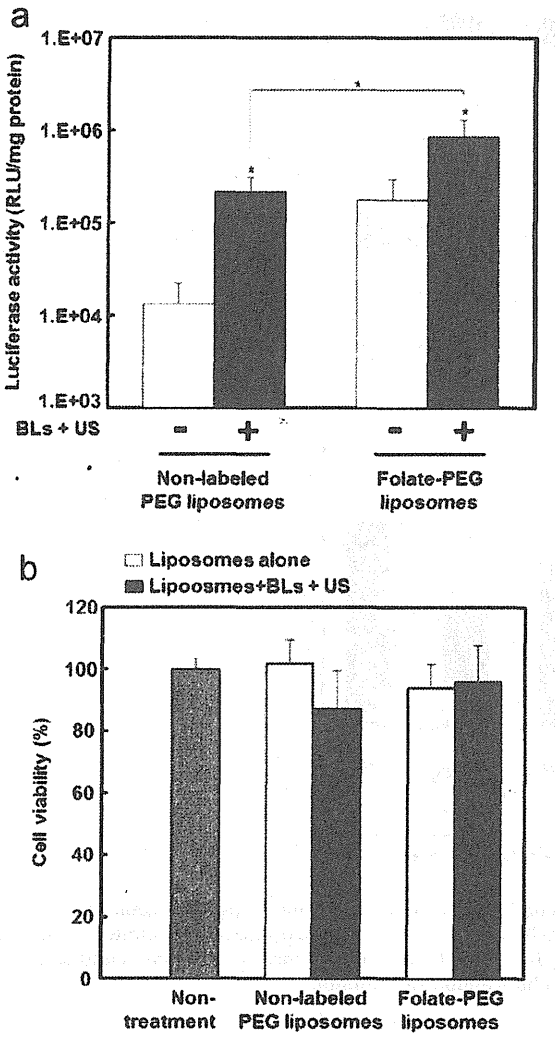

Figure 4. Enhanced gene transfection of folate-PEG liposomes by BLs and US exposure. KB cells were treated with non-labeled or folate-PEG liposomes for 4 h. The cells were washed and treated with BLs (60 μg) and US exposure. The cells were incubated for 20 h and then (a) luciferase activity was determined and (b) cell viability was measured using a WST-8 assay. Data are the means ± SD ($n = 4$). *$p < 0.05$ compared with folate-PEG liposomes alone.

BLs and US exposure enhance the direct internalization of folate-PEG liposomes. Therefore, we evaluated the involvement of folate-PEG liposomes attached to the surface of the cell membrane in enhanced gene transfection. KB cells were transfected with folate-PEG liposomes with or without BLs and US exposure at 37°C or 4°C, and the luciferase activity was measured. It is known that endocytosis is inhibited at 4°C. Therefore, folate-PEG liposomes cannot internalize into cells at 4°C. In contrast, Folate-PEG liposomes associate with cell membrane and internalize into cells at 37°C. When the cells were treated with folate-PEG liposomes with BLs and US exposure at 37°C, the luciferase activity was increased compared with that of the cells treated with folate-PEG liposomes alone ($p < 0.05$). In contrast, the luciferase activity did not change

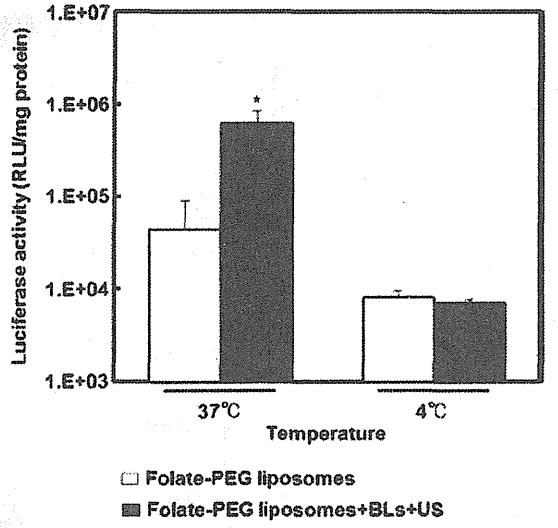

Figure 5. Effect of folate-PEG liposomes attached to the cell membrane on gene transfection. KB cells were preincubated for 30 min at either 37°C or 4°C before transfection and then treated with folate-PEG liposomes for a further 1 h at 37°C or 4°C. After incubation, the cells were washed and BLs were added. The cells were then exposed to US and cultured for 23 h. Luciferase activity was determined. Data are the means ± SD ($n = 4$). *$p < 0.05$ compared with folate-PEG liposomes alone.

in the cells treated with folate-PEG liposomes with BLs and US exposure at 4°C compared with the treatment of folate-PEG liposomes alone ($p > 0.05$) (Figure 5). This result indicated that folate-PEG liposomes attached to the cell membrane could not involve in the enhanced gene transfection by BLs and US exposure.

Discussion

Recently, it has been reported that endosomal escape is one of the most important steps in efficient gene delivery (Varga et al., 2005; Hama et al., 2006). We previously reported that BLs and US exposure could improve the endosomal escape of AG73-PEG liposomes, leading to increased transfection efficiency (Negishi et al., 2010b); however it is not well understood whether BLs and US exposure can enhance the transfection efficiency of carriers other than AG73-PEG liposomes, which are modified by several ligands, and internalize into cells via several receptors and endocytic pathways. In this study, we demonstrated that BLs and US exposure could enhance the gene transfection efficiency of folate-PEG liposomes.

Selective gene delivery has been considered a promising method. For this purpose, folate, RGD peptide, transferrin, and anisamide have been used as ligands to develop selective gene delivery carriers (Li et al., 2008; Ng et al., 2009; Koppu et al., 2010; Morris & Sharma 2011). Folate receptor is expected to be a specific receptor of cancer cells, and folate-modified carriers have been developed to achieve selective gene delivery. The majority of these carriers were internalized via endocytosis and required to deliver genes from endosomes to cytosol for

high gene expression. For efficient endosomal escape, some studies developed folate-modified carriers, which function to enhance endosomal escape (Shi et al., 2002); therefore, we chose folate-modified liposomes as a model to assess the utility of BLs and US exposure in general. In this study, to evaluate whether BLs and US exposure can be generally applied to gene delivery carriers to improve transfection efficiency, we examined whether BLs and US exposure can enhance the gene transfection efficiency of folate-PEG liposomes.

We previously reported that AG73-PEG liposomes were partially internalized into cells via clathrin-mediated endocytosis and syndecan, a heparan sulfate-containing transmembrane proteoglycan (Negishi et al., 2010b), whereas the cellular uptake pathway of folate-PEG liposomes was mainly raft-dependent endocytosis via folate receptor (Figures 1, 2). Our result also showed that BLs and US exposure could enhance the transfection efficiency of folate-PEG liposomes (Figure 4a). These results suggested that BLs and US exposure could enhance the transfection efficiency of carriers, which were internalized via various receptors and endocytic pathways in different cell lines; however, we could not assess the effect of BLs and US exposure on carriers, which internalized into cells via macropinocytosis. Further study is required of the involvement of the uptake pathway in enhanced gene transfection by BLs and US exposure.

On the other hand, it is reported that high transfection activity is required to overcome rate-limiting steps such as cellular internalization, endosomal escape, and nuclear transfer (Varga et al., 2005; Hama et al., 2006). PEG modification has the advantage of increasing the stability of carriers *in vivo*, but it is also believed to suppress cellular association and/or endosomal escape of carriers and to decrease gene expression (Zalipsky et al., 1999; Guo & Szoka, 2001; Shin et al., 2003; Walker et al., 2005; Hatakeyama et al., 2007). Indeed, the present study also showed that folate-PEG liposomes could not deliver genes into cytosol from endosomes sufficiently (Figure 3); therefore, endosomal escape is an important step to achieve efficient gene delivery. Our previous study demonstrated that BLs and US exposure could enhance the endosomal escape of AG73-PEG liposomes (Negishi et al., 2010b). In contrast, we and other groups reported that microbubbles and US exposure increased the permeability of the cell membrane, and delivered genes into the cytoplasm directly (Taniyama et al., 2002; Lentacker et al., 2009; Negishi et al., 2008; Suzuki et al., 2010; Negishi et al., 2011). Hence, we assessed the involvement of folate-PEG liposomes attached to the surface of cell membrane in enhanced gene transfection. Our results indicated that enhanced gene transfection by BLs and US exposure did not rely on folate-PEG liposomes associating with cell membrane (Figure 5). It was suggested that BLs and US exposure could affect intracellular trafficking of folate-PEG liposomes, leading to increased gene expression in the same manner as AG73-PEG liposomes.

When we compared Figure 3 and Figure 4, the transfection efficiency of folate-PEG liposomes with chloroquine was higher than that of the treatment with BLs and US exposure. It is possible that BLs and US exposure in further optimized condition may increase the transfection efficiency of folate-PEG liposomes up to that of chloroquine treatment. On the other hand, we need to concern that chloroquine cannot target some tissue and cells *in vivo*. In contrast, BLs and US exposure have advantage that the effect may be induced in the specific area focused US. Therefore, it is expected that BLs and US may enhance the transfection efficiency of gene delivery carriers in targeted tissues and cells.

However, we should investigate the more specific mechanism by which BLs and US exposure may affect directly intracellular vesicle morphology or induce several biological effects. It has been reported that microbubbles and US exposure induced the influx of calcium ions (Juffermans et al., 2008; Zhou et al., 2008; Kumon et al., 2009), and acidification of endosomes was regulated by calcium ions (Lelouvier et al., 2011); therefore, we will elucidate in a further study whether BLs and US exposure induce the influx of calcium ions and destabilize endosomes. We may also need to examine more clearly the effect of BLs and US exposure on transcription and other organelles. There is a possibility that several biological effects concerning gene transfection are induced by BLs and US exposure, and can affect, at least, folate-PEG liposomes internalized into cells (Figure 5) and improve intracellular trafficking of folate-PEG liposomes in the same manner as AG73-PEG liposomes.

We have reported that BLs and US exposure could enhance the transfection efficiency of AG73-PEG liposomes by about sixty-fold compared with AG73-PEG liposomes alone. On the other hand, the transfection efficiency of folate-PEG liposomes was enhanced up to five-fold by BLs and US exposure. These results suggested that BLs and US exposure had more influence on the transfection efficiency of AG73-PEG liposomes. The difference in the extent of enhanced transfection efficiency might be dependent on the different endocytic pathways and receptors that carriers mediate. The sensitivity of BLs and US exposure to different cell lines might also influence the efficacy of endosomal escape, leading to increased gene transfection; therefore, it is important, in order to achieve efficient gene transfection, that we understand the factors influencing the efficacy of endosomal escape. Although more study is required of the detailed mechanism of enhanced gene transfection, we expect that BLs and US exposure will be a promising method to achieve efficient endosomal escape, and this method may be applied in existing carriers, and peptide or protein delivery, which suffers from low endosomal escape.

Conclusions

We previously reported that BLs and US exposure could enhance endosomal escape and gene transfection of

AG73-PEG liposomes, which were internalized into cells via syndecan and partially clathrin-mediated endocytosis (Negishi et al., 2010b). In this study, we evaluated whether BLs and US exposure could be applied to other carriers, and assessed the utility of BLs and US exposure in general. The present study showed the enhanced transfection efficiency of folate-PEG liposomes, which are internalized into cells via folate receptor and raft-dependent endocytosis, when BLs and US exposure was applied. Our results suggested that BLs and US exposure could enhance endosomal escape and transfection efficiency of folate-PEG liposomes in the same manner as for AG73-PEG liposomes; thus, BLs and US exposure may be a promising method in general to achieve efficient gene transfection using various gene carriers.

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Declaration of interest

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The authors report no declaration of interest.

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Efficient Suppression of Murine Intracellular Adhesion Molecule-1 Using Ultrasound-Responsive and Mannose-Modified Lipoplexes Inhibits Acute Hepatic Inflammation

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Hepatitis is often associated with the overexpression of various adhesion molecules. In particular, intracellular adhesion molecule-1 (ICAM-1), which is expressed on hepatic endothelial cells (HECs) in the early stage of inflammation, is involved in serious illnesses. Therefore, ICAM-1 suppression in HECs enables the suppression of inflammatory responses. Here, we developed an ICAM-1 small interfering RNA (siRNA) transfer method using ultrasound (US)-responsive and mannose-modified liposome/ICAM-1 siRNA complexes (Man-PEG_{2000} bubble lipoplexes [Man-PEG_{2000} BLs]), and achieved efficient HEC-selective ICAM-1 siRNA delivery in combination with US exposure. Moreover, the sufficient ICAM-1 suppression effects were obtained via this ICAM-1 siRNA transfer *in vitro* and *in vivo*, and potent anti-inflammatory effects were observed in various types of inflammation, such as lipopolysaccharide, dimethylnitrosamine, carbon tetrachloride, and ischemia/reperfusion-induced inflammatory mouse models. *Conclusion*: HEC-selective and efficient ICAM-1 siRNA delivery using Man-PEG_{2000} BLs and US exposure enables suppression of various types of acute hepatic inflammation. This novel siRNA delivery method may offer a valuable system for medical treatment where the targeted cells are HECs. (HEPATOLOGY 2012;56:259-269)

Hepatitis resulting from conditions such as drug-induced hepatic inflammation and ischemia/reperfusion (IR)-induced liver injury followed by surgery is a major obstacle for medical treatment.[1,2] Moreover, it was reported that chronic hepatitis progresses to cirrhosis and liver cancer[3,4]; therefore, the prevention and early treatment of hepatitis are important for patients and medical professionals. Most drug-induced hepatitis is caused by nuclear factor-κB activation and proinflammatory cytokine production followed by various stimulations in medical treatments.[5] In IR-induced liver injury, a large amount of reactive oxygen species produced by IR stimulation is involved in the induction of inflammatory responses.[6] Although the mechanism for each inflammatory response is different, various adhesion molecules, such as vascular cell adhesion molecule (VCAM) and intracellular adhesion molecule (ICAM), are abundantly expressed on hepatic endothelial cells (HECs) in the early stage of inflammatory responses followed by various types of stimulation.[7] Among these, ICAM-1 is known as a major molecule that is highly involved in the adhesion, diapedesis, and tissue infiltration of leukocytes contributing to the deterioration in inflammatory responses.[8] During alcohol-

Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; BL, bubble lipoplex; CCl_4, carbon tetrachloride; DAPI, 4′,6-diamidino-2-phenylindole; DMN, dimethylnitrosamine; FITC, fluorescein isothiocyanate; H&E, hematoxylin and eosin; HEC, hepatic endothelial cell; ICAM, intracellular adhesion molecule; IFN-γ, interferon-γ; IL, interleukin; IR, ischemia/reperfusion; iv, intravenous; LPS, lipopolysaccharide; MCP-1, monocyte chemoattractant protein 1; MDA-5, melanoma differentiation-associated gene 5; mRNA, messenger RNA; RIG-1, retinoic acid-inducible gene 1; siRNA, small interfering ribonucleic acid; TLR, Toll-like receptor; TNF-α, tumor necrosis factor α; US, ultrasound.

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