reasons, PEG-liposomes have been known as drug, antigen, and gene delivery carriers.[13−17] To solve the above-mentioned issues of microbubbles, we previously developed PEG-modified liposomes entrapping echo-contrast, "Bubble liposomes" (BLs), which can function as a novel gene and siRNA delivery tool by applying them with US exposure *in vitro* and *in vivo*.[18−23] Furthermore, we showed that BLs with US exposure could achieve the delivery of basic fibroblast growth factor (bFGF)-expressing plasmid into skeletal muscle in a hindlimb ischemia model and increases in capillary density and blood flow.[24] The results were obtained by local injection of BLs and naked pDNA. To achieve gene delivery to deeper tissues, a systemic delivery system of pDNA is thought to be more effective; however, transfection efficiency is reduced if the BLs and pDNA are not colocalized in blood vessels, and it is therefore important to control the biodistribution of BLs and pDNA. In addition, pDNA is susceptible to nuclease degradation. To overcome these problems, the loading of pDNA onto BLs could be effective for gene delivery. Recently, it has been reported that pDNA combined with cationic lipid-shelled microbubbles by electrostatic charge coupling led to an increase in gene transfection *in vitro* and *in vivo*.[25−27] However, the microbubbles used in these reports have a size of 1−7 μm. These microbubbles show difficulty in penetrating deep into tissues; therefore, target tissues would be restricted. Nanosized Bubble liposomes could be a superior carrier for extensive delivery into tissues.

In the present study, we prepared nanosized pDNA-loaded BLs (p-BLs) using a cationic lipid to overcome these issues and investigated the effects of the amount of PEG in the BLs on their interaction with pDNA and the stability of pDNA in serum. We also assessed the feasibility and effectiveness of p-BLs with US exposure for gene therapy by trying to deliver a bFGF-expressing plasmid into skeletal muscle in a hindlimb ischemia model via tail vein injection.

EXPERIMENTAL SECTION

Preparation of Liposomes and BLs. To prepare liposomes for conventional BLs, 1,2-dipalmitoyl-*sn*-glycero-phosphatidylcholine (DPPC) and 1,2-distearoylphosphatidyle-thanolamine-methoxy-polyethylene glycol (PEG_{2000}) were mixed at a molar ratio of 94:6. Both lipids were purchased from NOF Corporation (Tokyo, Japan). 1,2-Dioleoyl-3-trimethylammonium-propane (DOTAP) and 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[methoxy(polyethylene glycol)-750] (PEG_{750}) from Avanti Polar Lipids (Alabaster, AL) were also used. Liposomes with various lipid compositions were prepared by a reverse phase evaporation method as described previously.[21] 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 (Nuclepore Track-Etch Membrane, 200 nm pore size; Whatman plc, U.K.). After sizing, the liposomes were passed through a sterile 0.45 μm syringe filter (Asahi Techno Glass Co., Chiba, Japan) to sterilize them. The lipid concentration was measured using the Phospholipid C test (Wako Pure Chemical Industries, Ltd., Osaka, Japan). BLs were prepared from liposomes and perfluoropropane gas (Takachiho Chemical Inc. Co. Ltd., Tokyo, Japan). First, 5 mL sterilized vials containing 2 mL of liposome suspension (lipid concentration: 1 mg/mL) were filled with perfluoropropane gas, capped, and then pressured with 7.5 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. The zeta potential and mean size of the BLs were determined using a light scattering method with a zeta potential/particle sizer (Nicomp 380ZLS, Santa Barbara, CA).

Plasmid DNA (pDNA). The plasmid pBLAST-hbFGF (InvivoGen Inc., San Diego, CA) is an expression vector encoding the human bFGF gene under the control of an EF-1α promoter. The plasmid pBLAST is used as an empty vector for the control. Fluorescein-labeled pDNA (Label IT Plasmid Delivery Control) was purchased from Mirus (Madison, WI).

Preparation of p-BLs. For the preparation of p-BLs, adequate amounts of pDNA were added to BLs and gently mixed. FITC-labeled pDNA and flow cytometry were used to examine the interaction between pDNA and BLs. The fluorescence intensity of p-BLs was analyzed using a FACSCanto (Becton Dickinson, San Jose, CA). To quantify the amount of pDNA loaded onto the BL surfaces, the BLs were centrifuged at 2,000 rpm for 1 min and the unbound pDNA was removed. The BL solution and aqueous solution containing unbound pDNA were then boiled for 5 min to solubilize Bubble liposomes and prevent background scattering. The optical density was measured at 260 nm using a spectrophotometer.

Stability of pDNA in Serum. The pDNA and p-BLs were incubated in 50% fetal bovine serum for 15−60 min. Serum was used without heat inactivation. To remove lipids and serum proteins, pDNA was extracted using a GenElute Mammalian Genome DNA Miniprep kit (Sigma-Aldrich, St. Louis, MO, USA). The stability of pDNA was confirmed by 1% agarose gel electrophoresis. The gel was stained with SYBER SAFE (Invitrogen Japan K.K., Tokyo, Japan) and visualized under ultraviolet light.

Hindlimb Ischemia Model. The ischemic hindlimb model was created in five-week-old male ICR mice as previously reported.[28] Briefly, animals were anesthetized, and a skin incision was made in the left hindlimb. After ligation of the proximal end of the femoral artery at the level of the inguinal ligament, the distal portion and all the side branches were dissected free and excised. The right hindlimb was kept intact to control the original blood flow. Measurements of the ischemic (left)/normal (right) limb blood flow ratio were performed for a set time using a laser Doppler blood flow meter (OMEGAFLO, FLO-C1).

***In Vivo* Gene Delivery into the Skeletal Muscle of Mice with BLs and US.** Ten and twelve days after ligation of the femoral artery, a mixture of 25 μL of pDNA (50 μg pBLAST-hbFGF or pBLAST as an control vector) and BL (200 μg) suspension was injected into the tail vein, and US exposure (1 MHz, 2.5 W/cm^2, 50% duty cycle, 96 s) was immediately applied at the site of hindlimb ischemia. A Sonitron 2000 (NEPA GENE, Co., Ltd.) was used as an ultrasound generator. Blood flow was measured several days after the second injection.

Real-Time PCR. Four days after the second injection, the mice were euthanized and sacrificed, and the thigh muscle in the US-exposed area was collected. Total RNA was extracted with RNAiso Plus (Takara Bio Inc., Japan) according to the manufacturer's instructions.

Complementary DNA was synthesized from 1 μg total RNA in a 20 μL reaction using Prime Script Reverse Transcriptase (Takara Bio Inc., Japan). Real-time RT-PCR was performed with an ABI PRISM 7000 Sequence Detection System instrument and software (Applied Biosystems Inc., Foster City, CA) using SYBR GreenER from Invitrogen. The sequences of primers were as follows: bFGF forward (5′-GTCACGGAAATACTCCAGTTGGT-3′), reverse (5′-CCCGTTTTGGATCCGAGTT-3′); HIF-1 forward (5′-CCACAGGACAGTACAGGATG-3′), reverse (5′-TCAAGTCGTGAATAATACC-3′); VEGF forward (5′-GGCTGCTGTAACGATGAAG-3′), reverse (5′-CTCTCTATGTGCTGGCTTTG-3′); PLGF forward (5′-GGATGTGCTCTGTGAATGC-3′), reverse (5′-CCTCTGAGTGGCTGGTTAC-3′); VEGFR1 forward (5′-ACATTGGTGGTGGCTGACTCTC-3′), reverse (5′-CCTCTCCTTCGGCTGGCATC-3′); VEGFR2 forward (5′-GCGGGCTCCTGACTACAC-3′), reverse (5′-CCAAATGCTCCACCAACTCTG-3′); Ang-1 forward (5′-CTACCAACAACAACAGCATCC-3′), reverse (5′-CTCCCTTTAGCAAAACACCTTC-3′); Ang-2 forward (5′-CTGTGCGGAAATCTTCAAGTC-3′), reverse (5′-TGCCATCTTCTCGGTGTT-3′); Tie-2 forward (5′-GTGTAGTGGACCAGAAGG-3′), reverse (5′-CTTGAGAGCAGAGGCATC-3′); SDF-1 forward (5′-GAGAGCCACATCGCCAGAG-3′), reverse (5′-TTTCGGGTCAATGCACACTTG-3′); CXCR4 forward (5′-AGCATGACGGACAAGTACC-3′), reverse (5′-GATGATATGGACAGCCTTACA-3′); and 18S forward (5′-GAATCGAACCCTGATTCCCCGTC-3′), reverse (5′-CGGCGACGACCCATTCGAAC-3′). As a control for RNA integrity and for assay normalization, 18S rRNA was used.

***In Vivo* Studies.** Animal use and relevant experimental procedures were approved by the Tokyo University of Pharmacy and Life Sciences Committee on the Care and Use of Laboratory Animals. All experimental protocols for animal studies were in accordance with the Principle of Laboratory Animal Care in Tokyo University of Pharmacy and Life Sciences.

Statistical Analyses. All data are the mean ± SD (n = 3–6). Data were considered significant when $P < 0.05$. The t-test was used to calculate statistical significance.

RESULTS

We initially investigated whether liposomes containing a cationic lipid, DOTAP, could entrap an US imaging gas as well as conventional BLs and whether pDNA could be loaded onto BLs using a fluorescence-activated cell sorter, the FACSCanto. As results, BLs containing DOTAP could effectively entrap the imaging gas and be used as ultrasound contrast agents (data not shown). As shown in Figure 1, BLs containing DOTAP were successfully loaded with pDNA; however, BLs not containing DOTAP could also interact with pDNA although the ratio of FITC-positive BLs was slightly decreased. To increase the amount of pDNA loaded onto BLs containing DOTAP, we attempted to prepare BLs containing different lengths of PEG and assessed the effect of PEG on BLs interactions with pDNA. As a result, the amount of pDNA loaded onto BLs was increased by adding short-length PEG (Figure 2). Thus, in all subsequent experiments BLs composed of DPPC, DOTAP, PEG_{2000}, and PEG_{750} (in a 79:15:2:4 molar ratio) were used. As shown in Table 1, there was almost no change in the size and zeta potential of the BLs after pDNA was added. We also examined the change in the amount of pDNA

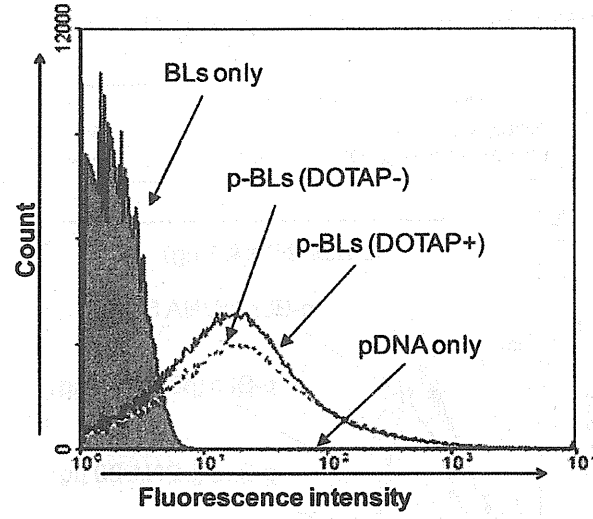

Figure 1. Interaction of pDNA with BLs. The interaction was examined by analyzing a mixture of FITC-pDNA (1 μg) and BLs (60 μg) with the FACSCanto.

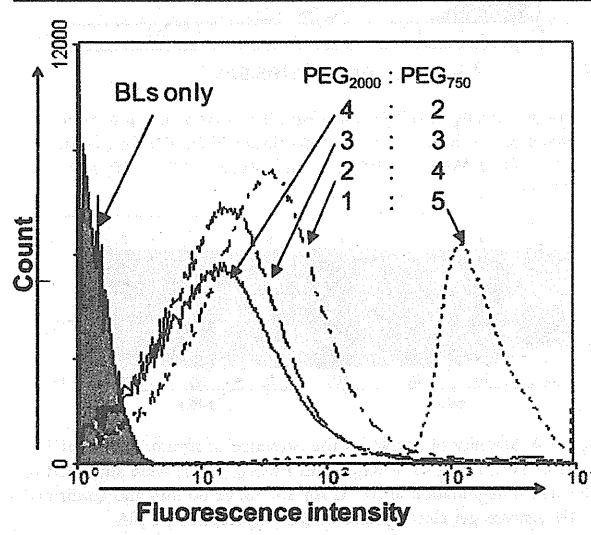

Figure 2. Interaction of pDNA with BLs and effects of PEG chain length on the interaction. The interaction was examined by analyzing a solution of p-BLs containing FITC-pDNA (1 μg) and various BLs (60 μg) with the FACSCanto.

bound to BLs when the concentration of the pDNA was increased. As shown in Figure 3, the amount of pDNA loaded increased in a dose-dependent manner.

We investigated the stability of pDNA in 50% serum. Figure 4 shows a digital photograph of free pDNA (lane 2–4) and p-BLs (lane 5–7) in the presence of serum for 15–60 min. Lane 1 is control pDNA. The high-mobility band was attributed to the most compact (supercoiled) form, whereas the less-intense band was considered to contain the nonsupercoiled content in the plasmid preparation. Loss of supercoil increased with longer incubation times; however, pDNA loaded onto BLs was more stable than free pDNA and was still present in a supercoiled conformation even after incubation for 60 min.

We attempted to deliver bFGF plasmid into the muscle of hindlimb ischemia model mice using p-BL and US exposure.

Table 1. Size (nm) and ζ Potential (mV) of BLs and p-BLs

lipid composition of BLs (molar ratio)	mean size (nm)		ζ potential (mV)	
	pDNA(−)	pDNA(+)	pDNA(−)	pDNA(+)
DPPC:PEG_{2000} = 94:6	615.1 ± 70.1	586.5 ± 73.5	−0.54	−0.43
DPPC:DOTAP:PEG_{2000}:PEG_{750} = 79:15:2:4	618.0 ± 81.0	532.6 ± 58.5	−0.18	−0.98

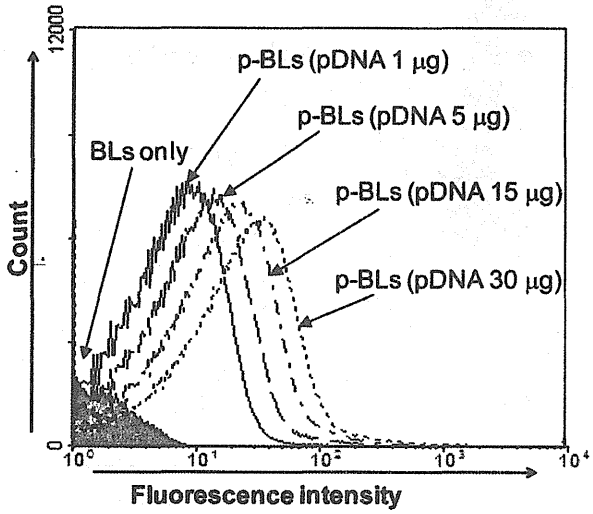

Figure 3. Loading of pDNA onto BLs. The interaction was examined by analyzing a solution of p-BLs containing FITC-pDNA (1–30 μg) and BLs (DOTAP (+), PEG_{2000} and PEG_{750} (2:4), 60 μg) with FACSCanto.

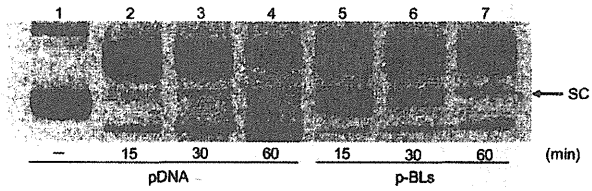

Figure 4. Stability of pDNA in the presence of serum. Naked pDNA or p-BLs (DOTAP (+), PEG_{2000} and PEG_{750} (2:4)) were subjected to 50% serum degradation at 37 °C for 15, 30, or 60 min and confirmed by 1% agarose gel electrophoresis. SC: supercoiled pDNA.

Four days after the second injection, we investigated the effects on various angiogenic genes by real-time PCR. As shown in Figure 5a, the amounts of mRNA of various angiogenic genes were high in the group treated with bFGF. In contrast, there were low levels in the group treated with the control plasmid. Two and four days after the second injection, we also measured the ischemic (left)/nonischemic (right) hindlimb blood flow ratio using a laser Doppler blood flow meter. Consistent with the induction of angiogenic factors, blood flow in the group treated with bFGF with p-BL and US exposure was significantly increased compared with the group treated with control plasmid and the group treated with PBS only (Figure 5b). These results suggest that the combination of p-BL and US exposure is an effective tool for a systemic gene delivery system.

DISCUSSION

Recently, a combination of microbubbles and US has been proposed as a less invasive and tissue-specific method of gene delivery.[5–12,29] We have previously shown that the combination

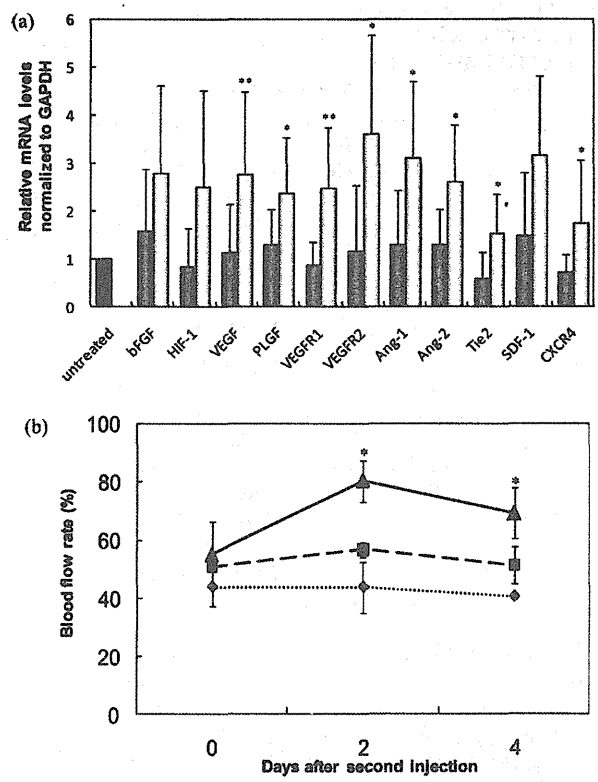

Figure 5. Therapeutic effects of bFGF gene transfer by p-BLs and US exposure on hindlimb ischemia mice. Ten days after femoral artery ligation, mice were treated with BLs and US (frequency, 1 MHz; duty, 50%; intensity, 2.5 W/cm^2; time, 96 s)-mediated bFGF gene transfer every 2 days with two administrations to hindlimb ischemia mice via tail vein injection. We injected the solution of pDNA (pBLAST or pBLAST-bFGF, 50 μg) and BLs (200 μg). (a) Effect of bFGF gene transfer p-BLs and US on mRNA expression of angiogenic genes. Four days after the second transfection, RNA was isolated from the thigh muscle and analyzed by real-time PCR. Black column: untreated group. Gray column: group treated with control plasmid using p-BLs and US. White column: group treated with bFGF using p-BLs and US. All data are reported as the mean ± SD (n = 5–6). (b) Effect of bFGF gene transfer p-BLs and US on recovery of blood flow. After the second transfection, blood flow was measured at 0–4 days using laser Doppler blood flow meter. Solid line: group treated with bFGF using p-BLs and US. Dashed line: group treated with control plasmid using p-BLs and US. Dotted line: untreated group. All data are reported as the mean ± SD (n = 3). ** indicates $P < 0.01$ compared with the group treated with control plasmid. * indicates $P < 0.05$ compared with the negative control.

of BL and US exposure was an effective gene delivery tool and could deliver pDNA into only US-exposed area of a tissue.[18–21,23,24,30] However, these results were obtained using a mixture of BLs and naked pDNA. Therefore, BLs and pDNA might not colocalize in blood vessels after intravenous administration. Free pDNA is susceptible to degradation by

deoxyribonuclease in serum and also rapidly eliminated from plasma when injected intravenously. Novel BLs interacting with pDNA were expected to resolve the above problems and lead to improved transfection efficiency *in vivo*. In this study, we prepared pDNA-loaded BLs (p-BLs) using DOTAP, a cationic lipid often used for gene delivery, as a more effective and efficient delivery tool for systemic injections.

We initially investigated the interaction between pDNA and BLs using flow cytometry. The BLs were detectable, although the fluorescence intensity was low. The fluorescein-labeled pDNA molecules were too small to be detected with flow cytometry; however, the pDNA-loaded BLs exhibited strong fluorescence. As results, the amount of pDNA loaded onto the surface of BLs was slightly increased by the presence of DOTAP and additionally increased by the presence of PEG_{750} (Figures 1, 2). It has been reported that the modification of liposomes with short and long PEG chains increases the fixed aqueous layer thickness.[31] The interaction could not be increased by using PEG_{750} without DOTAP (data not shown). We considered that structural changes in the PEG chain facilitated the interaction between cationic lipid and anionic pDNA. BLs containing many short PEG chains could be loaded with much more pDNA. Coating a liposome surface with PEG resulted in prolonged circulation time; however, short-length PEG reduces this effect.[14,32,33] Therefore, excessive reduction of PEG_{2000} could destabilize BLs *in vivo*. It has also been reported that the surface density and the length of PEG have effects on the encapsulation efficiency and permeability of drug.[34,35] Retaining the US imaging gas tended to be difficult. We therefore decided that the molar ratio of PEG_{2000} to PEG_{750} was 2:4. We also confirmed that not only pDNA but also siRNA can be loaded onto BLs (si-BLs) (data not shown). Interestingly, the ratio of short to long PEG chains had less effect on the amount of siRNA loaded onto BLs than pDNA. These results could be attributed to the molecular size of pDNA and siRNA.

We also investigated the stability of pDNA interacting with BLs in 50% serum. The presence of nuclease in the serum can degrade DNA, resulting in a loss of supercoil content.[36] It has also been reported that the pDNA-supercoiled structure should be retained to optimize cellular transfection efficiency.[37−39] However, pDNA held by BLs showed increased stability in 50% serum than free pDNA, although loss of supercoil increased with longer incubation times (Figure 4). In the solution of p-BLs, free pDNA was present with p-BLs; therefore the pDNA not held by BLs was degraded within a relatively short time.

For the integrity of pDNA in serum, pDNA should be stably held by BLs; however, pDNA held by BLs should be released from the surface of BLs after US exposure to be transfected into cells after intravenous injection. Prior to transfection experiments *in vivo*, we investigated the destruction efficiency of p-BLs under US exposure. The solution of p-BLs was exposed to US and analyzed using the FACSCanto. Unlike the solution of p-BLs before US exposure, no fluorescence was detected in the solution (data not shown). This result suggested that the US caused the release of pDNA from BLs.

We also examined whether p-BLs could be delivered to the ischemic hindlimb using a US imaging system. As a result, the US echo signal was detected 10−20 s after injection (data not shown). The hindlimb ischemia model used in this report was injected with p-BLs ten days after femoral artery ligation. The model showed the spontaneous recovery of 40−50% of the blood flow ratio which resulted from the angiogenic-induced enhancement of collateral blood flow. This result suggested that p-BLs could be used as a US contrast agent and be delivered to the ischemic hindlimb, in which blood flow was markedly decreased via systemic injection.

Recently, the delivery of naked pDNA encoding an angiogenic gene into ischemia has been reported in clinical trials; however, the transfection efficiency is still insufficient for effective angiogenesis without side effects.[40] We previously reported that BLs with US exposure could be a less invasive tool of gene delivery and achieve the delivery of a bFGF-expressing plasmid into skeletal muscle in a hindlimb ischemia model and increases capillary density and blood flow by intramuscular injection.[24] These results suggested that the combination of US and local injection of pDNA and BLs into muscle was an effective method of therapy for the ischemic hindlimb. However, a local injection could limit the region of gene transfection to myocytes and partial capillaries surrounding the injection site. Angiogenic gene therapy by systemic injection could be hypothesized to increase the accessibility of genes to deep tissues and vascular endothelial cells; therefore, we injected the solution of p-BLs via the tail vein, and the ischemic hindlimb was exposed to US. In experiments using pDNA encoding the firefly luciferase gene, this method was more effective than the conventional method by BLs not containing DOTAP (data not shown). This result suggests that the combination of p-BL and US exposure is an effective tool for a systemic gene delivery system. In fact, the amounts of mRNA of various angiogenic genes were high and blood flow recovered in the group treated with bFGF compared with the control plasmid (Figure 5a, b). It is thought that the transfected cells are vascular endothelial cells and surrounding myocytes. Cells mainly transfected by p-BLs and US remain unknown and should be clarified in the future. It has been reported that low-intensity US exposure could induce angiogenesis.[41−43] However, no significant recovery of the ischemic hindlimb was observed without bFGF plasmid under our US conditions. In experiments using pDNA encoding the firefly luciferase gene, no significant expression was observed in the US-unexposed area, normal hindlimb, liver, kidney, heart, spleen, and lung (data not shown). This result suggested that p-BLs developed in this study enabled site-specific pDNA delivery in combination with transdermal US exposure. Furthermore, microbubbles modified with an antibody and having a targeting function have recently been developed.[44−46] Liposomes can be easily modified to add a targeting function; thus, p-BLs using an antibody or peptide will enable more efficient pDNA delivery only to target cells in combination with US exposure and lead to beneficial clinical applications for various diseases.

In this study, we showed that BLs could efficiently load pDNA and protect pDNA against deoxyribonuclease degradation. Indeed, in the ischemic muscle, p-BLs could deliver bFGF-expressing pDNA at the US focused site, leading to the induction of angiogenic factors and the improvement of blood flow. These results suggest that the combination of p-BLs with US exposure may be useful for delivering pDNA to a tissue or organ via systemic injection and may be a less invasive therapeutic system for ischemic diseases.

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Notes

The authors declare no competing financial interest.

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Research Article

Selective Gene Transfer to the Retina Using Intravitreal Ultrasound Irradiation

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This paper aims to evaluate the efficacy of intravitreal ultrasound (US) irradiation for green fluorescent protein (GFP) plasmid transfer into the rabbit retina using a miniature US transducer. Intravitreal US irradiation was performed by a slight modification of the transconjunctival sutureless vitrectomy system utilizing a small probe. After vitrectomy, the US probe was inserted through a scleral incision. A mixture of GFP plasmid (50 μL) and bubble liposomes (BLs; 50 μL) was injected into the vitreous cavity, and US was generated to the retina using a SonoPore 4000. The control group was not exposed to US. After 72 h, the gene-transfer efficiency was quantified by counting the number of GFP-positive cells. The retinas that received plasmid, BL, and US showed a significant increase in the number (average ± SEM) of GFP-positive cells (32 ± 4.9; $n = 7$; $P < 0.01$). No GFP-positive cells were observed in the control eyes ($n = 7$). Intravitreal retinal US irradiation can transfer the GFP plasmid into the retina without causing any apparent damage. This procedure could be used to transfer genes and drugs directly to the retina and therefore has potential therapeutic value.

1. Introduction

Ultrasound (US) increases the permeability of the plasma membrane and reduces the thickness of the unstirred layer at the cell surface, thereby facilitating the entry of DNA into cells. Furthermore, a combination of low-intensity US and microbubble (MB) echocontrast agents allows direct DNA transfer into the cytosol through small pores in the cells caused by cavitation effects, and dramatically enhances gene transfection both *in vitro* and *in vivo* [1–4]. Previously, our group reported that combination of US and MB increases the induction efficiency of plasmid DNA in the surface of ocular tissues such as cornea, conjunctiva, and eyelid [1, 5, 6]. The retina deeper part of ocular tissue was more hard to deliver DNA because of difficulties of US exposure, we also demonstrated a possibility of transcorneal US irradiation with MB transfer of DNA plasmids into the retina (Sonoda S, et al. IOVS 2006;47:ARVO E-Abstract 828). However, the efficiency of DNA plasmid induction was not so high and the applications of US irradiation to retina were limited to external irradiation form the cornea due to US probe size. Furthermore the lack of targeting ability of this transcorneal method meant that unpredictable effects might occur in other tissues, such as the lens, iris, and ciliary body. Selective retinal transfection would thus be advantageous to improve induction efficiency and avoid unexpected US exposure.

A clinical application of a new therapeutic US method for treating thrombosis has been developed [7, 8]. This method employs a miniature US transducer at the tip of a MicroLysUS infusion catheter (EKOS Corp., Bothell, USA), which approaches the target site via arterial vessels, and has been shown to improve clinical outcomes [7–9]. We have explored the use of this concept to apply US at shorter distances with a smaller probe, which should allow us to irradiate US selectively and to minimise the damage to the other ocular tissues. Our group developed a tiny US probe as small as a 19-gauge needle which can insert to vitreous cavity and exposure US selectively to retina. The aim of

the present study was to evaluate the plasmid DNA deliver efficacy of intravitreal US irradiation using a miniature US transducer. This manuscript is the first attempt of intravitreal US irradiation to retina.

2. Methods

All of the animals were handled humanely in strict accordance with the Association for Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic and Vision Research, and with the approval of the ethics board of Kagoshima University, Japan. Male New Zealand albino rabbits (age = 14 weeks; body weight = 3 kg; KBT Oriental Co. Ltd., Saga, Japan) were anesthetised with an intramuscular injection of ketamine hydrochloride (14 mg/kg) and then xylazine hydrochloride (14 mg/kg). The procedures specifically followed the transconjunctival sutureless vitrectomy system (TSV) [10, 11]. Using a trocar cannula (25G trocar cannula system, Alcon, Fort Worth, Texas, USA), three incisions were made in the inferotemporal, superotemporal, and superonasal quadrants, and an infusion cannula was inserted into the inferotemporal incision (**Figure 1(a)**). Vitrectomy was performed with an accurus 800CS with a 25-gauge TSV (Alcon). The central and preretinal vitreous was excised to allow sufficient room for agent injections. Then, the superonasal incision was enlarged using a 19-gauge needle (Terumo, Tokyo, Japan) to allow the US probe to be inserted (Figures 1(b) and 1(f)).

A bubble liposome (BL) is a type of MB that has been developed by our group to allow more efficient gene transfer into a target site than conventional MBs [5, 6, 12–14]. The BLs were prepared following the methods described in our previous report [15]. Green fluorescent protein (GFP) coding plasmid (pEGFP-N2, Clontech, Mountain View, CA, USA; 50 μL) was mixed with BLs (50 μL), and then injected at a slow speed (for 30 s) into the generated preretinal space through the cannula, using a syringe with a 27-gauge blunt needle (Nipro, Osaka, Japan). During the injection, the US probe was positioned 1-2 mm from the central part of the optic disc of the retina, and US (frequency = 3 MHz; duty=6%; intensity = 0.15 W/cm^2; time = 60 s) was applied to the retina using a SonoPore 4000 (NEPA GENE, Chiba, Japan) (Figures 1(c) and 1(d)). The control group was treated using identical procedures but without US exposure (BL + plasmid). Immediately after the US exposure, we confirmed the rupture of the BLs, which indicated the occurrence of cavitation [1, 2, 16]. The sclera incision port, which was used for the US probe, was then sutured with 10-0 nylon (Alcon), and the other microcannulas that did not require sutures were removed (**Figure 1(e)**). Finally, antibiotic ointment was applied to the conjunctival sac (whole procedure movie; see supplementary file in supplementary material available online at doi:10.1155/2012/412752). We also confirmed the condition used plasmid alone following US irradiation (US + plasmid).

The eyes were enucleated 72 h after treatment, and immediately frozen in liquid nitrogen-cooled isopentane. Serial sections were sliced with a cryostat adjacent area of optic disc where US were exposed. The GFP was observed by laser confocal microscopy (FV-1000; Olympus, Tokyo, Japan). The gene-transfer efficiency was quantified by measuring the intensity of GFP-positive cells randomly selected four sections form each rabbit and calculated average number by three masked observers. Seven rabbit eye were treated for each condition.

Table 1: Quantification of gene-transfer efficiency by counting the number of GFP-positive cells.

Condition	Number of GFP positive cell (mean ± SEM)	Mann-Whitney U test
control (BL + *plasmid*)	No positive cell	
US + BL + plasmid	32.0 ± 4.9	$P < 0.01$ (versus control and US + plasmid)
US + plasmid	4.2 ± 2.7	$P < 0.01$ (versus control)

3. Results

No GFP-positive cells were observed in the control eyes (n = 7; **Figure 2(a)**); however, the retinas that received plasmid and US concomitantly with or without BL showed GFP-positive cells (**Figure 2(b)**). Importantly, the GFP-positive cells were limited to the area exposed to US, and were observed mainly in the outer nuclear layer. The average number of GFP-positive cells in BL + plasmid + US group was 32.0 ± 4.9 (mean ± SEM, n = 7) per visual field and 4.2 ± 2.7 (mean ± SEM, n = 7) in plasmid + US group with ×200 magnification (**Table 1**). Induction efficiency of BL + plasmid + US was a statistically significant increase ($P < 0.01$, Mann-Whitney U test) compared with both control and US + plasmid group.

Slit-lamp biomicroscopy examinations were performed 1 day and 3 days after treatment, and revealed no obvious tissue damage (i.e., an intact bulb shape, no cells in the anterior chamber, and no preretinal haemorrhage). Histological examinations by hematoxylin and eosin (H&E) staining showed no critical retinal damage in any of the treatment groups (**Figure 2(c)**).

4. Discussion

In this study, direct intravitreal retinal US irradiation transferred the GFP plasmid to the rabbit retina without any apparent tissue damage. Furthermore, combining the GFP plasmid with BLs greatly enhanced the gene delivery. To our knowledge, this is the first report to attempt intravitreal US irradiation with a 25-gauge TSV system. Diameter of US transducers commonly used for gene delivery were 3 mm and more, therefore only transcorneal US irradiation could be available on occasions when US was exposed to the retina, deeper portion of ocular tissue. Our preliminary study using transcorneal US exposure to retina showed (Sonoda S, et al. IOVS 2006;47:ARVO E-Abstract 828), in that study luciferase

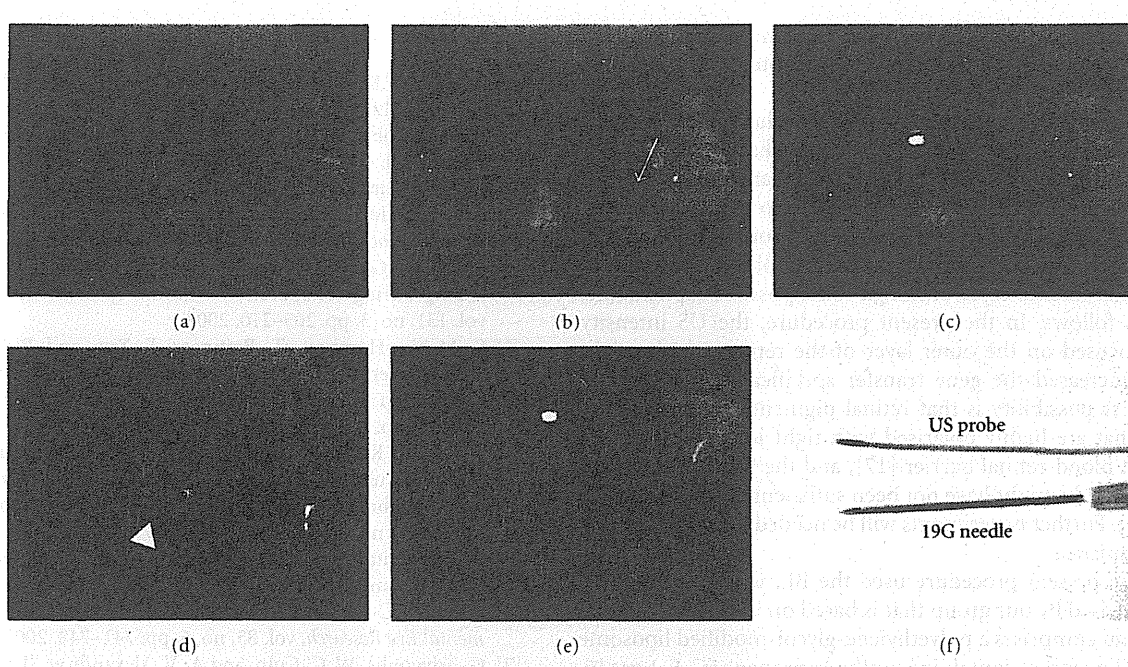

FIGURE 1: (a) Vitrectomy was performed with a 25-gauge vitrectomy system with rabbit eye. (b) Enlarge the superonasal incision with 19G needle (arrow) for the insertion of the US probe. (c) The eye ball had preserved intact after insertion of the US probe. (d) Bubble liposome was injected through the superotemporal port (arrowhead) and US irradiation (asterisk) was performed simultaneously. (e) The eye ball was kept intact after whole procedures. (f) The US probe size is as small as a 19G needle.

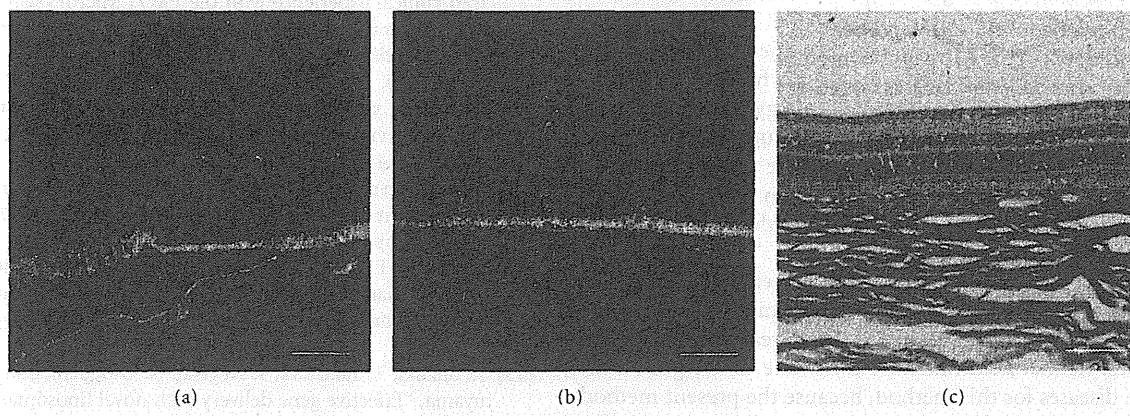

FIGURE 2: (a) No-GFP positive cells were observed in the control (plasmid + BL without US), (b) In the group treated with plasmid + BL + US, GFP-positive cells were observed exclusively in the area where US was exposed, and located in the outer nuclear layer. (c) H&E staining showed no obvious tissue damage. Bar equals 100 μm.

plasmid was employed instead of GFP, we could not compare accurately, scattered positive staining cells (11.3 ± 3.2, mean $\pm$ SEM, $n = 6$) were only observed in superficial layer of retina. In this study with intravitreal US irradiation, the average number of positive staining cells was obviously larger than transcorneal US exposure.

Our group had achieved to minimize the diameter of US probe which is enough small to insert vitreous cavity. On the contrary, the US probe, was not customized for the only purpose for intravitreal US irradiation, still remaining problems are such as ring sonography transducer that generate a 360° circumferential pulse and US intensity was not specially tuned for retina. Even this type of US probe could transfer GFP plasmid into retina. In other word, development of a US probe specialised for ocular tissue, for example, with optimal US intensity and unidirectional US irradiation towards the retina, might increase the gene-transfer efficiency. Furthermore, reducing the size of the US probe could avoid the need for additive sclera incision, reduce the procedure time, and potentiate the no-suture

minimal surgery of the 25 G TSV system. Taken together, Intravitreal US irradiation may have potential therapeutic value.

GFP-positive cells were observed exclusively in the area that was exposed to US. This indicates that intravitreal US exposure is more selective for the retina than the transcorneal method, thereby avoiding gene delivery to unexpected areas and minimizing the damage to the surrounding tissues. It is not clear why GFP-positive cells were observed mainly in the outer nuclear layer, although some possible explanations are as follows. In the present procedure, the US intensity was focused on the outer layer of the retina, which might have increased the gene transfer specifically to that layer. Another possibility is that retinal pigment epithelial (RPE) cells that are highly polarised with tight junctions produce a tight blood-retinal barrier [17], and the US intensity used in this study might have not been sufficient to break the RPE barrier. Further experiments will be needed to elucidate these mechanisms.

The present procedure used the BL, which is a type of MB devised by our group that is based on liposome technology, and comprises a polyethylene-glycol-modified liposome (PEG-liposome) containing perfluoropropane (C_3F_8) gas [6, 12, 15]. In the case of intravitreal US, only the combination of BL plus US exposure enhanced the gene-transfer efficiency. As described in previous reports [1, 2, 6], trapped gas inside the BL is cavitated by US, enhanced to form microjet flow, and allows easy gene entry into the cell [13, 15]. The BL has several advantages compared with the conventional and well-studied MB, the Optison (GE Healthcare, Little Chalfont, UK). The BL can be modified to incorporate additional components, such as targeting functions, as it was developed based on liposome technology, besides BL was incompatible with DNA plasmid and drugs easily [18, 19]. Furthermore, components of the BL are already in use in clinical applications, and US has also been used clinically for a long time [15, 20]. Based on these facts, the present method is likely to be safe for human treatment.

Again, the major advantage of this method was selective US irradiation in vitreous cavity with minimal side effect. This concept would be applicable to treat various retinal diseases. Retinal detachment (RD) is one of the good candidate diseases for this method, because the present method can be easily applied during vitrectomy and the gene transfer of neuroprotective or anti-apoptotic factor might prevent the further deterioration of vision [21, 22]. Now we established a rabbit RD model to evaluate the possibility of our method. If this concept would be proven by this model, the application would be widely expanded to such disease as retinitis pigmentosa and glaucoma.

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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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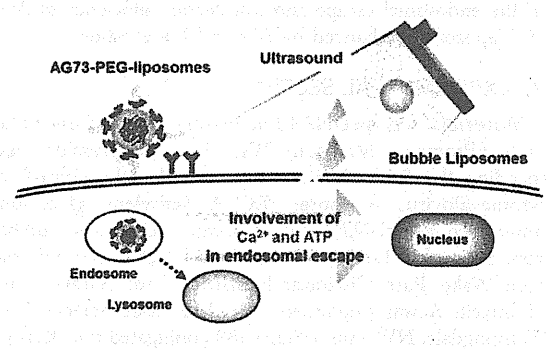
ABSTRACT: Recently, we reported the accelerated gene transfection efficiency of laminin-derived AG73-peptide-labeled polyethylene glycol-modified liposomes (AG73-PEG liposomes) and cell penetrating TAT-peptide labeled PEG liposomes using PEG-modified liposomes, which trap echo-contrast gas, "Bubble liposomes" (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 ($DSPE\text{-}PEG_{2000}$-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 $DSPE\text{-}PEG_{2000}$-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 ($DSPE\text{-}PEG_{2000}$-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

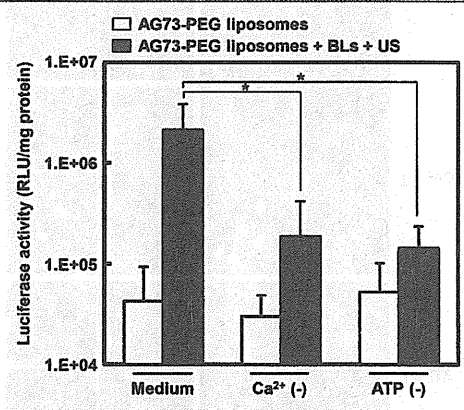

Figure 1. Effects of Ca^{2+} and ATP on gene expression by AG73-PEG liposomes with BLs and US exposure. 293T-Syn2 cells were treated with AG73-PEG liposomes for 4 h at 37 °C, and then cells were washed twice with Ca^{2+}-free DMEM containing 10 mM EGTA for a depleted Ca^{2+} condition. ATP was depleted 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

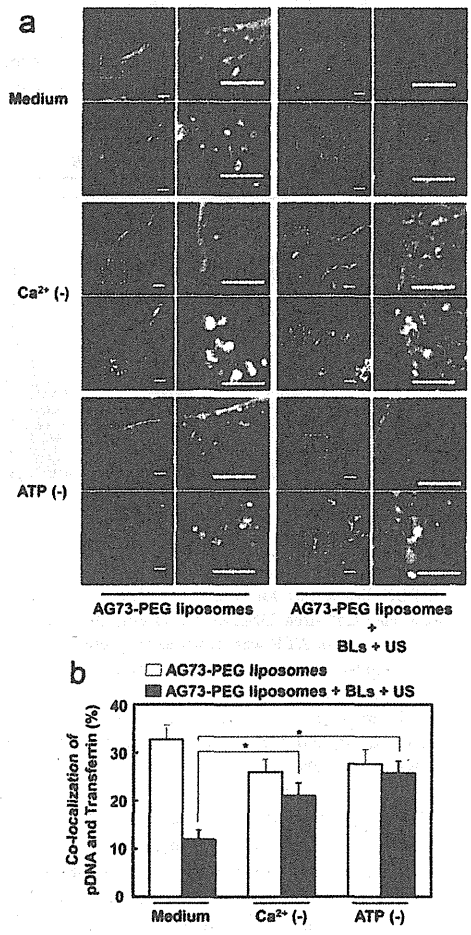

Figure 2. Effects of Ca^{2+} and ATP on intracellular localization of pDNA and endosomes. (a, b) The 293T-Syn2 cells were treated with AG73-PEG liposomes encapsulating Cy3-labeled pDNA (red) and Alexa Fluor 488-conjugated transferrin (green) for 4 h at 37 °C and then washed twice with Ca^{2+}-free DMEM 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

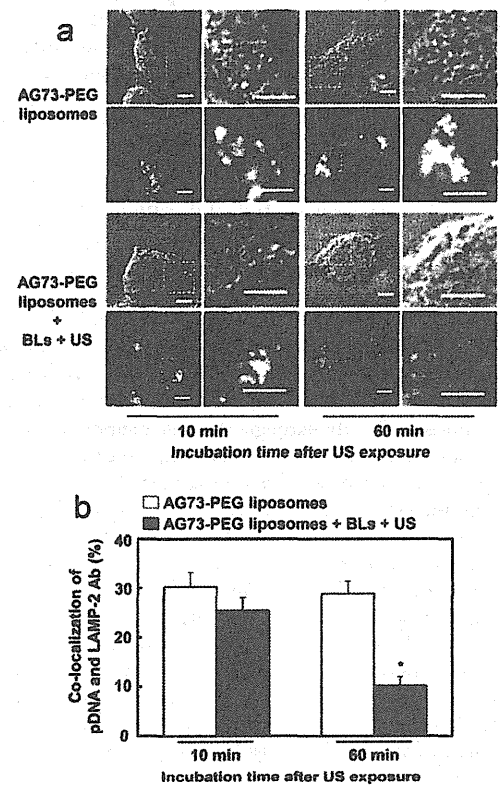

Figure 3. Effect of BLs and US exposure on intracellular localization of pDNA and lysosomes. The 293T-Syn2 cells were treated with AG73-PEG liposomes encapsulating Cy3-labeled pDNA (red) for 4 h at 37 °C. BLs (120 μg/mL) were added to cells followed by immediate US exposure. The cells were incubated 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.

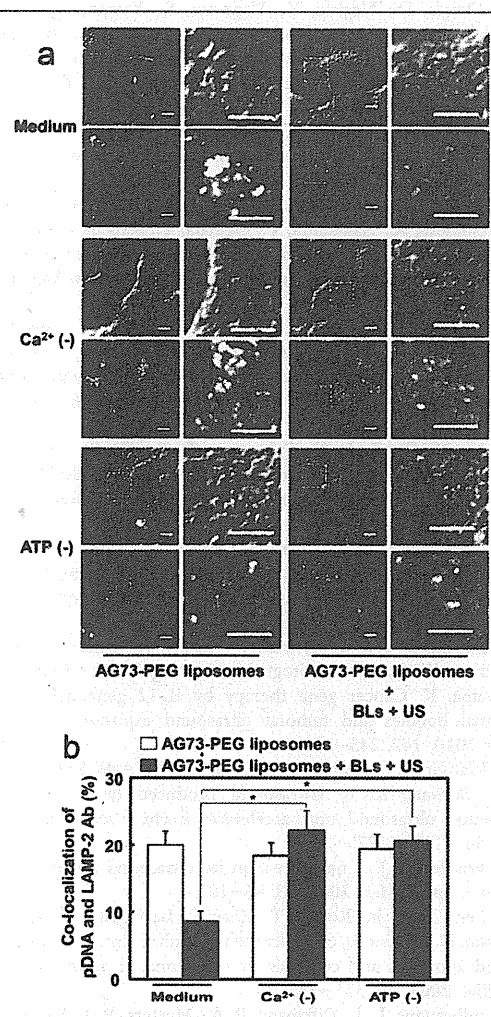

Figure 4. Effects of Ca^{2+} and ATP on intracellular localization of pDNA and lysosome. (a, b) The 293T-Syn2 cells were treated with AG73-PEG liposomes encapsulating Cy3-labeled pDNA (red) for 4 h at 37 °C and then washed twice with Ca^{2+}-free DMEM containing 10 mM EGTA to create Ca^{2+}-depleted 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).

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