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GENE DELIVERY

Tumor specific ultrasound enhanced gene transfer *in vivo* with novel liposomal bubbles

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Abstract

Bubble liposomes (liposomes which entrap an ultrasound imaging gas) may constitute a unique system for delivering various molecules efficiently into mammalian cells *in vitro*. In this study, Bubble liposomes were compared with cationic lipid (CL)–DNA complexes as potential gene delivery carriers into tumor *in vivo*. The delivery of genes by Bubble liposomes depended on the intensity of the applied ultrasound. Transfection efficiency plateaued at 0.7 W/cm^2 ultrasound intensity. Bubble liposomes efficiently transferred genes into cultured cells even when the cells were exposed to ultrasound for only 1 s. In addition, Bubble liposomes could introduce the luciferase gene more effectively than CL-DNA complexes into mouse ascites tumor cells and solid tumor tissue. We conclude that the combination of Bubble liposomes and ultrasound is a minimally-invasive and tumor specific gene transfer method *in vivo*.

Keywords: Liposomes; Bubble liposomes; Gene delivery; Ultrasound; Cancer

1. Introduction

In cancer gene therapy, it is important to develop the easy, safe, efficient, minimally-invasive and tissue-specific technologies of gene transfer into tumor tissue. Sonoporation is a method of gene delivery with ultrasound. Ultrasound increases the permeability of the plasma membrane and reduces the thickness of the unstirred layer of the cell surface, aiding DNA entry into cells [1,2]. Preliminary studies into the utility of ultrasound for gene delivery used frequencies in the range of 20–50 kHz [1,3]. However, these frequencies are also known to induce tissue damage and cavitation if not properly controlled [4–6]. To overcome this problem, several studies have used frequencies of 1–3 MHz, intensities of 0.5–2 W/cm^2, and pulse-modulation [7–9]. In a separate approach, a combination of therapeutic ultrasound and microbubble echo contrast agents was shown to enhance gene transfection efficiency [10–15] by effectively and directly transferring DNA into the cytosol. Microbubbles based on protein microspheres, and sugar microbubbles, are commercially available; however, although they encapsulate ultrasound contrast agents, they are too large (2–10 μm diameter) for intravascular application [16]. It has been reported that the *i.v.* injection of Optison without ultrasound exposure results in lethal embolisms in vital organs in mice [17]. Although a similar effect has not been observed in humans, it is possible that Optison can not pass through capillary vessels. Ideally, microbubbles should be smaller than red blood cells.

Liposomes can be used as drug, antigen and gene delivery carriers [18–26]. Based on liposome technology, we developed novel liposomal bubbles (Bubble liposomes) containing the ultrasound imaging gas, perfluoropropane. When coupled with ultrasound exposure, Bubble liposomes can be used as novel

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gene delivery agents [27]. In addition, we found out that the gene delivery was only observed at the site of ultrasound exposure. Therefore, using Bubble liposomes and ultrasound, we could establish minimally-invasive and tumor tissue-specific gene delivery. In the present study, the characteristics of Bubble liposomes as gene delivery vectors were studied, and gene transfection efficiencies into tumor *in vivo* were compared with lipofection using cationic liposomes, a common non-viral gene transfer method.

2. Materials and methods

2.1. Cells

African green monkey kidney fibroblast COS-7 cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma Chemical Co., St. Louis, MO) supplemented with 10% heat inactivated fetal Bovine serum (FBS, GIBCO, Invitrogen Co., Carlsbad, CA). Mouse Sarcoma-180 (S-180) cells were cultured in Eagle's medium (MEM; Sigma) supplemented with 10% heat inactivated FBS. All culture media contained 100 U/mL penicillin (Wako Pure Chemical Industries, Ltd., Osaka, Japan) and 100 μg/mL streptomycin (Wako).

2.2. Preparation of liposomes and Bubble liposomes

Liposomes composed of 1,2-distearoyl-sn-glycero-phosphatidylcholine (DSPC) (NOF Corporation, Tokyo, Japan) and 1,2-distearoyl-sn-glycero-3-phosphatidyl-ethanolamine-methoxy-polyethyleneglycol (DSPE-PEG (2 k)–OMe; NOF) (94:6 (m/m)) were prepared by reverse phase evaporation. In brief, all reagents (total lipid: 100 μmol) were dissolved in 8 mL of 1:1 (v/v) chloroform/diisopropyl ether, then 4 mL of PBS was added. The mixture was sonicated and evaporated at 65 °C. The solvent was completely removed, and the size of the liposomes was adjusted to less than 200 nm using an extruding apparatus (Northern Lipids Inc., Vancouver, BC) and sizing filters (pore sizes: 100 nm and 200 nm; Nuclepore Track-Etch Membrane, Whatman plc, UK). After sizing, the liposomes were sterilized by passing them through a 0.45 μm pore size filter (MILLEX HV filter unit, Durapore PVDF membrane, Millipore Corporation, MA). The liposome size was measured with dynamic light scattering (ELS-800, Otsuka Electronics Co., Ltd., Osaka, Japan). The average diameter of these liposomes were about 150–200 nm. Lipid concentration was measured with the Phospholipid C test wako (Wako Pure Chemical Industries). Bubble liposomes were prepared from the liposomes and perfluoropropane gas (Takachiho Chemical Ind. Co. Ltd., Tokyo, Japan). In brief, 5 mL sterilized vials containing 2 mL of the liposome suspension (lipid concentration: 1 mg/mL) were filled with perfluoropropane, capped and then supercharged with 7.5 mL of perfluoropropane. The vial was placed in a bath-type sonicator (42 kHz, 100 W; BRANSONIC 2510J-DTH, Branson Ultrasonics Co., Danbury, CT) under the condition of positive pressure with perfuluoropropane in the vial under the condition of positive pressure with perfuluoropropane in the vial for 5 min to form the Bubble liposomes.

2.3. Microscopic observation of Optison and Bubble liposomes and size distribution

Optison (NEPA GENE, CO., LTD., Chiba, Japan) or Bubble liposomes were placed on glass slides, covered with a cover slip and observed with a microscope (Leica MICROSYSTEMS, Wetzlar, Germany) using a darklite illuminator (NEPA GENE). The size distribution of Optison and Bubble liposomes was measured by dynamic light scattering (ELS-800).

2.4. Transmission electron microscopy of Bubble liposomes

Bubble liposomes were suspended into sodium alginate solution (0.2% (w/v) in PBS). This suspension was dropped into calcium chloride solution (100 mM) to hold Bubble liposomes within calcium alginate gel. Then, the beads of calcium alginate gel containing Bubble liposomes were prefixed with 2% glutaraldehyde solution in 0.1 M Cacodylate buffer, post-fixed with 2% OsO_4, dehydrated with an ethanol series, and then embedded in Epan812 (polymerized at 60 °C). Ultrathin sections were made with an ultramicrotome at a thickness of 60–80 nm. Ultrathin sections were mounted on 200 mesh copper grids. They were stained with 2% uranyl acetate for 5 min and Pb for 5 min. The samples were observed with JEOL JEM12000EX at 100 kV. The treatment after prefixation was carried out in Hanaichi Ultrastructure Research Institute Co.,Ltd (Aichi, Japan).

2.5. Transfection of plasmid DNA into cells using Bubble liposomes

Luciferase cording plasmid DNA (pCMV-Luc), COS-7 cells (1×10^5 cells) and Bubble liposomes (60 μg) were suspended in culture medium (500 μL) with 10% FBS in 2 mL polypropylene tubes. The suspension was ultrasonicated using a Sonopore 4000 (6 mm diameter probe; NEPA GENE) sonicator under various conditions. The cells were washed twice with PBS, resuspended in fresh culture medium and cultured in 48-well plates for 2 days.

2.6. Transfection of plasmid DNA into cells by lipofection

Plasmid DNA (pCMV-Luc, 0.25 μg) and Lipofectin (1.25 μg) (Invitrogen) were mixed and complexed according to the manufacturer's instructions. The complex was added to COS-7 cell suspensions (1×10^5 cells/500 μL/ tube) containing various concentrations of serum for 10 s. The cells were washed twice with PBS, resuspended in fresh culture medium and cultured in 48-well plates for 2 days.

2.7. In vivo gene delivery into mouse ascites tumor cells

S-180 cells (1×10^6 cells) were *i.p.* injected into ddY mice (4 weeks old, male) (Sankyo Labo Service Corporation, Tokyo, Japan) on day 0. When S-180 cells grew as the ascites tumor in mice after 8 days of the injection [28], the mice were anaesthetized with NEMBUTAL (50 mg/kg) (Dainippon

Sumitomo Pharma, Osaka, Japan), then injected with 510 μL of pCMV-Luc (10 μg) and Bubble liposomes (500 μg) in PBS. Ultrasound (frequency: 1 MHz, duty: 50%; intensity: 1.0 W/cm^2, time: 1 min) was transdermally applied to the abdominal area using a Sonopore 3000 ultrasonicator with a probe of diameter 20mm (NEPA GENE). In other experiments, pCMV-Luc (10μg) and Lipofectin (50μg) or Lipofectamine 2000 (50μg) were mixed and complexed according to the manufacturer's instructions. The complex was suspended in PBS (510μL) and injected into the peritoneal cavities of mice. After 2days, S-180 cells were recovered from the abdomens of the mice. Then, the recovered cells were lysed in the lysis buffer (0.1M Tris–HCl (pH 7.8), 0.1% Triton X-100, 2mM EDTA) and luciferase activity was determined.

2.8. In vivo gene delivery into mouse footpad solid tumor

S-180 cells (1×10^6 cells) were inoculated into the left footpad of ddY mice (5 weeks old, male). At day 4, when the thickness of the footpad was over 3.5 mm (normal thickness was about 2 mm), the left femoral artery was exposed. One hundred μL of pCMV-Luc (10 μg) with or without Bubble liposomes (100 μg) were injected into femoral artery using 30-gauge needle. In the same time, ultrasound (frequency: 0.7 MHz, duty: 50%; intensity: 1.2 W/cm^2, time: 2 min) was transdermally applied to the tumor tissue using a Sonopore 4000 ultrasonicator with a probe of diameter 8 mm (NEPAGENE). The needle hole was then closed with an adhesive agent (Aron Alpha; Sankyo, Tokyo, Japan) and skin was put in a suture. In other samples, pCMV-Luc (10 μg) and Lipofectamine 2000 (25 μg) (Invitrogen Corporation, Carlsbad, CA) were mixed and complexed according to manual of Lipofectamine 2000. The complex were suspended in PBS (100 μL) and injected into femoral artery of mice. After 2 days of injection, the mice were sacrificed and the tumor tissues were collected. Then, the tumor tissues were homogenated in the lysis buffer and luciferase activity was determined.

2.9. Luciferase assay

Luciferase activity was measured using a luciferase assay system (Promega, Madison, WI) and a luminometer (TD-20/20, Turner Designs, Sunnyvale, CA). Activity is reported in relative light units (RLU) per mg protein.

2.10. In vivo Luciferase imaging

The mice were anaesthetized and *i.p.* injected with D-luciferin (150 mg/kg) (Xenogen, Corporation, CA). After 10 min, luciferase expression was observed with *in vivo* luciferase imaging system (IVIS) (Xenogen Corporation).

2.11. Hemolysis assay

Mouse red blood cells (2.5×10^8 cells/500 μL) were exposed with ultrasound (frequency: 0.7 MHz, Duty: 50%, Intensity: 0.5–1.5 W/cm^2, Time: 10 s.) in absent or present of Bubble liposomes. The red blood cell suspension was centrifuged for 10 min at 3000 rpm. Then, absorbance ($A_{540\ nm}$) of the supernatant was measured. The rate of hemolysis was calculated as follows: % of hemolysis = ($A_{540\ nm}$ of experimental group $-A_{540\ nm}$ of non-treated group)/($A_{540\ nm}$ of hypotonic solution treated group $-A_{540\ nm}$ of non-treated group) × 100.

2.12. In vivo studies

All experimental protocols for animal studies were in accordance with the Principle of Laboratory Animal Care in Teikyo University.

2.13. Statistical analysis

Differences in luciferase activity between experimental groups were compared with non-repeated measures ANOVA and Dunnett's test.

3. Results and discussion

The use of non-viral vectors is attractive as a safe, clinically acceptable gene therapy technique. In addition, non-viral vectors should be easy to prepare and use. However, most non-viral vectors deliver plasmid DNA into cells via endocytosis, followed by plasmid DNA degradation in the endosomes. Consequently, non-viral vectors often result in low gene delivery efficiency. It has been reported that new types of non-viral vectors can induce the escape of genes from endosomes [29–31] and directly deliver genes into the cytosol via a fusion mechanism [28,32]. In addition, microbubbles and ultrasound have been investigated with a view to improving the transfection efficiency of non-viral vectors. Gene delivery using a combination of microbubbles such as Optison and ultrasound has been widely reported. In order for extracellular plasmid DNA to be directly and effectively delivered into the cytosol, transient pores in the cell membrane must be formed by cavitation. However, conventional microbubbles are very large, with most greater than 2 μm in diameter [16]. Actually, our observations of Optison using a microscope and a darklite illuminator showed some bubbles more than 10 μm in diameter (Fig. 1(a)). In the measurement of the size distribution, there were some large microbubbles (Fig. 1d)). Tsunoda et al. pointed out that these large bubbles might cause lethal embolism in some vital organs [17]. In contrast, most Bubble liposomes were much smaller than Optison, with average diameters less than 2 μm (Fig. 1(b, e)). The injection of 1 mg of Bubble liposomes into the tail veins of mice was not lethal (data not shown), suggesting that Bubble liposomes may not cause lethal embolism. To confirm the structure of Bubble liposomes, we observed Bubble liposome with transmission electron microscopy (Fig. 1(c)). Interestingly, there were nanobubbles into lipid bilayer. From this result, it was thought that Bubble liposomes were different from conventional microbubbles which was the echo gas wrapped with lipid mono-layer. Kodama T. et al. and Klibanov A.L. et al. reported about microbubbles using distearoylphospatidylcholine and PEG-

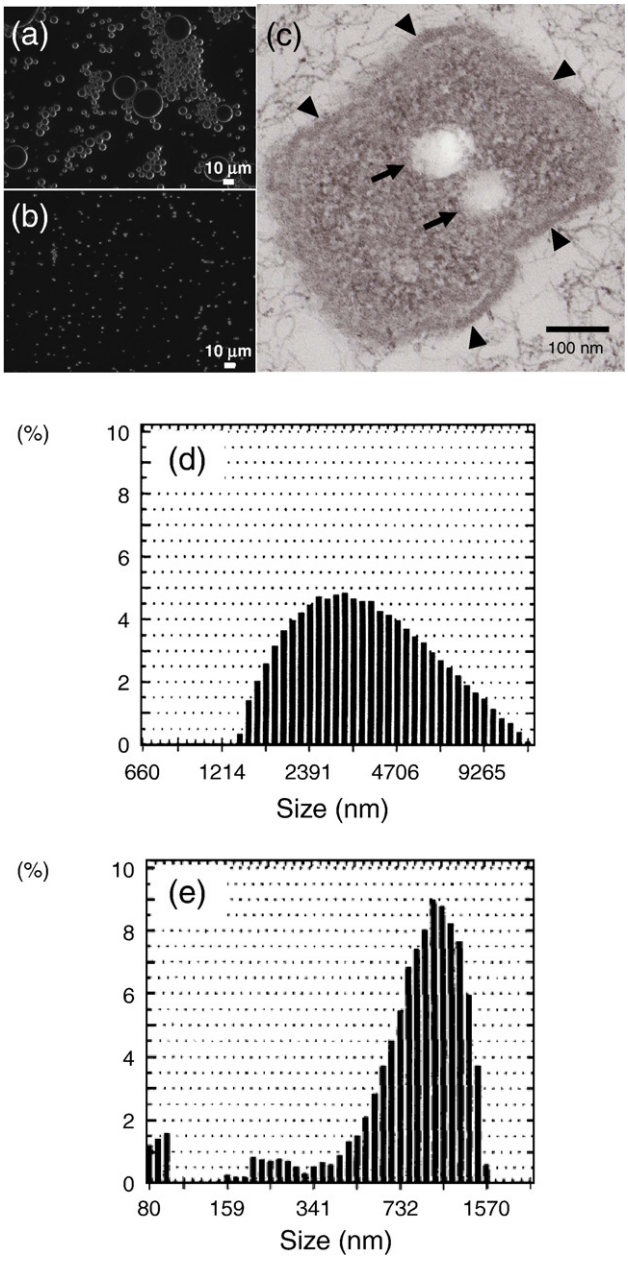

Fig. 1. Microscopy of Optison and Bubble liposomes. Optison (a) and Bubble liposomes (b) were observed with a microscope using a darklite illuminator. Original magnification ×400. Bubble liposomes (c) were observed with a transmission electric microscope at 100 kV. Original magnification ×50,000. Arrow head shows lipid bi-layer and arrow shows perfluoropropane nanobubble. The size distribution of Optison (d) and Bubble liposomes (e).

stearate [33,34]. These microbubbles were made by being stabilized hydrophobic echo gas with amphipathic molecules such as lipid and surfactant. In our method, it was thought that liposomes were reconstituted by sonication under the condition of supercharge with perfluoropropane in the 5 mL vial container. At the same time, perfluoropropane would be entrapped within lipids like micelles, which were made by DSPC and DSPE-PEG (2 k)-OMe from liposome composition, to form nanobubbles. The lipid nanobubbles were encapsulated within the reconstituted liposomes (Fig. 1(c)), which sizes were changed into around 1 μm (Fig. 1(b,e)) from 150–200 nm of original. In addition, we evaluated about the stability of Bubble liposomes by transfection efficiency with sonoporation (Fig. 2). The efficiency gradually decreased according to storage time. We also observed the aspect and ultrasound imaging of Bubble liposomes. The suspension of Bubble liposomes gradually became clear in aspects, resulted in decreasing the echo signal according to storage time (data not shown). These results suggested that perfluoropropane was gradually degassed from Bubble liposomes. Therefore, we used fresh Bubble liposomes in all experiments.

Previously, we reported that Bubble liposomes could induce cavitation and deliver plasmid DNA into various types of cells [27]. In order to examine what conditions are necessary for Bubble liposomes to efficiently deliver genes, transfection efficiency was assessed using Bubble liposomes combined with various levels of ultrasound exposure (Fig. 3(a)). COS-7 cells were exposed to various intensities of ultrasound in the presence of Bubble liposomes for 10 s. Gene transfection efficiency increased with increasing ultrasound intensity and reached a plateau at 0.7 W/cm^2. No cytotoxicity was evident even at 2.5 W/cm^2 (data not shown). The length of ultrasound exposure required to achieve gene expression was examined by measuring gene expression after 0, 1, 5 and 10 s of exposure (Fig. 3(b)). Surprisingly, gene expression was observed after 1 s of ultrasound exposure in the presence of Bubble liposomes. Transfection efficiency depended on ultrasound exposure time and reached a plateau after 5 s exposure. Efficiency was found to depend on both ultrasound intensity and exposure time (Fig. 3), indicating that Bubble liposomes can rapidly induce gene delivery while requiring only weak ultrasound, and without inducing cytotoxicity. Five seconds or 0.7 W/cm^2 of ultrasound exposure resulted in maximal gene expression, presumably due to bubble cavitation.

The transfection efficiency of some cationic non-viral vectors is significantly decreased in the presence of serum

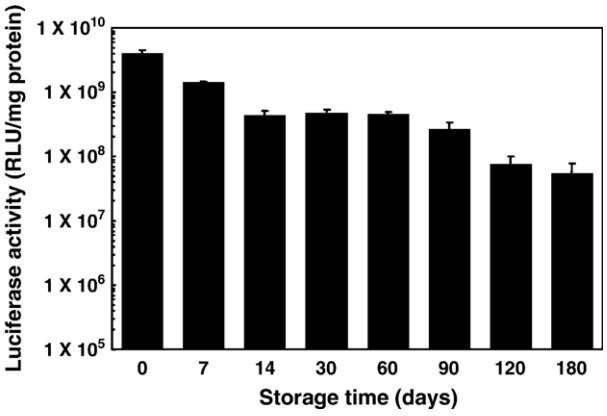

Fig. 2. Stability of Bubble liposomes. After preparation of Bubble liposomes, the vial containing Bubble liposomes was put in the refrigerator for each period. After storage, the transfection efficiency was measured with each samples. COS-7 cells (1×10^5 cells/500 μL) were mixed with pCMV-Luc (5 μg) and Bubble liposomes (60 μg). The cell mixture was exposed to ultrasound (frequency: 2 MHz, duty: 50%, burst rate: 2 Hz, intensity: 2.5 W/cm^2, time: 10 s.). The cells were washed and cultured for 2 days, then luciferase activity was determined as described in Materials and methods. Each bar represents the mean±S.D. for triplicate.

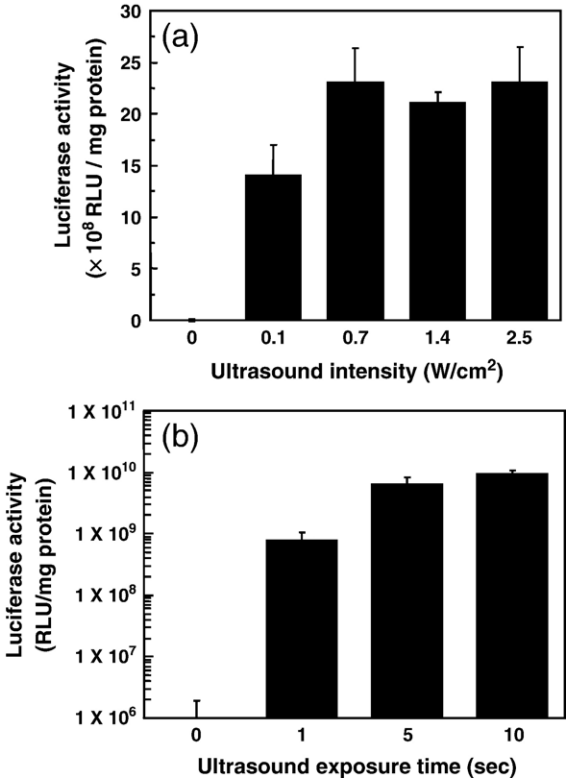

Fig. 3. Effect of ultrasound conditions on transfection efficiency with Bubble liposomes. COS-7 cells (1×10^5 cells/500 μL) were mixed with pCMV-Luc (5 μg) and Bubble liposomes (60 μg). The cell mixture was exposed to ultrasound (a): (frequency: 2 MHz, duty: 50%, burst rate: 2 Hz, intensity: 0–2.5 W/cm^2, time: 10 s.) or (b): (frequency: 2 MHz, duty: 50%, burst rate: 2 Hz, intensity: 2.5 W/cm^2, time: 0–10 s.). The cells were washed and cultured for 2 days, then luciferase activity was determined as described in Materials and methods. Each bar represents the mean±S.D. for triplicate.

due to an interaction between serum proteins and the cationic vectors [28]. Whereas, transfection efficiency with the combination of Bubble liposomes and ultrasound did not decrease even in the presence of 50% serum in *in vitro* study [27]. In the next examination, we examined whether Bubble liposomes could deliver plasmid DNA into S-180 ascites tumor cells in living animals after local injection (Fig. 4). In this examination, we compared the transfection efficiency with Bubble liposomes or cationic liposomes such as Lipofectin and Lipofectamine 2000. Luciferase expression was low in the mice treated with lipofectin-plasmid DNA complexes prepared by the traditional lipofection method, presumably because the complexes were associated with various proteins in the peritoneal cavity. On the other hand, luciferase expression increased in the mice treated with Lipofectamine 2000-plasmid DNA complexes compare with Lipofectin, because it was known that LF2000 was better than Lipofectin for gene delivery in the presence of serum. In addition, luciferase expression in mice treated with plasmid DNA, Bubble liposomes and ultrasound exposure was higher than that in the mice treated with Lipofectamine 2000-plasmid DNA complexes. This result supported the previous our report. In short, it was thought that Bubble liposomes and ultrasound was not affected by proteins existing in the peritoneal cavity and this method immediately and directly delivered plasmid DNA into cells with the mechanism which was not endocytosis pathway in lipofection method. We also confirmed that ultrasound combined with Bubble liposomes was effective at delivering genes to other tissues in the peritoneal cavity such as stomach, kidney, liver, spleen, intestine, diaphragm, pancreas, peritoneum and mesentery. Luciferase activity in these tissues was much lower than that observed in the S-180 cells (less than 130 RLU/mg protein).

Mizuguchi et al. reported about the effective cancer gene therapy by cytokine provision in the local area via gene delivery into arteries leading to tumor or arteries in tumor tissue [35]. Previously, we succeeded the gene delivery into artery of ultrasound exposure site with Bubble liposomes [27]. Therefore, we thought that our technology could be applied to establish the tumor tissue specific gene delivery. In this time, we attempted to deliver plasmid DNA to solid tumor via the injection into the artery that lead to tumor (Fig. 5). In Fig. 4, Lipofectin did not work well as gene delivery tool. In this study, we only used Lipofectamine 2000 as a control. In the mice treated with plasmid DNA and ultrasound, luciferase expression was same low level in the mice of plasmid DNA injection. And, luciferase expression was also low level in the mice treated with Lipofectamine 2000 and plasmid DNA complex, although the complex could be induced into S-180 ascites tumor cells. Generally, enough time is necessary for the complex to bind to cell surface and deliver plasmid DNA into cells. In this case, there was no time for the complex to retain in tumor tissue after injection because of blood stream and it would be resulted in

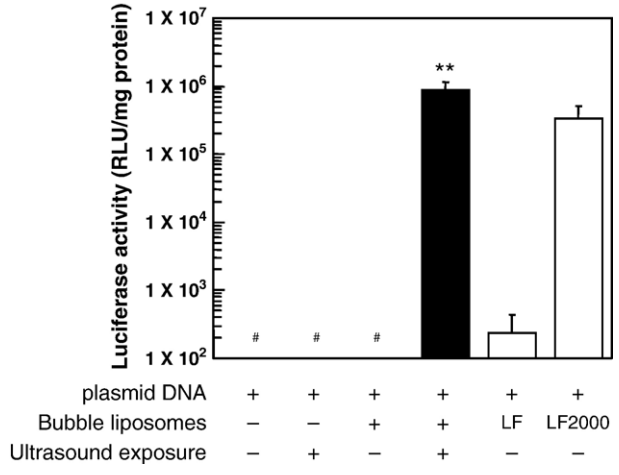

Fig. 4. *In vivo* gene delivery into mouse ascites tumor cells with Bubble liposomes. S-180 cells (1×10^6 cells) were i.p. injected into ddY mice. After 8 days, the mice were anaesthetized, then injected with 510 μL of pCMV-Luc (10 μg) and Bubble liposomes (500 μg) in PBS. Ultrasound (frequency: 1 MHz, duty: 50%; intensity: 1.0 W/cm^2, time: 1 min) was transdermally applied to the abdominal area. In another experiment, pCMV-Luc (10 μg) — Lipofectin (50 μg) or Lipofectamine 2000 (50 μg) complex was suspended in PBS (510 μL) and injected into the peritoneal cavity of mice. After 2 days, S-180 cells were recovered from the abdomens of the mice. Luciferase activity was determined as described in Materials and methods. Each bar represents the mean±S.D. for three to six mice/group. $^{**}P<0.01$ compared to the group treated with plasmid DNA, Bubble liposomes, ultrasound exposure or lipofection with Lipofectin or Lipofectamine 2000. LF, Lipofectin. LF2000, Lipofectamine 2000. #$<10^2$ RLU/mg protein.

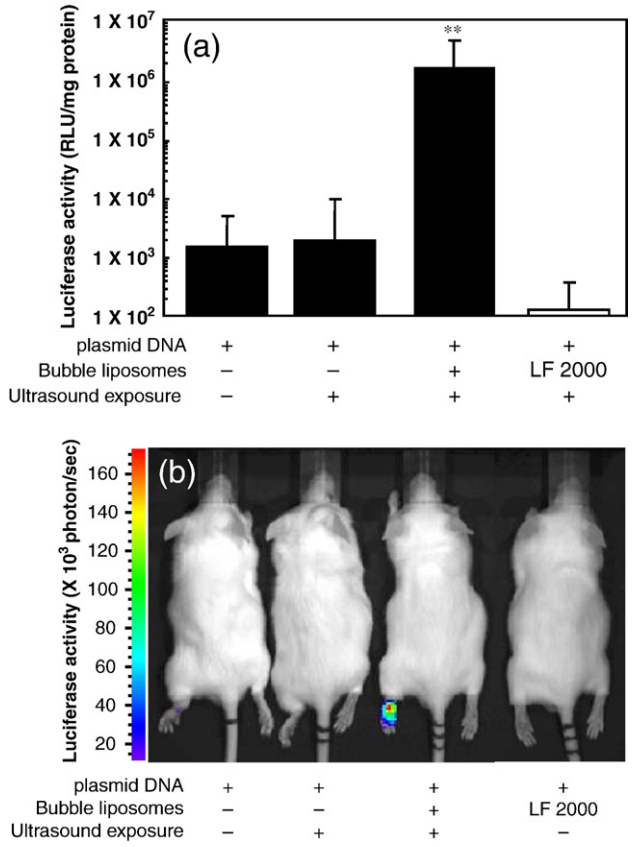

Fig. 5. *In vivo* gene delivery into mouse solid tumor with Bubble liposomes. S-180 cells (1×10^6 cells) were inoculated into left footpad of ddY mice. After 4 days, the mice were anaesthetized, then injected with 100 mL of pCMV-Luc (10 μg) in absent or present of Bubble liposomes (100 μg) in PBS. Ultrasound (frequency: 0.7 MHz, duty: 50%; intensity: 1.2 W/cm^2, time: 1 min) was transdermally exposed to tumor tissue. In another experiment, pCMV-Luc (10 μg) — Lipofectamine 2000 (25 μg) complex was suspended in PBS (100 μL) and injected into the left femoral artery. After 2 days, tumor tissue was recovered the mice. Luciferase activity was determined as described in Materials and methods. (a) Luciferase activity in solid tumor. Each bar represents the mean±S.D. for five mice/group. $^{**}P<0.01$ compared to the group treated with plasmid DNA, ultrasound exposure or Lipofectamine 2000. (b) *In vivo* luciferase imaging in the solid tumor bearing mice. The photon counts are indicated by the pseudo-color scales. LF 2000, Lipofectamine 2000.

low efficiency of transfection. On the other hand, luciferase expression in the combination of Bubble liposomes and ultrasound was much higher than that in other group (Fig. 5(a)). Koch et al. reported that the combination of ultrasound and microbubble (Levovist) enhanced lipoplex-mediated cell transfection efficiency *in vitro* and also severely damaged most cells. [36]. Therefore, we attempted to confirm the enhancement of transcfection efficiency with Lipofectamine 2000 by Bubble liposomes and ultrasound in the condition without cell damage. The transfection efficiency with lipoplex was not enhanced with Bubble liposomes and ultrasound *in vitro* and *in vivo* (data not shown). The size of Lipofectamine 2000-plasmid DNA complexes was larger than that of naked plasmid DNA by forming the spaghetti–meatball like structure. We guessed that it was difficult for the complexes to enter into cytosol via transient pore on the membrane with cavitation of Bubble liposomes in the condition without cell damage. In the Koch's report, ultrasound was exposed to *in vitro* cells for 60 s with Levovist (20 and 200 mg/mL). In this study, Bubble liposomes (1 mg/mL) were injected into the femoral artery. The concentration of Bubble liposomes would be much lower than that of Levovist because of the dilution of Bubble liposomes in the blood. In addition, the time of ultrasound exposure to Bubble liposomes was very short because of blood flow. Therefore, I thought that the transfection efficiency in the combination of cavitation with Bubble liposomes and lipoplexes was not enhanced. To evaluate gene expression site, we observed luciferase expression with luciferase *in vivo* imaging system (Fig. 5(b)). In the mice treated with Bubble liposomes and ultrasound, luciferase expression was observed in the tumor tissue because of inducing cavitation at ultrasound exposure site. Then, there were a possibility of hemolysis by the cavitation of Bubble liposomes in artery. We examined about hemolytic effect in the treatment of Bubble liposomes and ultrasound (Fig. 6). When the ultrasound was exposed to red blood cell with or without Bubble liposomes *in vitro*, serious hemolysis was not induced. These results suggested that this gene delivery system was important method to achieve tumor specific gene delivery without serious damage.

Plasmid DNA was effectively delivered into S-180 ascites tumor cells and solid tumor tissues with Bubble liposomes and ultrasound, although plasmid DNA did not form a complex with Bubble liposomes because Bubble liposomes were made of neutral charge lipids and modified polyethylene glycol on the surface, and existed free *in vivo*. These results could be explained from Fig. 3. In short, it is thought that Bubble liposomes can immediately and effectively deliver plasmid DNA into cells *in vivo* before the plasmid DNA is degraded by DNase. A mixture of plasmid DNA and Bubble liposomes was injected into mice, and the plasmid DNA was delivered to a specific area of the abdomen or solid tumor tissue by local exposure to ultrasound, suggesting that gene targeting can be induced at a site by exposure to ultrasound. In future studies, we intend to establish minimally-invasive and tissue-specific gene delivery with Bubble liposomes after systemic injection.

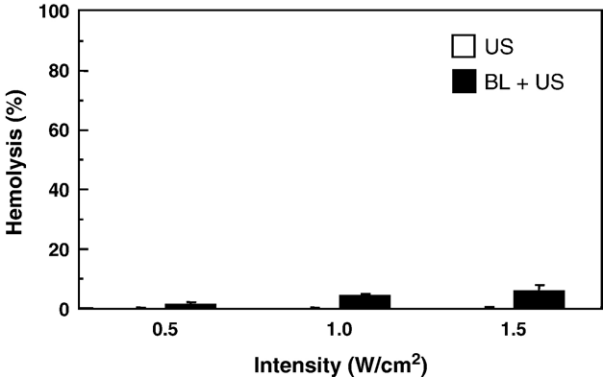

Fig. 6. Hemolysis of red blood cells by Bubble liposomes and ultrasound. Mouse red blood cells (2.5×10^8 cells/ 500 mL) were exposed with ultrasound (frequency: 0.7 MHz, Duty: 50%, Intensity: 0.5–1.5 W/cm^2, Time: 10 s.) in absent or present of Bubble liposomes. Hemolysis was assessed as described in Materials and methods. Each bar represents the mean±S.D. for triplicate.

The present study showed that Bubble liposomes can be a more effective gene delivery tools into tumor *in vivo* than conventional lipofection. Moreover, Bubble liposomes are an attractive gene delivery approach in cancer gene therapy as the method is minimally-invasive and tumor specific gene transfer, requiring only exposure to ultrasound applied to the surface of the body.

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