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Gene delivery by combination of novel liposomal bubbles with perfluoropropane and ultrasound

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Abstract

Microbubbles and ultrasound have recently been investigated with a view to improving the transfection efficiency of non-viral gene delivery systems. However, microbubbles are unstable and their targeting ability is insufficient for clinical use. To circumvent these problems, we developed novel polyethyleneglycol (PEG) modified liposomes (Bubble liposomes) containing perfluoropropane, which is an ultrasound imaging gas. Here, we used ultrasound to induce cavitation in Bubble liposomes and then investigated their ability to deliver genes in vitro and in vivo. Bubble liposomes could deliver plasmid DNA to many cell types without cytotoxicity. Additionally, in vivo gene delivery, Bubble liposomes were more effective delivery into femoral artery than lipofection method. Thus, Bubble liposomes might be efficient and novel non-viral tools for gene delivery.

Keywords: Liposomes; Gene delivery; Ultrasound; Cavitation; Non-viral vector

1. Introduction

Gene therapy has a potential in the treatment of cancer and diseases that are due to genomic causes. In addition, at present gene therapy is applied into cardiovascular diseases. Especially, in arteriosclerosis obliterans (ASO), vascular endothelial growth factor and hepatocyte growth factor (HGF) gene therapies have been reported to have beneficial effects. Viral vectors are efficient carriers of genes for transduction, but some problems have become evident [1–3]. Delivery vectors that are highly potent in terms of gene transduction efficiency should also be safe and easy to apply. Non-viral vectors have recently received focus as gene carriers [4], but their transduction efficiency is very low. Efforts have recently been directed towards improving this aspect [5–9].

Microbubbles, which are contrast agents for medical ultrasound imaging, improve transfection efficiency after ultrasound-induced cavitation [10–15]. However, microbubbles are generally unstable and their mean diameter of around 1–6 μm is too large for intravascular applications [16]. Moreover, functional particles such as targeting molecules are difficult to modify on the surface of microbubbles. Therefore, microbubbles should generally be smaller than red blood cells, stable after injection into the blood and ultimately, their surface should be easily modified with functional molecules for targeting.

Liposomes have some advantages as drug, antigen and gene delivery carriers [6,7,17–25]. Their size can be easily controlled and they can be modified to add a targeting function [20–24]. Therefore, we considered that Bubble liposomes could be novel gene delivery agents. Based on liposome technology, we developed novel Bubble liposomes containing the ultrasound

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imaging gas, perfluoropropane [26,27]. Here, we assessed the feasibility of Bubble liposomes for gene delivery after cavitation induced by ultrasound.

2. Materials and methods

2.1. Cells

The African green monkey kidney fibroblast cell line, COS-7, was 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). Meth-A fibrosarcoma cells and Jurkat cells, a human T cell line, were cultured with RPMI-1640 (Sigma Chemical Co., St. Louis, MO) supplemented with 10% heat-inactivated FBS. Colon 26 cells derived from a mouse colon adenocarcinoma, were cultured with RPMI-1640 (Sigma Chemical Co., St. Louis, MO) supplemented with 10% heat-inactivated FBS and 2.5% HEPES. B16BL6 cells were cultured with Eagle's medium (MEM) supplemented with 10% heat-inactivated FBS. Human umbilical vein endothelial cells (HUVEC, Kurabo Industries, Osaka, Japan) were cultured in a DMEM and medium 199 mixture with 15% heat-inactivated FBS, heparin (3.25 U/mL) and endothelial cell growth supplement (ECGS, Sigma Chemical Co., St. Louis, MO). All culture media contained 100 U/ml penicillin (Wako Pure Chemical Industries, Ltd., Osaka, Japan) and 100 μg/ml streptomycin (Wako Pure Chemical Industries, Ltd., Osaka, Japan).

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 (2k)-OMe) (NOF Corporation, Tokyo, Japan) (94:6 (m/m)) were prepared by reverse phase evaporation. In brief, all reagents were dissolved in 9:1 (v/v) chloroform/methanol. Physiological saline was added into the lipid solution. After that, 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 extruding equipment (Northern Lipids Inc., Vancouver, BC) and sizing filter (pore size: 200 nm, 100 nm) (Nuclepore Track-Etch Membrane, Whatman plc, UK). After sizing, liposomes were passed through a 0.45 μm pore size filter (MILLEX HV filter unit, Durapore PVDF membrane, Millipore Corporation, MA) to sterilize. Lipid concentration was measured with Phospholipid C test Wako (Wako Pure Chemical Industries, Ltd., Osaka, Japan). Bubble liposomes were prepared from liposomes and perfluoropropane gas (Takachiho Chemical Ind. Co. Ltd., Tokyo, Japan). In brief, 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 vial was placed in a bath-type sonicator (42 kHz, 100 W) (BRANSONIC 2510J-DTH, Branson Ultrasonics Co., Danbury, CT) for 5 min to form Bubble liposomes.

2.3. Ultrasound imaging in vitro

Bubble liposomes and PEG-liposomes (1 mg/mL, 200 μL) were placed into latex tubes filled with degassed PBS (10 mL) in a water bath. The probe (9 MHz) of an ultrasound imaging machine (UF-750XT, Fukuda Denshi Co Ltd., Tokyo, Japan) was positioned under the bath and Bubble liposomes and PEG-liposomes were imaged.

2.4. Cytotoxicity of Bubble liposomes and ultrasound to COS-7 cells

COS-7 cells (1×10^5 cells) and Bubble liposomes (60 μg) mixed with 500 μL of culture medium in 2 mL polypropylene tubes (BMBio, Tokyo, Japan) were exposed to ultrasound (frequency, 2 MHz; duty, 50%; burst rate, 2 Hz) for 10 s using a Sonopore 3000 (NEPA GENE, CO., LTD., Chiba, Japan). The cells were washed with culture medium and resuspended in 1 mL of the same medium. Cell suspensions (100 μL) were seeded in 96-well plates and incubated for 24 h. Cell viability was assayed using MTT [3-(4,5-s-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] (Dojindo, Kumamoto, Japan) as described by Mosmann with minor modifications [28]. Briefly, MTT (5 mg/mL, 10 μL) was added to each well and the cells were incubated at 37 °C for 4 h. The formazan product was dissolved in 100 μL of 10% sodium dodecyl sulfate (SDS) (Wako Pure Chemical Ind. Co., Ltd. Osaka, Japan) containing 15 mM HCl. Color intensity was measured using a microplate reader (POWERSCAN HT; Dainippon Pharmaceutical, Osaka, Japan) at test and reference wavelengths of 595 and 655 nm, respectively.

2.5. Damage to plasmid DNA caused by Bubble liposomes and ultrasound

Plasmid DNA (pCMV-Luc; 1 μg) dissolved in 500 μL of Opti-MEM (Invitrogen Corporation, Carlsbad, CA) was exposed to ultrasound with or without Bubble liposomes (60 μg) under the following conditions: frequency, 2 MHz; duty, 50%; intensity, 0, 0.1, 2.5, 4.5 and 6.0 W/cm^2, time, 0, 10, 30 s. As control, we used naked plasmid DNA with and without Bubble liposomes. In this group, plasmid DNA and Bubble liposomes were contacted for 30 s. Bubble liposomes were then removed using phenol/chloroform and plasmid DNA recovered by ethanol precipitation was dissolved in TE buffer and resolved by electrophoresis in 0.7% agarose gels.

2.6. Transfection of plasmid DNA into cells using Bubble liposomes

Plasmid DNA (pCMV-Luc or pEGFP-C1 (Clontech Laboratories, Inc., Mountain View, CA)), cells and Bubble liposomes were suspended in culture medium with 10% FBS in 2 mL polypropylene tubes. Ultrasound was exposed under various conditions through a probe placed in the suspension. The cells were washed twice with PBS and then resuspended in fresh culture medium. And the cells were cultured in 48-wells plate or chamber slide glass (ASAHI TECHNOGLASS CO., Chiba, Japan).

2.7. In vivo gene delivery into the femoral artery of mice

Three hundred μL of Plasmid DNA (pCMV-Luc; 10 μg) with or without Bubble liposomes (250 μg) suspension was injected into femoral artery of ddY mice (6 weeks age, male) using 30-gauge needle (M-S SURGICAL MFG. CO., LTD., Tokyo, Japan). In the same time, ultrasound (frequency, 1 MHz, duty, 50%; intensity, 1.0 W/cm^2, time, 2 min) was transdermally exposed to downstream of injection site. In other samples, plasmid DNA (pCMV-Luc, 10 μg) and Lipofectamine 2000 (50 μg) (Invitrogen Corporation, Carlsbad, CA) were mixed and complexed according to manual of Lipofectamine 2000. The complex was suspended in PBS (300 μL) and injected into femoral artery of mice. After 2 days of injection, the mice were sacrificed and the femoral artery of ultrasound exposure area was collected. Then, the artery was homogenated in the lysis buffer (0.1 M Tris-HCl (pH 7.8), 0.1% Triton X-100, 2 mM EDTA).

2.8. Measurement of luciferase and EGFP expression

Luciferase activity was measured using a luciferase assay system (Promega, Madison, WI) and a luminometer (TD-20/20, Turner Designs, Sunnyvale, CA, USA), is indicated as relative light units (RLU) per mg protein. In the Luciferase in vivo imaging, D-luciferin (150 mg/kg) was intraperitoneally injected into mice. After 10 min of injection, luciferase expression was imaged with luciferase in vivo imaging system (IVIS system 100, Xenogen Corporation, Alameda, CA). EGFP expression

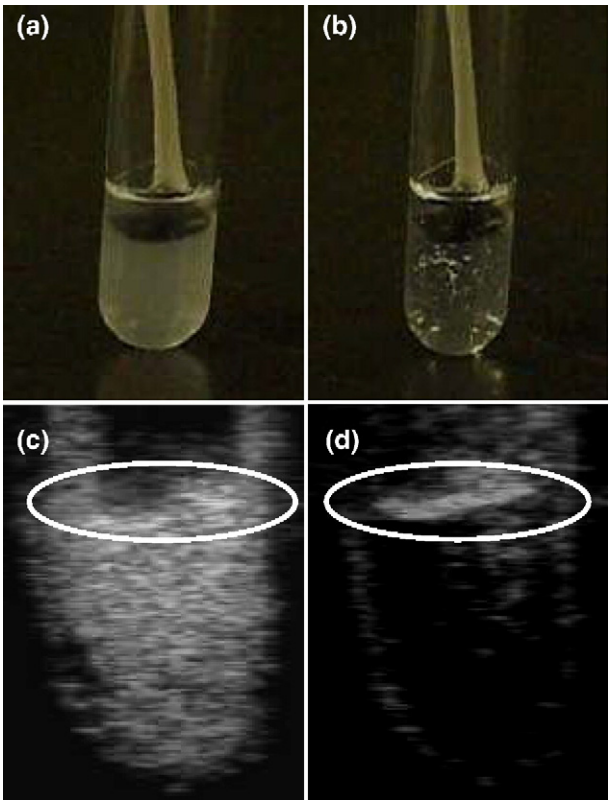

Fig. 2. Cavitation of Bubble liposomes exposed to ultrasound. Naked (a, b) and ultrasonographic (c, d) images of Bubble liposomes. Ultrasonic probe (circle) positioned in Bubble liposome suspension exposed 2.5 W/cm^2 of ultrasound for 10 s. Images were observed before (a, c) and after ultrasound (b, d).

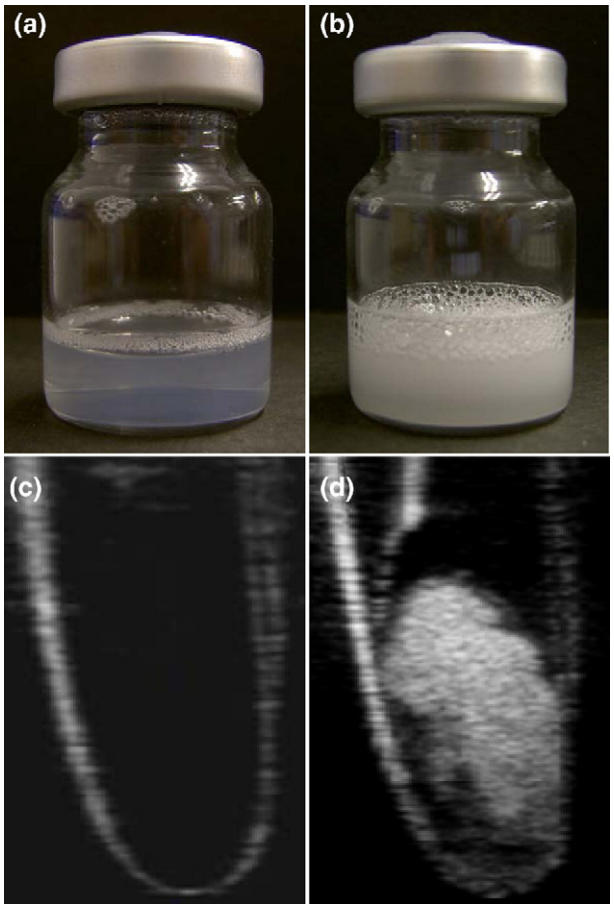

Fig. 1. Aspect and ultrasonography of PEG-liposomes and Bubble liposomes. Aspects of PEG-liposomes (a) and Bubble liposomes (b). PEG-liposomes sonicated with perfluoropropane gas became to Bubble liposomes in the vial. Ultrasonographic images of PEG- (c) and Bubble (d) liposomes.

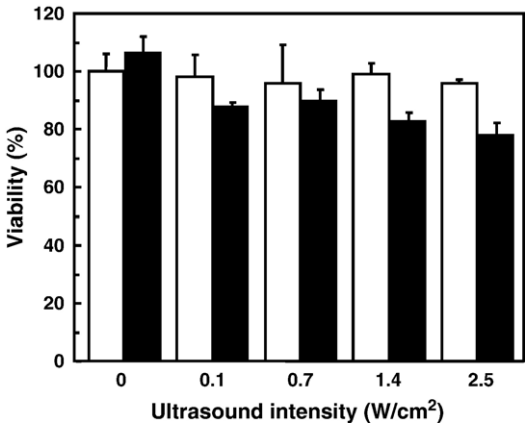

Fig. 3. Viability of COS-7 cells exposed with US and Bubble liposomes. COS-7 cells were exposed to ultrasound under various intensities with (■) or without (□) Bubble liposomes and then cultured for 24 h. Cell viability was assessed by MTT assays. Data are shown as means ± S.D. (n = 3).

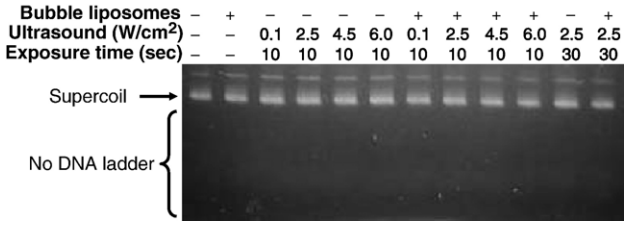

Fig. 4. Plasmid pCMV-Luc exposed to ultrasound under various conditions with or without Bubble liposomes. After exposure to ultrasound, Bubble liposomes were removed using phenol/chloroform and then pCMV-Luc was conventionally precipitated with ethanol. The precipitate was dissolved in TE buffer and resolved by electrophoresis in 0.7% agarose gels.

was observed with fluorescence microscopy (Leica MICROSYSTEMS, Wetzlar, Germany).

3. Results

3.1. Features of Bubble liposomes

Liposomes placed in vials that were supercharged with perfluoropropane gas were sonicated in a bath sonicator. The suspension of Bubble liposomes became cloudier than the original liposome suspension (Fig. 1). On the other hand, when vials were supercharged without perfluoropropane gas or with perfluoropropane gas at atmospheric pressure and then sonicated, the appearance of the liposomes did not change (data not shown). Therefore, sonicating the liposomes under high pressure with perfluoropropane gas was critical. Ultrasound imaging confirmed that the perfluoropropane gas was in fact trapped within the Bubble liposomes. Echo signals were apparently enhanced in Bubble liposomes compared with conventional PEG-liposomes. In addition, ultrasound (2 MHz, 2.5 W/cm^2) disrupted Bubble liposomes by inducing cavitations and then echo signals of these liposomes decreased (Fig. 2).

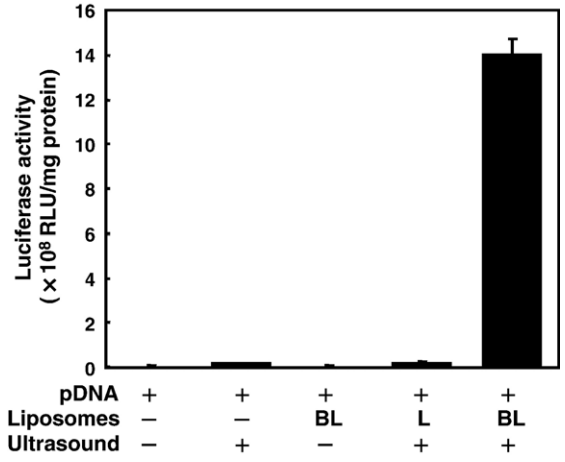

Fig. 5. Luciferase expression in COS-7 cells transfected by ultrasound with Bubble liposomes. COS-7 cells (1×10^5 cells/500 μL) mixed with pCMV-Luc (5 μg) and Bubble liposomes (60 μg) were exposed to ultrasound (frequency, 2 MHz; Duty, 50%; burst rate, 2 Hz; intensity, 2.5 W/cm^2; time 10 s). The cells were washed and cultured for 2 days and then luciferase activity was determined as described in Materials and methods. Data are shown as means ± S.D. (n=3). BL, Bubble liposomes; L, PEG-liposomes.

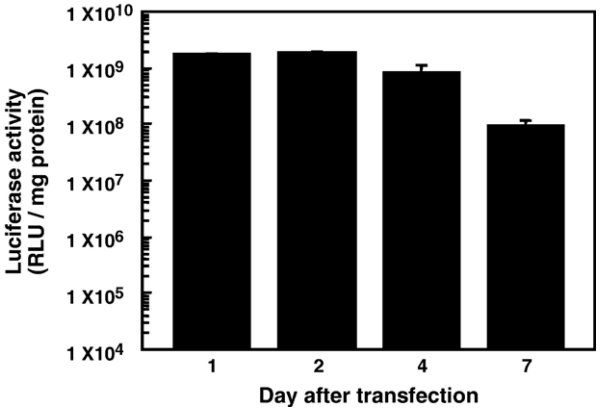

Fig. 6. Duration of luciferase expression in COS-7 cells transfected using ultrasound with Bubble liposomes. COS-7 cells (1×10^5 cells/500 μL) mixed with pCMV-Luc (5 μg) and Bubble liposomes (60 μg) were exposed to ultrasound under the following conditions: frequency, 2 MHz; duty, 50%; burst rate, 2 Hz; intensity, 2.5 W/cm^2; time 10 s). The cells were washed and cultured for 1, 2, 4, 7 days. After that, luciferase activity was determined as described in Materials and methods. Each data represents the mean ± S.D. (n=3).

Cavitations of the Bubble liposomes were also visually obvious since suspensions were clarified after exposure to ultrasound (Fig. 2).

3.2. Effects of cavitation induced in Bubble liposomes and ultrasound exposure on COS-7 cells and plasmid DNA

Heat and jet streams are generally induced with cavitation, which might damage cells and plasmid DNA. We therefore examined the effects of ultrasound on cells and plasmid DNA with or without Bubble liposomes. Ultrasound did not damage COS-7 cells in the absence of Bubble liposomes (Fig. 3) and only slightly affected the cells even when the amount of ultrasound was sufficient to induce cavitation of the Bubble liposomes. We also examined the effects of cavitation on plasmid DNA after ultrasound with and without Bubble liposomes using agarose gel electrophoresis (Fig. 4). The results showed that

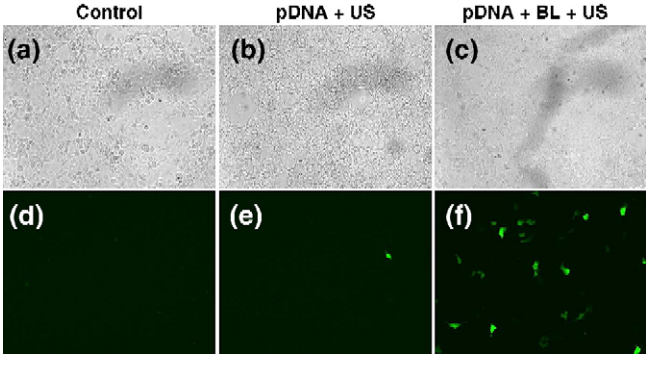

Fig. 7. EGFP expression in COS-7 cells transfected with Bubble liposomes and ultrasound exposure. COS-7 cells (1×10^5 cells/500 μL/tube) were mixed with pEGFP-C1 (5 μg) and Bubble liposomes (60 μg). The cell mixture was exposed to ultrasound (frequency, 2 MHz; duty, 50%; burst rate, 2 Hz; intensity, 2.5 W/cm^2; time 10 s). The cells were washed and cultured for 2 days. Thereafter, EGFP expression was examined by fluorescence microscopy original mgnification X100. Phase contrast, (a-c); Fluorecence (d-f). BL, Bubble liposomes; US, Ultrasound.

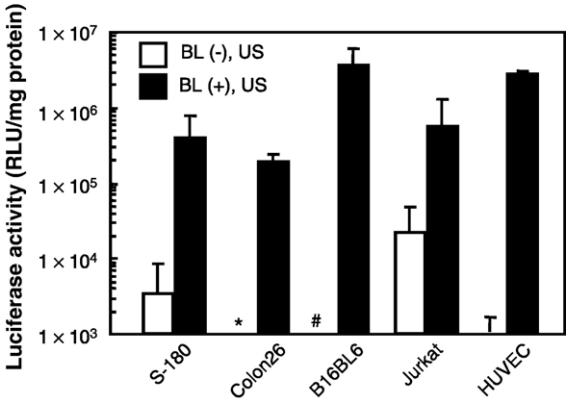

Fig. 8. Luciferase expression in various types of cells transfected using Bubble liposomes and ultrasound. Cells (1×10^5 cells/500 μL) mixed with pCMV-Luc (5 μg) and Bubble liposomes (60 μg) were exposed or not to ultrasound (frequency, 2 MHz; duty, 50%; burst rate, 2 Hz; intensity, 2.5 W/cm^2; time 10 s). The cells were washed and cultured for 2 days. Thereafter, luciferase activity was determined as described in Materials and methods. Data are shown as means±S.D. (n=3). BL, Bubble liposomes; US, Ultrasound. *<10^3 RLU/mg protein, #<10^0 RLU/mg protein.

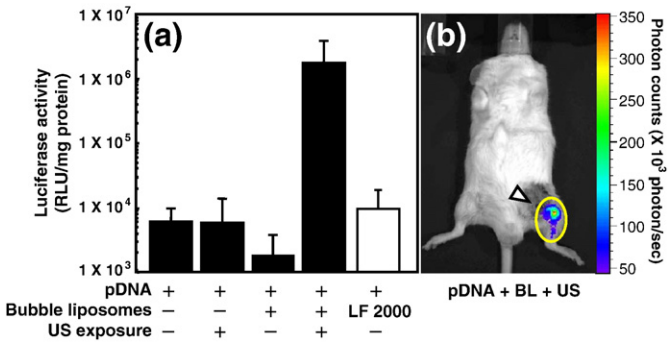

Fig. 10. Gene delivery to femoral artery with Bubble liposomes. Each sample containing plasmid DNA 10 μg was injected into femoral artery. In the same time, ultrasound (frequency, 1 MHz; duty, 50%; burst rate, 2 Hz; intensity, 1 W/cm^2; time 2 min) was exposed to the downstream area of injection site. (a) Luciferase expression in femoral artery of the ultrasound exposure area at 2 days after transfection, Luciferase expression was determined as described in Materials and methods. Data are shown as means±S.D. (n=5). (LF2000: Lipofectamine 2000) (b) In vivo luciferase imaging at 2 days after transfection in the mouse treated with plasmid DNA, Bubble liposomes and ultrasound exposure. The photon counts are indicated by the pseudo-color scales. Arrow head shows injection site and circle shows ultrasound exposure area BL, Bubble liposomes; US, Ultrasound.

10–30 s of ultrasound did not degrade plasmid DNA regardless of the presence or absence of Bubble liposomes.

3.3. Gene transduction with Bubble liposomes and ultrasound

We examined the transduction of naked plasmid DNA into COS-7 cells by Bubble liposomes and/or ultrasound (Fig. 5). Levels of luciferase expression were much higher after ultrasound in the presence, than in the absence of Bubble liposomes. We then examined the profile of gene expression with transfection using Bubble liposomes and ultrasound (Fig. 6). Gene expression peaked 2 days after transfection and then gradually decreased, but remained detectable at 7 days after transfection.

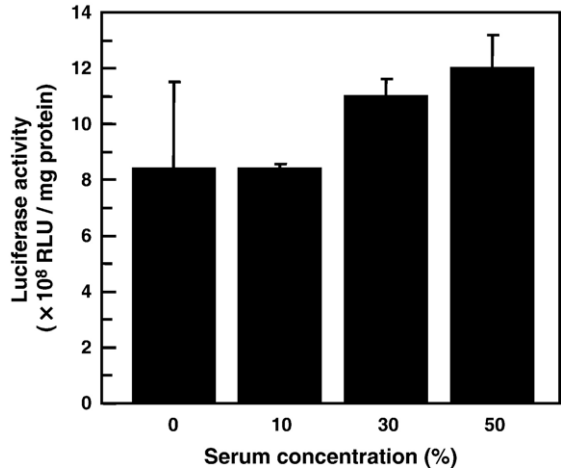

Fig. 9. Effect of serum on transfection efficiency of Bubble liposomes. COS-7 cells (1×10^5 cells/500 μL) mixed with pCMV-Luc (0.25 μg) and Bubble liposomes (60 μg) were exposed to ultrasound (frequency, 2 MHz; duty, 50%; burst rate, 2 Hz; intensity, 2.5 W/cm^2; time 10 s) in the absence or the presence of serum (0, 10, 30, 50%). The cells were washed and cultured for 2 days. Thereafter, luciferase activity was determined as described in Materials and methods. Data are shown as means±S.D. (n=3).

Fig. 7 shows the efficiency of transgene delivery using the EGFP gene expression system. The numbers of EGFP-positive cells significantly increased after ultrasound in the presence, compared with the absence of Bubble liposomes. We also examined the feasibility of gene transduction into S-180, Colon 26, B16BL6, Jurkat cells and human umbilical vein endothelial cells (HUVEC). Fig. 8 shows that Bubble liposomes with ultrasound more effectively transduced luciferase gene into all of these types of cells than ultrasound alone.

Considering in vivo gene delivery with Bubble liposomes, it is necessary to deliver plasmid DNA into cells in presence of serum. Then, we examined about the effect of serum on gene delivery with Bubble liposomes (Fig. 9). Gene expression with Bubble liposomes was not affected even in the presence of serum.

3.4. In vivo gene delivery with Bubble liposomes

To evaluate the ability of Bubble liposomes to in vivo gene delivery, we attempted to deliver plasmid DNA with Bubble liposomes into femoral artery. In this study, we also examined the gene delivery with conventional lipofection method (Fig. 10(a)). The gene expression with ultrasound or Bubble liposomes was low level. In addition, the gene expression was very low even in using Lipofectamine 2000. However, in the combination of Bubble liposomes and ultrasound exposure, gene expression was higher than other groups. And the gene expression was observed at only area of ultrasound exposure (Fig. 10 (b)).

4. Discussion

Plasmid DNA shows promise as a safe and clinically acceptable route for delivering gene therapy, but it must be effective and site-specific. Microbubbles and ultrasound have recently been proposed for gene delivery, since microbubble-

enhanced ultrasound can alter cell membrane permeability for a short time due to sonoporation, which allows extracellular macromolecules such as plasmid DNA to instantaneously enter cells without cytotoxicity [11–15,29,30]. Cavitation energy created by the collapse of the bubble has been considered as a key mechanism in intracellular delivery. This technique has been applied for site-specific intracellular delivery of macromolecules both *in vitro* and *in vivo* [11,16].

Microbubble reagents such as Optison, which are generally used in ultrasound imaging, could be used as gene delivery carriers together with ultrasound [11,16]. Although the mean diameter of Optison particles is about 2.0–4.5 μm, they contain bubbles of up to 32 μm in diameter, so Optison is too large to reach peripheral tissues. Tsunoda et al. reported that some mice died immediately after the administration of Optison i.v. even without sonication due to lethal embolisms in vital organs [31]. The same problem has not been reported in human, but there is a possibility that Optison cannot pass through capillary vessels. Optison were developed as ultrasound imaging agent. Thus, they had not been optimized for ultrasound imaging of peripheral tissues and gene delivery tool. Moreover, adding molecules with useful functions such as targeting is difficult because Optison is composed of albumin. We resolved these issues by developing Bubble liposomes that are derived by a novel method from liposomes. Suspensions of Bubble liposomes were cloudier than PEG-liposomes due to entrapment of perfluoropropane gas. Although the mean diameter of Bubble liposomes was about 1 μm according to dynamic light scattering, the suspension also contained many bubbles in the submicron range. Contrast microscopy showed that most of the Bubble liposomes were less than 3 μm in diameter. Moreover, 500 μg of Bubble liposomes (in terms of lipid amount) injected into the tail veins of mice, did not cause any deaths (data not shown), indicating that these novel liposomes would be safe for use *in vivo*. We are presently investigating the structure of Bubble liposomes by transmission electron microscopy.

Here, we investigated the feasibility of novel Bubble liposomes for gene delivery after ultrasound exposure. We initially examined whether perfluoropropane gas was associated with the Bubble liposomes. Ultrasound imaging revealed that echo signals are enhanced with Bubble liposomes compared with PEG-liposomes. In addition, Bubble liposomes collapsed after exposure to ultrasound, suggesting that ultrasound-induced cavitation. We then attempted to transduce plasmid DNA into cells using this feature of Bubble liposomes. Luciferase activity was very high after Bubble liposomes were exposed to ultrasound and cytotoxicity was absent. We examined the efficiency of transgene expression during transfection with plasmid DNA encoding EGFP. More cells were EGFP-positive in the presence, than in the absence of Bubble liposomes. In addition, the Bubble liposomes could transduce plasmid DNA into various tumor cells, T cell lines and endothelial cells. In general, transducing plasmid DNA into lymphocytes with non-viral vectors is difficult. Therefore, the transduction of plasmid DNA using Bubble liposomes into Jurkat cells, which are derived from T cell lines, is remarkable. In this gene delivery system, it is thought that gene expression is transient. To maintain gene expression for long time, it is necessary to repeat injection. Fortunately, Bubble liposomes were made of PEG-liposomes which were very low immunogenic. Therefore, it is thought that we could repeat injection of Bubble liposomes without reducing the ability of gene delivery in vivo.

In vivo gene delivery with Bubble liposomes and ultrasound, Bubble liposomes could effectively transduce plasmid DNA into the femoral artery. And this transfection efficiency of Bubble liposomes was higher than that of conventional lipofection methods using Lipofectamine 2000. This result suggested that Bubble liposomes could quickly transduce plasmid DNA into the artery by cavitation even under the condition of short contact time between Bubble liposomes and the endothelial cells and the existence of blood stream and serum. It was thought that plasmid DNA was transduced into endothelial cells in femoral artery because it was physiologically difficult for plasmid DNA and Bubble liposomes to extravasate from normal artery. In this study, mixture of plasmid DNA and Bubble liposomes was injected and we succeeded to deliver plasmid DNA in specific area by local exposure of ultrasound. Thus, gene expression depended on the site of ultrasound exposure. It was suggested that this system could induce gene targeting to the site where was exposed with ultrasound. In the future, we would like to establish non-invasive and tissue specific gene delivery with Bubble liposomes after systemic injection.

In this study, all data were obtained by using mixture of Bubble liposomes and plasmid DNA. Although gene expression was observed with mixture of plasmid DNA and Bubble liposomes and ultrasound exposure in femoral artery injection, it is important to control the biodistribution of both Bubble liposomes and plasmid DNA in systemic injection. In short, it is necessary for this gene delivery system to deliver both plasmid DNA and Bubble liposome to the same space. In addition, plasmid DNA is easily degraded with DNase. Therefore, to improve these problems, we are attempting to prepare the plasmid DNA entrapping type or complex type of Bubble liposomes.

We prepared Bubble liposomes that contained submicron-sized bubbles using a novel method. These novel liposomes induced cavitation upon exposure to ultrasound, which resulted in plasmid DNA transduction into cells in vitro and in vivo. These results suggested that our Bubble liposomes will be useful tools for gene delivery as well as being a universal ultrasound imaging agent.

5. Conclusion

This is the first report about the use of liposomal bubbles for gene delivery. In this study, we showed that combination of bubble liposomes and ultrasound exposure could be an effective and novel gene delivery method in vitro and in vivo. In the future, it is expected that bubble liposomes might be utilized as non-invasive gene delivery tools.

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References

[1] E. Check, Safety panel backs principle of gene-therapy trials, Nature 420 (2002) 595.
[2] E. Check, Second cancer case halts gene-therapy trials, Nature 421 (2003) 305.
[3] E. Marshall, Gene therapy death prompts review of adenovirus vector, Science 286 (1999) 2244–2245.
[4] C.C. Conwell, L. Huang, Recent progress in non-viral gene delivery, Non-viral Gene Therapy, Gene Design and Delivery, 2005, pp. 3–10.
[5] K. Wada, H. Arima, T. Tsutsumi, Y. Chihara, K. Hattori, F. Hirayama, K. Uekama, Improvement of gene delivery mediated by mannosylated dendrimer/alpha-cyclodextrin conjugates, J. Control. Release 104 (2005) 397–413.
[6] K. Kogure, R. Moriguchi, K. Sasaki, M. Ueno, S. Futaki, H. Harashima, Development of a non-viral multifunctional envelope-type nano device by a novel lipid film hydration method, J. Control. Release 98 (2004) 317–323.
[7] R. Moriguchi, K. Kogure, H. Akita, S. Futaki, M. Miyagishi, K. Taira, H. Harashima, A multifunctional envelope-type nano device for novel gene delivery of siRNA plasmids, Int. J. Pharm. 301 (2005) 277–285.
[8] H. Mizuguchi, T. Nakagawa, M. Nakanishi, S. Imazu, S. Nakagawa, T. Mayumi, Efficient gene transfer into mammalian cells using fusogenic liposome, Biochem. Biophys. Res. Commun. 218 (1996) 402–407.
[9] M. Fechheimer, J.F. Boylan, S. Parker, J.E. Sisken, G.L. Patel, S.G. Zimmer, Transfection of mammalian cells with plasmid DNA by scrape loading and sonication loading, Proc. Natl. Acad. Sci. U. S. A. 84 (1987) 8463–8467.
[10] W.J. Greenleaf, M.E. Bolander, G. Sarkar, M.B. Goldring, J.F. Greenleaf, Artificial cavitation nuclei significantly enhance acoustically induced cell transfection, Ultrasound Med. Biol. 24 (1998) 587–595.
[11] T. Li, K. Tachibana, M. Kuroki, M. Kuroki, Gene transfer with echo-enhanced contrast agents: comparison between Albunex, Optison, and Levovist in mice — initial results, Radiology 229 (2003) 423–428.
[12] Y. Taniyama, K. Tachibana, K. Hiraoka, M. Aoki, S. Yamamoto, K. Matsumoto, T. Nakamura, T. Ogihara, Y. Kaneda, R. Morishita, Development of safe and efficient novel nonviral gene transfer using ultrasound: enhancement of transfection efficiency of naked plasmid DNA in skeletal muscle, Gene Ther. 9 (2002) 372–380.
[13] Y. Taniyama, K. Tachibana, K. Hiraoka, T. Namba, K. Yamasaki, N. Hashiya, M. Aoki, T. Ogihara, K. Yasufumi, R. Morishita, Local delivery of plasmid DNA into rat carotid artery using ultrasound, Circulation 105 (2002) 1233–1239.
[14] S. Sonoda, K. Tachibana, E. Uchino, A. Okubo, M. Yamamoto, K. Sakoda, T. Hisatomi, K.H. Sonoda, Y. Negishi, Y. Izumi, S. Takao, T. Sakamoto, Gene transfer to corneal epithelium and keratocytes mediated by ultrasound with microbubbles, Investig. Ophthalmol. Vis. Sci. 47 (2006) 558–564.
[15] R.V. Shohet, S. Chen, Y.T. Zhou, Z. Wang, R.S. Meidell, R.H. Unger, P.A. Grayburn, Echocardiographic destruction of albumin microbubbles directs gene delivery to the myocardium, Circulation 101 (2000) 2554–2556.
[16] J.R. Lindner, Microbubbles in medical imaging: current applications and future directions, Nat. Rev. Drug. Discov. 3 (2004) 527–532.
[17] K. Kawamura, N. Kadowaki, R. Suzuki, S. Udagawa, S. Kasaoka, N. Utoguchi, T. Kitawaki, N. Sugimoto, N. Okada, K. Maruyama, T. Uchiyama, Dendritic cells that endocytosed antigen-containing IgG-liposomes elicit effective antitumor immunity, J. Immunother. 29 (2006) 165–174.
[18] H. Yanagie, K. Maruyama, T. Takizawa, O. Ishida, K. Ogura, T. Matsumoto, Y. Sakurai, T. Kobayashi, A. Shinohara, J. Rant, J. Skvarc, R. Ilic, G. Kuhne, M. Chiba, Y. Furuya, H. Sugiyama, T. Hisa, K. Ono, H. Kobayashi, M. Eriguchi, Application of boron-entrapped stealth liposomes to inhibition of growth of tumour cells in the in vivo boron neutron-capture therapy model, Biomed. Pharmacother. 60 (2006) 43–50.
[19] H. Yanagie, K. Ogura, K. Takagi, K. Maruyama, T. Matsumoto, Y. Sakurai, J. Skvarc, R. Illic, G. Kuhne, T. Hisa, I. Yoshizaki, K. Kono, Y. Furuya, H. Sugiyama, H. Kobayashi, K. Ono, K. Nakagawa, M. Eriguchi, Accumulation of boron compounds to tumor with polyethylene-glycol binding liposome by using neutron capture autoradiography, Appl. Radiat. Isotopes 61 (2004) 639–646.
[20] K. Maruyama, O. Ishida, S. Kasaoka, T. Takizawa, N. Utoguchi, A. Shinohara, M. Chiba, H. Kobayashi, M. Eriguchi, H. Yanagie, Intracellular targeting of sodium mercaptoundecahydrododecaborate (BSH) to solid tumors by transferrin-PEG liposomes, for boron neutron-capture therapy (BNCT), J. Control. Release 98 (2004) 195–207.
[21] M. Harata, Y. Soda, K. Tani, J. Ooi, T. Takizawa, M. Chen, Y. Bai, K. Izawa, S. Kobayashi, A. Tomonari, F. Nagamura, S. Takahashi, K. Uchimaru, T. Iseki, T. Tsuji, T.A. Takahashi, K. Sugita, S. Nakazawa, A. Tojo, K. Maruyama, S. Asano, CD19-targeting liposomes containing imatinib efficiently kill Philadelphia chromosome-positive acute lymphoblastic leukemia cells, Blood 104 (2004) 1442–1449.
[22] O. Ishida, K. Maruyama, H. Tanahashi, M. Iwatsuru, K. Sasaki, M. Eriguchi, H. Yanagie, Liposomes bearing polyethyleneglycol-coupled transferrin with intracellular targeting property to the solid tumors in vivo, Pharm. Res. 18 (2001) 1042–1048.
[23] K. Maruyama, E. Holmberg, S.J. Kennel, A. Klibanov, V.P. Torchilin, L. Huang, Characterization of in vivo immunoliposome targeting to pulmonary endothelium, J. Pharm. Sci. 79 (1990) 978–984.
[24] T. Mizoue, T. Horibe, K. Maruyama, T. Takizawa, M. Iwatsuru, K. Kono, H. Yanagie, F. Moriyasu, Targetability and intracellular delivery of anti-BCG antibody-modified, pH-sensitive fusogenic immunoliposomes to tumor cells, Int. J. Pharm. 237 (2002) 129–137.
[25] K. Maruyama, S.J. Kennel, L. Huang, Lipid composition is important for highly efficient target binding and retention of immunoliposomes, Proc. Natl. Acad. Sci. U. S. A. 87 (1990) 5744–5748.
[26] V. Mor-Avi, R.M. Lang, K.A. Robinson, C. Korcarz, A.F. Ng, P. Vignon, S. Akselrod, S.G. Shroff, Contrast echocardiographic quantification of regional myocardial perfusion: validation with an isolated rabbit heart model, J. Am. Soc. Echocardiogr. 9 (1996) 156–165.
[27] V. Mor-Avi, S.G. Shroff, K.A. Robinson, B.P. Cholley, A.F. Ng, R.M. Lang, Echocardiographic contrast agents and left ventricular contractility: evaluation using an isolated rabbit heart model, J. Am. Soc. Echocardiogr. 9 (1996) 452–461.
[28] T. Mosmann, Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays, J. Immunol. Methods 65 (1983) 55–63.
[29] M. Kinoshita, K. Hynynen, A novel method for the intracellular delivery of siRNA using microbubble-enhanced focused ultrasound, Biochem. Biophys. Res. Commun. 335 (2005) 393–399.
[30] M. Kinoshita, K. Hynynen, Intracellular delivery of Bak BH3 peptide by microbubble-enhanced ultrasound, Pharm. Res. 22 (2005) 716–720.
[31] S. Tsunoda, O. Mazda, Y. Oda, Y. Iida, S. Akabame, T. Kishida, M. Shin-Ya, H. Asada, S. Gojo, J. Imanishi, H. Matsubara, T. Yoshikawa, Sonoporation using microbubble BR14 promotes pDNA/siRNA transduction to murine heart, Biochem. Biophys. Res. Commun. 336 (2005) 118–127.