9. Taniyama Y, Tachibana K, Hiraoka K, Aoki M, Yamamoto S, Matsumoto K, *et al.* Development of safe and efficient novel nonviral gene transfer using ultrasound: enhancement of transfection efficiency of naked plasmid DNA in skeletal muscle. Gene Ther. 2002;9:372–80.
10. Taniyama Y, Tachibana K, Hiraoka K, Namba T, Yamasaki K, Hashiya N, *et al.* Local delivery of plasmid DNA into rat carotid artery using ultrasound. Circulation. 2002;105:1233–9.
11. Li T, Tachibana K, Kuroki M, Kuroki M. Gene transfer with echo-enhanced contrast agents:comparison between Albunex, Optison, and Levovist in mice–initial results. Radiology. 2003;229:423–8.
12. Unger EC, Porter T, Culp W, Labell R, Matsunaga T, Zutshi R. Therapeutic applications of lipid-coated microbubbles. Adv Drug Deliv Rev. 2004;56:1291–314.
13. Sonoda S, Tachibana K, Uchino E, Okubo A, Yamamoto M, Sakoda K, *et al.* Gene transfer to corneal epithelium and keratocytes mediated by ultrasound with microbubbles. Invest Ophthalmol Vis Sci. 2006;47:558–64.
14. Blume G, Cevc G. Liposomes for the sustained drug release in vivo. Biochim Biophys Acta. 1990;1029:91–7.
15. Allen TM, Hansen C, Martin F, Redemann C, Yau-Young A. Liposomes containing synthetic lipid derivatives of poly(ethylene glycol) show prolonged circulation half-lives in vivo. Biochim Biophys Acta. 1991;1066:29–36.
16. Maruyama K, Yuda T, Okamoto A, Kojima S, Suginaka A, Iwatsuru M. Prolonged circulation time in vivo of large unilamellar liposomes composed of distearoyl phosphatidylcholine and cholesterol containing amphipathic poly(ethylene glycol). Biochim Biophys Acta. 1992;1128:44–9.
17. Maruyama K, Ishida O, Kasaoka S, Takizawa T, Utoguchi N, Shinohara A, *et al.* Intracellular targeting of sodium mercaptoundecahydrododecaborate (BSH) to solid tumors by transferrin-PEG liposomes, for boron neutron-capture therapy (BNCT). J Control Release. 2004;98:195–207.
18. Negishi Y, Omata D, Iijima H, Hamano N, Endo Y, Suzuki R, Maruyama K, Nomizu M, and Aramaki Y, Preparation and Characterization of Laminin-derived Peptide AG73-coated Liposomes as a Selective Gene Delivery Tool. *Biol Pham. Bull. in press*
19. Suzuki R, Takizawa T, Negishi Y, Hagisawa K, Tanaka K, Sawamura K, *et al.* Gene delivery by combination of novel liposomal bubbles with perfluoropropane and ultrasound. J Control Release. 2007;117:130–6.
20. Suzuki R, Takizawa T, Negishi Y, Utoguchi N, Sawamura K, Tanaka K, *et al.* Tumor specific ultrasound enhanced gene transfer in vivo with novel liposomal bubbles. J Control Release. 2008;125:137–44.
21. Suzuki R, Takizawa T, Negishi Y, Utoguchi N, Maruyama K. Effective gene delivery with novel liposomal bubbles and ultrasonic destruction technology. Int J Pharm. 2008;354:49–55.
22. Negishi Y, Endo Y, Fukuyama T, Suzuki R, Takizawa T, Omata D, *et al.* Delivery of siRNA into the cytoplasm by liposomal bubbles and ultrasound. J Control Release. 2008;132:124–30.
23. Negishi Y, Omata D, Iijima H, Takabayashi Y, Suzuki K, Endo Y, *et al.* Enhanced laminin-derived peptide AG73-mediated liposomal gene transfer by bubble liposomes and ultrasound. Mol Pharm. 2010;7:217–26.
24. Suzuki R, Namai E, Oda Y, Nishiie N, Otake S, Koshima R, *et al.* Cancer gene therapy by IL-12 gene delivery using liposomal bubbles and tumoral ultrasound exposure. J Control Release. 2010;142:245–50.
25. Liu F, Huang L. A syringe electrode device for simultaneous injection of DNA and electrotransfer. Mol Ther. 2002;5:323–8.
26. Couffinhal T, Silver M, Zheng LP, Kearney M, Witzenbichler B, Isner JM. Mouse model of angiogenesis. Am J Pathol. 1998;152:1667–79.
27. Jang HS, Kim HJ, Kim JM, Lee YS, Kim KL, Kim JA, *et al.* A novel *ex Vivo* angiogenesis assay based on electroporation-mediated delivery of naked plasmid DNA to skeletal muscle. Mol Ther. 2004;9:464–74.
28. Koch S, Pohl P, Cobet U, Rainov NG. Ultrasound enhancement of liposome-mediated cell transfection is caused by cavitation effects. Ultrasound Med Biol. 2000;26:897–903.
29. Yang JP, Huang L. Overcoming the inhibitory effect of serum on lipofection by increasing the charge ratio of cationic liposome to DNA. Gene Ther. 1997;4:950–60.
30. Shah PB, Losordo DW. Non-viral vectors for gene therapy: clinical trials in cardiovascular disease. Adv Genet. 2005;54:339–61.
31. Emoto M, Tachibana K, Iwasaki H, Kawarabayashi T. Antitumor effect of TNP-470, an angiogenesis inhibitor, combined with ultrasound irradiation for human uterine sarcoma xenografts evaluated using contrast color Doppler ultrasound. Cancer Sci. 2007;98:929–35.
32. Barzelai S, Sharabani-Yosef O, Holbova R, Castel D, Walden R, Engelberg S, *et al.* Low-intensity ultrasound induces angiogenesis in rat hind-limb ischemia. Ultrasound Med Biol. 2006;32:139–45.

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Pharmaceutical nanotechnology

Efficient siRNA delivery using novel siRNA-loaded Bubble liposomes and ultrasound

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ABSTRACT

Recently, we developed novel polyethyleneglycol (PEG)-modified liposomes (Bubble liposomes; BLs) entrapping an ultrasound (US) imaging gas and reported that the combination of BLs and US was useful for the delivery of siRNA directly into the cytoplasm. However, the results were obtained using a mixture of BLs and naked siRNA. With systemic injections, it is important to control the biodistribution of both BLs and siRNA. In addition, the delivery of siRNA is affected by nuclease degradation after intravenous administration. In this study, we prepared novel siRNA-loaded BLs (si-BLs) using a cationic lipid, 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP). We demonstrated that siRNA could be loaded onto BLs containing DOTAP and that siRNA-loaded BLs were stable in serum. A specific gene-silencing effect was also achieved by transfection with si-BLs. Thus, the combination of si-BLs with US exposure can be used for delivery of siRNA to a specific tissue via systemic injection.

1. Introduction

RNA interference (RNAi) has potential application in the development of new therapies for malignant, infectious, and autoimmune diseases. Indeed, synthetic siRNAs are capable of knocking down targets *in vivo* (Frank-Kamenetsky et al., 2008; Halder et al., 2006; Kim et al., 2008; McCaffrey et al., 2002; Morrissey et al., 2005; Niu et al., 2006; Sato et al., 2008; Song et al., 2003; Takeshita et al., 2005; Xia et al., 2007). However, effective and nontoxic delivery is the major challenge to its implementation in a clinical setting.

One novel approach to the administration of a drug or gene is ultrasound (US)-enhanced delivery, which exploits cavitation bubbles produced by the pressure oscillations of US. US pressures above a certain threshold can cause oscillating bubbles to collapse violently, a process known as inertial cavitation. Inertial cavitation is believed to temporarily improve the permeability of cell membranes, enabling the transport of extracellular molecules into viable cells (Delius and Adams, 1999; Duvshani-Eshet and Machluf, 2005; Greenleaf et al., 1998; Holmes et al., 1992; Schratzberger et al., 2002). Furthermore, in combination with microbubbles, contrast agents for medical US imaging improve siRNA transfection efficiency (Du et al., 2011; Kinoshita and Hynynen, 2005; Otani et al., 2009; Tsunoda et al., 2005). However, microbubbles have problems with size, stability, and targeting functionality.

Polyethyleneglycol (PEG)-modified liposomes have excellent biocompatibility, stability, and a long circulation time and can be easily prepared in a variety of sizes and modified to add a targeting function. For these reasons, they are widely used as carriers of drugs, antigens, and genes (Allen et al., 1991; Blume and Cevc, 1990; Harata et al., 2004; Maruyama et al., 1992, 2004). Therefore, PEG-liposomes containing a US imaging gas could be used as novel gene delivery agents. We recently reported that "Bubble liposomes" (BLs) were suitable for gene delivery *in vitro* and *in vivo* (Negishi et al., 2011b,c; Suzuki et al., 2007, 2008a,b). Furthermore, we showed that the combination of BLs and US was also useful for the delivery of siRNA *in vitro* and *in vivo* and that siRNA was introduced directly into the cytoplasm (Negishi et al., 2008). However, the results were obtained using a mixture of BLs and naked siRNA. With systemic injections, transfection efficiency is reduced if the BLs and siRNA are not colocalized in blood vessels. Therefore, it is important to control the biodistribution of both BLs and siRNA. In addition, siRNA is degraded by nuclease and removed rapidly from the circulation after intravenous administration. To overcome these problems, the loading of siRNA onto BLs could be effective for siRNA delivery. Recently, it has been reported that PEGylated lipoplexes (PEG-siPlex) bound to microbubbles led to an increase in the local lipoplex concentration near the cell membrane and resulted in much higher transfection with siRNA in the presence of US (Lentacker et al., 2009; Vandenbroucke et al., 2008). It was also shown that the delivery of siRNA by siRNA-microbubble complexes

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was effective for transfection into arteries (Suzuki et al., 2010). However, microbubbles were used in these reports. Microbubbles have problems with size, stability, and targeting functionality as mentioned above. Therefore, we developed nanosized, siRNA-loaded BLs using cholesterol-conjugated siRNA (chol-si-BLs) and demonstrated that using chol-si-BLs led to the stability of siRNA (Negishi et al., 2011a). In this study, we prepared siRNA-loaded BLs (si-BLs) using a cationic lipid. Novel si-BLs were able easily prepared compared with chol-si-BLs. Additionally, this method may have widespread utility for drug delivery systems because it is applicable to various materials possessing negative electrical charges. We also investigated the effects of the amount of PEG in the BLs on their interaction with siRNA, the stability of siRNA in serum, and the gene-silencing effects of transfection with si-BLs and US.

2. Materials and methods

2.1. Cell lines and cultures

COS-7 cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Kohjin Bio Co. Ltd., Tokyo, Japan) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Equitech Bio Inc., Kerrville, TX), 100 units/mL penicillin, and 100 μg/mL streptomycin in a humidified atmosphere containing 5% CO_2 at 37 °C.

2.2. Preparation of liposomes and BLs

To prepare liposomes for conventional BLs, 1,2-dipalmitoyl-sn-glycero-phosphatidylcholine (DPPC) and 1,2-distearoylphosphatidylethanolamine-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 (Negishi et al., 2008). In brief, all reagents were dissolved in 1:1 (v/v) chloroform/diisopropylether. 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, UK). After being sized, 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 pressurized 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 the light-scattering method with a zeta potential/particle sizer (Nicomp 380ZLS, Santa Barbara, CA).

2.3. Ultrasound imaging of BLs

BLs diluted with PBS were dispensed into 6-well plates. B-mode recordings were made using a high-frequency ultrasound imaging system (NP60R-UBM, Nepa Gene, Co., Ltd., Chiba, Japan).

2.4. Plasmid DNA and siRNA

The plasmid pCMV-GL3, derived from pGL3-basic (Promega, Madison, WI), is an expression vector encoding the firefly luciferase gene under the control of a cytomegalovirus promoter. Small interfering RNA targeting luciferase (Luciferase GL3 siRNA; siGL3) and a nontargeting siRNA (Control (non-sil.) siRNA; siCont) were purchased from Qiagen K.K. (Tokyo, Japan). Their sequences were as follows: siGL3, *5′-CUUACGCUGAGUACUUCGAdTdT-3′* and *5′-UCGAAGUACUCAGCGUAAGdTdT-3′*; siCont, *5′-UUCUCCGAACGUGUCACGUdTdT-3′* and *5′-ACGUGACACGUUCGGAGAAdTdT-3′*. Nontargeting fluorescein-labeled siRNA (BLOCK-iT Fluorescent Oligo) was purchased from Invitrogen Japan K.K. (Tokyo, Japan).

2.5. Preparation of si-BLs

For the preparation of si-BLs, adequate amounts of siRNA were added to BLs and gently mixed. FITC-labeled siRNA and flow cytometry were used to examine the interaction between siRNA and BLs. The fluorescence intensity of si-BLs was analyzed using a FACSCanto (Becton Dickinson, San Jose, CA). To quantify the amount of siRNA loaded onto the BL surfaces, the BLs were centrifuged at 2000 rpm for 1 min and the unbound siRNA was removed. The BL solution and the aqueous solution containing the unbound siRNA were then boiled for 5 min after which the optical density was measured at 260 nm using a spectrophotometer.

2.6. Stability of siRNA in serum

The BLs, siRNA, and si-BLs were incubated in 50% serum for 15, 30, and 60 min. Serum was used without heat inactivation. The stability of the siRNA was confirmed by 15% polyacrylamide gel electrophoresis. The gel was stained with SYBR SAFE (Invitrogen Japan K.K., Tokyo, Japan) and visualized under ultraviolet light.

2.7. Transfection of siRNA into cells using BLs or si-BLs

The mixture of siRNA (final concentration 100 nM) and BLs or si-BLs (60 μg) in culture medium containing 10% FBS was added to the cells transfected with pDNA on the previous day. The cells were immediately exposed to US (frequency, 2 MHz; duty, 50%; burst rate, 2.0 Hz; intensity 2.0 W/cm^2) for 10 s through a 6-mm diameter probe placed in the well. A Sonopore 3000 (NEPA GENE, Co., Ltd., Chiba, Japan) was used to generate the US. The cells were washed twice with culture medium and cultured for two days.

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

2.8. Statistical analyses

All data are reported as the mean ± SD ($n = 4$). Data were considered significant when $P < 0.05$. The t-test was used to calculate statistical significance.

3. Results

3.1. Preparation of BLs containing DOTAP

Initial experiments were performed to investigate whether liposomes containing a cationic lipid, DOTAP, could entrap a US imaging gas as well as conventional BLs. We prepared liposomes containing DOTAP in various amounts and attempted to entrap the gas. The

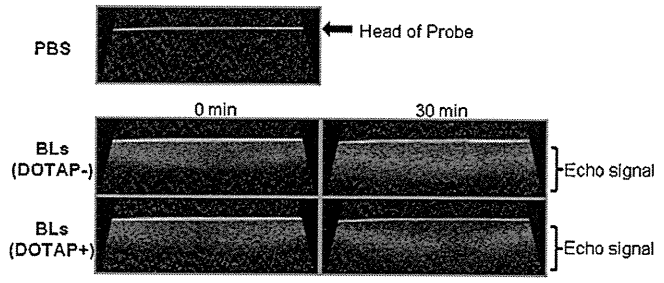

Fig. 1. Ultrasonographic images of a plate containing BLs with or without DOTAP.

liposomes containing up to 15 mol% DOTAP became cloudy, and we concluded that they could effectively entrap the imaging gas (data not shown). The liposomes containing more than 15 mol% DOTAP had difficulty entrapping the gas. We also examined BLs containing DOTAP using a high-frequency US imaging system. The system is a two-dimensional US image display composed of bright dots representing the US echoes. The brightness of each dot is determined by the amplitude of the returned echo signal. As shown in Fig. 1, the US echo signal was detected even 30 min later.

3.2. Effects of polyethyleneglycol on the interaction of siRNA with BLs

To assess whether siRNA could be loaded onto the surface of BLs, we used a fluorescence-activated cell sorter, the FACSCanto. We also prepared BLs containing different lengths of PEG to assess the effect of PEG on BL interactions with siRNA. As shown in Fig. 2, BLs not containing DOTAP were successfully loaded with siRNA. Approximately 40% of the BLs were FITC positive. Approximately 45% of the BLs containing DOTAP but not containing PEG_{750} were FITC positive. In contrast, BLs containing DOTAP and PEG_{750} were more heavily loaded with siRNA. Approximately 80% were FITC positive. Thus, in all subsequent experiments, BLs composed of DPPC, DOTAP, PEG_{2000}, and PEG_{750} (in a 79:15:3:3 molar ratio) were used.

Table 1
Size (nm) and zeta potential (mV) of BLs and si-BLs.

Lipid composition of BLs (molar ratio)	BLs	si-BLs
DPPC:PEG_{2000} = 94:6	528.3 nm	587.9 nm
DPPC:DOTAP:PEG_{2000}:PEG_{750} = 79:15:3:3	749.0 nm	862.2 nm
DPPC:PEG_{2000} = 94:6	−0.81 mV	−0.42 mV
DPPC:DOTAP:PEG_{2000}:PEG_{750} = 79:15:3:3	−0.20 mV	−0.13 mV

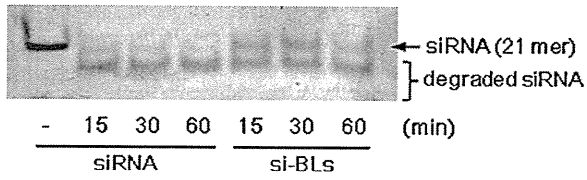

Fig. 3. Stability of siRNA in the presence of serum. Naked siRNA or si-BLs (DOTAP (+), PEG_{2000} and PEG_{750} (3:3)) were subjected to 50% serum degradation at 37 °C for 0.5 or 1 h and confirmed by 15% acrylamide gel electrophoresis.

As shown in Table 1, there was almost no change in the size and zeta potential of the BLs after siRNA was added.

We investigated the stability of siRNA in serum. Small interfering RNA held by BLs showed increased stability in 50% serum compared with free siRNA, although some siRNA was degraded (Fig. 3). We also examined the change in the amount of siRNA bound to BLs when the concentration of the siRNA was increased. As shown in Fig. 4, the amount siRNA loaded increased in a dose-dependent manner. We finally estimated that 60 μg of BLs could be loaded with at least 100 pmol of siRNA and that approximately 30% of the siRNA was bound to the lipid surface.

3.3. Transfection of siRNA into cells using BLs or si-BLs

Before the transfection experiments, we investigated the destruction efficiency of si-BLs under the US exposure. The solution of si-BLs was exposed to the same conditions used for *in vitro* transfection and was analyzed using the FACSCanto. Unlike the solution of si-BLs before US exposure, no fluorescence was detected in the

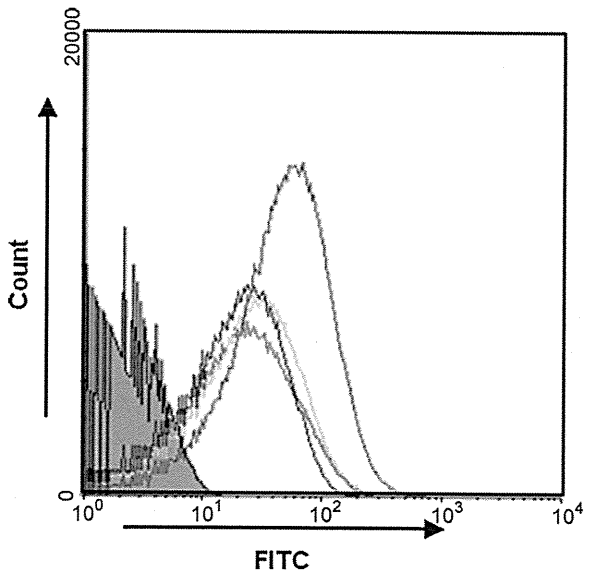

Fig. 2. Interaction of siRNA with BLs and the effects of PEG chain length on the interaction. The interaction was examined by analyzing a mixture of FITC-siRNA (50 pmol) and various BLs (60 μg) with the FACSCanto; gray area: BLs only; red curve: si-BLs (DOTAP (−), PEG_{2000} and PEG_{750} (molar ratio, 6:0)); green curve: si-BLs (DOTAP (+), PEG_{2000} and PEG_{750} (6:0)); blue curve: si-BLs (DOTAP (−), PEG_{2000} and PEG_{750} (3:3)); purple curve: si-BLs (DOTAP (+), PEG_{2000} and PEG_{750} (3:3)).

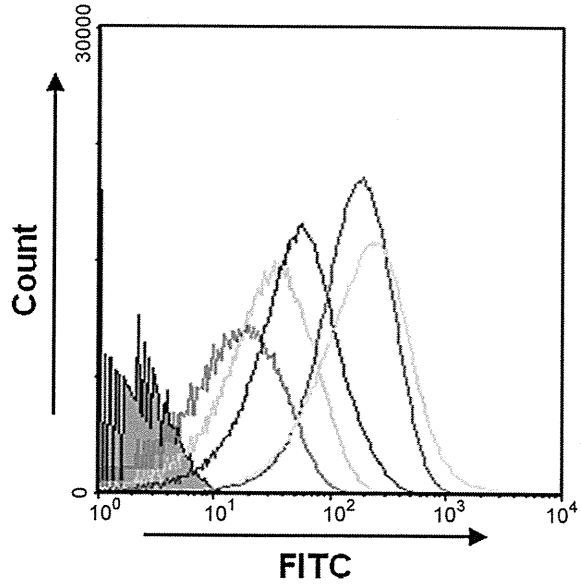

Fig. 4. Loading of siRNA onto BLs. The interaction was examined by analyzing a mixture of FITC-siRNA (12.5–200 pmol) and BLs (60 μg) containing DPPC, DOTAP, PEG_{2000} and PEG_{750} (79:15:3:3) with FACSCanto; gray area: BLs only; red curve: si-BLs (siRNA 12.5 pmol); green curve: si-BLs (siRNA 25 pmol); blue curve: si-BLs (siRNA 50 pmol); purple curve: s si-BLs (siRNA 100 pmol); light blue curve: si-BLs (siRNA 200 pmol).

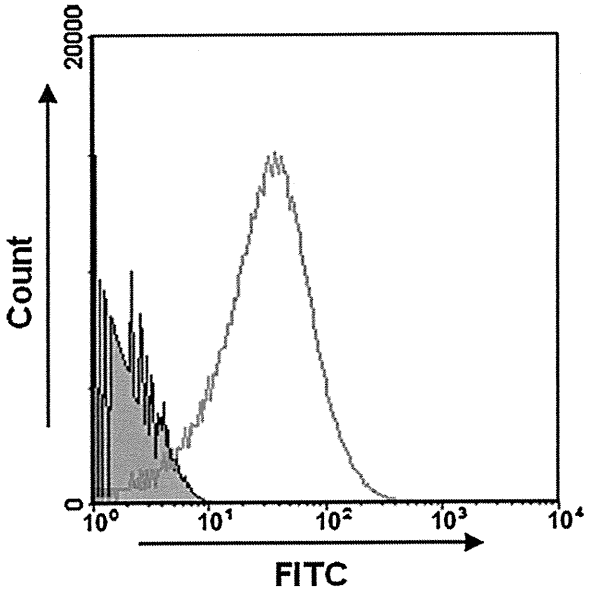

Fig. 5. Effects of US on si-BLs. The interaction was examined by analyzing a mixture of FITC-siRNA (50 pmol) and BLs (60 μg) containing DPPC, DOTAP, PEG_{2000}, and PEG_{750} (79:15:3:3) with the FACSCanto; gray area: BLs only; red curve: si-BLs; green curve: solution of si-BLs after US exposure (frequency, 2 MHz; duty, 50%; burst rate, 2.0 Hz; intensity, 2.0 W/cm^2; time, 10 s). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

solution (Fig. 5). This result suggested that the US caused the release of siRNA from the surface of the BLs.

To investigate the gene-silencing effects of siRNA transfection with si-BLs and US, cells transfected with pDNA encoding firefly luciferase (pCMV-GL3) on the previous day were added to BLs loaded with nontargeting control or luciferase-targeting siRNA (siCont or siGL3) and exposed to US (Fig. 6). Approximately 80% of luciferase expression was specifically blocked by siGL3 in the si-BLs-treated group and in the group treated with conventional BLs. Cytotoxicity was absent after the transfection with si-BLs and US (data not shown).

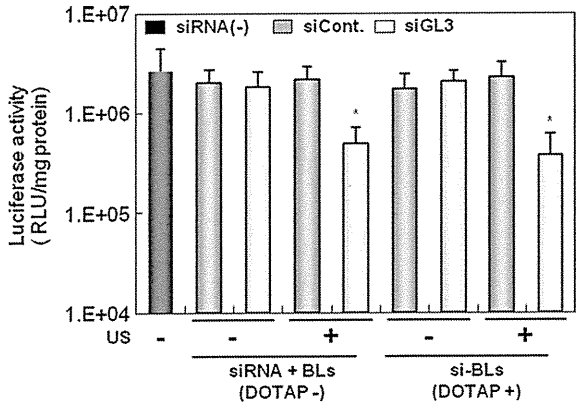

Fig. 6. Down-regulation of luciferase expression by siRNA with BL and US. COS-7 cells transfected with pCMV-GL3 on the previous day were added to siRNA (100 nM) and conventional BLs (DOTAP (−), PEG_{2000}, and PEG_{750} (molar ratio, 6:0)) or si-BLs (DOTAP (+), PEG_{2000}, and PEG_{750} (molar ratio, 3:3)) and applied. At 2 days posttransfection, luciferase expression was measured. siRNA(−); the group not transfected with siRNA, siCont; the group transfected with nontargeting siRNA (siCont), siGL3; the group transfected with siRNA targeting luciferase (siGL3). *P values <0.05 compared with the group transfected with siCont. All data are reported as the mean ± SD (n = 4).

4. Discussion

RNAi therapeutics have great potential for treating intractable diseases ranging from acquired diseases, such as viral infections, to purely genetic disorders. However, inefficient delivery into specific organs has hindered their clinical application.

Recently, a combination of microbubbles and US has been proposed as a less invasive and tissue-specific method of gene delivery. The combination produces transient changes in the permeability of the cell membrane and allows for the site-specific intracellular delivery of molecules such as dextran, pDNA, peptides, and siRNA both *in vitro* and *in vivo* (Du et al., 2011; Kinoshita and Hynynen, 2005; Li et al., 2003; Otani et al., 2009; Sonoda et al., 2006; Taniyama et al., 2002a,b; Tsunoda et al., 2005; Unger et al., 2004). However, because existing microbubbles have problems with size, stability, and targeting functionality, we developed liposomal bubbles (BLs). BLs are an effective and novel tool for gene and siRNA delivery *in vitro* and *in vivo* (Negishi et al., 2008, 2011b,c; Suzuki et al., 2007, 2008a,b). Our method using BLs and US did not involve endocytosis, and siRNA was directly introduced into the cytoplasm within a fairly short time. Thus, it seems unnecessary to consider the escape of siRNA from the endosome and the degradation of siRNA in lysosomes, although the endosomal escape is an important issue in other delivery tools. Furthermore transfection methods using physical energy other than US are expected and are currently being developed (Endoh and Ohtsuki, 2009; Kong et al., 2004; Oliveira et al., 2007; Schiffelers et al., 2005; Takei et al., 2008). These methods are difficult to apply to deep tissue. In contrast, US is able to control the accessible tissue sites by changing of the frequency and to reach the deep tissues. However, our previous results were obtained using a mixture of BLs and naked siRNA, which do not colocalize in blood vessels after intravenous administration. Additionally, siRNA is susceptible to degradation by nucleases and rapid removal from circulation. Consequently, these factors may cause a reduction in transfection efficiency. In this study, we prepared siRNA-loaded BLs (si-BLs) using a cationic lipid as a more effective, efficient delivery tool for systemic injections.

We initially attempted to entrap a US imaging gas in BLs containing DOTAP, a cationic lipid often used for gene delivery. Liposomes containing up to 15 mol% DOTAP did entrap the gas and could be used as ultrasound contrast agents. However, liposomes containing more than 15 mol% DOTAP had difficulty maintaining the gas. We also tested BLs containing DOTAP using a high-frequency US imaging system. An echo signal was detected as well as for BLs without DOTAP, although the imaging effect was slightly reduced 30 min later (Fig. 1). These results were assumed to be due to differences in the response to US and the stability of each BL type.

We used flow cytometry to examine the interaction between siRNA and BLs. The BLs were detectable, although the fluorescence intensity was low. The fluorescein-labeled siRNA molecules were too small to be detected with flow cytometry. However, the siRNA-loaded BLs exhibited strong fluorescence. We determined that siRNA could interact with BLs, and the interaction was due to the cationic charge of DOTAP. The amount of siRNA bound to the BLs was slightly increased in the presence of DOTAP. Furthermore, BLs containing both DOTAP and PEG_{750} could be loaded with much more siRNA (Fig. 2). BLs containing neither DOTAP nor PEG_{750} also loaded successfully with a certain amount of siRNA. These results suggested that siRNA could be loaded not only by the electrostatic interaction, but also by the fixed aqueous layer formed with PEG. It has been reported that the modification of liposomes with short and long PEG chains increases the fixed aqueous layer thickness (Sadzuka et al., 2002). We considered that the structural changes in the PEG chain facilitated interaction between the cationic lipid and anionic siRNA. Moreover, there were no significant changes in size after adding siRNA (Table 1). The data suggested that siRNA

was bound to the surface of BLs and that BLs did not aggregate. We also investigated the stability of siRNA interacting with BLs in 50% serum. Although some siRNA was degraded, siRNA held by BLs showed increased stability in 50% serum compared with free siRNA (Fig. 3). In the solution of si-BLs, free siRNA was present with si-BLs. Therefore, the siRNA not held by BLs was degraded. We examined the change in the amount of bound siRNA by adding various amounts of siRNA to BLs. As shown in Fig. 4, the amount of siRNA loaded onto BLs (60 μg) increased with siRNA addition in a dose-dependent manner up to 100 pmol.

We also investigated the effects of US exposure on si-BLs by analyzing the si-BL solution after exposure under the same conditions used for the *in vitro* transfection. No fluorescence was detected. Moreover, there were only a few detectable molecules in the solution of si-BLs after US exposure, and the histogram representing the results was almost parallel to the horizontal axis, similar to the solution of free siRNA (Fig. 5). This result suggested that US exposure collapsed si-BLs, releasing siRNA from the surface of the BLs. We confirmed that there was no damage to siRNA from US exposure by electrophoresis (data not shown). Undetectable fluorescence does not necessarily mean that siRNA were released from BLs: it is also possible that siRNA interacted with lipids or BLs that reverted to liposomes by degassing. However, the gene-silencing effects of siRNA transfection via si-BLs and US were comparable to those of siRNA transfection with conventional BLs and US (Fig. 6). Therefore, it appears that the exposure to US-induced cavitation, the release of siRNA from BLs, and the delivery of siRNA into the cytoplasm. We are currently developing BLs composed of lipids other than DPPC or DOTAP in attempts to form more stable and effective BLs. In the future, we will also examine siRNA delivery and disease-associated gene-silencing effects.

The preparation method of si-BLs developed in this study was easier than that of chol-si-BLs reported previously (Negishi et al., 2011a). Furthermore, BLs containing cationic lipid are expected to have widespread application to delivery tools of various molecules possessing negative electric charges. We confirmed that not only siRNA but also pDNA can be loaded onto BLs (p-BLs). Additionally, microbubbles conjugated to an antibody and having a targeting function have been developed recently (Behm et al., 2008; Leong-Poi et al., 2005; Palmowski et al., 2008). Liposomes can be easily modified to add a targeting function. Thus, the development of targeting si-BLs or p-BLs using an antibody or peptide is expected to lead to beneficial clinical applications for various diseases.

5. Conclusion

In this study, we showed that si-BLs could deliver siRNA as well as conventional BLs, although there remains room for improvement. Additionally, BLs containing a cationic lipid interacted with siRNA and protected the siRNA against nuclease degradation. These results suggest that si-BLs combined with US exposure may be useful for delivering siRNA to a tissue or organ via systemic injection.

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References

Allen, T.M., Hansen, C., Martin, F., Redemann, C., Yau-Young, A., 1991. Liposomes containing synthetic lipid derivatives of poly(ethylene glycol) show prolonged circulation half-lives in vivo. Biochim. Biophys. Acta 1066, 29–36.

Behm, C.Z., Kaufmann, B.A., Carr, C., Lankford, M., Sanders, J.M., Rose, C.E., Kaul, S., Lindner, J.R., 2008. Molecular imaging of endothelial vascular cell adhesion molecule-1 expression and inflammatory cell recruitment during vasculogenesis and ischemia-mediated arteriogenesis. Circulation 117, 2902–2911.

Blume, G., Cevc, G., 1990. Liposomes for the sustained drug release in vivo. Biochim. Biophys. Acta 1029, 91–97.

Delius, M., Adams, G., 1999. Shock wave permeabilization with ribosome inactivating proteins: a new approach to tumor therapy. Cancer Res. 59, 5227–5232.

Du, J., Shi, Q.S., Sun, Y., Liu, P.F., Zhu, M.J., Du, L.F., Duan, Y.R., 2011. Enhanced delivery of monomethoxypoly(ethylene glycol)-poly(lactic-co-glycolic acid)-poly l-lysine nanoparticles loading platelet-derived growth factor BB small interfering RNA by ultrasound and/or microbubbles to rat retinal pigment epithelium cells. J. Gene Med. 13, 312–323.

Duvshani-Eshet, M., Machluf, M., 2005. Therapeutic ultrasound optimization for gene delivery: a key factor achieving nuclear DNA localization. J. Control. Release 108, 513–528.

Endoh, T., Ohtsuki, T., 2009. Cellular siRNA delivery using cell-penetrating peptides modified for endosomal escape. Adv. Drug Deliv. Rev. 61, 704–709.

Frank-Kamenetsky, M., Grefhorst, A., Anderson, N.N., Racie, T.S., Bramlage, B., Akinc, A., Butler, D., Charisse, K., Dorkin, R., Fan, Y., Gamba-Vitalo, C., Hadwiger, P., Jayaraman, M., John, M., Jayaprakash, K.N., Maier, M., Nechev, L., Rajeev, K.G., Read, T., Röhl, I., Soutschek, J., Tan, P., Wong, J., Wang, G., Zimmermann, T., de Fougerolles, A., Vornlocher, H.P., Langer, R., Anderson, D.G., Manoharan, M., Koteliansky, V., Horton, J.D., Fitzgerald, K., 2008. Therapeutic RNAi targeting PCSK9 acutely lowers plasma cholesterol in rodents and LDL cholesterol in nonhuman primates. Proc. Natl. Acad. Sci. U.S.A. 105, 11915–11920.

Greenleaf, W.J., Bolander, M.E., Sarkar, G., Goldring, M.B., Greenleaf, J.F., 1998. Artificial cavitation nuclei significantly enhance acoustically induced cell transfection. Ultrasound Med. Biol. 24, 587–595.

Halder, J., Kamat, A.A., Landen, C.N., Han, L.Y., Lutgendorf, S.K., Lin, Y.G., Merritt, W.M., Jennings, N.B., Chavez-Reyes, A., Coleman, R.L., Gershenson, D.M., Schmandt, R., Cole, S.W., Lopez-Berestein, G., Sood, A.K., 2006. Focal adhesion kinase targeting using *in vivo* short interfering RNA delivery in neutral liposomes for ovarian carcinoma therapy. Clin Cancer Res. 12, 4916–4924.

Harata, M., Soda, Y., Tani, K., Ooi, J., Takizawa, T., Chen, M., Bai, Y., Izawa, K., Kobayashi, S., Tomonari, A., Nagamura, F., Takahashi, S., Uchimaru, K., Iseki, T., Tsuji, T., Takahashi, T.A., Sugita, K., Nakazawa, S., Tojo, A., Maruyama, K., Asano, S., 2004. CD19-targeting liposomes containing imatinib efficiently kill Philadelphia chromosome-positive acute lymphoblastic leukemia cells. Blood 104, 1442–1449.

Holmes, R.P., Yeaman, L.D., Taylor, R.G., McCullough, D.L., 1992. Altered neutrophil permeability following shock wave exposure *in vitro*. J. Urol. 147, 733–737.

Kim, S.H., Jeong, J.H., Lee, S.H., Kim, S.W., Park, T.G., 2008. Local and systemic delivery of VEGF siRNA using polyelectrolyte complex micelles for effective treatment of cancer. J. Control. Release 129, 107–116.

Kinoshita, M., Hynynen, K., 2005. A novel method for the intracellular delivery of siRNA using microbubble-enhanced focused ultrasound. Biochem. Biophys. Res. Commun. 335, 393–399.

Kong, X.C., Barzaghi, P., Ruegg, M.A., 2004. Inhibition of synapse assembly in mammalian muscle *in vivo* by RNA interference. EMBO Rep. 5, 183–188.

Lentacker, I., Wang, N., Vandenbroucke, R.E., Demeester, J., De Smedt, S.C., Sanders, N.N., 2009. Ultrasound exposure of lipoplex loaded microbubbles facilitates direct cytoplasmic entry of the lipoplexes. Mol. Pharm. 6, 457–467.

Leong-Poi, H., Christiansen, J., Heppner, P., Lewis, C.W., Klibanov, A.L., Kaul, S., Lindner, J.R., 2005. Assessment of endogenous and therapeutic arteriogenesis by contrast ultrasound molecular imaging of integrin expression. Circulation 111, 3248–3254.

Li, T., Tachibana, K., Kuroki, M., Kuroki, M., 2003. Gene transfer with echo-enhanced contrast agents: comparison between Albunex, Optison, and Levovist in mice—initial results. Radiology 229, 423–428.

Maruyama, K., Ishida, O., Kasaoka, S., Takizawa, T., Utoguchi, N., Shinohara, A., Chiba, M., Kobayashi, H., Eriguchi, M., Yanagie, H., 2004. Intracellular targeting of sodium mercaptoundecahydrododecaborate (BSH) to solid tumors by transferrin-PEG liposomes, for boron neutron-capture therapy (BNCT). J. Control. Release 98, 195–207.

Maruyama, K., Yuda, T., Okamoto, A., Kojima, S., Suginaka, A., Iwatsuru, M., 1992. Prolonged circulation time in vivo of large unilamellar liposomes composed of distearoyl phosphatidylcholine and cholesterol containing amphipathic poly(ethylene glycol). Biochim. Biophys. Acta 1128, 44–49.

McCaffrey, A.P., Meuse, L., Pham, T.T., Conklin, D.S., Hannon, G.J., Kay, M.A., 2002. RNA interference in adult mice. Nature 418, 38–39.

Morrissey, D.V., Lockridge, J.A., Shaw, L., Blanchard, K., Jensen, K., Breen, W., Hartsough, K., Machemer, L., Radka, S., Jadhav, V., Vaish, N., Zinnen, S., Vargeese, C.,

Bowman, K., Shaffer, C.S., Jeffs, L.B., Judge, A., MacLachlan, I., Polisky, B., 2005. Potent and persistent *in vivo* anti-HBV activity of chemically modified siRNAs. Nat. Biotechnol. 23, 1002–1007.

Negishi, Y., Endo-Takahashi, Y., Ishii, K., Suzuki, R., Oguri, Y., Murakami, T., Maruyama, K., Aramaki, Y., 2011a. Development of novel nucleic acid-loaded Bubble liposomes using cholesterol-conjugated siRNA. J. Drug Target 19, 830–836.

Negishi, Y., Endo, Y., Fukuyama, T., Suzuki, R., Takizawa, T., Omata, D., Maruyama, K., Aramaki, Y., 2008. Delivery of siRNA into the cytoplasm by liposomal bubbles and ultrasound. J. Control. Release 132, 124–130.

Negishi, Y., Matsuo, K., Endo-Takahashi, Y., Suzuki, K., Matsuki, Y., Takagi, N., Suzuki, R., Maruyama, K., Aramaki, Y., 2011b. Delivery of an angiogenic gene into ischemic muscle by novel bubble liposomes followed by ultrasound exposure. Pharm. Res. 28, 712–719.

Negishi, Y., Tsunoda, Y., Endo-Takahashi, Y., Oda, Y., Suzuki, R., Maruyama, K., Yamamoto, M., Aramaki, Y., 2011c. Local gene delivery system by bubble liposomes and ultrasound exposure into joint synovium. J. Drug Deliv. 2011, 203986.

Niu, X.Y., Peng, Z.L., Duan, W.Q., Wang, H., Wang, P., 2006. Inhibition of HPV 16 E6 oncogene expression by RNA interference *in vitro* and *in vivo*. Int. J. Gynecol. Cancer 16, 743–751.

Oliveira, S., Fretz, M.M., Høgset, A., Storm, G., Schiffelers, R.M., 2007. Photochemical internalization enhances silencing of epidermal growth factor receptor through improved endosomal escape of siRNA. Biochim. Biophys. Acta 1768, 1211–1217.

Otani, K., Yamahara, K., Ohnishi, S., Obata, H., Kitamura, S., Nagaya, N., 2009. Nonviral delivery of siRNA into mesenchymal stem cells by a combination of ultrasound and microbubbles. J. Control. Release 133, 146–153.

Palmowski, M., Huppert, J., Ladewig, G., Hauff, P., Reinhardt, M., Mueller, M.M., Woenne, E.C., Jenne, J.W., Maurer, M., Kauffmann, G.W., Semmler, W., Kiessling, F., 2008. Molecular profiling of angiogenesis with targeted ultrasound imaging: early assessment of antiangiogenic therapy effects. Mol. Cancer Ther. 7, 101–109.

Sadzuka, Y., Nakade, A., Hirama, R., Miyagishima, A., Nozawa, Y., Hirota, S., Sonobe, T., 2002. Effects of mixed polyethyleneglycol modification on fixed aqueous layer thickness and antitumor activity of doxorubicin containing liposome. Int. J. Pharm. 238, 171–180.

Sato, Y., Murase, K., Kato, J., Kobune, M., Sato, T., Kawano, Y., Takimoto, R., Takada, K., Miyanishi, K., Matsunaga, T., Takayama, T., Niitsu, Y., 2008. Resolution of liver cirrhosis using vitamin A-coupled liposomes to deliver siRNA against a collagen-specific chaperone. Nat. Biotechnol. 26, 431–442.

Schiffelers, R.M., Xu, J., Storm, G., Woodle, M.C., Scaria, P.V., 2005. Effects of treatment with small interfering RNA on joint inflammation in mice with collagen-induced arthritis. Arthritis Rheum. 52, 1314–1318.

Schratzberger, P., Krainin, J.G., Schratzberger, G., Silver, M., Ma, H., Kearney, M., Zuk, R.F., Brisken, A.F., Losordo, D.W., Isner, J.M., 2002. Transcutaneous ultrasound augments naked DNA transfection of skeletal muscle. Mol. Ther. 6, 576–583.

Song, E., Lee, S.K., Wang, J., Ince, N., Ouyang, N., Min, J., Chen, J., Shankar, P., Lieberman, J., 2003. RNA interference targeting Fas protects mice from fulminant hepatitis. Nat. Med. 9, 347–351.

Sonoda, S., Tachibana, K., Uchino, E., Okubo, A., Yamamoto, M., Sakoda, K., Hisatomi, T., Sonoda, K.H., Negishi, Y., Izumi, Y., Takao, S., Sakamoto, T., 2006. Gene transfer to corneal epithelium and keratocytes mediated by ultrasound with microbubbles. Invest. Ophthalmol. Vis. Sci. 47, 558–564.

Suzuki, J., Ogawa, M., Takayama, K., Taniyama, Y., Morishita, R., Hirata, Y., Nagai, R., Isobe, M., 2010. Ultrasound-microbubble-mediated intercellular adhesion molecule-1 small interfering ribonucleic acid transfection attenuates neointimal formation after arterial injury in mice. J. Am. Coll. Cardiol. 55, 904–913.

Suzuki, R., Takizawa, T., Negishi, Y., Hagisawa, K., Tanaka, K., Sawamura, K., Utoguchi, N., Nishioka, T., Maruyama, K., 2007. Gene delivery by combination of novel liposomal bubbles with perfluoropropane and ultrasound. J. Control. Release 117, 130–136.

Suzuki, R., Takizawa, T., Negishi, Y., Utoguchi, N., Maruyama, K., 2008a. Effective gene delivery with novel liposomal bubbles and ultrasonic destruction technology. Int. J. Pharm. 354, 49–55.

Suzuki, R., Takizawa, T., Negishi, Y., Utoguchi, N., Sawamura, K., Tanaka, K., Namai, E., Oda, Y., Matsumura, Y., Maruyama, K., 2008b. Tumor specific ultrasound enhanced gene transfer *in vivo* with novel liposomal bubbles. J. Control. Release 125, 137–144.

Takei, Y., Nemoto, T., Mu, P., Fujishima, T., Ishimoto, T., Hayakawa, Y., Yuzawa, Y., Matsuo, S., Muramatsu, T., Kadomatsu, K., 2008. *In vivo* silencing of a molecular target by short interfering RNA electroporation: tumor vascularization correlates to delivery efficiency. Mol. Cancer Ther. 7, 211–221.

Takeshita, F., Minakuchi, Y., Nagahara, S., Honma, K., Sasaki, H., Hirai, K., Teratani, T., Namatame, N., Yamamoto, Y., Hanai, K., Kato, T., Sano, A., Ochiya, T., 2005. Efficient delivery of small interfering RNA to bone-metastatic tumors by using atelocollagen *in vivo*. Proc. Natl. Acad. Sci. U.S.A. 102, 12177–12182.

Taniyama, Y., Tachibana, K., Hiraoka, K., Aoki, M., Yamamoto, S., Matsumoto, K., Nakamura, T., Ogihara, T., Kaneda, Y., Morishita, R., 2002a. 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, 372–380.

Taniyama, Y., Tachibana, K., Hiraoka, K., Namba, T., Yamasaki, K., Hashiya, N., Aoki, M., Ogihara, T., Yasufumi, K., Morishita, R., 2002b. Local delivery of plasmid DNA into rat carotid artery using ultrasound. Circulation 105, 1233–1239.

Tsunoda, S., Mazda, O., Oda, Y., Iida, Y., Akabame, S., Kishida, T., Shin-Ya, M., Asada, H., Gojo, S., Imanishi, J., Matsubara, H., Yoshikawa, T., 2005. Sonoporation using microbubble BR14 promotes pDNA/siRNA transduction to murine heart. Biochem. Biophys. Res. Commun. 336, 118–127.

Unger, E.C., Porter, T., Culp, W., Labell, R., Matsunaga, T., Zutshi, R., 2004. Therapeutic applications of lipid-coated microbubbles. Adv. Drug Deliv. Rev. 56, 1291–1314.

Vandenbroucke, R.E., Lentacker, I., Demeester, J., De Smedt, S.C., Sanders, N.N., 2008. Ultrasound assisted siRNA delivery using PEG-siPlex loaded microbubbles. J. Control. Release 126, 265–273.

Xia, C.F., Zhang, Y., Zhang, Y., Boado, R.J., Pardridge, W.M., 2007. Intravenous siRNA of brain cancer with receptor targeting and avidin-biotin technology. Pharm. Res. 24, 2309–2316.

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Note

Gene delivery system involving Bubble liposomes and ultrasound for the efficient *in vivo* delivery of genes into mouse tongue tissue

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ABSTRACT

Oral squamous cell carcinoma is the most common type of head and neck cancer. Recently, efficient, easy, and minimally invasive gene delivery methods are expected to be developed as cancer gene therapies. However, the optimal method for delivering therapeutic genes into oral tissue for cancer treatment has not been elucidated. Therefore, we hypothesized that the tongue is a good target tissue for gene delivery with Bubble liposomes and ultrasound. To assess this, we attempted to deliver a mixture of plasmid DNA encoding a luciferase or enhanced green fluorescent protein, and Bubble liposomes into murine tongue with or without ultrasound exposure. The ultrasound conditions were 1 MHz, 2 W/cm^2, 60 s, and duty cycle: 50%. The time-course of gene expression in the tongue was investigated with a luciferase assay and fluorescent microscopy. Luciferase expression was significantly increased in tongue transfected using Bubble liposomes and ultrasound compared with that of the tongue untreated with ultrasound, and this high level of luciferase activity was maintained for 2 weeks. From these results, Bubble liposomes can be used in combination with ultrasound to efficiently deliver plasmid DNA into the tongue *in vivo*. This technique is a highly promising approach for gene delivery into oral tissue.

1. Introduction

Although the most common types of oral disease are dental caries and periodontal disease, oral cancer such as squamous cell carcinoma (SCC) is associated with an unfavorable prognosis. Tongue SCC is the most common type of oral SCC, and metastasis to the lymph nodes and/or proximal tissues often occurs (Ohba et al., 2010; Shiga et al., 2007). The current treatments for tongue SCC include surgery, radiation therapy, and chemotherapy, all of which have severe side effects. Therefore, cancer cell-specific treatment that does not damage normal cells is desired. Recently, gene delivery to tumor cells such as using adenovirus-based p53 gene therapies has gained attention (Edelman and Nemunaitis, 2003; Huang et al., 2009). The two main gene carrier systems for gene therapy are viral vectors and non-viral delivery systems. Viral vectors are efficient carriers for gene transfection (Lundstrom, 2003), but some serious problems such as immunogenicity and toxicity have been reported (Check, 2002, 2003; Marshall, 1999). On the other hand, the transfection efficiency of non-viral methods remains a problem. Therefore, it is necessary to develop a safe and highly efficient gene transfer method.

Recently, it has been reported that the use of microbubbles in combination with low energy ultrasound (US) enhances transfection efficiency (Greenleaf et al., 1998; Shohet et al., 2000; Sonoda et al., 2006; Taniyama et al., 2002a,b). Regarding the orofacial area, there have been a few reports about gene delivery using microbubbles and US, for example, Sakai et al. (2009) and Chen et al. (2009) reported transient gene transfection in the target tissue using different microbubbles. However, prolonged gene expression is necessary in the clinical setting, and the size and stability of the microbubbles employed also needs to be improved. Previously, we developed "Bubble liposomes (BL)" as a novel gene delivery carrier system and reported that gene delivery using a combination of BL and US is safer and more efficient in both *in vitro* and *in vivo* compared to other non-viral methods (Negishi et al., 2008, 2011; Suzuki et al., 2007). However, there are no reports about gene delivery to

Abbreviations: SCC, squamous cell carcinoma; US, ultrasound; BL, Bubble liposome; PEG, polyethylene glycol; EBD, Evans blue dye; QOL, quality of life.

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oral tissue using this technique. Therefore, in the present study, we assessed whether efficient gene delivery into mouse tongue tissue could be achieved using BL and US.

2. Materials and methods

2.1. Animals

Five-week-old male ICR mice were used for all animal experiments (Tokyo Laboratory Animals Science, Tokyo, Japan). All studies were approved by the Animal Experiment Committee of Tokyo University of Pharmacy and Life Sciences. The mice were given feed and tap water *ad libitum* throughout the experimental period.

2.2. Preparation of Bubble liposomes

The BL were prepared using the previously described method (Negishi et al., 2008; Suzuki et al., 2007). In brief, PEG liposomes composed of 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) (NOF Corporation, Tokyo, Japan) and 1,2-distearoyl-sn-glycero-3-phosphatidyl-ethanolamine-polyethyleneglycol (DSPE-PEG_{2000}-OMe) (NOF Corporation) in a molar ratio of 94:6 were prepared using 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. Then, 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 Phospholipid C test Wako (Wako Pure Chemical Industries Ltd., Osaka, Japan), and BL were prepared from liposomes and perfluoropropane gas (Takachio Chemical Ind. Co. Ltd., Tokyo, Japan). First, 2-mL sterilized vials containing 0.8 mL of a liposome suspension (lipid concentration: 1 mg/mL) were filled with perfluoropropane gas, capped, and then pressurized with a further 3 mL of perfluoropropane gas. The vial was placed in a bath-type sonicator (42 kHz, 100 W) (Bransonic 2510j-DTH, Branson Ultrasonics Co., Danbury, CT, USA) for 5 min to form BL.

2.3. Plasmid DNA

Two reporter plasmids were used. The pcDNA3-Luc plasmid, which is derived from pGL3-basic (Promega, Madison, WI), is an expression vector encoding the firefly luciferase gene under the control of the cytomegalovirus promoter. The pEGFP-N3 plasmid (Clontech Laboratories, Inc., Mountain View, CA) is an expression vector encoding enhanced green fluorescent protein (EGFP) under the control of the cytomegalovirus promoter.

2.4. In vivo gene delivery using BL and US

ICR mice were anesthetized with 10 mg/mL pentobarbital throughout each procedure. A 20 μL mixture of pDNA (20 μg) and BL (10 μg) was injected into the tongue tissue of the mice using a 33-gauge syringe (Hamilton Company, USA), and US exposure (frequency: 1 MHz; duty: 50%; intensity: 2 W/cm^2; time: 60 s) was immediately applied to the injection site. A Sonitron 2000 (Nepa Gene Co., Ltd.) was used as an ultrasound generator. Several days after the injection, the mice were sacrificed, and the tongue tissue in the US-exposed area was collected and homogenized with Polytron (Kinematica, Inc., New York, USA). The cell lysate and tissue homogenates were prepared with a lysis buffer (0.1 M Tris–HCl (pH 7.8), 0.1% Triton X-100, and 2 mM EDTA). Luciferase activity was then measured using a luciferase assay system (Promega, Madison, WI) and a luminometer (LB96V, Belthold Japan Co. Ltd., Tokyo, Japan). Activity is indicated as relative light units (RLU) per mg of protein. To analyze EGFP expression, the collected tongue was fixed with paraformaldehyde and dehydrated in sucrose solution. The specimens were then embedded in OCT compound and immediately frozen at −80 °C. Serial 10 μm thick sections were then cut using a cryostat and observed with a fluorescence microscope (Axiovert 200 M, Carl Zeiss).

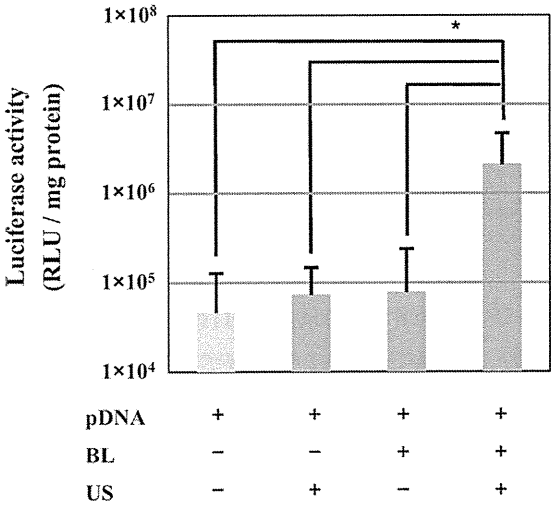

Fig. 1. Luciferase activity in tongue tissue transfected with a reporter gene using BL and US. Mice were subjected to BL and US-mediated luciferase gene transfer. Relative luciferase activity was determined on day 5 after transfection. The data are shown as the mean ± S.D. *$P < 0.05$, Mann–Whitney's U test ($n = 5$), compared to other groups. pDNA (pCMV-luciferase): 20 μg; BL: 10 μg; US conditions: frequency: 1 MHz, duty: 50%, and intensity: 2 W/cm^2, time: 60 s. BL, Bubble liposomes; US, ultrasound.

2.5. Tissue damage testing using Evans blue dye (EBD)

Tissue-damage testing using EBD was performed as reported previously (Liu and Huang, 2002). Briefly, EBD was dissolved in PBS (10 mg/mL) and sterilized using 0.2 μm membrane filters. The mice treated with pDNA, BL, and US were administered EBD (0.5 mg dye/10 g body weight) by tail vein injection and then sacrificed 1 day after the EBD injection. Their tongue tissues were collected, fixed with paraformaldehyde, embedded in OCT compound, and immediately frozen at −80 °C. Serial 10 μm thick sections were cut using a cryostat and observed with a fluorescence microscope (Axiovert 200 M, Carl Zeiss).

2.6. Statistical analysis

All data are shown as the mean ± S.D. ($n = 5$ or 6). Mann–Whitney's U test was used to determine the statistical significance of any differences. The differences detected in multiple comparison tests were assessed by two-way repeated-measures analysis of variance (ANOVA). Differences associated with a $P < 0.05$ were considered significant.

3. Results

We first tried to deliver naked pDNA into tongue tissue using BL and US under the conditions used in a previous study, in which naked pDNA was delivered into skeletal muscles (Negishi et al., 2011). Significantly increased gene expression was detected in the group treated with BL and US exposure (Fig. 1.); i.e., it was 12-fold higher than that of the group treated with pDNA alone. In the groups

treated with pDNA + BL and pDNA + US, the relative luciferase activity remained as low as that of the pDNA alone group.

Then, to optimize the conditions for *in vivo* gene delivery into tongue tissue, we examined three transfection condition parameters, the total pDNA, US intensity, and US exposure time. First, to assess whether the pDNA injection volume affected transfection efficiency, we adjusted it from 0.2 μg to 20 μg. As a result, the increase in luciferase activity was found to be dependent on the amount of pDNA, and the most significant increase in relative luciferase activity was detected at 20 μg pDNA (Fig. 2a.). Next, we investigated the relationship between US intensity and the transfection efficiency of gene delivery into tongue tissue. The US intensity was varied within the 0–4 W/cm^2 range. The relative luciferase activity was significantly higher in the groups treated with US intensities of 2.0 W/cm^2 and 4.0 W/cm^2 (Fig. 2b.). Moreover, we also examined the effect of the US exposure time and found that luciferase activity was highest when US was delivered for 60 s (Fig. 2c.). In contrast, when US was delivered for a longer period, the transfection efficiency tended to decrease. We further examined the duration of gene expression induced after treatment with BL and US exposure. As a result, we found that high luciferase activity was maintained for about 2 weeks (Fig. 3).

Next, the localization of EGFP-expressing cells and tissue damage after gene delivery with BL and US was observed with fluorescence microscopy. In histological observations, distinct EGFP expression was observed in the tongue tissue treated with BL and US (Fig. 4.). In the group treated with BL and US, there were many EGFP expressing cells throughout the muscle layer. In the other groups, only a few sporadically distributed cells were found to express EGFP. However, using a high US intensity to achieve efficient gene transfection leads to tissue damage (Duvshani-Eshet and Machluf, 2005; Kim et al., 1996). Therefore, to investigate the tissue damage caused by gene transfection, mice had EBD injected into their tail veins one day before they were euthanized, as enhanced EBD uptake indicates increased cell damage. As a result, we found that severe tissue damage was observed after the application of high intensity US (4.0 W/cm^2) or a US exposure time of 120 s or more (Fig. 5).

From these results, we suggest that the optimal conditions for gene delivery into the murine tongue using BL and US are as follows: total pDNA (2 μg/μL): 20 μg, US intensity: 2 W/cm^2, and US exposure time: 60 s.

In addition, *Sonazoid*™, a commercially available microbubble, has been used as an echo-contrast gas in clinical. We therefore also test the transfection efficacy of *Sonazoid*™ in the same experiment. However, the luciferase activity was moderate increase even in the combination of US exposure (data not shown).

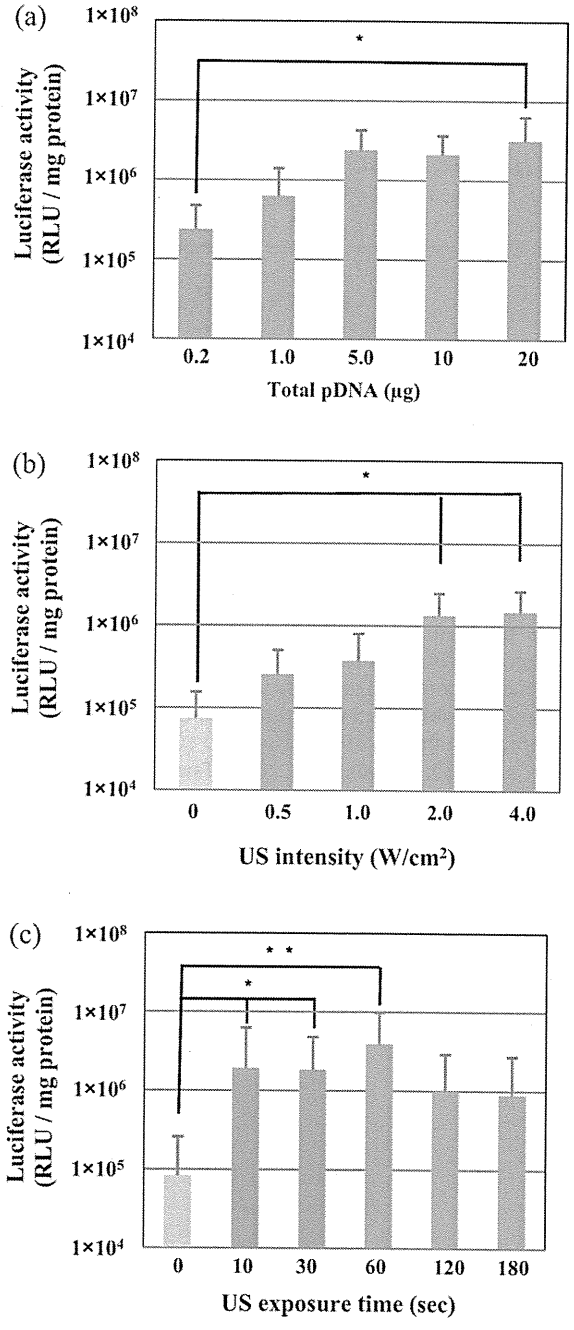

Fig. 2. Characteristics of ultrasound gene delivery systems using BL. To examine the optimal parameters for BL and US-mediated gene transfer into tongue tissue, the mice were subjected to various transfection conditions; i.e., by altering the amount of pDNA, US intensity, and US exposure time. The other conditions were as follows: US frequency: 1 MHz, duty: 50%. The data are shown as the mean ± S.D. (a) The variation in the gene expression level induced by changing the amount of pDNA. The amount of pDNA was changed from 0.2 μg to 20 μg. The total injection volume remained constant at 20 μL. *P < 0.05, Mann–Whitney's U test (n = 5), compared with 0.2 μg of pDNA. (b) The variation in the gene expression level induced by changes in the US intensity. The US intensity was set at 0, 0.5, 1.0, 2.0, or 4.0 W/cm^2. *P < 0.05, Mann–Whitney's U test (n = 5), compared with 0 W/cm^2 (no US exposure). (c) The variation in the gene expression level induced by changes in the US exposure time. The US duration was set at 0, 10, 30, 60, 120, or 180 s. *P < 0.05, **P < 0.01, Mann–Whitney's U test (n = 6), compared with 0 s (no US exposure).

4. Discussion

Gene therapy is expected to be clinically useful for treating genetic diseases, cancer, and/or infectious diseases. These diseases are also found in the orofacial area, and a number of studies have recently examined the usefulness of gene therapy for a variety of oral diseases. In those studies, gene delivery into the orofacial area was performed with viral vectors due to their high transfection efficiency. For example, it has been reported that reporter genes were transfected into rat salivary glands using several kinds of viral vector (Shai et al., 2002; Zheng and Baum, 2005). In addition, viruses are the most common transfer system used to deliver gene therapy to oral SCC (Ladeinde et al., 2005). However, viral systems are not perfect because of their safety and immunogenicity (Check, 2002, 2003; Marshall, 1999).

Therefore, many researchers have tried to establish non-viral gene delivery systems that combine high transfection efficiency with reduced invasiveness. Among these non-viral gene delivery methods, Fechheimer et al. (1987) first reported the US-mediated gene delivery technique, and since then gene transfer using ultrasonic waves has developed into a safe and non-viral gene transfection technology. A physical phenomenon known as

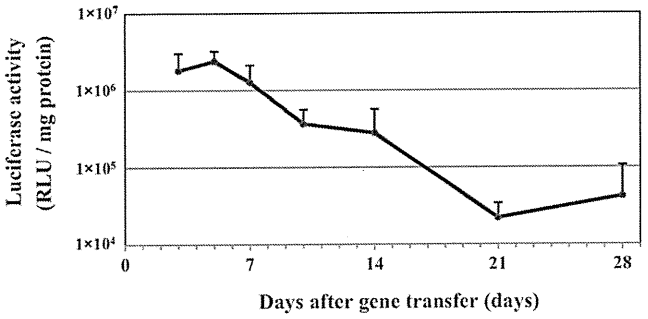

Fig. 3. Time-dependent changes in luciferase activity in tongue tissue transfected using BL and US. Relative luciferase activity was examined at 3, 5, 7, 10, 14, 21, and 28 days after the gene transfection. The transfection conditions were as follows: pDNA (pCMV-luciferase): 20 μg; BL: 10 μg; US conditions: frequency: 1 MHz, duty: 50%, intensity: 2 W/cm^2, and time: 60 s. The data are shown as the mean ± S.D.

cavitation is assumed to be the mechanism responsible for US-mediated gene delivery. When the "cavitation bubble" generated by US energy is destroyed, it produces a jet stream, which in turn produces transient pores in cell membranes, allowing extracellular plasmid DNA to enter the cytosol. In addition, it has been shown that transfection efficiency often improves in the presence of microbubbles, and the utility of their application has been demonstrated both *in vitro* and *in vivo* (Greenleaf et al., 1998; Shohet et al., 2000; Sonoda et al., 2006; Taniyama et al., 2002a,b).

Microbubbles were originally used as an ultrasonic contrast agent for clinical ultrasound diagnosis. A variety of microbubbles with different encapsulated gas types, shell materials, and diluents have been designed. The mean diameter of microbubbles that are marketed as echo-enhanced contrast agents is several micrometers. Several reports have compared the transfection efficiencies of these microbubbles during their use in combination with US (Alter et al., 2009; Hassan et al., 2009; Li et al., 2003; Wang et al., 2005).

However, the intravascular application of microbubbles is hindered by problems with their stability, targeting ability, and particle size. Thus, we developed and applied a new liposome composed of hydrophilic polyethylene glycol (PEG) as a drug delivery system. PEG-liposomes containing perfluoropropane gas are known as "Bubble liposomes (BL)". We have reported that this BL-mediated US gene transfer system enhances transfection efficiency both *in vitro* and *in vivo* (Negishi et al., 2008, 2010; Suzuki et al., 2007, 2008a,b, 2010). Negishi et al. (2011) used gene transfer methods involving BL and US to transfect genes into murine skeletal muscle and discussed their reasons for selecting skeletal muscle as a target tissue for gene therapy. They stated that as skeletal muscle cells are large and display stability and longevity, they are an attractive target tissue for gene therapy.

The generation and oscillation of "cavitation bubbles" after ultrasound exposure is influenced by the composition and pressure of the organs surrounding the transfection site. Therefore, it is important to optimize each transfection condition in the targeted tissue. The majority of tongue tissue is composed of skeletal muscle and is covered with a keratinized oral mucosa. In our histological observations, distinct EGFP expression was observed in the tongue muscle, as shown in Fig. 4. On the other hand, scattered EGFP expression was observed on the surface area of the mucoepithelial layer (data not shown). Moreover, a previous report described that repeated US exposure enhances transfection efficiency and prolonged gene expression compared with single US exposure (Bekeredjian et al., 2003). In this study, we showed that high luciferase activity was maintained for 2 weeks in murine tongue tissue treated with BL and single US exposure. Such persistent expression is thought to be suitable for therapy against tongue cancer. Whether the effect of the therapeutic gene needs to be continuous depends on the disease being targeted; for example, whether it is an infectious or genetic disease. Therefore, if the gene delivery method involving BL and US is to be applied to other oral tissues, it is very important to optimize the transfection conditions for long-term gene expression in the target tissue. Further

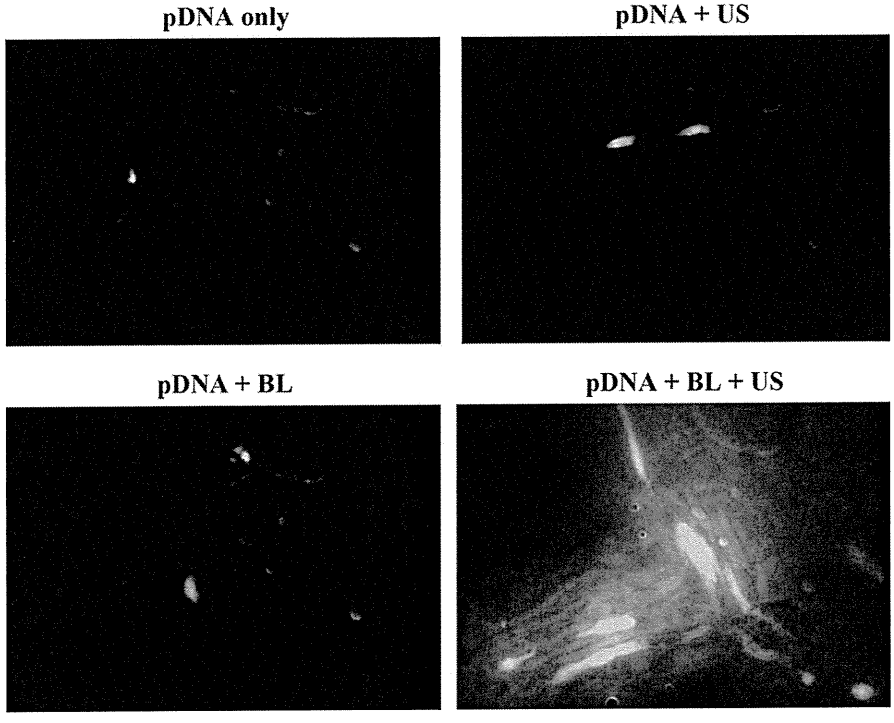

Fig. 4. EGFP expression in tongue tissue transfected with a reporter gene using BL and US. Mice were treated with BL and then subjected to US-mediated EGFP transfer into the tongue. On day 5 after the transfection, the tongue was sectioned into 10 μm thick slices using a cryostat, and EGFP expression was analyzed by fluorescent microscopy. Each of the gene transfer conditions is indicated above the pictures. Magnification: 200×. BL, Bubble liposomes; US, ultrasound.

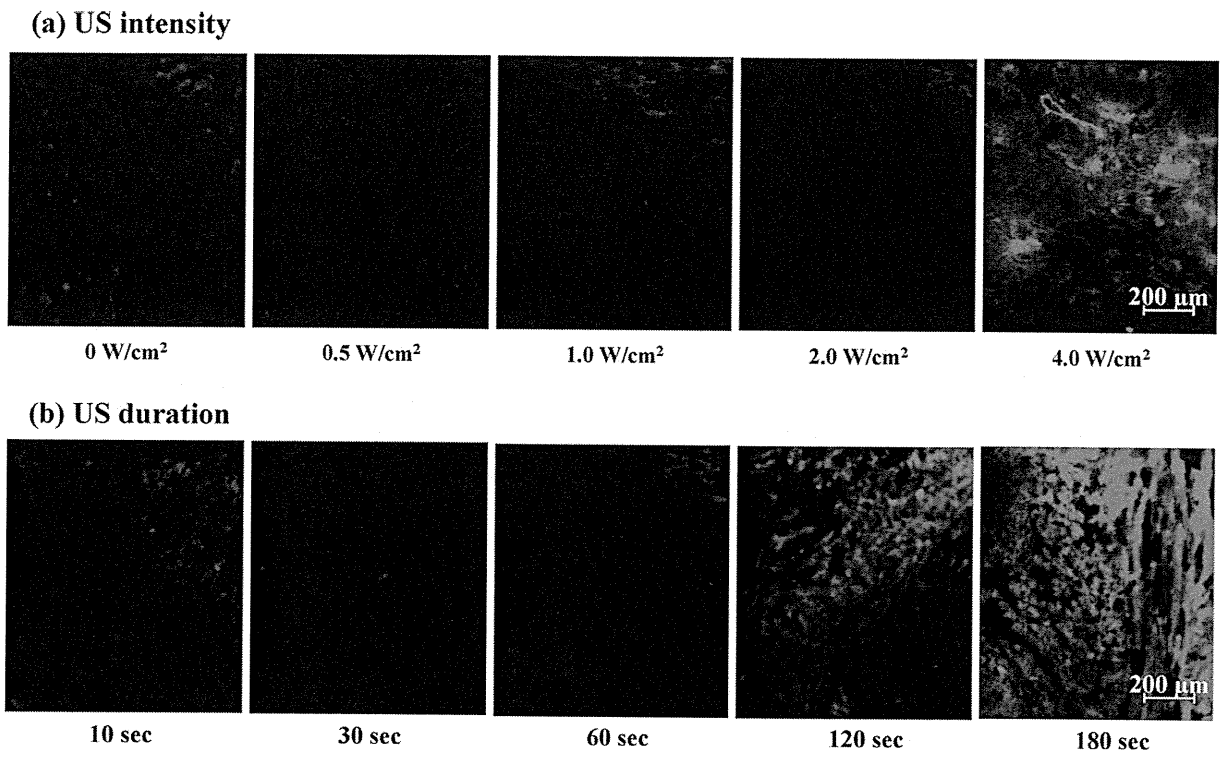

Fig. 5. Tissue-damage testing using EBD. To assess the tissue-damage by BL and US-mediated gene transfer into tongue tissue, the mice were treated pDNA with BL and various US exposure conditions. (a) At a frequency of 1 MHz with an intensity of 0, 0.5, 1.0, 2.0, or 4.0 W/cm^2 for 60 s (upper section). (b) At a frequency of 1 MHz with an intensity of 2.0 W/cm^2 for 10, 30, 60, 120, or 180 s (lower). Evans-blue fluorescence of 10 μm cryosections from the tongue was examined with fluorescence microscopy. Scale bar: 200 μm.

experiments using genes that encode therapeutic proteins are required to assess the clinical application of this US-mediated BL method.

This is the first report regarding gene transfer to tongue tissue using BL and US as a therapeutic method for diseases of the oral cavity. As mentioned above, tongue SCC is one of the most common forms of head and neck cancer; nevertheless, no standardized treatment strategy for this condition has been established (Shiga et al., 2007). Oral dysfunction and decreased quality of life (QOL) are often seen after surgical treatment in tongue SCC patients. Therefore, gene delivery systems involving BL and US exposure that enable cancer cell-specific treatment may improve the QOL of tongue SCC patients.

5. Conclusion

In conclusion, the results of this study suggest that our gene delivery method involving BL and US could be a useful treatment for patients with tongue SCC. This US-mediated BL technique is a highly promising approach for gene delivery into oral tissue.

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References

Alter, J., Sennoga, C.A., Lopes, D.M., Eckersley, R.J., Wells, D.J., 2009. Microbubble stability is a major determinant of the efficiency of ultrasound and microbubble mediated in vivo gene transfer. Ultrasound Med. Biol. 35, 976–984.
Bekeredjian, R., Chen, S., Frenkel, P.A., Grayburn, P.A., Shohet, R.V., 2003. Ultrasound-targeted microbubble destruction can repeatedly direct highly specific plasmid expression to the heart. Circulation 108, 1022–1026.
Check, E., 2002. Safety panel backs principle of gene-therapy trials. Nature 420, 595.
Check, E., 2003. Second cancer case halts gene-therapy trials. Nature 421, 305.
Chen, R., Chiba, M., Mori, S., Fukumoto, M., Kodama, T., 2009. Periodontal gene transfer by ultrasound and nano/microbubbles. J. Dent. Res. 88, 1008–1013.
Duvshani-Eshet, M., Machluf, M., 2005. Therapeutic ultrasound optimization for gene delivery: a key factor achieving nuclear DNA localization. J. Control. Release 108, 513–528.
Edelman, J., Nemunaitis, J., 2003. Adenoviral p53 gene therapy in squamous cell cancer of the head and neck region. Curr. Opin. Mol. Ther. 5, 611–617.
Fechheimer, M., Boylan, J.F., Parker, S., Sisken, J.E., Patel, G.L., Zimmer, S.G., 1987. Transfection of mammalian cells with plasmid DNA by scrape loading and sonication loading. Proc. Natl. Acad. Sci. U.S.A. 84, 8463–8467.
Greenleaf, W.J., Bolander, M.E., Sarkar, G., Goldring, M.B., Greenleaf, J.F., 1998. Artificial cavitation nuclei significantly enhance acoustically induced cell transfection. Ultrasound Med. Biol. 24, 587–595.
Hassan, M.A., Feril Jr., L.B., Suzuki, K., Kudo, N., Tachibana, K., Kondo, T., 2009. Evaluation and comparison of three novel microbubbles: enhancement of ultrasound-induced cell death and free radicals production. Ultrason. Sonochem. 16, 372–378.
Huang, P.I., Chang, J.F., Kirn, D.H., Liu, T.C., 2009. Targeted genetic and viral therapy for advanced head and neck cancers. Drug Discov. Today 14, 570–578.
Kim, H.J., Greenleaf, J.F., Kinnick, R.R., Bronk, J.T., Bolander, M.E., 1996. Ultrasound-mediated transfection of mammalian cells. Hum. Gene Ther. 7, 1339–1346.
Ladeinde, A.L., Ogunlewe, M.O., Adeyemo, W.L., Bamgbose, B.O., 2005. Gene therapy in the management of oral cancer: a review of recent developments. Niger. Postgrad. Med. J. 12, 18–22.
Li, T., Tachibana, K., Kuroki, M., 2003. Gene transfer with echo-enhanced contrast agents: comparison between Albunex, Optison, and Levovist in mice – initial results. Radiology 229, 423–428.
Liu, F., Huang, L., 2002. A syringe electrode device for simultaneous injection of DNA and electrotransfer. Mol. Ther. 5, 323–328.
Lundstrom, K., 2003. Latest development in viral vectors for gene therapy. Trends Biotechnol. 21, 117–122.
Marshall, E., 1999. Gene therapy death prompts review of adenovirus vector. Science 286, 2244–2245.

Negishi, Y., Endo, Y., Fukuyama, T., Suzuki, R., Takizawa, T., Omata, D., Maruyama, K., Aramaki, Y., 2008. Delivery of siRNA into the cytoplasm by liposomal bubbles and ultrasound. J. Control. Release 132, 124–130.

Negishi, Y., Matsuo, K., Endo-Takahashi, Y., Suzuki, K., Matsuki, Y., Takagi, N., Suzuki, R., Maruyama, K., Aramaki, Y., 2011. Delivery of an angiogenic gene into ischemic muscle by novel Bubble liposomes followed by ultrasound exposure. Pharm. Res. 28, 712–719.

Negishi, Y., Omata, D., Iijima, H., Takabayashi, Y., Suzuki, K., Endo, Y., Suzuki, R., Maruyama, K., Nomizu, M., Aramaki, Y., 2010. Enhanced laminin-derived peptide AG73-mediated liposomal gene transfer by Bubble liposomes and ultrasound. Mol. Pharmacol. 7, 217–226.

Ohba, T., Motoi, N., Kimura, Y., Okumura, S., Kawabata, K., Yoshizawa, Y., Inase, N., Ishikawa, Y., 2010. Cytokeratin expression profiling is useful for distinguishing between primary squamous cell carcinoma of the lung and pulmonary metastases from tongue cancer. Pathol. Int. 60, 575–580.

Sakai, T., Kawaguchi, M., Kosuge, Y., 2009. siRNA-mediated gene silencing in the salivary gland using in vivo microbubble-enhanced sonoporation. Oral Dis. 15, 505–511.

Shai, E., Falk, H., Honigman, A., Panet, A., Palmon, A., 2002. Gene transfer mediated by different viral vectors following direct cannulation of mouse submandibular salivary glands. Eur. J. Oral Sci. 110, 254–260.

Shiga, K., Ogawa, T., Sagai, S., Kato, K., Kobayashi, T., 2007. Management of the patients with early stage oral tongue cancers. Tohoku J. Exp. Med. 212, 389–396.

Shohet, R.V., Chen, S., Zhou, Y.T., Wang, Z., Meidell, R.S., Unger, R.H., Grayburn, P.A., 2000. Echocardiographic destruction of albumin microbubbles directs gene delivery to the myocardium. Circulation 101, 2554–2556.

Sonoda, S., Tachibana, K., Uchino, E., Okubo, A., Yamamoto, M., Sakoda, K., Hisatomi, T., Sonoda, K.H., Negishi, Y., Izumi, Y., Takao, S., Sakamoto, T., 2006. Gene transfer to corneal epithelium and keratocytes mediated by ultrasound with microbubbles. Invest. Ophthalmol. Vis. Sci. 47, 558–564.

Suzuki, R., Namai, E., Oda, Y., Nishiie, N., Otake, S., Koshima, R., Hirata, K., Taira, Y., Utoguchi, N., Negishi, Y., Nakagawa, S., Maruyama, K., 2010. Cancer gene therapy by IL-12 gene delivery using liposomal bubbles and tumoral ultrasound exposure. J. Control. Release 142, 245–250.

Suzuki, R., Takizawa, T., Negishi, Y., Hagisawa, K., Tanaka, K., Sawamura, K., Utoguchi, N., Nishioka, T., Maruyama, K., 2007. Gene delivery by combination of novel liposomal bubbles with perfluoropropane and ultrasound. J. Control. Release 117, 130–136.

Suzuki, R., Takizawa, T., Negishi, Y., Utoguchi, N., Maruyama, K., 2008a. Effective gene delivery with novel liposomal bubbles and ultrasonic destruction technology. Int. J. Pharm. 354, 49–55.

Suzuki, R., Takizawa, T., Negishi, Y., Utoguchi, N., Sawamura, K., Tanaka, K., Namai, E., Oda, Y., Matsumura, Y., Maruyama, K., 2008b. Tumor specific ultrasound enhanced gene transfer in vivo with novel liposomal bubbles. J. Control. Release 125, 137–144.

Taniyama, Y., Tachibana, K., Hiraoka, K., Aoki, M., Yamamoto, S., Matsumoto, K., Nakamura, T., Ogihara, T., Kaneda, Y., Morishita, R., 2002a. 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, 372–380.

Taniyama, Y., Tachibana, K., Hiraoka, K., Namba, T., Yamasaki, K., Hashiya, N., Aoki, M., Ogihara, T., Yasufumi, K., Morishita, R., 2002b. Local delivery of plasmid DNA into rat carotid artery using ultrasound. Circulation 105, 1233–1239.

Wang, X., Liang, H.D., Dong, B., Lu, Q.L., Blomley, M.J., 2005. Gene transfer with microbubble ultrasound and plasmid DNA into skeletal muscle of mice: comparison between commercially available microbubble contrast agents. Radiology 237, 224–229.

Zheng, C., Baum, B.J., 2005. Evaluation of viral and mammalian promoters for use in gene delivery to salivary glands. Mol. Ther. 12, 528–536.

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RESEARCH ARTICLE

Development of novel nucleic acid-loaded Bubble liposomes using cholesterol-conjugated siRNA

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Abstract

Recently, we developed polyethyleneglycol (PEG)-modified liposomes (Bubble liposomes; BLs) entrapping ultrasound (US) gas and reported that the combination of BLs and US exposure was an effective tool for the delivery of siRNA directly into cells and US-exposed tissues within a short period; however, the results were obtained using a mixture of BLs and naked siRNA. With systemic injections, it is important to control the biodistribution of both BLs and siRNA. In addition, the delivery of siRNA is affected by nuclease degradation and rapid removal from the circulation after intravenous administration. In this study, we attempted to prepare novel siRNA-loaded BLs (chol-si-BLs) using cholesterol-conjugated siRNA (chol-siRNA). We demonstrated that chol-siRNA could be loaded into BLs, leading to the stability of siRNA even in the presence of an RNase. The specific gene-silencing effect was also achieved by transfection with chol-si-BLs and US. Thus, the combination of chol-si-BLs with US exposure is expected to deliver siRNA into a specific tissue via systemic injection.

Keywords: siRNA delivery, ultrasound, microbubbles, chol-siRNA

Introduction

Recently, a combination of microbubbles and ultrasound (US) has been proposed as a less invasive and tissue-specific method of gene delivery. This combination produces transient changes in the permeability of the cell membrane and allows for the site-specific intracellular delivery of molecules such as dextran, pDNA, peptides, and siRNA both *in vitro* and *in vivo* (Taniyama et al., 2002a,b; Li et al., 2003; Unger et al., 2004; Kinoshita and Hynynen, 2005; Tsunoda et al., 2005; Sonoda et al., 2006); however, as existing microbubbles have issues with size, stability, and targeting function.

Polyethyleneglycol (PEG)-modified liposomes have excellent biocompatibility, stability, and a long circulation time, and can be easily prepared in a variety of sizes and modified to add a targeting function. For these reasons, they are widely used as carriers of drugs, antigens, and genes (Blume et al., 1990; Allen et al., 1991; Maruyama et al., 1992, 2004; Harata et al., 2004); therefore, PEG-liposomes containing a US-imaging gas could be used as novel gene delivery agents. We recently reported that "Bubble liposomes" (BLs) were suitable for gene delivery *in vitro* and *in vivo* (Suzuki et al., 2007, 2008a,b). Furthermore, we showed that BLs with US exposure was an effective tool for the delivery of siRNA *in vitro* and *in vivo* (Negishi et al., 2008); however, the results were obtained using a mixture of BLs and naked siRNA. With systemic injections, transfection efficiency is reduced if the BLs and siRNA are not colocalized in blood vessels; therefore, it is important to control the biodistribution of BLs and siRNA. In addition, siRNA is susceptible to nuclease degradation and rapid removal from the circulation after intravenous administration. In this study, we prepared siRNA-loaded BLs (chol-si-BLs) using cholesterol-conjugated siRNA (chol-siRNA) to overcome these issues. We also

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investigated the stability of chol-siRNA in the presence of RNase and the gene-silencing effects of transfection with chol-si-BLs and US.

Methods

Cell lines and cultures

COS-7 cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Kohjin Bio Co., Ltd., Tokyo, Japan) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Equitech Bio Inc., Kerrville, TX), 100 units/mL penicillin and 100 μg/mL streptomycin in a humidified atmosphere containing 5% CO_2 at 37°C. Colon26 cells stably expressing firefly luciferase (Colon26-Luc) were cultured in DMEM supplemented with 10% heat-inactivated FBS, 100 units/mL penicillin, 100 μg/mL streptomycin and 10 μg/mL puromycin in a humidified atmosphere containing 5% CO_2 at 37°C.

Preparation of liposomes and BLs

To prepare liposomes for conventional BLs, 1,2-dipalmitoyl-sn-glycero-phosphatidylcholine and 1,2-distearoylphosphatidylethanolamine-methoxy-polyethylene glycol (PEG_{2000}) were mixed at a molar ratio of 94:6. Both lipids were purchased from NOF Corporation (Tokyo, Japan). For the preparation of chol-si-BLs, an adequate amount of chol-siRNA was added to the lipid mixture. Liposomes were prepared by a reverse-phase evaporation method (REV method) as described previously (Negishi et al., 2008). In brief, all reagents were added to the solution of 1:1 (v/v) chloroform/diisopropylether. 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, UK). After being sized, the liposomes were passed through a sterile 0.45-μm syringe filter (Asahi Techno Glass Co., Chiba, Japan) for sterilization. To remove non-entrapped chol-siRNA, the liposomes were centrifuged twice at 1,00,000*g* for 1 h. 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 liposome suspension (lipid concentration: 1 mg/mL) were filled with perfluoropropane gas, capped, and then pressured with 7.5 mL 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 light scattering method with a zeta potential/particle sizer, (Nicomp 380ZLS, Santa Barbara, CA). Fluorescein isothiocyanate (FITC)-labeled siRNA and flow cytometry were used to examine the interaction between siRNA and BLs. The fluorescence intensity of chol-si-BLs was analyzed using a FACSCanto (Becton Dickinson, San Jose, CA).

Plasmid DNA and siRNA

The plasmid pCMV-GL3, derived from pGL3-basic (Promega, Madison, WI), is an expression vector encoding the firefly luciferase gene under the control of a cytomegalovirus promoter. Small interfering RNA targeting luciferase (siLuc) and a random control siRNA (siRandom) were modified with cholesterol (chol) at the 3′ end of the sense strand (Japan Bio Services Co., Ltd., Saitama, Japan). Their sequences were as follows: siLuc, sense: 5′-GAACUGUGUGUGAGAGGUCCU*chol-3′, and antisense: 5′-AGGACCUCUCACACACAGUUc*g*C-3′; siRandom, sense: 5′-AGCUAGUCGUAGUGGAUGCGU*chol-3′ and antisense: 5′-ACGCAUCCACUACGACUAG CUc*g*C-3′. Lower-case letters represent 2′-*O*-methyl-modified nucleotides; asterisks represent phosphorothioate linkages. For some experiments, siRNA with FITC label at the 5′-end of the antisense strand was used.

Stability of siRNA in serum

The chol-siRNA and chol-si-BLs were incubated in 0–5% RNase for 30 min. After incubation, chol-siRNA was extracted by phenol chloroform isoamyl alcohol. The stability of siRNA was confirmed by 20% acrylamide gel electrophoresis. The gel was stained with SYBER SAFE (Invitrogen Japan K.K., Tokyo, Japan) and visualized under ultraviolet light.

Transfection of siRNA and/or pDNA into cells using BLs or chol-si-BLs

COS-7 cells (3×10^4 cells/well) were seeded into the wells of a 48-well plate (Asahi Techno Glass Co., Chiba, Japan) on the day before transfection. Colon26-Luc cells (3×10^4 cells) were suspended in a tube on the day of transfection. Five micrograms of pDNA and 60 μg chol-si-BLs were mixed together with culture medium containing 10% FBS and added to the cells. The cells were immediately exposed to US (frequency, 2 MHz; duty, 50%; burst rate, 2.0 Hz; intensity, 2.5 W/cm^2) for 10 s through a 6-mm diameter probe placed in the well. A Sonopore 3000 (NEPA GENE, Co., Ltd., Chiba, Japan) was used to generate the US. The cells were washed twice with culture medium and cultured for 2 days.

To measure luciferase activity after transfection, cell lysate was prepared with a lysis buffer (0.1M Tris–HCl (pH 7.8), 0.1% Triton X-100, and 2 mM EDTA). Luciferase activity was measured using a luciferase assay kit (Promega, Madison, WI) and a luminometer (LB96V; Belthold Japan Co., Ltd., Tokyo, Japan). Activity was standardized as relative light units per mg of protein.

Statistical analyses

All data are the mean ± SD ($n=4$). Data were considered significant when $P<0.05$. The *t*-test was used to calculate statistical significance.

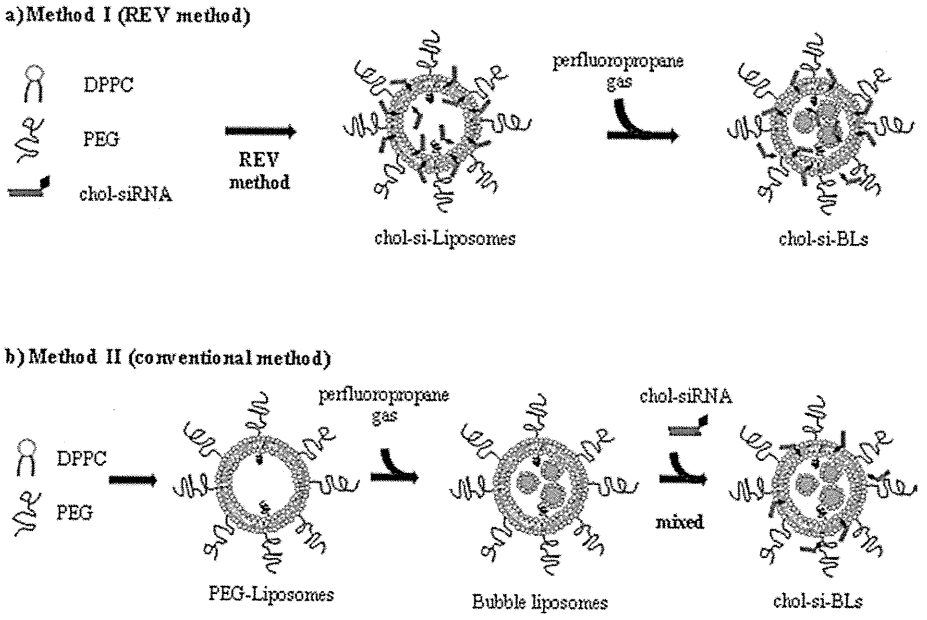

Figure 1. Scheme of chol-si-BLs prepared by two methods. (A) Method I; adding chol-siRNA to the lipid mixture and preparing liposomes by a REV method. (B) Method II; simply mixing BLs prepared by the conventional method and chol-siRNA.

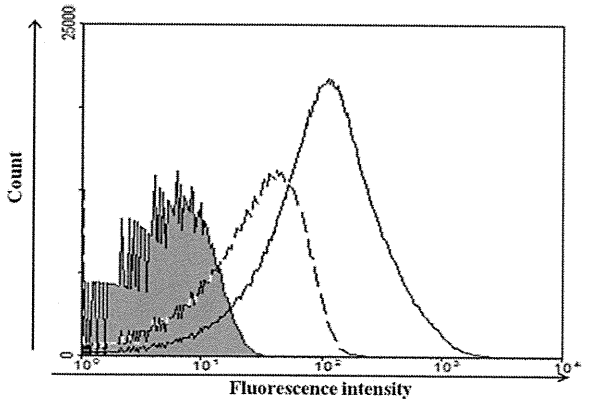

Figure 2. Interaction of chol-siRNA with BLs. The interaction was examined by analyzing a solution of chol-si-BLs with FACSCanto. Gray area: BLs only; solid line: chol-si-BLs prepared by Method I; dashed line: chol-si-BLs prepared by Method II; dotted line: chol-FITC-siRNA only.

Results

Preparation of chol-si-BLs

Initial experiments were performed to investigate the optimal method of preparation of chol-si-BLs (Figure 1). We attempted to add chol-siRNA to the lipid mixture, prepare liposomes by a REV method, and prepare BLs (Method I). We also attempted another method, that is, adding chol-siRNA to BLs prepared by the conventional method and mixing gently (Method II). To assess whether siRNA could be loaded into BLs prepared by each method, we used a fluorescence-activated cell sorter, the FACSCanto. As shown in Figure 2, BLs were successfully loaded with siRNA with both methods. The solution of chol-FITC-siRNA only could not be detected. Approximately 33% of the chol-si-BLs prepared by Method II were FITC positive. In contrast, the chol-si-BLs prepared by Method I were more heavily loaded with siRNA; approximately 80% were FITC positive. Thus, in all subsequent experiments, chol-si-BLs prepared by Method I were used. As shown in Table 1, there was almost no change in the size and zeta potential of the BLs after siRNA was added. We examined the change in the amount of siRNA bound to BLs when the concentration of siRNA was increased from 0.02 mol% to 0.4 mol% total lipids. As shown in Figure 3, the amount siRNA-loaded increased in a dose-dependent manner. We also investigated the stability of siRNA in the presence of RNase. The siRNA held by BLs showed increased stability in 0.1% RNase compared to free chol-siRNA, although some siRNA was degraded (Figure 4).

Co-transfection of pDNA and siRNA into cells using chol-si-BLs

To investigate the gene-silencing effects of siRNA transfection with chol-si-BLs and US, cells were co-transfected with pDNA encoding firefly luciferase (pCMV-GL3) and a random control or luciferase-targeting siRNA (siRandom or siLuc) in the concentration range of 0.02–0.4 mol% of total lipid (Figure 5A). Approximately 90% of luciferase expression was specifically blocked by siLuc of 0.1 mol% in the chol-si-BLs-treated group. We examined the effects of US exposure time on the downregulation of luciferase expression. As a result, transfection with chol-si-BLs and US exposure for only 5 s could deliver siRNA and pDNA into the cell; approximately 80% luciferase expression was specifically blocked (Figure 5B). The downregulation effects by siRNA were unaffected by the US exposure time, although luciferase expression was increased in

Table 1. Size (nm) and zeta potential (mV) of BLs and chol-si-BLs.

Lipid composition of BLs (molar ratio)	Mean size	zeta potential
BLs (DPPC: PEG2000 = 94:6)	519.2 ± 67.5 nm	−0.73 ± 0.12 mV
chol-si-BLs (DPPC:PEG2000: chol-siRNA = 94:6:0.1)	491.4 ± 55.9 nm	−0.47 ± 0.31 mV

DPPC, 1,2-dipalmitoyl-sn-glycero-phosphatidylcholine.

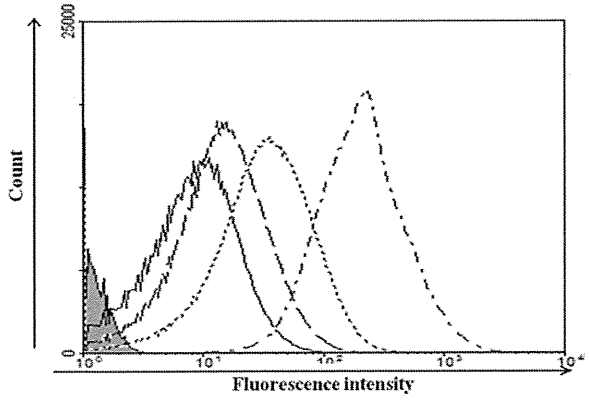

Figure 3. Loading of chol-siRNA into BLs. The interaction was examined by analyzing a solution of chol-si-BLs containing chol-FITC-siRNA (0.02–0.40 mol% of total lipid) with FACSCanto. Gray area: BLs only; solid line: chol-si-BLs (siRNA 0.02 mol%); dashed line: chol-si-BLs (siRNA 0.06 mol%); dotted line: chol-si-BLs (siRNA 0.10 mol%); dashed-dotted line: chol-si-BLs (siRNA 0.40 mol%).

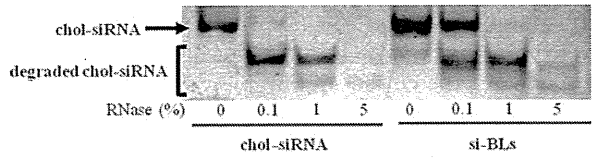

Figure 4. Stability of siRNA in the presence of RNase. Naked chol-siRNA or chol-si-BLs were subjected to 0–5% RNase degradation at 37°C for 30 min and confirmed by 20% acrylamide gel electrophoresis.

an US exposure time-dependent manner. Cytotoxicity was absent after US exposure within 10 s (Figure 5C). We also investigated the effects of US intensity ranging from 0.5 to 2.5 W/cm². The downregulation effects by siRNA were unaffected by the US intensity and cytotoxicity was absent after transfection (data not shown).

Transfection of siRNA using chol-si-BLs into stable cell lines

We performed siRNA transfection into Colon26-Luc to examine the effects of siRNA against endogenous gene expression. As shown in Figure 6A, there were no effects of specific gene-silencing; therefore, we attempted siRNA transfection into the cells in suspension. As a result, approximately 50% luciferase expression was specifically blocked by siLuc of 0.1 mol% in the chol-si-BLs-treated group (Figure 6B); however, the

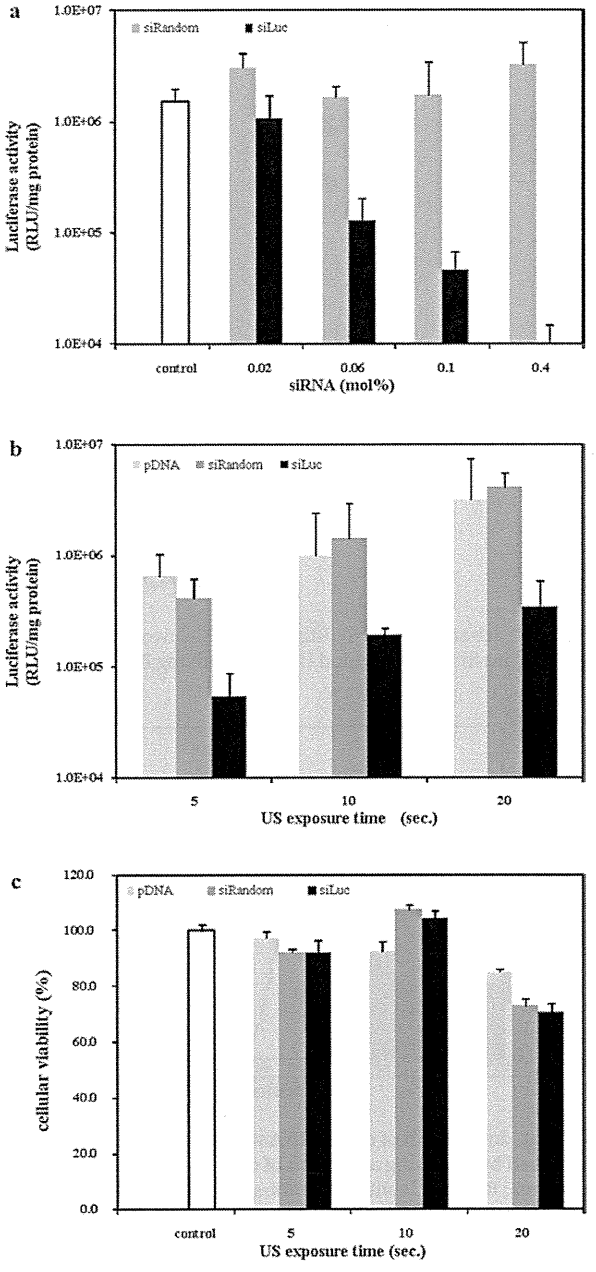

Figure 5. Downregulation of luciferase expression by siRNA. Luciferase expression in COS-7 cells transfected with pDNA and siRNA using chol-si-BLs and ultrasound (US) (frequency, 2 MHz; duty, 50%; burst rate, 2.0 Hz; intensity 2.5 W/cm²; time, 10 s) at 2-days post-transfection. (A) Effect of the amount of siRNA on gene-silencing effects. Control: group transfected with pCMV-GL3 only, siRandom: group transfected with pCMV-GL3 and non-targeting siRNA (siRandom), siLuc: group transfected with pCMV-GL3 and siRNA targeting luciferase (siLuc). (B) Effect of US exposure time on gene-silencing effects. (C) Effect of US exposure time on cellular viability. pDNA: group transfected with pCMV-GL3 only, siRandom: group transfected with pCMV-GL3 and non-targeting siRNA (siRandom), siLuc: group transfected with pCMV-GL3 and siRNA targeting luciferase (siLuc). All data are reported as the mean ± SD (n=4).

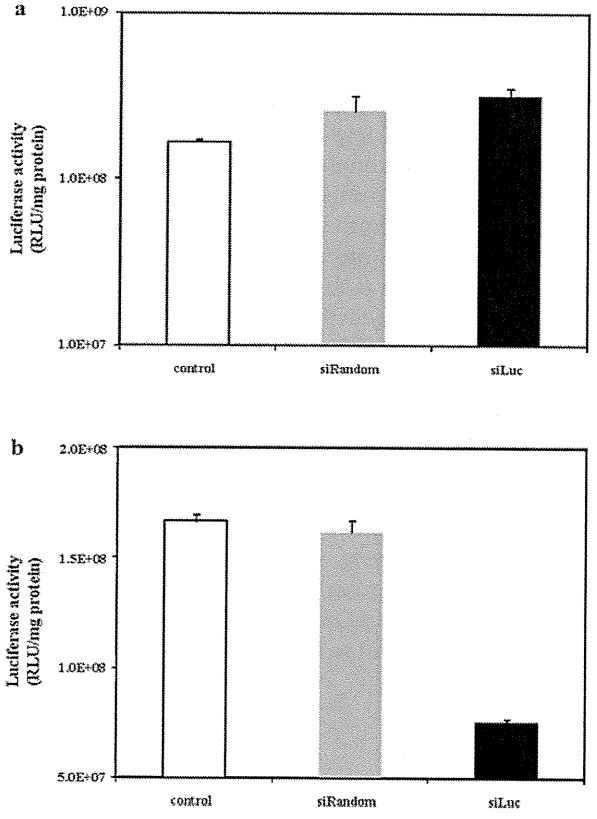

Figure 6. Downregulation of luciferase expression by siRNA. Luciferase expression in Colon26-Luc cells transfected with siRNA using chol-si-BLs and US at 2-days post-transfection. (A) Effect of chol-siRNA transfection into cells under adherent conditions. (B) Effect of chol-siRNA transfection into cells in suspension. Control: untreated group, siRandom: group transfected with non-targeting siRNA (siRandom), siLuc: group transfected with siRNA targeting luciferase (siLuc). All data are reported as the mean ± SD ($n=4$).

experimental conditions of transfection decreased cell viability (data not shown).

Discussion

The specific silencing of gene expression through RNAi has great potential to address previously untreatable diseases. The safe and effective delivery of siRNA, however, remains challenging. We have developed BLs and shown that they were an effective and novel tool for gene and siRNA delivery *in vitro* and *in vivo* (Suzuki et al., 2007, 2008a,b; Negishi et al., 2008). The siRNA transfection by the combination of BLs and US exposure delivers siRNA into only US-exposed area after systemic injection; however, our previous data were obtained using a mixture of BLs and naked siRNA. Therefore, BLs and siRNA did not colocalize in blood vessels after intravenous administration. Additionally, siRNA is susceptible to rapid removal from the circulation and degradation by nucleases, leading to a reduction in transfection efficiency *in vivo*. In this study, we prepared chol-si-BLs-containing chol-siRNA as a more effective, efficient delivery tool for systemic injections. Cholesterol is often used for the preparation of liposomes, and thus chol-siRNA seems to embed easily in the lipid bilayer.

We initially investigated the optimal method for preparation of chol-si-BLs, and then used flow cytometry to examine the interaction between siRNA and BLs. The BLs were detectable, although the fluorescence intensity was low. Fluorescein-labeled siRNA molecules were too small to be detected with flow cytometry; however, the chol-si-BLs exhibited strong fluorescence. We compared Method I (adding chol-siRNA to the lipid mixture and preparing liposomes by REV method) with Method II (simply mixing BLs and chol-siRNA). As shown in Figure 2, BLs could be loaded with much more chol-siRNA by Method I, although chol-siRNA could interact with BLs both methods were used. These results suggested that chol-siRNA of chol-si-BLs by Method I might not only be embedded in the lipid layer but also encapsulated in the liposomes. Additionally, we examined the change in the amount of bound chol-siRNA by adding various amounts of chol-siRNA to the lipid mixture. As shown in Figure 3, the amount of chol-siRNA loaded into BLs increased with chol-siRNA addition in a dose-dependent manner for up to 0.4 mol% of the total lipid content. We also investigated the stability of siRNA interacting with BLs in RNase. Although some siRNA was degraded, siRNA held by BLs showed increased stability in 0.1% RNase compared to free chol-siRNA (Figure 4). The chol-siRNA embedded in the outside lipid layer of BLs seemed susceptible to degradation by nucleases. It has been reported that the modification of liposomes with short and long PEG chains increases the fixed aqueous layer thickness (Sadzuka et al., 2002). In this study, we prepared BLs with only one type of PEG chain length; therefore, BLs with short and long PEG might improve the stability of siRNA. In Figure 4, the electrophoresis data also indicated that there was no damage to chol-siRNA from sonication for the preparation of chol-si-BLs. The fact was also supported by the gene-silencing effects by chol-siRNA in Figure 5 and 6.

We examine the gene-silencing effects of chol-siRNA transfected chol-si-BLs and US. The expression of luciferase in cells was efficiently inhibited in a siLuc dose-dependent manner (Figure 5A). The concentration range of 0.02–0.4 mol% of the total lipid, was equivalent to 25–500 nM. Approximately 80% luciferase expression was specifically blocked by siLuc of more than 0.06 mol%. The condition of US exposure affects the efficiency of US-mediated gene transfection (Duvshani-Eshet and Machluf, 2005; Mitragotri, 2005); therefore, we also investigated the effects of US exposure time on the downregulation of luciferase expression (Figure 5B and 5C). As a result, approximately 80% luciferase expression was specifically blocked and cytotoxicity was absent after transfection within 10 s. The downregulation effects by siRNA were unaffected by intensity of the US; thus, showing that the US condition (frequency, 2 MHz; duty, 50%; burst rate, 2.0 Hz; intensity, 2.5 W/cm^2; time,

10 s) were appropriate for siRNA transfection by chol-si-BLs. These results correlate with our previous study (Negishi et al., 2008). Interestingly, the downregulation effects by siRNA were unaffected by the US exposure time, although luciferase expression was increased in an US exposure time-dependent manner. There are two possible reasons for this result. First, the size of molecule may effect on the transport of molecules via transient pores by cavitation. Second, the US exposure time may effect on the transport amount of pDNA to the nucleus. Plasmid DNA needs to enter the nucleus for effective therapy. On the other hand, siRNA is known to function in the cytosol. The detailed mechanism of this transfection method remains unknown and should be studied in the future. It was also pointed out that the exposure to US induced cavitation, the release of siRNA from chol-si-BLs, and the delivery of siRNA into the cytoplasm within a fairly short time; however, it is necessary to perform the experiment with siRNA targeting endogenous genes considering the application of synthetic siRNA to the treatment of disease. We then attempted siRNA transfection into the cells stably expressing firefly luciferase, Colon26-Luc cells. There were no effects of specific gene-silencing by siRNA transfection under the same conditions used so far (Figure 6A). This result suggested that the amount of siRNA transfected into cells was insufficient for the downregulation of endogenous gene expression. We therefore changed the transfection conditions using cells in suspension, not in an adherent condition. As shown in Figure 6B, approximately 50% luciferase expression was specifically blocked, although cell viability was decreased. These data demonstrated that the suspended cells were more likely to be affected by US energy than adherent cells and the amount of siRNA transfected into cells was increased. It is expected that the combination of chol-si-BLs and US exposure will be a safe and an effective siRNA transfection system after a more detailed examination of the US conditions. Cholesterol is one of the major components of the eukaryotic cell membrane, and is highly distributed in plasma membranes (Lange et al., 1989). Cholesterol fits its non-polar part between alkyl chains of phospholipids, and is appropriate for incorporation into the phospholipid bilayer due to its easy-to-fit structure and its physical influence on membrane properties such as fluidity and permeability. Thus, cholesterol is often used to prepare liposomes as not only membrane models but also drug carriers. BLs stability was considered to be unaffected by the cholesterol content in this study (0.02–0.4 mol% of total lipid). Indeed, liposomes containing chol-siRNA entrapped the gas and could be used as US contrast agents as well as conventional BLs without cholesterol (data not shown). In addition, chol-siRNA might not be easy to release from the lipid bilayer, although it seemed to embed easily. The amount of chol-siRNA transfected into cells would be efficiently increased if its release rate from lipid by US exposure could be increased. In the future, we will examine siRNA delivery via systemic injection and its own disease-associated gene-silencing effects. It was reported that chol-siRNA could improve pharmacokinetic and cellular uptake in mice via systemic injection (Soutschek et al., 2004). The mechanisms of improved distribution and cellular uptake of siRNA through cholesterol conjugation were also demonstrated in a recent study; chol-siRNA seemed to be incorporate into circulating lipoprotein particles and was efficiently internalized via receptor-mediated processes (Wolfrum et al., 2007). Therefore, chol-siRNA alone tends to be delivered to limited tissues such as the liver, adrenal, and kidney; however, the chol-si-BLs developed in this study enabled site-specific siRNA delivery to other tissues in combination with transdermal US exposure. Additionally, microbubbles modified with an antibody and having a targeting function have recently been developed (Leong-Poi et al., 2005; Behm et al., 2008; Palmowski et al., 2008). Liposomes can be easily modified to add a targeting function; thus, chol-si-BLs using an antibody or peptide will enable more efficient siRNA delivery only to target cells in combination with US exposure and lead to beneficial clinical applications for various diseases.

Conclusion

In this study, we showed that BLs could efficiently load chol-siRNA and protect siRNA against nuclease degradation. Additionally, chol-si-BLs could deliver siRNA to cells, although there remains room for improvement. These results suggest that chol-si-BLs with US exposure may be useful for delivering siRNA to a tissue or organ via systemic injection.

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Declaration of interest

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References

Allen TM, Hansen C, Martin F, Redemann C, Yau-Young A. (1991). Liposomes containing synthetic lipid derivatives of poly(ethylene glycol) show prolonged circulation half-lives *in vivo*. Biochim Biophys Acta, 1066, 29–36.

Behm CZ, Kaufmann BA, Carr C, Lankford M, Sanders JM, Rose CE, Kaul S, Lindner JR. (2008). Molecular imaging of endothelial vascular cell adhesion molecule-1 expression and inflammatory cell recruitment during vasculogenesis and ischemia-mediated arteriogenesis. Circulation, 117, 2902–2911.

Blume G, Cevc G. (1990). Liposomes for the sustained drug release *in vivo*. Biochim Biophys Acta, 1029, 91–97.

Duvshani-Eshet M, Machluf M. (2005). Therapeutic ultrasound optimization for gene delivery: A key factor achieving nuclear DNA localization. J Control Release, 108, 513–528.

Harata M, Soda Y, Tani K, Ooi J, Takizawa T, Chen M, Bai Y, Izawa K, Kobayashi S, Tomonari A, Nagamura F, Takahashi S, Uchimaru K, Iseki T, Tsuji T, Takahashi TA, Sugita K, Nakazawa S, Tojo A, Maruyama K, Asano S. (2004). CD19-targeting liposomes containing imatinib efficiently kill Philadelphia chromosome-positive acute lymphoblastic leukemia cells. Blood, 104, 1442–1449.

Kinoshita M, Hynynen K. (2005). A novel method for the intracellular delivery of siRNA using microbubble-enhanced focused ultrasound. Biochem Biophys Res Commun, 335, 393–399.

Lange Y, Swaisgood MH, Ramos BV, Steck TL. (1989). Plasma membranes contain half the phospholipid and 90% of the cholesterol and sphingomyelin in cultured human fibroblasts. J Biol Chem, 264, 3786–3793.

Leong-Poi H, Christiansen J, Heppner P, Lewis CW, Klibanov AL, Kaul S, Lindner JR. (2005). Assessment of endogenous and therapeutic arteriogenesis by contrast ultrasound molecular imaging of integrin expression. Circulation, 111, 3248–3254.

Li T, Tachibana K, Kuroki M, Kuroki M. (2003). Gene transfer with echo-enhanced contrast agents: Comparison between Albunex, Optison, and Levovist in mice-initial results. Radiology, 229, 423–428.

Maruyama K, Ishida O, Kasaoka S, Takizawa T, Utoguchi N, Shinohara A, Chiba M, Kobayashi H, Eriguchi M, Yanagie H. (2004). Intracellular targeting of sodium mercaptoundecahydrododecaborate (BSH) to solid tumors by transferrin-PEG liposomes, for boron neutron-capture therapy (BNCT). J Control Release, 98, 195–207.

Maruyama K, Yuda T, Okamoto A, Kojima S, Suginaka A, Iwatsuru M. (1992). Prolonged circulation time *in vivo* of large unilamellar liposomes composed of distearoyl phosphatidylcholine and cholesterol containing amphipathic poly(ethylene glycol). Biochim Biophys Acta, 1128, 44–49.

Mitragotri S. (2005). Healing sound: The use of ultrasound in drug delivery and other therapeutic applications. Nat Rev Drug Discov, 4, 255–260.

Negishi Y, Endo Y, Fukuyama T, Suzuki R, Takizawa T, Omata D, Maruyama K, Aramaki Y. (2008). Delivery of siRNA into the cytoplasm by liposomal bubbles and ultrasound. J Control Release, 132, 124–130.

Palmowski M, Huppert J, Ladewig G, Hauff P, Reinhardt M, Mueller MM, Woenne EC, Jenne JW, Maurer M, Kauffmann GW, Semmler W, Kiessling F. (2008). Molecular profiling of angiogenesis with targeted ultrasound imaging: Early assessment of antiangiogenic therapy effects. Mol Cancer Ther, 7, 101–109.

Sadzuka Y, Nakade A, Hirama R, Miyagishima A, Nozawa Y, Hirota S, Sonobe T. (2002). Effects of mixed polyethyleneglycol modification on fixed aqueous layer thickness and antitumor activity of doxorubicin containing liposome. Int J Pharm, 238, 171–180.

Sonoda S, Tachibana K, Uchino E, Okubo A, Yamamoto M, Sakoda K, Hisatomi T, Sonoda KH, Negishi Y, Izumi Y, Takao S, Sakamoto T. (2006). Gene transfer to corneal epithelium and keratocytes mediated by ultrasound with microbubbles. Invest Ophthalmol Vis Sci, 47, 558–564.

Soutschek J, Akinc A, Bramlage B, Charisse K, Constien R, Donoghue M, Elbashir S, Geick A, Hadwiger P, Harborth J, John M, Kesavan V, Lavine G, Pandey RK, Racie T, Rajeev KG, Röhl I, Toudjarska I, Wang G, Wuschko S, Bumcrot D, Koteliansky V, Limmer S, Manoharan M, Vornlocher HP. (2004). Therapeutic silencing of an endogenous gene by systemic administration of modified siRNAs. Nature, 432, 173–178.

Suzuki R, Takizawa T, Negishi Y, Hagisawa K, Tanaka K, Sawamura K, Utoguchi N, Nishioka T, Maruyama K. (2007). Gene delivery by combination of novel liposomal bubbles with perfluoropropane and ultrasound. J Control Release, 117, 130–136.

Suzuki R, Takizawa T, Negishi Y, Utoguchi N, Maruyama K. (2008a). Effective gene delivery with novel liposomal bubbles and ultrasonic destruction technology. Int J Pharm, 354, 49–55.

Suzuki R, Takizawa T, Negishi Y, Utoguchi N, Sawamura K, Tanaka K, Namai E, Oda Y, Matsumura Y, Maruyama K. (2008b). Tumor specific ultrasound enhanced gene transfer *in vivo* with novel liposomal bubbles. J Control Release, 125, 137–144.

Taniyama Y, Tachibana K, Hiraoka K, Aoki M, Yamamoto S, Matsumoto K, Nakamura T, Ogihara T, Kaneda Y, Morishita R. (2002a). 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, 372–380.

Taniyama Y, Tachibana K, Hiraoka K, Namba T, Yamasaki K, Hashiya N, Aoki M, Ogihara T, Yasufumi K, Morishita R. (2002b). Local delivery of plasmid DNA into rat carotid artery using ultrasound. Circulation, 105, 1233–1239.

Tsunoda S, Mazda O, Oda Y, Iida Y, Akabame S, Kishida T, Shin-Ya M, Asada H, Gojo S, Imanishi J, Matsubara H, Yoshikawa T. (2005). Sonoporation using microbubble BR14 promotes pDNA/siRNA transduction to murine heart. Biochem Biophys Res Commun, 336, 118–127.

Unger EC, Porter T, Culp W, Labell R, Matsunaga T, Zutshi R. (2004). Therapeutic applications of lipid-coated microbubbles. Adv Drug Deliv Rev, 56, 1291–1314.

Wolfrum C, Shi S, Jayaprakash KN, Jayaraman M, Wang G, Pandey RK, Rajeev KG, Nakayama T, Charrise K, Ndungo EM, Zimmermann T, Koteliansky V, Manoharan M, Stoffel M. (2007). Mechanisms and optimization of *in vivo* delivery of lipophilic siRNAs. Nat Biotechnol, 25, 1149–1157.