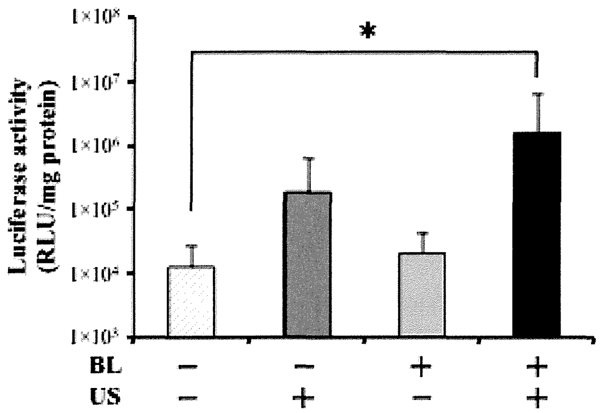

Fig. 3. Luciferase activity in gingival tissue transfected using Bubble liposomes (BL) and ultrasound (US) under optimized conditions. Rats were treated with BL and US-mediated luciferase gene delivery. Relative luciferase activity [measured as relative light units (RLU)] was determined 1 d after transfection. Data are shown as mean ± SD *$p < 0.05$, Mann–Whitney *U*-test ($n = 5$), significantly different from the group treated with a pDNA injection only. pDNA (pCMV-Luciferase), 10 μg; BL, 5 μg; US conditions: frequency, 1 MHz; duty cycle, 50%; intensity, 2.0 W/cm^2; time, 30 s.

enhance or suppress the generation of target proteins. Two main delivery carriers of genes – viral and nonviral vectors – are currently being used for this purpose. While viral vectors are known to be excellent carriers of genes, they are associated with immunogenicity and carcinogenicity (19–21). Therefore, many researchers have reported physical or chemical methods independent of virus. Since Fechheimer *et al.* (22) first reported the US-mediated gene-delivery technique in 1987, therapeutic US has been used as a convenient device to deliver genes or drugs into target tissues. The application of US provides precise target-directivity, with the delivery effect being observed in the US exposure area only. Thus, therapeutic molecules, such as genes, drugs, peptides or recombinant proteins, can be delivered by local administration into the target tissue with a cavitating jet generated by the physical action of US. Our results showed intense distinct expression of EGFP in the US exposure area, especially in the middle of the injected gingiva. When US is utilized to deliver genes or drugs, we can control the depth of focus and the exposure range by changing the wavelength or intensity. However, these parameters need to be optimized specifically for use in each type of target tissue, because an infinite combination of US parameters has been theoretically suggested.

Recently, transfection efficacy was shown to be enhanced by combining US energy with microbubbles (23–26). Microbubbles are known to serve as artificial cavitation nuclei and reduce the threshold of cavitation generated by US (27). We also developed a unique carrier, BL, and reported that the BL-mediated US gene-delivery system enhanced transfection efficiency both *in vitro* and *in vivo* (12–14,28–31). Chen *et al.* (32) demonstrated gene delivery into the gingiva of mice using original nano/microbubbles and US. They showed that delivery efficiency increased when mice were administered a luciferase gene by nano/microbubbles and US exposure; however, high luciferase activity was observed for 1 d only. Moreover, using histological observations, they showed that the transfected cells in gingival tissues were muscle cells. In our results, expression peaked 1 d after the gene-delivery procedure, and relative luciferase activity decreased after 3 d. A previous report showed that transfection efficiency was higher, and gene expression was longer, with repeated US exposure than with single US exposure (33). To extend the duration of the transfection effect while maintaining treatment efficacy, it may be necessary to repeat the delivery procedure. On the other hand, we previously reported, using the same delivery technique, that gene-transfection efficiency into the tongue tissue of mice was maintained for about 10 d with a single treatment (16). Skeletal muscle is one of the candidate target tissues of gene therapy, and stability and longevity after gene delivery is useful for the treatment of diseases. As the characteristics of the target cell may also affect the duration of gene expression, we need to investigate in detail whether sufficient treatment efficacy can be acquired in gingival tissue by gene delivery with BL and US.

As shown in Fig. 4, distinct EGFP-expressing cells were observed in both the gingival epithelium and the connective tissue layer. As the mixture of BL and plasmid DNA was almost wholly diffused in the injected labial gingiva, it is difficult to distinguish between delivery to epithelial tissue and to connective tissue. Although US may localize the delivery area to a specific part of the whole body, other devices are required to distinguish detailed objects, such as cells. For the delivery of anticancer agents, a study was performed using a more delicate technique involving the modification of targeting peptides on the surface of BL before transfection (34). Modified-BL may improve targeting ability at the cellular level. The type of target tissue and delivery efficiency may be affected by exposure to US energy or by the properties of BL, including size, lipid composition and encapsulating gas. Therefore, optimizing conditions in the BL and for the US-mediated delivery technique are necessary for specific tissues. In this study, we optimized the US parameter (2.0 W/cm^2, 30 s) to enhance the efficiency of delivery into gingival tissue. These optimized conditions show the same tendency also in our previous report (16). The characterization of the target tissue could have been influenced a delivery efficiency. For example, the alveolar bone exists just under the gingiva. As the depth of US propagation is different in soft tissue and hard tissue, the composition of target tissue and adjacent tissue may affect the biological response. Moreover, the heat generated by US

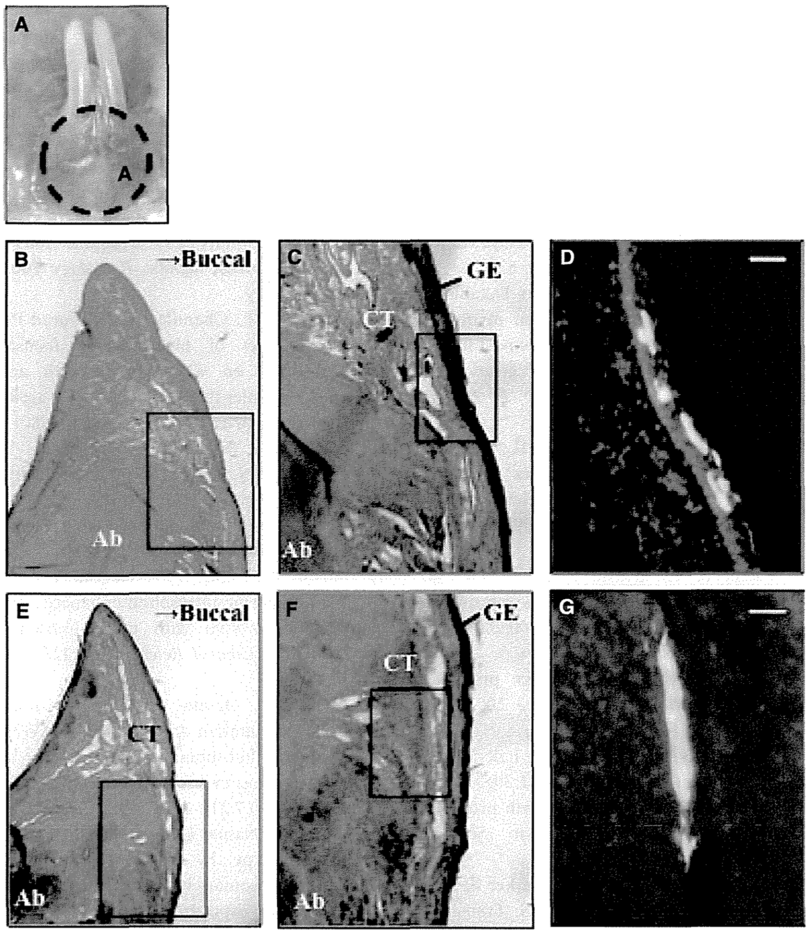

Fig. 4. Localization of enhanced green fluorescent protein (EGFP)-expressing cells. Gingiva treated with Bubble liposomes (BL) and ultrasound (US)-mediated gene delivery were inspected to identify the type of cells that expressed EGFP. Intense EGFP fluorescence was localized in the gingival epithelial layer and the connective tissue layer. No inflammatory cell infiltration was observed in transfected gingival tissues. (A) The transfection area is shown in the dotted line circle. (B, E) Hematoxylin and eosin (H&E) staining in the sagittal sections of rat lower gingiva transfected with BL using US. (C, F) Higher magnifications of B and E (square). (D) EGFP expression in the gingival epithelium layer at a higher magnification of C (square). (G) EGFP expression in the connective tissue layer at a higher magnification of F (square). Blue, DAPI (used for nuclear staining); green, EGFP. Ab, alveolar bone; CT, connective tissue; GE, gingival epithelium. Scale bar: 200 μm.

exposure may cause local tissue damage. Therefore, prolonged US exposure time may lead to decreased transfection efficiency.

Our study is the first to show gene expression in gingival epithelial cells and connective tissue cells, but not in skeletal muscle cells, using a delivery technique combining BL and US. A reporter plasmid was used in this study to examine whether effective gene delivery into the gingiva was achieved when BL and US were used together. Our technique, using BL and US to deliver plasmid DNA into periodontal tissue, is applicable not only for plasmids, but also for peptides, drugs and small interfering RNA. As such molecules have lower molecular mass values than plasmids, transfection may result in deeper penetration of such molecules into tissues, which suggests that our system may be a useful local drug-delivery system for periodontal therapy.

In conclusion, the results of this study demonstrated that the most efficient conditions for US energy for gene delivery into rat gingival tissue using BL and US were US intensity of 2.0 W/cm^2 and US exposure time of 30 s. In the future, our gene-delivery method using BL and US may become a beneficial treatment for patients with periodontitis.

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We are grateful to Dr Katsuro Tachibana (Department of Anatomy, School of Medicine, Fukuoka University) for his technical advice regarding the induction of cavitation with US, to Mr Yasuhiko Hayakawa and Mr Kosho Suzuki (NEPA GENE Co., Ltd.) for their technical advice regarding US exposure, and to Dr Kenji Mishima (Department of Oral Pathology and Diagnosis, School of Dentistry, Showa University) for his advice regarding histological observation. This study was supported, in part, by the Industrial Technology Research Grant Program (04A05010) from New Energy, the Industrial Technology Development Organization (NEDO) of Japan.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Histological analysis of local cell viability. Gingival sections (NADH-TR and H&E staining) at higher magnification from rats treated with BL and US-mediated gene delivery. The gingival tissues were examined histologically to assess cytotoxicity. (A): Untreated groups, the gingival epithelium area (a, b) and the connective tissue area (c, d). (B): BL+US groups, the gingival epithelium area (e, f) and the connective tissue area (g, h). pDNA (pCAG-EGFP): 10 μg; BL: 5 μg; US conditions: Frequency: 1 MHz, Duty cycle: 50%, Intensity: 2.0 W/cm^2, Time: 30 s. BL, Bubble liposomes; US, Ultrasound. Scale bar: 50 μm.

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ORIGINAL ARTICLE

Thrombus-targeted perfluorocarbon-containing liposomal bubbles for enhancement of ultrasonic thrombolysis: *in vitro* and *in vivo* study

K. HAGISAWA,* T. NISHIOKA,† R. SUZUKI,‡ K. MARUYAMA,‡ B. TAKASE,§ M. ISHIHARA,§ A. KURITA,§ N. YOSHIMOTO,† Y. NISHIDA,* K. IIDA,¶ H. LUO¶ and R. J. SIEGEL¶

**Department of Physiology, National Defense Medical College, Tokorozawa; †Division of Cardiology, Saitama Medical Center, Saitama Medical University, Kawagoe; ‡Department of Drug Delivery System, School of Pharmaceutical Sciences, Teikyo University, Tokyo; §Division of Biomedical Engineering, National Defense Medical College Research Institute, Tokorozawa, Japan; and ¶Cardiac Non-invasive Laboratory, Heart Institute, Cedars-Sinai Medical Center, Los Angeles, CA, USA*

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Summary. *Background:* External low-frequency ultrasound (USD) in combination with microbubbles has been reported to recanalize thrombotically occluded arteries in animal models. *Objective:* The purpose of this study was to examine the enhancing effect of thrombus-targeted bubble liposomes (BLs) developed for fresh thrombus imaging during ultrasonic thrombolysis. *Methods: In vitro*: after the administration of thrombus-targeted BLs or non-targeted BLs, the clot was exposed to low-frequency (27 kHz) USD for 5 min. *In vivo*: Rabbit iliofemoral arteries were thrombotically occluded, and an intravenous injection of either targeted BLs (n = 22) or non-targeted BLs (n = 22) was delivered. External low-frequency USD (low intensity, 1.4 W cm^{-2}, to 12 arteries, and high intensity, 4.0 W cm^{-2}, to 10 arteries, for both the targeted BL group and the non-targeted BL group) was applied to the thrombotically occluded arteries for 60 min. In another 10 rabbits, recombinant tissue-type plasminogen activator (rt-PA) was intravenously administered. *Results: In vitro*: the weight reduction rate of the clot with targeted BLs was significantly higher than that of the clot with non-targeted BLs. *In vivo*: TIMI grade 3 flow was present in a significantly higher number of rabbits with USD and targeted BLs than rabbits with USD and non-targeted BLs, or with rt-PA monotherapy. High-intensity USD exposure with targeted BLs achieved arterial recanalization in 90% of arteries, and the time to reperfusion was shorter than with rt-PA treatment (targeted BLs, 16.7 ± 5.0 min; rt-PA, 41.3 ± 14.4 min). *Conclusions:* Thrombus-targeted BLs developed for USD thrombus imaging enhance ultrasonic disruption of thrombus both *in vitro* and *in vivo*.

Keywords: drug targeting, liposomes, RGD peptide, thrombolytic therapy, ultrasound.

Correspondence: Toshihiko Nishioka, Division of Cardiology, Saitama Medical Center, Saitama Medical University, 1981, Kamoda, 350-8550 Kawagoe, Japan.
Tel.: +81 49 228 3587; fax: +81 49 226 5274.
E-mail: nishioka@saitama-med.ac.jp

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Introduction

Most life-threatening cardiovascular events, including acute coronary syndrome and ischemic stroke, are caused by arterial thrombosis. Acute ST-elevation myocardial infarction (STEMI) is characterized by atherosclerotic plaque rupture and occlusive thrombus formation associated with platelet aggregation [1,2]. Percutaneous coronary intervention (PCI) and fibrinolysis are the standard therapeutic strategies for recanalizing thrombotically occluded arteries in patients with STEMI [3]. Primary PCI is performed in most of the STEMI patients presenting to a PCI-capable facility, with a cardiac catheterization laboratory, an interventional cardiologist, and the appropriate specialized staff and equipment to perform acute PCI. Enzymatic fibrinolysis for the treatment of STEMI is less invasive and logistically more convenient; however, this option gives a lower initial recanalization rate, and a higher incidence of coronary reocclusion and life-threatening systemic bleeding, and may result in worse short-term and long-term clinical outcomes than direct PCI [4,5]. For ischemic stroke treatment, fibrinolysis is recommended only for selected patients who can be treated within 4.5 h of onset, owing to the substantial risk of intracranial hemorrhage [6–8].

To overcome the limitations of conventional fibrinolytic therapy, the cavitational and non-cavitational effects of ultrasound (USD) have been studied and tested in conjunction with thrombolytic agents to facilitate thrombus disruption [9–16]. Treatment without the use of a thrombolytic agent, but with the combination of echo contrast microbubbles and USD, has been found to be effective *in vitro* and *in vivo*. This has been theorized to be attributable to a lower cavitational threshold and enhanced microstreaming phenomena when microbubbles in conjunction with USD are used [17–23]. *In vivo*, transcutaneous USD in combination with microbubbles has been reported to recanalize thrombotically occluded iliofemoral, coronary and ascending pharyngeal arteries [18,20–22], reduce infarction size [23] and improve microvascular recovery [20] in animal models, without significant side effects. Clinical trials using transcutaneous USD with microbubbles in the setting of ischemic stroke have not been conducted; instead, a combination of USD, microbubbles and thrombolytic agents has been examined. This combination strategy improves recanalization rates and preserves brain function as compared with USD and thrombolytic agents without microbubbles [24–27]. However, an increased number of intracranial hemorrhages has also been reported [27].

In order to non-invasively detect thrombus location, we manufactured thrombus-targeted liposomal bubbles (bubble liposomes [BLs]), which we also expected to enhance ultrasonic clot disruption. These BLs may avoid the need for invasive angiography to identify the thrombotically occluded site prior to the application of therapeutic USD in *in vivo* studies [18,20–22]. The BL was composed of perfluorocarbon gas-containing nanosized liposomes with Arg-Gly-Asp (RGD) sequence peptides on their surface lipid layer, which attach glycoprotein IIb–IIIa complex on activated platelets and enhance the visualization of fresh thrombus by conventional diagnostic ultrasound examination [28]. We hypothesized that these thrombus-targeted BLs could also enhance disruption with the use of therapeutic external USD, and could be used to develop a fully non-invasive diagnostic and therapeutic system for the treatment of thrombotic vessel occlusion. The aim of this study was to examine the enhancing effect of the newly developed thrombus-targeted liposomal bubbles on ultrasonic disruption of the thrombus *in vitro* and *in vivo*.

Materials and methods

Preparation of thrombus-targeted BLs

Liposome-based perfluorocarbon-containing BLs were composed of 126 mg of 1,2-disteoyl-*sn*-glycero-phosphocholine (Avanti, Alabaster, AL, USA), 51 mg of 1,2-distearoyl-*sn*-glycero-3-phosphatidylethanolamine-*m*-polyethylene glycol 2000 with maleimide (Avanti), 30 mg of cholesterol (Sigma-Aldrich, Tokyo, Japan), CGGGRGDF peptide (Operon Biotechnologies, Tokyo, Japan), and perfluoropropane gas (Takachiho, Tokyo, Japan). We previously reported the manufacture of these liposome-based perfluorocarbon-containing BLs [28]. In brief, a mixture of all reagents except for CGGGRGDF peptide and perfluoropropane gas was dissolved in 2.0 mL of chloroform, and mixed with the same amount of di-isopropyl ether and normal saline. The mixture was sonicated, with a probe-type 19.5-kHz ultrasound device at 550 W (XL-2020 Sonicator; Misonix, Farmingdale, NY, USA), and then evaporated at 65 °C with a rotary evaporating system (Tokyo Rika, Tokyo, Japan). After the chemical solvent had been completely removed, the size of liposomes was adjusted to < 0.2 μm with extruding equipment and a membrane filter (Northern Lipids, Vancouver, Canada) with sizing filters. To the liposome liquid, 1 mg of linear octapeptide with the sequence CGGGRGDF (Operon Biotechnologies) was added, and allowed to conjugate to the maleimide on the liposomal surface via thio–ether covalent coupling at room temperature for 2 h. Gel filtration was then used to remove unreacted peptide fragments. The lipid concentration was measured with the Wako Phospholipid C test (Wako Pure Chemical Industries, Osaka, Japan) and the RGD-liposomes were diluted to a final concentration of 20 mg mL^{-1}. The RGD-liposomes were sealed in a 5-mL vial, and air was exchanged with perfluoropropane gas (Takachiho); this was followed by 20-kHz USD treatment with a bath-type sonicating system (Model 3510; Branson, Emerson, CT, USA) for 5 min to generate the RGD-BLs [28]. Sterile filtration (0.45 μm) was then performed to remove the expanded and oversized BLs. Non-targeted BLs were prepared with the same methods but without the addition of RGD peptide. The amount of perfluoropropane gas trapped in the BLs was estimated to be ~ 10 μL mg^{-1} lipid, and the diameter of each BL was 180 ± 44 nm as measured by dynamic laser light-scattering measurements with an ELS-800 particle analyzer (Otsuka Photonics, Tokyo, Japan).

Therapeutic USD system

For both *in vitro* and *in vivo* studies, two different USD systems (low intensity or high intensity) were used. For the low-intensity USD study, the Timi3 system was used (Timi3 Systems, Santa Clara, CA, USA). This device consisted of a low-frequency USD generator (maximum intensity, 1.4 W cm^{-2}) and a transducer that delivered 27 kHz of USD at a pulse rate of 25 Hz (acoustic pressure, 0.145 MPa; mechanical index, 1.4). For the high-intensity study, the therapeutic USD system was composed of a sine wave pulse generator (MG-422A; Anritsu, Tokyo, Japan), a radiofrequency power amplifier

(2100L; ENI, Rochester, NY, USA), and a prototype piezoelectric transducer (Fuji Ceramics, Shizuoka, Japan). The transducer consisted of 10 PZT disks (thickness, 4 mm) tightly bonded together. It was operated in a continuous-wave mode at a frequency of 27 kHz (acoustic pressure, 0.346 MPa; mechanical index, 3.2) and an intensity of 4.0 W cm^{-2} as measured by the calorimetric method [29].

Protocol for in vitro *study on human thrombi*

The following *in vitro* investigation conforms with the principles outlined in the Declaration of Helsinki, and the protocol was approved by the Ethical Committee of the National Defense Medical College. In total, 60 thrombi were used in this *in vitro* study. For the preparation of each thrombus, 9 mL of whole blood was collected in a test tube from a healthy volunteer, placed on a seesaw-type shaker, and allowed to coagulate at room temperature while being shaken and rotated at a speed of 60 r.p.m. for 1 h. Targeted BLs or non-targeted BLs (100 μL, 20 mg mL^{-1} lipid concentration, ~ 1.1% v/v) were added to the test tube 10 min after the initiation of coagulation. The formed thrombus was washed with normal saline, cut into small pieces, weighed on an electronic balance, and placed in a plastic test tube containing 2 mL of human plasma. Before the therapeutic USD exposure, attachment of the BLs on the clot was confirmed by conventional USD imaging, as well as by scanning electron microscopy [28]. The test tube was placed at 1 cm from the therapeutic USD transducer in a bath filled with degassed water. Thirty thrombi were exposed to low-intensity USD: 10 without BLs as controls, 10 with non-targeted BLs, and 10 with targeted BLs. Similarly, 30 thrombi were exposed to high-intensity USD: 10 without BLs as controls, 10 with non-targeted BLs, and 10 with targeted BLs. The water temperature was maintained at 37 °C. Each thrombus was exposed to low-intensity USD (27 kHz, 1.4 W cm^{-2}) or high-intensity USD (27 kHz, 4.0 W cm^{-2}) for 5 min. After USD exposure, the clot was weighed again. The thrombus weight reduction rate ([pre-treatment weight – post-treatment weight]/pre-treatment weight × 100 [%]) was calculated as an index of the thrombolytic effect.

In vivo *study protocol in an acute thrombotic occlusion model of a rabbit iliofemoral artery*

The animal protocol was approved by the Animal Care and Use Committee of the National Defense Medical College, and conformed with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication, 8th Edition, 2011).

A total of 54 New Zealand white rabbits (~ 2.4 kg) were used: 24 for the low-intensity USD study, 20 for the high-intensity USD study, and 10 for the fibrinolysis study. Each rabbit was anesthetized with 50 mg of ketamine and 20 mg of xylazine injected intramuscularly, and anesthesia was maintained with pentobarbital (15 mg kg^{-1}) delivered via a marginal ear vein. The adequacy of anesthesia was monitored by the loss of the ear pinch reflex. Anesthetized rabbits were placed on a warming plate to maintain the body temperature at 37 °C. Aseptic techniques were used for all surgical procedures. A 5Fr sheath was inserted into the right carotid artery, a balloon catheter was advanced to the right iliofemoral artery, and the intima was injured by balloon inflation and scratching. The balloon catheter was then pulled back, a 0.014-inch guide wire was positioned at the injured site, and electrical stimulation (3-V battery) was applied between the guide wire and skin electrode [18,21,28,30]. Thirty minutes later, the right iliofemoral artery was thrombotically occluded, and the arterial occlusion was confirmed by angiography. The thrombus was also imaged with a 9-MHz linear transducer and a conventional USD machine 1 min after BL injection (UF-750XT; Fukuda Denshi, Tokyo, Japan) (frame rate, 24–30/s; mechanical index, 0.3) (Fig. 1).

To determine the thrombolytic effect of targeted BLs and USD, we applied the low-intensity or high-intensity USD transcutaneously over the site of the rabbit iliofemoral arterial thrombus in combination with an intravenous injection of non-targeted BLs or targeted BLs. A total of 44 New Zealand white rabbits with iliofemoral

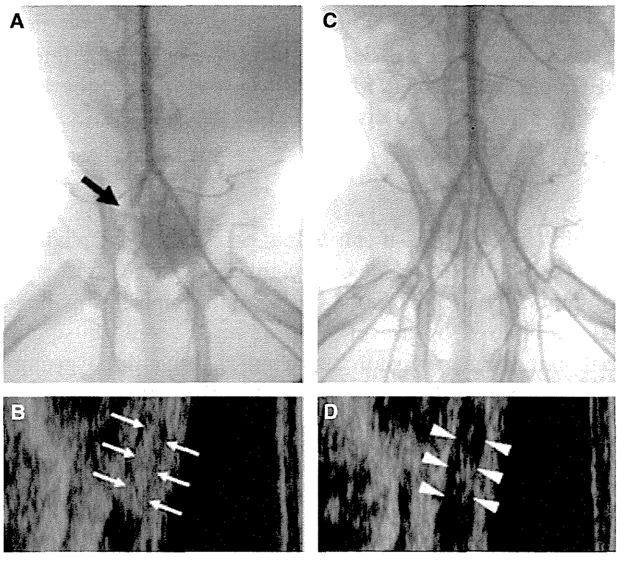

Fig. 1 . (A) Angiographic image of thrombotically occluded rabbit iliac artery (black arrow) after balloon inflation and electrical (3-V battery) stimulation. (B) In sonographic images, the targeted bubble liposomes (BLs) accumulated on the thrombus (white arrows). (C, D) After combination therapy with low-frequency ultrasound (USD) and thrombus-targeted BLs, TIMI grade 3 flow was achieved (C), and the high echogenic area within the iliac artery almost disappeared (white arrowheads) (D).

arterial occlusions were divided into two groups: 24 for low-intensity USD, and 20 for high-intensity USD. In both groups, non-targeted BLs were intravenously administered in half (12 or 10, respectively) of the rabbits, and targeted BLs were used in the other half (12 or 10, respectively) of the rabbits.

Before the USD exposure, 200 IU kg^{-1} heparin was injected intravenously. Angiography was performed every 15 min for a total of 90 min, and the TIMI blood flow grade was assessed [18,31]. Non-targeted or targeted BLs (1 mL, ~ 1.1% v/v) were intravenously injected every 15 min after the angiography. Subsequently, transcutaneous USD was applied externally over the site of the thrombus for 12 min. In the targeted BL and USD group, thrombus location was also confirmed with a diagnostic USD machine (UF-750XT; Fukuda Denshi) and with angiography (Fig. 1). BL injection and subsequent USD exposure were repeated four times for a total of 60 min. The vessel was observed for acute thrombotic reocclusion for the next 30 min. The recanalization (TIMI grade 3 flow) rate was calculated, and the reperfusion time was measured only in cases in which reperfusion was achieved. All of the angiographic assessments represented the consensus of two expert cardiologists (T.N. and B.T.) blinded to group allocation.

In 10 New Zealand white rabbits with iliofemoral arterial occlusions, 27 500 IU kg^{-1} recombinant tissue plasminogen activator (rt-PA) (Monteplase, Eizai, Tokyo, Japan) was intravenously administered within a period of 1 min immediately after intravenous injection of 200 IU kg^{-1} heparin. Angiography was repeated every 15 min for a total of 90 min, and the TIMI blood flow grade was assessed [18,31]. At the end of the experiment, all rabbits were killed with an overdose of pentobarbital (75 mg kg^{-1}) injected via a marginal ear vein.

Statistical analysis

Results are given as mean value ± 1 standard deviation. In the *in vitro* study, clot weight reduction rates were compared by use of ANOVA. *In vivo*, the reperfusion rates were compared by use of a 2 × 2 or a 2 × 3 chi-square test, and the reperfusion times among the three groups were compared by use of the Kruskal–Wallis test. A *P*-value of < 0.05 was considered to be statistically significant.

Results

In vitro *study*

The clot weight reduction rate achieved with low-intensity USD with targeted BLs was significantly higher than that with the non-targeted BLs and in the controls without BLs (25% ± 11% vs. 14% ± 9% and 9% ± 3%, respectively; $P < 0.01$). Non-targeted BLs gave no significant enhancement of clot weight reduction (Fig. 2). Similarly, the clot weight reduction rate achieved with high-intensity USD in combination with targeted BLs was significantly higher than that with non-targeted BLs and in the controls (65% ± 21% vs. 21% ± 9% and 21% ± 14%, respectively; $P < 0.01$). No significant enhancement of clot weight reduction was observed when non-targeted-BLs were used.

On comparison of the effects of low-intensity USD and high-intensity USD, the clot weight reduction rate was significantly higher in the high-intensity USD group than in the low-intensity USD group when USD was applied without BLs (21% ± 9% vs. 9% ± 3%) and with targeted BLs (65% ± 21% vs. 25% ± 11%).

In both groups, echo signal enhancement of all thrombi by targeted BLs declined to control and non-targeted BL levels after USD exposure.

In vivo *low-intensity USD study*

Rabbit iliofemoral arterial thrombus was clearly recognized with a conventional USD system with the targeted BLs, and also confirmed by angiography (Fig. 1). The Doppler signal enhancement of the iliofemoral arterial flow by both targeted and non-targeted BLs was observed even at 12 min after therapeutic USD irradiation. TIMI grade 3 flow was achieved in eight of 12 rabbits (67%) with targeted BL and USD exposure; however, TIMI grade 3 flow was achieved in only one of 12 rabbits (8%) in the non-targeted BL and USD group (Table 1; Fig. 3). These differences were statistically significant (targeted BLs, 67%; non-targeted BLs, 8%; $P = 0.003$). The time to reperfusion was ~ 40 min in the group with targeted

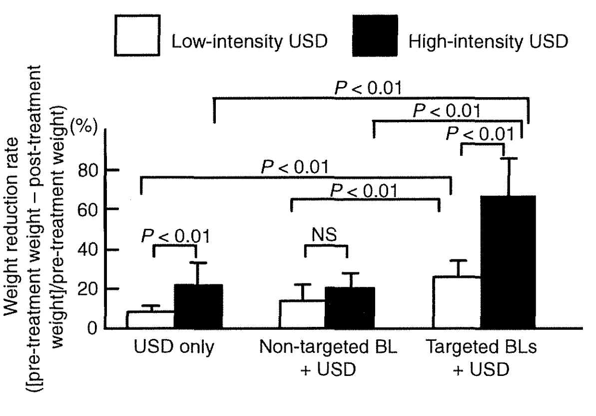

Fig. 2. The *in vitro* thrombus weight reduction rate ([pre-treatment weight − post-treatment weight]/pre-treatment weight × 100 [%]) obtained with low-frequency ultrasound (USD): high-intensity USD exposure for 5 min with the targeted bubble liposomes (BLs), with the non-targeted BLs, or without BLs ($P < 0.01$, $n = 10$, ANOVA); low-intensity USD exposure for 10 min with the targeted BLs, with the non-targeted BLs, or without BLs ($P < 0.01$, $n = 10$, ANOVA). The clot weight reduction rate increased with therapeutic USD with targeted BLs in each intensity study. NS, not significant.

BL and USD exposure (Table 1). There were three cases of blood flow reduction (TIMI grade 3 to 2) during the observation period in the targeted BL group (Fig. 3).

In vivo *high-intensity USD study and fibrinolysis study*

After exposure to low-frequency high-intensity transcutaneous USD, TIMI grade 3 flow was achieved in nine of 10 rabbits (90%) with targeted BLs. On the other hand, recanalization was achived in only two of 10 rabbits (20%) with USD and non-targeted BLs, and four of 10 arteries (40%) were recanalized with rt-PA monotherapy (Table 2; Fig. 4). These differences were statistically significant (targeted BLs, 90%; non-targeted BLs, 20%; rt-PA, 40%; $P = 0.004$). Moreover, the average reperfusion time for rabbits to achieve TIMI grade 3 flow was significantly shorter for those cases with high-intensity USD thrombolysis with targeted BLs than for those with high-intensity USD with non-targeted BLs or for those with ordinary thrombolysis with rt-PA (targeted BLs, 16.7 ± 5.0 min; non-targeted BLs, 60.0 ± 0 min; rt-PA, 41.3 ± 14.4 min; $P = 0.007$; $n = 10$) (Table 2). There were no cases of acute reocclusion, and all recanalized arteries maintained TIMI grade 3 flow, whereas two cases of acute reocclusion occurred during the procedure and the observation period in the non-targeted BL group (Fig. 4).

Table 1 Frequency of TIMI grade 3 flow (A) and time to each TIMI grade 3 flow (B) achieved with a combination of transcutaneous low-intensity ultrasound (USD) and targeted bubble liposomes (BLs) or non-targeted BLs

	Targeted BLs + USD	Non-targeted BLs + USD	*P*-value
(A)			
Frequency, no. (%)	8/12 (67)	1/12 (8)	0.003
(B)			
Mean time (min)	43.1 ± 20.3	60.0 ± 0	NA

NA, not applicable.

Discussion

This study demonstrated significant enhancement of ultrasonic thrombus disruption by the thrombus-targeted BLs and external low-frequency USD system *in vitro* and *in vivo*, and the possibility of a completely non-invasive treatment that combines the identification of thrombus position with rapid clot disruption. This study validates the use of the low-frequency USD system with targeted BLs for rapid, effective and comprehensive thrombolytic treatment. The combination of USD and targeted BLs played a primary role in this study in both the diagnosis and treatment of thrombotic vascular occlusions.

In many of the previous studies dealing with *in vivo* USD clot disruption and using microbubbles to reinforce the USD energy, invasive angiography was used to identify the precise location of the thrombus, and to guide the manipulation of the therapeutic USD probe [9,10,18,21]. Otherwise, transcranial Doppler was used to monitor deteriorated blood flow, or a large transducer was used to cover the area of vessel occlusion [25–27,32]. Recently, we developed thrombus-targeted liposomal bubbles for USD imaging of fresh thrombus. This *in vitro* and *in vivo*

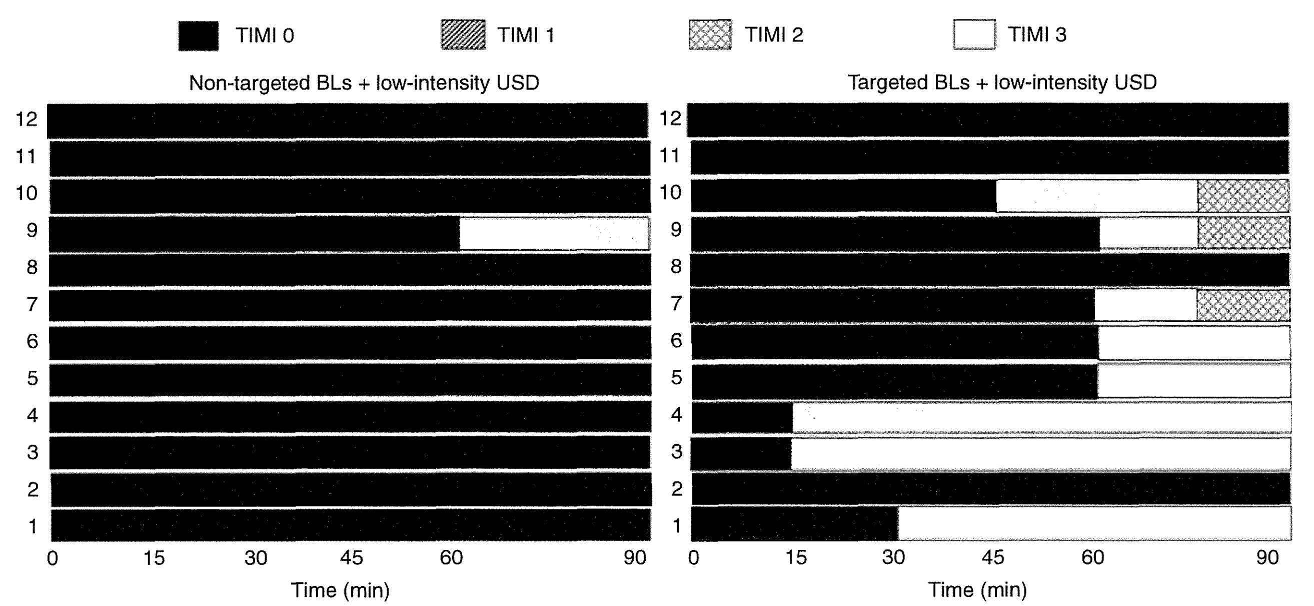

Fig. 3. Schematic presentation showing TIMI flow grades of the arteries treated with low-intensity ultrasound (USD) with non-targeted bubble liposomes (BLs) and targeted BLs. TIMI grade 3 flow was achieved in 67% of arteries with targeted BLs and in only 8% of arteries with non-targeted BLs ($P = 0.003$, 2 × 2 chi-square test).

study showed that the USD image of thrombus could be identified at a glance with a conventional diagnostic USD machine with the targeted BLs, as also found in our previous study [28]. This enhanced thrombus imaging facilitated successful USD thrombolysis without invasive angiography.

The targeted BLs significantly enhanced ultrasonic clot disruption *in vitro* and *in vivo* when used with both low-intensity and high-intensity USD. In particular, with high-intensity USD exposure with targeted BLs *in vivo*, arterial recanalization was achieved in 90% of acute thrombotically occluded rabbit iliofemoral arteries within 20 min from the beginning of the diagnostic procedure. This speed of treatment potentially surpasses that of the PCI procedures, which, on average, require at least 90 min to achieve reperfusion from arrival at the hospital [3]. This rapid and non-invasive therapy shows promise in acute cardiovascular medicine, as diagnostic and therapeutic USD equipment is compact in size, inexpensive, and does not require dedicated laboratory space and specialized PCI staff.

Table 2 Frequency of TIMI grade 3 flow (A) and time to each TIMI grade 3 flow (B) achieved with a combination of transcutaneous high-intensity ultrasound (USD) and targeted bubble liposomes (BLs) or non-targeted BLs, or recombinant tissue-type plasminogen activator (rt-PA) alone

	Targeted BLs + USD	Non-targeted BLs + USD	rt-PA	*P*-value
(A)				
Frequency, no. (%)	9/10 (90)	2/10 (20)	4/10 (40)	0.004
(B)				
Mean time (min)	16.7 ± 5.0	60.0 ± 0	41.3 ± 14.4	0.007

These results were equivalent to those of the sonothrombolysis study by Culp *et al.*, in which a combination of 2-MHz USD and eptifibatide-tagged microbubbles opened acute intracranial thrombotic occlusions in six of eight pigs without the use of a thrombolytic agent [22]. Recently, Alonso *et al.* reported that diagnostic 2-MHz USD in combination with abciximab immunobubbles induced thrombolysis (increased plasma D-dimer levels) without lytic agents in rats [33]. However, the arterial recanalization was not assessed, as a partial thrombotic occlusion model of the rat carotid artery was used. Xie *et al.* also reported that diagnostic USD (1.5 MHz) treatment with platelet-targeted microbubbles in combination with half-dose recombinant prourokinase gave a 53% coronary arterial recanalization rate at 30 min in pigs [20]. These studies demonstrate that clinically used diagnostic USD frequencies can be applied to thrombus dissolution with thrombus-targeted microbubbles. However, their thrombolytic effect was relatively limited, except for the intracranial model [22], presumably because the cavitational energy generated by high-frequency USD (MHz) and microbubbles was relatively low in the absence of a closed space such as a skull, where USD energy could be enhanced by standing wave formation [34]. To overcome this limitation, we used low-frequency (kHz) USD as a therapeutic device to achieve a higher recanalization rate.

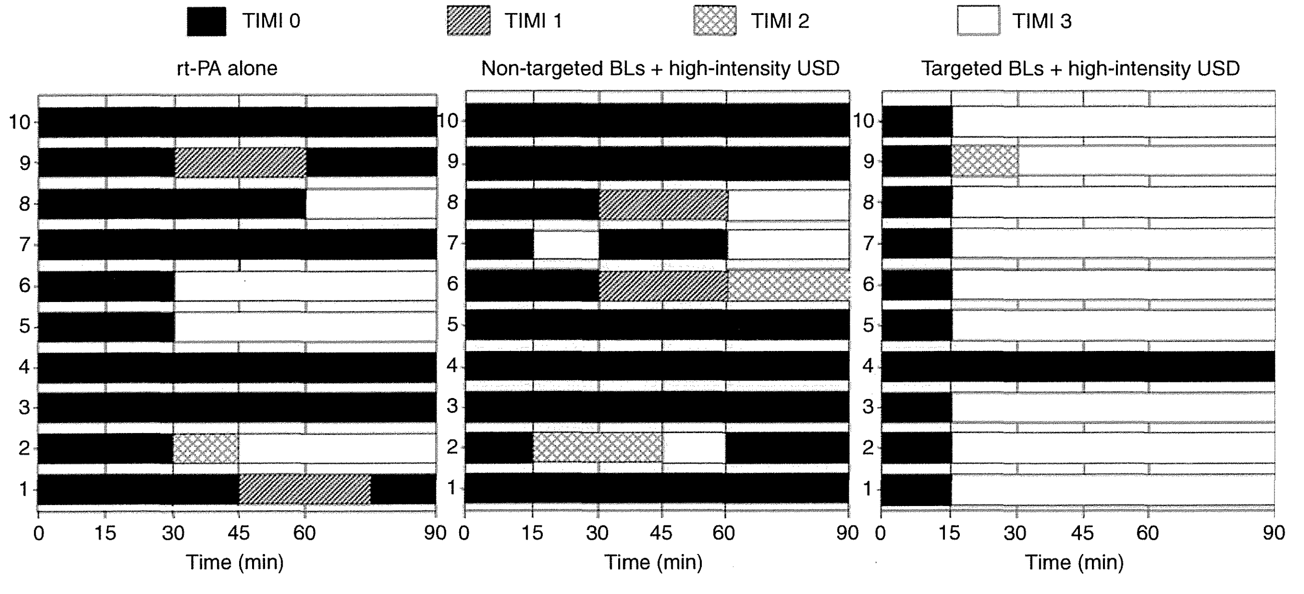

Fig. 4. Schematic presentation showing TIMI flow grades of the arteries treated with recombinant tissue-type plasminogen activator (rt-PA) alone, with high-intensity ultrasound (USD) with non-targeted bubble liposomes (BLs), and with high-intensity USD with targeted BLs. TIMI grade 3 flow was achieved in 90% of arteries treated with USD with targeted BLs, in only 20% of arteries treated with USD with non-targeted BLs, and in 40% of arteries treated with rt-PA monotherapy (P = 0.02, 2 × 3 chi-square test).

Low-frequency USD is advantageous, because it penetrates deeper into tissue and has less thermal effect than high-frequency USD [35]. Low-frequency USD has been reported to recanalize canine iliofemoral and coronary arteries without tissue damage [9,10,21,36], and has been safely applied in combination with thrombolytic agent in humans with STEMI [13], with a USD intensity as low as that used in our low-intensity USD study. However, there are still safety concerns regarding the application of high-intensity, low-frequency USD with microbubbles, and these should be clarified in a future study.

Nano-sized BLs could have some potential disadvantages. It is assumed that BLs release less energy than larger microbubbles when they collapse. In fact, the non-targeted BLs showed negligible enhancement of USD clot disruption *in vitro*. Moreover, only 8–20% cases of thrombosed rabbit iliofemoral arteries recanalized *in vivo* with non-targeted BLs. Theoretically, each BL itself is too small to reflect USD waves with the frequency used in this study. However, we previously demonstrated that the targeted BLs are highly concentrated around and within the thrombus, by using scanning electron microscopy, and that they markedly enhance ultrasonic thrombus imaging [28]. Consequently, as shown in this *in vitro* and *in vivo* study, we found marked enhancement of the thrombolytic effect by attaching the thrombus-targeting ligands on the same BL structure. The small size of the BLs, with a mean diameter of 180 nm, could also have some advantageous effects. A BL size of <1 μm ensures both a long *in vivo* circulation time and deep penetration into thrombi. The longer the circulation time, the more opportunity the targeted BL has to attach to the activated thrombus. Deep penetration into thrombi through the fibrin network allows for greater accumulation of targeted BLs within thrombi. These two features of small BLs were favorable, when USD energy was applied, for disruption and reduction of the culprit thrombus.

Liposomes are usually considered to be non-toxic, unless they are administered at very high doses [37]. Polyethylene glycol is also considered to be non-toxic, and is excreted unmetabolized in the urine [38]. The RGD peptide is an octapeptide, and is considered to be non-toxic and non-immunogenic [39,40]. Perfluoropropane is an inert gas, used as a constituent of commercially available echo contrast agents such as Optison and Definity [41], and is exhaled from the lungs [42]. Therefore, this echo contrast agent is generally considered to be non-toxic, although safety in humans remains to be demonstrated.

In a clinical setting, lower-intensity USD has some advantages over high-intensity USD in terms of safety. However, low-intensity USD exposure with targeted BLs achieved only a modest thrombolytic effect in this study. When low-intensity USD is used, an alternative approach is necessary to enhance the resonance phenomenon caused by the interaction between USD and the BLs. As the RGD peptide is not an ideal targeting ligand, because of its broad cross-reactivity with a number of integrins, one possible option is to achieve a higher concentration of the BLs on the thrombus by using a more effective targeting ligand. The other option is to use larger BLs, which generate a higher amount of cavitational energy during collapse. However, the BLs should be small enough to penetrate into the thrombus. Therefore, the most effective size of BLs remains to be determined. Another option is to combine targeted BL-enhanced USD thrombolysis with conventional fibrinolytic therapy. The dose of the fibrinolytic agent, such as tissue-type plasminogen activator, could be reduced with this strategy. Further study is needed to elucidate this issue.

The targeted BLs enhance imaging of the culprit thrombus and enable manipulation of the therapeutic USD probe, targeting and directing it towards the culprit thrombus. Furthermore, the combination treatment with targeted BLs and high-intensity, low-frequency USD achieved a 90% recanalization rate, which is markedly higher than that with rt-PA monotherapy. This method has the potential to be a reperfusion strategy that could be more rapid than any other method, including PCI. The absence of acute reocclusion with this therapeutic approach might be attributable to minimal mural thrombus being left in the culprit lesion. Further study is needed to identify the most suitable targeting ligand and BL size for generating the maximum thrombus disruption and achieving the most effective thrombolysis with low-intensity USD.

Limitations

There are some technical limitations regarding thrombus formation in both the *in vitro* study and the *in vivo* study. We prepared all *in vitro* clots from the blood of a single individual, to examine the effects of USD and targeted BLs on clots with homogeneous lytic activities. However, this could simultaneously be a limitation of this study, because individual lytic response can differ as a function of the different levels of inhibitory enzymes and/or varying concentrations of plasminogen. Moreover, *in vivo* hyperacute thrombi could be more fragile than those in clinical culprit lesions.

Reocclusion of the culprit artery was not observed after successful recanalization with low-frequency USD and targeted BLs. However, the observation period after recanalization in this study might not be long enough to exclude the possibility of reocclusion, which may occur later. There are some safety limitations. It is known from the simulation study of intracranial sonothrombolysis [34] that USD sometimes causes standing wave formation and unnecessary acoustic cavitation, especially in brain tissue, even outside the targeted clot. Regarding the coronary and peripheral arteries, low-frequency, high-intensity USD energy can be delivered transcutaneously for clot disruption without concomitant tissue damage in animal models, especially when coupled with the use of a cooling system to prevent thermal injury [9,10,21,36]. However, low-frequency USD could cause unexpected non-linear cavitational effects, and its safety has to be clarified in combination with microbubbles before clinical application.

Conclusions

Perfluorocarbon gas-containing liposomal nanobubbles with activated thrombus-targeting RGD peptides developed for USD thrombus imaging are novel echo contrast agents that can markedly enhance USD thrombolysis both *in vitro* and *in vivo*. The combination of USD and thrombus-targeted BLs could be used as an effective and completely non-invasive recanalization therapy that does not require angiography to detect acute thrombotic vessel occlusion or therapeutic thrombus dissolution.

Addendum

K. Hagisawa, T. Nishioka, and R. J. Siegel: conception and design; K. Hagisawa, T. Nishioka, R. Suzuki, B. Takase, K. Iida, and H. Luo: acquisition of data; K. Hagisawa, T. Nishioka, and A. Kurita: analysis and interpretation of data; K. Hagisawa, T. Nishioka, and R. J. Siegel: drafting of the manuscript; K. Maruyama, M. Ishihara, N. Yoshimoto, and Y. Nishida: supervision.

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Disclosure of Conflict of Interests

The authors state that they have no conflict of interest.

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Ultrasound-Mediated Gene Delivery Systems by AG73-Modified Bubble Liposomes

Yoichi Negishi,[1] Yuka Tsunoda,[1] Nobuhito Hamano,[1] Daiki Omata,[2] Yoko Endo-Takahashi,[1] Ryo Suzuki,[2] Kazuo Maruyama,[2] Motoyoshi Nomizu,[3] Yukihiko Aramaki[1]

[1]*Department of Drug Delivery and Molecular Biopharmaceutics, School of Pharmacy, Tokyo University of Pharmacy and Life Sciences, Hachioji, Tokyo, Japan*

[2]*Laboratory of Drug and Gene Delivery, Faculty of Pharma-Sciences, Teikyo University, Tokyo, Japan*

[3]*Department of Clinical Biochemistry, School of Pharmacy, Tokyo University of Pharmacy and Life Sciences, Hachioji, Tokyo, Japan*

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ABSTRACT:

Targeted gene delivery to neovascular vessels in tumors is considered a promising strategy for cancer therapy. We previously reported that "Bubble liposomes" (BLs), which are ultrasound (US) imaging gas-encapsulating liposomes, were suitable for US imaging and gene delivery. When BLs are exposed to US, the bubble is destroyed, creating a jet stream by cavitation, and resulting in the instantaneous ejection of extracellular plasmid DNA (pDNA) or other nucleic acids into the cytosol. We developed AG73 peptide-modified Bubble liposomes (AG73-BL) as a targeted US contrast agent, which was designed to attach to neovascular tumor vessels and to allow specific US detection of angiogenesis (Negishi et al., Biomaterials 2013, 34, 501–507). In this study, to evaluate the effectiveness of AG73-BL as a gene delivery tool for neovascular vessels, we examined the gene transfection efficiency of AG73-BL with US exposure in primary human endothelial cells (HUVEC). The transfection efficiency was significantly enhanced if the AG73-BL attached to the HUVEC was exposed to US compared to the BL-modified with no peptide or scrambled peptide. In addition, the cell viability was greater than 80% after transfection with AG73-BL. These results suggested that after the destruction of the AG73-BL with US exposure, a cavitation could be effectively induced by the US exposure against AG73-BL binding to the cell surface of the HUVEC, and the subsequent gene delivery into cells could be enhanced. Thus, AG73-BL may be useful for gene delivery as well as for US imaging of neovascular vessels.

Keywords: *AG73; Bubble liposome; gene delivery; syndecan; ultrasound imaging*

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Yoichi Negishi and Yuka Tsunoda contributed equally to this work.
Correspondence to: Yoichi Negishi, Department of Drug Delivery and Molecular Biopharmaceutics, School of Pharmacy, Tokyo University of Pharmacy and Life Sciences, Hachioji, Tokyo, Japan; e-mail: negishi@toyaku.ac.jp
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INTRODUCTION

Gene therapy is expected as a potential alternative to conventional therapeutic approach. However, safe and effective gene delivery techniques are required to realize its clinical application. Recently, among the various attempts to develop clinically applicable gene therapy, despite their relatively lower efficiency, nonviral methods are focused on safer alternatives in gene therapy to viral vectors. Among nonviral physical delivery methods,[1–4] it has been shown that ultrasound (US) exposure enhances the efficiency of drug or gene delivery into tissues and cells, a technique known as sonoporation.[3] It is believed that this effect transiently increases the permeability of cell membranes around by US cavitation activity, enabling the transport of extracellular molecules into viable cells.[2,5–8] Microbubbles can act as contrast agents for US imaging by enabling visualization of disease site and enhancing the delivery efficiency of drugs or genes by cavitation as a result of US exposure while reducing cellular damage.[9–15] Conventional microbubbles containing US echo-contrast gases are commercially available.[16,17] However, these microbubbles exhibit problems with size, stability, and targeting function. Liposomes—useful carriers of drugs, antigens, and genes—can easily be prepared in a variety of sizes and modified to add a targeting function.[18–22] Therefore, we assumed that polyethylene glycol-modified liposomes containing the US imaging gas (*perfluoropropane gas*) could be novel gene delivery carriers. We have previously developed novel liposomal bubbles, "Bubble liposomes" (BLs), which could solve the problems faced with microbubbles and which could feasibly be used for delivering genes in vitro and in vivo.[17,23–28]

There is currently a need for imaging angiogenesis both for diagnostic purposes and for assessing therapeutic effects. Targeted contrast US provides an opportunity when moieties such as peptides or monoclonal antibodies that bind to the endothelial cell surface are utilized to image angiogenesis.

Many types of peptides have been reported that specifically bind to tumor angiogenic endothelium, including the AG73 peptide, which is considered a ligand for syndecan.[29–32] Moreover, syndecan-2 is highly expressed in neovascular vessels.[33–36] We hypothesized that BLs targeted via linkage with AG73 would specifically adhere to the tumor angiogenic endothelium, and this selective adhesion would allow us to detect tumor angiogenesis ultrasonically. We have recently succeeded in developing AG73-modified BLs (AG73-BL) as a targeted US contrast agent.[37] If the AG73-BL is used for gene delivery in combination with US exposure, efficient cavitation may be induced at the surface of the target cells, leading to the delivery of extracellular pDNA into the cytosol. However, it is unknown whether a gene delivery system with the combination of AG73-BL and US exposure will be an effective method. Herein, we assessed the gene delivery ability of AG73-BL to HUVEC as a component cell of blood vessels.

MATERIALS AND METHODS

Materials

Dipalmitoylphosphatidylcholine (DPPC), 1,2-distearoylphosphatidylethanolamine-methoxy-polyethyleneglycol (DSPE-PEG$_{2000}$-OMe), and 1,2-distearoyl-*sn*-glycero-3-phosphatidylethanolamine-polyethyleneglycol-maleimide (DSPE-PEG$_{2000}$-Mal) were purchased from NOF Corporation (Tokyo, Japan). An Endothelial Cell Growth Medium Kit was purchased from Cell Applications, Inc. (San Diego, CA). All other materials were used without further purification.

Preparation of Liposomes and BLs

To prepare liposomes for the BLs, DPPC and DSPE-PEG$_{2000}$-OMe were mixed at a molar ratio of 94:6. The liposomes were prepared by a reverse-phase evaporation method, as described previously.[23,24] In brief, all the 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 extrusion 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 Technoglass, Chiba, Japan) for sterilization. For the fluorescent labeling of the lipid membrane,1,1-dioctadecyl-3,3,3,3-tetramethyl-indocarbocyanine perchlorate (DiI: 0.1 or 1 mol% of total lipids) was added. The lipid concentration was measured using the Phospholipid C test (Wako Pure Chemical Industries, Osaka, Japan). BLs were prepared from liposomes and perfluoropropane gas (Takachiho Chemical, Tokyo, Japan). First, 5 mL sterilized vials containing 2 mL of a liposome suspension (lipid concentration: 1 mg/mL) were filled with perfluoropropane gas, capped, and 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, Danbury, CT) for 5 min to form BL. The mean size of the BLs were determined using the light-scattering method with a zeta potential/particle sizer (Nicomp 380ZLS, Santa Barbara, CA). As shown in previous report, it was confirmed that the mean particle diameter of BLs ranged from 400 to 600 nm with a relatively narrow distribution after the encapsulation of the gas.[37]

Preparation of AG73 Peptide-Modified Liposomes and BLs

The Cys-AG73 peptide (CGG-RKRLQVQLSIRT) and a scrambled Cys-AG73T control peptide (CGG-LQQRRSVLRTKI) were synthesized manually using the 9-fluorenylmethoxycarbonyl (Fmoc)-based solid-phase strategy, prepared in the COOH terminal amide form, and purified by reverse-phase, high-performance liquid chromatography. The peptides were confirmed by an electrospray ionization mass spectrometer at the Central Analysis Center, Tokyo University of Pharmacy and Life Sciences. Liposomes composed of DPPC, DSPE-PEG$_{2000}$-OMe, and DSPE-PEG$_{2000}$-Mal at a molar ratio of 94:5.8:0.2 were prepared by a reverse-phase evaporation method. For the preparation of AG73 peptide-modified liposomes, adequate amounts of AG73 peptide were added to the liposomes and gently mixed, as described previously.[37] Briefly, for coupling, AG73 peptide at a molar ratio of fivefold DSPE-PEG$_{2000}$-Mal was added to the liposomes in the presence of tris(2-carboxyethyl)phosphine hydrochloride, and the mixture was incubated for 6 h at room temperature to conjugate the cysteine of the Cys-AG73 peptide with the maleimide of the liposomes through a thioether bond. The resulting AG73 peptide-conjugated liposomes (AG73-liposomes) were dialyzed to remove any excess peptide. The AG73-liposomes were modified with 6 mol% PEG and 0.2 mol% peptides. AG73 peptide-modified BLs (AG73-BL) were prepared from liposomes and perfluoropropane gas. The conjugation of AG73 to PEG liposomes has been confirmed by reverse-phase high-performance liquid chromatography (HPLC) analysis as shown in our previous report.[38] Results showed that Cys-AG73 peptide could be completely conjugated to DSPE-PEG2000-Mal. The average number of the peptides per BL was approximately 30 peptide molecules based on the calculation by using the above values and the assumption that the molecular weight of peptide is 2000; the average number of phospholipid molecules per liposome was estimated by the method of Enoch and Strittmatter.[39]

Cell Lines

Human umbilical vein endothelial cells (HUVECs) were purchased from Cell Applications and cultured using an Endothelial Cell Growth Medium Kit. All experiments were performed using HUVECs between passage 5 and 9.

Microscopic Analyses

The day before transfection, HUVEC (3×10^4 cells/well) were seeded in the wells of a 48-well plate (Asahi Technoglass, Chiba, Japan) and incubated for 18 h at 37°C in 5% CO_2. Thirty micrograms of AG73-BL was mixed with the culture medium and added to the cells. The plates were sealed with sterile tape and inverted for 5 min. The images of the cells bound with the BL, AG73-BL, or AG73T-BL were randomly captured with a microscope (Axiovert 200 M, Carl Zeiss) at 100× magnification. Total number of bound bubbles per cell (n = 300 cells per each treatment) in four images was counted. Number of each bubbles binding to one HUVEC are averaged.

Plasmid DNA

The plasmid pcDNA3-Luc, derived from pGL3-basic (Promega, Madison, WI), is an expression vector encoding the firefly luciferase gene under the control of a cytomegalovirus promoter.

Transfection of pDNA Using BL, AG73-BL, or AG73T-BL

The day before transfection, HUVEC (3×10^4 cells/well) were seeded in the wells of a 48-well plate (Asahi Technoglass, Chiba, Japan) and incubated for 18 h at 37°C in 5% CO_2. Ten to thirty micrograms of BL, AG73-BL, or AG73T-BL and 14 μg of pDNA were mixed with the culture medium and added to the cells. The plates were sealed with sterile tape and inverted for 5 min. After the interaction between each type of BL and the cells, the tape was removed and the cells were immediately exposed to US (frequency, 2 MHz; duty, 50%; burst rate, 2.0 Hz; intensity, 0.1 W/cm^2) for 10 s through a 6-mm-diameter probe placed in the well. A Sonopore 3000 sonicator (NEPA GENE, Chiba, Japan) was used to generate the US. The cells were washed twice with culture medium and cultured for 2 days. For the measurement of luciferase expression, cell lysate was prepared with a lysis buffer (0.1*M* Tris-HCl (pH 7.8), 0.1% Triton X-100, and 2 m*M* EDTA). Luciferase activity was measured using a luciferase assay system (Promega, Madison, WI) and a luminometer (LB96V, Belthold Japan, Tokyo, Japan). The activity is indicated as relative light units (RLUs) per mg of protein.

Cytotoxicity of BL, AG73-BL, or AG73T-BL and US to HUVEC

One day after transfection as described above, cell viability was assayed using Cell Counting Kit-8 (Dojindo, Kumamoto, Japan). Briefly, WST-8 (10 μL) was added to each well and the cells were incubated at 37°C for 2 h. The formazan product was dissolved in 10 μL of HCl (0.1*M*) to stop the reaction. The color intensity was measured using a microplate reader at test and reference wavelengths of 450 and 650 nm, respectively.

Statistical Analyses

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

RESULTS AND DISCUSSION

Adhesion of AG73 Peptide-Modified Bubble Liposomes (AG73-BL) to HUVECs

As shown in Figure 1, if BLs modified with a targeting ligand are prepared and used for gene delivery in combination with US exposure, after the binding of the BL onto the target cell and exposure to US, it is possible that efficient cavitation will occur just above the target cell membrane, leading to efficient gene delivery into the target cell. Indeed, we observed that gene delivery could be significantly enhanced when culture plate with adherence cell lines (HUVEC or COS7 cells) were mixed with the solution of BLs and pDNA, inverted for several minutes, and exposed to US (data not shown). In this situation, it is possible that BLs can be easily accessible to the surface of cell membrane because of the floating character of itself. Therefore, these results may suggest that destruction and cavitation of BLs by US exposure occur on the surface of cell membrane, leading to more efficient gene delivery.

More recently, we have developed AG73 peptide-modified Bubble liposomes (AG73-BL) as neovascular-targeting BLs to enhance the contrast image.[37] Therefore, we assumed that AG73-BL in combination with US exposure could induce cavitation directly above the target cell membrane. Prior to the transfection experiment using the AG73-BL, we first confirmed the adhesion of AG73-BL to HUVECs as a component cell of blood vessels in the culture plate. To confirm whether AG73-BL actually bind to the surface of HUVECs in the culture plate, AG73-BL was mixed with the culture medium and added to the cells. The plates were deaerated with the mixture, sealed with sterile tape, and inverted for 5 min to float and attach the AG73-BL onto the cell membranes. Then, the cells were observed under a microscope. As shown in Figure 2, we found that AG73-BL could bind to the cells. In contrast, nonlabeled BL (BL) or scrambled peptide-modified BLs (AG73T-BL) had

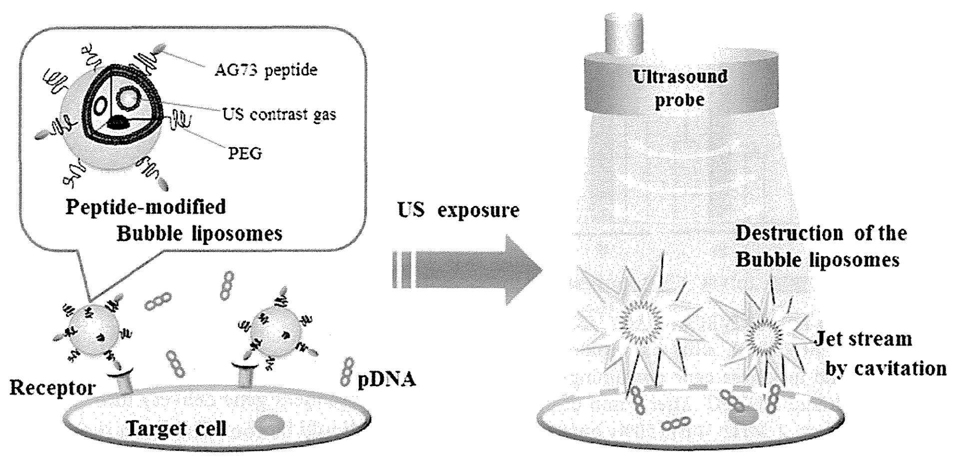

FIGURE 1 Scheme of gene transfection with AG73-BLs exposed to US. If AG73 peptide-modified BL, which can attach to the cell membrane of HUVECs, is used for gene delivery in combination with US exposure, after binding the AG73-BL onto the cell membrane of the HUVECs and exposing it to US, it may be possible for efficient cavitation to be induced on the target cell membrane, leading to efficient gene delivery into the target cell.

very low affinity for binding to the cells. The AG73 peptide is derived from laminin and serves as a ligand for syndecans, which are highly expressed on HUVECs.[35,36] The AG73 peptide also binds to the heparan sulfate side chains of syndecans.[30,31] Therefore, these results suggested that AG73-BL could effectively bind to HUVECs via the syndecan receptor.

Gene Transfection by the Combination of AG73-BL and US Exposure

Using luciferase-expressing pDNA to assess the gene delivery efficiency, we examined the gene delivery capacity of the AG73-BL into the cells in the combination with US exposure. As shown in Figure 3, highly efficient gene expression was observed when the AG73-BL and US exposure were applied. In contrast, lower gene expression was observed with the use of BL or AG73T-BL. We found that the cavitation induction on the cell surface by the combination of AG73-BL and US exposure was important in achieving efficient gene delivery. The cytotoxicity of the combination of AG73-BL and US exposure was determined using the WST assay. However, cell viability was more than 80% after each transfection (Figure 4). These results suggest that the AG73-BL has stronger adherence to the cells compared to BL or AG73T-BL, which lead to the inducement of efficient cavitation on the cell membrane and the delivery of plasmid DNA into the cells. In the case of systemic gene delivery, transfection efficiency is decreased if the BL and gene are not colocalized in blood vessels. Therefore, it is necessary to control the biodistribution of both the BL and the gene, and to consider the degradation of the gene by nuclease. To overcome these problems, we have developed plasmid DNA and siRNA-loaded Bubble liposomes containing cationic lipids that are capable of loading plasmid DNA or siRNA.[40–42] We

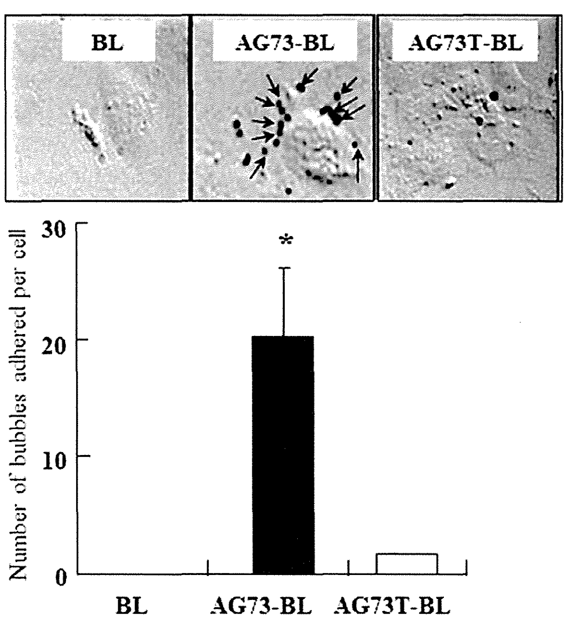

FIGURE 2 Comparison of the cell attachment properties of BL, AG73-BL, or AG73T-BL by microscopic analysis. BL, AG73-BL, or AG73T-BL was added to each HUVEC culture. After 5 min of incubation, each specimen was observed with bright field microscopy. The binding bubbles to HUVEC are indicated by arrows. Magnification: ×100. The number of bubbles binding to one HUVEC is measured. $^*P < 0.05$ compared with AG73T-BL or BL.

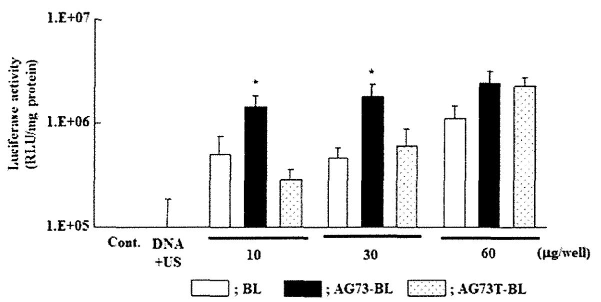

FIGURE 3 Gene transfection into HUVECs by AG73-BL and US exposure. BL, AG73-BL, or AG73T-BL with or without pDNA (CMV promoter/enhancer and luciferase gene containing plasmid DNA) was added to each cultured HUVEC. After 5 min of incubation, US was applied (frequency, 2 MHz; duty, 50%; burst rate, 2 Hz; intensity, 0.1 W/cm^2; time, 10 s). The cells were cultured for 48 h, and luciferase activity was determined. $^*P < 0.05$ compared with AG73T-BL or BL.

have also reported that newly developed AG73-BL can work as an US imaging agent that targets tumor vessels and allows imaging in in vivo studies.[37] Therefore, if AG73-BLs containing cationic lipids are prepared, they could become an ideal US-responsive and neovasculature-selective gene delivery tool when used in combination with US exposure and could lead to beneficial clinical applications for cancer therapy.

Recently, the term theranostics has been defined as a combined method of the modalities of diagnostic imaging and therapy, which enables simultaneous diagnosis and delivery of therapeutic drugs or genes.[43] Therefore, if BLs with cationic lipids are encapsulated with an echo-contrast gas, modified with a targeting ligand, and loaded with a therapeutic gene, the

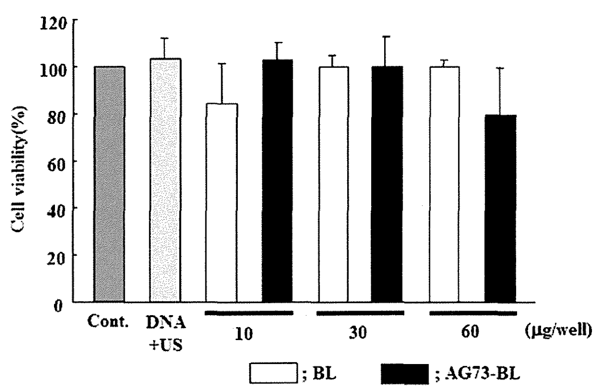

FIGURE 4 Cytotoxicity of BL or AG73-BL with US exposure. BL or AG73-BL was added to each cultured HUVEC. After 5 min of incubation, US was applied (frequency, 2 MHz; duty, 50%; burst rate, 2 Hz; intensity, 0.1 W/cm^2; time, 10 s). The cytotoxicity 1 day after transfection by the combination of AG73-BL and US exposure was determined using the WST assay. All data represent the mean $\pm$ SD (n = 4).

combination with US may enable target-tissue specific US imaging and gene therapy by the theranostic approach.

CONCLUSIONS

In this study, we showed that AG73-BL could stably and specifically attach onto the surface of HUVECs via a ligand for syndecans. Furthermore, the gene delivery efficiency was higher than that of control BL (nonlabeled BL or AG73T-BL), when US exposure was applied after the attachment. These results suggested that cavitation could be effectively induced by US exposure to AG73-BL binding to the cell surface of HUVECs, and the subsequent gene delivery into cells could be enhanced. AG73-BL should be considered as a more feasible gene delivery tool and an US contrast agents in vivo. Therefore, the combination of AG73-BL and US exposure may be useful for US imaging and the delivery of pDNA or other nucleic acid molecules to the neovasculature in tumors via systemic injection and may be applicable to a less invasive diagnostic and therapeutic system.

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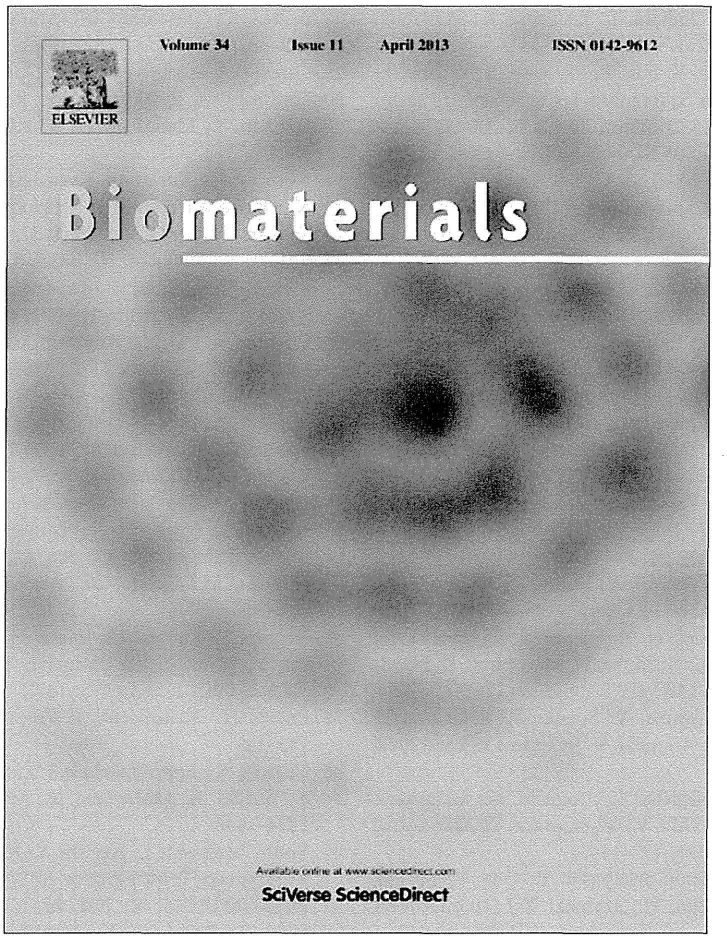
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pDNA-loaded Bubble liposomes as potential ultrasound imaging and gene delivery agents

Yoko Endo-Takahashi [a], Yoichi Negishi [a,*], Arisa Nakamura [a], Daichi Suzuki [a], Saori Ukai [a], Katsutoshi Sugimoto [b], Fuminori Moriyasu [b], Norio Takagi [c], Ryo Suzuki [d], Kazuo Maruyama [d], Yukihiko Aramaki [a]

[a] *Department of Drug Delivery and Molecular Biopharmaceutics, School of Pharmacy, Tokyo University of Pharmacy and Life Sciences, 1432-1 Horinouchi, Hachioji, Tokyo 192-0392, Japan*

[b] *Department of Gastroenterology and Hepatology, Tokyo Medical University, 6-7-1 Nishishinjuku, Shinjuku-ku, Tokyo 160-0023, Japan*

[c] *Department of Molecular and Cellular Pharmacology, School of Pharmacy, Tokyo University of Pharmacy and Life Sciences, 1432-1 Horinouchi, Hachioji, Tokyo 192-0392, Japan*

[d] *Laboratory of Drug and Gene Delivery, Faculty of Pharma Sciences, Teikyo University, 2-11-1 Kaga, Itabashi-ku, Tokyo 173-8605, Japan*

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Gene delivery

ABSTRACT

We have developed polyethyleneglycol (PEG)-modified liposomes (Bubble liposomes; BLs) that entrap ultrasound (US) contrast gas, and we have reported that the combination of BLs and US exposure was an effective tool for delivering pDNA and siRNA *in vitro* and *in vivo*. In this study, we prepared pDNA-loaded BLs using three types of cationic lipids to enhance the US imaging effect and the transfection efficiency via systemic injection. We investigated the US imaging abilities of these BLs, their protective effects on pDNA from serum component, and their transfection effects *in vitro* and *in vivo*. As a result, we demonstrated that the US imaging ability and transfection effect varied with lipid component and that p-BLs containing DSDAP could be the most stable and effective tool the among three types of p-BLs. Indeed, in ischemic muscle, p-BLs containing DSDAP could be detected using diagnostic US and could deliver bFGF-expressing pDNA using therapeutic US, leading to the induction of angiogenic factors and the improvement of blood flow. These results suggest that combining p-BLs with US exposure may be useful for stable US imaging and efficient gene delivery and may lead to the establishment of a theranostic approach, which is a combination of disease diagnosis and therapy.

1. Introduction

Various techniques using nanoparticles in combination with light, sound, and electromagnetic fields are currently being developed for both therapeutic and diagnostic application [1–9]. The term theranostic describes technology with concurrent and complementary diagnostic and therapeutic capabilities. Among all diagnostic imaging techniques, ultrasound (US) imaging has a unique advantage because it is real-time, low-cost, safe, and easy to incorporate into portable devices. In fact, US is used widely in clinical settings not only for diagnosis but also for therapy. With the use of microbubbles as US contrast agents the sensitivity of US imaging has been greatly improved. Furthermore, a combination of US and microbubbles has been proposed as a less invasive and tissue-specific method for 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, siRNA, and peptides both *in vitro* and *in vivo* [10–15].

Microbubbles hold significant potential for theranostic applications, given their propensity to be visualized *in vivo* with high sensitivity, their ability to improve drug delivery across biologic barriers, and the possibility of loading therapeutic molecules into or onto their shell. However, microbubbles have room for improvement in size, stability, and targeting function. To solve these issues, we previously developed "Bubble liposomes" (BLs). These are PEG-modified liposomes that contain echo-contrast gas, which can function as a gene and siRNA delivery tool with US exposure *in vitro* and *in vivo* [16–20]. Furthermore, to increase the efficiency of nucleic acid delivery via systemic injection, we prepared pDNA-loaded BLs (p-BLs) and siRNA-loaded BLs (si-BLs) using 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP), a cationic lipid often used for gene delivery [21,22]. These types of BLs could improve the stability of nucleic acids in the presence of serum so that the effects of nucleic acid delivery can be observed.

* Corresponding author. Tel./fax: +81 42 676 3183.
E-mail address: negishi@toyaku.ac.jp (Y. Negishi).

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