Table 2. Pharmacokinetic variables of analytes in plasma and tumor after an i.v. administration of NK012 or CPT-11 to nude mice bearing human colon cancer HT-29 cells (NK012, 30 mg/kg; CPT-11, 66.7 mg/kg)

	Test article		C_{max} (μg/mL)	T_{max} (h)	$T_{1/2z}$ (h)	AUC_{last} (μg h/mL)	AUC_{inf} (μg h/mL)	CL_{tot} (mL/h/kg)	V_{ss} (mL/kg)	MRT_{last} (h)	$MRT_{inf.}$ (h)
Plasma	NK012	P-b SN-38*	—†	—	31.4	5,000	5,010	5.99	40.4	6.68	6.74
		P-u SN-38‡	3.10	0.0833	61.7	15.5	15.8	—	—	10.8	15.3
	CPT-11	CPT-11	—	—	3.08	22.1	22.2	3,010	5,420	1.78	1.80
		SN-38	0.488	0.0833	3.76	1.10	1.11	—	—	3.82	4.04
Tumor	NK012	P-b SN-38	13.8	6	—	1,010	—	—	—	62.8	—
		P-u SN-38	0.188	6	—	10.2	—	—	—	58.1	—
	CPT-11	CPT-11	12.6	3	3.36	99.7	100	—	—	4.41	4.55
		SN-38	0.108	1	4.75	1.07	1.10	—	—	5.20	5.92

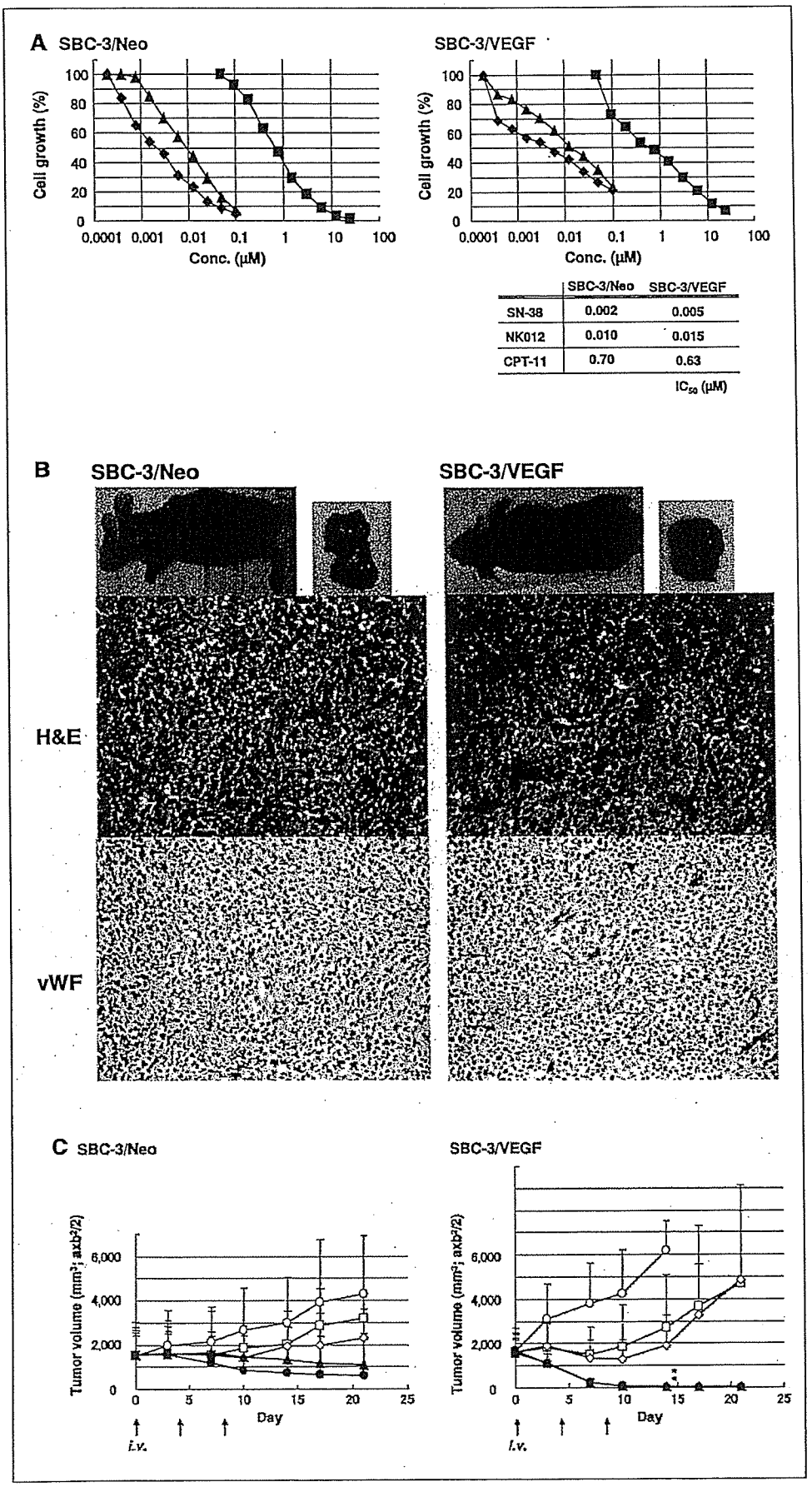
NOTE: Three female nude mice were used for the analysis of biodistribution of SN-38 and CPT-11 in plasma and tissues. Data were expressed as means.
*Polymer-bound SN-38; SN-38 remaining bound to PEG-PGlu.
†Not determined.
‡Polymer-unbound SN-38; free SN-38 from PEG-PGlu.

Several preclinical studies on cytotoxic agent–incorporating polymeric micelles show their advantage as anticancer agents *in vivo* as compared with drugs of small molecular size (19, 22, 23). Because the advantage of passive targeting has been explained by the enhanced permeability and retention theory, it is essential to elucidate the correlation between the effectiveness of micellar drugs and tumor hypervascularity and hyperpermeability. We hypothesized that a polymeric micelle–based drug carrier could increase its accumulation in the tumor site and could thus enhance the therapeutic efficacy in tumors with high vascularity. To ascertain the hypothesis, we used SBC-3/VEGF. We adopted a bulky tumor model for our *in vivo* experiment to clarify the difference in activity against SBC-3/Neo and SBC-3/VEGF tumors. Histologic examination of SBC-3/VEGF showed hypervascularity and prominent leakage of erythrocytes. On the other hand, SBC-3/Neo showed hypovascularity. Our *in vivo* experiment showed that NK012 obviously enhanced its antitumor activity in SBC-3/VEGF-inplanted mice and eradicated bulky masses. It was thought that the sensitivity of cells to NK012 might not change *in vivo* because the *in vitro* sensitivity of NK012 was almost equivalent between SBC-3/Neo and SBC-3/VEGF cells. When we compared the distribution of NK012 (free SN-38) in the tumor sites, significantly enhanced accumulation was observed in the SBC-3/VEGF tumors. This strongly suggested that the drug distribution throughout the tumor site was enhanced by the hypervascularity and hyperpermeability induced by VEGF, and, subsequently, higher antitumor activity was achieved. High vascular density and enhanced vascular permeability might also be favorable for drug delivery of low molecular weight drugs. However, the SN-38 concentration was not significantly high in SBC-3/VEGF tumors after the administration of CPT-11, and tumors exhibited rapid regrowth after the treatment. We assume that such conventional low molecular size anticancer agents almost disappear from the bloodstream without being subjected to the enhanced permeability and retention effect before they can reach the target organs (solid tumor). The fact of correlation between the blood vessel density in

Table 3. Tumor-to-plasma concentration ratio (Kp) of analytes after an i.v. administration of NK012 (30 mg/kg) to nude mice bearing human colon cancer HT-29 cells

Test article	Analyte		Time after administration (h)						
			0.0833	1	6	24	48	72	168
NK012	P-b SN-38*	Plasma (μg/mL)	612	410	254	23.3	1.25	0.278	0.0333
		Tumor (μg/g)	4.99	8.00	13.8	9.95	5.90	5.03	3.58
		Kp† (mL/g)	0.00815	0.0195	0.0543	0.427	4.72	18.1	108
	P-u SN-38‡	Plasma (μg/mL)	3.10	1.24	0.673	0.0717	0.0127	0.00925	0.00325
		Tumor (μg/g)	0.0763	0.187	0.188	0.0904	0.0531	0.0426	0.0358
		Kp (mL/g)	0.0246	0.151	0.279	1.26	4.18	4.61	11.0

NOTE: Data were expressed as means of three mice.
*Polymer-bound SN-38; SN-38 remaining bound to PEG-PGlu.
†Kp values were calculated on the mean concentrations of three mice.
‡Polymer-unbound SN-38; free SN-38 from PEG-PGlu.



	SBC-3/Neo	SBC-3/VEGF
SN-38	0.002	0.005
NK012	0.010	0.015
CPT-11	0.70	0.63

IC_{50} (μM)

Figure 4. Growth inhibitory effect of NK012, SN-38, and CPT-11 on SBC-3/Neo and SBC-3/VEGF cells. *A*, in *in vitro* experiment, the cells were exposed to the indicated concentrations of each drug for 72 hours. The growth inhibition curves and IC_{50} values for NK012 (▲), SN-38 (♦), and CPT-11 (■) are shown. *B*, representative photographs of massive tumors developed from SBC-3/Neo and SBC-3/VEGF at the time just before treatment initiation. Histologic (H&E, ×20) and immunohistochemical (von Willebrand factor, ×20) examinations for each tumor are shown. *C*, i.v. administration of NK012 or CPT-11 was started when the mean tumor volumes of groups reached a massive size of 1,500 mm^3. The mice were divided into test groups (O, control; □, CPT-11 15 mg/kg/d; ◇, CPT-11 30 mg/kg/d; ▲, NK012 10 mg/kg/d; ●, NK012 20 mg/kg/d). NK012 or CPT-11 was administered i.v. on days 0, 4, and 8. Each group consisted of four mice. *, $P < 0.05$.

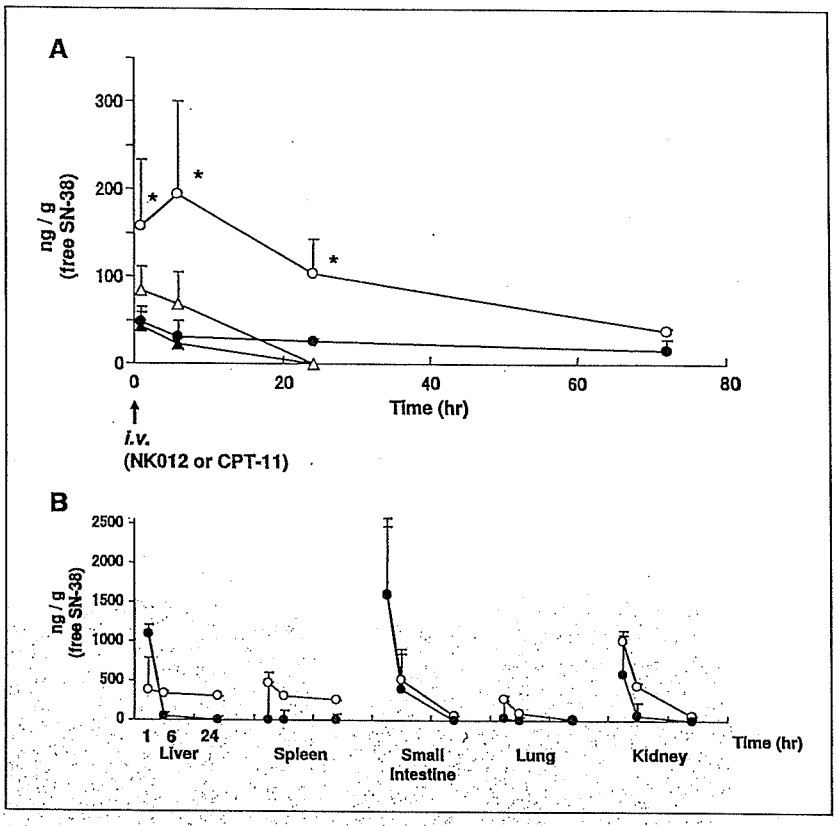


Figure 5. Tissue and tumor distribution of free SN-38 after administration of NK012 and CPT-11. *A*, time profile of free SN-38 concentration in SBC-3/Neo (●, NK012 20 mg/kg/d; ▲, CPT-11 30 mg/kg/d) and SBC-3/VEGF (○,NK012 20 mg/kg/d; △, CPT-11 30 mg/kg/d). NK012 on days 0 and 4 (96 hours) or CPT-11 on day 0 was administered. *, $P < 0.05$. *B*, tissue distribution of free SN-38 after single injection of NK012 at 30 mg/kg (○) and CPT-11 at 40 mg/kg (●).

the tumor mass and poor prognosis for survival in people with various types of cancers (25–28) supports the idea that low molecular weight drugs are not so effective in the treatment of solid tumors, which are rich in blood vessels.

Jain (35) reported that the convective passage of large drug molecules into the core of solid tumors could be impeded by abnormally high interstitial pressures in solid tumors. However, he also considered that low molecular weight anticancer agents might be harmful to normal organs because they can leak out of normal blood vessels freely; he finally concluded that one useful strategy for evading the barriers to drug dispersion would be to inject patients with drug carriers, such as liposome, filled with low molecular weight drugs. NK012 has the potential to allow the effective sustained release of SN-38 inside a tumor following the accumulation of NK012 into tumor tissue. As a matter of fact, substantial amount of SN-38 is expected to be released from the polymeric micelle. Consequently, released SN-38 becomes distributed throughout the tumor tissue and internalizes into cancer cells to kill them.

In recent years, the novel liposome-based formulation of SN-38 (LE-SN38) has been developed (36). LE-SN38 shows promising antitumor activity against various cancer cell lines (37, 38) and a clinical trial to assess its efficacy is now under way (39). The release of SN-38 from LE-SN38 is very slow as compared with NK012, ~1.9% of the drug being released from LE-SN38 in PBS buffer over 120 hours (36). The size of LE-N38 ranges from 150 to 200 nm. On the other hand, the particle size of NK012 is ~20 nm. Interestingly, Unezaki et al. (40) reported that fluorescence-labeled PEG liposomes were densely located outside the tumor vessels and stayed around the vessel walls for 2 days after i.v. injection. These data suggest that the PEG liposome is too large to move freely in the tumor interstitium and too stable to be released easily. The difference in size distribution and the character of the drug release between NK012 and LE-SN38 might influence their clinical effectiveness in the treatment of solid tumors.

One of the major toxicities associated with CPT-11 administration is severe diarrhea. Although the mechanism of the diarrhea has not yet been elucidated, one possible explanation is structural and functional injuries to the gastrointestinal tract owing to the mitotic inhibitory activity of SN-38 and CPT-11. It was reported that the number of episodes of diarrhea had a better correlation with the plasma AUC of SN-38 than with CPT-11 (41). In the present study, no difference in SN-38 accumulations in the small intestine was seen when equimolar NK012 (20 mg/kg) and CPT-11 (30 mg/kg) were administered. We also reported, using a rat mammary tumor model, that NK012 showed significant antitumor effect with diminishing incidence of diarrhea as compared with CPT-11 (42). These results suggest that diarrhea, one of the dose-limiting toxicities of CPT-11, is not augmented by the administration of NK012.

In conclusion, the present data suggest that NK012 possesses a treatment advantage over CPT-11, especially in hypervascular tumors such as renal cell carcinomas, medulloblastomas, and hepatocellular carcinomas. We have now started a phase I clinical trial for NK012 in patients with advanced solid tumors.

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

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Abstract

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

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

1. Introduction

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

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

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

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

2. Materials and methods

2.1. Cells

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

2.2. Preparation of liposomes and Bubble liposomes

Liposomes composed of 1,2-distearoyl-sn-glycero-phosphatidylcholine (DSPC) (NOF Corporation, Tokyo, Japan) and 1,2-distearoyl-sn-glycero-3-phosphatidyl-ethanolamine-methoxy-polyethyleneglycol (DSPE-PEG (2k)-OMe) (NOF Corporation, Tokyo, Japan) (94:6 (m/m)) were prepared by reverse phase evaporation. In brief, all reagents were dissolved in 9:1 (v/v) chloroform/methanol. Physiological saline was added into the lipid solution. After that, the mixture was sonicated and evaporated at 65 °C. The solvent was completely removed, and the size of the liposomes was adjusted to less than 200 nm using extruding equipment (Northern Lipids Inc., Vancouver, BC) and sizing filter (pore size: 200 nm, 100 nm) (Nuclepore Track-Etch Membrane, Whatman plc, UK). After sizing, liposomes were passed through a 0.45 μm pore size filter (MILLEX HV filter unit, Durapore PVDF membrane, Millipore Corporation, MA) to sterilize. Lipid concentration was measured with Phospholipid C test Wako (Wako Pure Chemical Industries, Ltd., Osaka, Japan). Bubble liposomes were prepared from liposomes and perfluoropropane gas (Takachiho Chemical Ind. Co. Ltd., Tokyo, Japan). In brief, 5 mL sterilized vials containing 2 mL of liposome suspension (lipid concentration: 1 mg/mL) were filled with perfluoropropane gas, capped and then pressured with 7.5 mL of perfluoropropane gas. The vial was placed in a bath-type sonicator (42 kHz, 100 W) (BRANSONIC 2510J-DTH, Branson Ultrasonics Co., Danbury, CT) for 5 min to form Bubble liposomes.

2.3. Ultrasound imaging in vitro

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

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

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

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

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

2.6. Transfection of plasmid DNA into cells using Bubble liposomes

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

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

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

2.8. Measurement of luciferase and EGFP expression

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

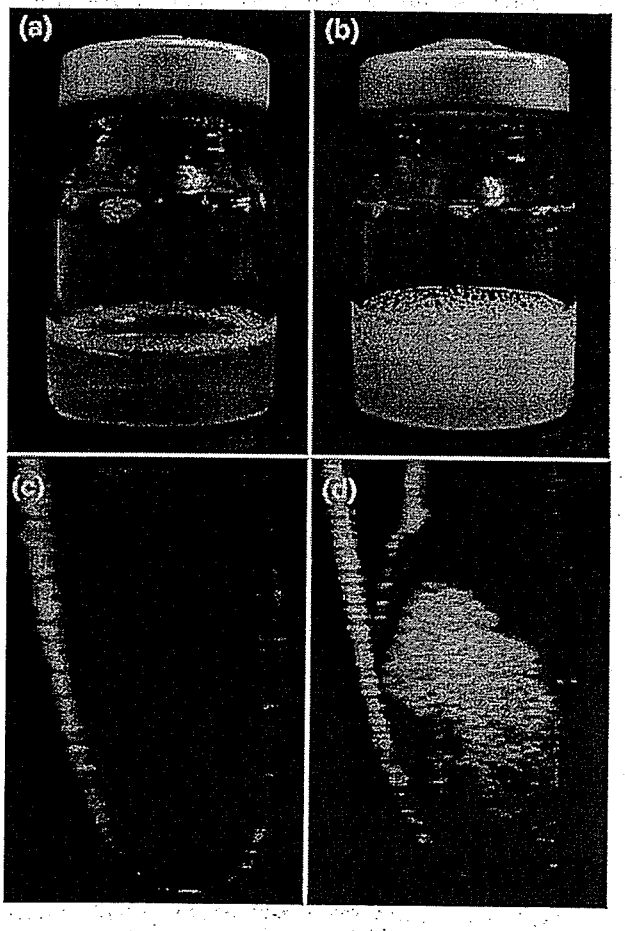

Fig. 1. Aspect and ultrasonography of PEG-liposomes and Bubble liposomes. Aspects of PEG-liposomes (a) and Bubble liposomes (b). PEG-liposomes sonicated with perfluoropropane gas became to Bubble liposomes in the vial. Ultrasonographic images of PEG- (c) and Bubble (d) liposomes.

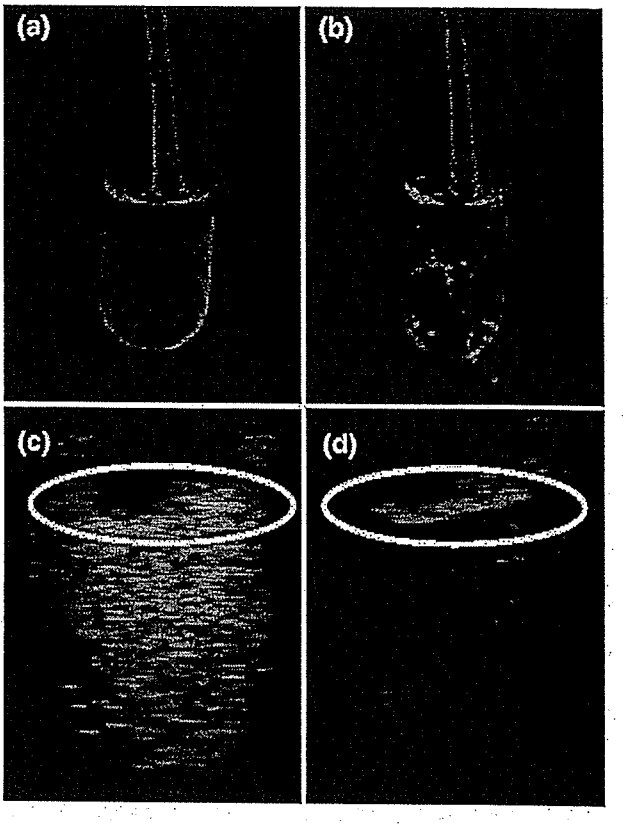

Fig. 2. Cavitation of Bubble liposomes exposed to ultrasound. Naked (a, b) and ultrasonographic (c, d) images of Bubble liposomes. Ultrasonic probe (circle) positioned in Bubble liposome suspension exposed 2.5 W/cm^2 of ultrasound for 10 s. Images were observed before (a, c) and after ultrasound (b,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

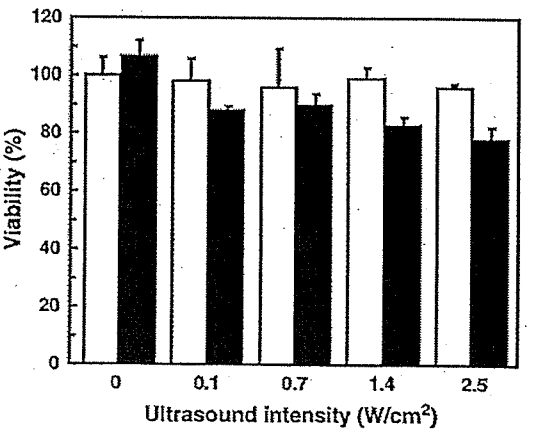

Fig. 3. Viability of COS-7 cells exposed with US and Bubble liposomes. COS-7 cells were exposed to ultrasound under various intensities with (■) or without (□) Bubble liposomes and then cultured for 24 h. Cell viability was assessed by MTT assays. Data are shown as means±S.D. (n=3).

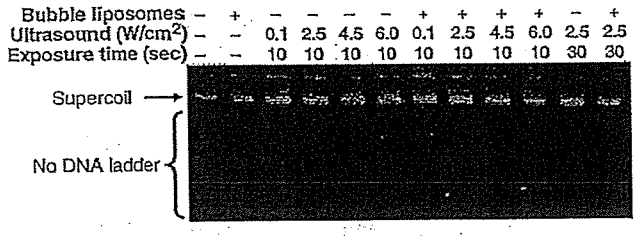

Fig. 4. Plasmid pCMV-Luc exposed to ultrasound under various conditions with or without Bubble liposomes. After exposure to ultrasound, Bubble liposomes were removed using phenol/chloroform and then pCMV-Luc was conventionally precipitated with ethanol. The precipitate was dissolved in TE buffer and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

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

3. Results

3.1. Features of Bubble liposomes

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

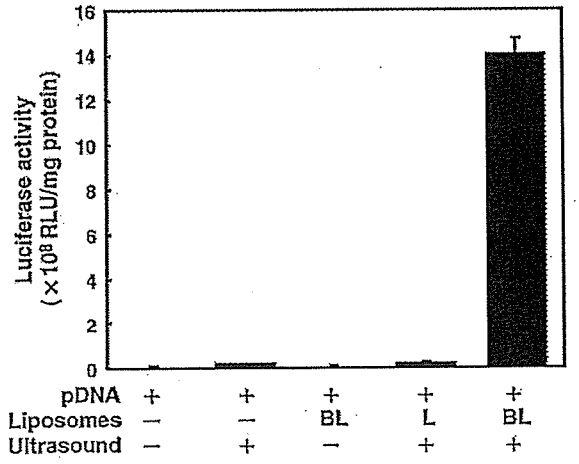

Fig. 5. Luciferase expression in COS-7 cells transfected by ultrasound with Bubble liposomes. COS-7 cells (1×10^5 cells/500 μL) mixed with pCMV-Luc (5 μg) and Bubble liposomes (60 μg) were exposed to ultrasound (frequency, 2 MHz; Duty, 50%; burst rate, 2 Hz; intensity, 2.5 W/cm^2; time 10 s). The cells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

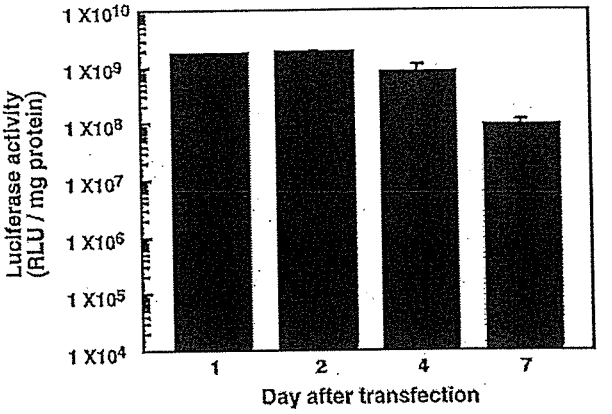

Fig. 6. Duration of luciferase expression in COS-7 cells transfected using ultrasound with Bubble liposomes. COS-7 cells (1×10^5 cells/500 μL) mixed with pCMV-Luc (5 μg) and Bubble liposomes (60 μg) were exposed to ultrasound under the following conditions: frequency, 2 MHz; duty, 50%; burst rate, 2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

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

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

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

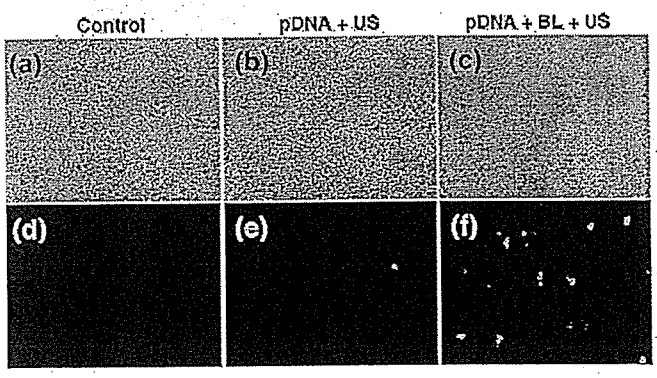

Fig. 7. EGFP expression in COS-7 cells transfected with Bubble liposomes and ultrasound exposure. COS-7 cells (1×10^5 cells/500 μL/tube) were mixed with pEGFP-C1 (5 μg) and Bubble liposomes (60 μg). The cell mixture was exposed to ultrasound (frequency, 2 MHz; duty, 50%; burst rate, 2 Hz; intensity,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

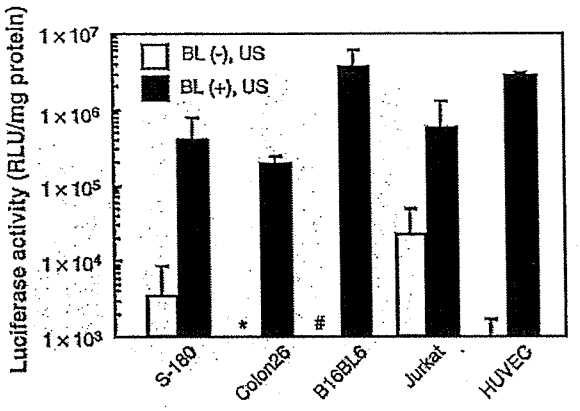

Fig. 8. Luciferase expression in various types of cells transfected using Bubble liposomes and ultrasound. Cells (1×10^5 cells/500 μL) mixed with pCMV-Luc (5 μg) and Bubble liposomes (60 μg) were exposed or not to ultrasound (frequency, 2 MHz; duty, 50%; burst rate, 2 Hz; intensity, 2.5 W/cm^2; time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

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

3.3. Gene transduction with Bubble liposomes and ultrasound

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

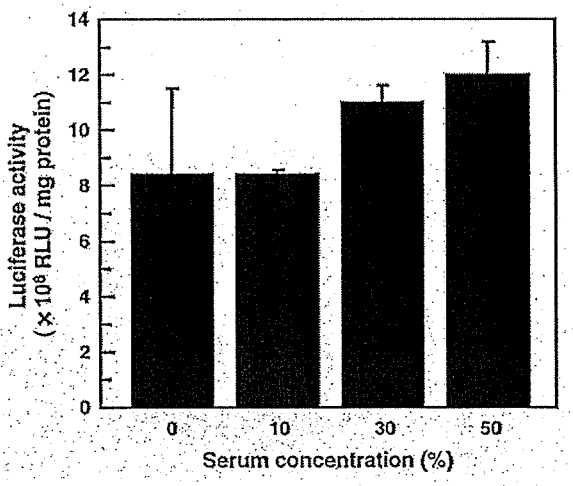

Fig. 9. Effect of serum on transfection efficiency of Bubble liposomes. COS-7 cells (1×10^5 cells/500 μL) mixed with pCMV-Luc (0.25 μg) and Bubble liposomes (60 μg) were exposed to ultrasound (frequency, 2 MHz; duty, 50%; burst rate, 2 Hz; intensity, 2.5 W/cm^2; time 10 s) in the absence or the presence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

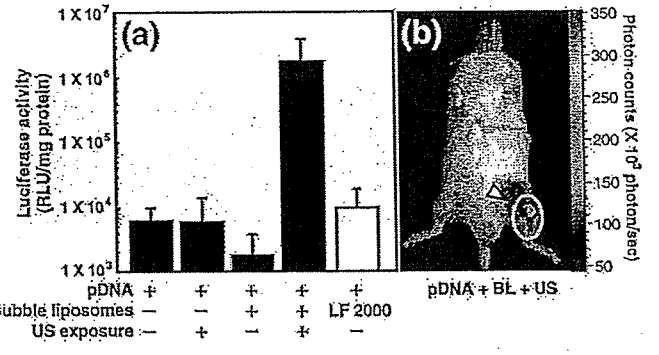

Fig. 10. Gene delivery to femoral artery with Bubble liposomes. Each sample containing plasmid DNA 10 μg was injected into femoral artery. In the same time, ultrasound (frequency, 1 MHz; duty, 50%; burst rate, 2 Hz; intensity, 1 W/cm^2; time 2 min) was exposed to the downstream area of injection site.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

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

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

3.4. In vivo gene delivery with Bubble liposomes

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

4. Discussion

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

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

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

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

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

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

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

5. Conclusion

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

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Development of the Liposomes Entrapped Ultrasound Imaging Gas ("Bubble Liposomes") as Novel Gene Delivery Carriers

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Abstract. Recently, microbubbles and ultrasound have been investigated with a view to improving the transfection efficiency of nonviral delivery systems for gene by cavitation. However, microbubbles had some problems in terms of stability and targeting ability. To solve these problems, we paid attention to liposomes that had many advantages such as stable and safe *in vivo* and easy to modify targeting ligand. Previously, we have represented that liposomes are good drug and gene delivery carriers. In addition, we developed that the liposomes ("Bubble liposomes") were entrapped with perfluoropropane known as ultrasound imaging gas. In this study, we assessed about feasibility of "Bubble liposomes" as gene delivery tool utilized cavitation by ultrasound irradiation. "Bubble liposomes" could effectively deliver plasmid DNA to cells by combination of ultrasound irradiation without cytototoxicity. This result suggested that "Bubble liposomes" might be a new class of tool for gene delivery.

Keywords: Liposome, Gene delivery
PACS: 87.16.Dg, 87.54.Hk, 87.57.-s

INTRODUCTION

Gene therapy has a potentiality for treatment of cancer and diseases owning to genomic defects. It is important to select gene delivery vector which have good potency in terms of gene transduction efficiency, safe and easy to apply. In this situation, non-viral vectors are drawing the attention. However, they suffer from low transduction efficiencies. To improve this problem, many researchers attempt to develop effective gene delivery tool.

Recently, it was reported that microbubbles, which were contrast agents for medical ultrasound imaging, improved the transfection efficiency by cavitation with ultrasound

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irradiation[1-3]. However, size of them is about 1-4 μm, it is too large to use for intravascular applications. It is necessary that they are held to a defined size, generally smaller than red blood cells and are sufficiently stable after injection into the blood. Additionally, it is known that microbubbles are difficult to modify on their surfaces such as addition of targeting function.

Previously, we developed the "Bubble liposomes" which were entrapped with perfluoropropane (PF) known as ultrasound imaging gas. The size of them was submicron, they could be used as intravascular application. In addition, it is easy to modify on liposome surface[4-7]. From the aspect of these, it is expected that "Bubble liposomes" might be a new type of bubble in medical field. Moreover, it is thought that cavitation would be induced by the ultrasound irradiation to "Bubble liposomes", and gene such as plasmid DNA could be delivered into cells. In this study, we assessed the feasibility of "Bubble liposomes" as gene delivery tool utilized cavitation by ultrasound irradiation.

CHARACTERISTICS OF "BUBBLE LIPOSOMES"

"Bubble liposomes" were made of conventional liposomes that were prepared with reverse phase evaporation method[8]. As shown Figure 1, "Bubble liposomes" became

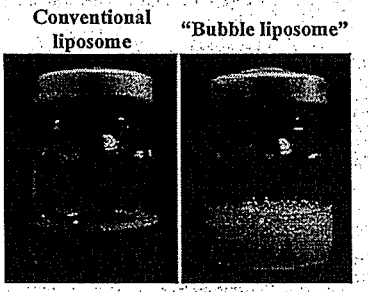

Figure 1. Appearance of conventional liposomes and " Bubble liposomes"

cloudy from conventional liposomes. This difference in appearance of these liposomes was thought to be related to PF gas entrapped within liposomes. To confirm the PF gas

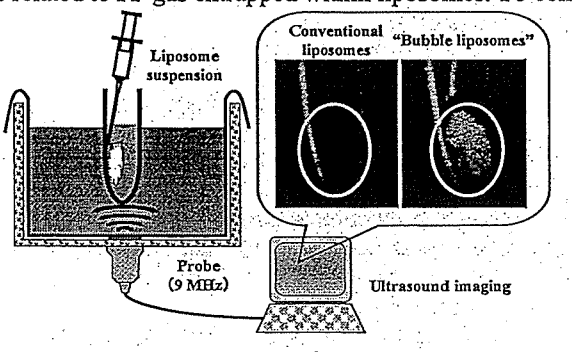

Figure 2. Ultrasound imaging of "Bubble liposomes"

within "Bubble liposome", they were imaged with a conventional ultrasound imaging machine (UF-750XT, Fukuda Denshi). Liposomes were injected into latex tube in water bath and imaged (Figure 2). "Bubble Liposomes" made ultrasound echo signal increased, but conventional liposomes did not so. Therefore, it confirmed that "Bubble liposomes" were including PF gas. In addition, it is expected that "Bubble liposomes" are able to be utilized as ultrasound imaging agents.

Next, we examined whether cavitation was induced with "Bubble liposomes" and ultrasound irradiation. Ultrasound irradiation was utilized with Sonoporation Gene Transfection System (SONOPORE, NEPA GENE CO., LTD.). The condition of ultrasound irradiation was shown Figure 3. When "Bubble liposomes" was irradiated

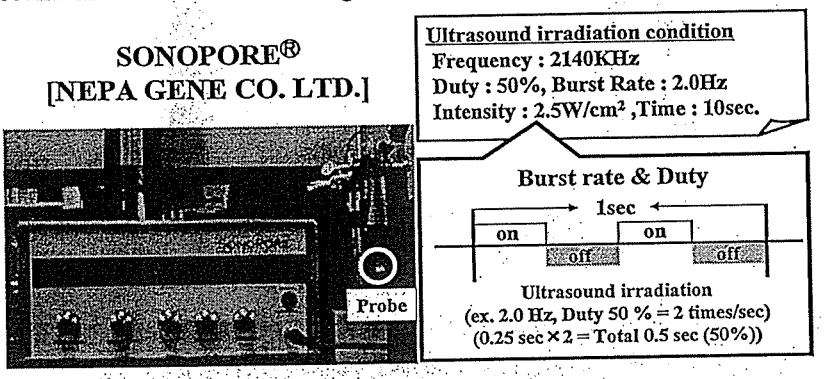

Figure 3. Condition of Ultrasound irradiation

with ultrasound, strength of ultrasound echo signal remarkably decreased with ultrasound imaging compared with ultrasound non-irradiated "Bubble liposomes" (Figure 4). This result suggested that cavitation was effectively induced by combination with "Bubble liposomes" and ultrasound irradiation. Therefore, it is thought that "Bubble liposomes" could be utilized as gene delivery carrier.

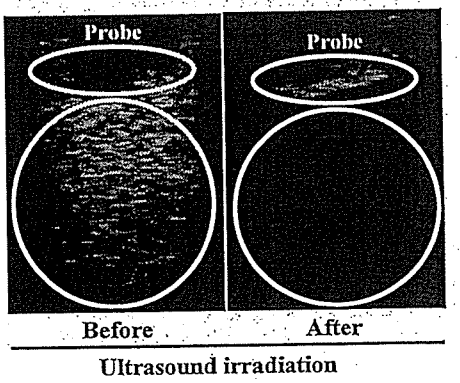

FIGURE 4. Ultrasound imaging of "Bubble liposomes" before and after ultrasound irradiation

"BUBBLE LIPOSOMES" AS GENE DELIVERY TOOL

Some researchers had reported about gene transfection utilized with microbubbles[2,3]. However, there was little of report about gene transfection with nano-bubbles. Thus, we examined about gene transfection with "Bubble liposomes" (FIGURE 5). When COS-7 cells were transfected with luciferase plasmid DNA

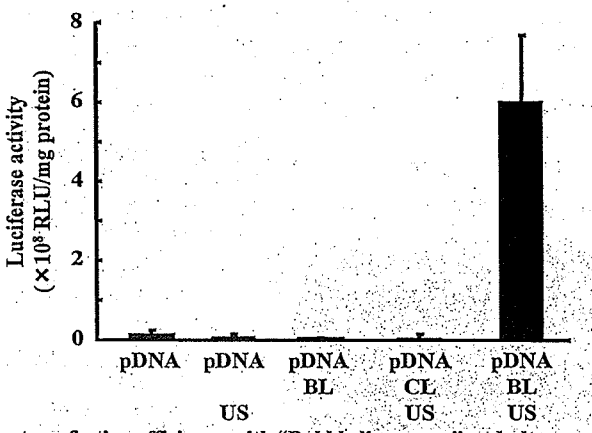

FIGURE 5. Gene transfection efficiency with "Bubble liposomes" and ultrasound irradiation

COS-7 cells were irradiated ultrasound with the condition as shown in FIGURE 3.
(BL: "Bubble liposomes", CL: Conventional liposomes, US: Ultrasound irradiation)

(pDNA) only or pDNA combined with ultrasound irradiation (general sonoporation), luciferase expression was not detectable. And it was also not detectable in the cells treated with mixture of pDNA and "Bubble liposomes" without ultrasound irradiation. In addition, pDNA was not transfected with conventional liposomes and ultrasound irradiation. On the other hand, combination of "Bubble liposomes" and ultrasound irradiation could effectively transfect pDNA into the cells due to induce the cavitation. Amazingly, the gene expression efficiency was very high with the ultrasound irradiation for 10 second though it was so short. Then, "Bubble liposomes" did not remarkably cytotoxicity (Data not shown). These results suggested that "Bubble liposomes" could be novel gene delivery carrier.

CONCLUSION

"Bubble liposomes" were imaged with ultrasound imaging machine. In addition, they could induce cavitation with ultrasound irradiation, and effectively and quickly introduce pDNA into the cells. Therefore, it was suggested that "Bubble liposomes" might be utilized as ultrasound imaging agent as well as gene delivery carrier.

ACKNOWLEDGMENTS

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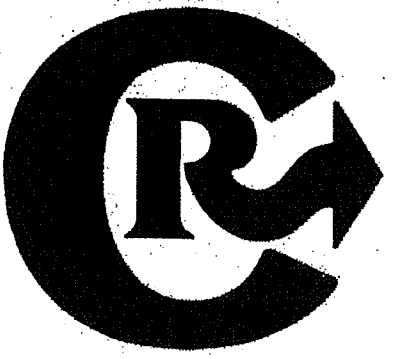

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Spinal gene transfer using ultrasound and microbubbles

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Abstract

Spinal gene therapy is a promising option for treating various spinal-related disorders. Several previous studies using viral vectors reported successful transfer of therapeutic genes into the spinal nerve system. However, because of the considerable immunogenicity related to the use of viruses, non-viral gene transfer still needs to be developed. One possible approach is the combined use of ultrasound and echo-contrast microbubbles. The present study shows that this method can be applied for targeted intrathecal gene delivery. We intrathecally injected a mixture of plasmid-DNA encoded with luciferase and commercially available albumin microbubbles by needle puncture at the lower lumbar intervertebral space in mice. Subsequent percutaneous ultrasonication on the lumbar vertebrae significantly enhanced the luciferase expression, analyzed by imaging luciferin bioluminescence, in the dorsal meningeal cells at the insonated region. No apparent neurological damages were induced by the present spinal interventions. In addition to the general benefits of the combined use of ultrasound and microbubbles, our approach can offer some advantages specific to spinal gene transfection including minimal invasiveness of simple percutaneous dural puncture, targetability due to the limited access of ultrasound waves through anatomical apertures of the vertebrae, and possible paracrine delivery of therapeutic molecules to the spinal nerve system.

Keywords: Gene therapy; Spinal disorder; Intrathecal delivery; Ultrasound; Microbubble

1. Introduction

Spinal gene transfer is expected to become a promising option for treating various spinal-related disorders including nerve injury, degenerative diseases, neoplasm, and chronic pain [1,2]. To date, the most widely used vehicles for gene delivery are viral vectors. Recent animal studies using adenovirus vectors indicated that direct spinal injection of specific growth-factor genes achieved functional recovery after acute spinal cord injury [3–5]. The feasibility of virus-mediated gene transfer to treat chronic pain has been also explored using the precursor genes of endogenous opioids [6,7]. The targeted spinal tissues for the opioid-gene transfer can be both meningeal and parenchyma cells. Among the utilized viral vectors that are mostly derived either from adenoviruses, adeno-associated viruses, herpes simplex viruses or retroviruses [8], herpes vectors may be the most promising for antinociceptive gene therapy because of its high selectivity to primary afferent neurons [2,9].

Despite such experimental successes in virus-mediated gene delivery, however, alternative non-viral transgene applications need to be developed because the clinical use of viral vectors is limited by such possible disadvantages as immunogenic properties, inflammatory responses, and the difficulty of producing large amounts of pure virus. In this context, it has been reported recently that the emission of high-pressure ultrasound in combination with echo-contrast agents, microbubbles, can facilitate gene transfection into cells [10]. Possible explanations for the mechanisms include the production of transient pores on the cell membranes as well as an increase in the

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membrane fluidity by impulsive pressures raised when microbubbles are disrupted by ultrasound [11,12]. The combined use of microbubbles and ultrasound for gene delivery has several advantages including low toxicity, low immunogenicity, low invasiveness, high target selectivity, and repeatable applicability [11]. In the present study, we focused on spinal gene delivery and demonstrated that percutaneous ultrasonication in combination with intrathecal microbubbles facilitated gene transfection in spinal meningeal cells in mice.

2. Materials and methods

2.1. Animals

The study was approved by the Animal Care Committee of Tohoku University Graduate School of Medicine. Male inbred BALB/c mice were purchased from the institutional breeding facilities at 5–6 weeks of age and maintained in an antigen- and virus-free room (22±1 °C, 60±10% relative humidity, 12 h/12 h light/dark cycle, food and water *ad libitum*). The mice were studied at 7–8 weeks of age.

2.2. Plasmid DNA

pCMV-luciferase-GL3 (pcLuc-GL3: 7.4 kb) was constructed by cloning the luciferase gene from the pGL3-Control Vector (Promega Corp., Madison, WI, USA) into pcDNA3 (5.4 kb) (Invitrogen, San Diego, CA, USA) at the *Hin*dIII and *Bam*HI sites. Plasmid DNA was purified with a QIAGEN plasmid isolation kit (QIAGEN, Hilden, Germany) and prepared at a final concentration of 1 mg/ml.

2.3. Intrathecal transfection of plasmid DNA

Mice were anesthetized with an intraperitoneal injection of sodium pentobarbital (80 mg/kg) and immobilized in a prone position on an acrylic plate. Intrathecal access was accomplished by percutaneous lumbar puncture through the 4/5th or 5/6th intervertebral space using a 27-gauge stainless-steel needle according to the Hylden and Wilcox technique with modification [13]. Dural penetration of the needle was confirmed by inspecting tail flicks of the mice. A total volume of 10 μl containing 5 μg plasmid and commercially available albumin-coated octa-fluoropropane gas microbubbles (MB), Optison™ (Amersham Health, Oslo, Norway; $5–8\times10^8$/ml, 3–4.5 μm in diameter), in phosphate buffered saline at a final MB concentration either 0, 20 or 50 v/v% was then injected slowly into the intrathecal space using a 50-μl microsyringe (Hamilton, Bonaduz, GR, Switzerland). A mixture of the plasmid with MB was prepared by gentle hand shaking immediately before injection. Immediately after the intrathecal injection, the mice were placed at a vertical position in a 37 °C water bath and dorsally insonated for 1 min at the vertebral region that had been injected using an ultrasound-emitting transducer (6 mm in diameter; Fuji Ceramics Co., Fujinomiya, Japan). The ultrasound (US) parameters used were as follows: central frequency, 950 kHz; duty ratio, 20%; the average intensity per cross section, 1.3 W/cm^2; acoustic pressure at a standoff distance of 1 mm from the transducer surface, 0.6 MPa; energy, 2.4 J/cm^2. After the insonation, the mice were dried and kept under a heat lamp until recovery from anesthesia.

2.4. Analysis of luciferase activity

Mice were killed by neck dislocation under deep anesthesia with isoflurane at 1, 3, 7 days after transfection of the luciferase gene. The spinal cord was harvested *en bloc* at the level of the lower thoracic to sacral region by careful dissection of the vertebrae and placed on a dish plate. Subsequently, the tissue was fully covered with Luciferin 30 mg in 1 ml PBS (Promega Co., Madison, WI, USA). Luciferin bioluminescence was immediately quantified as the luciferase activity using an *in vivo* imaging system (IVIS™, Xenogen Co., Alameda, CA, USA) [14].

2.5. Immunohistochemistry

The harvested spinal cords were preserved in 10% PFA for 4 h and then embedded in paraffin and sectioned. Sections (4 μm thickness) were evaluated for the presence of luciferase protein by immunostaining. The sections were deparaffinized in xylene for 5 min 3×, rehydrated through graded ethanol and equilibrated in PBS. The sections were incubated in 3% H_2O_2 for 30 min to dampen endogenous peroxidase activity. They were incubated for 30 min at room temperature with 10% normal goat serum (Nichirei Biosciences Inc., Tokyo) to reduce nonspecific protein binding. After a wash in PBS, the sections were incubated with biotin-labeled rabbit anti-luciferase antibody (0.5 g/ml) (Cortex Biochem, San Leandro, CA, USA) overnight at 4 °C. The following day, after three washes in PBS, immunoreactivity was detected using an anti-rabbit IgG Histofine SAB-PO(M) kit (Nichirei Biosciences Inc., Tokyo, Japan) and diaminobenzidine (DAB) as a chromogen according to the manufacturer's protocols. After color development, the spinal cord sections were counterstained with hematoxylin and were then dehydrated, cleared, and mounted on slides.

2.6. Assessment of post-transfectional spinal injury

2.6.1. Thermal nociception

For assessing the nociceptive responses to thermal stimuli after the intrathecal procedure, the paw withdrawal latencies following exposure to infra-red radiant heat were determined [15] using a commercial device (7370-Planter Test, Ugo Basille, Comerio, Italy) three days after the gene transfection. Mice were placed in a clear plastic chamber (210 mm× 105 mm× 130 mm) with a glass floor and allowed to acclimate for at least 5 min. After the acclimation period, radiant heat was projected to the hind paw and time count was started. The heat projection was made through a 5 mm× 10 mm aperture in the top of a movable case containing the radiant heat source that was positioned under the glass floor directly beneath the paw. The radiant heat source consisted of a high intensity projecting Halogen lamp bulb (8 V–50 W) located 40 mm below the floor. The time count was stopped when the mouse withdrew its paw. Mice were tested

with three determinations each at the right and left paw and were allowed to rest for at least 30 min between each session.

2.6.2. Rotarod

For assessing motor function after the intrathecal procedure, mice were tested using a rotarod (IITC; Life Science Instrument, Woodland Hills, CA, USA) three days after the gene transfection. The rod had a diameter of 3.8 cm and was accelerated from 0 to 30 rpm over a 17.5-s period. The total time that the mice remained on the rotarod was measured. The time count was stopped when mice fell from the rod or when they rotated around completely two times without walking [16]. Mice were tested with three trials and were allowed to rest for at least 30 min between each session.

2.7. Statistical analysis

All values are expressed as mean±SEM. Statistical analysis for the spinal luciferase activities was performed by one-factor analysis of variance (ANOVA) with Fisher's protected least significant difference test (Fisher's PLSD) as a post-hoc procedure. Unpaired Student-*T* test was used for the behavioral evaluations. Statistical significance was defined as $p<0.05$.

3. Results

3.1. Effects of microbubbles and ultrasound on spinal gene transfection

Fig. 1 shows representative views of luciferin bioluminescence in the mouse spinal cord obtained by the imaging system (sum of photon counts from a region of interest at 5 min is presented). The spinal luciferase activities determined one day after four different treatments (DNA alone, DNA+MB, DNA+US, and DNA+MB+US) are shown in Fig. 2a. The concentration of MB used was 50%. The luciferase activities in the treatments with DNA+MB and DNA+US were as low as that with DNA alone. In contrast, ultrasonication after the DNA+MB injection significantly increased the luciferase activity by approximately 25 fold compared to the other treatments ($p<0.001$).

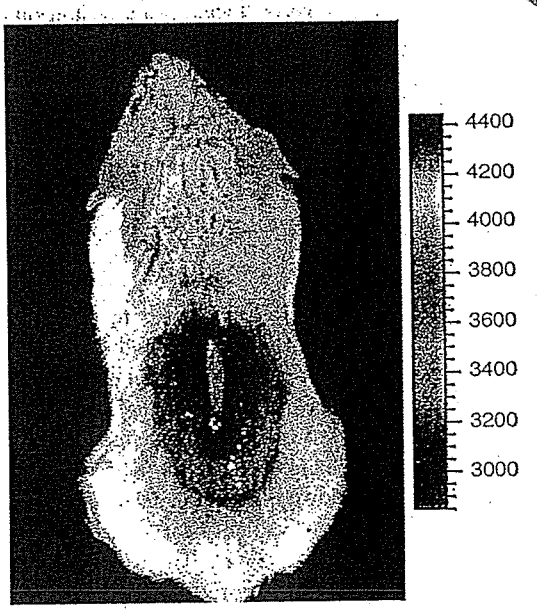

Fig. 1. Representative image showing luciferin bioluminescence (IVIS™, Xenogen Co., Alameda, CA, USA) in the spinal cord of BALB/c mice a day after the intrathecal injection of plasmid DNA and microbubbles (Optison™, Amersham Health, Oslo, Norway) followed by percutaneous ultrasonication. Imaging time is 5 min. Color bar units represent photons s^{-1} cm^{-2}.

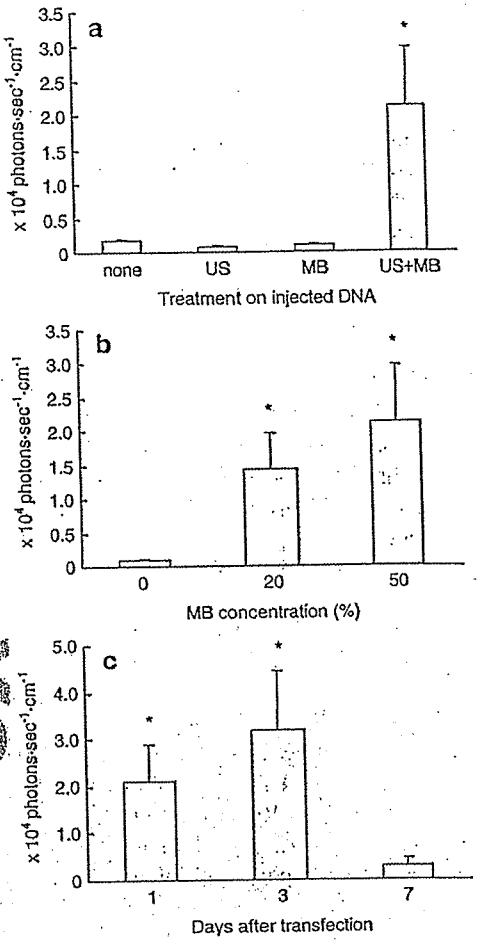

Fig. 2. Plasmid-derived spinal luciferase activity represented by luciferin bioluminescence in mice subjected to intrathecal gene delivery using ultrasound and microbubbles. The ultrasound parameters were as follows: central frequency, 950 kHz; duty ratio, 20%; the average intensity per cross section, 1.3 W/cm^2; acoustic pressure at a standoff distance of 1-mm from the transducer surface, 0.6 MPa; energy, 2.4 J/cm^2; exposure time, 1 min. (a) Treatment effects of ultrasound and 50% microbubbles one day after the application. Combined use of ultrasound and microbubbles significantly enhanced the gene transfection compared to the other treatments. (b) Effects of the microbubble concentration on the spinal gene expression. Microbubbles at concentrations of both 20 and 50% significantly enhanced the gene transfection one day after the application. No significant difference was found between the luciferase activities in mice treated with the two concentrations. (c) Time course of spinal gene expression in mice treated with ultrasound and 50% microbubbles. Luciferase activity significantly increased at 1 and 3 days after gene transfection which disappeared by the 7th day. No statistical difference was found between the gene expression at 1 and 3 days post-transfection. *$p<0.001$. $n=5$ in each group. US: ultrasound; MB: microbubble (Optison).