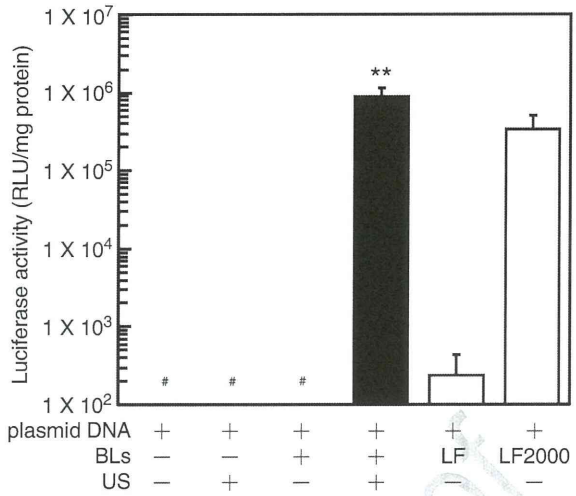

Fig. 4. In vivo gene delivery into mouse ascites tumor cells with Bubble liposomes. S-180 cells (1×10^6 cells) were i.p. injected into ddY mice. After 8 days, the mice were anaesthetized, then injected with 510 μL of pCMV-Luc (10 μg) and Bubble liposomes (500 μg) in PBS. Ultrasound (frequency: 1 MHz, duty: 50%; intensity: 1.0 W/cm^2, time: 1 min) was transdermally applied to the abdominal area. In another experiment, pCMV-Luc (10 μg) – Lipofectin (50 μg) or Lipofectamine 2000 (50 μg) complex was suspended in PBS (510 μL) and injected into the peritoneal cavity of mice. After 2 days, S-180 cells were recovered from the abdomens of the mice. Luciferase activity was determined, as described in Materials and Methods. Each bar represents the mean ± S.D. ($n = 3$–6). $^{**}P < 0.01$ compared to the group treated with plasmid DNA, Bubble liposomes, ultrasound exposure or lipofection with Lipofectin or Lipofectamine 2000. LF, Lipofectin. LF2000, Lipofectamine 2000. # < 10^2 RLU/mg protein

4. After 2 days of US exposure, ascites tumor cells were recovered from the abdomen of the mice. Then, the gene expression in the recovered cells was measured (see Fig. 5).

3.5.3. Gene Delivery for Solid Tumor (20)

1. S-180 cells (1×10^6 cells) were inoculated into the left footpad of ddY mice (5 weeks age, male).
2. At day 4, when the thickness of the footpad was over 3.5 mm (normal thickness was about 2 mm), the left femoral artery was exposed by operation.
3. BLs (100 μg) and plasmid DNA suspension (100 μL) were injected into the femoral artery using 30-gauge needle.
4. In the same time, US (frequency: 0.7 MHz, duty: 50%, intensity: 1.2 W/cm^2, time: 2 min) was transdermally exposed to the tumor tissue using Sonopore 3000 or 4000 with a probe (KP-T8) (diameter: 8 mm).
5. The needle hole was then closed with an adhesive agent and skin was put in a suture.

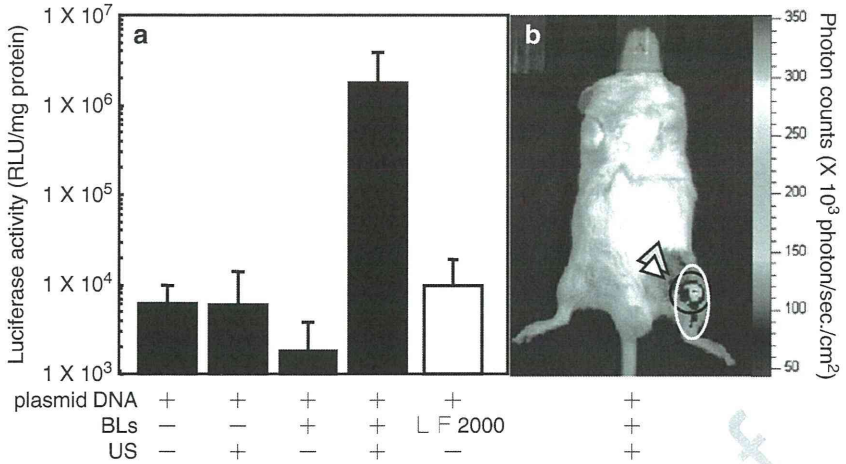

Fig. 5. Gene delivery to femoral artery with Bubble liposomes Each sample containing plasmid DNA 10 μg was injected into femoral artery. At the same time, ultrasound (frequency, 1 MHz; duty, 50%; burst rate, 2 Hz; intensity, 1 W/cm^2; time 2 min) was exposed to the downstream area of injection site. (**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 pseudocolor scales

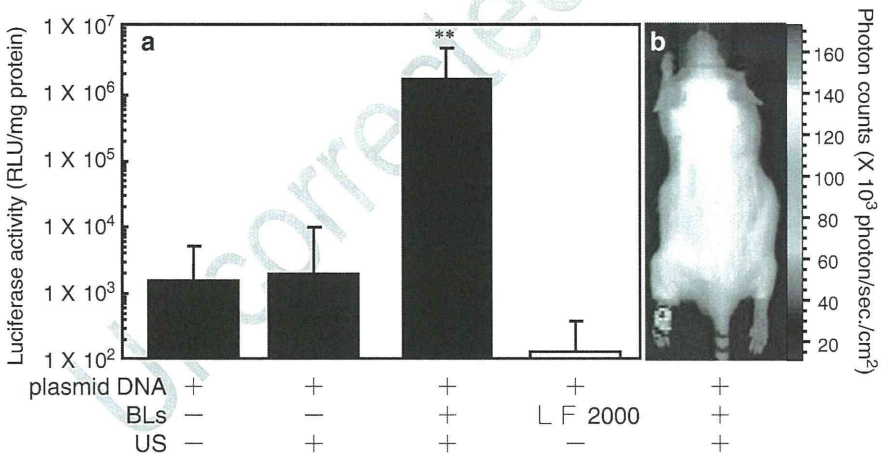

Fig. 6. In vivo gene delivery into mouse solid tumor with Bubble liposomes. S-180 cells (1×10^6 cells) were inoculated into left footpad of ddY mice. After 4 days, the mice were anaesthetized, then injected with 100 μL of pCMV-Luc (10 μg) in absence or presence of Bubble liposomes (100 μg) in PBS. Ultrasound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

6. After 2 days of US exposure, the mice were sacrificed and the tumor tissues were collected. Then, the gene expression of the tumor tissue was measured (see Fig. 6 and Note 8).

3.6. Measurement of Reporter Gene Expression

3.6.1. Luciferase Assay

1. The lysis buffer (0.1 M Tris–HCl (pH 7.8), 0.1% Triton X-100, 2 mM EDTA) was added to the sample cells in vitro or tissues in vivo. In the case of the tissues in vivo, they were homogenized before next step.
2. The cells or the homogenized tissues in lysis buffer were repeatedly frozen and thawed three times to completely disrupt the cell membranes.
3. After that, the lysate of the cells or tissues was centrifuged and the supernatant was collected in other tube.
4. Luciferase activity in the supernatant was measured using a luciferase assay system and a luminometer. The activity is reported in relative light units (RLU) per mg protein of cells or tissue.

3.6.2. In Vivo Luciferase Imaging

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

4. Notes

1. There are some important points to prepare BLs. The air in the vial containing the liposome suspension is completely replaced with perfluoropropane. After that, it needs to be supercharged in the vial with perfluoropropane. And the vial is sonicated with a bath-type sonicator (42 KHz, 100 W) (BRANSONIC 2510 J-DTH, Branson Ultrasonics). In this step, sonication power and the vial position in the bath are very important. Because we have experimented that BLs were not prepared using other type of bath sonicator (UC-1 (38 KHz, 80 W), IKEDA RIKA, Japan) with low intensity of ultrasound exposure. In addition, BLs were not prepared using other gas such as air, nitrogen gas or carbonic dioxide gas. Therefore, it thought that it is important for the preparation of BL to use hydrophobic gas such as perfluoropropane.
2. To fix BLs as a sample for transmission electron microscope, BLs were held within calcium alginate gel. The handling of BLs was improved by holding within the gel. The advantage for using this gel is to make the gel even at low temperature. Because BLs became unstable according to increasing temperature. Therefore, it is thought that the gel, such as agarose, which has gel point at high temperature is inappropriate for this purpose.
3. It was thought that liposomes were reconstituted by sonication under the condition of supercharge with perfluoropropane.

Then, perfluoropropane was entrapped within lipids like micelles. In addition, the lipid nanobubbles were encapsulated within liposomes. To confirm the structure of BLs, we observed BLs with transmission electron microscope. Interestingly, BLs had nanobubbles into lipid bilayer. Therefore, we called this "Bubble liposome" because of this structure. This structure of BLs was different from that of conventional microbubbles and nanobubbles which had lipid monolayer.

4. This protocol can be adapted for many other types of cell. In the gene transfection for adherent cells, the transfection efficiency in the condition of suspension was higher than that in the condition of adhesion on the culture plate. Although this result is unclear, it is thought that the distance between BLs and cells is important. Because BLs entrapping gas is easy to flow and result in getting away from the adherent cells on the plate.
5. In in vitro gene delivery, it is very important to fix the location of it, in order to reduce the experimental error of each data. The efficiency of this gene delivery was not affected even in the presence of serum. Moreover, the gene expression was observed even under the condition of US exposure for 1 s. From these results, it was suggested that this system could immediately deliver plasmid DNA into cells.
6. In in vivo gene delivery, echo jelly is necessary for US exposure to mice. Gene expression was observed in the arrested area of US exposure. Because it is thought that the mechanical effect based on the disruption of BLs by US exposure results in generation of some pores on plasma membrane of the cells in the area of US exposure.
7. This system is thought that there is not a serious damage for the cells in blood such as red blood cells by the disruption of BLs in blood stream by US exposure.
8. The transfection efficiency with the gene delivery system by sonoporation mechanism using BLs and US was higher than conventional lipofection method with Lipofectin and Lipofectamine 2000. Therefore, it is expected that this system might be an effective nonviral gene delivery system.

Acknowledgements

We are grateful to Dr. Katsuro Tachibana (Department of Anatomy, School of Medicine, Fukuoka University) for technical advice regarding the induction of cavitation with ultrasound, to Dr. Naoki Utoguchi, Mr. Yusuke Oda, Mr. Eisuke Namai,

Ms. Tomoko Takizawa, Ms. Kaori Sawamura and Ms. Kumiko Tanaka (Department of Pharmaceutics, School of Pharmaceutical Sciences, Teikyo University), Yoichi, Negishi (School of Pharmacy, Tokyo University of Pharmacy and Life Science), and Dr. Kosuke Hagisawa (Department of Medical Engineering, National Defense Medical College) for excellent technical advice and assistance, to Mr. Yasuhiko Hayakawa, Mr. Takahiro Yamauchi, and Mr. Kosho Suzuki (NEPAGENE Co., Ltd.) for technical advice regarding ultrasound exposure using Sonopore 3000 and 4000, and Sonitron 2000.

This study was supported by an Industrial Technology Research Grant in 2004 from NEDO, JSPS KAKENHI (16650126), MEXT KAKENHI (160700392, 19700423), a Research on Advanced Medical Technology (17070301) in Health and Labour Sciences Research Grants from Ministry of Health, Labour and Welfare, and the Program for Promotion of Fundamental Studies(07-24) in Health Sciences of the National Institute of Biomedical Innovation (NIBIO).

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Ultrasonics Sonochemistry 18 (2011) 425–430

Contents lists available at ScienceDirect

Ultrasonics Sonochemistry

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Synergistic effect of ultrasound and antibiotics against *Chlamydia trachomatis*-infected human epithelial cells in vitro

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ARTICLE INFO

Article history:
Received 12 April 2010
Received in revised form 12 July 2010
Accepted 21 July 2010
Available online 27 July 2010

Keywords:
Ultrasound
Nanobubbles
Antibiotic
Intracellular bacteria

ABSTRACT

To investigate whether or not the combined ultrasound and antibiotic treatment is effective against chlamydial infection, a new ultrasound exposure system was designed to treat chlamydia-infected cells. First, the minimum inhibitory concentrations of antibiotics against *Chlamydia trachomatis* were determined. Infected cultures were treated with antibiotics then sonicated at intensity of 0.15 or 0.44 W/cm^2 with or without Bubble liposomes. After 48 or 72 h after infection, chlamydial inclusions were stained and examined by fluorescence microscopy. The internalization of dextran–fluorescein conjugates by ultrasound irradiation with Bubble liposomes was observed by fluorescence microscopy. The results showed that application of nanobubble-enhanced ultrasound caused no significant effect on cell viability and chlamydial infectivity. However, Doxycycline (1/2 MIC) or CZX (1.0 μg/ml) in combination with nanobubble-enhanced ultrasound dramatically reduced the number of inclusions compared with that administered with antibiotics only. Bubble dose-dependent synergy was also observed. After ultrasound irradiation at intensity of 0.44 W/cm^2 on the presence of Bubble liposomes, 10% of HeLa cells were observed to have internalized the dextran molecules. This study suggests the possibility of using nanobubble-enhanced ultrasound to deliver antibiotic molecules into cells to eradiate intracellular bacteria, such as chlamydiae, without causing much damage to the cells itself.

1. Introduction

An obligate intracellular pathogen, *Chlamydia trachomatis*, is the most prevalent sexually transmitted bacterium worldwide [1]. *C. trachomatis* is a Gram-negative bacterium which has a unique biphasic developmental cycle characterized by an infectious but metabolically inactive extracellular form, called the 'elementary body', which initiates infection through the uptake by the host cell. Thereafter, elementary bodies differentiate into noninfectious but metabolically active forms, called the 'reticulate body', which proliferate within the inclusion. Reticulate bodies also differentiate back to elementary bodies before release at the end of the developmental cycle. At its sites of primary infection, *C. trachomatis* infects the urethral or cervical epithelium, causing acute urethritis or cervicitis [2]. These frequently progress into chronic inflammatory disease, the most significant of which, is chronic salpingitis, an inflammatory disease of fallopian tubes that can result in pelvic inflammatory disease, ectopic pregnancy, and tubal infertility [3].

The recommended antibiotic treatments for urogenital infections are a single dose of azithromycin or a 7-day course of doxycycline for management of active infections [4]. These regimens have been shown to result in satisfactory cure rates of acute infections [5,6]; however, chronic diseases (designated "persistent infection") have been suggested to be less responsive to antibiotic therapy [7].

Previous work has shown that some antibiotics treatment of *Pseudomonas aeruginosa* or *Escherichia coli* coupled with ultrasound irradiation enhances the bactericidal activity [8]. The more recent research has revealed that similar synergistic effects of combined ultrasound and antibiotic treatment are seen in both Gram-positive and Gram-negative bacteria with some antibiotics, especially the aminoglycosides [9]. It is not clear whether the combined ultrasound and antibiotic treatment are effective on intracellular pathogen, e.g. chlamydial infection. If an intracellular bacterial infection could be efficiently eradicated from an infected person, one could avoid chronic antibiotic treatments. In addition, this strategy of treatment could be beneficial in the management of chlamydial persistent diseases.

Here, we are studying the synergistic use of ultrasound and antibiotics to kill the *chlamydia*. This report presents results of

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doi:10.1016/j.ultsonch.2010.07.015

the first step in that research, which is investigation of the in vitro response of *C. trachomatis*-infected human epithelial cells to combination of ultrasound and two types of antibiotics.

2. Materials and methods

2.1. Chlamydial strain and cell lines

C. trachomatis serovar E/UW-5/Cx was prepared in McCoy cells and propagated according to a previously reported method [10]. The mouse fibroblast cell line McCoy cell (CRL 1696) and human epithelial cell line HeLa 229 cell (CLL 2.1) were maintained in Dulbecco's modified Eagle medium (DMEM, Invitrogen, Grand Island, NY, USA) supplemented with 10% heat-inactivated fetal calf serum (FCS, Invitrogen) and 100 μg/ml streptomycin.

2.2. Infection of HeLa cells

The HeLa cells were seeded into a 24-well plate with lumox™ fluorocarbon film base (optically clear, 50 μm-thin, gas permeable film, Greiner bio-one, Gottingen, Germany). Stocks of chlamydial strain were diluted with sucrose–phosphate–glutamate (SPG) medium [10]. Chlamydial suspensions of 0.5×10^4 inclusion-forming units (IFUs) in 0.25 ml SPG medium were inoculated onto the monolayer cultures of HeLa cells (1×10^4 cells/well). This is equivalent to a multiplicity of infection of 0.5. After incubation at 37 °C for 90 min, the inoculum was decanted, and the cells were washed in medium to remove the nonadsorbed chlamydiae and were then further incubated in 1 ml DMEM containing 1 μg/ml cycloheximide (Sigma Chemicals, St. Louis, MO, USA) and 2% FCS (maintenance medium).

2.3. Preparation of bubble liposome

Bubble liposomes were prepared according to a method previously described [11]. Liposomes composed of 1,2-distearoyl-sn-glycero-phosphatidylcholine (DSPC) (NOF Corp., Tokyo, Japan) and 1,2-distearoyl-snglycero-3-phosphatidyl-ethanolamine-methoxypolyethyleneglycol(DSPE-PEG(2k)-OMe, (PEG Mw = ca. 2000), NOF) (94: 6 (m/m)) were prepared by reverse phase evaporation. Briefly, all reagents (totallipid: 100 μmol) were dissolved in 8 ml of 1:1 (v/v) chloroform/diisopropyl ether, then 4 ml of phosphate buffered saline (PBS) were added. The mixture was sonicated and evaporated at 65 °C. The solvent was completely removed, and the size of the liposomes was adjusted to less than 200 nm using an extruding apparatus (Northern Lipids Inc., Vancouver, BC, Canada) and sizing filters (pore sizes: 100 and 200 nm; Nuclepore Track-Etch Membrane, Whatman plc, UK). After sizing, the liposomes were sterilized by passing them through a 0.45 μm pore size filter (MILLEX HV filter unit, Durapore PVDF membrane, Millipore Corp., MA, USA). The size of the liposomes was measured by dynamic light scattering (ELS-800, Otsuka Electronics Co., Ltd., Osaka, Japan). The average diameter of these liposomes was between 150 and 200 nm. Lipid concentration was measured using the Phospholipid C test (Wako Pure Chemical Industries). BLs were prepared from the liposomes and perfluoropropane gas (Takachiho Chemical Industrial Co., Ltd., Tokyo, Japan). Briefly, 5 ml sterilized vials containing 2 ml of the liposome suspension (lipid concentration: 2 mg/ml) were filled with perfluoropropane, capped, and then supercharged with 7.5 ml of perfluoropropane. The vial was placed in a bath-type sonicator (42 kHz, 100 W; BRANSONIC 2510J-DTH, Branson Ultrasonics Co., Danbury, CT, USA) for 5 min to form the BLs. In this method, the liposomes were reconstituted by sonication under the condition of supercharge with perfluoropropane in the 5 ml vial container. At the same time, perfluoropropane would be entrapped within lipids like micelles, which were made by DSPC and DSPE-PEG(2k)-OMe from liposome composition, to form nanobubbles. The lipid nanobubbles were encapsulated within the reconstituted liposomes, which sizes were changed into around 1 μm from 150 to 200 nm of original.

2.4. Immunofluorescence staining and fluorescence microscopy

At 48 or 72 h after infection, the infected monolayers were washed with PBS, and the cells were fixed with −20 °C chilled methanol. After the specimens had been dried, the inclusion bodies were stained with fluorescein isothiocyanate (FITC)-labeled monoclonal antibody against *C. trachomatis* lipopolysaccharides (Progen Biotechnik, Heidelberg, Germany) for 30 min at room temperature. The cells were rinsed with saline, and the films were cut off from the plate, and mounted in a 1:1 solution of PBS–glycerol. The antibody staining resulted in yellow–green chlamydial proteins, and Evans blue counterstaining yielded red eukaryotic cells. The formation of inclusions was assessed using a Zeiss Axiophot fluorescence microscope. The cells positive for inclusions are considered infected cells and infectivity was presented as the number of inclusion-forming units (IFUs).

2.5. Antibiotics and measurements of MICs

Doxycycline (DOX, Sigma Chemicals) and ceftizoxime (CZX, Fujisawa Yakuhin Kogyou, Tokyo, Japan) were obtained in powder form. Both antibiotics were diluted with saline, and were dissolved in maintenance medium at a concentration of 100 μg/ml and frozen at −80 °C until used. The minimum inhibitory concentrations (MICs) were determined using a method previously described [12]. Briefly, confluent monolayer cultures of cells in a 24-well flat-bottomed plate with 13-mm coverslips were inoculated by centrifugation and incubated in 1 ml of maintenance medium containing a serial dilution of antibiotics for 72 h. To determine the MICs, the cover slips were stained and observed as described in Immunofluorescence staining and fluorescence microscopy. The lowest concentration of the antimicrobial agent that completely inhibited the formation of visible chlamydial inclusions was determined as the MIC.

2.6. Ultrasound exposure

An acoustically transparent gel (Pharmaceutical Innovations Inc., Newark, NJ) was applied on the ultrasound probe before positioning the plate containing the sample on top of it (Fig. 1). Thera-

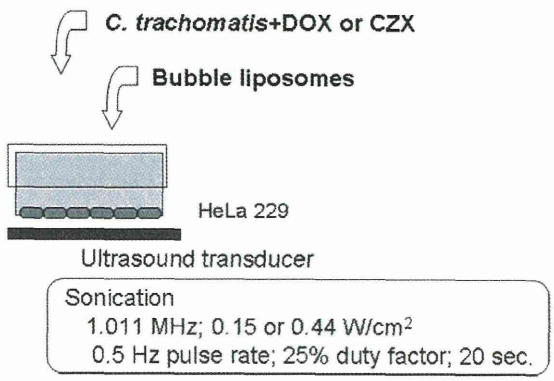

Fig. 1. Experimental design. Schematic drawing of the ultrasound setting. *C. trachomatis*-infected HeLa cells were exposed to ultrasound after addition of antibiotic and Bubble liposomes.