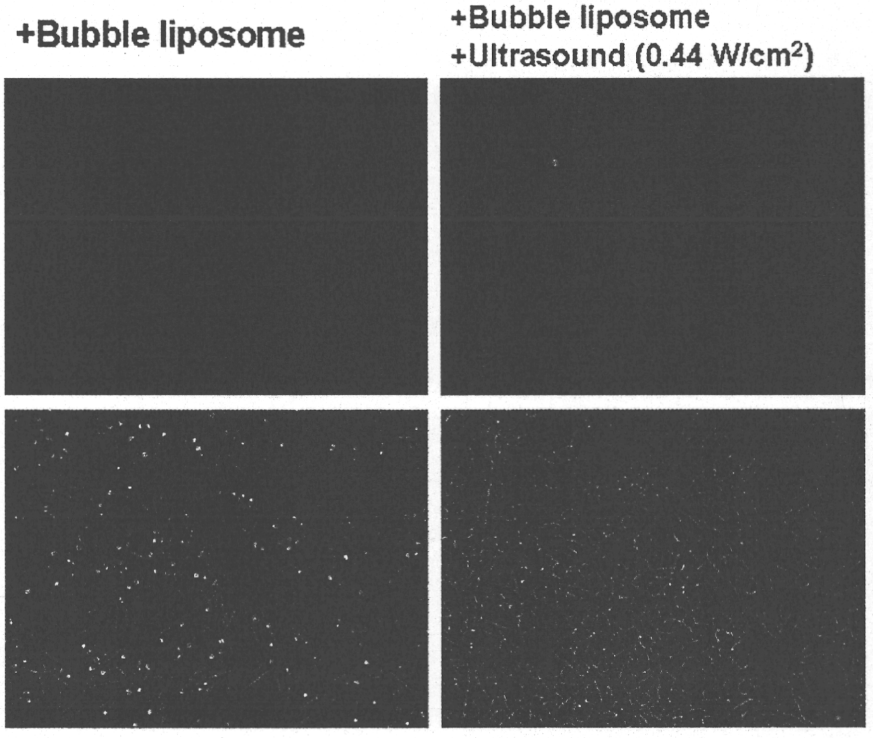

Fig. 6. Internalization of dextran–fluorescein conjugates by ultrasound irradiation with Bubble liposomes. Upper images show the detection of fluorescein into HeLa cells by fluorescence microscopy, and lower images show the condition of HeLa cells by light microscopy. After addition of dextran conjugates and Bubble liposomes on the cells, ultrasound was irradiated for 20 s or not.

hanced ultrasound to deliver antibiotic molecules into cells to eradicate intracellular bacteria without causing significant damage to the cells itself. This modality may become a new treatment modality for Chlamydial infection.

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References

[1] R.W. Peeling, R.C. Brunham, Chlamydiae as pathogens: new species and new issues, Emerg. Infect. Dis. 2 (1996) 307–319.
[2] J. Schachter, Infection and disease epidemiology, American Society for Microbiology, Washington DC, 1999.
[3] W. Cates Jr., J.N. Wasserheit, Genital chlamydial infections: epidemiology and reproductive sequelae, Am. J. Obstet. Gynecol. 164 (1991) 1771–1781.
[4] K.A. Workowski, S.M. Berman, CDC sexually transmitted diseases treatment guidelines, Clin. Infect. Dis. 35 (2002) S135–137.
[5] D.H. Martin, T.F. Mroczkowski, Z.A. Dalu, J. McCarty, R.B. Jones, S.J. Hopkins, R.B. Johnson, A controlled trial of a single dose of azithromycin for the treatment of chlamydial urethritis and cervicitis. The Azithromycin for Chlamydial Infections Study Group, N. Engl. J. Med. 327 (1992) 921–925.
[6] A. Nilsen, A. Halsos, A. Johansen, E. Hansen, E. Torud, D. Moseng, G. Anestad, G. Storvold, A double blind study of single dose azithromycin and doxycycline in the treatment of chlamydial urethritis in males, Genitourin. Med. 68 (1992) 325–327.
[7] J.D. Oriel, G.L. Ridgway, Epidemiology of chlamydial infection of the human genital tract: evidence for the existence of latent infections, Eur. J. Clin. Microbiol. 1 (1982) 69–75.
[8] W.G. Pitt, M.O. McBride, J.K. Lunceford, R.J. Roper, R.D. Sagers, Ultrasonic enhancement of antibiotic action on gram-negative bacteria, Antimicrob. Agents Chemother. 38 (1994) 2577–2582.
[9] A.M. Rediske, B.L. Roeder, M.K. Brown, J.L. Nelson, R.L. Robison, D.O. Draper, G.B. Schaalje, R.A. Robison, W.G. Pitt, Ultrasonic enhancement of antibiotic action on *Escherichia coli* biofilms: an in vivo model, Antimicrob. Agents Chemother. 43 (1999) 1211–1214.
[10] A. Nagayama, T. Nakao, H. Taen, In vitro activities of ofloxacin and four other new quinoline-carboxylic acids against *Chlamydia trachomatis*, Antimicrob. Agents Chemother. 32 (1988) 1735–1737.
[11] R. Suzuki, T. Takizawa, Y. Negishi, K. Hagisawa, K. Tanaka, K. Sawamura, N. Utoguchi, T. Nishioka, K. Maruyama, Gene delivery by combination of novel liposomal bubbles with perfluoropropane and ultrasound [see comment], J. Control. Release 117 (2007) 130–136.
[12] T. Notomi, Y. Ikeda, A. Nagayama, Minimum inhibitory and minimal lethal concentration against *Chlamydia trachomatis* dependent on the time of addition and the duration of the presence of antibiotics, Chemotherapy 45 (1999) 242–248.
[13] K. Laasila, L. Laasonen, M. Leirisalo-Repo, Antibiotic treatment and long term prognosis of reactive arthritis, Ann. Rheum. Dis. 62 (2003) 655–658.
[14] T. Yli-Kerttula, R. Luukkainen, U. Yli-Kerttula, T. Mottonen, M. Hakola, M. Korpela, M. Sanila, J. Parviainen, J. Uksila, R. Vainionpaa, A. Toivanen, Effect of a three month course of ciprofloxacin on the outcome of reactive arthritis, Ann. Rheum. Dis. 59 (2000) 565–570.
[15] M.U. Rahman, M.A. Cheema, H.R. Schumacher, A.P. Hudson, Molecular evidence for the presence of chlamydia in the synovium of patients with Reiter's syndrome, Arthritis Rheumatism 35 (1992) 521–529.
[16] J.S. Gaston, Immunological basis of Chlamydia induced reactive arthritis, Sex. Transm. Infect. 76 (2000) 156–161.
[17] H. Hanada, Y. Ikeda-Dantsuji, M. Naito, A. Nagayama, Infection of human fibroblast-like synovial cells with *Chlamydia trachomatis* results in persistent infection and interleukin-6 production, Microb. Pathog. 34 (2003) 57–63.
[18] N. Reveneau, D.D. Crane, E. Fischer, H.D. Caldwell, Bactericidal activity of first-choice antibiotics against gamma interferon-induced persistent infection of human epithelial cells by *Chlamydia trachomatis*, Antimicrob. Agents Chemother. 49 (2005) 1787–1793.
[19] H.R. Guzman, A.J. McNamara, D.X. Nguyen, M.R. Prausnitz, Bioeffects caused by changes in acoustic cavitation bubble density and cell concentration: a unified explanation based on cell-to-bubble ratio and blast radius, Ultrasound Med. Biol. 29 (2003) 1211–1222.
[20] L.B. Feril Jr., T. Kondo, Q.L. Zhao, R. Ogawa, K. Tachibana, N. Kudo, S. Fujimoto, S. Nakamura, Enhancement of ultrasound-induced apoptosis and cell lysis by echo-contrast agents, Ultrasound Med. Biol. 29 (2003) 331–337.
[21] L.B. Feril Jr., R. Ogawa, K. Tachibana, T. Kondo, Optimized ultrasound-mediated gene transfection in cancer cells, Cancer Sci. 97 (2006) 1111–1114.

[22] M. Ward, J. Wu, J.F. Chiu, Experimental study of the effects of Optison concentration on sonoporation in vitro, Ultrasound Med. Biol. 26 (2000) 1169–1175.
[23] C.X. Deng, F. Sieling, H. Pan, J. Cui, Ultrasound-induced cell membrane porosity, Ultrasound Med. Biol. 30 (2004) 519–526.
[24] K. Tachibana, T. Uchida, K. Ogawa, N. Yamashita, K. Tamura, Induction of cell-membrane porosity by ultrasound, Lancet 353 (1999) 1409.
[25] L.B. Feril Jr., K. Tachibana, T. Kondo, R. Ogawa, Q.-L. Zhao, Y. Yamaguchi, K. Ogawa, H. Endo, Y. Irie, Y. Harada, Hypotonia-induced cell swelling enhances ultrasound-induced mechanical damage to cancer cells, J. Med. Ultrasonics 37 (2010) 3–8.

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Cancer gene therapy by IL-12 gene delivery using liposomal bubbles and tumoral ultrasound exposure

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ABSTRACT

Interleukin-12 (IL-12) gene therapy is expected to be effective against cancers because it primes the immune system for cancer cells. In this therapy, it is important to induce IL-12 gene expression in the tumor tissue. Sonoporation is an attractive technique for developing non-invasive and non-viral gene delivery systems, but simple sonoporation using only ultrasound is not an effective cancer gene therapy because of the low efficiency of gene delivery. We addressed this problem by combining ultrasound and novel ultrasound-sensitive liposomes (Bubble liposomes) which contain the ultrasound imaging gas perfluoropropane. Our previous work showed that this is an effective gene delivery system, and that Bubble liposome collapse (cavitation) is induced by ultrasound exposure. In this study, we assessed the utility of this system in cancer gene therapy using IL-12 corded plasmid DNA. The combination of Bubble liposomes and ultrasound dramatically suppressed tumor growth. This therapeutic effect was T-cell dependent, requiring mainly $CD8^+$ T lymphocytes in the effector phase, as confirmed by a mouse *in vivo* depletion assay. In addition, migration of $CD8^+$ T cells was observed in the mice, indicating that the combination of Bubble liposomes and ultrasound is a good non-viral vector system in IL-12 cancer gene therapy.

1. Introduction

Interleukin 12 (IL-12), a heterodimeric protein composed of p35 and p40 subunits [1,2], is produced by antigen-presenting cells such as dendritic cells and macrophages. IL-12 has a variety of immunomodulatory anti-tumor effects including induction of interferon-γ (IFN-γ secretion by stimulation of T cells and natural killer (NK) cells, and promotion of cytotoxic T lymphocyte (CTL) maturation [3,4]. In addition, IL-12 induces antiangiogenic effects mainly through IFN-γ-dependent production of the chemokine, interferon-inducible protein-10 (IP-10) [5], suggesting that IL-12 would be an effective anti-tumor agent. Although the systemic administration of IL-12 has been shown to suppress tumor growth, clinical trials were interrupted because of fatal adverse effects [6,7]. On the other hand, local administration of IL-12 into tumors is accepted as a more effective immunotherapeutic approach because of reduced systemic toxicity [8]. In particular, gene therapy by intratumoral injection of the IL-12 gene is expected to be an effective cancer therapy because it would lead to the locally sustained release of IL-12 in the tumor [9].

In cancer gene therapy, it is important to develop easy, safe, efficient and minimally-invasive techniques for transferring genes into tumor tissue. Non-viral gene therapy has many advantages over gene therapy, including ease of plasmid DNA production, lower toxicity and immunogenicity, and lower cost. Many researchers have attempted to develop non-viral gene delivery carriers such as lipids and polymers [10–13]. In addition, there is wide interest in the potential of therapeutic ultrasound for enhancing the efficiency of gene delivery [14,15]. In particular, a physical method using ultrasound combined with nano/microbubbles has many of the desired characteristics for an ideal gene therapy, including low toxicity, the potential for repeated applications, organ specificity, and broad applicability to acoustically accessible organs [16,17]. Ultrasound can create transient nonlethal perforations in cell membranes [18], allowing extracellular plasmid DNA to be directly delivered into the cytosol. The main mechanism of gene delivery is thought to be acoustic cavitation using nano/microbubbles as cavitation nuclei. Based on liposome technology, we previously developed novel liposomal bubbles (Bubble liposomes) containing lipid micelles of the ultrasound imaging gas, perfluoropropane [19–23]. When coupled with ultrasound exposure, Bubble liposomes could

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be used as novel gene delivery agents *in vitro* and *in vivo* [19,20,24]. In addition, we found that gene delivery into femoral artery with this method was much more efficient than the conventional lipofection method using Lipofectamine 2000 [19]. And there is little report about cancer gene therapy using nano/microbubbles and ultrasound. Therefore, it is expected that gene delivery using Bubble liposomes and ultrasound will be an effective non-viral vector system for cancer gene therapy. In this study, we assessed this gene delivery system in cancer gene therapy using IL-12 gene.

2. Materials and methods

2.1. Cells and animals

Murine ovarian carcinoma OV-HM cells were kindly provided by Dr. Hiromi Fujiwara. An ovarian tumor OV2944, was induced in a female (C57BL/6 X C3H/He) F_1 mice by giving a single whole-body neutron irradiation, and a cloned line with highly metastatic property (designated OV-HM) was isolated from the parental line [25]. OV-HM cells were grown in RPMI-1640 (Sigma Chemical Co., St. Louis, MO) containing 100 U/ml penicillin (Wako Pure Chemical Industries, Osaka, Japan) and 100 μg/ml streptomycin (Wako Pure Chemical Industries) supplemented with 10% heat-inactivated fetal bovine serum (FBS, GIBCO, Invitrogen Co., Carlsbad, CA). B6C3F1 female mice were obtained from Sankyo Labo Service Corporation, Inc. (Tokyo, Japan) and used at 6 weeks of age. All of the experimental procedures were performed in accordance with the Teikyo University guidelines for the welfare of animals in studies of experimental neoplasia.

2.2. Preparation of Bubble liposomes

Liposomes composed of 1,2-distearoyl-sn-glycero-phosphatidyl-choline (DSPC) (NOF Corp., Tokyo, Japan) and 1,2-distearoyl-sn-glycero-3-phosphatidyl-ethanolamine s-methoxypolyethyleneglycol (DSPE-PEG(2 k)-OMe, (PEG, Mw = ca. 2000); NOF) (94:6 (m/m)) were prepared by reverse phase evaporation. Briefly, all reagents (total lipid: 100 μmol) were dissolved in 8 ml of 1:1 (v/v) chloroform/diisopropyl ether, and then 4 ml of phosphate buffered saline (PBS) was added. The mixture was sonicated and evaporated at 65 °C. The organic 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, 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). 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). Bubble liposomes 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 2510 J-DTH, Branson Ultrasonics Co., Danbury, CT) for 5 min to form the Bubble liposomes. In this method, the liposomes were reconstituted by sonication and supercharged with perfluoropropane in the 5-mL vial container. Perfluoropropane was entrapped within the lipid micelles, comprising DSPC and DSPE-PEG (2 k)-OMe, to form nanobubbles. The lipid nanobubbles were encapsulated within the reconstituted liposomes, which now ranged in size from around 500 nm–1 μm, compared to 150–200 nm before supercharging with perfluoproropane.

2.3. Plasmid DNA vector construction

The plasmid pCMV-Luc contained the firefly luciferase gene of pGL3-control (Promega) at the *Hin*dIII/*Xba*I site of the pcDNA3 vector (Invitrogen). This plasmid was an expression vector encoding the firefly luciferase gene under the control of a cytomegalovirus promoter. The plasmid pCMV-IL12 contained murine IL-12, derived from mIL-12 BIA/pBluescript II KS(−) (kindly provided by Dr. Yamamoto, Department of Immunology, Graduate School of Pharmaceutical Sciences, Osaka University, Japan) in the *Xho*I/*Not*I site of the pHMCMV5 vector. This expression vector encoded the murine IL-12 gene under the control of a cytomegalovirus promoter [26].

2.4. Intratumoral administration of plasmid DNA

B6C3F1 mice were intradermally inoculated with OV-HM cells (1×10^6 cells/mouse) in the flank. After 7 days, a suspension (25 μL/mouse) of Bubble liposomes (2.5 μg) and pCMV-Luc or pCMV-IL12 (10 μg) was injected into the tumor, and ultrasound (1 MHz, 0.7 W/cm^2, 60 s) was transdermally applied to the tumor tissue. A conventional lipofection method was also investigated. A suspension (25 μL/mouse) of Lipofectamine 2000 (20 μg) and pCMV-Luc or pCMV-IL12 (10 μg) were incubated together for 20 min to allow them to complex. Then the complex was injected into the tumor. All treatment groups consisted of five mice.

2.5. Luciferase assay

Each day after the pCMV-Luc injection, mice were sacrificed and the tumor tissue was recovered. The tumor tissue was homogenized in lysis buffer (0.1% Triton X-100, 0.1 M Tris–HCl pH 7.8, 2 mM EDTA) and frozen (−80 °C) and thawed at room temperature twice. The homogenized tumor tissue was centrifuged (12,000 rpm, 4 °C, 10 min) and the supernatant was recovered for the luciferase assay. Luciferase activity was measured using a luciferase assay system (Promega) and luminometer (TD-20/20, Turner Designs, Sunnyvale, CA). The activity was measured as relative light units (RLU) per milligram protein.

2.6. Reverse transcription-polymerase chain reaction (RT-PCR) analysis for IL-12 expression in tumor tissues

OV-HM tumors were collected 1 or 2 days after intratumoral injection of pCMV-IL12 and Bubble liposomes, and total RNA was isolated using ISOGEN according to the manufacturer's instructions and dissolved with 20 μL TE buffer. RT proceeded for 60 min at 42 °C in 20 μl reaction mixture containing 1 μg total RNA treated with DNase I, 5 mM $MgCl_2$, RNA PCR buffer (Takara Bio, Kyoto, Japan), 1 mM dNTP mix, 0.125 μM Oligo dT-Adaptor primer (Takara Bio), 0.5 U/μL RNase inhibitor and 0.25 U/μL AMV reverse transcriptase XL (Takara Bio). PCR amplification of IL-12 and β-actin transcripts was performed in 20 μL reaction mixture containing 2 μL RT-material, PCR buffer, 0.5 U Takara Ex Taq HS, 0.2 mM dNTP, 2.5 mM $MgCl_2$, and 0.4 mM primers. The sequences of the specific primers were as follows: murine IL-12: forward, 5′-ctc acc tgt gac acg cct ga-3′; reverse, 5′-cag gac act gaa tac ttc tc-3′; and murine β-actin: forward, 5′-tgt gat ggt ggg aat ggg tca g-3′; reverse, 5′-ttt gat gtc acg cac gat ttc c-3′. After denaturation for 5 min at 95 °C, three sequential steps, denaturation for 45 s at 95 °C, annealing for 60 s at 48 °C, and extension for 2 min at 72 °C, were repeated for 40 cycles, with a final extension step for 4 min at 72 °C.

EZ Load (Bio-Rad Laboratories, Inc., Tokyo, Japan) was used as a 100-bp molecular ladder. The PCR products were electrophoresed through a 2% agarose gel, stained with ethidium bromide, and visualized under ultraviolet radiation. The expected PCR product sizes were 430 bp (IL-12) and 514 bp (β-actin).

2.7. Anti-tumor effect of intratumoral administration on IL-12 gene expression in mice

B6C3F1 mice were intradermally inoculated with OV-HM cells (1×10^6 cells/mouse) into the flank. For single therapy, established tumors with diameters of 8–10 mm were injected with a suspension (25 μL/mouse) of Bubble liposomes (2.5 μg) and pCMV-IL12 (10 μg), and ultrasound (1 MHz, 0.7 W/cm^2, 60 s) was transdermally applied to the tumor tissue. We also examined the intratumoral injection of a complex (25 μL/mouse) of Lipofectamine 2000 (20 μg) and pCMV-IL12 (10 μg) as a conventional lipofection method for comparison. For repetitive therapy, the mice were treated as above on days 0, 2, 5, 7, 9 and 12 after first treatment. The anti-tumor effects were evaluated by measuring tumor volume. Tumor volume was calculated using the formula: (major axis × minor axis^2) × 0.5. All data are expressed as relative tumor volume to that before the first treatment. All treated groups contained five mice.

2.8. In vivo depletion analysis

GK1.5 hybridoma (rat anti-mouse CD4 mAb) and 53-6.72 hybridoma (rat anti-mouse CD8 mAb) were purchased from American Type Culture Collection (ATCC) (Manassas, VA). Ascites from BALB/c nude mice intraperitoneally injected with each hybridoma were collected, and antibodies were purified using a protein A column (GE Healthcare, Pollards Wood, UK). Mice bearing OV-HM were intratumorally injected with pCMV-IL12 and Bubble liposomes on days 0, 2, 5, 7, 9 and 12 after the first treatment. Additionally, the mice were intraperitoneally injected four times on days −3, 4, 11 and 18 after the first treatment with 100 μg/mouse of anti-mouse CD8 mAb for CD8$^+$ cells or anti-mouse CD4 antibody for CD4$^+$ cells, or on days −3, −2, −1, 0, 5, 10, 15 and 20 after the first treatment with 200 μg/mouse of anti asialoGM1 mAb (Wako Pure Chemical Industries) for NK cells. The depletion of T-cell subsets and NK cells was confirmed by flow cytometric analysis of peripheral blood. Tumor growth was monitored as described above.

2.9. Immunohistochemical analysis

B6C3F1 mice were intradermally inoculated with OV-HM cells (1×10^6 cells/mouse) into the flank. After 7, 9 or12 days, a suspension (25 μL/mouse) of Bubble liposomes (2.5 μg) and pCMV-IL12 (10 μg) was injected into the tumor, and ultrasound (1 MHz, 0.7 W/cm^2, 60 s) was transdermally applied to the tumor tissue. After 13 days of tumor inoculation, the mice were sacrificed, and the tumor tissue was dissected and then embedded in the OCT compound. Frozen sections (10 μm thick) were fixed with 4% paraformaldehyde at 4 °C for 10 min, and treated with 0.3% H_2O_2 in methanol:PBS (1:1) for 15 min and 1.5% normal cow serum in PBS for 10 min at room temperature. The sections were treated with rat anti-mouse CD8 mAbs (1:100) or rat anti-mouse perforin mAbs (1:100) (Kamiya Biomedical Co., Seattle, WA) in PBS containing 0.1% BSA at 4 °C overnight. The section was washed and treated with horse radish peroxidase-conjugated goat anti-rat IgG Abs (1:500) in PBS containing 0.1% BSA at room temperature for 2 h. The diaminobenzidine-reaction system (Vector Laboratories, Burlingame, CA) was used to stain the sections. We also stained the sections with hematoxylin solution for counterstaining. The samples were observed with a microscope (IX-71, Olympus, Tokyo, Japan).

2.10. Statistical analysis

Differences between experimental groups were compared with non-repeated measures ANOVA and Dunnett's test.

3. Results

3.1. Bubble liposomes and ultrasound-mediated gene delivery into solid tumors

To evaluate the effectiveness of gene delivery with Bubble liposomes and ultrasound into OV-HM solid tumors, we utilized the luciferase reporter gene expression assay (Fig. 1a). The effectiveness of gene delivery with conventional lipofection using Lipofectamine 2000 was also examined. Luciferase expression with ultrasound or Bubble liposomes was low, and even lower with Lipofectamine 2000. On the other hand, luciferase expression with the combination of Bubble liposomes and ultrasound exposure was higher than in the other groups. Therefore, the profile of luciferase expression was measured in mice treated with Bubble liposomes and ultrasound. Luciferase expression gradually decreased after transfection (Fig. 1b), with the elimination rate constant (Ke) and half period ($T_{1/2}$) of gene expression being 1.26 days^{-1} and 0.54 days, respectively.

3.2. IL-12 gene expression in solid tumors transfected with IL-12 corded plasmid DNA using Bubble liposomes and ultrasound

To assess IL-12 expression in solid tumors transfected with IL-12 corded plasmid DNA (pCMV-IL12), the expression of IL-12p40 mRNA was examined with RT-PCR (Fig. 2). No expression of IL-12p40 mRNA in solid tumors transfected with pCMV-IL12 was observed. A small amount of IL-12p40 mRNA was expressed in solid tumors transfected with pCMV-IL12 using Lipofectamine 2000 on 1 day after gene transfection. On the other hand, the expression of IL-12p40 mRNA was observed in solid tumors transfected with pCMV-IL12 using Bubble liposomes and ultrasound for at least 2 days after gene transfection. This result indicates that IL-12 is expressed more effectively in solid tumors transfected using Bubble liposomes and ultrasound than using Lipofectamine 2000.

3.3. Anti-tumor effect of IL-12 gene delivery with Bubble liposomes and ultrasound

First, we examined the effect of a single delivery of IL-12 gene (Fig. 3a). Gene delivery using Bubble liposomes, ultrasound or

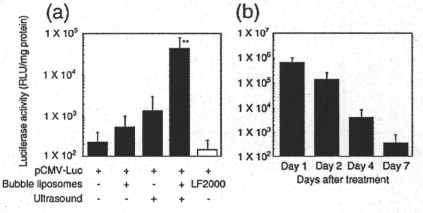

Fig. 1. Gene expression profiles in solid tumors following gene delivery with Bubble liposomes and ultrasound. Comparison of gene expression efficiency by gene delivery with each gene delivery method. B6C3F1 mice were intradermally inoculated with 1×10^6 OV-HM cells into the flank. Seven days after inoculation, the tumors were injected with pCMV-Luc (10 μg) using Bubble liposomes (2.5 μg) and/or ultrasound (1 MHz, 0.7 W/cm^2, 1 min), or Lipofectamine 2000 as a conventional lipofection method. (a) Two days after gene delivery, the mice were sacrificed and luciferase expression was measured in the solid tumor tissue. The data represent means ± SD ($n=3$). (b) Time course of luciferase expression after gene delivery with Bubble liposomes and ultrasound. Luciferase expression was measured at each time point after pCMV-Luc delivery into the solid tumor with Bubble liposomes and ultrasound exposure. **$P<0.01$ compared to the group treated with plasmid DNA, Bubble liposomes, ultrasound or LF2000. LF2000: Lipofectamine 2000.

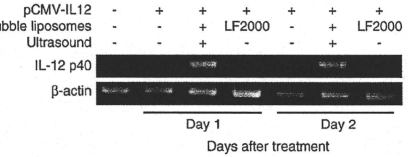

Fig. 2. RT-PCR analysis of IL-12 expression in solid tumors after pCMV-IL12 gene delivery. B6C3F1 mice were intradermally inoculated with 1×10^6 OV-HM cells into the flank. Seven days after inoculation, the tumors were injected with pCMV-IL12 (10 μg) using Bubble liposomes (2.5 μg) and/or ultrasound (1 MHz, 0.7 W/cm^2, 1 min), or Lipofectamine 2000 as a conventional lipofection method. One or 2 days after gene delivery, the mice were sacrificed, total RNA was prepared from the tumors, then RT-PCR was performed using a specific IL-12 primer as described in Materials and Methods. The PCR products were electrophoresed though a 3% agarose gel and stained with EtBr. To ensure the quality of the procedure, RT-PCR was performed on the same sample using a specific β-actin primer. LF2000: Lipofectamine 2000.

Lipofectamine 2000 showed no apparent anti-tumor effect, as was found for pCMV-Luc intratumoral delivery using Bubble liposomes and ultrasound. In contrast, the growth of OV-HM tumors was dramatically suppressed in mice treated by pCMV-IL12 intratumoral delivery using Bubble liposomes and ultrasound; however, complete regression was not observed. Thus, we examined the effect of repetitive IL-12 gene therapy to obtain more effective therapeutic effects (Fig. 3b). Gene delivery using Bubble liposomes, ultrasound or Lipofectamine 2000 showed no apparent anti-tumor effect, even in repetitive therapy. In contrast, IL-12 gene delivery using the combination of Bubble liposomes and ultrasound effectively suppressed tumors, and complete regression occurred in 80% of the tumor-bearing mice. There was no decrease in body weight of these mice as a side effect of IL-12 cancer therapy (data not shown). In addition, this group of mice demonstrated prolonged survival, indicating that OV-HM cells were effectively killed by IL-12 gene therapy with Bubble liposomes and ultrasound.

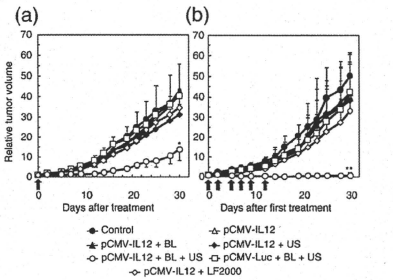

Fig. 3. Anti-tumor effect of IL-12 gene delivery. B6C3F1 mice were intradermally inoculated with 1×10^6 OV-HM cells into the flank. (a) Single gene therapy. (b) Repetitive gene therapy. Each day (arrows) after first treatment, the tumors were injected with pCMV-IL12 (10 μg) using Bubble liposomes (2.5 μg) and/or ultrasound (1 MHz, 0.7 W/cm^2, 1 min), or Lipofectamine 2000 as a conventional lipofection method. The volume of the growing tumors was calculated by: (tumor volume; mm^3) = (major axis; mm) × (minor axis; mm)^2 × 0.5). The data are represented as tumor volume relative to the tumor volume on the first day of treatment (day 7 after tumor inoculation). Arrow shows days of treatment. Each point represents the mean ± SD ($n = 5$). *$P < 0.05$ or **$P < 0.01$ compared to the group treated with pCMV-IL12, pCMV-IL12 + BL, US or LF2000 or pCMV-Luc + BL + US. BL: Bubble liposomes, US: Ultrasound, LF2000: Lipofectamine 2000.

3.4. Determination of immune subsets responsible for tumor regression induced by IL-12 gene delivery using Bubble liposomes and ultrasound

To investigate the anti-tumor mechanism of intratumoral pCMV-IL12 delivery using Bubble liposomes and ultrasound, we examined the individual contribution of CD4$^+$ and CD8$^+$ T cells and NK cells (Fig. 4). The anti-tumor effects of pCMV-IL12 delivery using Bubble liposomes and ultrasound were attenuated by the depletion of CD8$^+$ T cells and CD4$^+$ T cells. The depletion of CD8$^+$ T cells effectively blocked the anti-tumor effect. Also, the anti-tumor effect was blocked in mice that were depleted of both CD4$^+$ and CD8$^+$ T cells. On the other hand, the tumor growth suppressing effects were not affected by NK cell depletion. Therefore, we concluded that CD8$^+$ CTLs, activated by the helper function of CD4$^+$ T cells, were the predominant effector cells in this therapeutic system. CD4$^+$ cells alone also partly contributed to the enhanced anti-tumor effect.

To investigate the infiltration of CD8$^+$ T cells into tumor tissues containing the IL-12 gene delivered using Bubble liposomes and ultrasound, tumor tissues were subjected to immunohistochemical staining for CD8 (Fig. 5a–c). Tumor tissue from untreated mice, or mice treated with the IL-12 gene delivered using Bubble liposomes and ultrasound, showed increased accumulation of CD8$^+$ T cells compared to control mice treated with the luciferase gene, delivered using Bubble liposomes and ultrasound. In addition, we examined the activation states of tumor-infiltrating T cells by immunohistochemical analysis for perforin, the major cytotoxic molecule in activated CTLs (Fig. 5d–f). Tumor tissue to which the IL-12 gene had been delivered using Bubble liposomes and ultrasound exhibited significantly higher numbers of perforin-positive cells than non-treated tumor tissue, or tissue treated with luciferase gene.

4. Discussion

There are two main therapeutic strategies in cancer gene therapy. One approach is to cause a direct effect on cancer cells by delivering suicide genes such as herpes simplex virus thymidine kinase, [27] siRNA for oncogenes, [28] and proteins associated with the cell cycle [29] and apoptosis [30,31]. In this approach, it is necessary to deliver the therapeutic gene into most of the cancer cells to induce cytotoxicity. The second approach is indirect and activates anti-tumor immunity mediated by the delivery of a cytokine gene such as IL-12. In such cytokine gene therapy, the therapeutic gene does not have to be delivered into all the cancer cells since the cytokine is secreted from the cells. Therefore, a local supply of IL-12 in tumors is

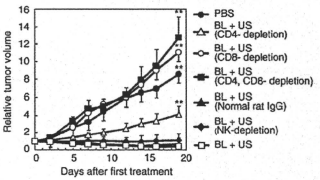

Fig. 4. Determination of immune subsets responsible for the anti-tumor effect induced by IL-12 gene delivery with Bubble liposomes and ultrasound. B6C3F1 mice were intradermally inoculated with 1×10^6 OV-HM cells into the flank. For depletion of CD4$^+$ T cells, CD8$^+$ T cells or NK cells in the mice, GK1.5 ascites (anti-CD4), 53-6.72 ascites (anti-CD8) or anti-asialoGM1 antisera was intraperitoneally injected as described in Materials and methods. On 0, 2, 5, 7, 9 or 12 days after first treatment, IL-12 gene therapy was performed with Bubble liposomes and ultrasound. The data represent the tumor volume relative to the tumor volume on the first day of treatment (day 7 after tumor inoculation). Each point represents the mean ± SD ($n = 5$). **$P < 0.01$ compared to the group treated with BL + US (Non-depletion). BL: Bubble liposomes, US: Ultrasound.

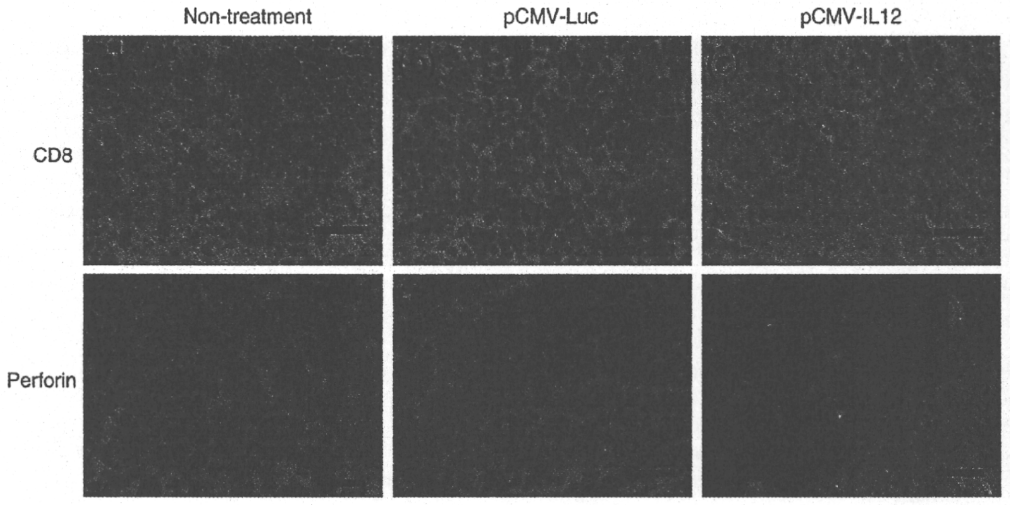

Fig. 5. Images of infiltrating T cells in OV-HM tumors following pCMV-IL12 delivery using Bubble liposomes and ultrasound. B6C3F1 mice were intradermally inoculated with 1×10^6 OV-HM cells into the flank. Seven, 9 or12 days after tumor inoculation, the tumors were injected with pCMV-Luc (b and e) or pCMV-IL12 (c and f) using Bubble liposomes and ultrasound. On day 13 after tumor inoculation, immunohistochemical staining against CD8 (a, b and c) and perforin (d, e and f) was performed using frozen tumor sections. Scale bar shows 10 μm.

an effective immunotherapeutic approach with reduced systemic adverse effects. Many anti-tumor effects depend on this method for IL-12 gene delivery. Viral vector systems have high potency and are effective for gene delivery, but gene therapy using viral vector systems may have associated safety issues [32]. Although transfection efficiency of most non-viral vector systems is lower, they are generally considered safer than viral vector systems [33]. Therefore, non-viral vector systems are preferred to cancer gene therapy using cytokines because it is not necessary to deliver the cytokine gene into all the cells. There have been many recent reports about gene delivery using the combination of nano/microbubbles and ultrasound [16–18], but most of them only confirmed the efficiency of gene expression using reporter genes. On the other hand, there have been few reports regarding therapeutic effects using sonoporation technology in cancer gene therapy. In this study, we assessed the effectiveness of the combination of Bubble liposomes and ultrasound as a non-viral vector system for effective cancer gene therapy, using plasmid DNA expressing IL-12, a potent primer of anti-tumor immunity.

IL-12 expression by IL-12 corded plasmid DNA delivery with the combination of Bubble liposomes and ultrasound was higher than that with a conventional lipofection method using Lipofectamine 2000 (Fig. 2). The therapeutic effect of IL-12 cancer gene therapy using each gene delivery method was compared (Fig. 3). IL-12 gene delivery with Lipofectamine 2000 did not suppress tumor growth, whereas gene delivery using a combination of Bubble liposomes and ultrasound effectively suppressed tumor growth. We originally thought that this was due to the level of IL-12 expression (Fig. 2), but complete tumor rejection was not observed in any mice treated with IL-12 gene delivery using Bubble liposomes and ultrasound (Fig. 3a), perhaps because the IL-12 gene is only transiently expressed in the tumor tissue. To address this problem, the IL-12 gene was repeatedly delivered using Bubble liposomes and ultrasound: as shown in Fig. 3b, the tumors were completely rejected. This complete rejection was attributed to the maintenance of therapeutic IL-12 levels in the tumor tissue. On the other hand, we could not observe anti-tumor effect in the luciferase corded plasmid DNA (pCMV-Luc) delivery with Bubble liposomes and ultrasound. This result not only suggests that IL-12 expression was important to suppress tumor growth but also suggests that there was no effect on tumor suppression by the combination of Bubble liposomes and ultrasound. In addition, we used Lipofectamine 2000 as control because gene transfection with intraperitoneal injection of Lipofectamine 2000 could effectively deliver into ascites tumor cells in our previous report [20]. Therefore, in this case of local tumoral injection, we thought that Lipofectamine 2000 could also be utilized as control vector system. Moreover, our collaborator reported about anti-tumor effects by single intratumoral injection of IL-12 expressing RGD fiber mutant adenovirus vector in OV-HM tumor bearing mice. In the report, tumor growth was suppressed and the tumor regression rate was about 40% [9]. In our study, although effective tumor growth suppression was observed in the therapeutic mice by single injection of IL-12 gene with Bubble liposomes and ultrasound, the tumor regressing mice were not observed. In this single therapy, our system was not equal to adenovirus vector in terms of tumor regression rate. On the other hand, the tumor regression rate in our repetitive therapy reached to 80%. And anti-tumor effect by repetitive therapy in our gene delivery system could go beyond that by single therapy with adenovirus vector. From these results, it was thought that the combination of Bubble liposomes and ultrasound might be a useful non-viral vector system for cancer gene therapy.

The anti-tumor effect by gene delivery with the combination of Bubble liposomes and ultrasound completely disappeared in mice lacking $CD8^+$ T lymphocytes (Fig. 4). Therefore, in this IL-12 gene therapy, $CD8^+$ T lymphocytes play a major role in suppressing tumor growth, suggesting that the combination of Bubble liposomes and ultrasound can effectively induce sufficient IL-12 expression to cause anti-tumor immune responses. In the Fig. 4, $CD8^+$ T lymphocytes depletion and $CD4^+$ and $CD8^+$ T lymphocytes depletion rather enhanced tumor growth. We thought that this reason was a same phenomenon as increasing the frequency of tumor generation according to decrease anti-tumor activity of immune competent cells by immunosuppressive agents. In brief, the balance of tumor growth and tumor rejection by immune system trend toward tumor growth by the depletion of $CD4^+$ and $CD8^+$ T lymphocytes. Therefore, it was thought that tumor growth was accelerated by the depletion. In other report, same phenomenon was observed [34]. The invasion of many $CD8^+$ T lymphocytes was observed in tumor tissue from mice treated with the IL-12 gene, Bubble liposomes and ultrasound (Fig. 5c), and perforin-positive cells were also observed in this tumor tissue (Fig. 5f). These results suggest that the expression of IL-12 genes, delivered using Bubble liposomes and ultrasound, primed the anti-tumor immunity, causing the tumor cells to be rejected. In this study, we did not measure the IL-12 concentration in the tumor tissue, but Colombo et al. reported that 30–80 pg/ml IL-12 at the tumor site can induce 40% regression of

C26 colon carcinoma [8]. Although the therapeutic effect depends on the type of cancer, we estimate that IL-12 concentrations of the order of tens of pg/ml are expressed at the tumor site using the present therapeutic system.

In conclusion, we demonstrated that the combination of Bubble liposomes and ultrasound effectively delivers the IL-12 gene into tumor tissue, and that local IL-12 production induces an immune response to the tumor cells. Therefore, the combination of Bubble liposomes and ultrasound could be a useful non-viral vector system in cancer gene therapy.

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References

[1] U. Gubler, A.O. Chua, D.S. Schoenhaut, C.M. Dwyer, W. McComas, R. Motyka, N. Nabavi, A.G. Wolitzky, P.M. Quinn, P.C. Familletti, Coexpression of two distinct genes is required to generate secreted bioactive cytotoxic lymphocyte maturation factor, Proc Natl Acad Sci U S A 88 (1991) 4143–4147.

[2] S.F. Wolf, P.A. Temple, M. Kobayashi, D. Young, M. Dicig, L. Lowe, R. Dzialo, L. Fitz, C. Ferenz, R.M. Hewick, Cloning of cDNA for natural killer cell stimulatory factor, a heterodimeric cytokine with multiple biologic effects on T and natural killer cells, J Immunol 146 (1991) 3074–3081.

[3] M.J. Brunda, Interleukin-12, J Leukoc Biol 55 (1994) 280–288.

[4] C.L. Nastala, H.D. Edington, T.G. McKinney, H. Tahara, M.A. Nalesnik, M.J. Brunda, M.K. Gately, S.F. Wolf, R.D. Schreiber, W.J. Storkus, Recombinant IL-12 administration induces tumor regression in association with IFN-gamma production, J Immunol 153 (1994) 1697–1706.

[5] W.G. Yu, M. Ogawa, J. Mu, K. Umehara, T. Tsujimura, H. Fujiwara, T. Hamaoka, IL-12-induced tumor regression correlates with in situ activity of IFN-gamma produced by tumor-infiltrating cells and its secondary induction of anti-tumor pathways, J Leukoc Biol 62 (1997) 450–457.

[6] M.B. Atkins, M.J. Robertson, M. Gordon, M.T. Lotze, M. DeCoste, J.S. DuBois, J. Ritz, A.B. Sandler, H.D. Edington, P.D. Garzone, J.W. Mier, C.M. Canning, L. Battiato, H. Tahara, M.L. Sherman, Phase I evaluation of intravenous recombinant human interleukin 12 in patients with advanced malignancies, Clin Cancer Res 3 (1997) 409–417.

[7] J.P. Leonard, M.L. Sherman, G.L. Fisher, L.J. Buchanan, G. Larsen, M.B. Atkins, J.A. Sosman, J.P. Dutcher, N.J. Vogelzang, J.L. Ryan, Effects of single-dose interleukin-12 exposure on interleukin-12-associated toxicity and interferon-gamma production, Blood 90 (1997) 2541–2548.

[8] M.P. Colombo, M. Vagliani, F. Spreafico, M. Parenza, C. Chiodoni, C. Melani, A. Stoppacciaro, Amount of interleukin 12 available at the tumor site is critical for tumor regression, Cancer Res 56 (1996) 2531–2534.

[9] J.Q. Gao, T. Sugita, N. Kanagawa, K. Iida, Y. Eto, Y. Motomura, H. Mizuguchi, Y. Tsutsumi, T. Hayakawa, T. Mayumi, S. Nakagawa, A single intratumoral injection of a fiber-mutant adenoviral vector encoding interleukin 12 induces remarkable anti-tumor and anti-metastatic activity in mice with Meth-A fibrosarcoma, Biochem Biophys Res Commun 328 (2005) 1043–1050.

[10] Y. Gao, Z. Xu, S. Chen, W. Gu, L. Chen, Y. Li, Arginine-chitosan/DNA self-assemble nanoparticles for gene delivery: in vitro characteristics and transfection efficiency, Int J Pharm 359 (2008) 241–246.

[11] H. Hatakeyama, H. Akita, K. Kogure, M. Oishi, Y. Nagasaki, Y. Kihira, M. Ueno, H. Kobayashi, H. Kikuchi, H. Harashima, Development of a novel systemic gene delivery system for cancer therapy with a tumor-specific cleavable PEG-lipid, Gene Ther 14 (2007) 68–77.

[12] T. Montier, T. Benvegnu, P.A. Jaffres, J.J. Yaouanc, P. Lehn, Progress in cationic lipid-mediated gene transfection: a series of bio-inspired lipids as an example, Curr Gene Ther 8 (2008) 296–312.

[13] M. Morille, C. Passirani, A. Vonarbourg, A. Clavreul, J.P. Benoit, Progress in developing cationic vectors for non-viral systemic gene therapy against cancer, Biomaterials 29 (2008) 3477–3496.

[14] M. Fechheimer, J.F. Boylan, S. Parker, J.E. Sisken, G.L. Patel, S.G. Zimmer, Transfection of mammalian cells with plasmid DNA by scrape loading and sonication loading, Proc Natl Acad Sci U S A 84 (1987) 8463–8467.

[15] M.W. Miller, D.L. Miller, A.A. Brayman, A review of in vitro bioeffects of inertial ultrasonic cavitation from a mechanistic perspective, Ultrasound Med Biol 22 (1996) 1131–1154.

[16] C.M. Newman, T. Bettinger, Gene therapy progress and prospects: ultrasound for gene transfer, Gene Ther 14 (2007) 465–475.

[17] Z.P. Shen, A.A. Brayman, L. Chen, C.H. Miao, Ultrasound with microbubbles enhances gene expression of plasmid DNA in the liver via intraportal delivery, Gene Ther 15 (2008) 1147–1155.

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

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

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

[21] R. Suzuki, Y. Oda, N. Utoguchi, E. Namai, Y. Taira, N. Okada, N. Kadowaki, T. Kodama, K. Tachibana, K. Maruyama, A novel strategy utilizing ultrasound for antigen delivery in dendritic cell-based cancer immunotherapy, J Control Release 133 (2009) 198–205.

[22] T. Yamashita, S. Sonoda, R. Suzuki, N. Arimura, K. Tachibana, K. Maruyama, T. Sakamoto, A novel bubble liposome and ultrasound-mediated gene transfer to ocular surface: RC-1 cells in vitro and conjunctiva in vivo, Exp Eye Res 85 (2007) 741–748.

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

[24] K. Un, S. Kawakami, R. Suzuki, K. Maruyama, F. Yamashita, M. Hashida, Enhanced transfection efficiency into macrophages and dendritic cells by the combination method using mannosylated lipoplexes and Bubble liposomes with ultrasound exposure Hum Gene Ther. (2009) In press.

[25] M. Hashimoto, O. Niwa, Y. Nitta, M. Takeichi, K. Yokoro, Unstable expression of E-cadherin adhesion molecules in metastatic ovarian tumor cells, Jpn J Cancer Res 80 (1989) 459–463.

[26] Y. Okada, N. Okada, S. Nakagawa, H. Mizuguchi, M. Kanehira, N. Nishino, K. Takahashi, N. Mizuno, T. Hayakawa, T. Mayumi, Fiber-mutant technique can augment gene transduction efficacy and anti-tumor effects against established murine melanoma by cytokine-gene therapy using adenovirus vectors, Cancer Lett 177 (2002) 57–63.

[27] C. Fillat, M. Carrio, A. Cascante, B. Sangro, Suicide gene therapy mediated by the Herpes Simplex virus thymidine kinase gene/Ganciclovir system: fifteen years of application, Curr Gene Ther 3 (2003) 13–26.

[28] X.H. Shi, Z.Y. Liang, X.Y. Ren, T.H. Liu, Combined silencing of K-ras and Akt2 oncogenes achieves synergistic effects in inhibiting pancreatic cancer cell growth in vitro and in vivo, Cancer Gene Ther 16 (2009) 227–236.

[29] M. Nogawa, T. Yuasa, S. Kimura, M. Tanaka, J. Kuroda, K. Sato, A. Yokota, H. Segawa, Y. Toda, S. Kageyama, T. Yoshiki, Y. Okada, T. Maekawa, Intravesical administration of small interfering RNA targeting PLK-1 successfully prevents the growth of bladder cancer, J Clin Invest 115 (2005) 978–985.

[30] C.W. Beh, W.Y. Seow, Y. Wang, Y. Zhang, Z.Y. Ong, P.L. Ee, Y.Y. Yang, Efficient delivery of Bcl-2-targeted siRNA using cationic polymer nanoparticles: down-regulating mRNA expression level and sensitizing cancer cells to anticancer drug, Biomacromolecules 10 (2009) 41–48.

[31] M. Folini, M. Pennati, N. Zaffaroni, RNA interference-mediated validation of genes involved in telomere maintenance and evasion of apoptosis as cancer therapeutic targets, Methods Mol Biol 487 (2009) 303–330.

[32] S. Lehrman, Virus treatment questioned after gene therapy death, Nature 401 (1999) 517–518.

[33] C.C. Conwell, L. Huang, Recent advances in non-viral gene delivery, Adv Genet 53PA (2005) 1–18.

[34] N. Kanagawa, J.Q. Gao, Y. Motomura, T. Yanagawa, Y. Mukai, Y. Yoshioka, N. Okada, S. Nakagawa, Antitumor mechanism of intratumoral injection with IL-12-expressing adenoviral vector against IL-12-unresponsive tumor, Biochem Biophys Res Commun 372 (2008) 821–825.