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Suppression of Melanoma Growth and Metastasis by DNA Vaccination Using an Ultrasound-Responsive and Mannose-Modified Gene Carrier

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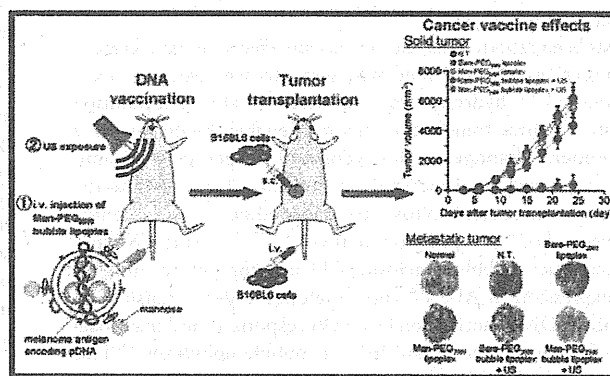
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Supporting Information

ABSTRACT: DNA vaccination has attracted much attention as a promising therapy for the prevention of metastasis and relapse of malignant tumors, especially highly metastatic tumors such as melanoma. However, it is difficult to achieve a potent cancer vaccine effect by DNA vaccination, since the number of dendritic cells, which are the major targeted cells of DNA vaccination, is very few. Here, we developed a DNA vaccination for metastatic and relapsed melanoma by ultrasound (US)-responsive and antigen presenting cell (APC)-selective gene carriers reported previously, named Man-PEG₂₀₀₀ bubble lipoplexes. Following immunization using US exposure and Man-PEG₂₀₀₀ bubble lipoplexes constructed with pUb-M, which expresses ubiquitylated melanoma-specific antigens (gp100 and TRP-2), the secretion of Th1 cytokines (IFN- γ and TNF- α) and the activities of cytotoxic T lymphocytes (CTLs) were specifically enhanced in the presence of B16BL6 melanoma antigens. Moreover, we succeeded in obtaining potent and sustained DNA vaccine effects against solid and metastatic tumor derived from B16BL6 melanoma specifically. The findings obtained from this study suggest that the gene transfection method using Man-PEG₂₀₀₀ bubble lipoplexes and US exposure could be suitable for DNA vaccination aimed at the prevention of metastatic and relapsed cancer.

KEYWORDS: mannose modification, bubble lipoplex, ultrasound, DNA vaccination, melanoma



INTRODUCTION

Melanoma is a neoplasm arising within epidermal melanocytes of the skin, and one of several cancers exhibiting the increasing incidence in recent years.¹ Early stage melanoma is curable, but melanoma metastasis and relapse occur frequently in the patients following treatments such as surgery, and the prognosis for patients with metastatic melanoma is poor.^{2,3} Although systemic therapy induces complete therapeutic responses in a minority of patients, metastatic melanoma is a devastating illness and treatment options are limited; therefore, there is a need for an effective therapy for metastatic melanoma.

Cancer vaccination has attracted much attention as a promising therapy for the prevention of tumor growth and metastasis, because it is based on an immune response provided by the cancer antigen, and consequently, its therapeutic effects are specific to the targeted cancer cells and the adverse effects followed by

cancer vaccination are low.^{4,5} In particular, it has been reported that DNA vaccination, which uses an exogenous gene encoding cancer antigen, can induce not only humoral immunity but also cellular immunity and, moreover, can induce cancer-specific CTLs with potent antitumor activities.^{6–9} In a variety of cancers, since melanoma is known to exhibit inherent immunogenicity and the identification of melanoma-specific antigen is proceeding, such as gp100, melanoma-antigen recognized by T cells-1 (MART-1) and tyrosinase-related protein (TRP),^{10–13} it is considered that DNA vaccination against melanoma is suitable for not only the

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prevention of metastasis and relapse but also the suppression of tumor growth.

To achieve potent therapeutic effects by DNA vaccination against cancer, it is essential to transfer the antigen-coding gene selectively and efficiently into APCs such as macrophages and dendritic cells, which play a pivotal role in the initiation, programming and regulation of cancer-specific immune responses.^{14,15} Our group has also developed mannose-modified liposome/plasmid DNA (pDNA) complexes (mannose-modified lipoplexes) for APC-selective gene transfer via mannose receptors expressing on APCs, and obtained APC-selective gene expression in the liver and spleen by mannose-modified lipoplexes.^{16,17} Moreover, our group also succeeded in obtaining DNA vaccine effects against cancer by intraperitoneal administration of mannose-modified lipoplexes constructed with tumor-specific antigen coding pDNA, such as ovalbumin (OVA) and melanoma-related antigens.^{18,19} However, the gene transfection efficiency into APCs was lower than that in other cells,²⁰ therefore, it could be difficult to induce a potent cancer vaccine effect for the prevention of metastasis and relapse by DNA vaccination using conventional lipofection methods.

It has been reported that cancer vaccine effects can be enhanced by physical stimulation-mediated gene transfer such as electroporation,^{21,22} hydrodynamic injection^{23,24} and sonoporation methods.²⁵ These transfection methods enable the delivery of a large amount of antigen-coding gene and antigen peptides into APCs, since exogenous materials are directly introduced into the cytoplasm without endocytosis in these methods.^{26–29} Recently, we have applied "sonoporation methods"^{25,29–31} using US exposure and microbubbles enclosing US imaging gas to enhance gene expression in APCs³² and developed a gene transfection method for DNA vaccination using US-responsive and mannose-modified gene carriers, Man-PEG₂₀₀₀ bubble lipoplexes.³³ This method enables APC-selective and -efficient gene expression, and moreover, effective vaccine effects against OVA-expressing cancer cells were obtained by applying this method to DNA vaccination using OVA-encoding pDNA.³³ However, the antigenicity of OVA is extremely high compared with other antigens,³⁴ and it is difficult to extrapolate the result obtained by DNA vaccination against OVA-expressing cells to actual cancer therapy, since OVA-expressing cells are transfectant constructed by gene transfer. Therefore, it is unclear if DNA vaccination by gene transfer using Man-PEG₂₀₀₀ bubble lipoplexes and US exposure is effective against cancer, i.e. melanoma, with metastatic properties.

In this study, we examined DNA vaccine effects against melanoma by transfection of pUb-M, coexpressing ubiquitylated gp100 and TRP-2, using Man-PEG₂₀₀₀ bubble lipoplexes and US exposure. First, we examined the level of gene expression in the splenic dendritic cells by gene transfer using Man-PEG₂₀₀₀ bubble lipoplexes constructed with pUb-M and US exposure. Second, we studied the characteristics of cytokine secretion and the induction of CTL activities against B16BL6 cell-derived melanoma by DNA vaccination using Man-PEG₂₀₀₀ bubble lipoplexes constructed with pUb-M and US exposure. Then, we investigated the cancer vaccine effects against solid and metastatic tumors derived from B16BL6 cells by DNA vaccination using Man-PEG₂₀₀₀ bubble lipoplexes and US exposure. Finally, we evaluated the duration of cancer vaccine effects against solid and metastatic melanoma after pUb-M transfection using Man-PEG₂₀₀₀ bubble lipoplexes and US exposure.

EXPERIMENTAL SECTION

Materials. 1,2-Stearoyl-3-trimethylammoniumpropane (DSTAP), 1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC) and 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[amino-(polyethylene glycol)-2000] (NH₂-PEG₂₀₀₀-DSPE) were purchased from Avanti Polar Lipids Inc. (Alabaster, AL, USA), Sigma Chemicals Inc. (St. Louis, MO, USA) and NOF Co. (Tokyo, Japan), respectively. Anti-CD11c monoclonal antibody (N418)-labeled magnetic beads were obtained from Miltenyi Biotec Inc. (Auburn, CA, USA). Fetal bovine serum (FBS) was purchased from Equitech-bio Inc. (Kerrville, TX, USA). RPMI-1640 and Dulbecco's modified Eagle's medium (DMEM) were purchased from Nissui Pharmaceutical Co., Ltd. (Tokyo, Japan). All other chemicals were of the highest purity available.

pDNA, Cell Lines and Mice. pUb-M containing murine melanoma glycoprotein-100_{25–33} (gp100) and tyrosinase-related protein-2_{181–188} (TRP-2) peptide epitopes was kindly provided by Prof. R. A. Reisfeld.³⁵ The B16BL6 melanoma cells, colon-26 adenocarcinoma cells and EL4 lymphoma cells were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). The B16BL6/Luc cells and colon-26/Luc cells, which are cell lines expressing firefly luciferase stably, were established as previously reported.^{36,37} The B16BL6 cells and EL4 cells were cultured in DMEM, and the colon-26 cells were cultured in RPMI-1640 at 37 °C in 5% CO₂. Both media were supplemented with 10% FBS, 100 IU/mL penicillin, 100 µg/mL streptomycin, and 2 mM L-glutamine. Female C57BL/6 mice (6 weeks old) and female Balb/c mice (6 weeks old) were purchased from the Shizuoka Agricultural Cooperative Association for Laboratory Animals (Shizuoka, Japan). All animal experiments were carried out in accordance with the Principles of Laboratory Animal Care as adopted and propagated by the U.S. National Institutes of Health and the Guidelines for Animal Experiments of Kyoto University.

Construction of Man-PEG₂₀₀₀ Bubble Lipoplexes. Man-PEG₂₀₀₀ bubble lipoplexes were constructed according to our previous report.³³ Briefly, DSTAP, DSPC and NH₂-PEG₂₀₀₀-DSPE or mannose-modified PEG₂₀₀₀-DSPE were mixed in chloroform at a molar ratio of 7:2:1 to produce the liposomes for bubble lipoplexes. The mixture for construction of liposomes was dried by evaporation and vacuum desiccated, and the resultant lipid film was resuspended in sterile 5% dextrose. After hydration for 30 min at 65 °C, the dispersion was sonicated for 10 min in a bath sonicator and for 3 min in a tip sonicator to produce liposomes. Then, the liposomes were sterilized by passage through a 0.45 µm filter (Nihon-Millipore, Tokyo, Japan). The lipoplexes were prepared by gently mixing equal volumes of pDNA and liposome solution at a charge ratio of 1.0:2.3 (–:+) . To enclose US imaging gas in lipoplexes, the prepared lipoplexes were pressured with perfluoropropane gas (Takachiho Chemical Industries Co., Ltd., Tokyo, Japan) and sonicated using a bath-type sonicator (AS ONE Co., Osaka, Japan) for 5 min. The particle sizes and zeta potentials of the liposomes/lipoplexes were determined by a Zetasizer Nano ZS instrument (Malvern Instrument, Ltd., Worcestershire, U.K.).

In Vivo Gene Transfection Method. Six week old C57BL/6 female mice were intravenously injected with 400 µL of bubble lipoplexes via the tail vein using a 26 gauge syringe needle at a dose of 50 µg of pDNA. At 5 min after the injection of the bubble lipoplexes, US (frequency, 1.045 MHz; duty, 50%; burst rate,

Table 1. Particle Sizes and Zeta Potentials of Lipoplexes and Bubble Lipoplexes Constructed with pUb-M^a

	particle size (nm)	zeta-potential (mV)
Bare-PEG ₂₀₀₀ lipoplex (DSTAP:DSPC:NH ₂ -PEG ₂₀₀₀ :DSPE = 7:2:1 (mol))	144 ± 13	45.7 ± 4.5
Man-PEG ₂₀₀₀ lipoplex (DSTAP:DSPC:Man-PEG ₂₀₀₀ :DSPE = 7:2:1 (mol))	143 ± 10	44.5 ± 5.8
Bare-PEG ₂₀₀₀ bubble lipoplex (DSTAP:DSPC:NH ₂ -PEG ₂₀₀₀ :DSPE = 7:2:1 (mol))	557 ± 20	46.7 ± 4.2
Man-PEG ₂₀₀₀ bubble lipoplex (DSTAP:DSPC:Man-PEG ₂₀₀₀ :DSPE = 7:2:1 (mol))	555 ± 19	45.1 ± 2.2

^a Each value represents the mean ± SD (*n* = 3).

10 Hz; intensity 1.0 W/cm²; time, 2 min) was exposed transdermally to the abdominal area using a Sonopore-4000 sonicator with a probe of diameter 20 mm. At predetermined times after injection, mice were sacrificed and spleens were collected for each experiment. In the intradermal transfection study, mice were intradermally injected with 200 μ L of bubble lipoplexes at a dose of 50 μ g of pDNA. At 5 min after the injection of the bubble lipoplexes, US (frequency, 2.062 MHz; duty, 50%; burst rate, 10 Hz; intensity 4.0 W/cm²; time, 2 min) was directly exposed to the injected site using a probe of diameter 6 mm. In the intrasplenic transfection, mice were directly injected into the spleen with 200 μ L of bubble lipoplexes at a dose of 50 μ g of pDNA. At 5 min after the injection of the bubble lipoplexes, US (frequency, 2.062 MHz; duty, 50%; burst rate, 10 Hz; intensity 4.0 W/cm²; time, 2 min) was directly exposed to the spleen using a probe of diameter 6 mm.

Measurement of the Level of mRNA Expression. Total RNA was isolated from the spleen using a GenElute Mammalian Total RNA Miniprep Kit (Sigma-Aldrich, St. Louis, MO, USA). Reverse transcription of mRNA was carried out using a PrimeScript RT reagent Kit (Takara Bio Inc., Shiga, Japan). The detection of the Ub-M cDNA was carried out by real-time PCR using SYBR Premix Ex Taq (Takara Bio Inc., Shiga, Japan) and Lightcycler Quick System 350S (Roche Diagnostics, Indianapolis, IN, USA) with primers. The primers for Ub-M, gp100, TRP-2 and GAPDH cDNA were constructed as follows: primer for Ub-M cDNA, 5'-GAG CCC AGT GAC ACC ATA GA-3' (forward) and 5'-GTG CAG GGT GGA CTC TTT CT-3' (reverse); primer for gp100, 5'-GCA CCC AAC TTG TTG TTC CT-3' (forward) and 5'-GTG CTA CCA TGT GGC ATT TG-3' (reverse); primer for TRP-2, 5'-CTT CCT AAC CGC AGA GCA AC-3' (forward) and 5'-CAG GTA GGA GCA TGC TAG GC-3' (reverse); primer for GAPDH, 5'-TCT CCT GCG ACT TCA ACA-3' (forward) and 5'-GCT GTA GCC GTA TTC ATT GT-3' (reverse) (Sigma-Aldrich, St. Louis, MO, USA). The mRNA copy numbers were calculated for each sample from the standard curve using the instrument software ("Arithmetic Fit Point analysis" for the Lightcycler). Results were expressed as relative copy numbers calculated relative to GAPDH mRNA (copy numbers of Ub-M, gp100 and TRP-2 mRNA/copy numbers of GAPDH mRNA).

Isolation of Splenic CD11c⁺ Cells (Dendritic Cells) in Mice. At 6 h after transfection, spleens were harvested and spleen cells were suspended in ice-cold RPMI-1640 medium on ice. Then, red blood cells were removed by incubation with hemolytic reagent (0.15 M NH₄Cl, 10 mM KHCO₃, 0.1 mM EDTA) for 3 min at room temperature. CD11c⁺ and CD11c⁻ cells were separated by magnetic cell sorting with an auto MACS (Miltenyi Biotec Inc., Auburn, CA, USA) following the manufacturer's instructions.

Evaluation of Antigen-Specific Cytokine Secretion. To prepare the tumor cell lysates (B16BL6 cells, EL4 cells and colon-26 cells), the cells were scraped from the plates and suspended in

lysis buffer (0.05% Triton X-100, 2 mM EDTA, 0.1 M Tris, pH 7.8). After three cycles of freezing and thawing, the lysates were centrifuged at 10000g, 4 °C for 10 min and the resultant supernatants were collected. The protein concentration of cell lysates was determined with a Protein Quantification Kit (Dojindo Molecular Technologies, Inc., Tokyo, Japan). At 2 weeks after the last immunization, the splenic cells collected from immunized mice were plated in 96-well plates and incubated for 72 h at 37 °C in the presence or absence of tumor cell lysates (100 μ g of proteins). IFN- γ , TNF- α , IL-4 and IL-6 in the culture medium were measured using a suitable commercial ELISA Kit (Bay Bioscience Co., Ltd., Hyogo, Japan).

CTL Assay. At 2 weeks after the last immunization, the splenic cells collected from immunized mice were plated in 6-well plates and coincubated with mitomycin C-treated tumor cells (B16BL6 cells, EL4 cells and colon-26 cells) for 4 days. After 4 days of coincubation, nonadherent cells were harvested, washed and plated in 96-well plates with target cells (B16BL6 cells, EL4 cells and colon-26 cells) at various effector cell/target cell (E/T) ratios. The target tumor cells were labeled with ⁵¹Cr by incubating with Na₂⁵¹CrO₄ (PerkinElmer, Inc., MA, USA) in culture medium for 1 h at 37 °C. At 4 h after incubation, the plates were centrifuged, and the supernatant in each well was collected and the radioactivity of released ⁵¹Cr was measured in a gamma counter. The percentage of ⁵¹Cr release was calculated as follows: specific lysis (%) = [(experimental ⁵¹Cr release - spontaneous ⁵¹Cr release) / (maximum ⁵¹Cr release - spontaneous ⁵¹Cr release)] × 100.

Therapeutic Experiments in Solid Tumor Models. At 2 weeks after the last immunization or on the immunization day, B16BL6 cells, EL4 cells and colon-26 cells were transplanted subcutaneously into the back of the mice (1 × 10⁶ cells). The tumor size was measured with calipers in two dimensions, and the tumor volume was calculated using the following equation: volume (mm³) = $\pi/6$ × longer diameter × (shorter diameter)². The survival of the mice was monitored up to 100 days after the transplantation of tumor cells.

Therapeutic Experiments in Lung Metastatic Tumor Models. At 2 weeks after the last immunization or on the immunization day, B16BL6 cells or colon-26 cells were intravenously administered via the tail vein (1 × 10⁵ cells) and the survival of the mice was monitored up to 100 days after administration of the tumor cells. To evaluate metastasis, B16BL6/Luc cells or colon-26/Luc cells were intravenously administered via the tail vein (1 × 10⁵ cells). At 14 days after the administration of the tumor cells, the number of B16BL6/Luc cells and colon-26/Luc cells in the lung was quantitatively evaluated by measuring luciferase activity as previously reported.^{36,37}

Statistical Analysis. Results were presented as the mean ± SD of more than three experiments. Analysis of variance (ANOVA) was used to test the statistical significance of differences among groups. Two-group comparisons were performed by the Student's *t* test. Multiple comparisons between control groups and other groups were performed by the Dunnett's test, and multiple

comparisons between all groups were performed by the Tukey–Kramer test.

RESULTS

Physicochemical Properties of Bubble Lipoplexes Constructed with pUb-M. The physicochemical properties of lipoplexes and bubble lipoplexes constructed with pUb-M used in all experiments were evaluated by measuring the particle sizes and zeta potentials. The mean particle sizes and zeta potentials of nonmodified PEG₂₀₀₀-lipoplexes (Bare-PEG₂₀₀₀ lipoplexes) and mannose-conjugated PEG₂₀₀₀-lipoplexes (Man-PEG₂₀₀₀ lipoplexes) were 144 ± 13 nm, 45.7 ± 4.5 mV and 143 ± 10 nm, 44.5 ± 5.8 mV, respectively (Table 1). Moreover, the mean particle sizes and zeta potentials of nonmodified bubble lipoplexes (Bare-PEG₂₀₀₀ bubble lipoplexes) and Man-PEG₂₀₀₀ bubble lipoplexes were 557 ± 20 nm, 46.7 ± 4.2 mV and 555 ± 19 nm, 45.1 ± 2.2 mV, respectively (Table 1). These results corresponded to our previous reports using other pDNA,³³ suggesting that pDNA had no effect on the physicochemical properties of Man-PEG₂₀₀₀ bubble lipoplexes.

Splenic Dendritic Cell-Selective and -Efficient Gene Expression by Gene Transfer Using Man-PEG₂₀₀₀ Bubble Lipoplexes and US Exposure. First, to investigate the level of gene expression by Man-PEG₂₀₀₀ bubble lipoplexes and US exposure in the spleen, we measured the relative mRNA copy numbers of Ub-M after transfection. As shown in Figures 1A and 1B, the level of Ub-M mRNA expression obtained by Man-PEG₂₀₀₀ bubble lipoplexes and US exposure reached a peak at 6 h after transfection. Moreover, that level of Ub-M mRNA expression was markedly higher than that obtained by Bare- and Man-PEG₂₀₀₀ lipoplexes, and significantly higher than that obtained by Bare-PEG₂₀₀₀ bubble lipoplexes and US exposure. Then, we investigated the mannose receptor-expressing cell selectivity of Ub-M mRNA expression obtained by gene transfer using Man-PEG₂₀₀₀ bubble lipoplexes and US exposure. In the spleen, the relative mRNA copy numbers of Ub-M in CD11c⁺ cells was significantly higher than that in CD11c⁻ cells following transfection using Man-PEG₂₀₀₀ bubble lipoplexes and US exposure (Figure 1C). On the other hand, no selective gene expression in CD11c⁺ cells was observed by gene transfer using Bare-PEG₂₀₀₀ bubble lipoplexes and US exposure (Figure 1C).

Antigen-Stimulatory Th1 Cytokine Secretion from the Splenic Cells Immunized by Man-PEG₂₀₀₀ Bubble Lipoplexes and US Exposure. To evaluate the melanoma-specific cytokine secretion from immunized splenic cells, splenic cells immunized by pUb-M were incubated with each tumor cell-lysate in vitro, and then, Th1 and Th2 cytokines secreted in the supernatants were measured. Following investigation of the expression level of gp100 and TRP-2, a melanoma-specific antigen, in each cell used in this study, the expression of gp100 and TRP-2 was only detected in B16BL6 cells which are melanoma cell lines (Supplementary Figure 1 in the Supporting Information). As results of the immunization according to the protocol shown in Figure 2A, the splenic cells immunized by Man-PEG₂₀₀₀ bubble lipoplexes and US exposure secreted the highest amount of IFN- γ and TNF- α , which are Th1 cytokines, in the presence of B16BL6 cell lysates (Figures 2B and 2C). On the other hand, the secretion of Th1 cytokines (IFN- γ and TNF- α) was lower in all the groups in the presence of EL4 and colon-26 cell lysates. Moreover, the secretion of IL-4 and IL-6, which are Th2 cytokines, was also lower in all the groups in the presence of each cell lysate (Figures 2D and 2E). These observations suggest that pUb-M transfer by

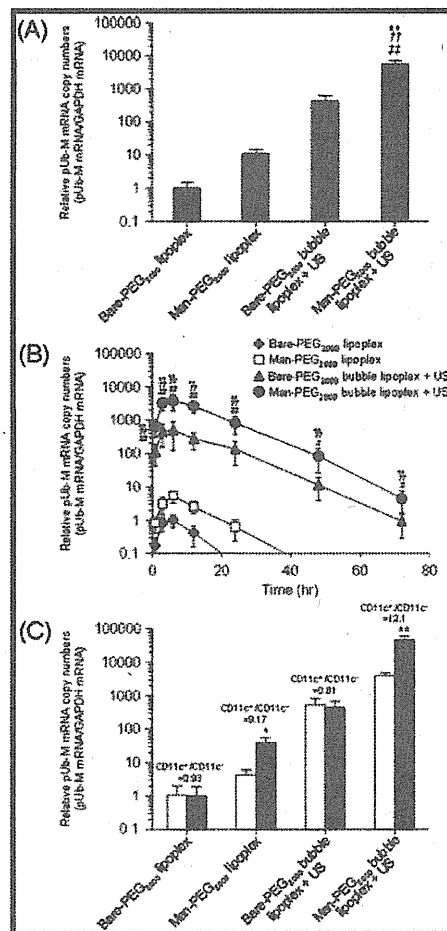


Figure 1. Enhanced Ub-M mRNA expression in the spleen and the splenic dendritic cells (CD11c⁺ cells) by Man-PEG₂₀₀₀ bubble lipoplexes constructed with pUb-M and US exposure in vivo. (A) The level of Ub-M mRNA expression obtained by Bare-PEG₂₀₀₀ lipoplexes, Man-PEG₂₀₀₀ lipoplexes, Bare-PEG₂₀₀₀ bubble lipoplexes with US exposure and Man-PEG₂₀₀₀ bubble lipoplexes with US exposure (50 μ g of pDNA) in the spleen at 6 h after transfection. Each value represents the mean \pm SD ($n = 4$). ** $p < 0.01$, compared with Bare-PEG₂₀₀₀ lipoplex; †† $p < 0.01$, compared with Man-PEG₂₀₀₀ lipoplex; ††† $p < 0.01$, compared with Bare-PEG₂₀₀₀ bubble lipoplex + US. (B) Time-course of Ub-M mRNA expression in the spleen after transfection by Bare-PEG₂₀₀₀ lipoplexes, Man-PEG₂₀₀₀ lipoplexes, Bare-PEG₂₀₀₀ bubble lipoplexes with US exposure and Man-PEG₂₀₀₀ bubble lipoplexes with US exposure (50 μ g of pDNA). Each value represents the mean \pm SD ($n = 4$). ** $p < 0.01$, compared with the corresponding group of Bare-PEG₂₀₀₀ lipoplex; †† $p < 0.01$, compared with the corresponding group of Man-PEG₂₀₀₀ lipoplex; * $p < 0.05$; ††† $p < 0.01$, compared with the corresponding group of Bare-PEG₂₀₀₀ bubble lipoplex + US. (C) Splenic cellular localization of Ub-M mRNA expression at 6 h after transfection by Bare-PEG₂₀₀₀ lipoplexes, Man-PEG₂₀₀₀ lipoplexes, Bare-PEG₂₀₀₀ bubble lipoplexes with US exposure and Man-PEG₂₀₀₀ bubble lipoplexes with US exposure (50 μ g of pDNA). Each value represents the mean \pm SD ($n = 4$). * $p < 0.05$; ** $p < 0.01$, compared with the corresponding group of CD11c⁻ cells.

Man-PEG₂₀₀₀ bubble lipoplexes and US exposure significantly enhances the differentiation of helper T cells into Th1.

Induction of Melanoma-Specific CTLs by pUb-M Transfer Using Man-PEG₂₀₀₀ Bubble Lipoplexes and US Exposure. We investigated the melanoma-specific CTL activities in the

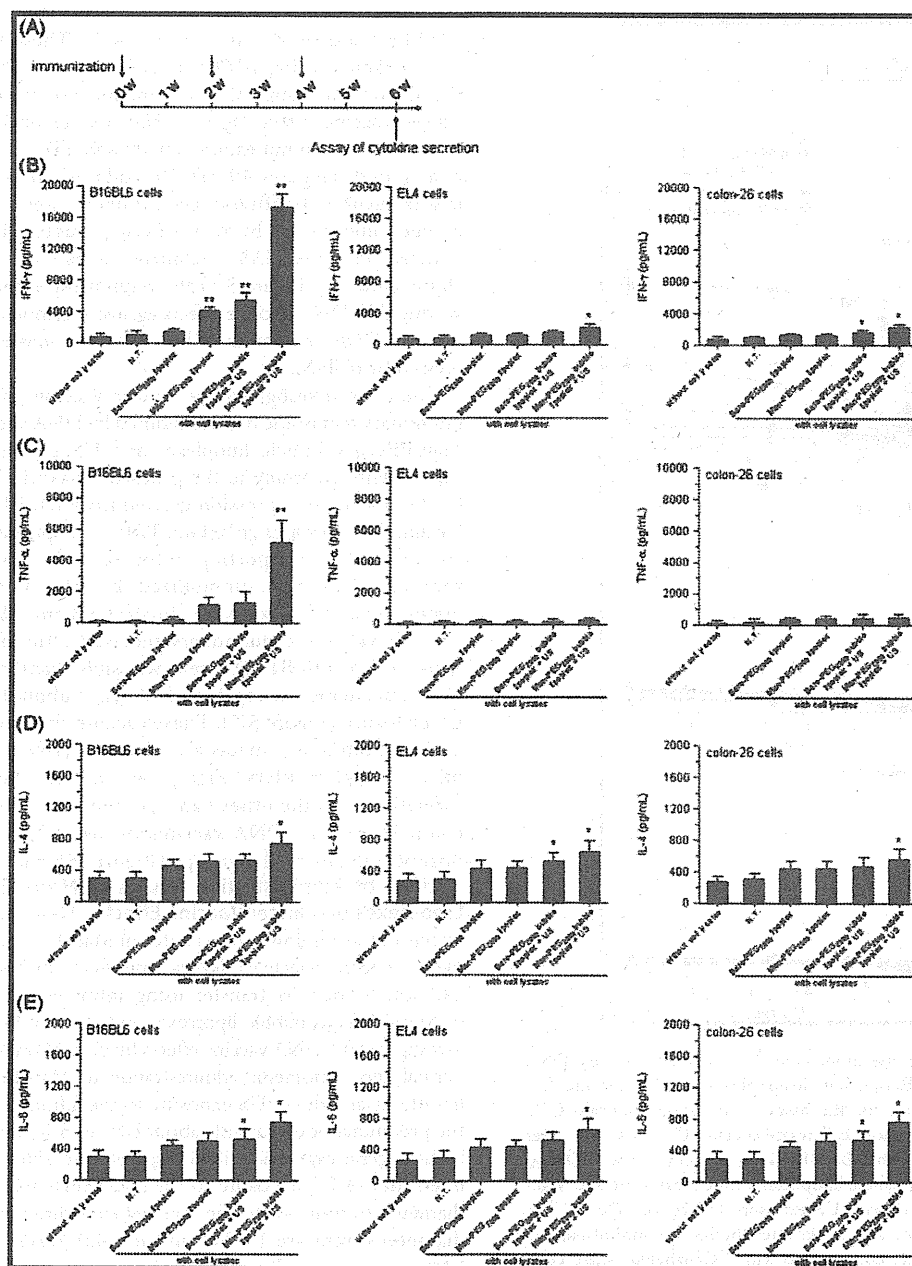


Figure 2. Melanoma-stimulatory cytokine secretion characteristics by DNA vaccination using Man-PEG₂₀₀₀ bubble lipoplexes constructed with pUb-M and US exposure. (A) Schedule of immunization for the evaluation of melanoma-stimulatory cytokine secretion characteristics. (B–E) Each cancer cell lysate-specific IFN- γ (B), TNF- α (C), IL-4 (D) and IL-6 (E) secretion from the splenic cells immunized three times biweekly with Bare-PEG₂₀₀₀ lipoplexes, Man-PEG₂₀₀₀ lipoplexes, Bare-PEG₂₀₀₀ bubble lipoplexes with US exposure and Man-PEG₂₀₀₀ bubble lipoplexes with US exposure (50 μ g of pDNA). The splenic cells were collected at 2 weeks after the last immunization. After the immunized splenic cells were cultured for 72 h in the presence of each cancer cell lysate (100 μ g protein), IFN- γ , TNF- α , IL-4 and IL-6 secreted in the medium were measured by ELISA. Each value represents the mean \pm SD ($n = 4$). * $p < 0.05$; ** $p < 0.01$, compared with the corresponding “without cell lysate” group.

splenic cells immunized by pUb-M. This experiment was performed according to the protocol shown in Figure 3A. As shown in Figure 3B, the splenic cells immunized by Man-PEG₂₀₀₀ bubble lipoplexes and US exposure showed the highest CTL activities of all groups stimulated by B16BL6 cells. In contrast, no CTL activity was obtained in all groups stimulated by EL4 and colon-26 cells (Figures 3C and 3D). These results suggest that melanoma-specific CTLs are induced effectively in the splenic

cells transfected pUb-M by Man-PEG₂₀₀₀ bubble lipoplexes and US exposure.

Cancer Vaccine Effects against Melanoma-Derived Solid and Metastatic Tumors by DNA Vaccination Using Man-PEG₂₀₀₀ Bubble Lipoplexes and US Exposure. Cancer vaccine effects against solid and metastatic tumors obtained by DNA vaccination using Man-PEG₂₀₀₀ lipoplexes and US exposure were examined. First, we evaluated the level of gp100 and

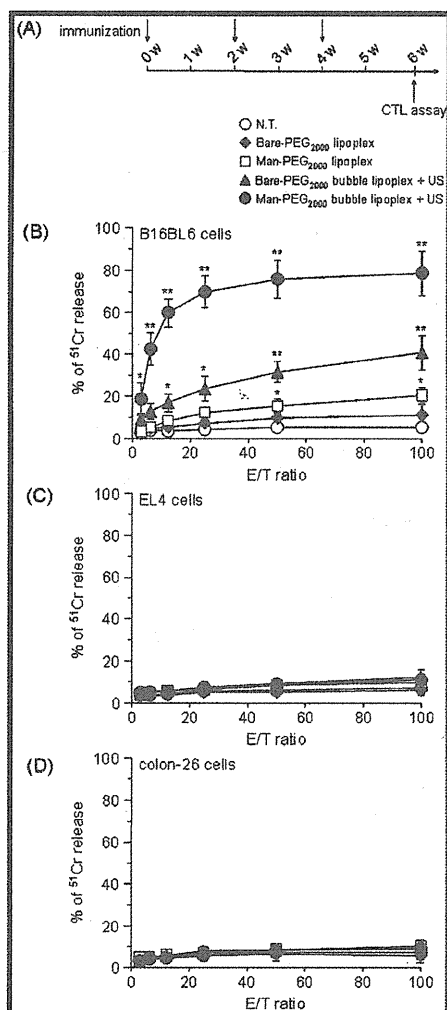


Figure 3. Evaluation of melanoma-specific CTL activities by DNA-vaccination using Man-PEG₂₀₀₀ bubble lipoplexes and US exposure. (A) Schedule of immunization for the assay of melanoma-specific CTL activities. (B–D) Each cancer cell lysate-specific CTL activities after immunization three times with Bare-PEG₂₀₀₀ lipoplexes, Man-PEG₂₀₀₀ lipoplexes, Bare-PEG₂₀₀₀ bubble lipoplexes with US exposure and Man-PEG₂₀₀₀ bubble lipoplexes with US exposure (50 μ g of pDNA). The splenic cells were collected at 2 weeks after the last immunization, and then, the splenic cells were cocultured with ⁵¹Cr-labeled cancer cells. CTL activities against B16BL6 cells (B), EL4 cells (C) and colon-26 cells (D) in the immunized splenic cells were determined by ⁵¹Cr release assay. Each value represents the mean \pm SD ($n = 4$). * $p < 0.05$; ** $p < 0.01$, compared with the corresponding "N.T." (no treatment) group.

TRP-2 expression in each tumor used in this study, and confirmed that the expression of gp100 and TRP-2 was only detected in B16BL6 tumor (Supplementary Figure 2 in the Supporting Information). Following investigation of cancer vaccine effects against solid tumors according to the protocol shown in Figure 4A, B16BL6-transplanted tumor growth was significantly suppressed in mice immunized with Man-PEG₂₀₀₀ bubble lipoplexes constructed with pUb-M and US exposure (Figures 4B and 4D). Moreover, the survival of B16BL6-transplanted mice was significantly prolonged by DNA vaccination using Man-PEG₂₀₀₀ bubble lipoplexes constructed with pUb-M and US exposure, and complete tumor-rejection was observed in 7/10 of

B16BL6-transplanted mice (Figure 4C). These vaccine effects were obtained against B16F1-transplanted mice (Supplementary Figure 4B in the Supporting Information); on the other hand, no cancer vaccine effects against EL4 and colon-26 cell-derived tumors, which do not express gp100 and TRP-2, were observed in all groups (Figures 4B–D). In addition, these DNA vaccine effects against B16BL6-derived tumors were not observed in mice immunized by Man-PEG₂₀₀₀ bubble lipoplexes constructed with pcDNA3.1 (control vector) and US exposure (Supplementary Figure 3 in the Supporting Information), suggesting that DNA vaccine effects against melanoma are attributed to not pDNA transfer itself but melanoma-related antigens expressed by pUb-M.

Then, we investigated the cancer vaccine effects against a pulmonary metastatic tumor obtained by DNA vaccination using Man-PEG₂₀₀₀ bubble lipoplexes and US exposure. Following experiments according to the protocol shown in Figure 5A, the level of luciferase expression derived from B16BL6/Luc cells in the lung, which express gp100 and TRP-2 (Supplementary Figures 1 and 2 in the Supporting Information), was significantly suppressed in mice immunized by Man-PEG₂₀₀₀ bubble lipoplexes and US exposure (Figures 5B and 5D). Moreover, the survival of the pulmonary metastatic tumor model mice constructed with B16BL6 cells was significantly prolonged by DNA vaccination using Man-PEG₂₀₀₀ bubble lipoplexes and US exposure (Figure 5C). These vaccine effects were obtained against pulmonary metastatic B16F1-derived tumor model mice (Supplementary Figure 4C in the Supporting Information); on the other hand, no therapeutic effects against colon-26 cells by DNA vaccination using this method were observed in any of the groups (Figures 5B–D).

Effect of Administration Routes of Man-PEG₂₀₀₀ Bubble Lipoplexes on Cancer Vaccine Effects. Next, we evaluated the effects of the administration routes of Man-PEG₂₀₀₀ bubble lipoplexes to obtain effective DNA vaccine effects. In this experiment, in addition to pUb-M transfer using intravenous administration of Man-PEG₂₀₀₀ bubble lipoplexes and external US exposure, we investigated the DNA vaccine effects by pUb-M transfer using intradermal and intrasplenic administration of Man-PEG₂₀₀₀ bubble lipoplexes and direct US exposure to the administration sites. In the preliminary experiments about US intensity for obtaining the highest gene expression in the spleen and skin, the optimized intensities of US exposure to the abdominal area by a probe of diameter 20 mm and to the injected sites directly by a probe of diameter 6 mm are 1.0 W/cm² and 4.0 W/cm², respectively (data not shown). Based on these investigations, we used the different US intensity depending on the probe size and US-exposed sites in this study. Following immunization against melanoma according to the protocol shown in Figure 6A, B16BL6-transplanted tumor growth was suppressed the best in mice transfected with pUb-M using intravenous injection of Man-PEG₂₀₀₀ bubble lipoplexes and external US exposure (Figure 6B). Moreover, the survival of B16BL6-transplanted mice was also prolonged the best by DNA vaccination using intravenous injection of Man-PEG₂₀₀₀ bubble lipoplexes and external US exposure (Figure 6C).

Duration of DNA Vaccine Effects by Man-PEG₂₀₀₀ Bubble Lipoplexes and US Exposure. Finally, to investigate the duration of DNA vaccine effects following pUb-M transfer using Man-PEG₂₀₀₀ bubble lipoplexes and US exposure, B16BL6 cells were retransplanted in mice in which first-transplanted tumors derived from B16BL6 cells were completely rejected by DNA

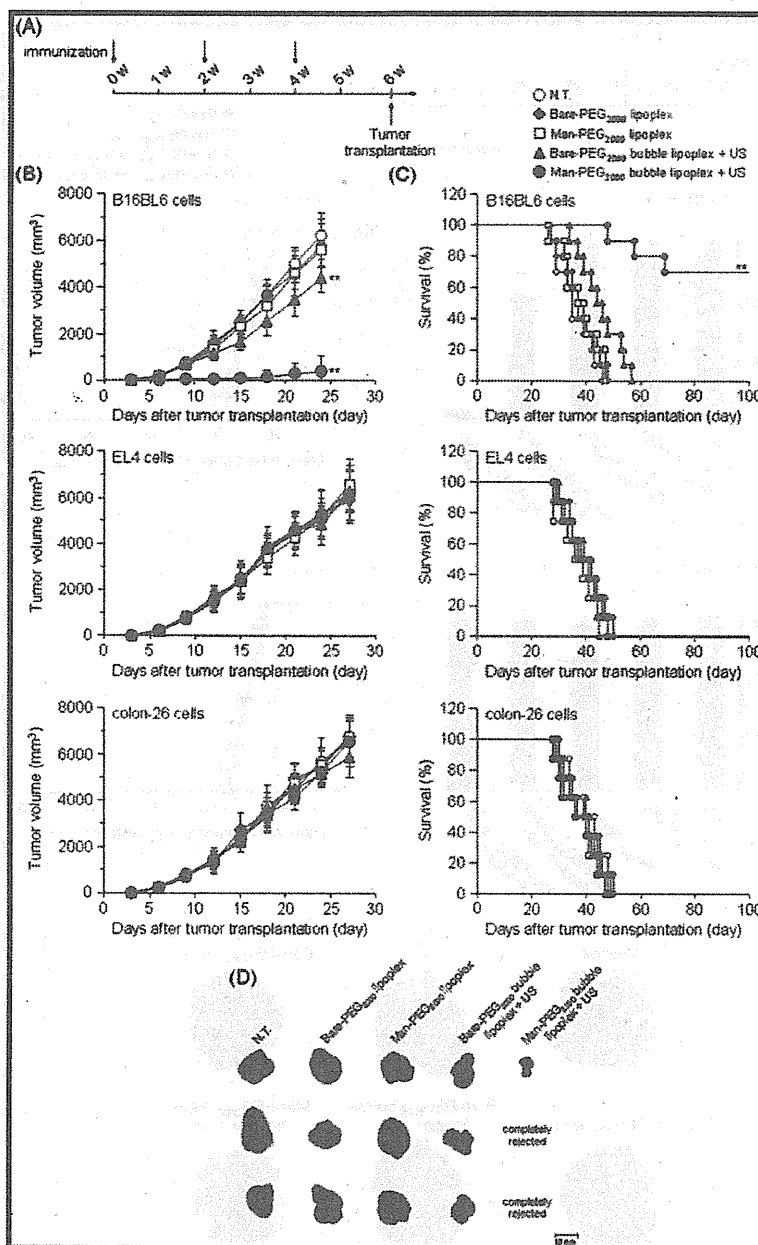


Figure 4. Cancer vaccine effects against solid tumors by DNA vaccination using Man-PEG₂₀₀₀ bubble lipoplexes and US exposure. (A) Schedule of therapeutic experiments on solid tumors. (B, C) The suppressing effects of tumor growth against solid tumors (B) and the prolonging effects of survival in tumor-transplanted mice (C) by DNA vaccination using Bare-PEG₂₀₀₀ lipoplexes, Man-PEG₂₀₀₀ lipoplexes, Bare-PEG₂₀₀₀ bubble lipoplexes with US exposure and Man-PEG₂₀₀₀ bubble lipoplexes with US exposure (50 μ g of pDNA). Two weeks after the last immunization, B16BL6 cells, EL4 cells and colon-26 cells (1×10^6 cells) were transplanted subcutaneously into the back of mice ($n = 8-10$). The tumor volume was evaluated (each value represents the mean \pm SD), and the survival was monitored up to 100 days after the tumor transplantation. ** $p < 0.01$, compared with the corresponding "N.T." (no treatment) group. (D) Photograph of a B16BL6 cell-derived solid tumor at 15 days after the tumor transplantation in mice immunized by each transfection method ($n = 3$).

vaccination using Man-PEG₂₀₀₀ bubble lipoplexes and US exposure at 100 days after the first transplantation (Figure 7A). As shown in Figure 7B, compared with N.T. mice, the second-transplanted tumor growth derived from B16BL6 cells was markedly suppressed and the survival of B16BL6-transplanted mice was significantly prolonged. In addition, we also evaluated the duration of DNA vaccine effects against a pulmonary metastatic tumor. Following intravenous injection of B16BL6/Luc cells into

mice at 100 days after the last immunization (Figure 7C), the level of luciferase expression derived from B16BL6/Luc cells in the lung was significantly suppressed and the survival of pulmonary metastatic tumor model mice constructed with B16BL6 cells was significantly prolonged in mice transfected with pUb-M using Man-PEG₂₀₀₀ bubble lipoplexes and US exposure (Figure 7D). These results suggest that DNA vaccine effects by pUb-M transfer using Man-PEG₂₀₀₀ bubble lipoplexes and US exposure

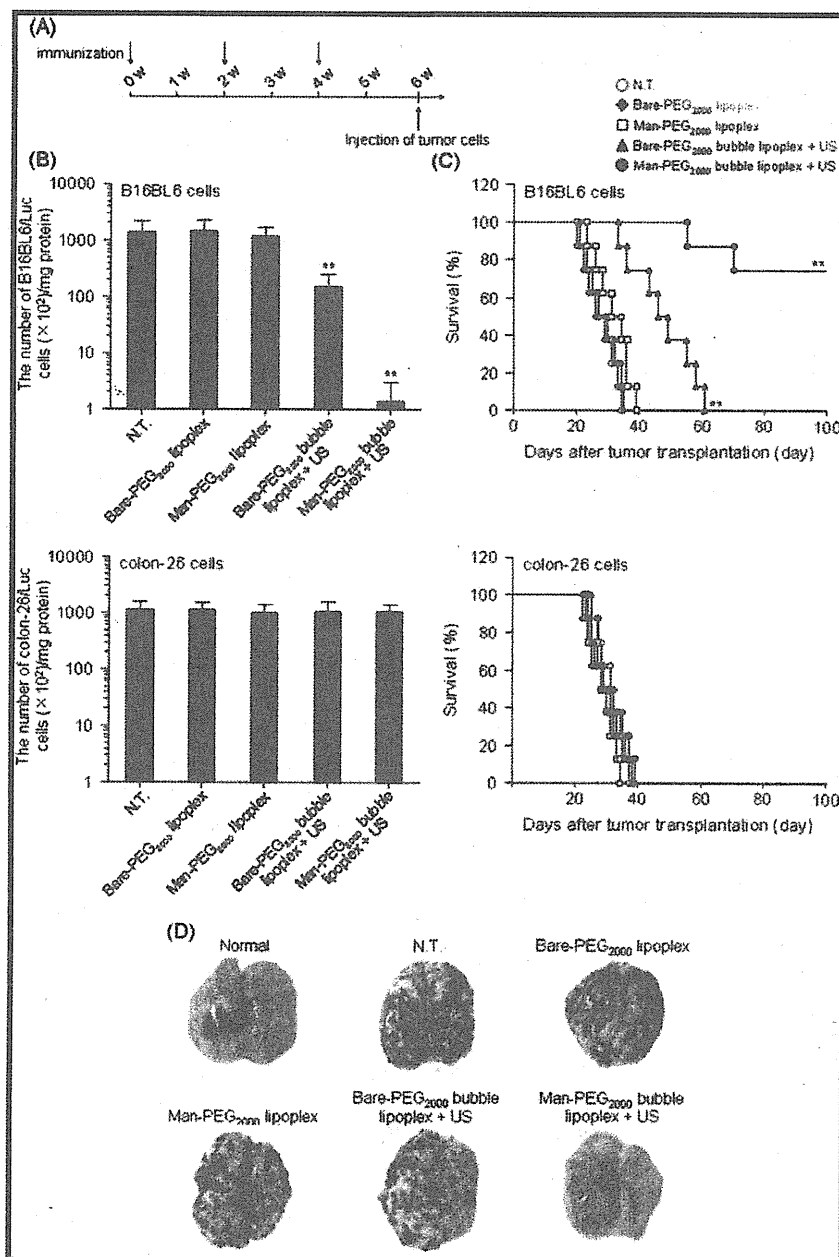


Figure 5. Cancer vaccine effects against pulmonary metastatic tumors by DNA vaccination using Man-PEG₂₀₀₀ bubble lipoplexes and US exposure. (A) Schedule of therapeutic experiments involving pulmonary metastatic tumors. (B, C) The suppressing effects of pulmonary metastatic tumors (B) and the prolonging of survival (C) by DNA vaccination using Bare-PEG₂₀₀₀ lipoplexes, Man-PEG₂₀₀₀ lipoplexes, Bare-PEG₂₀₀₀ bubble lipoplexes with US exposure and Man-PEG₂₀₀₀ bubble lipoplexes with US exposure (50 μ g of pDNA). Two weeks after the last immunization, B16BL6/Luc and colon-26/Luc cells (for the evaluation of tumor metastasis) and B16BL6 and colon-26 cells (for the evaluation of survival) were injected intravenously (1×10^5 cells) into mice. The pulmonary metastatic tumors at 14 days after the tumor injection were evaluated by the luciferase activity ($n = 5$, each value represents the mean \pm SD), and the survival was monitored up to 100 days after the tumor injection ($n = 8$). ** $p < 0.01$, compared with the corresponding "N.T." (no treatment) group. (D) Photograph of a B16BL6-derived pulmonary metastatic tumor at 14 days after the tumor injection in mice immunized by each transfection method.

were sustained for at least 100 days against both solid and metastatic tumors.

DISCUSSION

The prognosis is poor for patients with melanoma, who exhibit a high rate of metastasis and relapse; therefore, the development

of therapy for suppressing this melanoma metastasis and relapse is required.^{2,3} It has been reported that DNA vaccination is effective for the prevention of metastasis and relapse,^{5,7,8} and especially the application of DNA vaccination against melanoma has been focused since the identification of cancer antigens such as gp100, MART-1 and TRP is proceeding in melanoma.^{10–13}

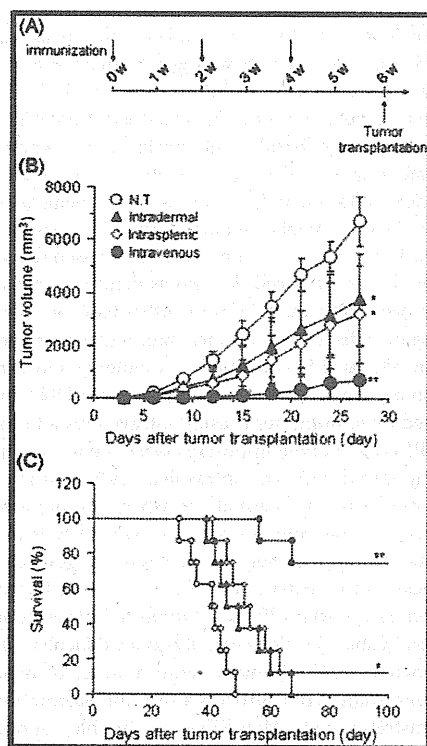


Figure 6. Effects of administration routes of Man-PEG₂₀₀₀ bubble lipoplexes on DNA vaccine effects. (A) Schedule of therapeutic experiments. (B, C) The suppressing effects of tumor growth against solid tumors (B) and the prolonging of survival in tumor-transplanted mice (C) by DNA vaccination using various administration routes of Man-PEG₂₀₀₀ bubble lipoplexes (50 μ g of pDNA) and US exposure. Man-PEG₂₀₀₀ bubble lipoplexes were given by intradermal, intrasplenic and intravenous administration into mice, and US was exposed to the injected site directly or to the abdominal area externally. Two weeks after the last immunization, B16BL6 cells (1×10^6 cells) were transplanted subcutaneously into the back of mice ($n = 8$). The tumor volume was evaluated (each value represents the mean \pm SD), and the survival was monitored up to 100 days after the tumor transplantation. * $p < 0.05$; ** $p < 0.01$, compared with the corresponding "N.T." (no treatment) group.

On the other hand, it is essential to transfer effectively into APCs such as dendritic cells to obtain potent therapeutic effects by DNA vaccination.^{14,15} In the present study, we applied an APC-selective and -efficient gene transfection method using Man-PEG₂₀₀₀ bubble lipoplexes constructed with gp100 and TRP-2-encoding pDNA and US exposure to DNA vaccination against melanoma with metastatic and relapsed properties.

The delivery of antigen-encoding gene into the dendritic cells, known as a major target cells for cancer immunotherapy, is necessary to achieve potent therapeutic effects with DNA vaccination.^{14,15} However, it seems that the number of dendritic cells distributed in organs, such as spleen and skin, is low for DNA vaccination.³⁸ Moreover, gene transfection efficiency in dendritic cells is low,²⁰ because dendritic cells are poorly dividing cells^{39,40} and immune effector cells are highly sensitive to cationic lipids.⁴¹ To overcome these obstacles, gene transfection methods using external physical stimulation, such as electroporation, hydrodynamic injection and sonoporation, have been investigated for cancer vaccination.^{21–25} In particular, sonoporation methods using microbubbles and US exposure are expected to be suitable

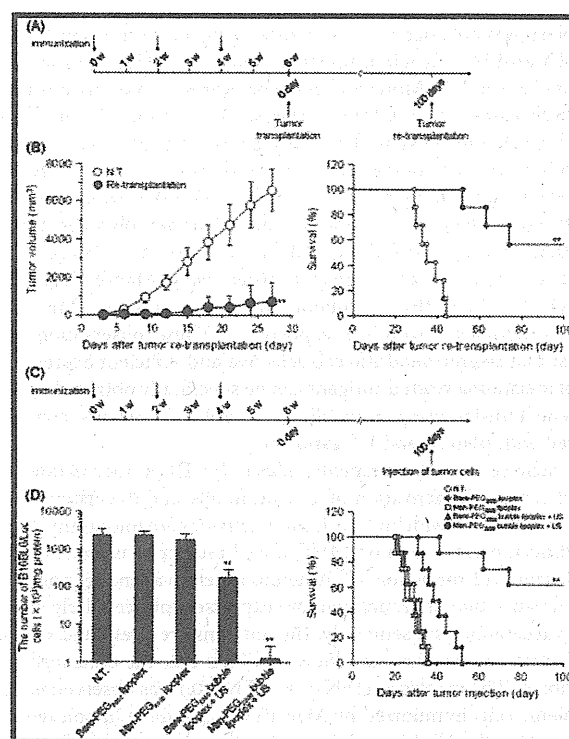


Figure 7. Duration of DNA vaccine effects by Man-PEG₂₀₀₀ bubble lipoplexes and US exposure. (A) Schedule of therapeutic experiments against solid tumors. At 100 days after first transplantation of B16BL6 cells into mice immunized three times by Man-PEG₂₀₀₀ bubble lipoplexes and US exposure, B16BL6 cells (1×10^6 cells) were retransplanted subcutaneously into the back of mice who completely rejected the first transplanted tumors ($n = 7$). (B) The suppressing effects of tumor growth against solid tumors and the prolonging of survival in tumor-transplanted mice by DNA vaccination using Man-PEG₂₀₀₀ bubble lipoplexes and US exposure (50 μ g of pDNA). The tumor volume was evaluated (each value represents the mean \pm SD), and the survival was monitored up to 100 days after the tumor retransplantation. (C) Schedule of therapeutic experiments against metastatic tumors. At 100 days after the last immunization using Bare-PEG₂₀₀₀ lipoplexes, Man-PEG₂₀₀₀ lipoplexes, Bare-PEG₂₀₀₀ bubble lipoplexes with US exposure and Man-PEG₂₀₀₀ bubble lipoplexes with US exposure (50 μ g of pDNA), B16BL6 cells (for the evaluation of tumor metastasis) and B16BL6 cells (for the evaluation of survival days) were injected intravenously (1×10^5 cells) into mice. (D) The suppressing effects of B16BL6 cell-derived pulmonary metastatic tumors and the prolonging of survival by DNA vaccination using Bare-PEG₂₀₀₀ lipoplexes, Man-PEG₂₀₀₀ lipoplexes, Bare-PEG₂₀₀₀ bubble lipoplexes with US exposure and Man-PEG₂₀₀₀ bubble lipoplexes with US exposure (50 μ g of pDNA). The pulmonary metastatic tumors at 14 days after the injection of B16BL6 cells were evaluated by the luciferase activity ($n = 4$), and the survival was monitored up to 100 days ($n = 8$). ** $p < 0.01$, compared with the corresponding "N.T." (no treatment) group.

as a gene transfection method for DNA vaccination in clinical situation, because microbubbles and US exposure systems have been used for diagnostic imaging^{42,43} and calculus fragmentation^{44,45} in clinical situation. We have developed a gene transfection method using Man-PEG₂₀₀₀ bubble lipoplexes and US exposure, and succeeded in obtaining APC-selective and -efficient gene expression following experiments using luciferase-encoding pDNA.³³ In this study using pUB-M which expresses melanoma-related antigens (gp100 and TRP-2), a high level of expression in

the spleen was obtained by gene transfer using intravenous injection of Man-PEG₂₀₀₀ bubble lipoplexes and external US exposure (Figures 1A and 1B). Moreover, this gene expression was obtained selectively in the splenic CD11c⁺ cells, known as dendritic cells⁴⁶ (Figure 1C), and these findings corresponded to those in our previous reports of using firefly luciferase-encoding pDNA.³³ In our previous report using pCMV-Luc and pCMV-OVA, we showed that the enhanced gene expression in the spleen was obtained by gene transfer using Man-PEG₂₀₀₀ bubble lipoplexes and US exposure, and not observed in gene transfer using Man-PEG₂₀₀₀ lipoplexes or Man-PEG₂₀₀₀ bubble lipoplexes only and Man-PEG₂₀₀₀ lipoplexes with US exposure.³³ These observations suggest that splenic dendritic cell-selective and -efficient expression of melanoma-related antigens can be specifically obtained by the gene transfer using Man-PEG₂₀₀₀ bubble lipoplexes constructed with pUb-M and US exposure.

To achieve potent therapeutic effects by DNA vaccination against cancer, the activation of Th1 immunity and the effective induction of CTLs with high antitumor activities are important.⁴⁷ The antigen presentation on MHC class I molecules is essential for efficient CTL induction.^{6,8,9} Antigens function as endogenous antigens since the cancer antigens are expressed intracellularly in DNA vaccination; consequently, the antigens are presented on MHC class I molecules.⁸ As shown in Figure 2, the enhanced secretion of Th1 cytokines (IFN- γ and TNF- α) was observed in the splenic cells immunized by Man-PEG₂₀₀₀ bubble lipoplexes constructed with pUb-M and US exposure by addition of B16BL6 cell lysates, compared with that of Th2 cytokines (IL-4 and IL-6). Moreover, the effective induction of CTLs against B16BL6 cells was also observed by DNA vaccination using Man-PEG₂₀₀₀ bubble lipoplexes and US exposure (Figure 3). Recently, we have reported that the antigen presentation on MHC class I molecules was also observed in DNA vaccination using Man-PEG₂₀₀₀ bubble lipoplexes constructed with OVA-encoding pDNA.³³ These results suggest that antigen presentation of melanoma antigens on MHC class I molecules is responsible for the enhanced secretion of Th1 cytokines stimulated by B16BL6 cell and the induction of CTLs against B16BL6 cells in this study. As shown in Figure 4, the growth of B16BL6 cell-derived tumors was suppressed and the survival of B16BL6 cell-transplanted mice was prolonged by DNA vaccination using Man-PEG₂₀₀₀ bubble lipoplexes and US exposure. Since the prognosis of patients with melanoma is poor, because of the high metastatic properties of melanoma as mentioned above,^{2,3} we also investigated the vaccine effects against metastatic melanoma by DNA vaccination using this method. As shown in Figure 5, B16BL6 cell-derived pulmonary metastasis constructed by intravenous injection of tumor cells was suppressed by DNA vaccination using this gene transfection method. These DNA vaccine effects followed by Man-PEG₂₀₀₀ bubble lipoplexes constructed with pUb-M and US exposure were obtained against not only B16BL6 cells but also B16F1-derived tumors (Supplementary Figure 4 in the Supporting Information), suggesting that this DNA vaccination might be potent against various types of melanoma. On the other hand, the potent therapeutic effects against B16BL6-derived solid and metastatic tumor transplanted mice were not observed in DNA vaccination using Man-PEG₂₀₀₀ bubble lipoplexes constructed with pUb-M and US exposure (data not shown). These findings suggest that the optimized duration for immunization is essential for obtaining potent antitumor effects by DNA vaccination using Man-PEG₂₀₀₀ bubble lipoplexes and US exposure, and DNA vaccination using Man-PEG₂₀₀₀ bubble lipoplexes and US

exposure may be suitable for the prevention of cancer metastasis and relapse. In addition, these vaccine effects against solid and metastatic tumors were sustained for at least 100 days (Figure 7). These observations lead us to believe that the enhanced secretion of Th1 cytokines and the induction of B16BL6 cell-specific CTLs contribute to the effective and long-term DNA vaccine effects against solid and metastatic tumors, following pUb-M transfer into splenic dendritic cells using Man-PEG₂₀₀₀ bubble lipoplexes and US exposure.

Intradermal and intrasplenic routes are widely used to transfer pDNA into the Langerhans cells known as dendritic cells in the skin or splenic dendritic cells.^{48,49} On the other hand, we obtained potent therapeutic effects by DNA vaccination using intravenous administration of Man-PEG₂₀₀₀ bubble lipoplexes and external US exposure in this study. As shown in Figure 6, the DNA vaccine effects obtained by immunization using intravenous administration of Man-PEG₂₀₀₀ bubble lipoplexes were higher, compared with that using intradermal and intrasplenic administration. In the gene transfer using intradermal/intrasplenic administration of Man-PEG₂₀₀₀ bubble lipoplexes, it is assumed that the diffusibility of Man-PEG₂₀₀₀ bubble lipoplexes is not good and the delivering efficiency to the dendritic cells may be low, because of the large particle size of Man-PEG₂₀₀₀ bubble lipoplexes (approximately 500 nm (Table 1)). Therefore, it may be difficult to deliver the antigen-encoding pDNA into a large number of dendritic cells in the gene transfection process using intradermal/intrasplenic administration of Man-PEG₂₀₀₀ bubble lipoplexes. On the other hand, when Man-PEG₂₀₀₀ bubble lipoplexes were administered intravenously, the antigen-encoding pDNA may be delivered into a large number of dendritic cells widely distributed in the spleen through the blood vessels. Therefore, it is assumed that potent vaccine effects are obtained by gene transfer in the dendritic cells widely distributed in the spleen. These results suggest that the intravenous administration of Man-PEG₂₀₀₀ bubble lipoplexes is suitable to obtain high therapeutic effects by DNA vaccination using Man-PEG₂₀₀₀ bubble lipoplexes and US exposure.

In this study, melanoma-specific vaccine effects were induced by DNA vaccination using Man-PEG₂₀₀₀ bubble lipoplexes and US exposure, and moreover, intravenous administration of Man-PEG₂₀₀₀ bubble lipoplexes was found to be suitable for DNA vaccination using this method in mice (Figure 6). For clinical application to achieve efficient DNA vaccination, Man-PEG₂₀₀₀ bubble lipoplexes need to be delivered to the spleen efficiently at a low dose. Recently, a medical catheter, which possesses a device to inject the microbubbles and to expose US, has been developed for the treatment of thrombolysis in clinical situation.^{44,45} During treatment, this catheter is positioned within the lesion sites via the vessels, and various types of drugs, such as the lytic agents and microbubbles, are infused simultaneously with US exposure. Since this system may enable the local injection of Man-PEG₂₀₀₀ bubble lipoplexes and direct US exposure to the spleen by catheter delivery via the blood vessels, more potent DNA vaccine effects against melanoma are expected to be obtained at a low dose of Man-PEG₂₀₀₀ bubble lipoplexes by applying this catheter-based US system in the future.

CONCLUSION

In the present study, we developed DNA vaccination using Man-PEG₂₀₀₀ bubble lipoplexes constructed with pUb-M encoding ubiquitylated melanoma-specific antigens (gp100 and TRP-2) and US exposure, and succeeded in obtaining potent DNA vaccine effects against solid and metastatic cancers derived from B16BL6

melanoma specifically. Moreover, its vaccine effects against melanoma were sustained for 100 days at least. The findings obtained in this study suggest that the gene transfection method using Man-PEG₂₀₀₀ bubble lipoplexes and US exposure could be suitable for DNA vaccination aimed at the prevention of metastatic and relapsed cancer.

■ ASSOCIATED CONTENT

Supporting Information. Additional figures as discussed in the text. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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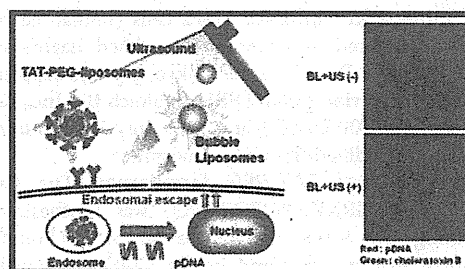
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Bubble Liposomes and Ultrasound Promoted Endosomal Escape of TAT-PEG Liposomes as Gene Delivery Carriers

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ABSTRACT: We have previously developed laminin-derived AG73 peptide-labeled poly(ethylene glycol)-modified liposomes (AG73-PEG liposomes) for selective cancer gene therapy and reported that Bubble liposomes (BLs) and ultrasound (US) exposure could accelerate the endosomal escape of AG73-PEG liposomes, leading to the enhancement of transfection efficiency; however, it is still unclear whether BLs and US exposure can also enhance the transfection efficiency of other vectors. We therefore assessed the effect of BLs and US exposure on the gene transfection efficiency of *trans*-activating transcription factor (TAT) peptide modified PEG liposomes. Although TAT-PEG liposomes were efficiently internalized into cells, the efficacy of endosomal escape was insufficient. The transfection efficiencies of TAT-PEG liposomes were enhanced by about 30-fold when BLs and US exposure were used. We also confirmed that BLs and US exposure could not enhance the direct transportation of TAT-PEG liposomes into cells. Confocal microscopy showed that BLs and US exposure promoted endosomal escape of TAT-PEG liposomes. Our results suggested that BLs and US exposure could enhance transfection efficiency by promoting endosomal escape, which was independent of modified molecules of carriers. Thus, BLs and US exposure can be a useful tool to achieve efficient gene transfection by improving endosomal escape of various carriers.

KEYWORDS: Bubble liposomes, gene delivery, TAT peptide, ultrasound



INTRODUCTION

Successful gene therapy depends on the efficient and safe delivery of genes into the desired tissues and cells. It is therefore necessary to develop efficient delivery vectors or methods for gene therapy. Nonviral vectors, such as cationic lipids or polymers, continue to be an attractive alternative to viral vectors due to their safety and convenient large-scale production, but their relatively low transfection efficiency compared with viral vectors is a major disadvantage.¹ In nonviral gene therapy, high transfection activity is required to improve the rate-limiting steps such as cellular internalization, endosomal escape, nuclear transfer, and intranuclear transcription.^{2,3} In these steps, endosomal escape is considered one of the most important steps. When the vector cannot overcome this process, the cargo is degraded in lysosomes, leading to decreased gene transfection efficiency. For efficient endosomal escape, some studies have developed carriers equipped with functions such as pH sensitivity,^{4,5} temperature dependence,⁶ or photosensitivity.⁷

We have previously developed laminin-derived AG73 peptide-labeled poly(ethylene glycol) modified liposomes (AG73-PEG liposomes) for selective cancer gene therapy.⁸ We also reported that echo-contrast gas-entrapping PEG liposomes, called "Bubble liposomes" (BLs), and ultrasound (US) exposure could accelerate the endosomal escape of AG73-PEG liposomes, leading to

enhanced transfection efficiency.⁹ It is expected that BLs and US exposure may promote the endosomal escape of various carriers and enhance their transfection efficiency; however, it is still unclear whether BLs and US exposure can enhance the transfection efficiency of vectors other than AG73-PEG liposomes, and the effect of BLs and US exposure on the transfection efficiency and endosomal escape of other functional molecule modified gene delivery carriers is not clearly understood.

Cell-penetrating peptides (CPPs), such as TAT and R8 peptides, are able to facilitate penetration through cell membranes and translocate different cargo into cells.¹⁰ The TAT peptide, derived from a human immunodeficiency virus *trans*-acting transcriptional activator, has been studied to achieve highly efficient gene delivery and to develop TAT-modified liposomes and a polyplex.^{11,12} The majority of these carriers were internalized via endocytosis and were required to achieve endosomal escape for efficient gene transfection.¹³ Additionally, TAT-modified carriers equipped with components enhancing endosomal escape have been developed.¹⁴

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We therefore prepared TAT-modified liposomes as a model to evaluate the effect of BLs and US exposure on gene transfection efficiency via endosomal escape.

In this study, to assess the utility of BLs and US exposure for efficient gene delivery in general, we focused on TAT peptide and evaluated the effect of BLs and US exposure on the gene transfection efficiency of TAT-modified liposomes.

EXPERIMENTAL SECTION

Materials. The plasmid pCMV-Luc is an expression vector encoding the firefly luciferase gene under the control of cytomegalovirus promoter. Fluorescein isothiocyanate-conjugated cholera toxin B subunit (FITC-CTB), chloroquine, chlorpromazine and protamine were purchased from Sigma (St. Louis, MO). Cy3-labeled pDNA was purchased from Mirus Bio, LLC (Madison, WI). Genistein was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Amiloride was purchased from Calbiochem (San Diego, CA).

Cell Lines and Cultures. HeLa cells (human cervical cell line) were cultured in Dulbecco's modified Eagle's medium (DMEM; Kohjin Bio Co. Ltd., Tokyo, Japan), supplemented with 10% fetal bovine serum (FBS; Equitech Bio Inc., Kerrville, TX), penicillin (100 U/mL), and streptomycin (100 µg/mL) at 37 °C in a humidified 5% CO₂ atmosphere.

Preparation of TAT-PEG Liposomes. The Cys-TAT peptide (CGG-GRKKRRQRRPPQ) was synthesized manually using the 9-fluorenylmethoxycarbonyl (Fmoc)-based solid-phase strategy, prepared in the COOH-terminal amide form and purified by reverse-phase high performance liquid chromatography. Liposomes were prepared by the hydration method. pDNA diluted in 10 mM HEPES buffer (pH 7.4) was condensed using protamine (*N/P* = 5.0). The complex of pDNA and protamine was added to a lipid film composed of 1,2-dioleoyl-*sn*-glycero-3-phospho-*rac*-1-glycerol (DOPG) (AVANTI Polar Lipids Inc., Alabaster, AL), 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE) (AVANTI Polar Lipids, Inc.), and 1,2-distearoyl-*sn*-glycero-3-phosphatidylethanolamine-polyethyleneglycol-maleimide (DSPE-PEG₂₀₀₀-Mal) (NOF Corporation, Tokyo, Japan) in a molar ratio of 2:9:0.57, followed by incubation for 10 min at room temperature to hydrate the lipids. The solution was sonicated for 5 min in a bath-type sonicator (42 kHz, 100 W) (2510J-DTH; Branson Ultrasonic Co., Danbury, CT). For coupling, TAT peptide, at a molar ratio of 5-fold DSPE-PEG₂₀₀₀-Mal, was added to the PEG liposomes, and the mixture was incubated for 6 h at room temperature to conjugate the cysteine of the Cys-TAT peptide with the maleimide of the PEG liposomes using a thioether bond. The resulting TAT-peptide conjugated PEG liposomes (TAT-PEG liposomes) were dialyzed to remove any excess peptide. TAT-PEG liposomes were modified with 5 mol % PEG and 3 mol % peptides of total lipid. The particle size and ζ-potential of prepared liposomes were measured by NICOMP 380 ZLS (Particle Sizing Systems, Santa Barbara, CA).

Preparation of Bubble Liposomes. PEG liposomes composed of 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) (NOF Corporation) and 1,2-distearoyl-*sn*-glycero-3-phosphatidylethanolamine-poly(ethylene glycol) (DSPE-PEG₂₀₀₀-OMe) (NOF Corporation) in a molar ratio of 94:6 were prepared by the reverse-phase evaporation method. In brief, all reagents were dissolved in 1:1 (v/v) chloroform/diisopropyl ether. Phosphate-buffered saline was added to the lipid solution, and the mixture was sonicated and then evaporated at 47 °C. The organic solvent was completely

removed, and the size of the liposomes was adjusted to less than 200 nm using extruding equipment and a sizing filter (pore size: 200 nm) (Nuclepore Track-Etch Membrane; Whatman Plc, UK). The lipid concentration was measured using a phospholipid C test Wako (Wako Pure Chemical Industries, Ltd., Osaka, Japan). BLs were prepared from liposomes and perfluoropropane gas (Takachio Chemical Ind. Col. Ltd., Tokyo, Japan). First, 2 mL sterilized vials containing 0.8 mL of liposome suspension (lipid concentration: 1 mg/mL) were filled with perfluoropropane gas, capped, and then pressurized with a further 3 mL of perfluoropropane gas. The vial was placed in a bath-type sonicator (42 kHz, 100 W) (2510J-DTH; Branson Ultrasonics Co.) for 5 min to form BLs.

Transfection of pDNA into Cells Using TAT-PEG Liposomes. The day before the experiments, HeLa cells (3×10^4) were seeded in a 48-well plate. The cells were treated with TAT-PEG liposomes (encapsulated pDNA: 3 µg/mL) in serum-free medium for 4 h at 37 °C. After replacement with fresh medium, the cells were cultured for 20 h, and then luciferase activity was measured.

Transfection of pDNA into Cells by Combination of TAT-PEG Liposomes with BLs and US Exposure. The day before the experiments, HeLa cells (3×10^4) were seeded in a 48-well plate. The cells were treated with TAT-PEG liposomes (encapsulated pDNA: 3 µg/mL) in serum-free medium for 4 h at 37 °C. After incubation, the cells were washed twice within 10 min to remove any excess TAT-PEG liposomes that were not associated with the cells, and BLs (120 µg/mL) were added. Within 2 min, US exposure was applied through a 6 mm diameter probe placed in the well (frequency, 2 MHz; duty, 50%; burst rate, 2 Hz; intensity, 1.0 W/cm²; time, 10 s). A Sonopore 3000 (NEPA GENE Co. Ltd., Chiba, Japan) was used to generate the US exposure. The cells were cultured for 20 h; then luciferase activity was determined, and cell viability was measured using a WST-8 assay (Cell Counting Kit-8; Dojindo Laboratories, Kumamoto, Japan).

Measurement of Luciferase Expression. Cell lysate was prepared with lysis buffer (0.1 M Tris-HCl (pH 7.8), 0.1% Triton X-100, and 2 mM EDTA). Luciferase activity was measured using a luciferase assay system (Promega) and a luminometer (LB96 V; Berthold Japan Co. Ltd., Tokyo, Japan). Activity is indicated as relative light units (RLU) per milligrams of protein.

Flow Cytometry Analysis. The day before the experiments, HeLa cells were seeded in a 12-well plate. Then, 0.2 mol % DiI-labeled TAT-PEG liposomes (pDNA: 3 µg/mL) were added to the cells and incubated for 1 h at 37 °C. The cells were collected, and the fluorescence intensities were measured by flow cytometry (FACSCanto; BD Biosciences, Franklin Lakes, NJ) to evaluate the cellular association of liposomes.

To examine the effect of BLs and US exposure on cellular uptake of pDNA, TAT-PEG liposomes (encapsulating Cy3-labeled pDNA: 3 µg/mL) were added to cells and incubated for 4 h at 37 °C. After incubation, the cells were washed twice, and BLs (120 µg/mL) were added. Then, US exposure was applied (frequency, 2 MHz; duty, 50%; burst rate, 2 Hz; intensity, 1.0 W/cm²; time, 10 s). Subsequently, the cells were incubated for 10 or 60 min, and then the cells were collected by trypsinization and washed with PBS supplemented with heparin (50 µg/mL) three times to remove TAT-PEG liposomes and pDNA bound to the cell surface. The fluorescence intensity was measured by flow cytometry.

Confocal Laser Scanning Microscopy (CLSM). HeLa cells were seeded a day before the experiments. HeLa cells were

treated with TAT-PEG liposomes (Cy3-labeled pDNA: 3 $\mu\text{g}/\text{mL}$) and FITC-CTB (10 $\mu\text{g}/\text{mL}$) for 1 h at 37 $^{\circ}\text{C}$. After incubation, the cells were washed, and BLs (120 $\mu\text{g}/\text{mL}$) were added. US exposure was then applied (frequency, 2028 kHz; duty, 50%; burst rate, 2.0 Hz; intensity, 1.0 W/cm^2 ; time, 10 s). Subsequently, the cells were incubated for 10, 60, or 180 min and then fixed with 4% paraformaldehyde for 1 h at 4 $^{\circ}\text{C}$. CLSM was then performed (FV1000D; Olympus Corporation, Tokyo, Japan).

RESULTS

Characterization of Prepared TAT-PEG Liposomes. We evaluated the average size and zeta potential of prepared TAT-PEG liposomes, which were about 130 nm with a slight positive charge (Table 1).

Table 1. Characteristics of Prepared Liposomes^a

prepared liposomes	PEG liposomes	TAT-PEG liposomes
particle size (nm)	132.1 \pm 6.5	122.5 \pm 10.5
ζ potential (mV)	3.17 \pm 1.7	7.91 \pm 1.6

^aData are the means and SD of three different determinations.

Cellular Association of TAT-PEG Liposomes. We first confirmed the effect of TAT peptide coating on the cellular association of liposomes and examined the association of TAT-PEG liposomes with HeLa cells. The cells were incubated with DiI-labeled liposomes for 1 h at 37 $^{\circ}\text{C}$, and fluorescence intensity was determined by flow cytometry. The cellular internalization of TAT-PEG liposomes was observed by confocal laser scanning microscopy (CLSM). The cells treated with TAT-PEG liposomes showed increased fluorescence intensities compared with nonlabeled PEG liposomes (Figure 1A). In cells treated with TAT-PEG liposomes, the fluorescence of liposomes was observed in the cytoplasm, whereas it was weak in the cytoplasm of cells treated with nonlabeled PEG liposomes (Figure 1B). Furthermore, we investigated the cellular uptake pathway of TAT-PEG liposomes. Inhibitors that block clathrin-mediated endocytosis, raft-dependent endocytosis, and macropinocytosis were used to determine the cellular uptake pathway of TAT-PEG liposomes. Clathrin-mediated endocytosis was inhibited by chlorpromazine, which prevents the assembly of coated pits at the plasma membrane.¹⁵ Raft-dependent endocytosis was inhibited by genistein, which is a tyrosine

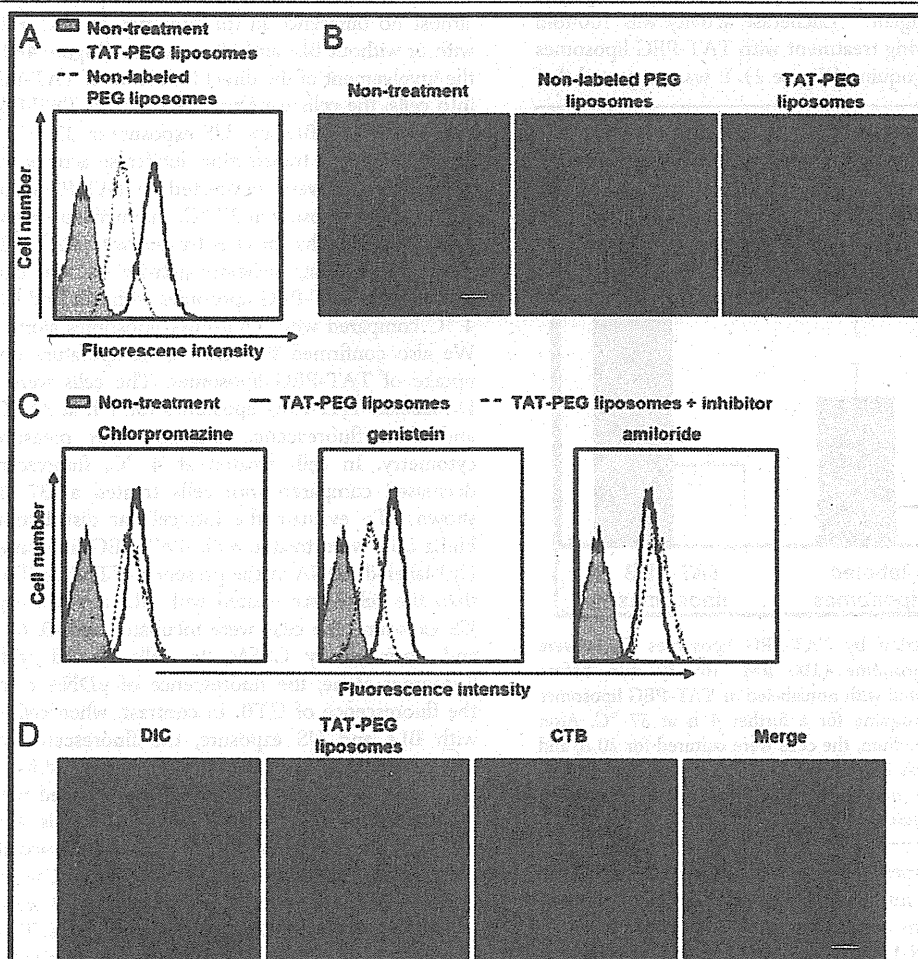


Figure 1. Cellular association of TAT-PEG liposomes. (A, B) HeLa cells were treated with DiI-labeled nonlabeled or TAT-PEG liposomes for 1 h at 37 $^{\circ}\text{C}$. (A) The fluorescence intensity was measured by flow cytometry. (B) Cells were observed by CLSM. The scale bar represents 10 μm . (C) Cells were incubated with chlorpromazine (10 $\mu\text{g}/\text{mL}$), genistein (400 μM), or amiloride (1 mM) for 30 min and then treated with DiI-labeled TAT-PEG liposomes in the presence of an endocytic inhibitor for a further 1 h at 37 $^{\circ}\text{C}$. Fluorescence intensities were measured by flow cytometry. (D) Cells were treated with DiI-labeled nonlabeled or TAT-PEG liposomes in the presence of FITC-CTB (10 $\mu\text{g}/\text{mL}$) for 1 h at 37 $^{\circ}\text{C}$. Cells were observed by CLSM. The scale bar represents 10 μm .

kinase inhibitor.¹⁶ We also used amiloride, a specific inhibitor of the Na^+/H^+ exchange required for macropinocytosis.¹⁷ Flow cytometry analysis showed that the fluorescence intensity of TAT-PEG liposomes in the cells was decreased when cells were treated with genistein. In contrast, the fluorescence intensity of TAT-PEG liposomes in the cells was not changed when cells were treated with chlorpromazine or amiloride (Figure 1C). Furthermore, to elucidate the intracellular localization of TAT-PEG liposomes, the cells were treated with DiI-labeled TAT-PEG liposomes and FITC-cholera toxin B subunit (FITC-CTB), a marker of raft-dependent endocytosis,¹⁸ and then observed by CLSM. As a result, the fluorescence of TAT-PEG liposomes was colocalized with the fluorescence of CTB in cells treated with TAT-PEG liposomes and CTB for 1 h (Figure 1D).

Gene Transfection by TAT-PEG Liposomes. Although TAT-PEG liposomes could be internalized efficiently into cells via raft-dependent endocytosis, it was necessary to achieve high gene expression so that genes in the endosome were delivered into the cytoplasm. To assess the ability of endosomal escape in TAT-PEG liposomes, the cells were treated with TAT-PEG liposomes in the presence of chloroquine, which is recognized as an endosomolytic agent.¹⁹ Luciferase activity was 100-fold higher than that following treatment with TAT-PEG liposomes in the absence of chloroquine (Figure 2). It was suggested that

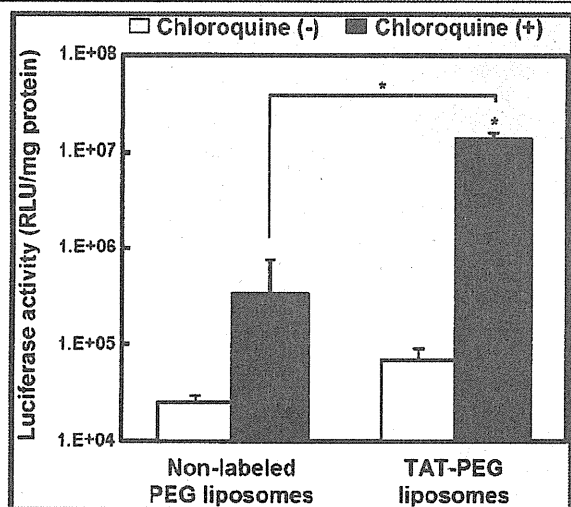


Figure 2. Gene transfection by TAT-PEG liposomes. Cells were preincubated with chloroquine (100 μM) for 30 min before transfection and then treated with nonlabeled or TAT-PEG liposomes in the presence of chloroquine for a further 4 h at 37 °C. After replacement with fresh medium, the cells were cultured for 20 h, and then luciferase activity was determined. Scale bars represent 10 μm . Data are the means \pm SD ($n = 4$). * $p < 0.05$ compared with treatment in the absence of chloroquine.

the TAT-PEG liposomes prepared in this study could be efficiently internalized into cells but might not release genes into the cytoplasm from endosomes.

Effects of BLs and US Exposure on the Transfection Efficiency of TAT-PEG Liposomes. To investigate the effect of BLs and US exposure on TAT-mediated liposomal gene transfection, HeLa cells were treated with TAT-PEG liposomes for 4 h at 37 °C in a serum-free medium, and then the cells were treated with BLs and US exposure. After treatment with TAT-PEG liposomes, luciferase activity was enhanced up to

30-fold by BLs and US exposure compared with TAT-PEG liposomes alone. Furthermore, the combination of TAT-PEG liposomes with BLs and US exposure had about 10-fold higher luciferase activity than nonlabeled PEG liposomes with BLs and US exposure (Figure 3A). We also examined the transfection efficiency by treating TAT-PEG liposomes with US in the absence of BLs. As a result, the transfection efficiency was barely enhanced by treatment with TAT-PEG liposomes with US compared with TAT-PEG liposomes alone (Figure 3B). The cytotoxicity of the combination of TAT-PEG liposomes with or without BLs and US exposure was determined using a WST-8 assay. The cell viability was more than 80% even after each transfection (Figure 3C,D). It was suggested that BLs and US exposure could enhance the transfection efficiency of TAT-PEG liposomes without significant cytotoxicity.

Mechanism of Gene Transfection by TAT-PEG Liposomes with BLs and US Exposure. We examined the effects of BLs and US exposure on the cellular uptake of TAT-PEG liposomes. Flow cytometry analysis was performed to measure the fluorescence intensity of Cy3-labeled pDNA in cells transfected by TAT-PEG liposomes with or without BLs and US exposure. As a result, the cellular uptake of pDNA showed almost no difference in the presence of TAT-PEG liposomes with or without BLs and US exposure (Figure 4A). To evaluate the involvement of the direct induction of TAT-PEG liposomes into cells, the cells were transfected with TAT-PEG liposomes with or without BLs and US exposure at 37 or 4 °C. Twenty-three hours after transfection, luciferase activity was measured. When the cells were transfected by TAT-PEG liposomes with BLs and US exposure at 37 °C, the luciferase activity increased compared with that of cells treated with TAT-PEG liposomes alone. In contrast, luciferase activity did not change in cells treated with TAT-PEG liposomes with BLs and US exposure at 4 °C compared with TAT-PEG liposomes alone (Figure 4B). We also confirmed the effect of temperature on the cellular uptake of TAT-PEG liposomes. The cells were treated with DiI-labeled TAT-PEG liposomes for 1 h at 37 °C or at 4 °C, and then fluorescence intensity was measured by flow cytometry. In cells treated at 4 °C, fluorescence intensity decreased compared with cells treated at 37 °C (data not shown). To evaluate the intracellular distribution of pDNA, HeLa cells were treated with TAT-PEG liposomes containing Cy3-labeled pDNA in the presence of FITC-CTB for 1 h, and then the cells were treated with BLs and US exposure. After US exposure, the cells were incubated for 10, 60, or 180 min and observed by CLSM. In cells treated with TAT-PEG liposomes alone, the fluorescence of pDNA colocalized with the fluorescence of CTB. In contrast, when cells were treated with BLs and US exposure, the fluorescence of CTB was dispersed widely in cells that were incubated for 10 min after US exposure (Figure 4C). We also confirmed the intracellular distribution of pDNA and CTB in living cells. As a result, the distribution of pDNA and CTB in living cells was similar to that of fixed cells (data not shown). Furthermore, we examined the intracellular distribution of pDNA and CTB treated by TAT-PEG liposomes with US in the absence of BLs. The intracellular distribution of pDNA and CTB showed almost no difference between the treatment of TAT-PEG liposomes alone and TAT-PEG liposomes with US exposure without BLs (data not shown).

It was suggested that BLs and US exposure could affect the intracellular trafficking of pDNA and enhance the transfection efficacy of TAT-PEG liposomes.

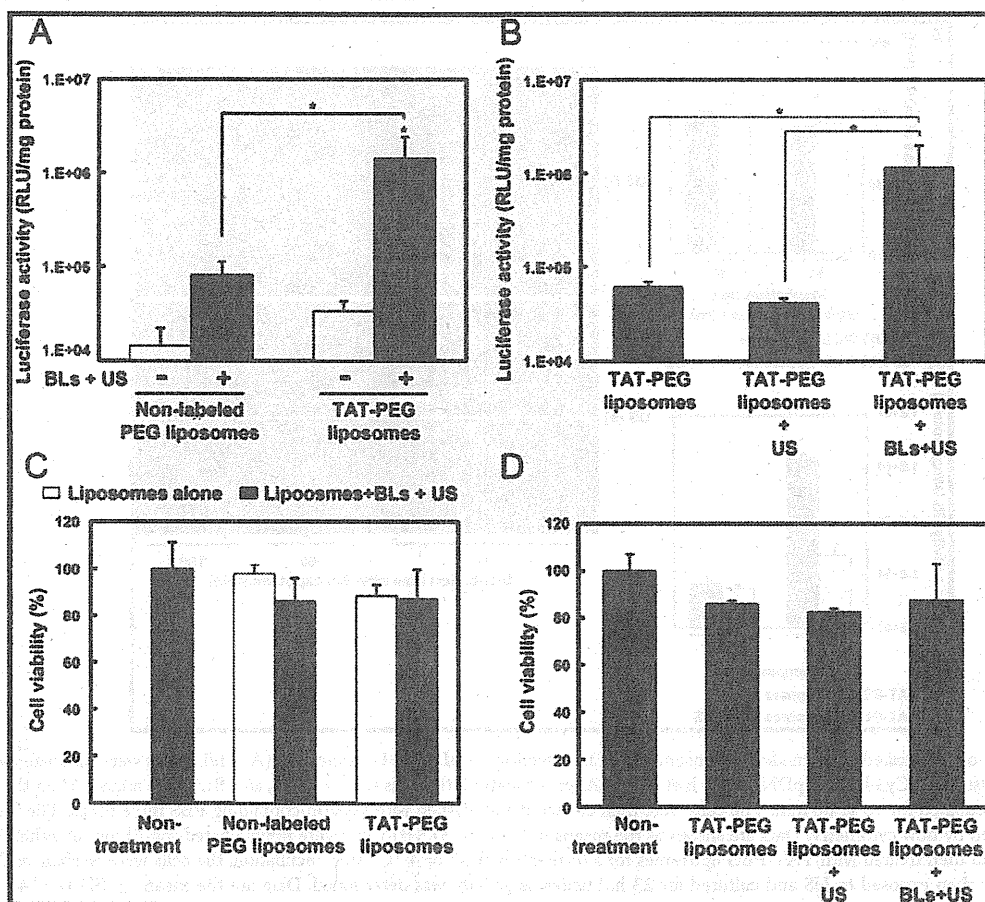


Figure 3. Effect of BLs and US exposure on TAT-mediated liposomal gene transfection. HeLa cells were treated with nonlabeled or TAT-PEG liposomes for 4 h. The cells were then washed and treated with or without BLs (120 μ g/mL) and US exposure. They were incubated for 20 h; then (A, B) luciferase activity was determined, and (C, D) cell viability was measured using a WST-8 assay. Data are the means \pm SD ($n = 4$). * $p < 0.05$ compared with TAT-PEG liposomes alone.

DISCUSSION

Recent studies have suggested that endosomal escape is important to achieve efficient gene delivery.^{2,3} We have previously reported that BLs and US exposure could improve the transfection efficiency of laminin-derived AG73-PEG liposomes containing pDNA by promoting endosomal escape.⁹ In this report, we demonstrated that BLs and US exposure could enhance not only the transfection efficiency of AG73-PEG liposomes but also that of TAT-PEG liposomes.

For efficient gene delivery, various moieties were used to develop carriers which enhance cellular internalization or selectivity. CPPs, such as TAT, R8, or penetratin, were used to achieve efficient gene internalization.^{12,20,21} On the other hand, for selective gene delivery, folate, transferrin, RGD, or anisamide was used as a ligand.^{22–25} These moieties were associated with a specific receptor and internalized via several endocytoses. TAT peptide was associated with heparan sulfate proteoglycan, which has been controversial.²⁶ In addition, some studies have developed TAT peptide-modified carriers, which were equipped with components enhancing endosomal escape;¹⁴ therefore, we focused on TAT peptide and evaluated whether BLs and US exposure can enhance the transfection efficiency of TAT peptide-modified carriers to demonstrate the utility of BLs and US exposure in general. The present results showed that BLs and US exposure could enhance the gene

transfection efficiency of TAT-PEG liposomes (Figure 3A). Furthermore, although we have previously reported that AG73-PEG liposomes were partially internalized via clathrin-mediated endocytosis,⁹ the TAT-PEG liposomes prepared in this study were mostly internalized via a raft-dependent endocytic pathway (Figure 1C). These results suggested that BLs and US exposure could enhance the transfection efficiency of vectors, which were internalized via various receptor and endocytic pathways; however, further studies are needed to evaluate the effect of BLs and US exposure on the transfection efficiency of vectors, which were internalized via various endocytic pathways, such as macropinocytosis.

For successful gene therapy, nonviral vectors could be needed to overcome rate-limiting steps, such as cellular internalization, endosomal escape, and nuclear transfer.^{2,3} Endosomal escape is considered to be one of the most important steps. Although PEG modification was considered a useful component to increase the stability of vectors in vivo, it also inhibited endosomal escape, leading to decreased gene expression.^{27,28} Our results also showed that TAT-PEG liposomes could not escape from endosomes to the cytosol efficiently (Figures 1D, 2). We previously reported that BLs and US exposure could enhance the endosomal escape of AG73-PEG liposomes.⁹ We therefore further confirmed the effect of BLs and US exposure on endosomal escape of TAT-PEG liposomes. We and other

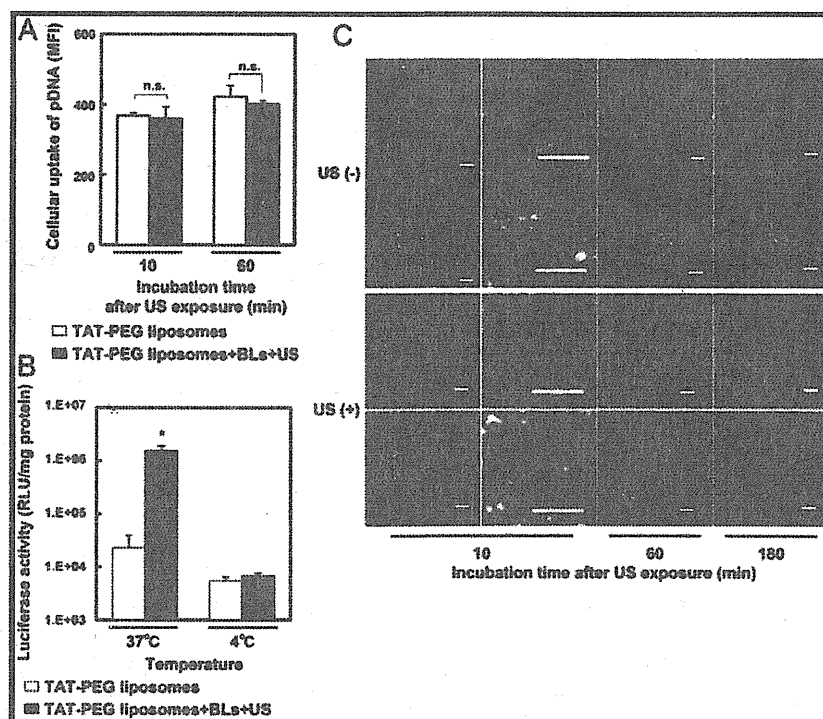


Figure 4. Mechanism of accelerated TAT-mediated liposomal gene transfection by BLs and US exposure. (A) HeLa cells were incubated with TAT-PEG liposomes encapsulating Cy3-labeled pDNA for 4 h at 37 °C. After incubation, the cells were washed, and BLs were added. Then the cells were exposed to US and incubated for 10 or 60 min. The cells were then collected and washed with heparin-containing PBS three times. The fluorescence intensity was measured by flow cytometry. Data are shown as the means \pm SD ($n = 3$). (B) Cells were preincubated for 30 min at either 37 or 4 °C before transfection and then treated with TAT-PEG liposomes for a further 1 h at 37 or 4 °C. After incubation, the cells were washed, and BLs were added. The cells were then exposed to US and cultured for 23 h. Luciferase activity was determined. Data are the means \pm SD ($n = 4$). * $p < 0.05$ compared with TAT-PEG liposomes alone. (C) Cells were treated with TAT-PEG liposomes encapsulating Cy3-labeled pDNA and FITC-CTB (10 $\mu\text{g}/\text{mL}$) for 1 h at 37 °C. After incubation, the cells were washed, and BLs were added. The cells were then exposed to US, incubated for 10, 60, 180 min, and then fixed with 4% paraformaldehyde for 1 h at 4 °C and observed by CLSM. The areas surrounded by dotted line are shown as enlarged images. Scale bars represent 5 μm .

groups have reported that the combination of BLs or microbubbles with US exposure could increase cell membrane permeability and deliver genes into the cytosol directly;^{29–33} however, our results indicated that enhanced transfection efficiency did not rely on the increase of the direct cellular uptake of TAT-PEG liposomes, which is associated with the cell membrane (Figure 4A). In addition, CLSM analysis showed that BLs and US exposure could affect intracellular trafficking of TAT-PEG liposomes. Although endocytic vesicles labeled with FITC-CTB were observed as punctuate structures, when BLs and US exposure was applied, it was observed that FITC-CTB diffused into the cytosol (Figure 4B). These results suggested that BLs and US exposure could accelerate endosomal escape of TAT-PEG liposomes. We also examined whether sonazoid (Daiichi-Sankyo Pharmaceuticals, Tokyo, Japan) and US exposure could enhance the transfection efficiency of TAT-PEG liposomes. Sonazoid consists of perfluorobutane gas microbubbles stabilized by a monolayer membrane of hydrogenated egg phosphatidyl serine.³⁴ As a result, the transfection efficiency of TAT-PEG liposomes was enhanced by sonazoid and US exposure (data not shown). This result suggested that microbubbles and US exposure could enhance the gene transfection efficiency of gene delivery carriers.

We also prepared folate-PEG liposomes containing pDNA and examined whether BLs and US exposure could enhance the transfection efficiency of folate-PEG liposomes. Folate,

a high-affinity ligand for folate receptor, has been widely used as a ligand for selective gene delivery, and folate-modified carriers required various components enhancing endosomal escape to achieve high gene transfection efficiency.³⁵ We confirmed that folate-PEG liposomes had relatively low transfection efficiency because of the lower ability of endosomal escape, but when BLs and US exposure was used with folate-PEG liposomes, the transfection efficiency of folate-PEG liposomes was enhanced (data not shown). These findings also suggested that BLs and US exposure could enhance the endosomal escape of gene delivery vectors, leading to increased gene expression.

We have reported that the transfection efficiency of AG73-PEG liposomes using BLs and US exposure was enhanced 60-fold,⁹ whereas that of TAT-PEG liposomes was up-regulated 12-fold (Figure 3A). These results suggested that BLs and US exposure could easily influence the intracellular trafficking of AG73-PEG liposomes compared to TAT-PEG liposomes. The different efficacy of endosomal escape between AG73-PEG liposomes and TAT-PEG liposomes might be dependent on the difference of the receptor, endocytic pathway of carrier, and the type of cells. The responsibility of BLs and US exposure to individual cells might also affect the efficiency of endosomal escape, leading to different transfection efficiencies; therefore, it is important to clarify the mechanism of the different efficacy of endosomal escape of these carriers. However, we expect that this method of promoting endosomal escape using BLs and US

exposure may also be applied to existing carriers for drug, peptide, or protein delivery, which have low intracellular delivery efficacy due to poor endosomal escape.

In further studies, we will attempt to demonstrate the detailed mechanism of enhanced endosomal escape of carriers by treatment with BLs and US exposure. It has been demonstrated that microbubbles and US exposure induce several biological effects, such as influx of calcium ions or generation of reactive oxygen species.^{36–39} It has been also reported that endosomal acidification is adjusted by calcium ions;⁴⁰ therefore, we will assess whether the influx of calcium ions induced by BLs and US exposure affects endosomal acidification and function, leading to the destabilization of endosomes and enhancement of endosomal escape. On the other hand, we may also need to elucidate more clearly the effect of BLs and US exposure on transcription and other organelles. Although it is possible that BLs and US exposure may induce several biological effects involved in gene expression, BLs and US exposure could affect the intracellular distribution of pDNA and CTB (Figure 4); therefore, our results suggested that BLs and US exposure could certainly improve at least the endosomal escape of TAT-PEG liposomes.

In conclusion, as schematically shown in Figure 5, TAT-PEG liposomes were internalized into cells via HSPG and raft-

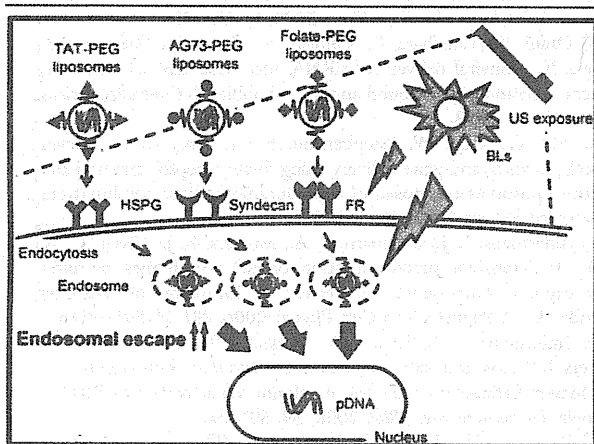


Figure 5. Diagram of enhanced gene delivery by BLs and US exposure. Several moiety-modified gene delivery carriers were internalized into cells via receptors and the endocytic pathway. When BLs and US exposure were applied, endosomal escape was enhanced, leading to increased transfection efficiency, which was independent of the receptor and endocytic pathway of carriers. HSPG, heparan sulfate proteoglycan; FR, folate receptor; US, ultrasound; BLs, Bubble liposomes; PEG, poly(ethylene glycol).

dependent endocytosis. On the other hand, AG73-PEG liposomes and folate-PEG liposomes were internalized via syndecan-2 and folate receptor, respectively. When BLs and US exposure were applied, endosomal escape was enhanced, leading to increased transfection efficiency of these carriers. These results suggested that BLs and US exposure could enhance transfection efficiency by promoting endosomal escape, which was independent of the receptors and endocytic pathway of carriers. Thus, BLs and US exposure can be useful tools to achieve efficient gene transfection by improving endosomal escape using various carriers.

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ABBREVIATIONS

BLs, Bubble liposomes; CTB, cholera toxin B subunit; DOPE, 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine; DOPG, 1,2-dioleoyl-*sn*-glycero-3-phospho-*rac*-1-glycerol; DSPE, 1,2-distearoyl-*sn*-glycero-3-phosphatidyl-ethanolamine; FBS, fetal bovine serum; Fmoc, fluorenylmethoxycarbonyl; Mal, maleimide; pDNA, plasmid DNA; PEG, poly(ethylene glycol); US, ultrasound

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