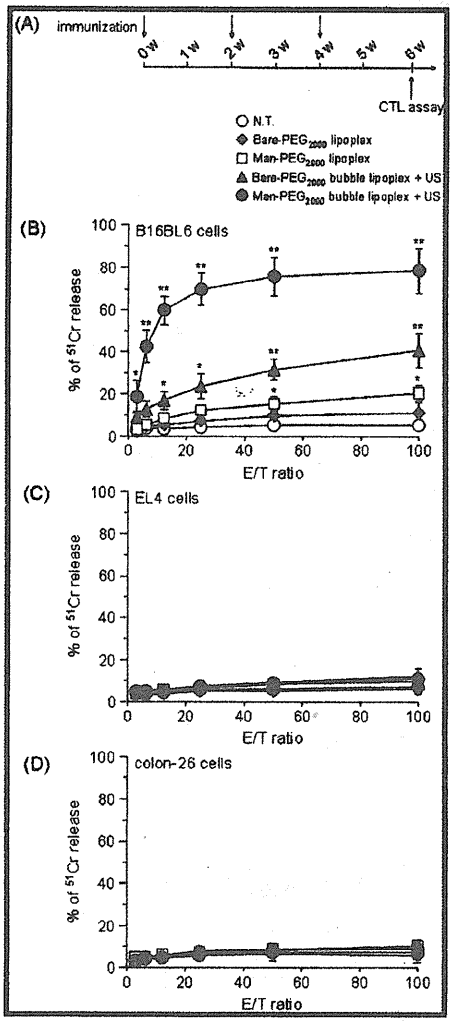

Figure 3. Evaluation of melanoma-specific CTL activities by DNA vaccination using Man-PEG_{2000} bubble lipoplexes and US exposure. (A) Schedule of immunization for the assay of melanoma-specific CTL activities. (B–D) Each cancer cell lysate-specific CTL activities after immunization three times with Bare-PEG_{2000} lipoplexes, Man-PEG_{2000} lipoplexes, Bare-PEG_{2000} bubble lipoplexes with US exposure and Man-PEG_{2000} bubble lipoplexes with US exposure (50 μg of pDNA). The splenic cells were collected at 2 weeks after the last immunization, and then, the splenic cells were coincubated with ^{51}Cr-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 ^{51}Cr release assay. Each value represents the mean ± 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-PEG_{2000} 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-PEG_{2000} 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-PEG_{2000} 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-PEG_{2000} 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-PEG_{2000} 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-PEG_{2000} 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-PEG_{2000} Bubble Lipoplexes on Cancer Vaccine Effects. Next, we evaluated the effects of the administration routes of Man-PEG_{2000} bubble lipoplexes to obtain effective DNA vaccine effects. In this experiment, in addition to pUb-M transfer using intravenous administration of Man-PEG_{2000} bubble lipoplexes and external US exposure, we investigated the DNA vaccine effects by pUb-M transfer using intradermal and intrasplenic administration of Man-PEG_{2000} 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/cm^2 and 4.0 W/cm^2, 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-PEG_{2000} 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-PEG_{2000} bubble lipoplexes and external US exposure (Figure 6C).

Duration of DNA Vaccine Effects by Man-PEG_{2000} Bubble Lipoplexes and US Exposure. Finally, to investigate the duration of DNA vaccine effects following pUb-M transfer using Man-PEG_{2000} 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

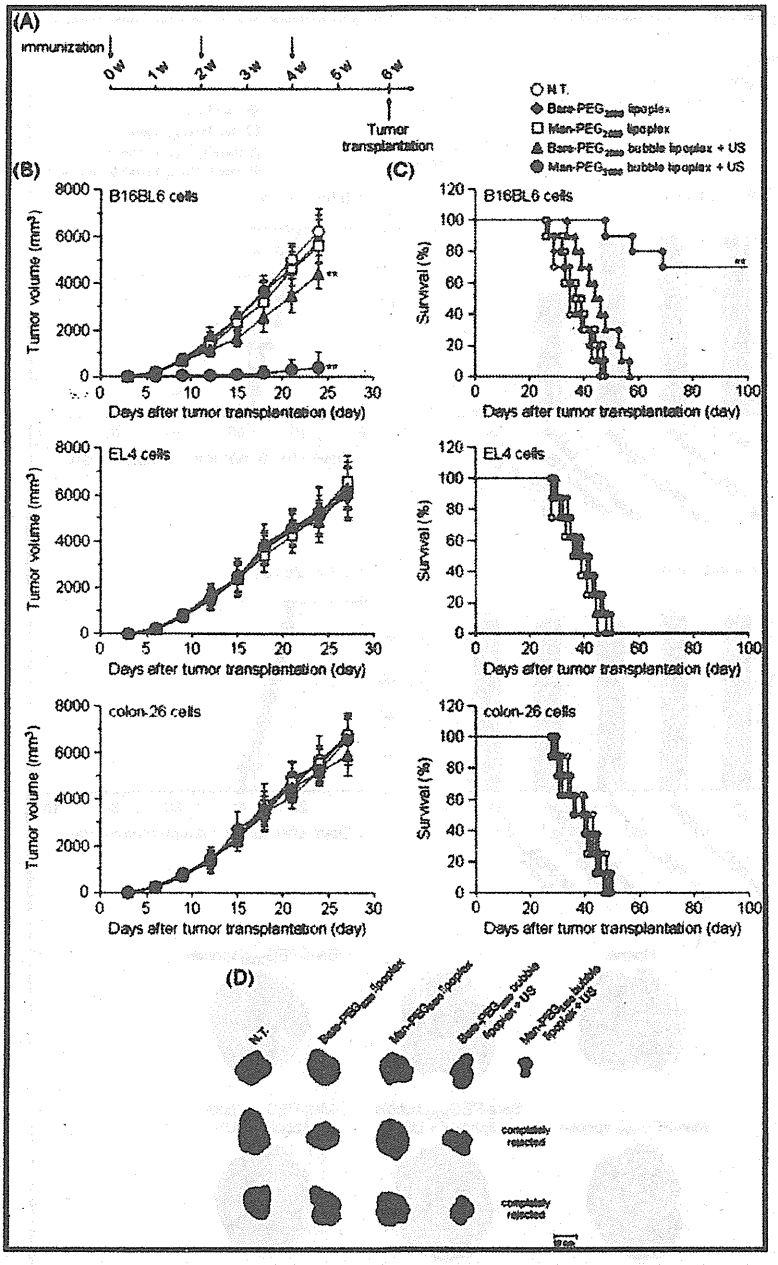

Figure 4. Cancer vaccine effects against solid tumors by DNA vaccination using Man-PEG_{2000} bubble lipoplexes and US exposure. (A) Schedule of therapeutic experiments on solid tumors. (B, C) The suppressing effects of tumor growth against solid tumors (B) and the prolonging effects of survival in tumor-transplanted mice (C) by DNA vaccination using Bare-PEG_{2000} lipoplexes, Man-PEG_{2000} lipoplexes, Bare-PEG_{2000} bubble lipoplexes with US exposure and Man-PEG_{2000} bubble lipoplexes with US exposure (50 μg 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 ± 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-PEG_{2000} 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-PEG_{2000} bubble lipoplexes and US exposure (Figure 7D). These results suggest that DNA vaccine effects by pUb-M transfer using Man-PEG_{2000} bubble lipoplexes and US exposure

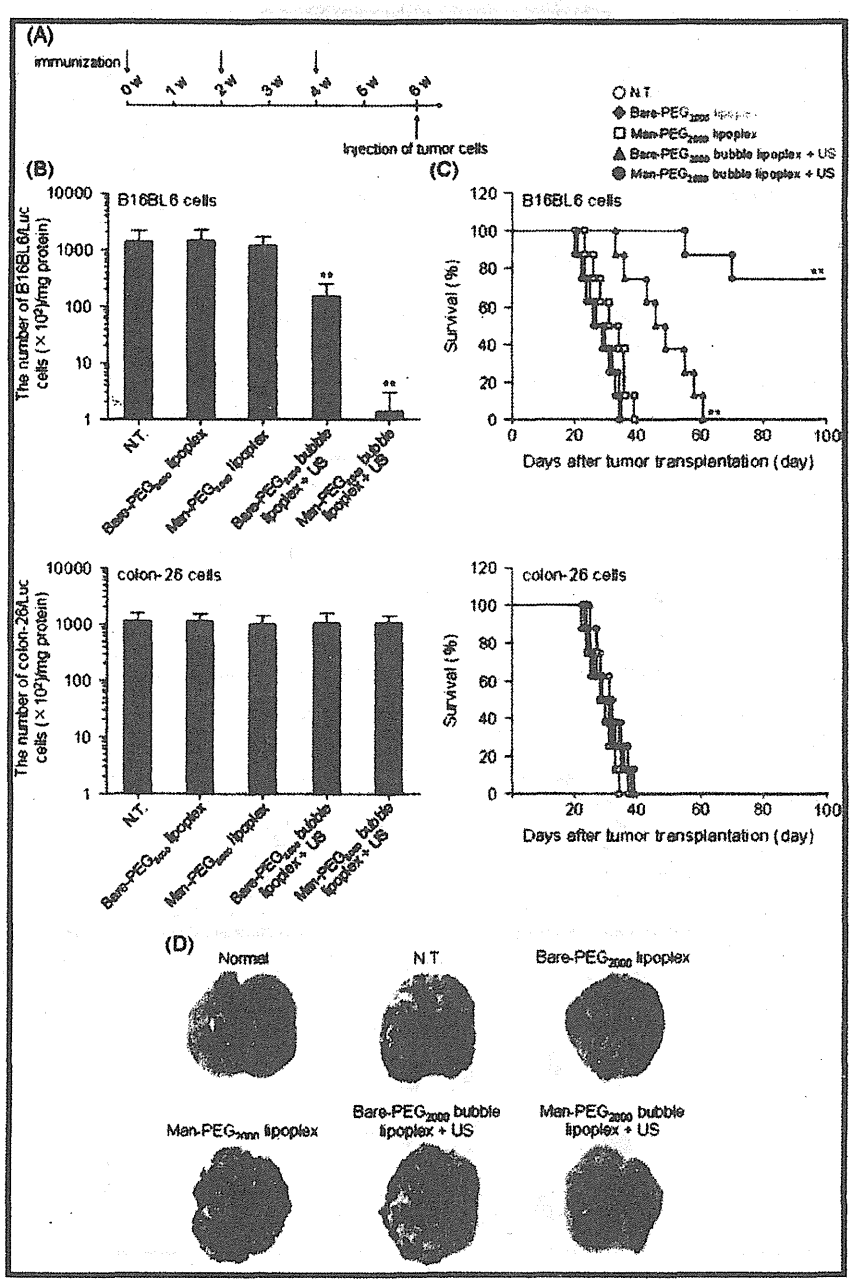

Figure 5. Cancer vaccine effects against pulmonary metastatic tumors by DNA vaccination using Man-PEG$_{2000}$ bubble lipoplexes and US exposure. (A) Schedule of therapeutic experiments involving pulmonary metastatic tumors. (B, C) The suppressing effects of pulmonary metastatic tumors (B) and the prolonging of survival (C) by DNA vaccination using Bare-PEG$_{2000}$ lipoplexes, Man-PEG$_{2000}$ lipoplexes, Bare-PEG$_{2000}$ bubble lipoplexes with US exposure and Man-PEG$_{2000}$ bubble lipoplexes with US exposure (50 μg 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 ± 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]

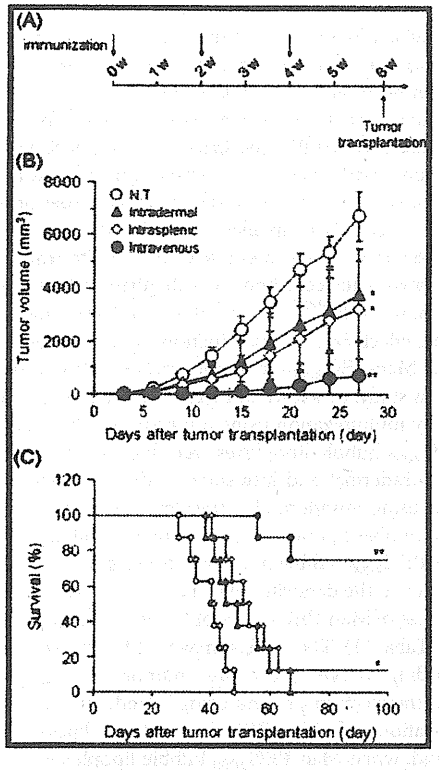

Figure 6. Effects of administration routes of Man-PEG_{2000} bubble lipoplexes on DNA vaccine effects. (A) Schedule of therapeutic experiments. (B, C) The suppressing effects of tumor growth against solid tumors (B) and the prolonging of survival in tumor-transplanted mice (C) by DNA vaccination using various administration routes of Man-PEG_{2000} bubble lipoplexes (50 μg of pDNA) and US exposure. Man-PEG_{2000} 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 ± 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.

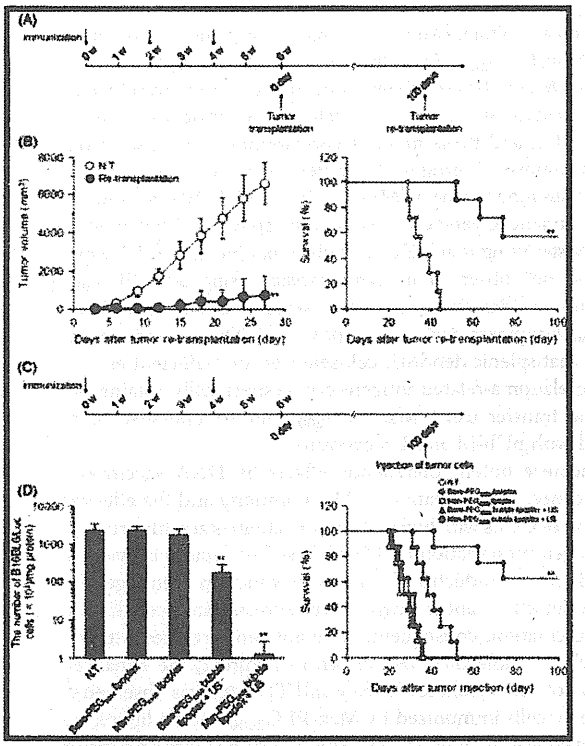

Figure 7. Duration of DNA vaccine effects by Man-PEG_{2000} bubble lipoplexes and US exposure. (A) Schedule of therapeutic experiments against solid tumors. At 100 days after first transplantation of B16BL6 cells into mice immunized three times by Man-PEG_{2000} bubble lipoplexes and US exposure, B16BL6 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-PEG_{2000} bubble lipoplexes and US exposure (50 μg of pDNA). The tumor volume was evaluated (each value represents the mean ± 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-PEG_{2000} lipoplexes, Man-PEG_{2000} lipoplexes, Bare-PEG_{2000} bubble lipoplexes with US exposure and Man-PEG_{2000} bubble lipoplexes with US exposure (50 μg 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-PEG_{2000} lipoplexes, Man-PEG_{2000} lipoplexes, Bare-PEG_{2000} bubble lipoplexes with US exposure and Man-PEG_{2000} bubble lipoplexes with US exposure (50 μg 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.

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-PEG_{2000} 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 electropolation, 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 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-PEG_{2000} 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-PEG_{2000} 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 cells[46] (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-PEG_{2000} bubble lipoplexes and US exposure, and not observed in gene transfer using Man-PEG_{2000} lipoplexes or Man-PEG_{2000} bubble lipoplexes only and Man-PEG_{2000} 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-PEG_{2000} 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-PEG_{2000} 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-PEG_{2000} 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-PEG_{2000} 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-PEG_{2000} 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-PEG_{2000} 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-PEG_{2000} 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-PEG_{2000} bubble lipoplexes and US exposure, and DNA vaccination using Man-PEG_{2000} 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-PEG_{2000} 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-PEG_{2000} 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-PEG_{2000} bubble lipoplexes were higher, compared with that using intradermal and intrasplenic administration. In the gene transfer using intradermal/intrasplenic administration of Man-PEG_{2000} bubble lipoplexes, it is assumed that the diffusibility of Man-PEG_{2000} bubble lipoplexes is not good and the delivering efficiency to the dendritic cells may be low, because of the large particle size of Man-PEG_{2000} 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-PEG_{2000} bubble lipoplexes. On the other hand, when Man-PEG_{2000} 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-PEG_{2000} bubble lipoplexes is suitable to obtain high therapeutic effects by DNA vaccination using Man-PEG_{2000} bubble lipoplexes and US exposure.

In this study, melanoma-specific vaccine effects were induced by DNA vaccination using Man-PEG_{2000} bubble lipoplexes and US exposure, and moreover, intravenous administration of ManPEG_{2000} 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-PEG_{2000} 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-PEG_{2000} 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-PEG_{2000} bubble lipoplexes by applying this catheter-based US system in the future.

CONCLUSION

In the present study, we developed DNA vaccination using Man-PEG_{2000} 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-PEG_{2000} 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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■ ACKNOWLEDGMENT

The authors are very grateful to Prof. R. A. Reisfeld (Department of Immunology, The Scripps Research Institute, USA) for providing pUb-M for this research. This work was supported in part by a Grant-in-Aid for Young Scientists (A) from the Ministry of Education, Culture, Sports, Science and Technology of Japan, and by Health and Labour Sciences Research Grants for Research on Noninvasive and Minimally Invasive Medical Devices from the Ministry of Health, Labour and Welfare of Japan, and by the Programs for Promotion of Fundamental Studies in Health Sciences of the National Institute of Biomedical Innovation (NIBIO), and by the Japan Society for the Promotion of Sciences (JSPS) through a JSPS Research Fellowship for Young Scientists.

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

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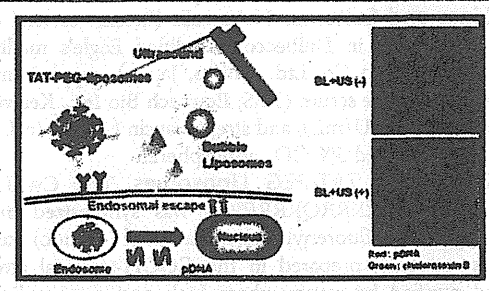
ABSTRACT: We have previously developed laminin-derived AG73 peptide-labeled poly(ethylene glycol)-modified liposomes (AG73-PEG liposomes) for selective cancer gene therapy and reported that Bubble liposomes (BLs) and ultrasound (US) exposure could accelerate the endosomal escape of AG73-PEG liposomes, 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 transcriptor (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]

Received: July 13, 2011
Revised: October 6, 2011
Accepted: October 24, 2011
Published: October 24, 2011

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% CO_2 atmosphere.

Preparation of TAT-PEG Liposomes. The Cys-TAT peptide (CGG-GRKKRRQRRRPPQ) 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-phophatidylethanolamine-polyethyleneglycol-maleimide (DSPE-PEG_{2000}-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; Bransonic Ultrasonic Co., Danbury, CT). For coupling, TAT peptide, at a molar ratio of 5-fold DSPE-PEG_{2000}-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-PEG_{2000}-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/cm^2; 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/cm^2; 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 μg/mL) and FITC-CTB (10 μg/mL) for 1 h at 37 °C. After incubation, the cells were washed, and BLs (120 μg/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 °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 ± 6.5	122.5 ± 10.5
ζ potential (mV)	3.17 ± 1.7	7.91 ± 1.6

[a]Data 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 °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 thyrosine

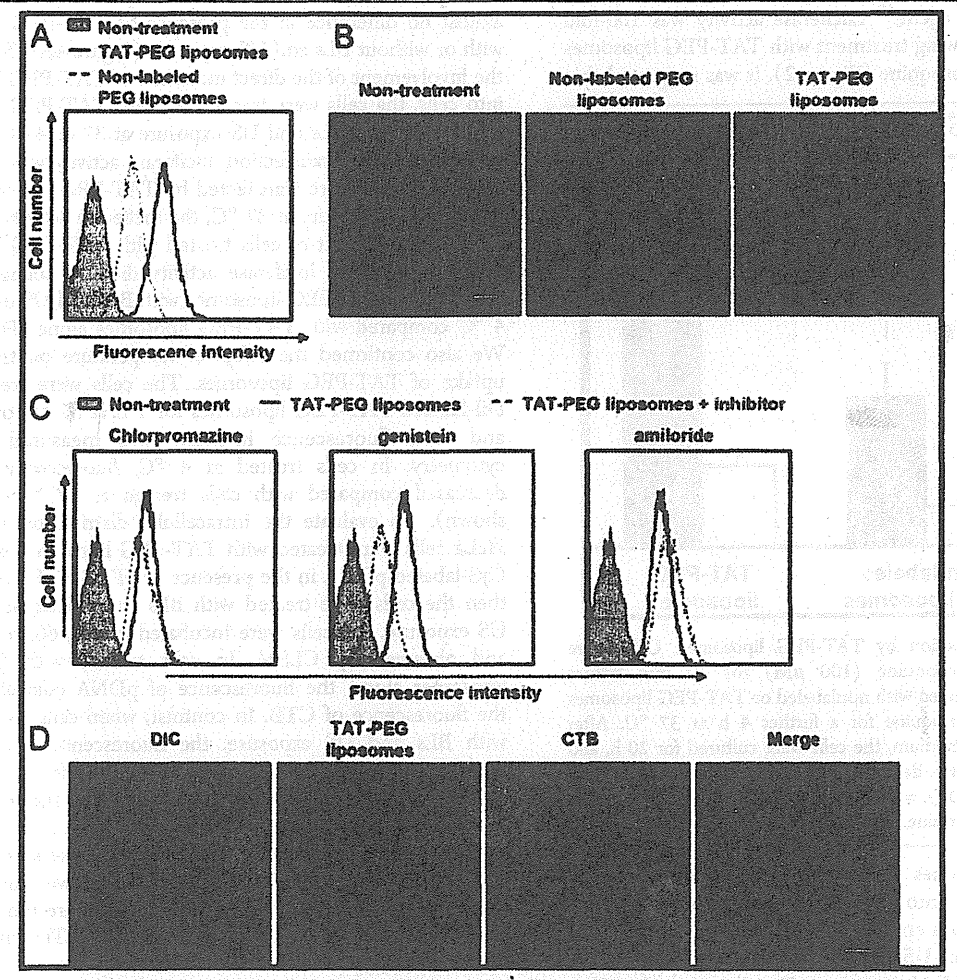


Figure 1. Cellular association of TAT-PEG liposomes. (A, B) HeLa cells were treated with DiI-labeled nonlabeled or TAT-PEG liposomes for 1 h at 37 °C. (A) The fluorescence intensity was measured by flow cytometry. (B) Cells were observed by CLSM. The scale bar represents 10 μm. (C) Cells were incubated with chlorpromazine (10 μg/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 °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 μg/mL) for 1 h at 37 °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

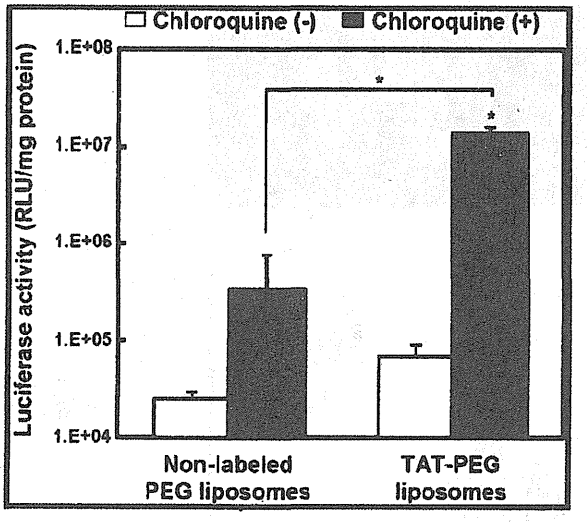


Figure 2. Gene transfection by TAT-PEG liposomes. Cells were preincubated with chloroquine (100 μM) for 30 min before transfection and then treated with nonlabeled or TAT-PEG liposomes in the presence of chloroquine for a further 4 h at 37 °C. After replacement with fresh medium, the cells were cultured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

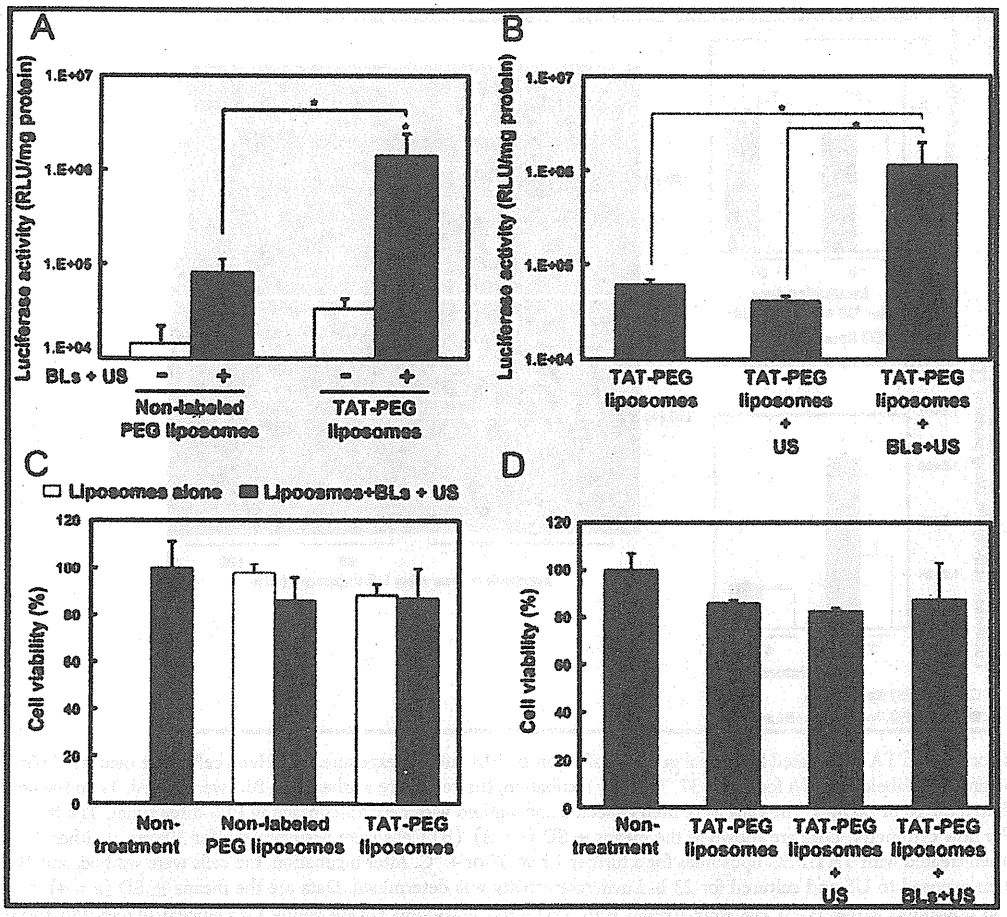


Figure 3. Effect of BLs and US exposure on TAT-mediated liposomal gene transfection. HeLa cells were treated with nonlabeled or TAT-PEG liposomes for 4 h. The cells were then washed and treated with or without BLs (120 μg/mL) and US exposure. They were incubated for 20 h; then (A, B) luciferase activity was determined, and (C, D) cell viability was measured using a WST-8 assay. Data are the means ± 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

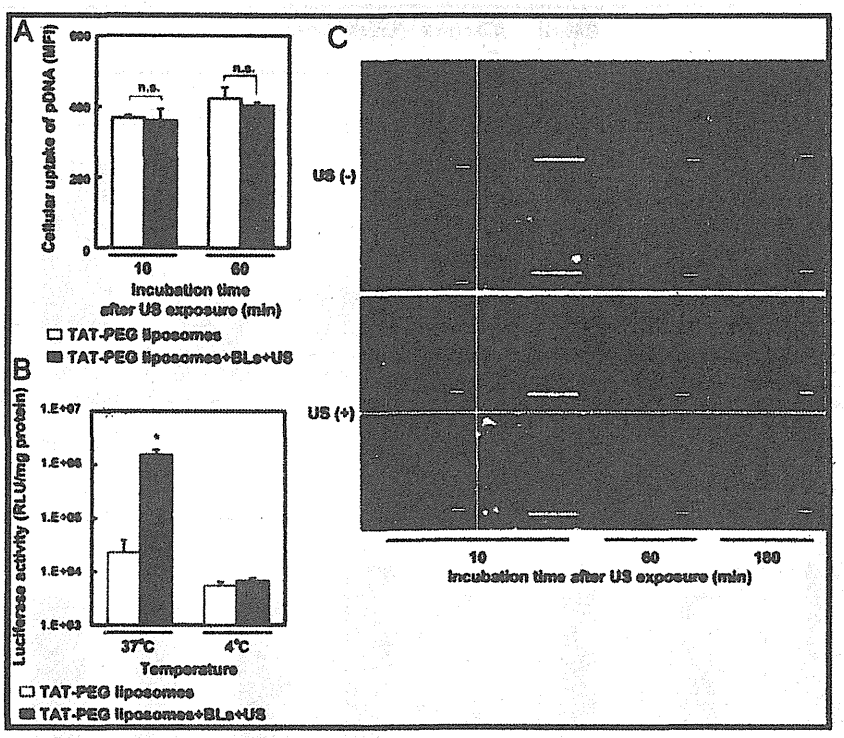


Figure 4. Mechanism of accelerated TAT-mediated liposomal gene transfection by BLs and US exposure. (A) HeLa cells were incubated with TAT-PEG liposomes encapsulating Cy3-labeled pDNA for 4 h at 37 °C. After incubation, the cells were washed, and BLs were added. Then the cells were exposed to US and 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 ± 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 ± 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 μg/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 sonazaoid 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 lisposomes 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-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.

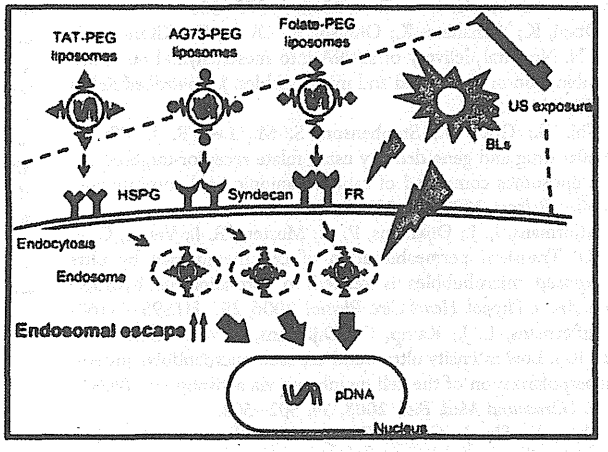


Figure 5. Diagram of enhanced gene delivery by BLs and US exposure. Several moiety-modified gene delivery carriers were internalized into cells via receptors and the endocytic pathway. When BLs and US exposure were applied, endosomal escape was enhanced, leading to increased transfection efficiency, 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).

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ACKNOWLEDGMENTS

We are grateful to Dr. Katsuro Tachibana (Department of Anatomy, School of Medicine, Fukuoka University) for technical advice regarding the induction of cavitation with US and to Mr. Yasuhiko Hayakawa, Mr. Takahiro Yamauchi, and Mr. Kosho Suzuki (NEPA GENE CO., LTD.) for technical advice regarding exposure to US. This study was supported by an Industrial Technology Research Grant (04A05010) from the New Energy and Industrial Technology Development Organization (NEDO) of Japan, Grant-in-Aid for Exploratory Research (18650146) and Grant-in-Aid for Scientific Research (B) (20300179) from the Japan Society for the Promotion of Science, and by a grant for private universities provided by the Promotion and Mutual Aid Corporation for Private Schools of Japan.

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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Pharm Res (2011) 28:712–719
DOI 10.1007/s11095-010-0286-4

RESEARCH PAPER

Delivery of an Angiogenic Gene into Ischemic Muscle by Novel Bubble Liposomes Followed by Ultrasound Exposure

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Received: 31 July 2010 / Accepted: 15 September 2010 / Published online: 8 October 2010

ABSTRACT

Purpose To develop a safe and efficient gene delivery system into skeletal muscle using the combination of Bubble liposomes (BL) and ultrasound (US) exposure, and to assess the feasibility and the effectiveness of BL for angiogenic gene delivery in clinical use.

Methods A solution of luciferase-expressing plasmid DNA (pDNA) and BL was injected into the tibialis (TA) muscle, and US was immediately applied to the injection site. The transfection efficiency was estimated by a luciferase assay. The ischemic hindlimb was also treated with BL and US-mediated intramuscular gene transfer of bFGF-expressing plasmid DNA. Capillary vessels were assessed using immunostaining. The blood flow was determined using a laser Doppler blood flow meter.

Results Highly efficient gene transfer could be achieved in the muscle transfected with BLs, and US mediated the gene transfer. Capillary vessels were enhanced in the treatment groups with this gene transfer method. The blood flow in the treated groups with this gene transfer method quickly recovered compared to other treatment groups (non-treated, bFGF alone, or bFGF+US).

Conclusion The gene transfer system into skeletal muscle using the combination of BL and US exposure could be an effective means for angiogenic gene therapy in limb ischemia.

KEY WORDS angiogenesis · bubble liposomes · gene delivery · ultrasound

INTRODUCTION

Skeletal muscle is a candidate target tissue for the gene therapy of both muscle (*e.g.*, Duschenne Muscular dystrophy) and non-muscle disorders (*e.g.*, cancer, ischemia, or arthritis). Its usefulness is due mainly to its stability and longevity after a gene transfer, which make it a good target tissue for gene therapy via the production of therapeutic proteins such as cytoskeletal proteins, trophic factors, or hormones. To achieve successful gene therapy in a clinical setting, it is critical that gene delivery systems be safe, easy to apply, and provide therapeutic transgene expression. Several previous studies using viral vectors reported the successful transfer of therapeutic genes into the target cells, but because of the considerable immunogenicity related to the use of viruses, non-viral gene transfer still needs to be developed (1). Recently, among physical non-viral gene transfer methods, it has been shown that therapeutic ultrasound enables genes to permeate cell membranes. The mechanism of gene transfer is believed to be involved in an acoustic cavitation (2–6). However, to achieve efficient gene transfer, a high

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intensity of US is required, which leads to tissue damage (7,8). In contrast, low-intensity US in combination with microbubbles has recently acquired much attention as a safe method of gene delivery (9–13). However, microbubbles have problems with size, stability, and targeting function. Liposomes have been known as drug, antigen, and gene delivery carriers (14–18). To solve the above-mentioned issues of microbubbles, we previously developed the polyethyleneglycol (PEG)-modified liposomes entrapping echo-contrast, "bubble liposomes" (BL), which can function as a novel gene delivery tool by applying them with US exposure (19–24).

In the present study, we developed a safe and efficient gene delivery system into skeletal muscle using the combination of BL and US exposure. We assessed the feasibility and the effectiveness of BL for gene therapy by trying to deliver a bFGF-expressing plasmid into skeletal muscle in a hindlimb ischemia model through the combination of BL and US exposure.

MATERIALS AND METHODS

Materials

Preparation of Bubble Liposomes

Bubble liposomes were prepared by the previously described methods (19,22). Briefly, PEG liposomes composed of 1, 2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) (NOF Corporation, Tokyo, Japan) and 1,2-distearoyl-*sn*-glycero-3-phosphatidyl-ethanolamine-polyethyleneglycol (DSPE-PEG_{2000}-OMe) (NOF corporation, Tokyo, Japan) in a molar ratio of 94:6 were prepared by a 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). BL were prepared from liposomes and perfluoropropane gas (Takachio Chemical Ind. Co. 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) (BRANSONIC 2510j-DTH, Branson Ultrasonics Co., Danbury, CT, USA) for 5 min to form BL.

Plasmid DNA (pDNA)

The plasmid pcDNA3-Luc, derived from pGL3-basic (Promega, Madison, WI), is an expression vector encoding the firefly luciferase gene under the control of a cytomegalovirus promoter. The plasmid pEGFP-N3 (Clontech Laboratories, Inc., Mountain View, CA) is an expression vector encoding the enhanced green fluorescein protein under the control of a cytomegalovirus promoter. The plasmid pBLAST-hbFGF (InvivoGen Inc.) is an expression vector encoding human bFGF under the control of an EF-1α promoter.

In Vivo Gene Delivery into the Skeletal Muscle of Mice with BL and US

ICR mice (5 weeks old, male) were anesthetized with pentobarbital throughout each procedure. A 40 μl suspension of pDNA (10 μg) and BL (30 μg) was injected into the tibialis (TA) muscle of the ICR mice, and US exposure (frequency: 1 MHz; duty: 50%; intensity: 2 W/cm^2; time: 60 s) was immediately applied at the injection site. A Sonitron 2000 (NEPA GENE, CO, LTD) was used as an ultrasound generator. Several days after the injection, the mice were euthanized and sacrificed, and the tibialis muscle in the US-exposed area was collected and homogenized. The cell lysate and tissue homogenates were prepared with a 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, Madison, WI) and a luminometer (LB96V, Belthold Japan Co. Ltd., Tokyo, Japan). The activity is indicated as relative light units (RLU) per mg of protein. For analyzing EGFP expression, the treated muscle was fixed with paraformaldehyde and dehydrated in a sucrose solution. The specimens were embedded in an OCT compound and immediately frozen at −80°C. Serial sections 8 μm thick were cut by cryostat and observed with a fluorescence microscope (Axiovert 200 M, Carl Zeiss).

In Vivo Luciferase Imaging

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

Tissue-Damage Testing Using Evans-Blue Dye (EBD)

Tissue-damage testing using EBD was performed as previously reported (25). Briefly, EBD was dissolved in PBS (10 mg/ml) and sterilized by using 0.2 μm membrane filters. Mice treated with pDNA, BL, and US exposure were administered with the dissolved EBD (0.5 mg dye per

10 g body weight) by tail vein injection. The mice were sacrificed 1 day after dye injection. The TA muscles were removed and photographed using a digital camera. The TA muscles were embedded in an OCT compound and immediately frozen at –80°C. Serial sections 10 μm thick were cut by cryostat and observed with a fluorescence microscope (Axiovert 200 M, Carl Zeiss).

Hindlimb Ischemia Model

The ischemic hindlimb model was created in five-week-old male ICR mice as previously reported (26). Briefly, animals were anesthetized, and a skin incision was made in the left hindlimb. After ligation of the proximal end of the femoral artery at the level of the inguinal ligament, the distal portion and all the side branches were dissected free and excised. The right hindlimb was kept intact to control the original blood flow. Immediately after ischemia was induced, a mixture of 40 μl of a pDNA (10 μg of pBLAST-hbFGF or pBLAST as an control vector) and BL (30 μg) suspension was injected into the adductor muscle of the ischemia mice, and US exposure (1 MHz, 2 w/cm^2, 50% duty cycle, 60 s) was immediately applied at the injection site. Measurements of the ischemic (left)/normal (right) limb blood flow ratio were performed for a set time using a laser Doppler blood flow meter (OMEGAFLO, FLO-C1).

bFGF ELISA

bFGF secretion was determined as previously reported (27). Briefly, 5- to 6-week-old male ICR mice were anesthetized by intraperitoneal injection of pentobarbital. The leg was shaved and depilated to expose the tibialis anterior muscle. Ten micrograms of DNA in a 40 μL bubble liposome or PBS solution were injected into the tibialis anterior muscle. After DNA injection, US exposure was applied. The tibialis anterior muscle was collected 2 days after the DNA injection. The muscle was washed three times in 3 mL of PBS to remove debris and blood. The washed muscle was placed in a 24-well plate coating growth factor reduced Matrigel (BD Biosciences) and incubated at 37°C. The muscle was grown in 1.5 mL of M199 medium containing 2% fetal bovine serum, 100 U/mL penicillin, and 100 mg/mL streptomycin. The levels of secreted cytokines in the conditioned media of the explants cultures were measured using human bFGF ELISA (R&D Systems), according to the manufacturer's instructions.

Immunohistochemistry

The ischemic thigh muscles were perfused on day 14 with PBS and 4% paraformaldehyde and embedded in paraffin. Muscle sections (4 μm) were stained with anti-CD31 antibody (BD pharmingen) overnight at 4°C. We then incubated the sections with Alexa Fluor 488 rabbit anti-rat IgG (Molecular Probes).

In Vivo Studies

Animal use and relevant experimental procedures were approved by the Tokyo University of Pharmacy and Life Science Committee and Teikyo University on the Care and Use of Laboratory Animals. All experimental protocols for animal studies were in accordance with the Principle of Laboratory Animal Care in Teikyo University.

Statistical Analyses

All data are shown as the mean±SD (n=4 or 6). Data were considered significant when $P<0.05$. The t-test was used to calculate statistical significance.

RESULTS

In Vivo Gene Delivery into the Skeletal Muscle of Mice with BL and US Exposure

It has been reported that microbubbles improve tissue permeability by cavitation upon US exposure. We first tried to deliver the naked pDNA (pCMV-Luc) into tibialis muscle using BL and US. A solution of pDNA and BL was injected into TA muscle, and US was immediately applied to the injection site, as shown in Fig. 1. As a result, the relative luciferase activity was high in the group treated using the pDNA plasmid with BL and US exposure. In contrast, there was low activity in the groups treated with pDNA alone, pDNA+BL, or pDNA+US. The luciferase activity in the group receiving a combination of BL with US exposure was 200- or 20-fold higher than that of the group treated with pDNA alone or pDNA + US, respectively (Fig. 1a). We next investigated whether their gene expression was derived from muscle cells. In a similar fashion, the EGFP expression plasmid (pEGFP-N3) was delivered into TA muscle, and 5 days after the gene delivery, the EGFP expression was sectioned and examined by fluorescent microscopy. As shown in Fig. 1b, the intramuscular gene delivery of the EGFP expression plasmid by BL and US exposure was present in a wide area of the positive muscle fibers of EGFP. In contrast, in the muscle specimens of the other treated groups (pDNA alone, pDNA+BL, or pDNA+US), very little expression was shown (Fig. 1b). We also observed the luciferase gene expression area in the whole body using an *in vivo* luciferase imaging system at 5 days after the transfection into the muscle treated with pDNA, BL, and US exposure.

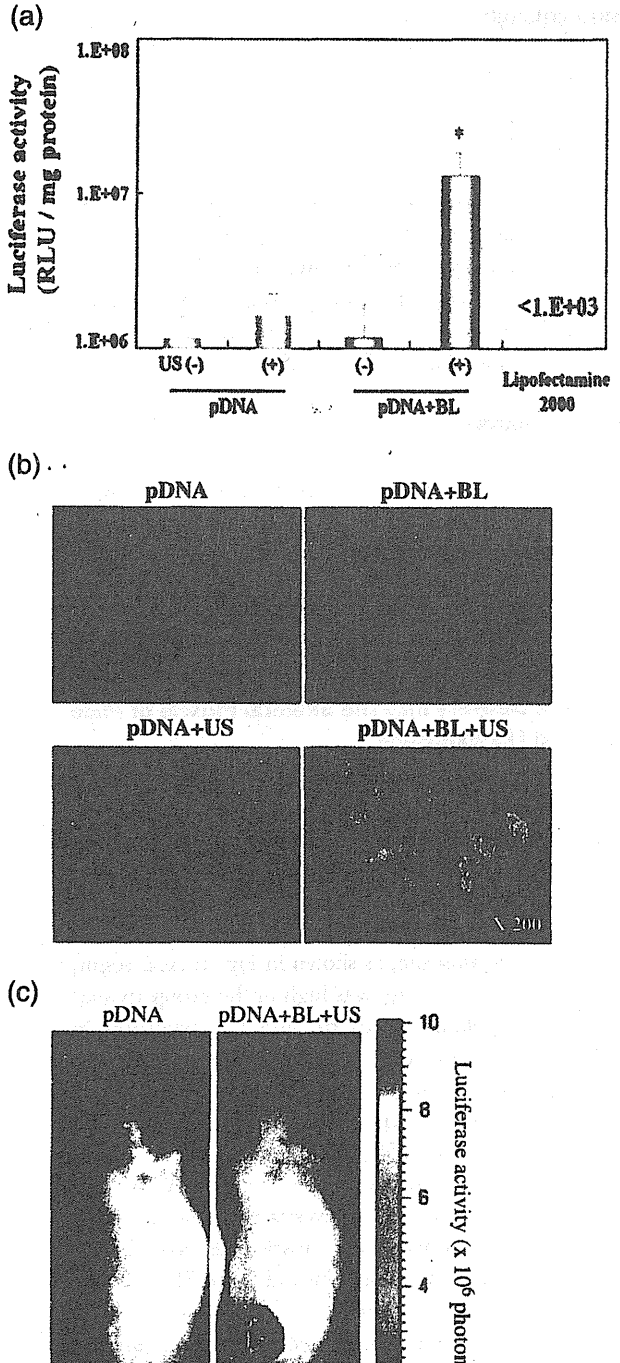

Fig. 1 Reporter gene expression after BL and US-mediated gene transfer compared with Lipofectamine 2000. (a) Luciferase expression after BL and US-mediated gene transfer compared with Lipofectamine 2000. Mice were treated with BL and US-mediated intramuscular luciferase gene transfer or Lipofectamine 2000. Five days after transfection, luciferase expression was determined. In another experiment, a pDNA (pCMV-Luc (10 μg))-Lipofectamine 2000 (25 μg) complex was suspended in PBS and injected into the left femoral artery. *$P < 0.01$ compared to the group of pDNA alone, pDNA + US, pDNA + BL, or Lipofectamine 2000 with BL. pDNA (pCMV-Luciferase): 10 μg, BL: 30 μg. US exposure (Frequency: 1 MHz, Duty: 50%, Intensity: 2 W/cm^2, Time: 60 s). (b) EGFP expression after BL and US-mediated gene transfer. Mice were treated with BL and US-mediated intramuscular EGFP gene transfer. Five days after transfection, EGFP expression was analyzed by fluorescent microscopy. Each of the gene transfer conditions are indicated above the pictures. Magnification: x 200. (c) photon counts are indicated by the pseudo-color scales.

Although the level of gene expression gradually decreased 2 weeks after the transfection using BL and US exposure, the moderate gene expression persisted for 4 weeks after the transfection (data not shown). The gene expression was restricted to the area of US exposure (Fig. 1c). This suggested that the combination of BL and US exposure facilitated the efficient transfection of pDNA into the muscle due to the induction of cavitation. We also assessed the tissue damage by testing EBD uptake in the muscle transfected with the BL and US exposure; however, significant tissue damage was not observed at the US condition (frequency: 1 MHz; duty: 50%; intensity: 2 W/cm^2; time: 60 s), even in the presence of the cavitation by BL and US exposure (Fig. 2b, e). When a higher US intensisty (4 W/cm^2) was applied, significant tissue damage was detected (Fig. 2c, f).

In Vivo Effects of the bFGF Expression System

We next attempted to deliver bFGF plasmid into tibialis muscle using BL and US and determine the bFGF protein expression in explant culture medium. The amount of bFGF protein was high in the group treated with bFGF plasmid with BL and US exposure. In contrast, there was low expression in the group treated with bFGF plasmid alone, or bFGF plasmid+US (Fig. 3). We further investigated the capillary density in order to know the effects of BL and US-mediated gene delivery with bFGF plasmid injected intramuscularly into hindlimb ischemia model mice. In the treatment group with BL and US-mediated gene transfer, their capillary vessels with CD31 positive cells were significantly increased compared to the treatment group of the control plasmid (empty vector), the bFGF plasmid alone, or bFGF plasmid + US (Fig. 4a, b). Measurements of the ischemic (left)/non-ischemic (right) hindlimb blood flow ratio were further performed for a period of time using a laser Doppler blood flow meter. Consistent with this induction of angiogenesis, the blood flow in the group treated with the bFGF plasmid with BL and US exposure was significantly increased compared with the group treated with the control plasmid (empty vector), the bFGF plasmid alone, bFGF plasmid + US (Fig. 5). Although we also examined the blood flow ratio after treatment with US exposure alone or BL with US exposure

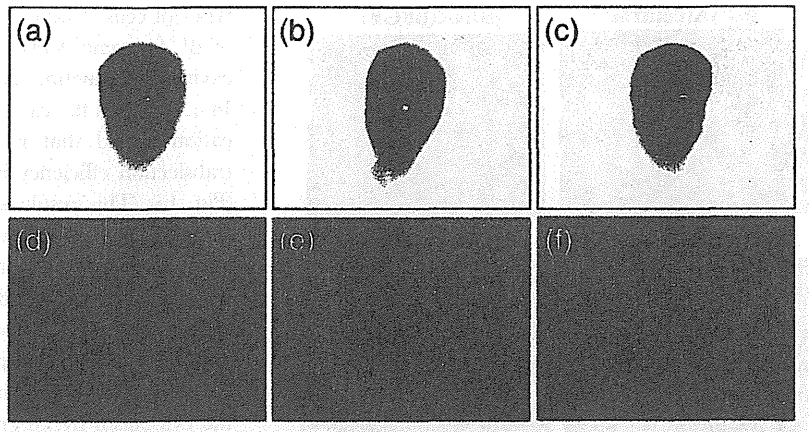

Fig. 2 Tissue-damage testing using EBD. pDNA alone without BL and US exposure (a, d). pDNA with BL and US exposure condition at a frequency of 1 MHz with an intensity of 2 W/cm^2 (b, e), or 4 W/cm^2 (c, f) for 60 s. The TA muscles were photographed using a digital camera (a, b, and c). Evans-blue fluorescence of 10 μm cryosections from the TA muscles was examined with fluorescence microscopy (d, e, and f). Magnification: × 100.

to the ischemic limb muscle, their blood flow ratio still remained in the 20 to 40% range. These results suggest that intramuscular injection of bFGF as an angiogenic gene with bubble liposomes followed by US exposure enabled us to improve an angiogenesis in the ischemic muscle.

DISCUSSION

The gene delivery of naked plasmid DNA is a feasible technique for non-viral gene therapy in a safe clinical use; however, a higher efficiency of site-specific delivery is required to achieve therapeutic effects in patients. In this view, we previously reported that BL is an efficient gene delivery tool (24,28,29). However, it is not enough to say that BL is a feasible and effective tool to carry out gene therapy to treat diseases. Here we demonstrate the development of a safe and efficient gene delivery system into skeletal muscle using the combination of BL and US exposure, and we assess the feasibility and the effectiveness of BL for angiogenic gene delivery. We therefore examined the potential ability of BL with US exposure to deliver a gene into skeletal muscle and its applicability for therapeutic angiogenesis in ischemic model. By using BL with US exposure, we first performed a transfer of luciferase-expressing plasmid DNA as a reporter plasmid into the TA muscle of mice. The remarkable gene expression could be enhanced efficiently only with the combination of both BL and US exposure when compared with other treatments (Fig. 1a). Exceeding our expectations, their gene expression was 200-fold higher than that of the plasmid DNA injection alone. When compared to Optison, one of the currently existing microbubbles (9–11), with US exposure, however, the gene transfer efficacy of BL was almost same as when using Optison (data not shown). Previously, our reports have demonstrated that the gene transfection efficiency *in vitro* could be affected with increasing the US intensity and the exposure time (20). The transfection efficiency increased with an increasing intensity of ultrasound and reached a plateau at 2 W/cm^2. No significant damage was observed under these conditions (Fig. 2b, e). When a higher intensity of US (4 W/cm^2) during the gene transfer with BL was applied to improve the transfection efficiency, the gene expression was conversely diminished (data not shown), and significant damage was also observed (Fig. 2c, f). This treatment caused significant tissue damage, probably due to the temperature elevation in the US exposure site. In this experiment, we therefore employed an US condition (frequency: 1 MHz; intensity: 2 W/cm^2; duty cycle: 50%; US exposure time: 1 min) that was in terms with a safety profile. As shown in Fig. 1b, the number of EGFP-positive muscle fibers could be apparently enhanced by the combination of BL and US

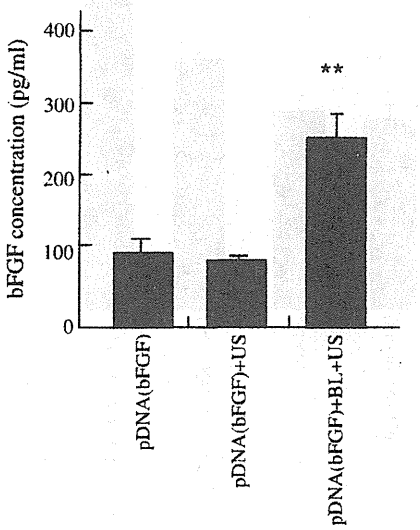

Fig. 3 bFGF protein expression after BL and US-mediated bFGF gene transfer. Mice were treated with BL and US-mediated intramuscular bFGF gene transfer. Two days after transfection, the muscle was collected and placed into Matrigel coating plates. After 3 days, the secreted bFGF protein expression was determined by ELISA. $^{**}P < 0.05$ *vs.* other treatment groups. pDNA (pBLAST-bFGF): 10 μg, BL: 30 μg, US exposure (Frequency: 1 MHz, Duty: 50%, Intensity: 2 W/cm^2, Time: 60 s).