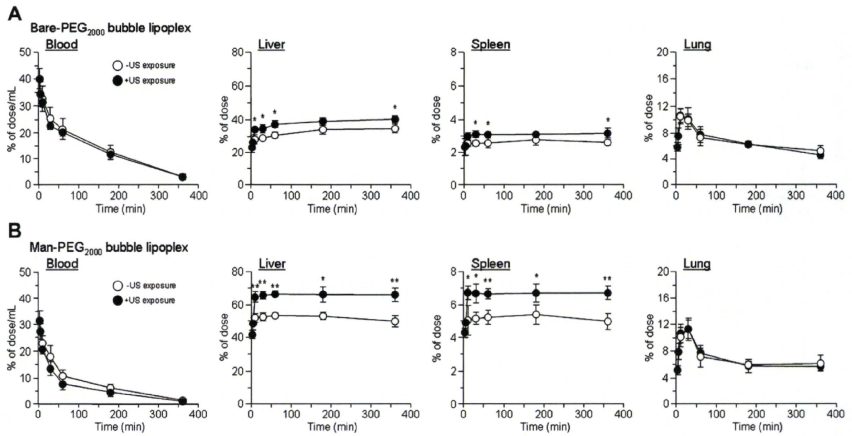

Fig. 7. Tissue distribution of pDNA by Man-PEG$_{2000}$ bubble lipoplexes and US exposure. Tissue distribution after intravenous administration of (A) Bare-PEG$_{2000}$ bubble lipoplexes and (B) Man-PEG$_{2000}$ bubble lipoplexes (50 μg pDNA) with or without US exposure in mice. US was exposed at 5 min after intravenous administration of bubble lipoplexes. Each value represents the mean ± SD ($n = 3$). *$p < 0.05$; **$p < 0.01$, compared with the corresponding group of US exposure.

pCMV-OVA and US exposure significantly enhances the differentiation of helper T cells to Th1 cells, which are pivotal cells for the activation of cytotoxic T lymphocytes (CTL) with high anti-tumor activity, by OVA stimulation.

3.11. Antigen-expressing cell-specific CTL activity in immunized splenic cells

Next, we assessed the CTL activity in the splenic cells harvested from mice immunized by Man-PEG$_{2000}$ bubble lipoplexes and US exposure. Following experiments according to the protocol shown in Fig. 9B, the splenic cells immunized by Man-PEG$_{2000}$ bubble lipoplexes constructed with pCMV-OVA and US exposure showed the highest CTL activity in all groups against E.G7-OVA cells which are the lymphoma cells expressing OVA (Fig. 9D). In contrast, the CTL activity was not observed in EL4 cells which are the lymphoma cells not expressing OVA in all groups (Fig. 9D). These results indicate that the splenic cells immunized by Man-PEG$_{2000}$ bubble lipoplexes constructed with pCMV-OVA and US exposure induce the OVA-expressing cell-specific CTL activity.

3.12. Therapeutic effects against antigen-expressing tumor by DNA vaccination

Finally, we investigated the anti-tumor effects by DNA vaccination using Man-PEG$_{2000}$ bubble lipoplexes and US exposure. Following experiments according to the protocol shown in Fig. 10A, significantly high anti-tumor effects against E.G7-OVA cells were observed in mice immunized by Man-PEG$_{2000}$ bubble lipoplexes constructed with pCMV-OVA and US exposure (Fig. 10B). However, in mice transplanted EL4 cells, no anti-tumor effects were observed in any of the groups (Fig. 10C). Moreover, we investigated the maintenance of DNA vaccine effects following administration of Man-PEG$_{2000}$ bubble lipoplexes and US exposure. According to the protocol shown in Fig. 11A, E.G7-OVA cells were re-transplanted into mice which first-transplanted tumors were completely rejected by DNA vaccination using Man-PEG$_{2000}$ bubble lipoplexes and US exposure. As results, high anti-tumor effects were observed in mice following re-transplantation of E.G7-OVA cells (Fig. 11B); therefore it was demonstrated that DNA vaccine effects obtained by Man-PEG$_{2000}$ bubble lipoplexes constructed with pCMV-OVA and US exposure were maintained for at least 80 days.

4. Discussion

To obtain high therapeutic effects by DNA vaccination using tumor-specific antigen-coding gene, it is essential to transfer the gene selectively and efficiently into the APCs, such as macrophages and dendritic cells [31,32]. However, it is difficult to transfer the gene into the APCs selectively because of the number of APCs is limited in the organ [33]. Since the APCs are expressed a large number of mannose receptors [28,29], we and other groups have developed mannose-modified non-viral carriers for gene delivery to the APCs [7,25,34]. On the other hand, our group also reported that the gene transfection efficiency in the APCs was lower than that in other cells [35]; therefore it is difficult to achieve high gene transfection efficiency to induce high therapeutic effects by DNA vaccination in vivo. In the present study, to establish an APC-selective and efficient gene delivery system, we developed US-responsive and mannose-modified carriers, named Man-PEG$_{2000}$ bubble lipoplexes, which had selectivity to the APCs and responded to US exposure. The gene delivery system using Man-PEG$_{2000}$ bubble lipoplexes and US exposure enabled to achieve markedly high gene expression in macrophages and dendritic cells selectively in vivo, in spite of the handy system used intravenous administration and external US exposure. Moreover, we succeeded in obtaining high anti-tumor effects by applying this method to DNA vaccine therapy using OVA-expressing pDNA.

Firstly, since PEG$_{2000}$-modification is necessary to enclose US imaging gas stably [12], we prepared Man-PEG$_{2000}$ lipoplexes

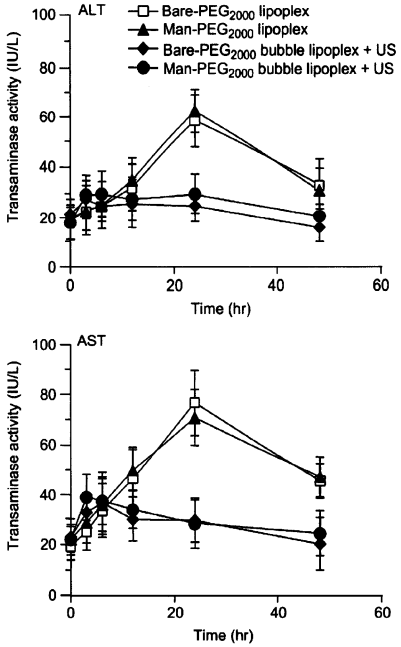

Fig. 8. Liver toxicity by gene transfection using Man-PEG_{2000} bubble lipoplexes and US exposure. Time-course of serum transaminase activities after transfection by Bare-PEG_{2000} lipoplexes, Man-PEG_{2000} lipoplexes, Bare-PEG_{2000} bubble lipoplexes with US exposure and Man-PEG_{2000} bubble lipoplexes with US exposure (50 μg pDNA). Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) in the serum were measured at predetermined times after transfection. Each value represents the mean ± SD ($n = 4$).

containing Man-PEG_{2000} lipids. This Man-PEG_{2000} lipoplexes exhibited mannose receptor-expressing cell-selective gene expression both in vitro and vivo (Fig. 2). On the other hand, the level of gene expression by Man-PEG_{2000} lipoplexes was lower than that by mannosylated lipoplexes without PEG-modification, as reported previously by our group [1,25]. However, this result was considered to be contributed by the reduced interaction with the cell membrane and the reduction of endosomal escape efficiency by PEG_{2000}-modification [36,37]. In the sonoporation method, Tachibana et al. demonstrated that a transient pore is created on the cell membrane followed by the degradation of microbubbles [38]. Then, nucleic acids, such as pDNA, siRNA and oligonucleotides, are introduced into the cell through the generated pore [13,15,16]. Consequently, since the nucleic acids are directly introduced into cytoplasm in the sonoporation method [13,14], it is considered that the low level of transfection efficiency obtained by Man-PEG_{2000} lipoplexes can be overcome by applying sonoporation method. As shown in Figs. 3 and 4, a large amount of pDNA is directly introduced into the cytoplasm and high level of gene expression is observed by gene transfection using Man-PEG_{2000} bubble lipoplexes and US exposure. Therefore, by delivering pDNA to the APCs using Man-PEG_{2000} bubble lipoplexes, it is suggested that high level of gene expression in the APCs can easily achieve by following US exposure in this gene transfection method.

In this study, the level of gene expression obtained by transfection using Man-PEG_{2000} bubble lipoplexes and US exposure was higher than that obtained by Man-PEG_{2000} lipoplexes or Bare-PEG_{2000} bubble lipoplexes with US exposure in the liver and spleen (Fig. 5). Moreover, gene expression by Man-PEG_{2000} bubble lipoplexes and US exposure was observed selectively in the hepatic NPCs and the splenic dendritic cells (Fig. 6), known as mannose receptor-expressing cells [28–30]. Although this selectivity of gene expression was the same as that obtained by mannosylated lipoplexes reported previously by our group [1,25], this level of gene expression was markedly higher. It is considered that this enhanced and cell-selective gene expression is contributed by the increase of interaction with mannose receptor-expressing cells by mannose modification (Supplementary Fig. 1), by the improvement of delivering efficiency of nucleic acids to the targeted organs (Fig. 7) and by the direct introduction of nucleic acids into the cytoplasm of targeted cells followed by US exposure to Man-PEG_{2000} bubble lipoplexes (Figs. 3C and 4B and Supplementary Fig. 4). Moreover, the enhanced gene expression was not observed in the lung, kidney and spleen (Fig. 5G and H). It is guessed that the reason why the enhanced gene expression was not observed in the lung is because US is not spread to the thoracic cavity by the diaphragm, and the reason why the enhanced gene expression was not observed in the kidney and heart was because the distributed amounts of bubble lipoplexes were markedly small. In addition, since the particle size of bubble lipoplexes (approximately 500 nm) is suitable for delivery to the liver and spleen, compared with stabilized liposomes (approximately 100 nm) [39], the gene transfection system using Man-PEG_{2000} bubble lipoplexes and US exposure is a suitable method for the selective delivery of nucleic acids into the mannose receptor-expressing cells in the liver and spleen.

On the other hand, the liver toxicity followed by gene transfection using Man-PEG_{2000} bubble lipoplexes and US exposure was lower than that by Man-PEG_{2000} lipoplexes (Fig. 8). It was reported that the CpG motifs in the pDNA sequence are recognized to Toll-like receptor 9 (TLR9) in the endosomes [40,41]; therefore it has been considered that the production of proinflammatory cytokines, such as TNF-α, IFN-γ and IL-12, could be induced in the lipofection method using liposomes and emulsions, and these cytokines cause liver injury [42]. However, in the gene transfection using Man-PEG_{2000} bubble lipoplexes and US exposure, a large amount of pDNA was directly introduced into the cytoplasm not-mediated endocytosis (Figs. 3C and 4B and Supplementary Fig. 4). Therefore, it is considered that pDNA is not recognized to TLR9 in the endosomes, and consequently liver toxicity followed by transfection using Man-PEG_{2000} bubble lipoplexes and US exposure is low.

In the previous study [16], we developed combination-use method using mannosylated lipoplexes [1] and BLs [12] with US exposure to achieve targeted cell-selective gene transfer. However, this combination-use method is complicated because of the necessity of twice injection of mannosylated lipoplexes and BLs, therefore it is difficult to apply for medical treatments using multiple injection. Moreover, it is considered that the difference of in-vivo distribution characteristics between mannosylated lipoplexes and BLs might be decreased its transfection efficacy. On the other hand, this transfection method using Man-PEG_{2000} bubble lipoplexes and US exposure is handy because of using only once injection of Man-PEG_{2000} bubble lipoplexes and external US exposure. In addition, this method using Man-PEG_{2000} bubble lipoplexes and US exposure overcame the difference of in-vivo distribution of formulations, which might lead to the decrease of

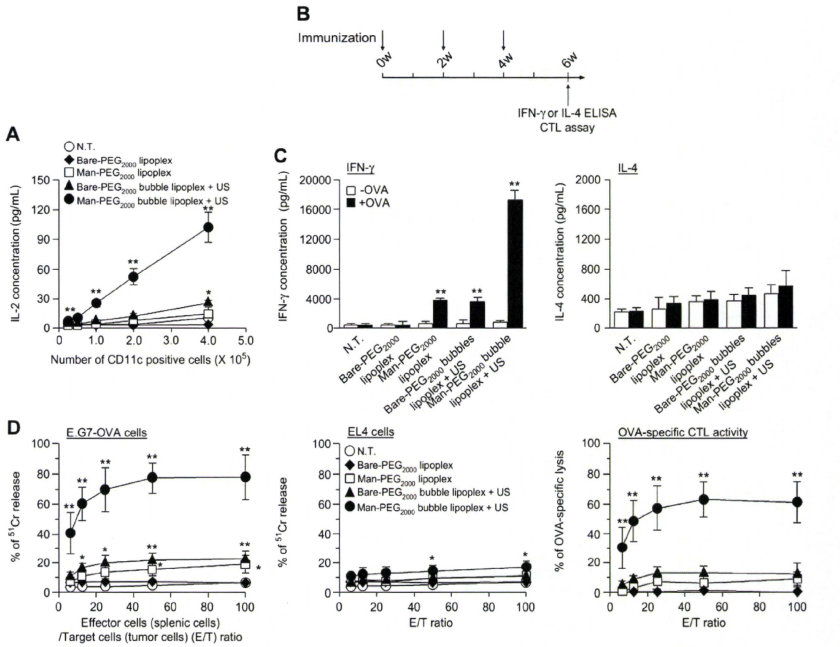

Fig. 9. Cytokine secretion characteristics and CTL activities by DNA vaccination using Man-PEG_{2000} bubble lipoplexes and US exposure. (A) OVA presentation on MHC class I molecules in the splenic $CD11c^+$ cells at 24 h after transfection by Bare-PEG_{2000} lipoplexes, Man-PEG_{2000} lipoplexes, Bare-PEG_{2000} bubble lipoplexes with US exposure and Man-PEG_{2000} bubble lipoplexes with US exposure (50 μg pDNA). OVA presentation on MHC class I molecules was determined by IL-2 level secreted from CD8-OVA1.3 cells co-incubated with the $CD11c^+$ cells isolated from once-immunized mice. Each value represents the mean ± SD ($n = 4$). $^*p < 0.05$; $^{**}p < 0.01$, compared with the corresponding group of N.T. (B) Schedule of immunization for OVA-specific cytokine secretion experiments and CTL assay. (C) OVA-specific IFN-γ and IL-4 secretion from the splenic cells immunized three times biweekly by 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 pDNA). The splenic cells were collected at 2 weeks after last immunization. After the immunized splenic cells were cultured for 72 h in the absence or presence of 100 μg OVA, IFN-γ and IL-4 secreted in the medium were measured by ELISA. Each value represents the mean + SD ($n = 4$). $^{**}p < 0.01$, compared with the corresponding group of OVA. (D) OVA-specific CTL activities after immunization three times by 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 pDNA). CTL activities to E.G7-OVA and EL4 cells 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 group of N.T. N.T., non-treatment.

transfection efficiency. In fact, the level of gene expression by this method was higher than that by combination-use method reported previously in the targeted organs (liver and spleen) (Fig. 5) and targeted cells (hepatic NPC and splenic dendritic cells) (Fig. 6); therefore this gene transfection method using Man-PEG_{2000} bubble lipoplexes and US exposure is more suitable for APC-selective gene transfer in vivo.

Since APC-selective and efficient gene expression was observed by transfection using Man-PEG_{2000} bubble lipoplexes and US exposure, effective therapeutic effects are to be expected by applying this transfection method to DNA vaccine therapy, which the targeted cells are the APCs, using tumor-specific antigen-coding pDNA [31,32]. However, since the level of gene expression by transfection using Man-PEG_{2000} bubble lipoplexes and US exposure was reduced sequentially (Supplementary Fig. 6), multiple transfections are essential to obtain more effective vaccine effects against cancer (Supplementary Fig. 7). On the other hand, a 2 week interval was needed to achieve the same level of gene expression by lipoplexes or bubble lipoplexes with US exposure as former transfection in the spleen (Supplementary Fig. 7B and C). Meyer et al. reported that the optimal transfection interval was necessary to achieve high gene expression by the second transfection using lipofection methods because of IFN-γ secretion by intravenous administration of lipoplexes [43]; therefore it is considered that a similar phenomenon is contributed to the sonoporation methods using microbubbles constructed with phospholipids. Based on these findings, we performed the optimization of immunization times (Supplementary Fig. 7) and determined the optimal immunization schedule as shown in Figs. 9B, 10A and 11A.

In DNA vaccine therapy, unlike cancer immunotherapy using tumor-specific antigenic peptides, the peptides expressed as gene products in the cells act as the internal antigen. Since the internal antigens are presented on MHC class I molecules, the strong activation of CTL and high anti-tumor effects are expected in DNA vaccination therapy [44,45]. In this study, by applying this gene transfection method to DNA vaccine therapy using OVA-expressing

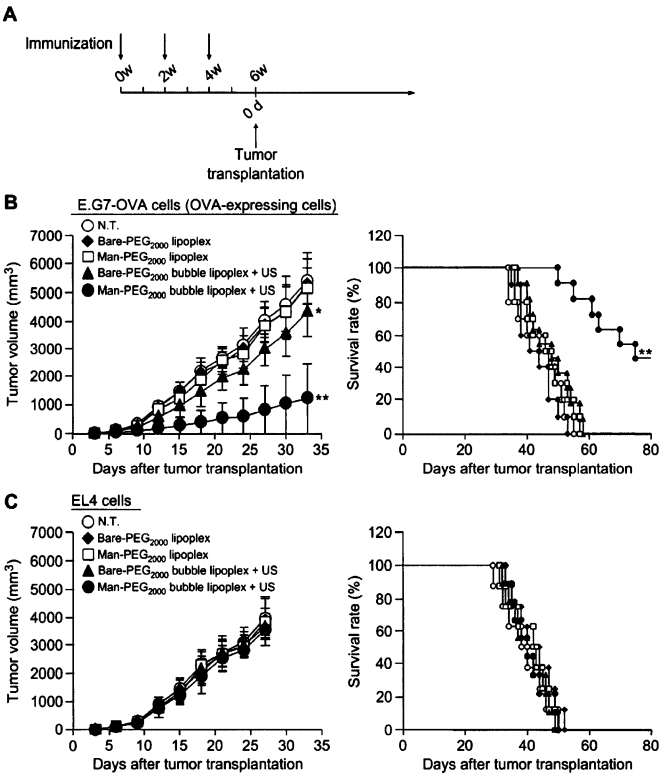

Fig. 10. Anti-tumor effects by DNA vaccination using Man-PEG_{2000} bubble lipoplexes and US exposure. (A) Schedule of immunization for experiments of therapeutic effects. (B, C) Anti-tumor effects by immunization using Bare-PEG_{2000} lipoplexes, Man-PEG_{2000} lipoplexes, Bare-PEG_{2000} bubble lipoplexes with US exposure and Man-PEG_{2000} bubble lipoplexes with US exposure (50 μg pDNA). Two weeks after last immunization, (B) E.G7-OVA cells or (C) EL4 cells (1×10^6 cells) were transplanted subcutaneously into the back of mice ($n = 8–11$). The tumor volume was evaluated (each value represents the mean ± SD) and the survival was monitored up to 80 days after the tumor transplantation. $^*p < 0.05$; $^{**}p < 0.01$, compared with the corresponding group of N.T. N.T., non-treatment.

pDNA, i) the presentation of OVA-peptides on MHC class I molecules of splenic dendritic cells, ii) OVA-specific Th1 cytokine secretion from splenic cells by OVA stimulation and iii) marked activation of CTL against OVA-expressing tumor were observed by gene transfection using Man-PEG_{2000} bubble lipoplexes constructed with pCMV-OVA and US exposure (Fig. 9). Moreover, high and long-term anti-tumor effects against OVA-expressing tumor were observed in mice transfected by Man-PEG_{2000} bubble lipoplexes constructed with pCMV-OVA and US exposure (Figs. 10 and 11). It is considered that these results are contributed by APS-selective and efficient gene transfection efficiency using Man-PEG_{2000} bubble lipoplexes and US exposure. Although more detailed examination by pDNA encoding other tumor-specific antigens, such as gp100 in melanoma or PSA in prostate cancer [45], is necessary, this transfection method by Man-PEG_{2000} bubble lipoplexes and US exposure might be available for DNA vaccine therapy.

The gene transfection method using Man-PEG_{2000} bubble lipoplexes and US exposure was enabled selective and efficient gene transfer to the mannose receptor-expressing cells in the liver such as Kupffer cells and hepatic endothelial cells, which are components of the APCs (Fig. 6A). Therefore, this method is also suitable for anti-inflammatory therapy targeted to Kupffer cells and hepatic endothelial cells, known to play a key role in inflammation [46,47]. In spite of low liver toxicity, since this gene transfection system showed NPC-selective and efficient gene expression in the liver (Fig. 8), better therapeutic effects could be expected by Man-PEG_{2000} bubble lipoplexes constructed with

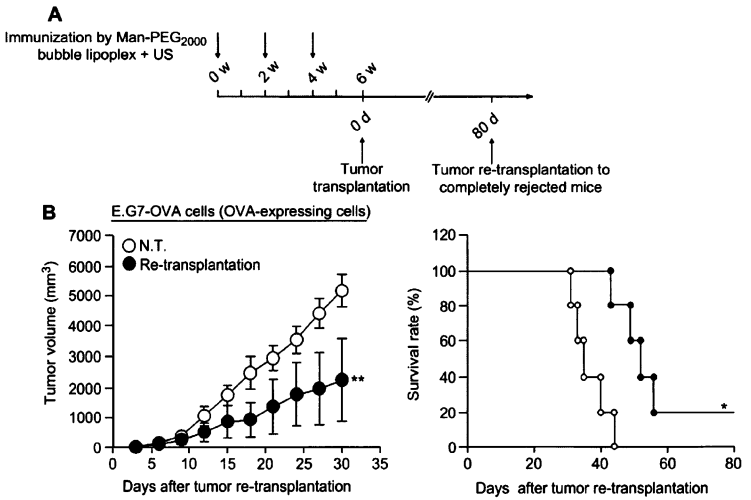

Fig. 11. Maintaining of Anti-tumor effects by DNA vaccination using Man-PEG_{2000} bubble lipoplexes and US exposure. At 80 days after first transplantation of E.G7-OVA cells to immunized mice three times by Man-PEG_{2000} bubble lipoplexes and US exposure, E.G7-OVA cells (1×10^6 cells) were re-transplanted subcutaneously into the back of mice which the first-transplanted tumors were completely rejected ($n = 5$). The tumor volume was evaluated (each value represents the mean ± SD) and the survival was monitored up to 80 days after the tumor re-transplantation. $^*p < 0.05$; $^{**}p < 0.01$, compared with the corresponding group of N.T. N.T., non-treatment.

various types of nucleic acids, such as NF-κB decoy [48], ICAM-1 antisense oligonucleotides [49], with low doses of nucleic acids. Moreover, organ-specific gene expression was observed in US-exposed organ by exposing US to the organ directly after intravenous administration of Man-PEG_{2000} bubble lipoplexes (Supplementary Fig. 5); therefore the beforehand knockdown of inflammatory factors such as NF-κB or ICAM-1 by Man-PEG_{2000} bubble lipoplexes and US exposure might be available for the prevention of ischemia reperfusion injury, a major problem in living donor liver transplantation.

5. Conclusion

In this study, we developed the gene transfection method using Man-PEG_{2000} bubble lipoplexes and US exposure. This transfection method enabled APC-selective and efficient gene expression, and moreover, effective anti-tumor effects was obtained by applying this method to DNA vaccine therapy against cancer. This method could be widely used in a variety of targeted cell-selective and efficient gene transfection methods by substituting mannose with various ligands reported previously [2–6]. In addition, in this gene transfection method, pDNA can directly introduce the nucleic acids into the cells through the transient pores created by US-responsive degradation of bubble lipoplexes, therefore this method could apply to many ligands which are not taken up via endocytosis. These findings make a valuable contribution to overcome the poor introducing efficiency into cytoplasm which is a major obstacle for gene delivery by non-viral vectors, and show that this method is an effective method for in-vivo gene delivery.

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Appendix. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.biomaterials.2010.06.058.

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Supplementary Data

Development of an ultrasound-responsive and mannose-modified gene carrier for DNA vaccine therapy

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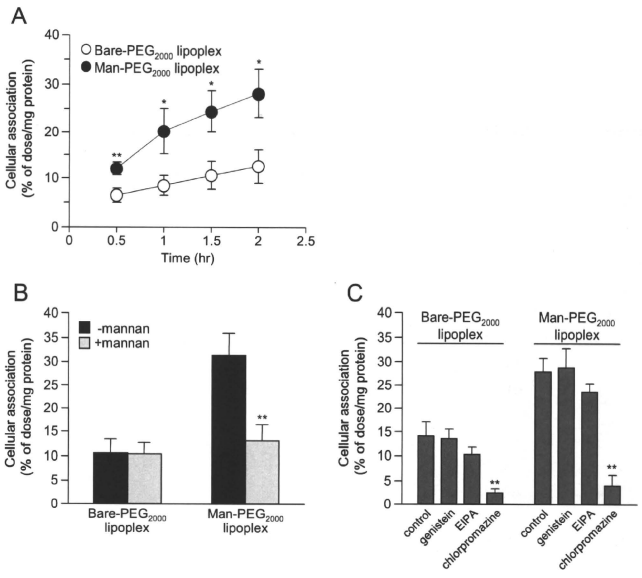
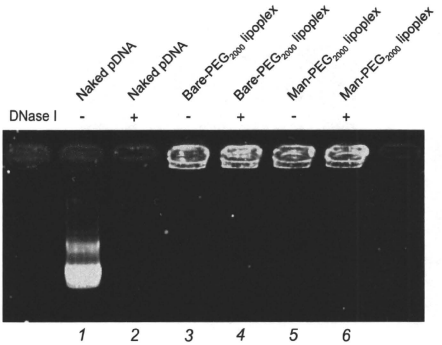
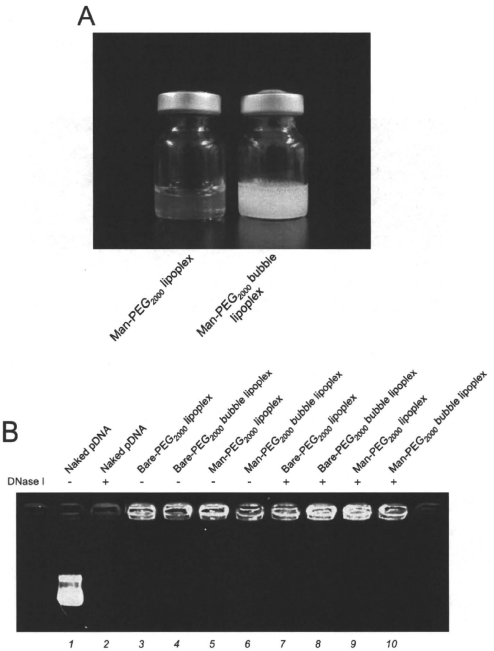


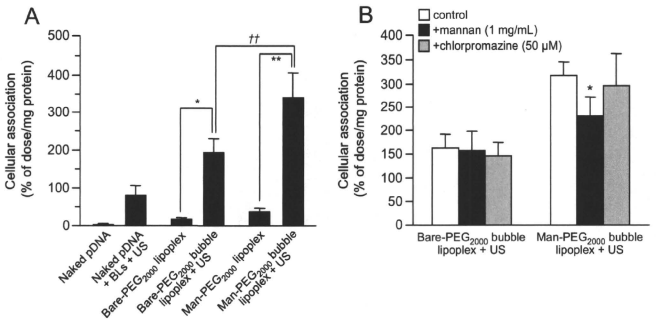
Supplementary Fig. 1. The cellular association of pDNA obtained by Bare-PEG_{2000} lipoplexes and Man-PEG_{2000} lipoplexes in mouse cultured macrophages. (A) Time-course of the cellular association of pDNA obtained by Bare-PEG_{2000} lipoplexes and Man-PEG_{2000} lipoplexes constructed with ^{32}P-labeled pDNA (5 μg pDNA). Each value represents the mean ± SD ($n = 3$). * $p < 0.05$; ** $p < 0.01$, compared with the corresponding group of Bare-PEG_{2000} lipoplex. (B) Cellular association of pDNA obtained by Bare-PEG_{2000} lipoplexes and Man-PEG_{2000} lipoplexes constructed with ^{32}P-labeled pDNA (5 μg pDNA) in the absence or presence of 1 mg/mL mannan at 2 hr after addition of each lipoplex. Each value represents the mean + SD ($n = 3$). ** $p < 0.01$, compared with the corresponding group of -mannan. (C) Inhibition of cellular association of pDNA obtained by Bare-PEG_{2000} lipoplexes and Man-PEG_{2000} lipoplexes constructed with ^{32}P-labeled pDNA (5 μg pDNA) in addition of various endocytosis inhibitors at 2 hr after addition of each lipoplex. Each value represents the mean + SD ($n = 3$). ** $p < 0.01$, compared with the corresponding group of control.



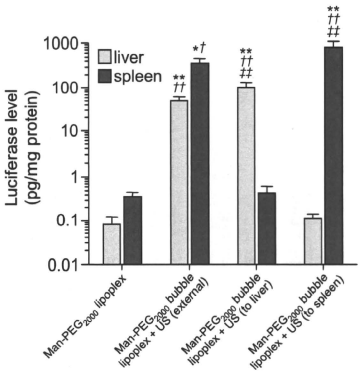
Supplementary Fig. 2. DNase I protection assay. Naked pDNA, Bare-PEG_{2000} lipoplexes and Man-PEG_{2000} lipoplexes were treated with DNase I at 37 °C for 15 min, and subjected to agarose gel electrophoresis. The samples were as follows: naked pDNA (lane 1 and 2); Bare-PEG_{2000} lipoplexes (lane 3 and 4); Man-PEG_{2000} lipoplexes (lane 5 and 6); non-treated with DNase I (lane 1, 3 and 5); treated with DNase I (lane 2, 4 and 6).



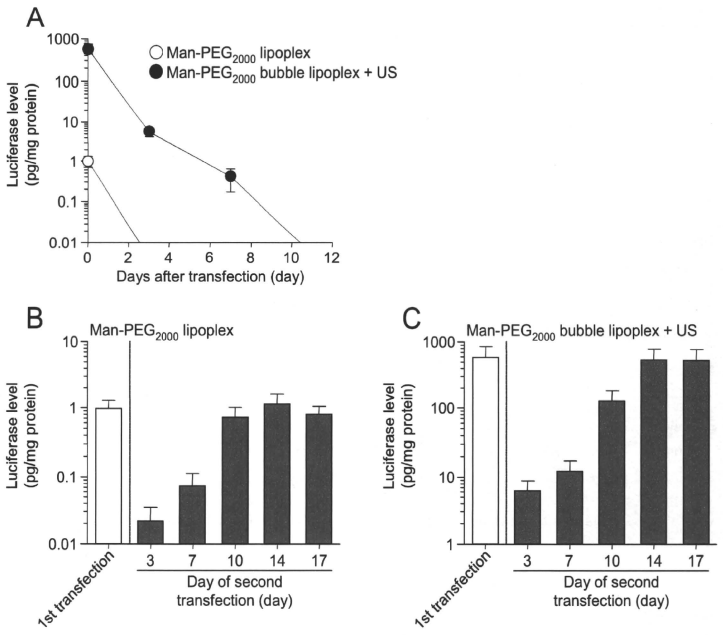
Supplementary Fig. 3. Photographs of Man-PEG_{2000} bubble lipoplexes and stability of Bare-PEG_{2000} bubble lipoplexes and Man-PEG_{2000} bubble lipoplexes against nuclease. (A) Man-PEG_{2000} bubble lipoplexes (right) were prepared by sonication of Man-PEG_{2000} lipoplexes (left) with supercharged perfluoropropane gas. (B) DNase I protection assay. Naked pDNA and lipoplexes were treated with DNase I, and then subjected to agarose gel electrophoresis. The samples were as follows: naked pDNA (lane 1 and 2); Bare-PEG_{2000} lipoplex (lane 3 and 7); Bare-PEG_{2000} bubble lipoplex (lane 4 and 8); Man-PEG_{2000} lipoplex (lane 5 and 9); Man-PEG_{2000} bubble lipoplex (lane 6 and 10); non-treated with DNase I (lane 1 and 3-6); treated with DNase I (lane 2 and 7-10).



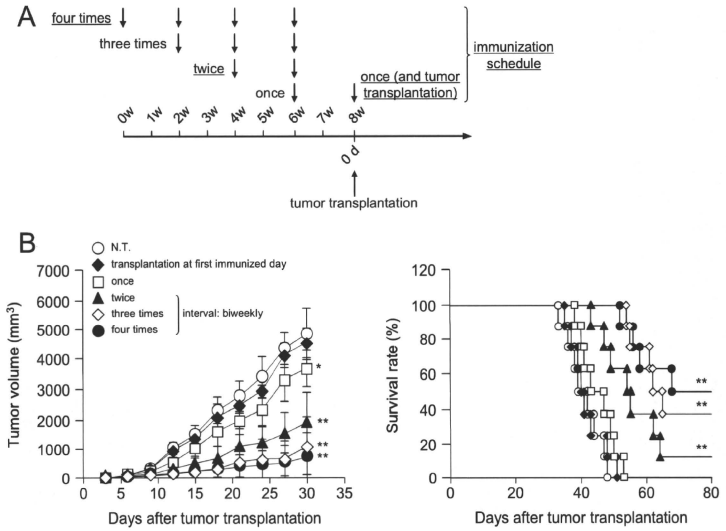
Supplementary Fig. 4. Enhancement of cellular association of pDNA by Man-PEG_{2000} bubble lipoplexes and US exposure in vitro. (A) Cellular association of ^{32}P-labeled pDNA by transfection using naked pDNA, naked pDNA + BLs with US exposure, Bare-PEG_{2000} lipoplexes, Bare-PEG_{2000} bubble lipoplexes with US exposure, Man-PEG_{2000} lipoplexes and Man-PEG_{2000} bubble lipoplexes with US exposure (5 μg pDNA) at 2 hr after addition of each lipoplex. Each value represents the mean + SD ($n = 3$). Significant difference; * $p < 0.05$; **, †† $p < 0.01$. (B) Cellular association by Bare-PEG_{2000} bubble lipoplexes with US exposure and Man-PEG_{2000} bubble lipoplexes with US exposure (5 μg ^{32}P-labeled pDNA) at 2 hr after addition of each lipoplex. Each value represents the mean + SD ($n = 3$). * $p < 0.05$, compared with the corresponding group of control.



Supplementary Fig. 5. The level of luciferase expression by Man-PEG_{2000} bubble lipoplexes and direct US exposure. Four-week-old ICR female mice received an intravenous injection of 400 μL Man-PEG_{2000} bubble lipoplexes using a 26-gauge syringe needle at a dose of 50 μg pDNA. After injection of Man-PEG_{2000} bubble lipoplexes, mice were operated the abdominal area under anesthesia. At 5 min after injection of Man-PEG_{2000} bubble lipoplexes, the organ was exposed directly to US using a Sonopore-4000 sonicator. US exposure conditions for the liver: frequency, 1.045 MHz; duty, 50%; burst rate, 10 Hz; intensity 1.0 W/cm^2; time, 2 min by using a 20 mm diameter probe, and for the spleen: frequency, 2.062 MHz; duty, 50%; burst rate, 10 Hz; intensity 4.0 W/cm^2; time, 2 min using a 6 mm diameter probe. At 6 hr after injection, mice were sacrificed and their organs collected for each experiment. The level of luciferase expression in the liver and spleen was determined at 6 hr after injection of Man-PEG_{2000} bubble lipoplexes. Each value represents the mean + SD ($n = 4$). * $p < 0.05$; ** $p < 0.01$, compared with the corresponding group of Man-PEG_{2000} lipoplex, † $p < 0.05$; †† $p < 0.01$, compared with the corresponding group of non-exposed US in each organ. ‡‡ $p < 0.01$, compared with the corresponding group of externally exposed US.



Supplementary Fig. 6. Repeated transfection by Man-PEG_{2000} bubble lipoplexes and US exposure. (A) Time-course of gene expression in the spleen by Man-PEG_{2000} lipoplexes and Man-PEG_{2000} bubble lipoplexes with US exposure. Each value represents the mean ± SD (n = 4). (B, C) The effect of transfection interval between first and second transfection on the level of gene expression by repeated transfection using (B) Man-PEG_{2000} lipoplexes and (C) Man-PEG_{2000} bubble lipoplexes with US exposure in the spleen. Mice were received the second transfection at 3, 7, 10, 14 and 17 days after first transfection. Each value represents the mean + SD (n = 4). The level of gene expression was sequentially decreased after transfection using Man-PEG_{2000} lipoplexes or Man-PEG_{2000} bubble lipoplexes with US exposure (Supplementary Fig. 6A). Therefore, it is considered that multiple transfections are essential to obtain more effective vaccine effects against cancer. However, following optimization of transfection interval, the levels of gene expression obtained by Man-PEG_{2000} lipoplexes or Man-PEG_{2000} bubble lipoplexes with US exposure in the second transfection were lower than that in first transfection when second transfection was performed at 3rd or 7th day after first transfection (Supplementary Figs. 6B and 6C). In this study, the level of gene expression in the second transfection reached to the same level as gene expression in the first transfection when second transfection was performed at 14th day after first transfection (Supplementary Figs. 6B and 6C).



Supplementary Fig. 7. Optimization of immunization times by Man-PEG_{2000} bubble lipoplexes and US exposure. (A) Schedule of optimization of immunization times by Man-PEG_{2000} bubble lipoplexes (50 μg pDNA) and US exposure. (B) Effects of the immunization times by Man-PEG_{2000} bubble lipoplexes and US exposure on DNA vaccine effects. Mice were immunized once, twice, three times and four times biweekly. At the immunized day or 2 weeks after last immunization, E.G7-OVA cells (1×10^6 cells) were transplanted subcutaneously into the back of mice. The tumor volume was calculated as follows: (long diameter) × (short diameter)^2 × 0.50. Each value represents the mean ± SD ($n = 8$) and the survival was monitored up to 80 days after tumor transplantation ($n = 8$). * $p < 0.05$; ** $p < 0.01$, compared with the corresponding group of N.T.. N.T., non-treatment.

Particle sizes and zeta potentials of liposomes and lipoplexes.

	Particle size (nm)	Zeta-potential (mV)
Bare-PEG_{2000} liposome (DSTAP:DSPC:NH_2-PEG_{2000}-DSPE=7:2:1 (mol))	96.7±2.2	61.9±1.4
Man-PEG_{2000} liposome (DSTAP:DSPC:Man-PEG_{2000}-DSPE=7:2:1 (mol))	95.6±2.5	63.0±2.4
Bare-PEG_{2000} lipoplex (DSTAP:DSPC:NH_2-PEG_{2000}-DSPE=7:2:1 (mol))	155.3±6.7	42.3±2.5
Man-PEG_{2000} lipoplex (DSTAP:DSPC:Man-PEG_{2000}-DSPE=7:2:1 (mol))	157.7±9.9	44.6±2.4

Supplementary Table 1. Particle sizes and zeta potentials of liposomes and lipoplexes. Each value represents the mean ± SD ($n = 3$).

Optimal lipid composition of lipoplexes to enclose the US imaging gas stably.

cationic lipid	neutral lipid	enclosing of US imaging gas
DSTAP	DSPC	+
	DPPC	+
	DOPE	-
	DOPC	-
DOTAP	DSPC	-
	DPPC	-
	DOPE	-
	DOPC	-
DOTMA	DSPC	-
	DPPC	-
	DOPE	-
	DOPC	-

Supplementary Table 2. Optimal lipid composition of lipoplexes to enclose US imaging gas stably. To optimize the lipid composition of lipoplexes to enclose US imaging gas (perfluoropropane gas) stably, lipoplexes constructed with several types of phospholipids were prepared and evaluated whether US imaging gas was enclosed into prepared lipoplexed stably or not. The enclosing of US imaging gas was identified by the cloudiness of lipoplex solution. The lipid composition of lipoplexes was cationic lipid:neutral lipid:Man-PEG_{2000}-DSPE (7:2:1 (molar ratio)), and the charge ratio of pDNA (pCMV-Luc) and liposomes was 1.0:2.3 (-:+). US imaging gas was enclosed as described in *Materials and Methods* section. +; enclosing US imaging gas stably, -; not enclosing US imaging gas.

Cationic lipids:

DSTAP; 1,2-stearoyl-3-trimethylammoniumpropane (18/0 (carbon chain/the number of double bonds), saturated, Tm > 50 °C),

DOTAP; 1,2-dioleoyl-3-trimethylammoniumpropane (18/1, unsaturated, Tm < 0 °C),

DOTMA; 1,2-di-*o*-octadecenyl-3-trimethylammonium propane (18/1, unsaturated, Tm < 0 °C).

Neutral lipids:

DSPC; 1,2-distearoyl-*sn*-glycero-3-phosphocholine (18/0, saturated, Tm = 55 °C),

DPPC; 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (16/0, saturated, Tm = 41 °C),

DOPE; 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (18/1, unsaturated, Tm = -16 °C),

DOPC; 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (18/1, unsaturated, Tm = -20 °C).

Particle sizes and zeta potentials of bubble liposomes and bubble lipoplexes.

	Particle size (nm)	Zeta-potential (mV)
Bare-PEG_{2000} bubble liposome (DSTAP:DSPC:NH_2-PEG_{2000}-DSPE=7:2:1 (mol))	504±7.4	61.4±2.6
Man-PEG_{2000} bubble liposome (DSTAP:DSPC:Man-PEG_{2000}-DSPE=7:2:1 (mol))	505±21	60.8±1.8
Bare-PEG_{2000} bubble lipoplex (DSTAP:DSPC:NH_2-PEG_{2000}-DSPE=7:2:1 (mol))	568±13	45.3±3.1
Man-PEG_{2000} bubble lipoplex (DSTAP:DSPC:Man-PEG_{2000}-DSPE=7:2:1 (mol))	569±12	44.8±2.1

Supplementary Table 3. Particle sizes and zeta potentials of bubble liposomes and bubble lipoplexes. Each value represents the mean ± SD (n = 3).

Supplementary Materials and Methods

Gel electrophoresis of pDNA.

15 μL of naked pDNA and lipoplexes (1.5 μg pDNA) were incubated with 5 μL DNase I solution (10 μg/mL in DNase I buffer consisted of 100 mM Tris, 25 mM $MgCl_2$ and 5 mM $CaCl_2$) for 15 min at 37 °C. The digestion by DNase I was terminated by addition of 100 mM EDTA (5 μL). Electrophoresis was carried out with 1% agarose gel prepared with 1 × TAE buffer at a constant voltage of 100 V for 30 min. The band of DNA was detected by ethidium bromide and visualized using a LAS-3000 imaging system (FUJIFILM Co., Tokyo, Japan).

Evaluation of cellular association of pDNA.

The cultured macrophages were plated on a 12-well culture plate at a density of 5×10^5 cells/3.8 cm^2. After 72 hr incubation, the macrophages were washed twice with Hank's balanced salt solution (HBSS, pH 7.4), and the culture medium was replaced with 1 mL HBSS containing lipoplexes constructed with ^{32}P-labeled pDNA ([α-^{32}P]-dCTP, PerkinElmer, Inc., MA, USA). At predetermined time after incubation, the solution was immediately removed and the macrophages were washed five times with ice-cold HBSS. The macrophages were solubilized with 0.3 M NaOH solution containing 10% Triton X-100, and the resultant cell lysates were dissolved in Soluene-350 (PerkinElmer, Inc., MA, USA). Then, the resultant lysates were decolorized with isopropanol and 30% H_2O_2, and neutralized with 5 N HCl. The radioactivity of ^{32}P-labeled pDNA was measured in scintillation counter (LSA-500, Beckman Coulter, Inc., CA, USA) after addition of Clear-Sol I solution. The protein concentration was determined with a Protein Quantification Kit (Dojindo Molecular Technologies, Inc., Tokyo, Japan).

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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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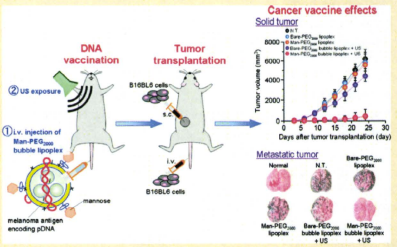
Supporting Information

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-PEG_{2000} bubble lipoplexes. Following immunization using US exposure and Man-PEG_{2000} 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-PEG_{2000} 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 APCs[32] and developed a gene transfection method for DNA vaccination using US-responsive and mannose-modified gene carriers, Man-PEG_{2000} 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-PEG_{2000} 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-PEG_{2000} bubble lipoplexes and US exposure. First, we examined the level of gene expression in the splenic dendritic cells by gene transfer using Man-PEG_{2000} 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-PEG_{2000} 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-PEG_{2000} 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-PEG_{2000} 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] (NH_2-PEG_{2000}-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% CO_2. 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-PEG_{2000} Bubble Lipoplexes. Man-PEG_{2000} bubble lipoplexes were constructed according to our previous report.[33] Briefly, DSTAP, DSPC and NH_2-PEG_{2000}-DSPE or mannose-modified PEG_{2000}-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,