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Secure Splenic Delivery of Plasmid DNA and Its Application to DNA Vaccine

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In this experiment, we developed a novel safe and effective gene delivery vector coated with γ-polyglutamic acid (γ-PGA-coated complexes). The γ-PGA-coated complex was composed of chiseled spherical nano-particles with anionic charges. The plasmid DNA/polyethyleneimine complex (non-coated complex) showed high transgene efficiency in the spleen and lung after intravenous administration in mice, with high liver toxicity and lethality. On the other hand, γ-PGA-coated complex selectively showed high transgene efficiency in the spleen without such toxicity. Furthermore, the γ-PGA-coated complex highly accumulated and showed high gene expression in the marginal zone of the spleen. Those results strongly indicated that γ-PGA-coated complex was suitable as a DNA vaccine vector. We therefore applied γ-PGA-coated complex to melanoma DNA vaccine, pUb-M. The γ-PGA-coated complex containing pUb-M significantly inhibited the growth and metastasis of a melanoma cell line, B16-F10 cells. In conclusion, we developed a splenic gene vector, γ-PGA-coated complex, as a novel technology for clinical vaccination.

Key words gene delivery; ternary complex; spleen; DNA vaccine; melanoma

Gene therapy is a new therapeutic strategy that offers the promise of treating intractable disease such as cancer, infectious diseases, innate immunodeficiency, and cardiovascular diseases.[1–3] The success of gene therapy largely depends on the delivery efficiency of the therapeutic gene. Gene delivery vectors are categorized as viral or non-viral vectors. Although viral vectors enable highly efficient gene delivery, they cause associated immune responses and oncogenic transformations with several reported fatal cases in clinical trials.[4,5] Non-viral gene delivery mediated by cationic liposomal and polymeric vectors has emerged as an attractive alternative because of their low immunogenicity, a clear structure, and easy modeling.[6,7]

Among non-viral gene vectors, polyethyleneimine (PEI) is a popular cationic polymer and show high gene expression in *in vitro* and *in vivo*, because of specific mechanisms such as binding to the cell surface, being taken up by the endocytotic pathway, and release of plasmid DNA (pDNA) from endosomes *via* the so-called "proton sponge mechanism." On the other hand, PEI caused nonspecific gene expressions, high cytotoxicity, and aggregation with blood components because of their strong cationic charge.

Recharging cationic complex with anionic compound was reported to be a promising method for overcoming these toxicities. In the previous study, we found that coating biodegradable anionic polymers reduced the cytotoxicity and agglutination of pDNA/PEI complex. Among them, a ternary complex coated with γ-polyglutamic acid (γ-PGA) and chondroitin sulfate showed strong gene expression *in vitro*, regardless of their safety.[8,9] Secure and effective gene delivery vector such as a ternary complex coated with γ-PGA suggests its suitability for *in vivo* gene delivery.

In the present study, we evaluated gene expression and cytotoxicity of γ-PGA-coated complex *in vivo* and discovered that the γ-PGA-coated complex showed selectively strong gene expression in the marginal zone of the spleen after safe intravenous administrations into mice. We therefore applied this splenic delivery system to DNA vaccination for melanoma. We used pUb-M which expresses melanoma-related antigen (gp100 and tyrosinase-related protein 2 (TRP2)) as melanoma DNA vaccine. The complex was constructed with pUb-M, PEI, and γ-PGA. DNA vaccination markedly suppressed tumor growth and metastasis without liver injury and acute toxicity, which are observed in commonly used vectors. We demonstrated a novel splenic delivery system may be applied to a DNA vaccine.

MATERIALS AND METHODS

Chemicals PEI (branched form, average molecular weight 25000), G418, and fluorescein isothiocyanate (FITC) were purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.). γ-PGA was provided by Yakult Pharmaceutical Industry Co., Ltd. (Tokyo, Japan). FITC–PEI was prepared in our laboratory. Briefly, PEI and FITC were dissolved in dimethylsulfoxide and stirred overnight at room temperature in the dark. FITC–PEI was purified by gel filtration. Almost 1.0% PEI nitrogen was labeled with FITC. All other chemicals were of the highest purity available.

Construction of pDNA Plasmid DNA encoding luciferase reporter gene (pCMV)-luciferase (pCMV-Luc) was constructed by subcloning the *Hin*dIII/*Xba*I firefly luciferase cDNA fragment from the pGL3-control vector (Promega, Madison, WI, U.S.A.) into the polylinker of the pcDNA3 vector (Invitrogen, Carlsbad, CA, U.S.A.). Red fluorescent protein encoding pDNA (ptdTomato-N1) was purchased from Clon-

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tech (Palo Alto, CA, U.S.A.). pUb-M was kindly provided by Prof. Reisfeld.[10] The pDNA was amplified using an EndoFree Plasmid Giga Kit (QIAGEN GmbH, Hilden, Germany).

Cells The mouse melanoma cell line, B16-F10 cells, was obtained from the Cell Resource Center for Biomedical Research Institute of Development, Aging and Cancer Tohoku University, Japan. B16-F10 cells were maintained in RPMI 1640 supplemented with 10% fetal bovine serum (FBS) and antibiotics under a humidified atmosphere of 5% CO_2 in air at 37°C. B16-F10 cells expressing luciferase regularly (B16-F10-Luc cells) were prepared in our laboratory. Briefly, to establish B16-F10-Luc cells, B16-F10 cells were transfected with pCMV-Luc and selected by G418.

Preparation of Complexes For the preparation of the non-coated complex, pDNA solution and PEI solution (pH 7.4) were mixed by pipetting thoroughly and left for 15 min at room temperature. The γ-PGA-coated complex was constructed by mixing γ-PGA and non-coated complex with pipetting and left for another 15 min at room temperature. In this study, we constructed complexes at a theoretical charge ratio: pDNA phosphate–PEI nitrogen–γ-PGA carboxylate=1 : 8 : 0 or 1 : 8 : 6, according to the previous report.[8]

Physicochemical Property of the Complexes Transmission electron microscopy (JEM-1210; JEOL, Tokyo, Japan) was used to observe the configuration of the complexes. The particle size and ζ-potential of the complexes were measured using a Zetasizer Nano ZS (Malvern Instruments, Ltd., Malvern, U.K.). The number-fractioned mean diameter is shown.

Animals Animal care and experimental procedures were performed in accordance with the Guidelines for Animal Experimentation of Nagasaki University with approval from the Institutional Animal Care and Use Committee. Male ddY mice and C57BL/6Cr mice (5 weeks old) were purchased from Japan SLC (Shizuoka, Japan).

***In Vivo* Transfection Experiments** The mice were injected intravenously with non-coated and γ-PGA-coated complexes containing 40 μg pDNA at a volume of 200 μL per mouse. At 6, 12, and 24 h after injection, the mice were sacrificed and the liver, kidney, spleen, heart, and lung were dissected. The tissues were homogenized in lysis buffer (pH 7.8 and 0.1 M Tris/HCl buffer containing 0.05% Triton X-100 and 2 mM ethylenediaminetetraacetic acid (EDTA)). The homogenates were centrifuged at 15000 rpm (Kubota 3700; Kubota, Tokyo, Japan) for 5 min. Ten microliters of supernatants were mixed with 50 μL luciferase assay buffer (Picagene, Toyo Ink, Tokyo, Japan) and the light produced was immediately measured using a luminometer (Lumat LB 9507, EG & G Berthold, Bad Wildbad, Germany). Luciferase activity is indicated as relative light units (RLUs) per gram of tissue.

To visualize the accumulation and gene expression of the complexes, the mice were administrated with γ-PGA-coated complexes constructed with ptdTomato-N1 and FITC–PEI as described above. Twenty-four hours after injection, the spleen was dissected. Sectioning and staining were entrusted to GenoStaff as described above. The relative levels of FITC–PEI and ptdTomato-N1 expressions in the spleen were characterized using fluorescent microscopy (10× magnification, Leica MZ16, Leica Microsystems, Tokyo, Japan).

Vaccination and Tumor Growth Inhibition The mice were injected intravenously with 60 μg naked pUb-M, γ-PGA-coated complexes containing 60 μg pCMV-Luc, and γ-PGA-coated complexes containing 60 μg pUb-M 4 times biweekly. Control mice were administrated 5% glucose solution. For the assessment of tumor growth, two weeks after the last administration, mice were administrated with 1×10^5 B16-F10 cells subcutaneously and tumor growth was monitored. Tumor volume (mm^3) was calculated as follows: major axis×minor axis$^2\div2$.

For the assessment of metastasis, the mice were injected intravenously with 5% glucose solution and γ-PGA-coated complexes containing 60 μg pUb-M 4 times biweekly. Two weeks after the last immunization, mice were administrated with 1×10^5 B16-F10-Luc cells intravenously and lung metastasis and survival rate were monitored. For evaluation of lung metastasis, three weeks after administration, some mice were sacrificed, the lung was dissected and luciferase activities were evaluated as described above.

***In Vivo* Toxicological Experiments** The ddY mice were injected intravenously with non-coated and γ-PGA-coated complexes containing 60 μg pDNA. At 6 h after injection, the mice were sacrificed and blood samples were obtained. The activities of aspartate transaminase (AST) and alanine transaminase (ALT) in the serum were determined with biochemical test kits (Wako Pure Chemical Industries, Ltd., Osaka, Japan).

The complexes were administrated daily to the mice for 7 d and the liver was dissected 24 h after the last administration. The samples were fixed in 20% formalin and then sliced and stained with hematoxylin–eosin (HE) for morphologic examination. Sectioning and staining were entrusted to GenoStaff (Tokyo, Japan). To assess their lethality, complexes containing 200 μg pDNA were administrated to the mice and their survival was observed for 2 weeks.

Statistical Analysis Statistical significance between two groups was identified by the Mann–Whitney *U*-test. Multiple comparisons among groups were made by Dunnett's pairwise multiple comparison *t*-test. Statistical significance of survivals and survival time were identified by chi-square test and log-rank test, respectively.

RESULTS

Physicochemical Properties of the Complexes Physicochemical properties of γ-PGA-coated complexes were compared to non-coated complexes. Transmission electron microscopy (TEM) images, particle size, and ζ-potential of non-coated and γ-PGA-coated complexes are shown in Fig. 1 and Table 1. Non-coated complex was observed as clumped nano-particles in the TEM of 102.8±2.9 nm particle size and +50.2±0.4 mV ζ-potential (Fig. 1a), while γ-PGA-coated complex had chiseled spherical nano-particles of 76.4±0.4 nm particle size and −36.7±0.7 mV ζ-potential (Fig. 1b).

***In Vivo* Transgene Efficiency of the Complexes** Mice were intravenously administrated complexes containing pCMV-Luc and their transgene efficiency in organs such as the liver, kidney, spleen, heart, and lung was evaluated at 6, 12, and 24 h after administration (Figs. 2a–c, respectively). Non-coated complex showed high transgene efficiency at more than 1.0×10^6 RLU/g tissue in the spleen and lung; however, γ-PGA-coated complex showed significantly lower transgene efficiency than non-coated complex in the lung ($p<0.05$). At the same time, γ-PGA-coated complex showed high gene

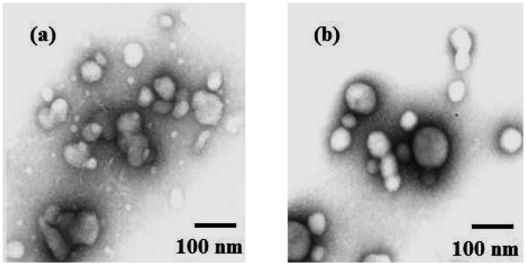

Fig. 1. Transmission Electron Microscopy (TEM) Image of Non-coated Complex (a) and γ-PGA-Coated Complex (b)

Table 1. Sizes and ζ-Potentials of the Complexes

Complex	Size (nm)	ζ-Potential (mV)
Non-coated complex	102.8±2.9	+50.2±0.4
γ-PGA-coated complex	76.4±0.4	−36.7±0.7

Data are the mean±S.E.M.

expression only in the spleen and it was significantly higher than non-coated complex at 6 h after administration ($p<0.05$). For detailed assessment of the gene delivery of γ-PGA-coated complex in the spleen, γ-PGA-coated complex containing ptdTomato-N1 and FITC–PEI was administrated to mice and the spleen was dissected 24 h after administration (Fig. 3). HE-stained section is showed in Fig. 3d: blue-stained and red-stained regions are white and red pulp, respectively. The marginal zone is the region at the interface between the red pulp and the white pulp of the spleen. Accumulation (green dot, Fig. 3a) and gene expression (red dot, Fig. 3b) of the complex were synchronously observed as orange dots (Fig. 3c) in the marginal zone of the spleen.

Suppression Effect of the Complexes on Melanoma γ-PGA-coated complex containing melanoma DNA vaccine, pUb-M, was administrated to mice and the immune response against melanoma cell line B16-F10 was evaluated with intradermal transplant and metastatic models (Figs. 4, 5, and 6). Naked pUb-M and γ-PGA-coated complex containing pCMV-Luc did not inhibit tumor growth in the intradermal transplant model. On the other hand, γ-PGA-coated complex containing pUb-M significantly inhibited tumor growth ($p<0.01$, Fig. 4). In the metastatic model, γ-PGA-coated complex containing pUb-M also inhibited the lung metastasis of B16-F10 cells and metastasis cell number was significantly decreased ($p<0.05$, Fig. 5). Furthermore, γ-PGA-coated complex containing pUb-M significantly improved the survival time of metastasis model mice ($p<0.01$, Fig. 6).

***In Vivo* Toxicity of the Complexes** Each complex was administrated to mice and the *in vivo* toxicity of the complexes, such as liver toxicity and lethality, was evaluated. Non-coated complex markedly increased AST and ALT, as shown in Figs. 7a and 7b. On the other hand, mice injected with γ-PGA-coated complex showed lower AST and ALT than with non-coated complex. Furthermore, the effect of multiple doses of the complexes was determined by histological experiments (Fig. 8). The liver of non-coated complex-treated mice showed high hepatopathy (Fig. 8a) and necrosis in the HE-stained section (Figs. 8b, c); however, γ-PGA-coated complex did not cause these toxicities (Figs. 8d–f). A large amount of the complexes was administrated to mice and their survival

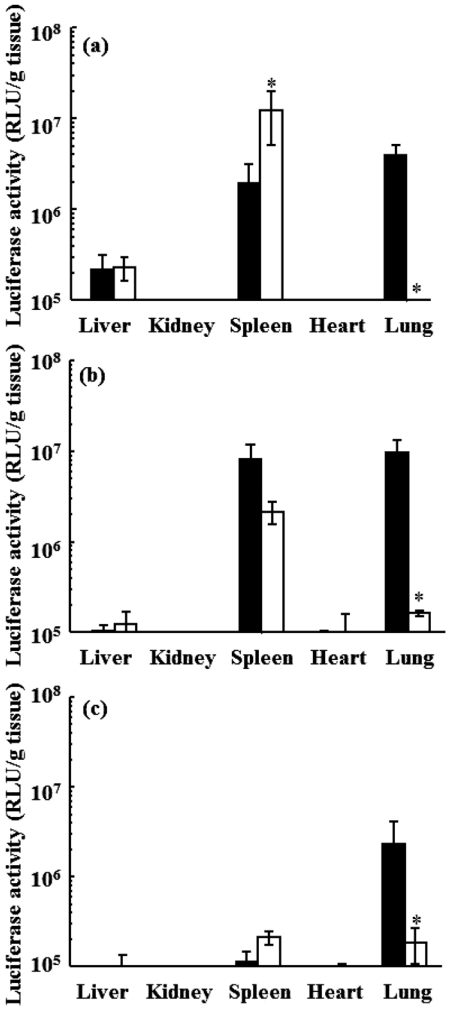

Fig. 2. Transfection Efficiency of the Complexes 6 (a), 12 (b), and 24 (c) h after Administration

Mice were administrated non-coated complex (■) and γ-PGA-coated complex (□) containing pCMV-Luc. At the appropriate time after administration, mice were sacrificed and the liver, kidney, spleen, heart, and lung were dissected. Gene expressions were determined as luciferase activity. Data are the mean±S.E.M. *$p<0.05$ *vs.* control.

was observed (Table 2). All mice administrated γ-PGA-coated complex survived; however, approximately 56% mice receiving non-coated complex died ($p<0.05$).

DISCUSSION

Generally, DNA is fragile under *in vivo* conditions; however, cationic polymer or liposome can stabilize DNA by encapsulating it in nanoparticles. In a previous study, we confirmed the stability of γ-PGA-coated complex by agarose electrophoresis. Furthermore, we observed the complexes with TEM, as shown in Fig. 1. The γ-PGA-coated complexes were observed as nano-sized particles with anionic charges. The particle size of γ-PGA-coated complexes is smaller than non-coated complex. The γ-PGA may be able to strongly compact the PEI complex by the electrostatic interaction. The overall charge ratio of γ-PGA-coated complex is +1; however, the ζ-potential of the ternary complex was apparently negative, suggesting the concentrated distribution of anionic polymers outside of the particles (Table 1).

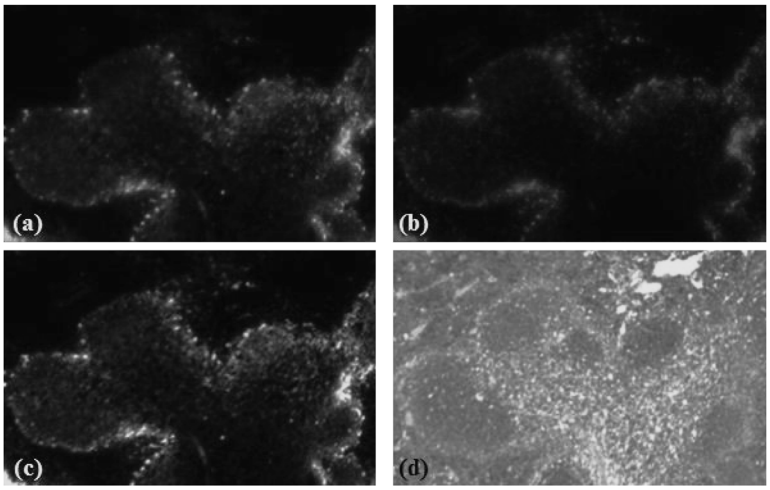

Fig. 3. Observation of a Spleen Section Dissected from a Mouse Administrated γ-PGA-Coated Complex

The mouse was administrated γ-PGA-coated complex containing ptdTomato-N1 and FITC–PEI. Twenty-four hours after administration, the spleen was removed. The localization of FITC–PEI (a), gene expressions of ptdTomato (b), merged picture (c), and HE-stained section (d) are shown (100× magnifications). (Color images were converted into gray scale.)

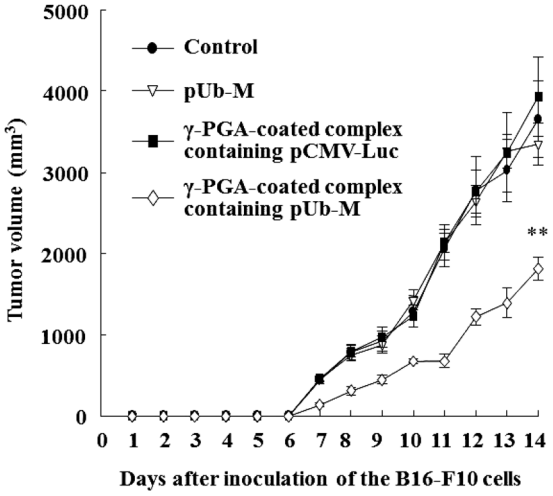

Fig. 4. Tumor Growth of Tumor-Bearing Mice Administrated Various Melanoma Vaccines

Mice were administrated the various vaccines biweekly 4 times. Two weeks after the last administration, B16-F10 cells were administrated to mice intradermally and tumor growth was monitored. Data are the mean±S.E.M. $^{**}p<0.01$ *vs.* control.

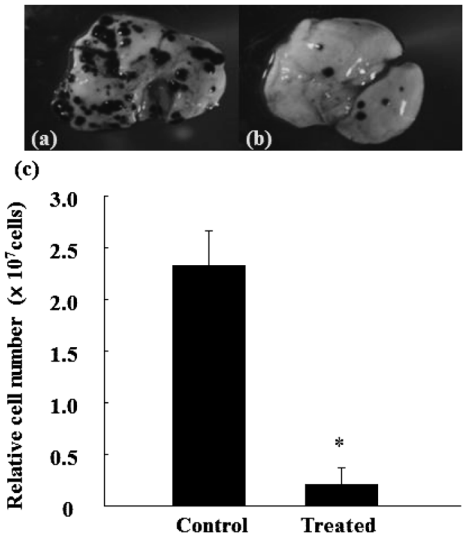

Fig. 5. Lung Metastasis of Tumor-Bearing Mice Administrated γ-PGA-Coated Complex Containing pUb-M

Mice were administrated the γ-PGA-coated complex containing pUb-M biweekly 4 times. Two weeks after the last administration, B16-F10-Luc cells were administrated to mice intravenously and the lungs were dissected three weeks after administration. The lungs were observed (a and b) and homogenized for luciferase assay (c). (a); control mice (b); the mice treated with γ-PGA-coated complex containing pUb-M. The relative cell number was calculated from luciferase activity in the lung (c). Data are the mean±S.E.M. $^{*}p<0.05$ *vs.* control.

Anionic particles are not usually taken up well by cells because they repulse the cellular membrane electrostatically. In previous reports, γ-PGA complex showed significantly higher uptake than the non-coated complex. Also, we found that γ-PGA complex was taken up by γ-PGA-specific receptor-mediated endocytotic pathway.[8,11] We discovered that the γ-PGA-coated complex showed high gene expression selectively in the marginal zone of the spleen (Figs. 2, 3). Sutherland *et al.* reported that poly-γ-D-glutamic acid (γDPGA), which is a capsular component of *Bacillus anthracis*, was mainly accumulated in the spleen and liver after intravenous injection into mice.[12,13] By the same mechanism, the γ-PGA-coated complex should accumulate and show high gene expressions in the spleen. The spleen is the largest secondary immune organ in the body and is responsible for initiating immune reactions to blood-borne antigens and for filtering the blood of foreign material and old or damaged red blood cells.[14] Dysfunction of the spleen results in an increased risk of infection.

The splenic gene delivery system is a promising approach for DNA vaccination or gene therapy for splenic disease. In particular, vaccinations are now attracting attention to prevent various infectious diseases and to treat cancer and autoimmune diseases. Furthermore, DNA vaccines have distinct advantages such as ease of rapid mass production and low cost for worldwide usage, even in impoverished regions.[15,16] A novel approach to improve the efficacy of DNA vaccines is to develop a vaccine vector which enables the delivery of DNA to antigen-presenting cells (APCs) effectively.[17,18] The marginal zone of the spleen is known to be rich in APCs, such as dendritic cells and macrophages.[19] The γ-PGA-coated complex should improve the transgene efficiency of DNA vac-

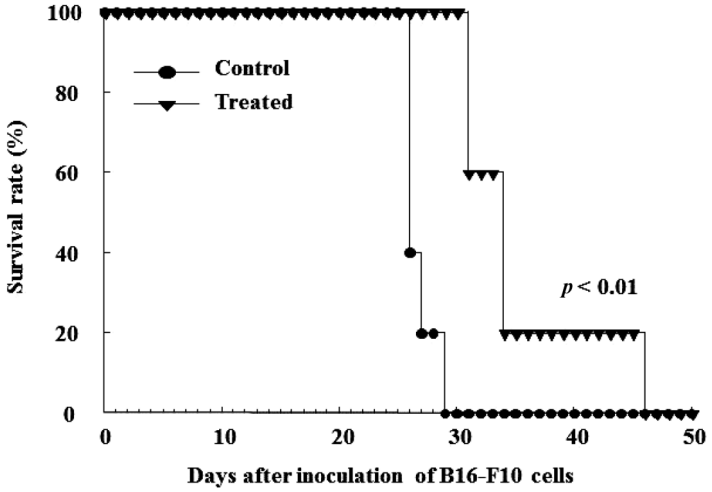

Fig. 6. Effects of γ-PGA-Coated Complex Containing pUb-M on Survival Time of Tumor-Bearing Mice

Mice were administrated the γ-PGA-coated complex containing pUb-M biweekly 4 times. Two weeks after the last administration, B16-F10 cells were administrated to mice intravenously and survival was monitored.

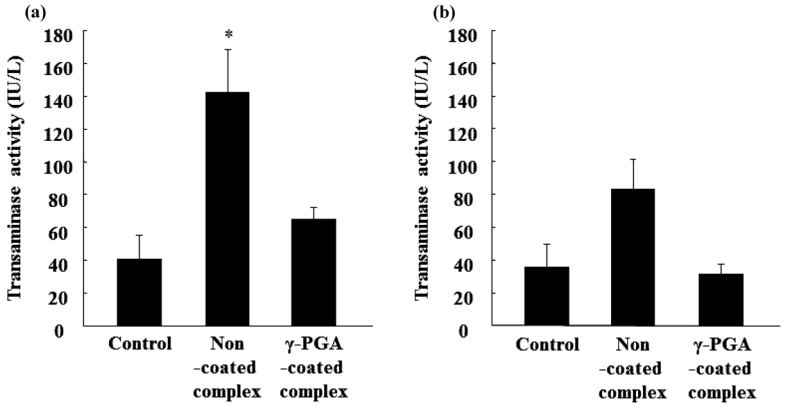

Fig. 7. AST (a) and ALT (b) Values of Mice Injected with Each Complex

The mice were administrated with each complex. Six hours after administration, blood was collected and serum AST and ALT values were measured. Each value is the mean±S.E.M. *$p<0.05$ *vs.* control.

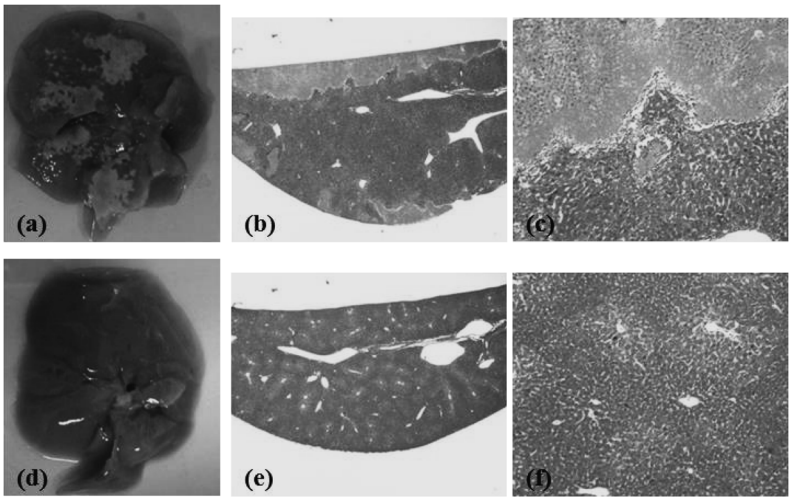

Fig. 8. Observation of the Liver Dissected from Mice Administrated Non-coated Complex (a–c) and γ-PGA-Coated Complex (d–f)

The mice were administrated daily with each complex for 7 d. Twenty-four hours after final administration, the mice were sacrificed and the liver was dissected. The livers (a and d) and HE-stained sections (b, c, e, and f) were observed. (b) and (e): 20 ×, (c) and (f): 100× magnifications.

Table 2. Survival and Death of Mice Treated with the Complexes

	Non-coated complex	γ-PGA-coated complex
Dead	5	0
Alive	4	10

cine on APCs in the marginal zone of the spleen with higher immune responses.

Melanoma is a malignant neoplasm of melanocytes most frequently arising from the skin and is known as highly metastatic cancer that is markedly resistant to conventional therapy.[20–23] On the other hand, melanoma is among the most immunogenic of all solid cancers and some antigens against melanoma, such as tyrosinase-related protein families, glycoprotein 100, and melanoma-associated antigen families have been reported.[24,25] Therefore, it is considered that DNA vaccine against melanoma is suitable for not only the prevention of metastasis and relapse but also suppression of tumor growth. To achieve potent therapeutic effects by DNA vaccine against cancer, it is essential to transfer the antigen-coding gene selectively and efficiently into APCs. In this experiment, we successfully developed a new type of safe DNA vaccine that effectively suppressed the growth of melanoma cell line B16-F10 after intravenous administrations (Fig. 4). Furthermore, it effectively suppressed the metastasis of B16-F10 cells administered intravenously to mice and improved the survival time of metastasis model mice (Figs. 5, 6). This new type of vaccination was effective without any adjuvant.

The commonly used non-viral gene delivery vector with cationic charges is reported to show high cytotoxicity and aggregation.[26,27] In previous studies, we confirmed that γ-PGA-coated complexes did not show cytotoxicity and agglutination, whereas the non-coated complex did.[8,11] Furthermore, it was previously reported that the PEI-mediated gene delivery vector caused liver necrosis and shock after intravenous administration.[28] So, we could not administer the non-coated complex in this melanoma vaccine study. Actually, the non-coated complex showed high liver toxicity and approximately 56% mice administrated a large amount of non-coated complex died (Figs. 7, 8, and Table 2). At the same time, γ-PGA-coated complex did not cause liver toxicity or death. The γ-PGA is a water-soluble, nontoxic, nonimmunogenic exopolymer.[29] Those results could be explained by γ-PGA on the surface of the complex being a biocompatible polymer, reducing the cationic charge of non-coated complex, and defusing interactions with the cellular membrane and biological components.

For the development of novel vaccines, *in vivo* safety is fundamental, and effective uptake of the vaccine into APCs enables the manufacture of a large amount of vaccine from a small amount of antigen. The γ-PGA-coated complex could deliver DNA vaccine to APCs in the marginal zone of the spleen effectively and safely. Thus, we developed a safe and effective splenic gene delivery vector, γ-PGA-coated complex, and showed its possibility of DNA vaccination.

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