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Splenic Gene Delivery System Using Self-assembling Nano-complex with Phosphatidylserine Analog

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The recognition of phosphatidylserine on the erythrocyte membrane mediates erythrophagocytosis by resident spleen macrophages. The application of phosphatidylserine to a gene vector may be a novel approach for splenic drug delivery. Therefore, we chose 1,2-dioleoyl-*sn*-glycero-3-phospho-L-serin (DOPS) as an analogue of phosphatidylserine for splenic gene delivery of plasmid DNA (pDNA). In the present study, we successfully prepared a stable pDNA ternary complex using DOPS and polyethyleneimine (PEI) and evaluated its efficacy and safety. The pDNA/PEI complex had a positive charge and showed high transgene efficacy, although it caused cytotoxicity and agglutination. The addition of DOPS changed the ζ-potential of the pDNA/PEI complex to negative. It is known that anionic complexes are not taken up well by cells. Surprisingly, however, the pDNA/PEI/DOPS complex showed relatively high transgene efficacy *in vitro*. Fluorescence microscope observation revealed that the pDNA/PEI/DOPS complex internalized the cells while maintaining the complex formation. The injection of the pDNA/PEI complex killed most mice within 24 h at high doses, although all mice in the pDNA/PEI/DOPS complex group survived. The ternary complex with DOPS showed markedly better safety compared with the pDNA/PEI complex. The pDNA/PEI/DOPS complex showed high gene expression selectively in the spleen after intravenous injection into mice. Thus the ternary complex with DOPS can be used to deliver pDNA to the spleen, in which immune cells are abundant. It appears to have an excellent safety level, although further study to determine the mechanism of action is necessary.

Key words gene delivery; spleen; 1,2-dioleoyl-*sn*-glycero-3-phospo-L-serin; plasmid DNA; ternary complex

In gene delivery, non-viral vectors, including cationic polymers, have several advantages, *e.g.*, they are non-immunogenic; cause few acute toxicities; and their structural and chemical properties can be tightly controlled, which allows vehicles that are suitable for mass production to be designed.[1,2] Among non-viral gene vectors, polyethylenimine (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.

Phosphatidylserine is a biocompatible phospholipid that has a negative charge at biological pH and is a component of the cellular membrane. In normal cells, phosphatidylserines are restricted in the inner leaflet of the cellular membrane by the function of ATP-dependent transporters.[3–5] In contrast, in apoptotic cells or aging erythrocytes, phosphatidylserines exposed on the cell surface was recognized and ingested by dendritic cells and macrophages.[6–8] The spleen is rich in immune cells such as macrophages and dendritic cells, and a functional spleen is essential for the immune response.[9,10] Splenic hyperfunction, which is caused by splenomegaly, presents with symptoms such as anemia or a bleeding tendency.[11]

The application of this phosphatidylserine to the gene vector may be novel approaches to improve the uptake of DNA by spleen. Therefore, we chose 1,2-dioleoyl-*sn*-glycero-3-phospho-L-serin (DOPS) as an analog of phosphatidylserine to splenic gene delivery of pDNA. In the present study, we successfully prepared stable pDNA ternary complexes using DOPS and PEI and evaluated their efficacy and safety.

MATERIALS AND METHODS

Chemicals PEI (branched form, average molecular weight 25000) and rhodamine B isothiocyanate were purchased from Aldrich Chemical Co. (Milwaukee, WI, U.S.A.). DOPS was obtained from NOF Co. (Tokyo, Japan). Fetal bovine serum (FBS) was purchased from Biological Industries Ltd. (Kibbutz Beit Haemek, Israel). RPMI 1640, Opti-MEM I, antibiotics (penicillin 100 U/mL and streptomycin 100 μg/mL), and other culture reagents were obtained from GIBCO BRL (Grand Island, NY, U.S.A.). The 2-(4-iodophenyl)-3-(4-nitrophenyl)-2*H*-tetrazolium, monosodium salt (WST-1) and 1-methoxy-5-methylphenazinium methylsulfate (1-methoxy PMS) were obtained from Dojindo Laboratories (Kumamoto, Japan). Hoechst 33342 and *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine, triethylammonium salt (NBD-PE) were purchased from Molecular Probes (Leiden, the Netherlands). Rhodamine-PEI (Rh-PEI) and fluorescein isothiocyanate (FITC)-PEI were prepared in our laboratory. Briefly, PEI and rhodamine B isothiocyanate or FITC were dissolved in dimethylsulfoxide and stirred overnight at room temperature in the dark. Rh-PEI and FITC-PEI were purified by gel filtration. Then, 1.5% and 1.0% of PEI nitrogen were labeled with rhodamine B and FITC, respectively. All other

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chemicals were of the highest purity available.

Construction of pDNA The 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 Clontech (Palo Alto, CA, U.S.A.). The pDNA was amplified using an EndoFree Plasmid Giga Kit (QIAGEN GmbH, Hilden, Germany). The pDNA was dissolved in 5% dextrose solution to 1 mg/mL and stored at −80°C until analysis.

Preparation of Complexes An appropriate amount of stock PEI solution (pH 7.4) was mixed with a diluted solution of pDNA by pipetting thoroughly to prepare the pDNA/PEI complex and was incubated for 15 min at room temperature.

DOPS liposomes were prepared according to a previous report.[12] The lipids were dissolved in chloroform and dried as a thin film in a test tube using an evaporator at 25°C, and then vacuum-desiccated for approximately 3 h. The film was resuspended in 5% sterile dextrose. After hydration, the dispersions were sonicated at 100 W (sonicator; Ohtake Works Co., Tokyo, Japan) for 3 min on ice. The resulting liposomes were extruded 11 times through double-stacked 100 nm polycarbonate membrane filters.

The DOPS liposome solution was mixed with the pDNA/PEI complex solution by pipetting thoroughly and was incubated for a further 15 min at room temperature. In this study, we constructed these complexes at a theoretical charge ratio: phosphate of pDNA: nitrogen of PEI: phosphate of DOPS=1 : 8 : 0 (pDNA/PEI complex), 1 : 8 : 2 (pDNA/PEI/DOPS2 complex), 1 : 8 : 4 (pDNA/PEI/DOPS4 complex), 1 : 8 : 6 (pDNA/PEI/DOPS6 complex), and 1 : 8 : 8 (pDNA/PEI/DOPS8 complex).

For the evaluation of intracellular distribution, DOPS liposomes containing 0.05 mol % of NBD-PE were constructed.

Physicochemical Properties 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.

To determine complex formations, 10 μL aliquots of complex solutions containing 1 μg pDNA were mixed with 2 μL loading buffer (30% glycerol and 0.2% bromophenol blue) and loaded onto a 0.8% agarose gel. Electrophoresis (i-Mupid J; Cosmo Bio, Tokyo, Japan) was carried out at 50 V in running buffer solution (40 mM Tris–HCl, 40 mM acetic acid, and 1 mM ethylenediaminetetraacetic acid (EDTA)) for 60 min. The retardation of pDNA was visualized with ethidium bromide staining.

Cell Culture 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. The cells were maintained in RPMI 1640 supplemented with 10% FBS and antibiotics (culture medium) under a humidified atmosphere of 5% CO_2 in air at 37°C.

WST-1 Assay Cytotoxicity tests of various complexes on B16-F10 cells were carried out using a WST-1 commercially available cell proliferation reagent. WST-1 reagent was prepared (5 mM WST-1 and 0.2 mM 1-methoxy PMS in PBS) and filtered through a 0.22 μm filter (Millex-GP; Millipore Co, Bedford, MA, U.S.A.) just before the experiments. B16-F10 cells were plated on 96 well plates (Becton-Dickinson, Franklin Lakes, NJ, U.S.A.) at a density of 3.0×10^3 cells/well in the culture medium. Each complex containing 1 μg pDNA in 100 μL Opti-MEM I (transfection medium) was added to each well and incubated for 2 h. After incubation, the medium was replaced with 100 μL culture medium and incubated for another 22 h. The medium was replaced with 100 μL culture medium, and 10 μL WST-1 reagent was added to each well. The cells were incubated for an additional 2 h at 37°C, and absorbance was measured at a wavelength of 450 nm with a reference wavelength of 630 nm, using a microplate reader (Multiskan Spectrum; Thermo Fisher Scientific, Inc., Waltham, MA, U.S.A.). The results are shown as a percentage of untreated cells.

***In Vitro* Transfection Experiment** The cells were plated on 24-well plates (Becton-Dickinson) at a density of 1.0×10^4 cells/well and cultivated in 0.5 mL culture medium. In the transfection experiment, after 24 h preincubation, the medium was replaced with 0.5 mL transfection medium, and each complex containing 0.5 μg pDNA was added to the cells and incubated for 4 h. After transfection, the medium was replaced with culture medium, and cells were cultured for a further 20 h at 37°C. After 20 h incubation, the cells were washed with PBS and then lysed in 100 μL lysis buffer (pH 7.8 and 0.1 M Tris–HCl buffer containing 0.05% Triton X-100 and 2 mM EDTA). Ten microliters of lysate samples 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). The protein content of the lysate was determined by the Bradford assay using BSA as the standard. Absorbance was measured using a microplate reader at 595 nm. Luciferase activity was indicated as relative light units (RLU) per mg protein.

Intracellular Distribution of Ternary Complex To observe the intracellular distribution of pDNA/PEI/DOPS6 complex, B16-F10 cells were transfected as described above with pDNA complexed with Rh-PEI and DOPS liposome including NBD-PE. At 20 h after transfection, cells were incubated with Hoechst 33342 containing culture medium for 30 min to visualize nuclei and then the medium was replaced, and fluorescence distribution of Rh-PEI, NBD-PE labeled DOPS liposome, and Hoechst 33342 was observed with fluorescent microscopy (400× magnification; BIOREVO BZ-9000; KEYENCE, Osaka, Japan). The tone of each image was adjusted and overlapped to give a merged picture by digital processing.

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 (5–6 weeks old) were purchased from Japan SLC (Shizuoka, Japan). After shipping, mice were acclimatized to the environment for at least one day before the experiments.

Agglutination Study Erythrocytes from mice were washed three times at 4°C by centrifugation at 5000 rpm (Kubota 3700; Kubota, Tokyo, Japan) for 5 min and then resuspended in PBS. A 2% (v/v) stock suspension was prepared for the agglutination study. The pDNA/PEI complex and pDNA/PEI/DOPS complexes were added to the erythrocytes (complexes: stock suspension=1 : 1). The suspensions were

incubated for 15 min at room temperature. A 10 μL sample of suspension was placed on a glass plate, and agglutination was observed by microscopy (400× magnification).

***In Vivo* Study** To evaluate the acute toxicity of pDNA/PEI and pDNA/PEI/DOPS6 complexes, each complex containing 200 μg pDNA at a volume of 1.5 mL per mouse was slowly injected into mice *via* the tail vein. The same volume of saline was also injected as a control. Twenty four hours after administration, surviving mice were counted. Damage to the liver and lung was observed macroscopically.

To examine the transgene efficacy of pDNA/PEI/DOPS6 complex, the mice were injected intravenously with pDNA/PEI/DOPS6 complex containing 40 μg pDNA at a volume of 300 μL per mouse. At 6 h following injection, the mice were sacrificed, and the liver, kidney, spleen, heart, and lung were dissected. The tissues were homogenized in lysis buffer. The homogenates were centrifuged at 15000 rpm for 5 min. The supernatants were used for luciferase assays, as described above. Luciferase activity was indicated as RLU per gram of tissue.

To visualize the accumulation and gene expression of the complexes, the mice were administered pDNA/PEI/DOPS6 complex constructed with ptdTomato-N1 and FITC-PEI. Twenty-four hours after injection, the spleen, liver, and lung were dissected. The samples were fixed in 20% formalin and then sliced and stained with hematoxylin–eosin for morphologic examination. Sectioning and staining were entrusted to GenoStaff (Tokyo, Japan). The relative levels of FITC-PEI and ptdTomato-N1 expressions in the spleen, liver, and lung were characterized using fluorescent microscopy (10× magnification, Leica MZ16; Leica Microsystems, Tokyo, Japan).

Statistical Analysis Statistical comparisons were made with Dunnett's or Tukey test after an ANOVA.

Table 1. Particle Size and ζ-Potential of the Complexes

Complex	Size (nm)	ζ-Potential (mV)
pDNA/PEI	40.9±4.0	+45.9±0.7
pDNA/PEI/DOPS2	88.6±0.8**	+46.2±0.1
pDNA/PEI/DOPS4	142.9±10.7**	−12.3±0.2**
pDNA/PEI/DOPS6	66.0±1.1*	−29.8±0.9**
pDNA/PEI/DOPS8	48.4±0.1	−33.3±0.4**

Each value represunts the mean±S.E. of three experiments.
*: $p<0.05$, **: $p<0.01$ *vs.* pDNA/PEI.

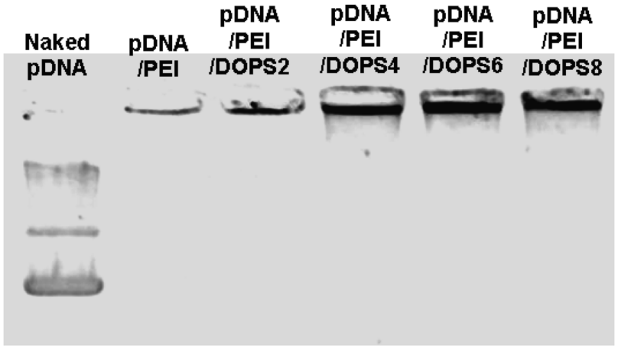

Fig. 1. Effect of DOPS Liposomes on Electrophoretic Migration of pDNA through an Agarose Gel

Each complex was loaded onto agarose gel, and electrophoresis was carried out. Retardation of pDNA was visualized using ethidium bromide.

RESULTS

Physicochemical Properties and Electrophoresis Assay The sizes and ζ-potentials of various complexes are summarized in Table 1. The pDNA/PEI complex showed 40.9±4.0 nm particle size. The complexes with DOPS had significantly larger particles than pDNA/PEI complex ($p<0.01$). Except for pDNA/PEI/DOPS4 complex, all complexes had less than 100 nm particles. The pDNA/PEI and pDNA/PEI/DOPS2 complexes showed positive ζ-potentials. The further addition of DOPS to pDNA/PEI complex decreased ζ-potentials. The pDNA/PEI/DOPS4, pDNA/PEI/DOPS6, and pDNA/PEI/DOPS8 complexes showed an anionic charge.

Complex formations were examined using a gel retardation assay (Fig. 1). Naked pDNA was detected as bands on agarose gel. On the other hand, in the lanes of pDNA/PEI complex and pDNA/PEI/DOPS2-8 complexes, no band was detected.

Cellular Toxicity For the evaluation of cytotoxicity, each complex was added to B16-F10 cells and cell viability was evaluated by WST-1 assay (Fig. 2). The pDNA/PEI complex showed significantly higher cellular toxicity than the control ($p<0.01$). On the other hand, the addition of DOPS decreased the cytotoxicity of pDNA/PEI complex, and pDNA/PEI/DOPS complexes with a charge ratio greater than 2 did not substan-

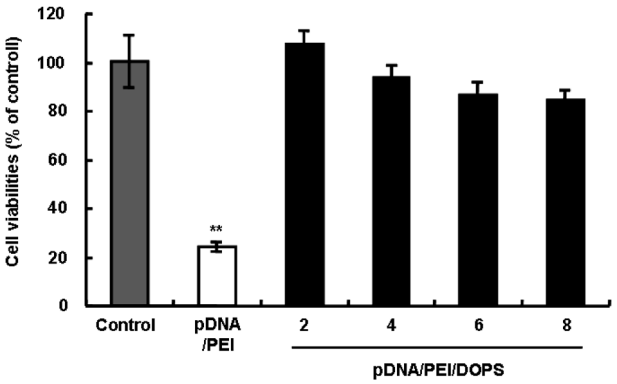

Fig. 2. Cytotoxicity Tests of Anionic Ternary Complexes and pDNA/PEI Complexes on B16-F10 Cells

Viability of cells treated with each complex was measured by the WST-1 assay. Cells were incubated with various complexes for 2 h, and cell viability was measured 22 h after treatment. Data are the percentage to untreated cells. Each value is the mean±S.E. of twelve experiments. **: $p<0.01$ *vs.* control.

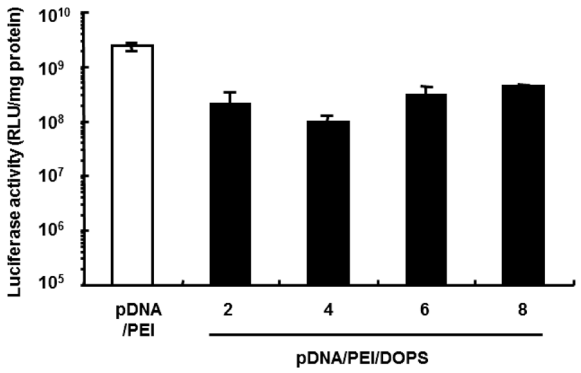

Fig. 3. Transfection Efficacy of Anionic Ternary Complexes and pDNA/PEI Complexes

B16-F10 cells were transfected with complexes containing pCMV-Luc. Twenty hours after transfection, luciferase activity was evaluated. Each value is the mean±S.E. of three experiments.

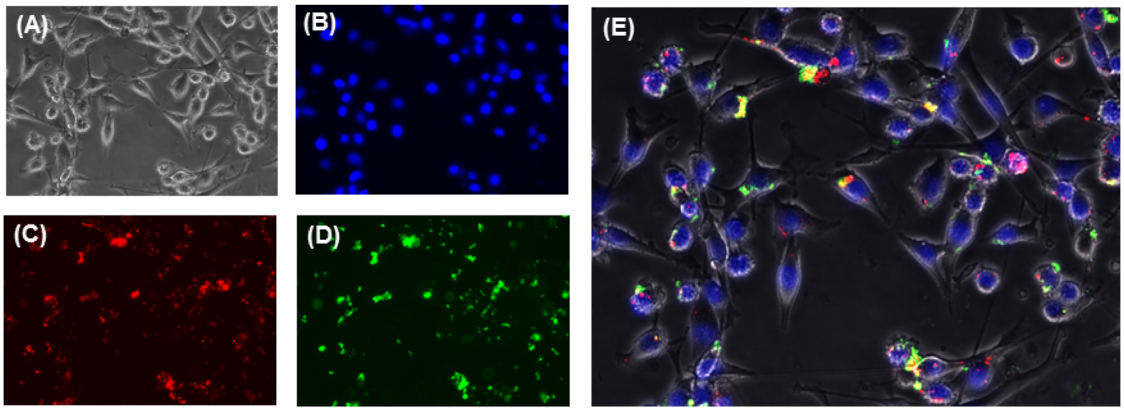

Fig. 4. Intracellular Distribution of pDNA/PEI/DOPS6 Complex

(A) Phase contrast image; (B) Nuclei staining with Hoechst 33342; (C) Rh-PEI; (D) NBD-PE-labeled DOPS liposome; (E) Merged image (400× magnification).

tially affect the cell viability of B16-F10 cells.

***In Vitro* Transfection Experiment** B16-F10 cells were transfected with various complexes, and luciferase activities were evaluated (Fig. 3). The pDNA/PEI complex had high transgene efficacy (2.45×10^9 RLU/mg protein). The pDNA/PEI/DOPS complexes also showed high transgene efficacies ($1.01–4.32\times10^8$ RLU/mg protein), although their transgene efficacies were lower than those of the pDNA/PEI complex. On the basis of the results of physicochemical properties, the WST-1 assay, and *in vitro* transfection experiments, we performed further studies on the properties of the pDNA/PEI/DOPS6 complexes.

Intracellular Distribution of Ternary Complex The intracellular distribution of pDNA/PEI/DOPS6 complex was observed with fluorescent microscopy in B16-F10 cells. The pDNA/PEI/DOPS6 complex was visualized by Rh-PEI, NBD-PE, and Hoechst 33342 (Fig. 4). Red dots of Rh-PEI and green dots of NBD-PE-labeled DOPS liposomes were located together mainly near the blue dots of cell nuclei. Synchronized red dots with green dots were seen as orange or yellow dots in merged images, as shown in Fig. 4E.

Agglutination Study Before the *in vivo* study, the pDNA/PEI or pDNA/PEI/DOPS6 complex was added to erythrocytes to evaluate agglutination (Fig. 5). The pDNA/PEI complex showed severe agglutination on microscopy but no agglutination was observed in the pDNA/PEI/DOPS6 complex.

***In Vivo* Study** *In vivo* acute toxicity of complexes was evaluated in mice. Surviving mice were counted 24 h after intravenous administration of pDNA/PEI or pDNA/PEI/DOPS6 complexes to ddY mice. All mice survived the administration of pDNA/PEI/DOPS6 complex or saline although 8 of 9 mice died after administration of pDNA/PEI complex (Fig. 6A). Damage to the liver and lung was observed macroscopically in surviving mice (Figs. 6B, C). The liver and lung of one surviving mouse administered pDNA/PEI complex were reddish and damaged with thrombi. In contrast, no severe damage was shown in the liver and lung of mice administered pDNA/PEI/DOPS6 complex or saline.

The pDNA/PEI/DOPS6 complex was administered to ddY mice, and their transgene efficacies in the liver, kidney, spleen, heart, and lung were evaluated (Fig. 7). The pDNA/PEI/DOPS6 complex showed markedly high gene expression selectively in the spleen. pDNA/PEI/DOPS6 complex containing ptdTomato-N1 and FITC-PEI was also administrated to ddY mice to visualize cellular uptake and gene expression. The liver, spleen and lung were dissected 24 h after injection and were observed with fluorescent microscopy (Fig. 8). Accumulation (green dot) and gene expression (red dot) of the complex were synchronously observed as orange dots in the spleen and liver.

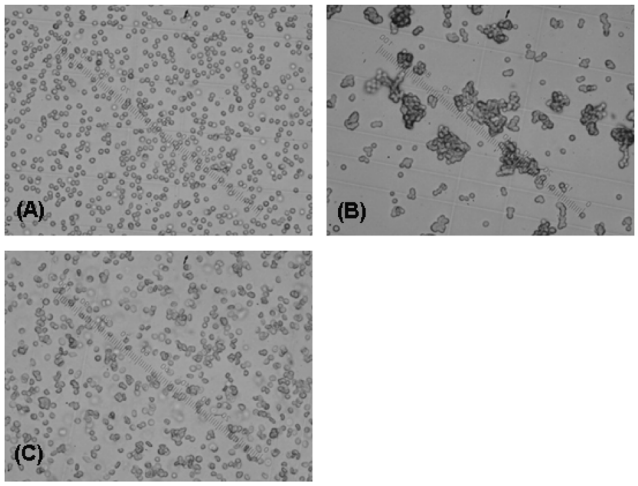

Fig. 5. Agglutination of Complexes with Erythrocytes

Each complex was added to erythrocytes, and agglutination was observed by microscopy (400× magnification). (A) PBS; (B) pDNA/PEI complex; (C) pDNA/PEI/DOPS 6 complex.

DISCUSSION

In the apoptosis process, asymmetry of lipid bilayers was disrupted and phosphatidylserine, which is usually restricted to the inner membrane layer, appeared on the surface of the membrane.[3–5] This exposed phosphatidylserine is recognized by phagocytes *via* multiple receptors and apoptotic cells are engulfed.[6–8] Similar phenomena are seen when removing aging erythrocytes.[9] Macrophages or immature dendritic cells in the spleen perform these functions.[13,14] We focused on this phenomenon and hypothesized that pDNA vector with phosphatidylserine analog must be taken up by the spleen and would be useful for gene therapy.

Therefore, we tried to develop a self-assembling complex with pDNA and DOPS as phosphatidylserine analog; however, it is difficult to assemble the complex with a negatively

(A)

	Saline	pDNA/PEI	pDNA/PEI/DOPS6
Dead	0	8	0
Alive	10	1	10
Total	10	9	10

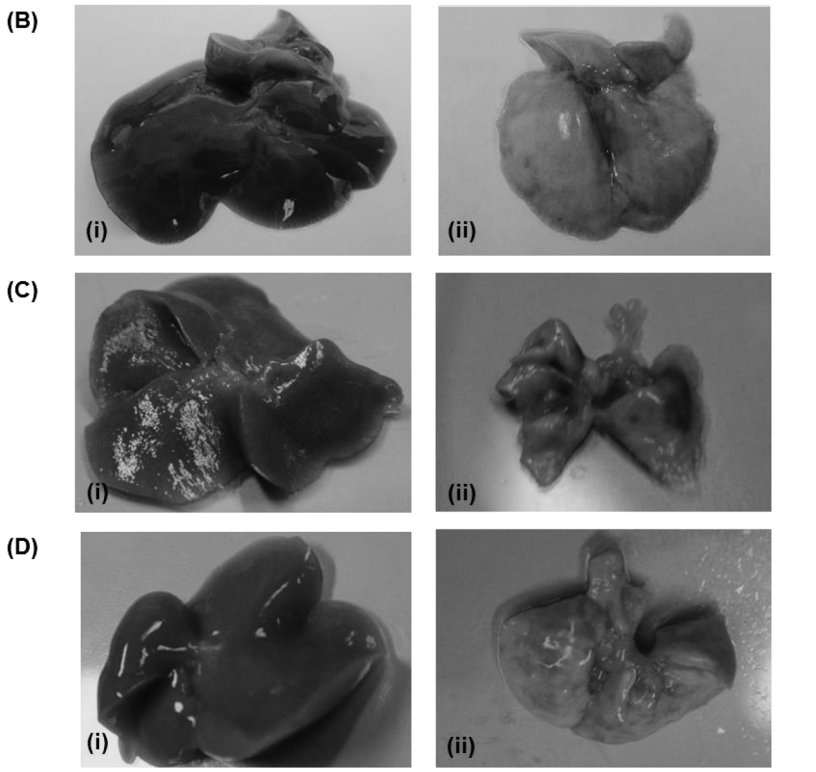

Fig. 6. Evaluation of Acute Toxicity of Complexes

Death of mice 24 h after administration of saline and each complex (A) and macroscopic observations of the liver (i) and lung (ii) dissected from saline-treated mouse (B), pDNA/PEI complex-treated mouse (C), and pDNA/PEI/DOPS6 complex-treated mouse (D).

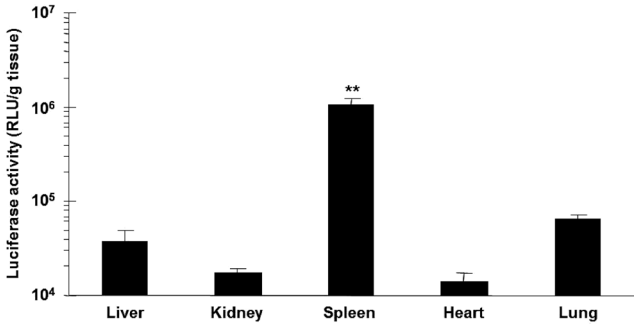

Fig. 7. Transfection Efficacy of pDNA/PEI/DOPS6 Complex in Mice

The complex containing pCMV-Luc was administrated intravenously to mice (40 μg pDNA per mouse). At 6 h after administration, mice were sacrificed and each organ was dissected for quantification of luciferase activity. Each value is the mean±S.E. of ten experiments. **: $p<0.01$ *vs.* other organs.

charged pDNA and negatively charged lipid, DOPS. We therefore developed a method using PEI to associate pDNA with DOPS. PEI is a common cationic polymer forming a complex with pDNA and is reported to show high gene expression *in vitro* and *in vivo*.[15–17] We used pDNA/PEI complex at a charge ratio of 1 : 8 among various charge ratios because of its high transgene efficacy shown in preliminary experiments. The pDNA/PEI complex was mixed with DOPS as liposomes.

The DOPS liposomes were prepared by thin-film hydration with the sonication method and had approximately 50 nm particle size and −55 mV ζ-potential. The pDNA/PEI complex had 40.9±4.0 nm particle size and +45.9±0.7 mV ζ-potential (Table 1). The addition of DOPS to the pDNA/PEI complex increased the particle size and decreased the ζ-potential, reaching a plateau at a charge ratio of 1 : 8 : 6 (−29.8 to −33.3 mV). These results suggested that the proportion of free DOPS-liposomes was minimal for the pDNA/PEI/DOPS6 complex. In addition, size distribution results did not show small particles of free DOPS-liposomes in this condition (data not shown). Therefore, we think the characteristics of complex were hardly influenced by free DOPS-liposomes. Also, the low ζ-potential suggested that DOPS liposomes were situated on the surface of the complex. Most ternary complexes were below 100 nm (nano-size) except for pDNA/PEI/DOPS4 complex. Negative DOPS liposomes may attenuate the compaction of the pDNA/PEI complex. In the preliminary experiment, a negative polymer such as heparin dissociated the pDNA/PEI complex and released pDNA. The retention of pDNA in the ternary complexes, however, was confirmed by the gel retardation assay (Fig. 1). We successfully prepared nano-sized and negative charged ternary complex, which were constructed with pDNA, PEI, and DOPS by electrostatic and hydrophobic bonds.

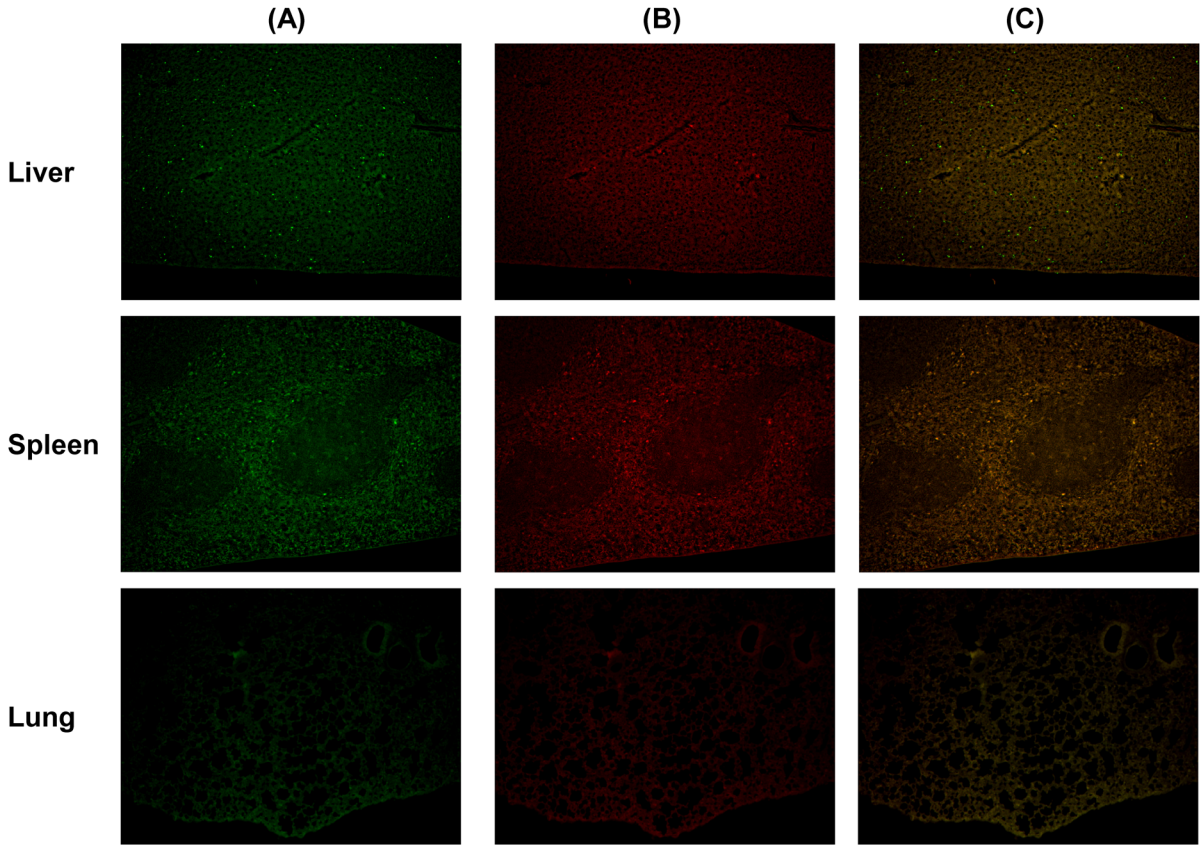

Fig. 8. Observation of the Liver, Spleen, and Lung Dissected from a Mouse Administered pDNA/PEI/DOPS6 Complex

The mouse was administered pDNA/PEI/DOPS6 complex containing ptdTomato-N1 and FITC-PEI. Twenty-four hours after administration, the liver, spleen, and lung were removed. The localization of FITC-PEI (A), gene expressions of ptdTomato (B), and the merged picture (C) are shown.

On the other hand, most anionic complexes are well known not to be taken up well by cells because they repulse the cellular membrane electrostatically. Therefore, high transgene efficacy cannot be expected by a negatively charged vector of pDNA; however, surprisingly, pDNA/PEI/DOPS complexes showed high transgene efficacy although their transgene efficacy was lower than that of pDNA/PEI complex (Fig. 3). Furthermore, we visualized the intracellular distribution of the pDNA/PEI/DOPS complex in B16-F10 cells by using Rh-PEI, NBD-PE-labeled DOPS liposome, and Hoechst 33342. Our complexes were constructed using electrostatic interaction, rather than a chemical approach. The preparation of complexes is greatly influenced by the amount of fluorescence. In the preliminary study, we prepared several fluorescently-labeled pDNA, PEI, and DOPS and constructed several complexes. Among them, we selected the complexes which are stable particles physicochemically and have high fluorescence detection sensitivity. PEI and DOPS of the ternary complex were located together mainly near the nuclei inside cells, suggesting that the nanoparticle internalized the cell, maintaining ternary complex formation and achieving gene expression (Fig. 4). Also, the red, green, and yellow signals were 80% of cells in the visual field. Some researchers reported that anionic charged gene vector modified with anionic ligands such as hyaluronic acids (HA) and folic acid (FA) showed high transgene expression.[18,19] These vectors were taken up by HA or FA receptor-mediated endocytosis. The anionic pDNA/PEI/DOPS complex also may be specifically taken up *via* a DOPS-related pathway although further studies are necessary to confirm the mechanism.

In the preliminary study, pDNA/PEI complexes showed no cytotoxicity under the transfection experiment conditions. However, the pDNA/PEI complex showed severe cytotoxicity and agglutination (Figs. 2, 5) under the severe conditions. The injection of pDNA/PEI complex killed most mice within 24 h at a high dose (Fig. 6), although all mice survived after the injection of pDNA/PEI complex under the *in vivo* transgene expression experiment conditions (data not shown). One surviving mouse showed severe damage to the liver and lung. There are many reports about the severe cytotoxicity of pDNA/PEI complex because of its positive charge, which causes strong interactions with the negatively charged cellular membrane.[20–22] Cationic gene vectors was also reported to interact with erythrocytes, resulting in adverse events, such as embolism or inflammatory reactions.[20,23]

Surface modifications of cationic complexes with anionic polymers, such as hyaluronic acid and glycosaminoglycans have been used to overcome their toxicities. The pDNA/PEI/DOPS complexes with negative surface charge also showed no cytotoxicity and reduced agglutination activities (Figs. 2, 5) even under the severe conditions. All mice also survived the high-dose pDNA/PEI/DOPS6 complex. Many types of phosphatidylserine analogs are also common and safe supplements.[24–26] The pDNA/PEI/DOPS6 complex is therefore expected to be a highly safe and effective gene delivery vector for *in vivo* use. In fact, pDNA/PEI/DOPS6 complex showed markedly high transgene efficacy in the spleen 6 h and 24 h after intravenous administration to ddY mice (Figs. 7, 8).

On the other hand, we previously reported that pDNA/PEI showed high gene expression in the liver, spleen, and lung non-selectively.[27] The spleen is rich in immune cells such as macrophages and dendritic cells, which play a crucial role in immune function and the removal of aging erythrocytes.[9,10,13] We also reported that a pDNA/PEI/γ-polyglutamic acid complex, which also induced strong gene expression (more than 1×10^6 RLU/g tissue) in the spleen, was an effective DNA vaccine against malaria and melanoma.[27,28] pDNA/PEI/DOPS complex also showed gene expression at about 1×10^6 RLU/g tissue in the spleen. The pDNA/PEI/DOPS complex might be effective as DNA vaccine, although further study is necessary.

Thus, we successfully developed a ternary complex with DOPS that is safe with high transgene efficacy under *in vitro* and *in vivo* conditions. This complex is expected to be a useful tool for spleen-specific gene delivery.

CONCLUSION

We developed a stable pDNA/PEI/DOPS complex as a promising gene delivery system to the spleen. The pDNA/PEI/DOPS complex showed excellent safety *in vitro* and *in vivo*. Fluorescence microscope observation revealed that the pDNA/PEI/DOPS complex internalized the cell, maintaining ternary complex formation. After intravenous injection, the pDNA/PEI/DOPS complex showed markedly high transgene efficacy in the spleen. This complex may be useful for gene therapy, although, further study, including transfection experiment using other cell lines, which are non-immune cells such as HeLa and NIH3T3 cells, is necessary to reveal the cell-selectivity of pDNA/PEI/DOPS complexes.

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Conflict of Interest The authors declare no conflict of interest.

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