Ternary Complex of Plasmid DNA Electrostatically Assembled with Polyamidoamine Dendrimer and Chondroitin Sulfate for Effective and Secure Gene Delivery

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The purpose of this study was to develop a ternary complex of plasmid DNA (pDNA) electrostatically assembled with polyamidoamine (PAMAM) dendrimer and chondroitin sulfate (CS) for effective and secure gene delivery. PAMAM dendrimers are new cationic polymers that are expected to be used as gene delivery vectors. However, cationic non-viral gene vectors showed cytotoxicity by binding to negative cellular membranes. We therefore prepared a ternary complex by adding CS, an anionic polymer, and examined its usefulness. The pDNA/PAMAM dendrimer complex (PAMAM dendriplex) and the PAMAM dendriplex coated by CS (CS complex) showed nanoparticles with positive ζ-potential and negative ζ-potential, respectively. The CS complex had no cytotoxicity against B16-F10 cells and no agglutination activity, although severe cytotoxicity and high agglutination were observed in the PAMAM dendriplex. As a result of an *in vitro* gene expression study of B16-F10 cells, not only the PAMAM dendriplex but also the CS complex showed high transfection efficiency. The transfection efficiency of the CS complex was significantly inhibited by clathrin-mediated endocytosis inhibitor (chlorpromazine), caveolae-mediated endocytosis inhibitor (genistein), and hypothermia. Tail-vein injection of the CS complex into mice led to significantly higher gene expression in the spleen than the PAMAM dendriplex. Thus, the ternary complex of pDNA electrostatically assembled with PAMAM denriplex and CS showed safe high gene expression in the spleen. This vector is expected to be useful for useful gene delivery.

Key words ternary complex; chondroitin sulfate; polyamidoamine dendrimer; gene delivery

Gene therapy is expected to be an effective method to treat cancer, infection, innate immunodeficiency and cardiovascular diseases.[1–4] The success of gene therapy is largely dependent on the development of vectors capable of effectively delivering foreign genes into targeted cells. One major approach in gene therapy is based on cationic polymers, such as polyethylenimine (PEI), polylysine, polyarginine, and chitosan.[5–8] When cationic polymer-encapsulated plasmid DNA (pDNA) makes association with the cell surface, it enters the cells by endocytosis.

Polyamidoamine (PAMAM) dendrimers are new cationic polymers, which are highly branched radial polymers that have specific and systematically variable size, shape and chemical structure. Their radical structure contains a 2-carbon ethylenediamine core and primary amino groups on the surface. Successive generations (G) have increasing diameter and double the surface functional amino groups of the preceding generation.[9,10] PAMAM dendrimers can be used as carriers for pDNA and show high transfection efficiency.[11,12] The pDNA/PAMAM dendrimer complexes (PAMAM dendriplex) are formed by electrostatic interactions and initiate cell entry through binding to anionic phospholipids on the cell membrane. With increasing generations of dendrimers, the PAMAM dendriplex showed higher transfection efficiency, which depended on the charge ratio and concentration.[11,13]

On the other hand, cationic non-viral gene vectors show cytotoxicity by binding non-specifically to negatively charged proteoglycans on cell membranes.[14] Cationic non-viral gene vectors also agglutinate with negatively charged blood components, such as erythrocytes and serum albumin. These agglutinations often cause adverse events, such as rapid elimination, embolization, and inflammatory reactions. The toxic pathway of the cationic PAMAM dendriplex has been confirmed as localization in the mitochondria, leading to reactive oxygen species (ROS) production and resulting in oxidative stress, apoptosis, and DNA damage.[15,16]

One promising approach for overcoming the disadvantages of cationic vectors is capsulation of the cationic vector with anionic polymer electrostatically.[17,18] Encapsulation of the cationic vector with anionic polymer was reported to show high transfection efficiency and safe vectors.[19,20] Among anionic polymers, chondroitin sulfate (CS) is highly biocompatible and has been used for tablets, eye drops, cosmetics, and medical applications.[21–25] Moreover, some reports have shown that CS has anti-inflammatory activities and anticancer activities.[26–28] Therefore, we developed a new gene vector of the PAMAM dendriplex coated by CS (CS complex) and evaluated its usefulness.

MATERIALS AND METHODS

Chemicals PAMAM dendrimer based on an ethylenediamine core of five generation, G5 PAMAM dendrimer (molecular weight (MW) 28826 Da, 128 N-terminal amines) was purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.). The CS (average molecular weight (MW) 22925 Da and specific dispersion (MW/MN 1.5)) and bovine serum albumin (BSA) were also purchased from Sigma-Aldrich. Fetal bovine serum (FBS) was obtained from Biosource International Inc. (Camarillo, CA, U.S.A.). 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,

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NY, U.S.A.). The 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt (WST-1), and 1-methoxy-5-methylphenazinium methylsulfate (1-methoxy PMS) were obtained from Dojindo Laboratories (Kumamoto, Japan).

Preparation of pDNA pCMV-Luc was constructed by subcloning the HindIII/XbaI firefly luciferase cDNA fragment from the pGL3-control vector (Promega, Madison, WI, U.S.A.) into the polylinker of the pcDNA vector (Invitrogen, Carlsbad, 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 as 1 mg/mL and stored at $-80°C$ until analysis.

Preparation of Complexes The theoretical charge ratio of cationic dendrimer to pDNA was calculated as the molar ratio of PAMAM dendrimers nitrogen to pDNA phosphate. To prepare the PAMAM dendriplex, an appropriate amount of stock polymer solution was mixed with a diluted solution of pDNA by pipetting thoroughly at charge ratios of 0.5, 1, 2, 4, 6, and 8 for the PAMAM dendriplex and left for 30 min at room temperature. For pharmaceutical modification, CS was added to PAMAM dendriplex 4 at charge ratios of 4, 6, 8, 10, 12, 14, 16, and 18; and left for another 30 min at room temperature. The theoretical charge ratio of anionic polymer to pDNA was calculated as the molar ratio of CS to pDNA phosphate.

Physicochemical Property of Complexes The particle sizes and ζ-potentials of various complexes were measured using a Zetasizer Nano ZS (Malvern Instruments, Ltd., Malvern, Worcestershire, U.K.). The number-fractioned mean diameter is shown.

To determine complex formation, 10 μL aliquots of various 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 100 V in running buffer solution (40 mM Tris–HCl, 40 mM acetic acid, and 1 mM etylenediaminetetraacetic acid (EDTA)). The retardation of pDNA was visualized with ethidium bromide using a FluorChem Imaging System (Alpha Innotech, CA, U.S.A.).

Cell Culture The mouse melanoma cell line, B16-F10 cells, was obtained from the Cell Resource Center for Biomedical Research (Tohoku University, Japan). B16-F10 cells were maintained in culture medium (RPMI 1640 supplemented with 10% FBS and antibiotics) under a humidified atmosphere of 5% CO_2 in air at 37°C.

In Vitro **Transfection Experiments** B16-F10 cells were plated on 24-well plates (Becton-Dickinson, Franklin Lakes, NJ, U.S.A.) at a density of 1.0×10^4 cells/well and cultivated in 500 μL culture medium.

In the transfection experiment, after 24 h pre-incubation, the medium was replaced with 500 μL Opti-MEM® I medium and each complex containing 1 μg pDNA was added to the cells and incubated for 2 h. Each complex was also incubated at 4°C for 2 h to determine the low temperature effect. After transfection, the medium was replaced with culture medium and cells were cultured for a further 22 h at 37°C. After 22 h incubation, the cells were washed with phosphate buffered saline (PBS) and then lysed in 100 μL lysis buffer (pH 7.8 and 0.1 M Tris–HCl buffer containing 0.05% Triron 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 a standard. Absorbance was measured using a microplate reader at 595 nm. Luciferase activity was indicated as relative light units (RLU) per mg protein.

For the inhibitor study, the cells were pre-treated with 14 μM chlorpromazine (CPZ) as an inhibitor of clathrin-mediated endocytosis, 2 mM amiloride as an inhibitor of macropinocytosis, or 200 μM genistein as an inhibitor of caveolae-mediated endocytosis for 30 min. After pre-treatment with inhibitors, PAMAM dendriplex 4 and CS complex 12 were added to the cells and incubated for 2 h. After transfection, medium was replaced with culture medium and cells were cultured for a further 22 h at 37°C, and then the luciferase activities were determined as described above.

WST-1 Assay Cytotoxicity tests of various complexes on B16-F10 cells were carried out using a WST-1 commercially available cell proliferation reagent. The assay is based on cleavage of the tetrazolium salt WST-1 by active mitochondria to produce a soluble colored formazan salt. B16-F10 cells were plated on a 96-well plate (Becton-Dickinson) at a density 3.0×10^3 cells/well and cultured in 100 μL culture medium. Complexes containing 1 μg pDNA in 100 μL Opti-MEM® I were added to each well and incubated for 2 h. After transfection, the medium was removed and the cells were cultured for a further 22 h at 37°C with culture medium. After incubation, the medium was then replaced with 100 μL culture medium and 10 μL WST-1 mixture solution (4.95 mM WST-1 and 0.2 mM 1-methoxy PMS) was added to each well and incubated for an additional 2 h at 37°C. The absorbance in each well was measured at 450 nm with a reference of 630 nm, using a microplate reader. The results are shown as a percentage of untreated cells (control).

In Vivo **Gene Expression Experiment** Animal care and experimental procedures were performed in accordance with the Guidelines for Animal Experimentation of Nagasaki University with approval of 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 week before the experiments.

The complexes including 40 μg pDNA were prepared for *in vivo* gene expression experiments. A 300 μL sample of complexes was injected into the mice *via* the tail vein and the liver, kidneys, spleen, heart, and lungs of the mice were dissected 6 h after the injection. The tissues were centrifuged at 15000 rpm for 5 min and the supernatants were used for luciferase assays. Luciferase activity is indicated as RLU per gram of tissue.

Agglutination Study Erythrocytes from mice were washed three times as 4°C by centrifugation at 5000 rpm (Kubota 3700; Kubota, Tokyo, Japan) for 5 min and resuspended in PBS. A 2% (v/v) stock suspension of erythrocytes was prepared for the agglutination study. The PAMAM dendriplex 4 and CS complex 12 were added to the erythrocyte suspension and incubated for 15 min at room temperature. A 10 μL sample was placed on a glass plate and agglutination was observed by microscopy (400× magnification).

Statistical Analysis Results are expressed as the mean± standard deviation of at least three experiments. Statistical analysis was performed using Student's t-test. A $p < 0.05$ was considered to indicate significance.

RESULTS

Physicochemical Properties of PAMAM Dendriplex The particle size and ζ-potentials of PAMAM dendriplexes were measured by a Zetasizer Nano ZS and are shown in Table 1. PAMAM dendriplex 0.5 was 149.3 nm particle size and −22.2 mV ζ-potential. PAMAM dendriplexes 1, 2, 4, 6, and 8 were <100 nm particle size and a positive surface charge. Release of pDNA from PAMAM dendriplexes was not determined by electrophoresis analysis (data not shown).

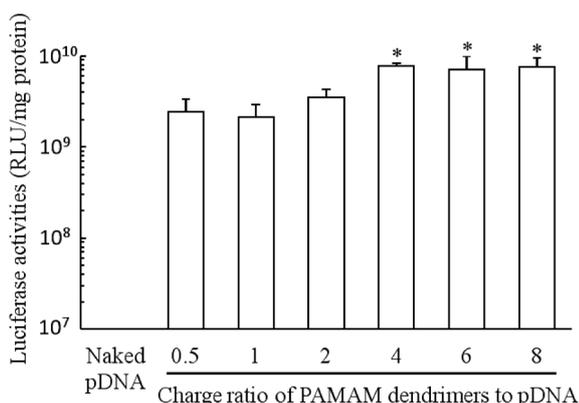***In Vitro* Transfection Experiments of PAMAM Den-**

Fig. 1. *In Vitro* Transfection Efficiency of Each PAMAM Dendriplex at Various Charge Ratios (0.5, 1, 2, 4, 6, and 8) and Naked pDNA

B16-F10 cells were transfected with complexes containing pCMV-Luc. At 22 h after transfection, luciferase activity was evaluated. Each bar represents the mean±standard deviation ($n=4$). *$p < 0.05$ *vs.* PAMAM complex 0.5.

driplex The B16-F10 cells were transfected with various PAMAM dendriplexes and their luciferase activities were determined chemiluminescently (Fig. 1). PAMAM dendriplexes showed high luciferase activity of $>1.0 \times 10^9$ RLU/mg protein, although no activity was observed in naked pDNA. PAMAM dendriplexes 4, 6, and 8 were significantly higher transfection efficiency than PAMAM dendriplex 0.5. We developed ternary complexes including CS based on PAMAM dendriplex 4.

Physicochemical Properties of CS Complex Ternary complexes based on PAMAM dendriplex 4 were prepared at various amounts of CS and their physicochemical properties were determined by a Zetasizer Nano ZS (Table 2). CS complex 4 was 183.9 nm particle size and 28.3 mV ζ-potential. The particle size of CS complex 6 was not determined because of aggregation. CS complexes 12, 14, 16, and 18 were <100 nm particle size and had a strong negative surface charge.

Release of pDNA from CS complexes was examined by electrophoresis analysis (Fig. 2). Naked pDNA was detected as a band on agarose gel. No pDNA was released from PAMAM dendriplex 4 and CS complexes.

***In Vitro* Transfection and Cytotoxicity of CS Complex** B16-F10 cells were incubated with CS complexes for 2 h and their luciferase activity was measured. The *in vitro* transgene efficiencies of CS complexes at various charge ratios are shown as RLU per mg protein in Fig. 3. CS complexes 4, 6, and 8 showed significantly lower transfection efficiency than PAMAM dendriplex 4 ($p < 0.05$). CS complexes 12, 14, 16, and 18, however, showed high luciferase activity of $>1.0 \times 10^9$ RLU/mg protein, which was equivalent to PAMAM dendriplex 4. Among them, CS complex 12 had the highest luciferase activity.

The cytotoxicity of each complex was compared in B16-F10 cells and the cell viability is shown in Fig. 4 by WST-1 assay. PAMAM dendriplex 4 showed significantly higher cytotoxicity than the control ($p < 0.05$). On the other hand, no cytotox-

Table 1. Particle Size and ζ-Potential of PAMAM Dendriplex

Complex	Charge ratio of PAMAM dendrimer to pDNA	Particle size (nm)	ζ-Potential (mV)
PAMAM dendriplex 0.5	0.5	149.3±11.1	−22.2±2.4
PAMAM dendriplex 1	1	64.5±37.2	23.7±1.4
PAMAM dendriplex 2	2	52.3±2.5	25.8±2.7
PAMAM dendriplex 4	4	59.3±4.2	28.2±0.6
PAMAM dendriplex 6	6	60.6±2.2	29.8±1.9
PAMAM dendriplex 8	8	58.1±2.8	22.9±3.3

Each value is the mean±standard deviation ($n=3$).

Table 2. Particle Size and ζ-Potential of CS Complex

Complex	Charge ratio of CS to pDNA	Particle size (nm)	ζ-Potential (mV)
CS complex 4	4	183.9±2.1	28.3±1.3
CS complex 6	6	N.D.	5.7±0.1
CS complex 8	8	199.2±4.7	−18.2±0.6
CS complex 10	10	110.4±0.8	−28.8±1.2
CS complex 12	12	69.0±1.2	−29.2±2.3
CS complex 14	14	72.5±0.5	−29.8±0.2
CS complex 16	16	71.9±0.5	−30.9±0.6
CS complex 18	18	73.7±1.5	−34.4±2.0

Each value is the mean±S.D. ($n=3$).
Not determined (N.D.).

icity was observed in CS complexes at any charge ratio. The effect of endocytotic inhibitors on transfection efficiency was examined in CS complex 12, which showed high transfection efficiency and no cytotoxicity.

Inhibition Study of *in Vitro* Transfection Inhibition studies were performed with various inhibitory agents. We examined the effect of endocytotic inhibitors on the high transfection efficiency of PAMAM dendriplex 4 (Fig. 5A) and CS complex 12 (Fig. 5B). PAMAM dendriplex 4 showed significantly lower transfection efficiency at 4°C. The transfection efficiency of PAMAM dendriplex 4 was decreased by genistein and amiloride ($p<0.05$). CPZ did not decrease transfection efficiency. On the other hand, the transfection efficiency of CS complex 12 was significantly decreased by genistein

and CPZ ($p<0.05$). Amiloride did not decrease transfection efficiency.

Agglutination Study of PAMAM Dendriplex and CS Complex Agglutination activities were examined in PAMAM dendriplex 4 and CS complex 12. The erythrocytes were observed by microscopy after 30 min incubation with the complex and are shown in Fig. 6. PAMAM dendriplex 4 showed severe agglutination (Fig. 6A), although CS complex 12 showed no agglutination (Fig. 6B).

***In Vivo* Gene Expression of PAMAM Dendriplex and CS Complex** In the preliminary experiments, high gene expression was observed 6 h after intravenous administration of the complexes. PAMAM dendriplex 4 and CS complex 12 were intravenously administered to ddY mice. Luciferase activities in the liver, kidney, spleen, heart, and lung were chemiluminescently determined as *in vivo* gene expression 6 h after their administration (Fig. 7). PAMAM dendriplex 4 showed high gene expression $>1.0\times10^5$ RLU/g tissue in the kidney, spleen, heart, and lung. PAMAM dendriplex 4 showed the highest gene expression in the spleen. On the other hand, CS complex 12 showed high gene expression $>1.0\times10^5$ RLU/g tissue in the liver, spleen and lung. In particular, CS complex 12 showed significantly higher gene expression in the spleen than PAMAM dendriplex 4.
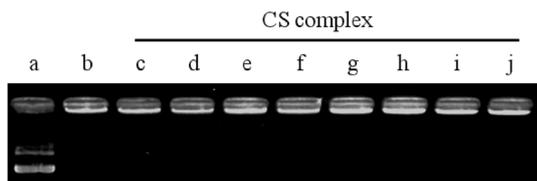

Fig. 2. Electrophoresis Analysis of Naked DNA (a), PAMAM Dendriplex 4 (b), and CS Complex at a Charge Ratio of 4 (c), 6 (d), 8 (e), 10 (f), 12 (g), 14 (h), 16 (i), and 18 (j)

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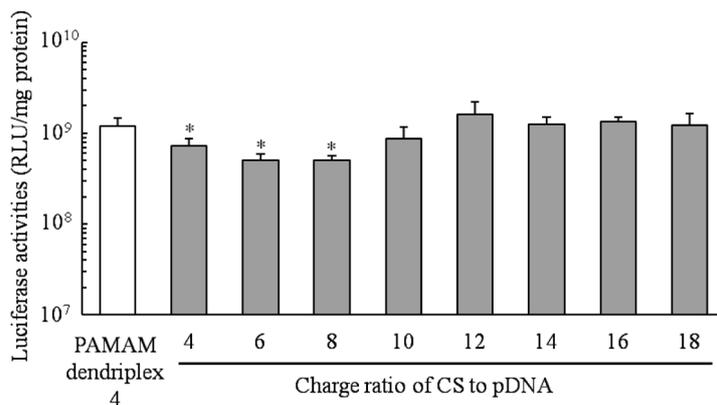

Fig. 3. *In Vitro* Transfection Efficiency of Each CS Complex (■) at Various Charge Ratios (4, 6, 8, 10, 12, 14, 16, and 18) and PAMAM Dendriplex 4 (□)

B16-F10 cells were transfected with complexes containing pCMV-Luc. At 22 h after transfection, luciferase activity was evaluated. Each bar represents the mean±standard deviation ($n=4$). *$p<0.05$ *vs.* PAMAM dendriplex 4.
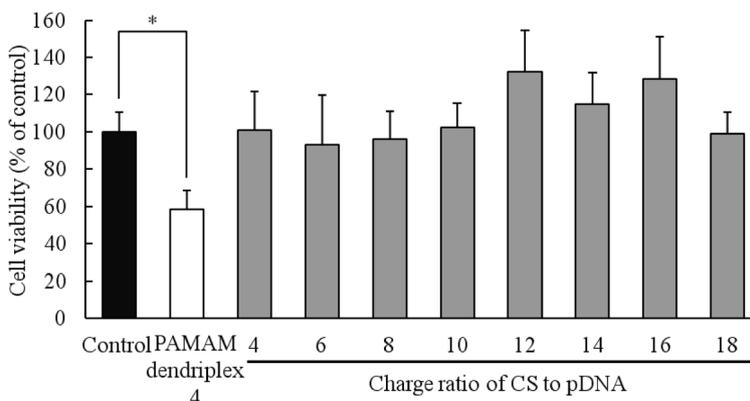

Fig. 4. Cytotoxicity of CS Complex (■) at Various Charge Ratios (4, 6, 8, 10, 12, 14, 16, and 18) and PAMAM Dendriplex 4 (□)

Viability of B16-F10 cells treated with the complexes was measured by WST-1 assay. Cells were incubated with the complexes for 2 h and cell viability was evaluated 24 h after treatment. Each bar represents the mean±standard deviation ($n=8$). *$p<0.05$ *vs.* control (■).
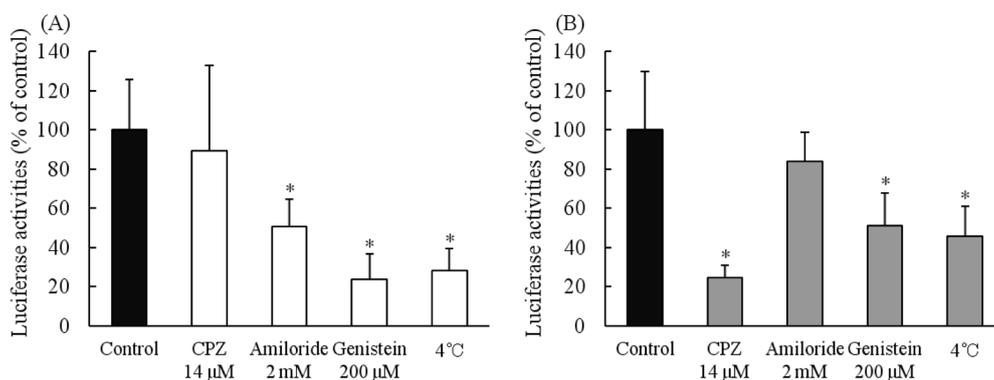
Fig. 5. Influence of Endocytotic Inhibitors on the Transfection Efficiency of PAMAM Dendriplex 4 (A, □) and CS Complex 12 (B, ■)

Each complex was transfected in medium with various endocytotic inhibitors. Each complex was also incubated at 4°C for 2h to determine the low temperature effect. At 24h after transfection, luciferase activity was evaluated. Each bar represents the mean±standard deviation (n=4). *p<0.05 vs. control (■).
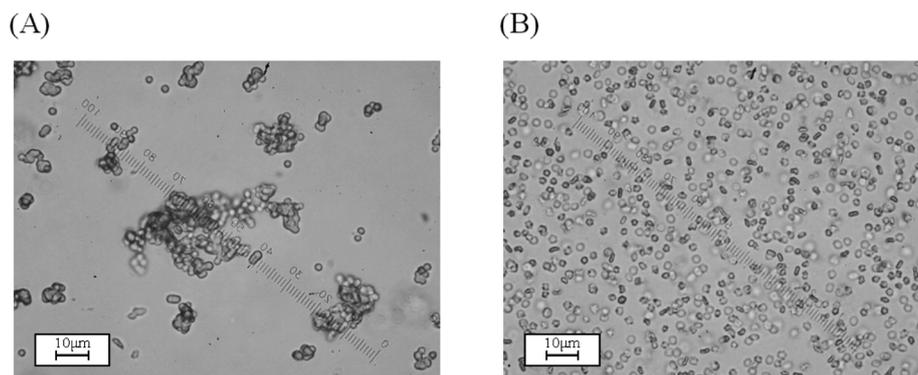

Fig. 6. Interaction of PAMAM Dendriplex 4 (A) and CS Complex 12 (B) with Erythrocytes

The complexes were added to erythrocytes, and agglutination was assessed. Agglutination was observed by phase microscopy (400× magnification).
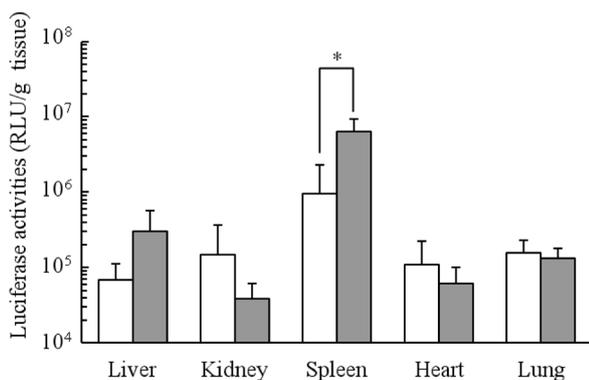

Fig. 7. In Vivo Gene Expression of Each Complex Administered to Mice

PAMAM dendriplex 4 (■) and CS complex 12 (□) were injected intravenously into mice (40 μg pDNA per mouse). At 6h after administration, mice were sacrificed and each organ was dissected to quantify luciferase activity. Each bar represents the mean±standard deviation (n=5). *p<0.05 vs. PAMAM dendriplex 4.

DISCUSSION

Successful gene therapy relies on the development of delivery gene vectors with high transfection efficiency and low cytotoxicity. Non-viral vectors with cationic polymers and cationic lipids have several advantages, including non-immunogenicity, low acute toxicity, and flexibility, to design a vehicle with well-defined structural and chemical properties to yield mass production.[29,30] Cationic polymers and cationic lipids can electrostatically bind to pDNA to form polyplexes and lipoplexes, and show high transgene efficiency under in vitro and in vivo conditions.[31]

Dendrimers are a new class of polymer that constitute hyperbranched macromolecules composed of layers of monomer units radiating from a central core.[32,33] PAMAM dendrimers are based on an ethylene diamine core and an amidoamine repeat branching structure.[34] PAMAM dendrimers form complexes with pDNA through sequence-independent electrostatic interactions between negatively charged phosphate groups of the nucleic acid and positively charged primary amino groups on the dendrimer surface, giving rise to a particle termed a dendriplex. Navarro and Tros de Ilarduya[13] reported that the dendriplex of pDNA with PAMAM dendrimers was 100–113 nm particle size and had 6–18 mV ζ-potential in the charge ratio range of 2–10 of PAMAM to pDNA.

We also formed the PAMAM dendriplex at various charge ratios, which was <100 nm particle size and had a positive surface charge (Table 1). The PAMAM dendriplex was found not to release pDNA by electrophoresis analysis (data not shown), indicating strong compaction of pDNA by PAMAM dendrimers. The PAMAM dendriplex was reported to be highly effective in protecting pDNA from attack by DNase I and had low toxicity.[13]

The present PAMAM dendriplex showed high transfection efficiency in B16-F10 cells (Fig. 1). The positive charge on the particle surface ensured binding to the negatively charged cellular membrane and was taken up by the cells via adsorptive

endocytosis or phagocytosis.[29,35] The buried tertiary amino groups of PAMAM dendrimer acted as a proton sponge in endosomes and enhanced the release of pDNA into cytoplasm.[16,36] Kukowska-Latallo *et al.*[11] synthesized twenty different types of PAMAM dendrimers and elucidated the efficiency of pDNA transfection by determining the expression of the reporter protein. The PAMAM dendriplex achieved highly efficient transfection with minimal cytotoxicity in a broad range of eukaryotic cells and cell lines. The capability of the PAMAM dendrimer to transfect cells appeared to depend on the size, shape, and number of primary amino groups on the surface of the polymer. The gene transfection of the PAMAM dendrimer was enhanced with the addition of chloroquine, indicating endosomal localization of the complexes.

The cytotoxicity of PAMAM dendriplex to B16-F10 cells and its agglutination are shown in Fig. 4 and Fig. 6A. A cytotoxicity study of PAMAM dendrimers as gene vectors has been previously reported, whereby the toxicity of PAMAM dendrimers increased with increasing dendrimer generation.[37,38] The cationic non-viral gene vectors showed cytotoxicity and agglutination by the strong affinity of positively charged particles to the cellular membrane and erythrocytes.[39,40] The PAMAM dendriplex induced oxidative stress by producing ROS and led to a cytotoxic response.[15,16,41]

CS is widely distributed in animal tissues and possibly plays an important role in different types of metabolic reactions as well as being a protective agent of joints, the internal wall of blood vessels, skin, and bones.[42,43] In addition, it was reported to reduce cell damage by scavenging free radicals, inhibiting the activation of apoptosis, and eliciting an anti-inflammatory effect at the synovial membrane and chondrocyte level.[26,44,45] These cytoprotective actions of CS may reduce the toxicity of PAMAM dendrimers.

Therefore, CS was added to PAMAM dendriplex and a stable complex was successfully constructed without pDNA release, except for CS complex 6 (Fig. 2). The increase of particle size by aggregation was observed around neutral of ζ-potential. The aggregation may cause to the large particles of CS complex 4–10. The prepared CS complex showed no cytotoxicity and no agglutination (Figs. 4, 6B), which may be caused by their negative ζ-potential. On the other hand, generally, anionic complexes are not taken up by cells because they repulse the cellular membrane electrostatically. The CS complex, however, showed high transfection efficiency (Fig. 3). Transfection efficiency of CS complex transiently decreased at charge ratio 4–8 but the effect equivalent to PAMAM dendriplex was shown by charge ratio >10. Hagiwara *et al.*[20] reported that molecular weight of CS can affect transfection efficiency of complexes. Only commercial CS was used in the present study. The influence of CS molecular weight on transfection efficiency may be important in further experiments. In the preliminary study, the transfection efficiency of CS complex was decreased by the addition of excess CS concentration-dependently (data not shown). The results indicated that CS complex with a negative charge was taken up by cells through a particular mechanism.

The influence of uptake inhibitors on transfection efficiency was elucidated in PAMAM dendriplex 4 and CS complex 12, which showed high transfection efficiency in B16-F10 cells. A caveolae-mediated endocytosis inhibitor (genistein), hypothermia, and macropinocytosis inhibitor (amiloride) significantly inhibited the transfection efficiency of PAMAM dendriplex (Fig. 5A). The complex of pDNA and PEI was also reported to abolish transfection efficiency by caveolae-mediated endocytosis inhibitors (filipin and genistein) in both A549 and HeLa cells, whereas inhibition of clathrin-mediated inhibitors (CPZ and potassium depletion) did not have any effect on transfection.[46] These results strongly indicate that PAMAM dendriplex was predominantly taken up by caveolae-mediated endocytosis.

In contrast, the transfection efficiency of CS complex 12 was significantly decreased by a clathrin-mediated endocytosis inhibitor (CPZ), caveolae-mediated endocytosis inhibitor (genistein), and hypothermia ($p < 0.05$). A macropinocytosis inhibitor (amiloride) did not decrease transfection efficiency (Fig. 5B). Lo *et al.*[47] reported that pDNA/CS-modified PEI was primarily taken up by U87 cells based on clathrin-mediated endocytosis. These results suggest that CS-coated particles were predominantly taken up by clathrin-mediated endocytosis. Hagiwara *et al.*[20] however, showed that the complex formed by combining pDNA/chitosan complex and CS (pDNA/chitosan/CS complex, diameter 186.3 nm) was taken up by COS7 cells *via* macropinocytosis. These conflicting results may be explained by the different particle sizes. Tang and Szoka[29] suggested that complexes with diameters of ≤100 nm correspond to the apparent diameter of the coated pit in receptor-mediated endocytosis. The pDNA/chitosan/CS complex may be too large to be taken up by the cells *via* clathrin-mediated endocytosis.

Tail-vein injection of PAMAM dendriplex into mice led to high gene expression in the spleen and lung.[19,48] In the present study, we observed that PAMAM dendriplex 4 showed high gene expression $>1.0 \times 10^5$ RLU/g tissue in the kidney, spleen, heart, and lung. PAMAM dendriplex 4 showed highest gene expression in the spleen (Fig. 7). High gene expression of PAMAM dendriplex in the lung may be explained by agglutination with blood components.

CS complex 12 also showed high gene expression $>1.0 \times 10^5$ RLU/g tissue in the liver, spleen, and lung. In particular, CS complex 12 showed significantly higher gene expression in the spleen than PAMAM dendriplex 4 (Fig. 7). CS complex 12 may be recognized by the spleen as a polysaccharide and increase the intensity of the complex. Kaplan *et al.*[49] reported that pneumococcal polysaccharide type 2 and 3 were localized in splenic macrophages, Kupffer cells in the liver, and inguinal lymph node macrophages.

Thus, we successfully developed a useful gene delivery vector. CS complex 12 showed high transgene efficiency in B16-F10 cells. High gene expression in the spleen was observed after its intravenous administration to mice. No cytotoxicity or agglutination was observed in CS complex 12. Although further study is necessary, CS complex 12 is expected to be used for gene therapy.

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