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Structural advantage of dendritic poly(L-lysine) for gene delivery into cells

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Abstract—This study aimed to investigate the relationships between structures of gene carrier molecules and their activities for gene delivery into cells. We compared 2 types of poly(L-lysine) as carriers, that is, dendritic poly(L-lysine) (KG6) and linear poly(L-lysine) (PLL). KG6 formed a neutral DNA complex, and its DNA compaction level was weaker than that of PLL. The amount of DNA binding and uptake into cells mediated by PLL was 4-fold higher than that with KG6. However, KG6-mediated gene expression was 100-fold higher than that by PLL. Since pK_a values of terminal amines of KG6 were lowered even though small amounts of DNA were internalized into cells, sufficient DNA amounts for effective gene expression escaped to the cytosol due to the proton sponge effect in the endosome. In addition, weakly compacted DNA with KG6 was advantageous in accessing RNA polymerase in the cell nucleus. On the other hand, PLL did not show the proton sponge effect in the endosome and resulted in strong compaction of DNA. Even though large DNA amounts were internalized into cells, most of the DNA would not take part in gene expression systems in the nucleus. Amount of induced cytokine production after intravenous injection of DNA complexes with KG6 and PLL was low, and was similar to the case when DNA was injected alone. Therefore, no significant difference in effects on cytokine production was observed between KG6 and PLL.

1. Introduction

Developments in molecular biology and molecular therapy require more efficient, more functional, and safe gene delivery techniques into cells. In the field of molecular biology, in order to understand functions of unknown genes and their networks, transfection of genes and delivery of siRNA into cells by convenient methods with high efficiency and low cytotoxicity have become key parameters.[1–3] In the field of molecular therapy, therapeutic gene, siRNA-encoding plasmid DNA, and siRNA itself should be efficiently delivered into target cells without toxicity.[4] At present, viral-mediated delivery has attracted increasing attention and has been used because of its high efficiency. However, with regard to safety, convenience, and distribution of commercial products, nonviral delivery systems such as the use of cationic polymers and cationic lipid-mediated nucleic acid delivery are strongly expected to form the basis of molecular therapy.[5]

Several compounds that can be used as nonviral gene carrier molecules have been reported. The use of cationic lipids was first reported by Felgner et al.[6] in 1987. In 1990s, relationships between lipid structures and transfection were studied enthusiastically. Cationic lipids are composed of three components: a cationic head group that binds DNA, a hydrophobic region that forms the liposome structure, and a linker region linking the first two components. In previous studies, several combinations of lipid structures were tested, and structures were optimized.[7] It was reported that the DNA complex contained in the cationic liposome was internalized by endocytosis. In the endosome, the cationic

Abbreviations: DMEM, Dulbecco's modified Eagle's medium; KG6, dendritic poly(L-lysine) of the 6th generation; PEI, polyethyleneimine; PLL, linear poly(L-lysine).

Keywords: Cytokine; Dendrimer; Gene delivery; Gene therapy; Poly(L-lysine); Transfection.

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lipid and/or the helper lipid perturbs the endosomal membrane. Fusion of lipids of the DNA complex and the endosomal membrane subsequently results in endosomal escape of DNA into the cytosol.[8] The use of DNA molecules complexed with cationic lipids resulted in strong expression of genes of interest mainly in lungs after intravenous injection into mice.[9,10] Complexes are rapidly cleared from the blood circulation, and are mainly internalized into endothelial cells and macrophages in the microvasculature of lungs.[11] It was reported that cytokines such as TNF-α, IL-12, and INF-γ were induced after intravenous injection of such DNA-lipid complexes.[12–14]

Poly(L-lysine) is a cationic polymer that has been commonly used as gene carrier. In 1987, Wu and Wu reported that targeted delivery of DNA to hepatoma cells was achieved using poly(L-lysine) modified with asialoglycoproteins as a gene carrier.[15] Thereafter, several ligands were used to modify poly(L-lysine) and oligolysine.[16] To obtain prolonged circulation in blood flow and targeted gene delivery in vivo, PEG modification of poly(L-lysine) and oligolysine has been performed.[17,18] In the case of PEG-modified poly(L-lysine), poly(L-lysine) forms a stable core-shell type complex, that is, the condensed complex of poly(L-lysine) and the DNA chain forms the core, and PEG coats the core to form the shell. Ligand modification of PEG-coated DNA complexes enables targeted gene delivery into target sites after systemic injection in vivo.[19,20] Effects of the molecular weight of poly(L-lysine) on efficiency of gene delivery were examined.[21,22] It was shown that the longer the chain length (hundreds of thousands of kDa) of the L-lysine entity, the more stable the complex was in blood, and the longer gene expression lasted in vitro and in vivo.

Polyethyleneimine (PEI) is also a well-known gene carrier. PEI can be of two forms: linear and branched. DNA binding ability and transfection efficiency of PEI depend on its molecular weight and its topology. Low molecular weight (<2 kDa) branched PEI molecules showed no transfection efficiency, although they showed DNA binding ability. In the case of larger PEI molecules, for example, linear 22 kDa, branched 25, 50, and 800 kDa, high transfection efficiencies comparable to those of cationic lipids were observed in vitro and in vivo.[23] Complex formation with PEI is influenced by salt concentration. Linear PEI (22 kDa) forms small and dispersed complexes with DNA in 5% glucose (no salt), and shows high efficiencies in vitro and in vivo, although large aggregates are formed in saline.[24] In contrast, branched PEI (25 kDa) can form small complexes even in saline, but transfection is not efficient especially in vivo.[25] Physicochemical characteristics of PEI could influence stability of complexes and transfection efficiencies in vitro and in vivo, however, practical relationships between topology of PEI and its function as a gene carrier are still unknown.

In 2002, we reported that monodispersed dendritic poly(L-lysine) was applicable as a gene carrier for in vitro transfection.[26] When comparing the 1st to 7th generations of dendrimers, the 6th generation (KG6) was sufficient to show high gene transfection ability. Transfection efficiency was not seriously reduced, even when 50% serum was added to the transfection medium. The ζ-potential of DNA complexes with KG6 was kept at a neutral or slightly positive value (+3 mV) even when the charge ratio (±) was increased to 8, supporting its stability in the presence of high concentration of serum. In addition, DNA complexes could circulate in the blood flow for a long time, that is, for at least 3 h after intravenous injection into mice.[27] The stealth character provided by neutral and hydrophilic surfaces is a key factor for prolonged circulation of DNA complexes in the blood flow. All these characteristics observed for KG6 indicated that DNA complexes with KG6 could be advantageous for in vivo gene delivery. On the other hand, linear poly(L-lysine) does not show such low ζ-potential of DNA complexes and a high transfection efficiency in vitro.

What is the key factor responsible for differences in physicochemical properties and transfection efficiencies of different DNA complexes? To date, individual researchers have solely investigated their own carriers for gene delivery in different assay systems, for example, differences in sample concentration, pH, buffer, cell type, cell number, cell condition, mouse type, injection volume, route, etc., and have not made comparisons with other carriers within the same study. Therefore, even though we are interested in identifying relationships between structure and function of different carriers, we cannot directly compare data reported from individual papers. Even for PEI, no study on structure-function relationships has been reported. In the present study, we focused on the structural differences between dendritic poly(L-lysine) and linear poly(L-lysine), and investigated relationships between the physicochemical properties of DNA complexes, gene transfection in vitro, and cytokine induction in vivo. This will provide important insights into the development of future gene carriers for the next generations.

2. Results and discussion

2.1. Structures of dendritic poly(L-lysine) and linear poly(L-lysine)

Dendritic poly(L-lysine) of the 6th generation (KG6) is a dendrimer consisting of L-lysine residues as a branch unit (Fig. 1A). KG6 has 128 amines and 126 lysine residues in one molecule. For comparison purposes, we chose to use linear poly(L-lysine) (M_w 15,000–30,000) (PLL) purchased from Sigma–Aldrich which has about 170 amines and lysine residues in the molecule (Fig. 1B).

2.2. Physicochemical characteristics of DNA complexes with poly(L-lysine)

ζ-Potentials of DNA complexes with KG6 and PLL at an amine/phosphate (N/P) ratio of 8 in DMEM were compared. As shown in Figure 2A, surface charge of the KG6–DNA complex was almost neutral

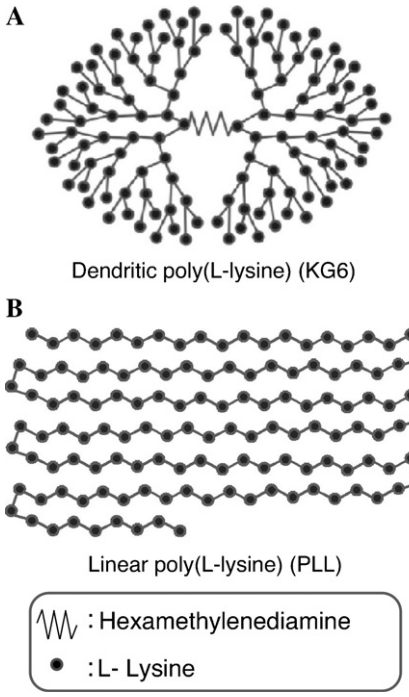

Figure 1. Structures of dendritic poly(L-lysine) of the 6th generation (KG6) (A) and linear poly(L-lysine) (PLL) (B). KG6 has 128 amines and 126 lysine residues in one molecule. PLL has about 170 amines and lysine residues in one molecule.

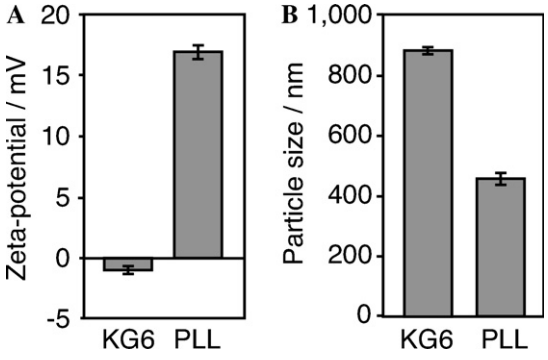

Figure 2. ζ-Potentials (A) and particle sizes (B) of DNA complexes with KG6 and PLL. Samples were prepared by mixing 1 μg plasmid DNA with KG6 or PLL at an N/P ratio of 8 in 1 mL DMEM. After incubation for 15 min at room temperature, zeta-potentials and particle sizes of DNA complexes were measured. Data represent mean values for $n = 3$, and bars are standard errors of means.

(−1.0 ± 0.3 mV), while that of PLL–DNA complex was significantly positive (+16.8 ± 0.5 mV), as previously reported.[26] Evaluation of sizes of DNA complexes with KG6 and PLL in DMEM showed that KG6 formed complexes of 887 ± 19 nm, which were larger than those with PLL (457 ± 20 nm, Fig. 2B). Since the surface of KG6–DNA complexes was neutral, electric repulsion between complexes was decreased, and complexes would grow larger than those with PLL.

Strength of DNA compaction was evaluated using an ethidium bromide titration assay.[28] KG6 and PLL bound to DNA in DMEM, leading to exclusion of ethidium bromide intercalation, and a decrease in fluorescence (Fig. 3). With KG6, the fluorescence intensity versus charge ratio curve flattened out at an N/P ratio of 2 and reached a plateau at 40%. With PLL, the fluorescence intensity versus charge ratio curve decreased, reached a plateau at an N/P ratio of 2, and the level was maintained at 20%. Since KG6 could not exclude ethidium bromide very strongly even at an N/P ratio of 16 compared to PLL, this indicated that DNA compaction mediated by KG6 was weaker than that with PLL. In a previous report, we showed that KG6 decreased ethidium bromide fluorescence level down to 20%.[26] In that study, we used PBS as solvent. In the present study, we used DMEM, and we found that the compaction level was weaker than that in the case with PBS. It is likely that other components, such

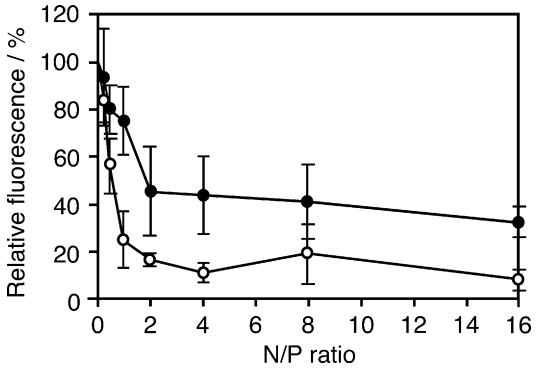

Figure 3. Fluorescence titration assay. Plasmid DNA was mixed with KG6 or PLL in DMEM at various N/P ratios. After standing for 15 min at room temperature, ethidium bromide was added to the complex mixtures. Fluorescence was measured using a spectrophotometer by exciting at 530 nm while monitoring emission at 590 nm. Closed circles and open circles indicate KG6–DNA complexes and PLL–DNA complexes, respectively. Data represent mean values for $n = 3$, and bars are standard errors of means.

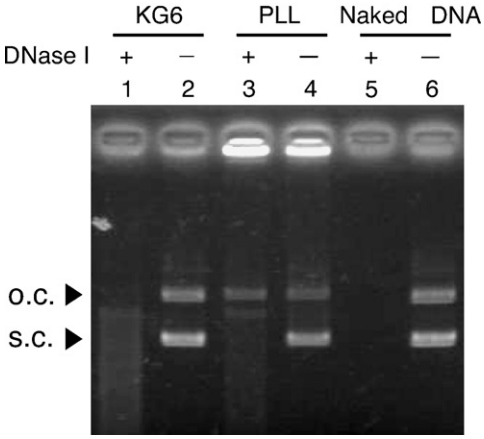

Figure 4. DNase protection assay. DNA complexes with KG6 or PLL were treated with DNase I and were subsequently heated at 80 °C to inactivate DNase. Samples were then treated with heparin to dissociate DNA complexes. Samples were subjected to agarose gel electrophoresis. Lane 1, KG6–DNA complexes treated with DNase I; lane 2, KG6–DNA complexes; lane 3, PLL–DNA complexes treated with DNase I; lane 4, PLL–DNA complexes; lane 5, naked plasmid treated with DNase I; lane 6, naked plasmid. Positions of open-coiled (o.c.) and supercoiled (s.c.) forms are indicated.

as $NaHCO_3$ (3700 mg/L), D-glucose (1000 mg/L), L-glutamine (584 mg/L), $MgSO_4$ (400 mg/L), $CaCl_2$ (200 mg/L), L-lysine hydrochloride (146 mg/L), sodium pyruvate (110 mg/L), several amino acids and vitamins, in DMEM affect the DNA compaction. Actually, we found that some components in DMEM affected ζ-potential and stability of complexes (data not shown), however, we have not identified these components yet.

The weaker compaction with KG6 was also supported by results obtained with a DNase I protection assay (Fig. 4). Although PLL–DNA complexes resisted DNase I digestion (Fig. 4, lane 3), DNA bound to KG6 at an N/P ratio of 8 in DMEM was moderately digested, then a smear was observed at the lower positions in the agarose gel (Fig. 4, lane 1). In addition, strong fluorescence originated from DNA–PLL complexes, which was not dissociated by addition of excess heparin after DNase digestion, was observed in the wells of the gel (Fig. 4, lanes 3 and 4). PLL–DNA complexes were also resistant to exchange between DNA and the anionic polymer heparin, although KG6–DNA complexes were completely dissociated by addition of heparin (Fig. 4, lane 2).

2.3. DNA binding and uptake into cells

CHO cells were incubated with DNA complexes at an N/P ratio of 8 in DMEM for 3 h. After washing with PBS, DNA labeled with tetramethylrhodamine was observed using fluorescence microscopy (Fig. 5). DNA complexes of KG6 and PLL were observed in cells as 300–600 and 700–1100 nm dots, respectively. We could not distinguish locations of fluorescence, that is, whether they existed inside or outside the cells. In order to quantify DNA amounts, cells were lysed, and real-time PCR was performed (Fig. 6A). Amount of DNA mediated by PLL was 4-fold higher than that with KG6. It is reasonable to think that the positive ζ-potential of PLL–DNA complexes is advantageous in allowing nonspecific binding to the cell surface and uptake into cells.

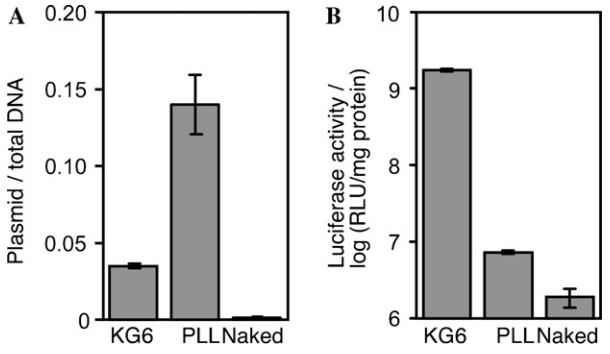

Figure 6. DNA binding and uptake into cells, and transfection efficiencies mediated by KG6 and PLL. (A) Amounts of DNA binding and uptake into cells. Cells were incubated with KG6 or PLL DNA complexes at an N/P ratio of 8 for 3 h at 37 °C. Cell harvesting and DNA extraction from cells were performed 24 h post-transfection. Amounts of plasmid DNA in CHO cells were estimated by quantitative real-time PCR. Amounts of plasmid DNA were standardized against total DNA amounts in CHO cells. (B) Transfection efficiencies of KG6 or PLL into CHO cells. Cells were incubated with KG6 or PLL DNA complexes at an N/P ratio of 8 for 3 h at 37 °C. Cell harvesting and luciferase assays were performed 24 h post-transfection. Data represent mean values for $n = 3$, and bars are standard errors of means.

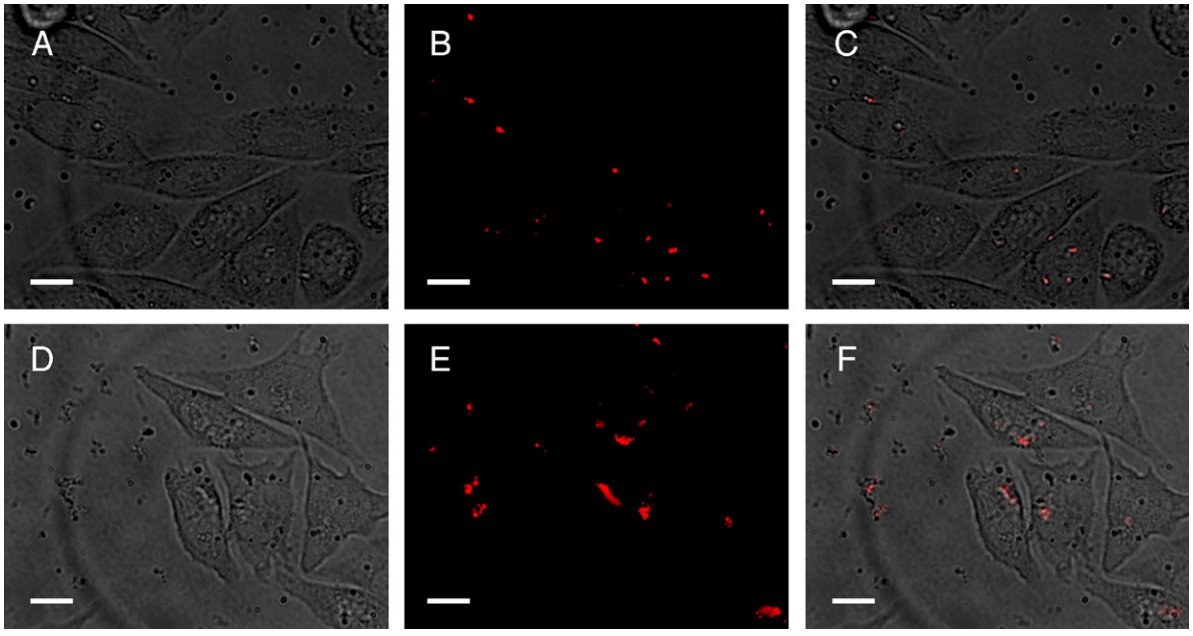

Figure 5. DNA uptake and distribution of TM-rhodamine-labeled DNA in cells. Tetramethylrhodamine-labeled plasmid DNA (0.25 μg) and plasmid (2.25 μg) were added to 250 μL DMEM containing KG6 or PLL at an N/P ratio of 8. After standing for 15 min at room temperature, the complex solution was added to cells. After incubation for 3 h at 37 °C, cells were washed with PBS and were observed by fluorescence microscopy. Top panel: KG6–DNA complexes, (A) transmission image, (B) fluorescence image, and (C) merged image of A and B. Bottom panel: PLL–DNA complexes, (D) transmission image, (E) fluorescence image, (F) merged image of D and E. All bars indicate 5 μm.

Table 1. Pro-inflammatory cytokine production after intravenous injection of DNA complexes with KG6 or PLL

Formulation (pg/mL)	DNA alone		PLL		KG6		DOTAP/cholesterol[a]	
	2 h	6 h	2 h	6 h	2 h	6 h	2 h	6 h
TNF-α	N.D.	97.6 ± 29.6	232.3 ± 79.1	72.5 ± 31.7	235.1 ± 86.2	115.2 ± 31.5	2600 ± 1000	400 ± 100
IFN-γ	N.D.	629.5 ± 135.8	N.D.	608.7 ± 225.4	N.D.	511.8 ± 236.2	N.D.	46,000 ± 13,000
IL-1β	33.7 ± 10.2	21.0 ± 39.8	70.6 ± 32.2	185.7 ± 43.5	16.6 ± 5.8	29.9 ± 18.9	—	—
IL-12	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	400 ± 100	1800 ± 500

Data represent mean values for $n = 3$, and standard errors of means.
N.D., not detected.
Lower limits of detections of TNF-α, IFN-γ, IL-1β, and IL-12 were 23.4, 9.4, 7.8, and 11.7 pg/mL (the lowest concentration of the standards), respectively.
[a] Nine hundred nanomole of liposomes and 25 μg plasmid in PBS.[31]

2.4. Efficiencies of gene expression mediated by the two different types of poly(L-lysine)

Gene expressions mediated by KG6 and PLL were evaluated by measuring enzyme activity of luciferase encoded in plasmid DNA as reporter gene (Fig. 6B). Gene expression mediated by KG6 was 100-fold higher than that by PLL.

What is the important step for efficient gene expression? In the case of KG6, amount of binding or internalized DNA was lower than that of PLL (Fig. 6A). We suggested that since pK_a values of terminal amines of KG6 were reduced to less than 6,[26] this lowered pK_a of amine resulted in a proton sponge effect in the endosomal compartment.[29] The proton sponge effect is a hypothesis of osmotic endosomal disruption. When polycations with a low pK_a are used as gene carrier and internalized into the endosome, their amino groups are protonated with influxing protons and endosomal acidification is suppressed. Influx of chloride ions with accumulations of protons increases endosomal osmotic pressure, then disrupts endosome, finally. Therefore, highly efficient endosomal escape mediated by KG6 was possible, leading to a higher gene expression level than that with PLL. On the other hand, in the case of PLL, a larger amount of DNA could be internalized into cells, however, most of the DNA could not escape from the endosomal compartment, and would be degraded, finally.

Another possibility is that DNA compaction by KG6 was weaker than that by PLL (Figs. 3 and 4), indicating that RNA polymerase could easily access the weakly compacted DNA compared to strongly compacted DNA. If DNA translocates into the cell nucleus as DNA complexed with poly(L-lysine), it is clear that weakly compacted and easy chain-exchangeable complexes are advantageous for gene expression.

2.5. Cytokine induction after intravenous injection of DNA complexes

Induction of cytokines by administration of DNA-gene carrier molecules is an important factor when planning safe nonviral gene therapy. In order to identify effects of physicochemical characteristics of DNA complexes on cytokine induction, we evaluated inductions of pro-inflammatory cytokines such as TNF-α, IFN-γ, IL-1β, and IL-12 in blood after intravenous injection of DNA complexes of KG6 and PLL (Table 1). Induction of TNF-α 2 h after injection of DNA complexes with KG6 and PLL was significantly higher than when DNA was injected alone. However, 6 h after injection, TNF-α level was reduced to the same level as in the case with DNA alone. For other cytokines, that is, IFN-γ, IL-1β, and IL-12, no difference was observed between DNA alone, KG6-DNA complexes, and PLL–DNA complexes. Since basal levels of the pro-inflammatory cytokines, especially TNF-α, IFN-γ, and IL-1β, are less than lower limits of detections (23.4, 9.4, and 7.8 pg/mL, respectively), the induction of cytokines productions might be due to the CpG motif in the plasmid DNA.[14,30] Furthermore, cytokine response in the case of PLL was the same as that of KG6, indicating that physicochemical characteristics of the DNA complexes did not affect inflammatory responses. On the other hand, the cationic liposome system involving DOTAP/cholesterol liposomes induced extremely higher levels of cytokines.[31] Compared to the DOTAP/cholesterol liposome system, poly(L-lysine)-mediated gene delivery is adequate for cases where cytokine induction should be avoided.

3. Conclusion

In order to identify relationships between structure and activity of carrier molecules for gene delivery into cells, two types of poly(L-lysine), that is, KG6 and PLL, were compared. KG6 was advantageous for endosomal escape due to the proton sponge effect, and for transcription of DNA, resulting from weak compaction of DNA by KG6, even though total amount of DNA binding and KG6-mediated uptake into cells were lower than those with PLL. There was no difference in cytokine induction triggered by intravenous injections of DNA complexes of KG6 and PLL. Both of them did not considerably induce production of cytokines compared to cationic lipid systems.

4. Experimental

4.1. Chemicals

Dendritic poly(L-lysine) (KG6) was synthesized as previously described.[26] Poly(L-lysine) hydrochloride (M_w 15,000–30,000) was purchased from Sigma–Aldrich

(St. Louis, MO, USA). Plasmid DNA (PGV-C/CMV) was constructed by subcloning the *Bgl*II/*Hin*dIII CMV promoter fragment from pRc/CMV vector (Invitrogen, Carlsbad, CA, USA) into the *Bgl*II/*Hin*dIII fragment of PGV-C (Toyo Ink., Japan). Plasmid DNA was amplified in the *Escherichia coli* strain DH5α, isolated and purified using the QIAGEN Plasmid Mega Kit (Qiagen GmbH, Hilden, Germany). Plasmid DNA was labeled using the *Label* IT® TM-Rhodamine Labeling Kit (Mirus, Madison, WI, USA). DMEM (Dulbecco's modified Eagle's medium) and FBS (fetal bovine serum) were purchased from IWAKI GLASS (Chiba, Japan).

4.2. Cell culture

CHO cells (Chinese hamster ovary, RCB Accession No. RCB0285) were purchased from RIKEN Cell Bank (Tsukuba, Japan). Cells were cultivated in DMEM supplemented with 10% FBS and antibiotics at 37 °C in a humidified atmosphere containing 5% CO_2. Cells were plated at a density of 5.0×10^5 cells/well 24 h prior to transfection in a 24-well plate.

4.3. Particle sizes and ζ-potentials of DNA complexes

Dynamic light scattering, which is also known as photon correlation spectroscopy (PCS) and quasi-elastic light scattering (QELS), measures the time-dependent fluctuations in the intensity of scattered light that occurs because the particles in suspension are undergoing Brownian motion. The velocity of this Brownian motion is measured and is called the translational diffusion coefficient. This diffusion coefficient can be converted into particle size using the Stokes–Einstein equation. ζ-Potential measurement is provided by the electrophoretic mobility. Since frequency shift of irradiated laser light is proportional to the mobility that is a function of the particle surface potential, the frequency shift can be converted into the surface charge on the particle. Here, samples were prepared by mixing 1.0 μg PGV-C/CMV with KG6 or PLL at an N/P ratio of 8 in 1 mL DMEM. After incubation for 15 min at room temperature, particle sizes and ζ-potentials of DNA complexes were measured using the Malvern Zeta sizer Nano-ZS.

4.4. Fluorescence titration assay

PGV-C/CMV (2.5 μg) was mixed with KG6 or PLL in 990-μL reactions, in which N/P ratios were 0, 0.25, 0.5, 1, 2, 4, 8, and 16, respectively. After standing for 15 min at room temperature, 20 μL ethidium bromide solution (20 μg/mL) was added to the complex solutions. Fluorescence of the intercalated dye was measured on a spectrofluorophotometer RF-5300PC (Shimadzu, Japan) by exciting at 530 nm while monitoring emission at 590 nm, with slits set at 20 nm. Fluorescence intensities of blanks were subtracted from all values before data analysis.

4.5. DNase protection assay

Tests were performed according to procedures reported previously.[32] PGV-C/CMV (1.0 μg) or DNA complexes with KG6 or PLL at an N/P ratio of 8 were prepared in a final volume of 50 μL by standing for 10 min at room temperature. Then, 0.3 Kunitz U DNase I (3 μL) (Sigma, St. Louis, MO) was added. Mixtures were incubated for 10 min at 37 °C and heated for 10 min at 80 °C to inactivate the enzyme. Finally, 10 μL of 5 mg/mL heparin (Sigma, St. Louis, MO) was added to each tube, and tubes were incubated for 3 h at room temperature to allow complete dissociation of complexes. Samples were visualized using 0.2 μg DNA per lane on a 1.0%-agarose gel, stained with ethidium bromide.

4.6. Localization of rhodamine-labeled plasmid DNA

Tetramethylrhodamine-labeled PGV-C/CMV (0.25 μg) and PGV-C/CMV (2.25 μg) were added to 250 μL DMEM containing KG6 or PLL at an N/P ratio of 8 after standing for 15 min at room temperature, and this complex solution was added to cells. After incubation for 3 h at 37 °C, excess of complex solutions was aspirated, and cells were washed 3 times with 500 μL PBS. Fluorescence microscopy was performed with the Olympus IX-70.

4.7. Quantification of intracellular plasmid DNA

CHO cells in a 24-well plate were washed with 400 μL PBS. DNA complexes were prepared by mixing 2.5 μg PGV-C/CMV with KG6 or PLL at an N/P ratio of 8 in 250 μL serum-free DMEM. After standing for 15 min at room temperature, the complex solution was poured onto the cells. After incubation for 3 h at 37 °C, medium was aspirated, cells were washed 3 times with 300 μL PBS, and solution was replaced with 1 mL DMEM containing 10% FBS. After incubation for 24 h at 37 °C, excess complex solution was aspirated, and cells were washed 3 times with 300 μL PBS, followed by addition of 200 μL PBS to each well. Extraction of DNA was performed as described in protocols of the High Pure PCR Template Preparation Kit (Roche Diagnostics GmbH, Mannheim, Germany). Amounts of PGV-C/CMV in CHO cells were estimated by quantitative real-time PCR (LightCycler 1.5, Roche Diagnostics) in which a part of the luciferase region of PGV-C/CMV in extracted DNA samples was amplified by PCR. Sequences of PCR primers were: forward, 5′-aagatggaaccgctgaga-3′; reverse, 3′-cttctgccaaccgacg-5′. Each reaction mixture (20 μL) for PCR consisted of 2 μL DNA template (1 ng/μL), 0.8 μL primers (10 μM stock solutions), and 10 μL SYBR® Premix Ex Taq™ (TaKaRa Bio, Tokyo, Japan). PCR was performed with the following cycles: denaturation at 95 °C for 10 s; and 40 cycles at 95 °C for 0 s, 60 °C for 5 s for annealing, and 72 °C for 10 s for extension. Amounts of PGV-C/CMV were standardized against total DNA amounts extracted from CHO cells.

4.8. Gene transfection ability

CHO cells in the 24-well plate were washed with 400 μL PBS. DNA complexes were prepared by mixing 2.5 μg PGV-C/CMV with KG6 or PLL at an N/P ratio of 8 in 250 μL serum-free DMEM. After

standing for 15 min at room temperature, the complex solution was poured onto the cells. After incubation for 3 h at 37 °C, the complex solution was replaced with 1 mL DMEM containing 10% FBS, and incubation was continued for 24 h at 37 °C. The harvested cells were lysed with 200 μL Glo Lysis Buffer (Promega, Madison, WI, USA), and cell lysates were centrifuged at 12,000 rpm for 1 min at 4 °C. Luciferase activity in the supernatants was evaluated with a luciferase assay system (Promega, Madison, WI, USA) and a luminometer (Minilumat LB9506, Berthold, Germany). Protein concentrations of cell lysates were measured using the PIERCE BCA Protein Assay Kit (PIERCE, Rockford, IL, USA). Light unit values represent specific luciferase activities (RLU/mg protein) standardized against total protein content of cell lysates. Measurement of gene transfer efficiency was performed in triplicates.

4.9. Evaluation of immune response after intravenous administration of DNA complexes

Fifty microliters of KG6 or PLL solution was added to 100 μL of 10% dextrose and 50 μL of 0.5 mg/mL PGV-C/CMV at an N/P ratio of 8. After 10-min incubation at room temperature, 200 μL of the complex solution was intravenously injected to mice. After intravenous administration, blood samples were collected at appropriate time points. Blood was allowed to clot overnight at 4 °C and was then centrifuged at 2000*g* for 20 min at 4 °C. Concentrations of proinflammatory cytokines (TNF-α, IL-1β, IL-12, and IFN-γ) were analyzed using ELISA kits (mouse cytokine immunoassay kit, R&D Systems, Minneapolis, MN, USA). Animal experiments were performed according to the Guidelines for Animal care and Use committee, Kyushu University.

Acknowledgments

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References and notes

1. Gilmore, I. R.; Fox, S. P.; Hollins, A. J.; Sohail, M.; Akhtar, S. *J. Drug. Target.* **2004**, *12*, 315–340.
2. Scherr, M.; Eder, M. *Curr. Opin. Mol. Ther.* **2004**, *6*, 129–135.
3. Vanhecke, D.; Janitz, M. *Oncogene* **2004**, *23*, 8353–8358.
4. Ryther, R. C.; Flynt, A. S.; Phillips, J. A., 3rd; Patton, J. G. *Gene Ther.* **2005**, *12*, 5–11.
5. Niidome, T.; Huang, L. *Gene Ther.* **2002**, *9*, 1647–1652.
6. Felgner, P. L.; Gadek, T. R.; Holm, M.; Roman, R.; Chan, H. W.; Wenz, M.; Northrop, J. P.; Ringold, G. M.; Danielsen, M. *Proc. Natl. Acad. Sci. U.S.A.* **1987**, *84*, 7413–7417.
7. Marshall, J.; Yew, N. S.; Eastman, S. J.; Jiang, C.; Scheule, R. K.; Cheng, S. H. In *Nonviral Vectors for Gene Therapy*; Huang, L., Hung, M. C., Wagner, E., Eds.; Academic Press: USA, 1999; pp 39–68.
8. Xu, Y.; Szoka, F. C., Jr. *Biochemistry* **1996**, *35*, 5616–5623.
9. Brigham, K. L.; Meyrick, B.; Christman, B.; Magnuson, M.; King, G.; Berry, L. C., Jr. *Am. J. Med. Sci.* **1989**, *298*, 278–281.
10. Song, Y. K.; Liu, F.; Chu, S.; Liu, D. *Hum. Gene Ther.* **1997**, *8*, 1585–1594.
11. McLean, J. W.; Fox, E. A.; Baluk, P.; Bolton, P. B.; Haskell, A.; Pearlman, R.; Thurston, G.; Umemoto, E. Y.; McDonald, D. M. *Am. J. Physiol.* **1997**, *273*, H387–H404.
12. Scheule, R. K.; St George, J. A.; Bagley, R. G.; Marshall, J.; Kaplan, J. M.; Akita, G. Y.; Wang, K. X.; Lee, E. R.; Harris, D. J.; Jiang, C.; Yew, N. S.; Smith, A. E.; Cheng, S. H. *Hum. Gene Ther.* **1997**, *8*, 689–707.
13. Freimark, B. D.; Blezinger, H. P.; Florack, V. J.; Nordstrom, J. L.; Long, S. D.; Deshpande, D. S.; Nochumson, S.; Petrak, K. L. *J. Immunol.* **1998**, *160*, 4580–4586.
14. Li, S.; Wu, S. P.; Whitmore, M.; Loeffert, E. J.; Wang, L.; Watkins, S. C.; Pitt, B. R.; Huang, L. *Am. J. Physiol.* **1999**, *276*, L796–L804.
15. Wu, G. Y.; Wu, C. H. *J. Biol. Chem.* **1987**, *262*, 4429–4432.
16. Wagner, E. In *Nonviral vectors for gene therapy*; Huang, L., Hung, M. C., Wagner, E., Eds.; Academic Press: USA, 1999; pp 207–227.
17. Harada-Shiba, M.; Yamauchi, K.; Harada, A.; Takamisawa, I.; Shimokado, K.; Kataoka, K. *Gene Ther.* **2002**, *9*, 407–414.
18. Kwok, K. Y.; McKenzie, D. L.; Evers, D. L.; Rice, K. G. *J. Pharm. Sci.* **1999**, *88*, 996–1003.
19. Collard, W. T.; Yang, Y.; Kwok, K. Y.; Park, Y.; Rice, K. G. *J. Pharm. Sci.* **2000**, *89*, 499–512.
20. Kwok, K. Y.; Park, Y.; Yang, Y.; McKenzie, D. L.; Liu, Y.; Rice, K. G. *J. Pharm. Sci.* **2003**, *92*, 1174–1185.
21. Ziady, A. G.; Ferkol, T.; Dawson, D. V.; Perlmutter, D. H.; Davis, P. B. *J. Biol. Chem.* **1999**, *274*, 4908–4916.
22. Ward, C. M.; Read, M. L.; Seymour, L. W. *Blood* **2001**, *97*, 2221–2229.
23. Abdallah, B.; Hassan, A.; Benoist, C.; Goula, D.; Behr, J. P.; Demeneix, B. A. *Hum. Gene Ther.* **1996**, *7*, 1947–1954.
24. Goula, D.; Remy, J. S.; Erbacher, P.; Wasowicz, M.; Levi, G.; Abdallah, B.; Demeneix, B. A. *Gene Ther.* **1998**, *5*, 712–717.
25. Wightman, L.; Kircheis, R.; Rossler, V.; Carotta, S.; Ruzicka, R.; Kursa, M.; Wagner, E. *J. Gene Med.* **2001**, *3*, 362–372.
26. Ohsaki, M.; Okuda, T.; Wada, A.; Hirayama, T.; Niidome, T.; Aoyagi, H. *Bioconjugate Chem.* **2002**, *13*, 510–517.
27. Kawano, T.; Okuda, T.; Aoyagi, H.; Niidome, T. *J. Controlled Release* **2004**, *99*, 329–337.
28. Wadhwa, M. S.; Collard, W. T.; Adami, R. C.; McKenzie, D. L.; Rice, K. G. *Bioconjugate Chem.* **1997**, *8*, 81–88.
29. Boussif, O.; Lezoualc'h, F.; Zanta, M. A.; Mergny, M. D.; Scherman, D.; Demeneix, B.; Behr, J. P. *Proc. Natl. Acad. Sci. U.S.A.* **1995**, *92*, 7297–7301.
30. Yew, N. S.; Zhao, H.; Wu, I-H.; Song, A.; Tousignant, J. D.; Przybylska, M.; Cheng, S. H. *Mol. Ther.* **2000**, *1*, 255–262.
31. Tan, Y.; Liu, F.; Li, Z.; Huang, L. *Mol. Ther.* **2001**, *3*, 673–682.
32. Gebhart, C. L.; Sriadibhatla, S.; Vinogradov, S.; Lemieux, P.; Alakhov, V.; Kabanov, A. V. *Bioconjugate Chem.* **2002**, *13*, 937–944.