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Amphiphilic Peptides With Arginines and Valines for the Delivery of Plasmid DNA

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ABSTRACT

A non-toxic and efficient gene carrier is one requirement for clinical gene therapy. In this study, amphiphilic peptides composed of arginines and valines were synthesized and characterized as plasmid DNA (pDNA) carriers. The peptides have a cationic region containing 1–4 arginines and a hydrophobic region containing 6 valines. The arginine–valine peptides (RV peptides) formed micelles in aqueous solution with a critical micelle concentration (CMC) of 1.35 mg/ml. In gel retardation assay, the RV peptides retarded all pDNA at weight ratios (pDNA:RV peptide) of 1:3 for R1V6, 1:2 for R2V6 and R3V6, and 1:1 for R4V6. A heparin competition assay showed that the R3V6 peptide formed tighter complexes with pDNA than poly-L-lysine (PLL). In vitro transfection assay into HEK293 cells showed that the R1V6 and R2V6 peptides had the highest transfection efficiencies at 1:30 weight ratios (pDNA:RV peptide), while the R3V6 and R4V6 peptides had the highest efficiencies at 1:20 weight ratios. Under optimal conditions, the R3V6 peptide had the highest transfection efficiency of all the RV peptides and PLL. MTT assay showed that the RV peptides did not have any detectable toxicity to cells. Therefore, the RV peptide may be useful for the development of non-toxic gene carriers. J. Cell. Biochem. 112: 1458–1466, 2011.

KEY WORDS: AMPHIPHILE; ARGININE; MICELLE; PLASMID DNA DELIVERY; TRANSFECTION

INTRODUCTION

Gene delivery, in which DNAs or RNAs are delivered into the cells in order to elicit therapeutic effects, is a promising strategy for treating hard-to-cure diseases. A number of carriers have been developed for gene delivery [Han et al., 2000; Niidome and Huang, 2002; Kang et al., 2005]. The carriers are generally classified into two groups, viral and non-viral, each with its own pros and cons. For instance, viral carriers have higher transfection efficiency, but can be cytotoxic, may elicit an immune response or can be integrated into the host chromosome [Kang et al., 2005]. On the contrary, non-viral carriers have low toxicity and immunogenicity compared with those of viral carriers [Kang et al., 2005]. However, the low transfection efficiencies of non-viral carriers limit their clinical applications. Also, viral and non-viral carriers have different gene delivery efficiencies depending on tissue type or delivery route. Therefore, gene carriers should be selected carefully, depending on the disease and administration route.

Due to the advantages of non-viral carriers, various studies have attempted to develop efficient non-viral carriers including cationic liposomes, cationic polymers, and their derivatives [Kang et al., 2005]. In addition, physical methods such as electroporation and sonoporation have been developed [Niidome and Huang, 2002]. Peptide-based carriers have also been developed as gene carriers. Poly-L-lysine (PLL) is a typical peptide carrier with positive charges on the ε-primary amine groups and the ability to form a small complex through electrostatic interaction with the negatively charged phosphate groups in the DNA backbone. PLL has been conjugated to specific ligands to target gene delivery [Trubetskoy et al., 1992; Mislick et al., 1995; Choi et al., 1998b; Suh et al., 2001]. In vitro and in vivo experiments showed that ligands conjugated to PLL could deliver DNA to specific tissues [Wu and Wu, 1988; Hashida et al., 1998; Nishikawa et al., 1998]. However, PLL is toxic to cells, which limits its clinical application [Choi et al., 1998a]. Therefore, many researchers have focused on developing non-toxic carriers, and a number of strategies for reducing cytotoxicity have

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been developed. To reduce the cytotoxicity of PLL, polyethylene glycol (PEG) has been conjugated to PLL [Katayose and Kataoka, 1997; Choi et al., 1998a; Lee and Kim, 2005]. Conjugating PEG to PLL not only reduced PLL cytotoxicity, but also increased the solubility of the PLL/DNA complex. Another method for reducing cytotoxicity is to introduce degradable bonds into the polymer backbone. For example, ester bonds were introduced at the amide bonds of PLL, resulting in synthesis of poly[α-(4-aminobutyl)-L-glycolic acid] (PAGA) [Lim et al., 2000].

Cationic polymers may be cytotoxic due to their high cationic charges [Lee and Kim, 2005]. Therefore, reducing the cationic charges on a carrier may reduce its cytotoxicity. In a previous study, we showed that natural peptides such as high mobility group box-1 (HMGB-1) had a lower charge density and was less cytotoxicity than PLL [Kim et al., 2008; Han et al., 2009]. Another important approach to reduce cytotoxicity is using short peptides. In the current study, we synthesized short peptides composed of 1–4 arginines and 6 valines. The peptides contain only arginine (R) and valine (V) and were named 'RV peptides.' These peptides formed micelles in aqueous solution. The peptide micelles were evaluated as gene carriers in terms of cytotoxicity and gene delivery efficiency. The results suggest that the RV peptides may be useful as non-toxic gene carriers.

MATERIALS AND METHODS

SYNTHESIS OF RV PEPTIDES

The peptides with 1–4 arginines and 6 valines were synthesized chemically and were purified using C18 reverse-phase chromatography (Peptron Co., Daejeon, South Korea). The peptides were dissolved in water at 4 mg/ml and were stored at −70°C.

CRITICAL MICELLE CONCENTRATION

The critical micelle concentration (CMC) of R3V6 peptide (MW 1,080) was measured via spectrofluorometry using 1,6-diphenyl-1,3,5-hexatriene (DPH). The R3V6 peptide solutions containing 4 μM DPH were prepared at various concentrations (0–4 mg/ml). The solutions were incubated for 16 h at 4°C. The DPH emission spectra were recorded at 428 nm using a spectrofluorometer, with excitation at 355 nm (Spectra max M2, Molecular devices, USA).

PREPARATION OF PCMV-LUC/RV PEPTIDE COMPLEXES

pCMV-Luc was constructed by inserting the luciferase cDNA at the HinDIII and XbaI sites of pcDNA3. pCMV-Luc was propagated in DH5α *E. coli* and was prepared using the Qiagen Maxi-prep kit (Invitrogen, Carlsbad, CA).

pCMV-Luc/RV peptide complexes were prepared at various weight ratios in 5% glucose. pCMV-Luc/PLL complex was prepared at a 2:1 weight ratio, based on a previous report [Lee et al., 2001]. pCMV-Luc and lipofectamine (Invitrogen) were mixed according to the manufacturer's instructions. pCMV-Luc/peptides mixtures were incubated at room temperature for 15 min.

GEL RETARDATION ASSAY

Complex formation between pCMV-Luc and RV peptides was verified through a gel retardation assay. A fixed amount of pCMV-Luc (0.5 μg) was mixed with increasing amounts of the RV peptides in 5% (w/v) glucose. The mixtures were analyzed on a 1% agarose gel in the presence of ethidium bromide. The pCMV-Luc was visualized using a UV transilluminator.

HEPARIN COMPETITION ASSAY

The stability of the pCMV-Luc/RV peptide complex was evaluated by heparin competition assay [Liu and Reineke, 2005]. The pCMV-Luc/R3V6 peptide and pCMV-Luc/PLL complexes were prepared at 1:20 and 1:2 weight ratios (1:6,000 and 1:30 molar ratios), respectively (pCMV-Luc:peptide). Two micrograms of pCMV-Luc was mixed with 40 μg of R3V6 peptide or 4 μg of PLL to form complexes in 20 μl 5% glucose solution. Increasing amounts of heparin (Sigma, St. Louis, MO) were added to the pCMV-Luc/peptide complex. The samples were analyzed on a 1% agarose gel containing ethidium bromide. The pCMV-Luc was visualized using a UV transilluminator.

DNASE I PROTECTION ASSAY

Ten micrograms of pCMV-Luc was mixed with 200 μg of R3V6 peptide in phosphate buffered saline (PBS). DNase I (10 unit, Promega, Madison, WI) was added to the complex solution. After incubation for 30 or 60 min at 37°C, 100 μl of the mixture was mixed with 100 μl of 2× stop solution (80 mM EDTA and 2% SDS) to terminate DNase I reaction and dissociate DNA from R3V6 peptide. The dissociated DNA was analyzed by agarose gel electrophoresis.

FACS ASSAY

The complex of R3V6 peptide with fluorescein amidite (FAM)-labeled oligodeoxynucleotide (0.7 μg, 100 pmol, MW 7,240) was transfected to cultured C6 cells and incubated for 24 h at 37°C. The DNA/R3V6 complex was prepared at a 1:20 weight ratio and DNA/lipofectamine complex was prepared according to the manufacturer's instructions provided in the technical manual. The transfected C6 cells were harvested and washed with PBS. The suspended cells were centrifuged at 2,000 rpm for 5 min. The C6 cells were resuspended in fluorescence-activated cell sorting (FACS) buffer (PBS with 0.02% NaN_3, 0.2% FBS) and centrifuged at 2,000 rpm for 5 min. After supernatant was removed, C6 cells were resuspended in fixing buffer (FACS buffer with 1% formaldehyde). Flow cytometry was performed using the BD FACS Calibur TM (BD Biosciences Immunocytometry Systems, San Jose, CA).

CELL CULTURE AND IN VITRO TRANSFECTION

Human embryonic kidney 293 (HEK293) and A7R5 rat aortic smooth muscle cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Invitrogen) containing 1% PS and 10% FBS at 37° in 5% CO_2 atmosphere. The cells were seeded in six-well plates at 4×10^4 cells/well for luciferase assays and were incubated for 24 h at 37°C. The pCMV-Luc/RV peptide complexes were prepared at various weight ratios by mixing 0.5 μg of pCMV-Luc with increasing amounts of RV peptide in 5% glucose solution. The pCMV-Luc/PLL (20 kDa) complex was prepared at a 1:2 weight ratio and the pCMV-Luc/lipofectamine complex was mixed according to the manufacturer's instructions. The complexes were added to the cells in fetal bovine serum (FBS)-free medium. After 4 h, the medium

was exchanged with DMEM containing 10% (v/v) FBS. The cells were incubated for an additional 24 h at 37°C and harvested for luciferase assays.

LUCIFERASE ASSAY

After transfection and incubation, the cells were washed twice with 1 ml of PBS, and 120 μl of reporter lysis buffer (Promega) was added to each well. The cell extracts were harvested and transferred to microcentrifuge tubes. The extracts were centrifuged at 13,000 rpm for 3 min to remove cell debris. Luciferase activities of the samples were measured using a 96-well plate luminometer (Berthold Detection System GmbH, Pforzheim, Germany). Protein concentrations of the extracts were measured using a BCA assay kit (Pierce, Rockford, IL). The final luciferase activities were calculated as relative light units (RLU) per mg total protein.

CYTOTOXICITY

The cytotoxicity of the RV peptide was measured by MTT assay as described previously [Benns et al., 2001, 2002; Han et al., 2001; Lee et al., 2001, 2003; Kim et al., 2009a]. HEK293 cells were seeded at density of 1×10^4 cells/well in 24-well microassay plates and incubated for 24 h before transfection. The pCMV-Luc/RV peptides complexes were prepared at their optimal weight ratios for the highest transfection. The pCMV-Luc/PLL complex was prepared at a 1:2 weight ratio (DNA:peptide) and the pCMV-Luc/lipofectamine complex was mixed according to the manufacturer's instruction. The PLL and lipofectamine were used as controls. The complexes were added to the cells in serum-free DMEM and were incubated with the cells for 4 h. After incubation, the medium was exchanged with 0.5 ml of fresh DMEM containing 1% PS and 10% FBS. The cells were incubated for an additional 24 h at 37° in a 5% CO_2 incubator. After the transfection, 40 μl of 2 mg/ml MTT solution in PBS was added to the wells. The plates were incubated for 4 h at 37°C. The MTT medium was removed, and 750 μl of dimethyl sulfoxide was added to each well to dissolve the formazan crystals formed by the live cells. The cell survival was evaluated by measuring the absorbance at 570 nm. Cell viability (%) was calculated according to the following equation:

$$\text{Cell viability (\%)} = \left(\text{OD}_{570\,\text{nm (sample)}}/\text{OD}_{570\,\text{nm (control)}}\right) \times 100,$$

where $\text{OD}_{570\text{nm(sample)}}$ represents the wells treated with pCMV-Luc/peptides complexes and $\text{OD}_{570\text{nm(control)}}$ represents the wells treated with 5% glucose solution.

STATISTICAL ANALYSIS

Statistical analysis was conducted using ANOVA followed by the Newman–Keuls test. All data were presented as average ± SEM, and P values less than 0.05 were considered statistically different.

RESULTS

DESIGN AND PHYSICAL CHARACTERIZATION OF RV PEPTIDES

The RV peptides were designed with amphiphilic characteristics in order to form micelles formation in aqueous solution (Fig. 1). The RV peptides had 2–5 positive charges with 1–4 arginines. The RV peptides were designed to have a low cytotoxicity with a small number of positive charges. In addition, the RV peptides in micelles may behave like larger molecules as micelles and have high transfection efficiency.

To confirm that the RV peptides formed micelles in aqueous solution, the CMC of RV peptides in 5% glucose was measured using DPH (Fig. 2). Since the R3V6 peptides showed the highest transfection efficiency in the following experiments, its CMC was measured as a representative value. The results showed that the CMC of R3V6 peptide was 1.35 mg/ml (Fig. 2).

To confirm that the RV peptides formed complexes with pDNA, gel retardation assays were performed. A fixed amount of pCMV-Luc was mixed with increasing amounts of the RV peptide. As shown in Figure 3, R1V6 peptides completely retarded pCMV-Luc at a 1:3 weight ratio (pDNA:peptide). The R4V6 peptides completely retarded pCMV-Luc at a 1:1 weight ratio, while the R2V6 and R3V6 peptides did the same at weight ratios of 1:2. The RV peptides with more

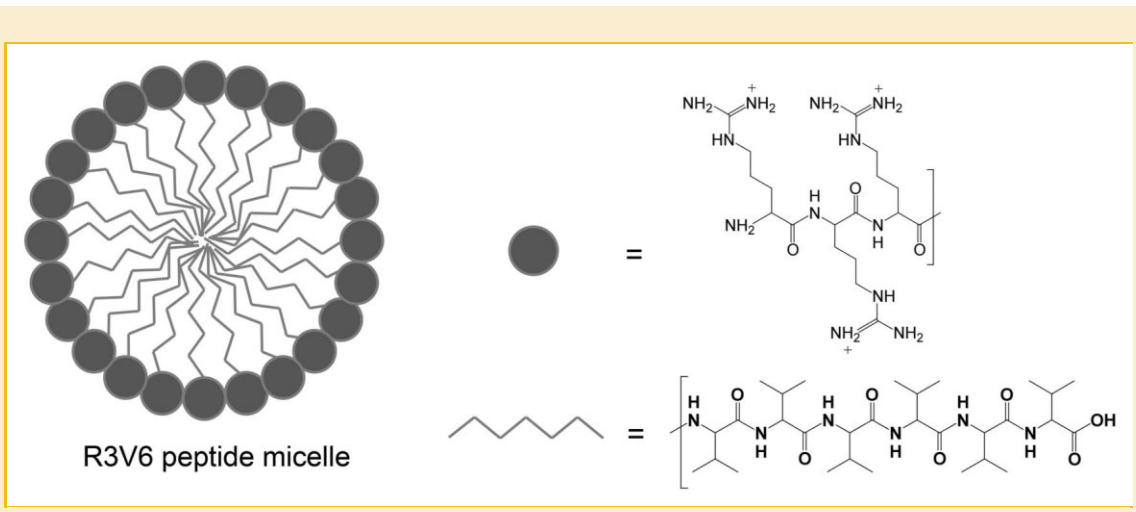

Fig. 1. Schematic representations of RV peptide micelles.

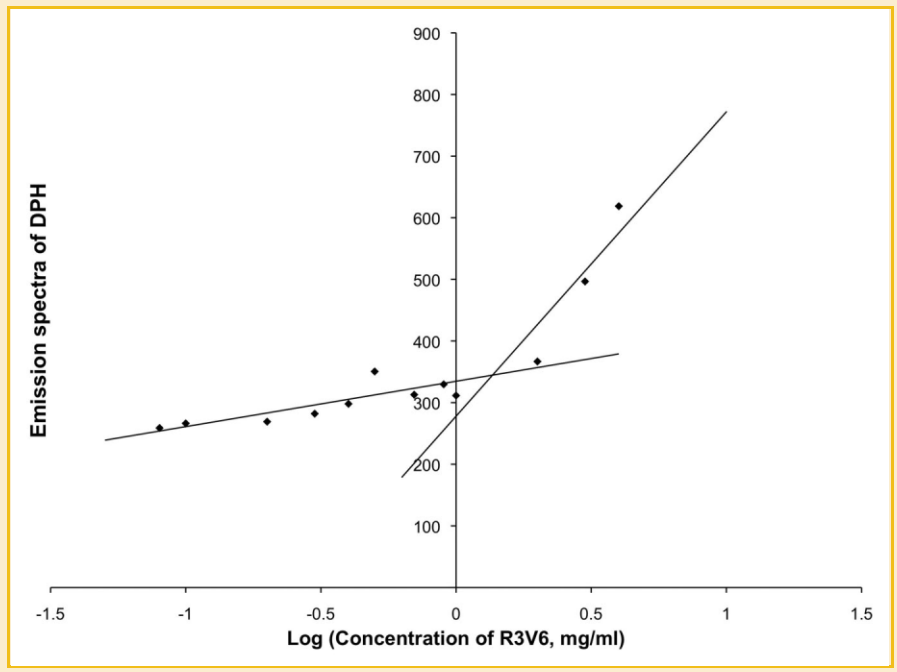

Fig. 2. Critical micelle concentration CMC of the R3V6 peptide was measured by spectrofluorometry. The R3V6 peptide solutions containing 4 μM DPH were prepared at various concentrations. The emission spectrum of DPH was recorded at 428 nm after an excitation at 355 nm using a spectrofluorometer.

positive charges retarded pCMV-Luc more efficiently. These results confirmed that peptides with more positive charge can more easily and tightly form pDNA:peptide complexes.

Heparin is highly negatively charged and will compete with DNA for the positively charged peptides. In heparin competition assay, the pCMV-Luc/R3V6 peptide and pCMV-Luc/PLL complexes were prepared at their optimal condition for transfection. The pCMV-Luc/PLL complex released pDNA in the presence of 16 μg heparin (Fig. 4); however, pCMV-Luc/R3V6 complexes remained associated at the same heparin concentration. The pCMV-Luc/R3V6 complexes

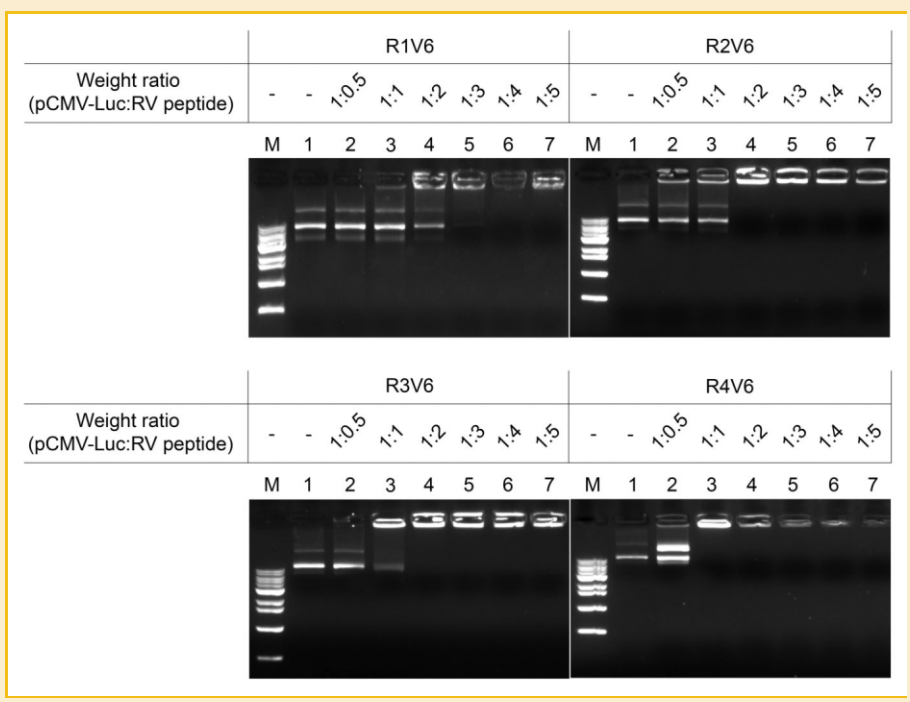

Fig. 3. Gel retardation assay. The formation of the pCMV-Luc/RV peptide complexes was verified via gel retardation assay. A fixed amount of pCMV-Luc (0.5 μg) was mixed with increasing amounts of RV peptides in 5% (w/v) glucose. The mixtures were analyzed in a 1% agarose gel with ethidium bromide.

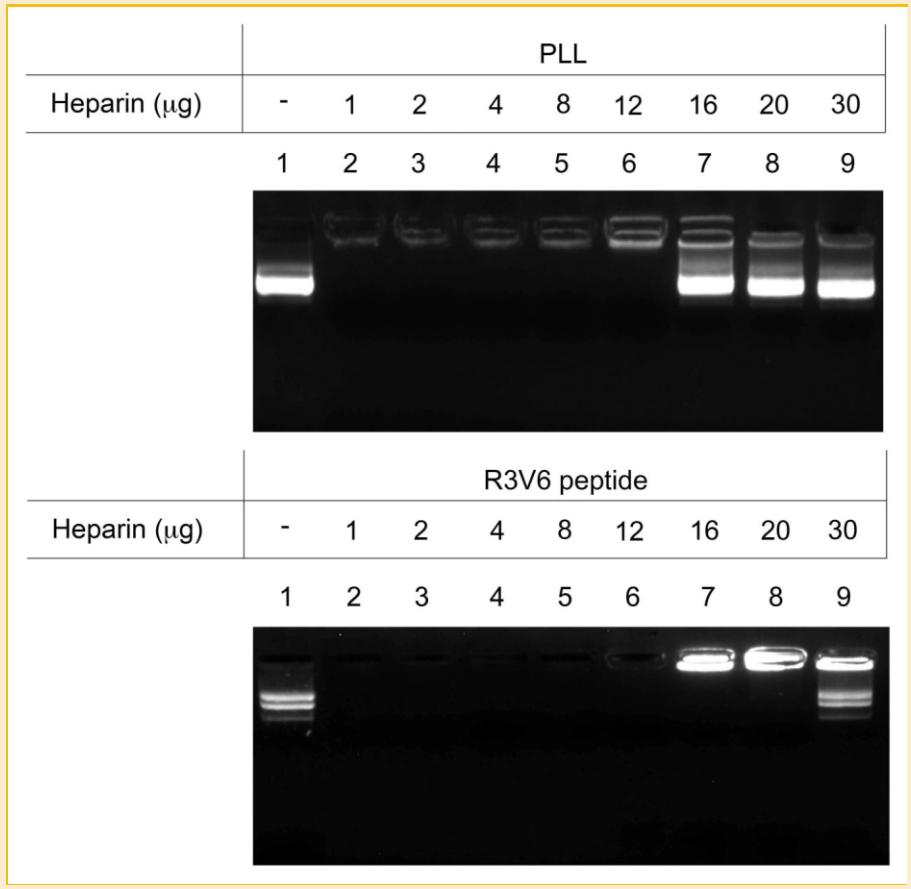

Fig. 4. Heparin competition assay. The stabilities of the pCMV-Luc/RV peptide complexes were evaluated by a heparin competition assay. The pCMV-Luc/R3V6 peptide and pCMV-Luc/PLL complexes were prepared as described. Increasing amounts of heparin were added to the complexes, and the samples were analyzed in 1% agarose gel containing ethidium bromide.

began to dissociate in 30 μg heparin. The results showed that pDNA form more stable complexes with the R3V6 peptides than PLL at their optimal conditions for transfection.

DNase I protection assay was performed to evaluate DNA protection ability of the R3V6 peptide. The pCMV-Luc/R3V6 complex was prepared at a 1:20 weight ratio. Naked pCMV-Luc was used as a control. The complex solutions were incubated with DNase I for 30 or 60 min at 37°C. As a result, naked pCMV-Luc was degraded completely by DNase I (Fig. 5, lanes 1–3). However, the R3V6 peptide protected pCMV-Luc over 60 min (Fig. 5, lanes 4–6).

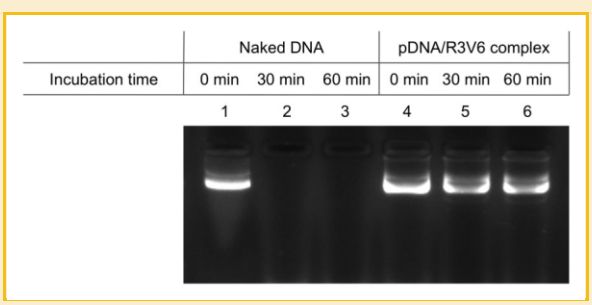

Fig. 5. DNase I protection assay pCMV-Luc/R3V6 complexes were prepared at a 1:20 weight ratio. DNase I was added to the complex or naked DNA solution. DNase I reaction was terminated by addition of 2× stop solution. pDNA was analyzed by 1% agarose gel electrophoresis.

IN VITRO TRANSFECTION ASSAY

To evaluate the intracellular uptake of DNA/R3V6 complex, FAM-labeled DNA/R3V6 peptide complex was transfected into C6 cell and the transfection efficiency was analyzed by flow cytometry. While lipofectamine showed approximately a cellular uptake efficiency of 51%, R3V6 had a uptake efficiency of 18% (Fig. 6). R3V6 peptide had lower efficiency than lipofectamine in terms of intracellular uptake, which is consistent with the results of luciferase assay. However, R3V6 peptide had a significant effect on DNA uptake, compared with naked DNA.

To evaluate the transfection efficiencies of RV peptides, in vitro transfection assays were performed with the RV peptides. The pCMV-Luc/RV peptide complexes were prepared and transfected into HEK293 cells at various weight ratios. The R1V6 and R2V6 peptides showed the highest transfection efficiencies at 1:30 weight ratios (pDNA:peptide) (Fig. 7), while the R3V6 and R4V6 peptides had the highest transfection efficiencies at 1:20 weight ratios (Fig. 7). Especially, the R4V6 peptide showed marked difference in the transfection efficiency between 1:10 and 1:20 weight ratios

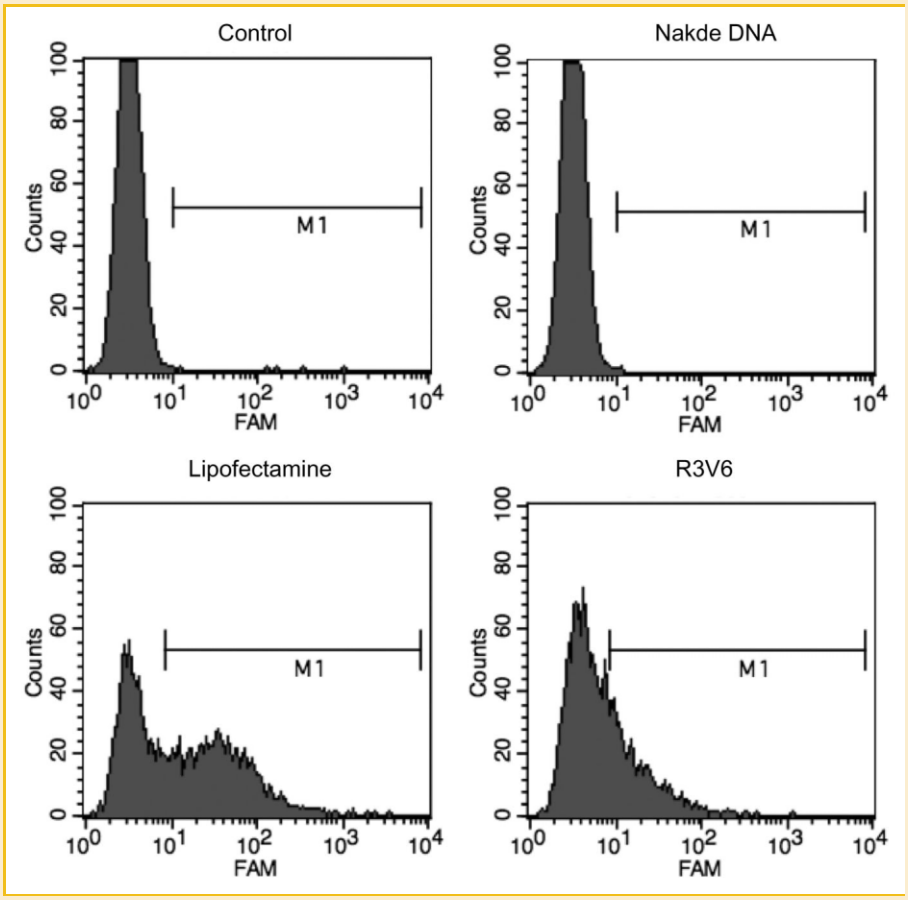

Fig. 6. Cellular uptake of DNA by RV peptide. The complex of R3V6 peptide with FAM-labeled oligodeoxynucleotide was transfected to C6 cells and incubated for 24 h at 37°C. Lipofectamine was used as a control carrier. The efficiency of cellular uptake was measured by flow cytometry.

(Fig. 7D). The R3V6 and R4V6 peptides reached their maximum transfection efficiencies at lower peptide concentrations than the R1V6 and R2V6 peptides. The RV peptides with more positive charges may form stable complexes with pDNA more efficiently than the RV peptides with fewer positive charges.

The transfection efficiencies of the RV peptides were compared with each other. Each RV peptide was complexed with pCMV-Luc at the optimal weight ratio. PLL, a common peptide based gene carrier, and lipofectamine, a commercial liposome, were used as positive controls. The complexes were transfected into HEK293 and A7R5 cells. The R3V6 peptide had higher transfection efficiency than other RV peptides in both HEK293 (Fig. 8A) and A7R5 cells (Fig. 8B). Although the R3V6 peptide had lower transfection efficiency than lipofectamine, it had higher transfection efficiency than PLL in 293 cells (Fig. 8C) and similar efficiency to PLL in A7R5 cells (Fig. 8D).

CYTOTOXICITY

The cytotoxicities of the RV peptides were evaluated by MTT assay. PLL and lipofectamine were used as controls. The pCMV-Luc/RV peptide complexes were prepared at the optimal weight ratios and transfected into HEK293 cells. The results of the MTT assay showed that no RV peptides had any detectable toxicity, while viabilities of the PLL and lipofectamine treated cells were approximately 70% and 60%, respectively (Fig. 9). These results confirm that the RV peptides are not toxic to cells.

DISCUSSION

In this research, we have shown that the RV peptides composed of hydrophilic arginines and hydrophobic valines formed stable complexes with pDNA and delivered pDNA to cells more efficiently than PLL. Furthermore, the RV peptides are less cytotoxic than PLL. In vitro transfection assays showed that the R3V6 peptides are the most efficient RV peptide in HEK293 cells.

The RV peptides consist of 7–10 amino acids, with 1–4 of them carrying positive charges. Because of the small number of positive charges, RV peptides had negligible cellular toxicity (Fig. 9). The cytotoxicities of cationic polymers such as polyethylenimine (PEI) and PLL are due to the high positive charge of the pDNA/polymer complex. In a previous report, it was shown that pDNA/PEI complexes might aggregate on and rupture the cellular membrane surfaces [Fischer et al., 1999]. In this experiment, PLL with a molecular weight of 20 kDa was used as a control. The degree of PLL polymerization was around 136, suggesting about 136 positive charges per molecule. However, the R3V6 peptide has much lower

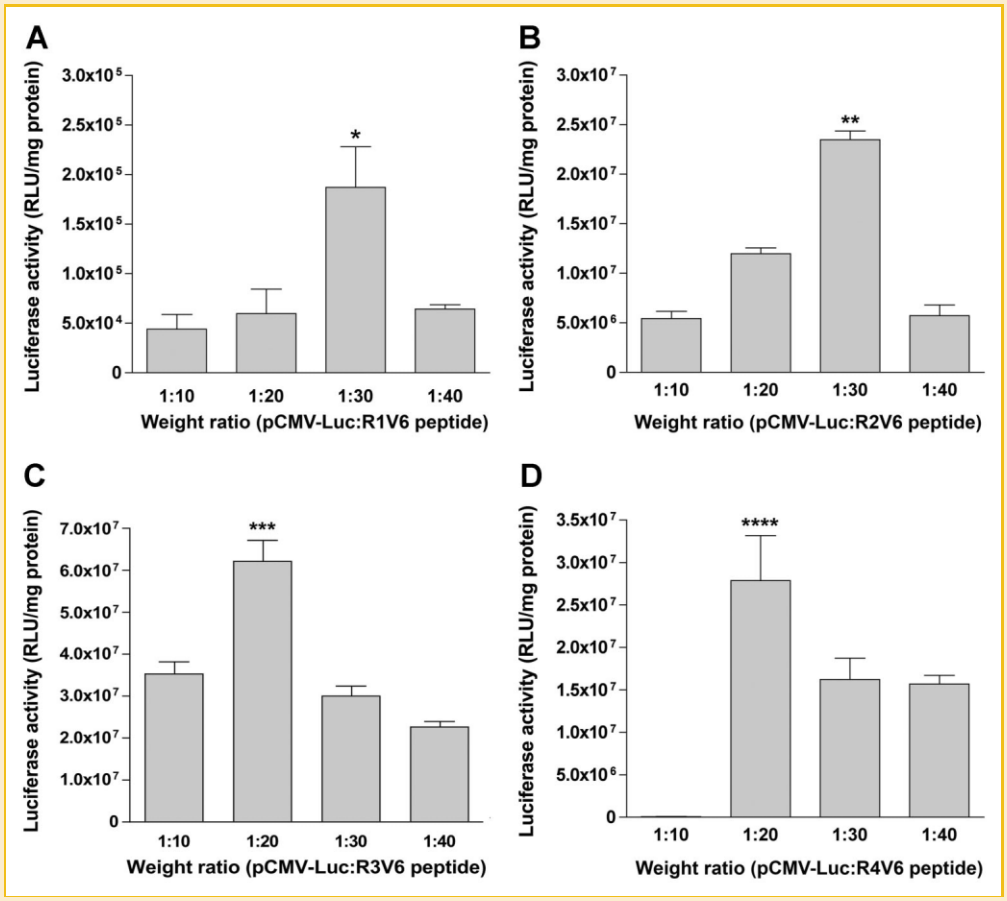

Fig. 7. Transfection efficiencies of the RV peptides depending on weight ratio. The pDNA/RV peptide complexes were prepared at various weight ratios and transfected into HEK293 cells. Transfection efficiency was measured by luciferase assay. (A) R1V6, (B) R2V6, (C) R3V6, and (D) R4V6. The data are expressed as mean values (±standard deviation) of quadruplicate experiments. *, **, ***, ****$P < 0.05$ as compared with 1:10, 1:20, and 1:40.

positive charge than PLL. Considering the low charge density of the R3V6 peptides, they were expected to be less toxic than PLL.

The R3V6 peptides formed complexes with pDNA more stably than PLL. A heparin competition assay confirmed that the pDNA/R3V6 peptides complexes were more stable than the pDNA/PLL complexes (Fig. 4). In addition, R3V6 peptides had higher transfection efficiency than that of PLL. This enhanced transfection efficiency may be due to micelle formation. Another possibility is a cell penetrating peptide (CPP) effect imparted by the arginines. Short arginine-rich peptides such as tat and oligoarginine R9 have been reported to act as CPPs [Deshayes et al., 2005; Abes et al., 2007]. However, most CPP effects have been observed in linear peptides. Recently, a spherical arginine-rich polymer has been reported to have CPP and nuclear targeting effects [Kim et al., 2009b]. Polyamidoamine dendrimers conjugated with arginines (PAMAM-R) had higher transfection efficiency than PAMAM due to CPP and nuclear targeting effects [Kim et al., 2009b]. The R3V6 peptides may form spherical complexes with pDNA, which then act as CPPs. Further study to identify this effect will be interesting.

Peptide micelles as gene carriers have been previously developed using longer peptides, usually composed of lysine or histidine [Kichler et al., 2006; Morris et al., 2007; Wiradharma et al., 2009]. One of the peptide micelles contained hydrophilic segments with 15–30 positive amino acids and hydrophobic segments with 15–30 alanines, tryptophanes, or phenylalanines [Morris et al., 2007]. The peptides voluntarily formed a micelle structure in aqueous solution and complexed with pDNA. The transfection efficiency of the micelles was even higher than that of lipofectamine or PEI. Furthermore, these peptides micelles were able to load a hydrophobic drug within their hydrophobic cores. The micelles loaded with a hydrophobic anti-cancer drug, doxorubicine, delivered both the drug and DNA to tumors [Wiradharma et al., 2009]. However, the toxicities of peptides/pDNA complexes should be minimized for clinical applications. The RV peptides micelles had fewer positive charges than the previously developed peptide micelles and did not have any detectable cytotoxicity.

Synthetic short peptides have been attractive gene carriers, since short peptides have low cytotoxicity and may not induce an immune response. In addition, they are easily degraded in the cells. In our current study, the RV peptides had a moderate transfection efficiency and low cytotoxicity. The R3V6 peptides had higher transfection efficiency and lower cytotoxicity than PLL in HEK293 cells. PLL has been modified in various ways for efficient and targeted delivery. Targeting ligands promoted effective binding,

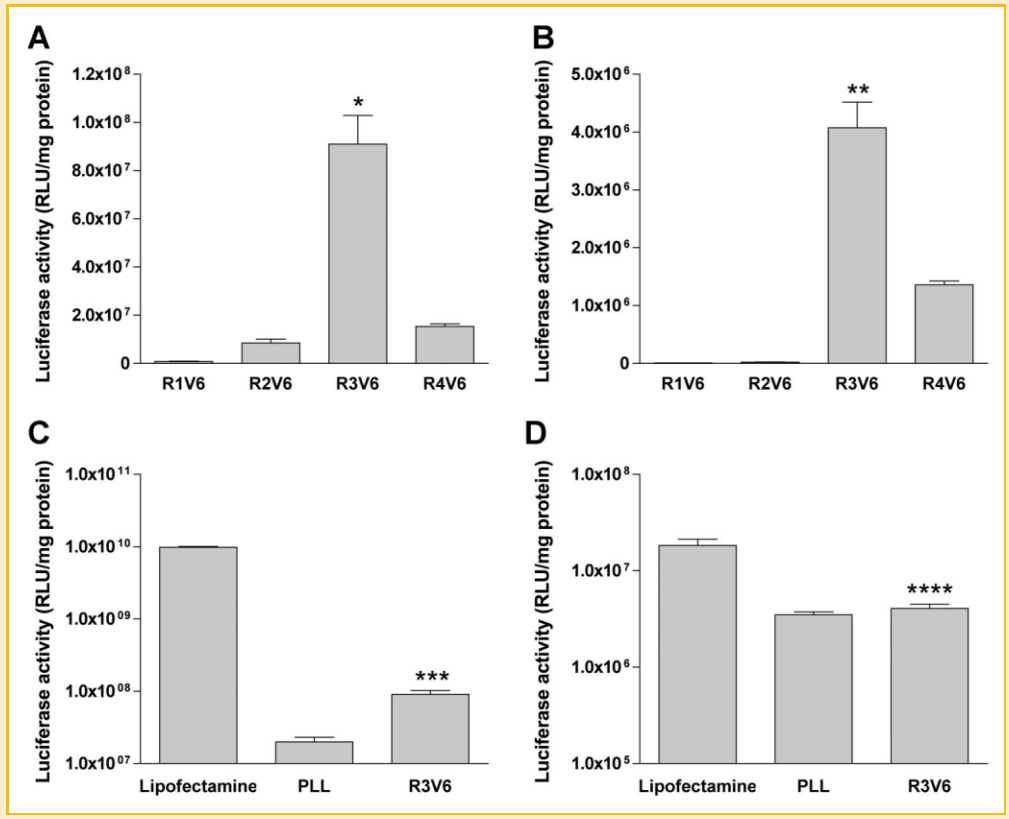

Fig. 8. Comparison of transfection efficiencies of RV peptides. The pDNA/RV peptides complexes were prepared at the optimal weight ratio and were compared each other in HEK293 (A) and A7R5 cells (B). The transfection efficiency of the R3V6 peptide was compared with those of PLL and lipofectamine in HEK293 (C) and A7R5 cells (D). Transfection efficiency was measured by luciferase assay. The data are expressed as mean values (±standard deviation) of quadruplicate experiments. *, $^{**}P < 0.05$ as compared with R1V6, R2V6, and R3V6. $^{***}P < 0.05$ as compared with lipofectamine and PLL. $^{****}P < 0.05$ as compared with lipofectamine.

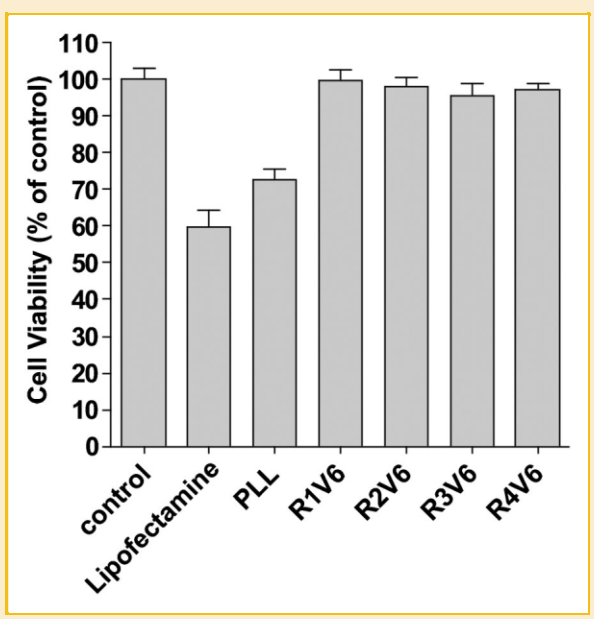

Fig. 9. Cytotoxicities of the RV peptides. The pDNA/RV peptides, pDNA/PLL, and pDNA/lipofectamine complexes were transfected into HEK293 cells. After transfection, cell viability was measured by MTT assay. The data are expressed as mean values (±standard deviation) of quadruplicate experiments.

internalization, and delivery to the target cells. Like PLL, the RV peptides may be more applicable after conjugation with various ligands. Decoration of the peptide with ligands may allow a targeting delivery to specific types of cells, which may reduce the side effects and increase the efficiency of gene delivery. Therefore, with low cytotoxicity and moderate transfection efficiency, the RV peptides may be useful for development of safe gene carriers.

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