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R3V6 Amphiphilic Peptide with High Mobility Group Box 1A Domain as an Efficient Carrier for Gene Delivery

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The R3V6 peptide includes a hydrophilic arginine stretch and a hydrophobic valine stretch. In previous studies, the R3V6 peptide was evaluated as a gene carrier and was found to have low cytotoxicity. However, the transfection efficiency of R3V6 was lower than that of poly-L-lysine (PLL) in N2A neuroblastoma cells. In this study, the transfection efficiency of R3V6 was improved in combination with high mobility group box 1A domain (HMGA). HMGA is originated from the nuclear protein and has many positively-charged amino acids. Therefore, HMGA binds to DNA *via* charge interaction. In addition, HMGA has a nuclear localization signal peptide and may increase the delivery efficiency of DNA into the nucleus. The ternary complex with HMGA, R3V6, and DNA was prepared and evaluated as a gene carrier. First, the HMGA/DNA complex was prepared with a negative surface charge. Then, R3V6 was added to the complex to coat the negative charges of the HMGA/DNA complex, forming the ternary complex of HMGA, R3V6, and DNA. A physical characterization study showed that the ternary complex was more stable than the PLL/DNA complex. The HMGA/R3V6/DNA complex had a higher transfection efficiency than the PLL/DNA, HMGA/DNA, or R3V6/DNA complexes in N2A cells. Furthermore, the HMGA/R3V6/DNA complex was not toxic to cells. Therefore, the HMGA/R3V6/DNA complex may be a useful gene delivery carrier.

Key Words : High mobility group box 1A (HMGA), DNA delivery, Transfection, Cytotoxicity

Introduction

Gene therapy is a promising strategy to treat hard-to-cure diseases by delivering therapeutic genes and can be defined as a method to supply genetic materials to patient cells. The purpose of gene therapy is to overcome the problems that are related with the direct administration of therapeutic proteins, such as low bioavailability, systemic toxicity, and instability. Current gene therapy systems are composed of three key factors. One is a therapeutic gene, which encodes the specific therapeutic protein that can correct or modulate a disease. Another factor is a gene expression system that controls the transcription or translation of a therapeutic gene within the target cells. The final factor is a gene delivery carrier that can transfer the therapeutic gene to a specific location.

Gene delivery carriers should have two functions: to protect a therapeutic gene from premature degradation and to deliver the gene to a target cell. A number of carriers have been developed for gene therapy to satisfy these two conditions. The gene carriers are classified into two groups, viral and non-viral vectors.[1-3] Viral vectors have been widely investigated because they have higher gene delivery efficiencies than non-viral vectors. However, viral vectors have problems such as cytotoxicity, oncogenic recombination, and immunogenicity. Some viral vectors may integrate their genomes into the host chromosomes and permanently alter the host's genetic structure. In addition, some viral vectors cannot bypass the host's defense mechanism and may induce a high immune response. In contrast, non-viral carriers have low cytotoxicity and low immunogenicity.[1,2] However, the low delivery efficiencies of non-viral carriers limit their therapeutic applications. Therefore, one of the goals in developing non-viral carriers is to improve their transfection efficiencies. As examples of non-viral vectors, poly-L-lysine (PLL) and polyethylenimine (PEI) have been widely investigated as non-viral gene delivery carriers.[4,5] However, the therapeutic applications of PLL and PEI are limited, due to their cytotoxicity.[6] This implies that another goal for non-viral carriers is low cytotoxicity.

R3V6 peptides are composed of an arginine stretch and a valine stretch. R3V6 formed a micelle structure in aqueous solution. An *in vitro* study showed that the R3V6 peptide had lower cytotoxicity than PLL and PEI.[7] However, the gene delivery efficiency of the R3V6 peptide was lower than that of PLL in N2A neuroblastoma cells, suggesting that R3V6 should be improved to be useful for therapeutic gene delivery.

In our previous study, a recombinant high mobility group box 1A domain (HMGA) was expressed in bacteria and purified by affinity chromatography.[8] It has been reported that wild-type high mobility group box 1 (HMGB-1) can non-specifically bind minor grooves of DNA and induce a strong DNA bend. HMGB-1 has an A box, B box, and acidic tail. The A box and B box are DNA-binding domains with positive charges. Specifically, the A box has a nuclear localization sequence (NLS). These characteristics suggest that HMGB-1 may be a useful DNA carrier.[9,10] However, its acidic tail has a high aspartate and glutamate content with a negative charge, which interferes with DNA and peptide interactions. Therefore, the acidic tail was eliminated from

HMGB-1 to improve the transfection efficiency of the HMGB-1/DNA complexes.[10,11] In addition, the B box has a pro-inflammatory domain, which has a deleterious effect in gene delivery. Thus, the recombinant HMGA without the B box or the acidic tail was evaluated as a DNA carrier.[8,11] However, HMGA had a lower transfection efficiency than PLL and lipofectamine in N2A cells. We hypothesized that the low transfection efficiency of HMGA may be due to its low charge density. If this is the case, the positive charges may be supplied by R3V6, which can increase the stability of the HMGA/DNA complex. Therefore, the combination of R3V6 and HMGA may increase the transfection efficiency of HMGA by increasing the stability of the complex. Furthermore, the NLS in HMGA may be useful to increase the transfection efficiency of R3V6. To confirm the synergistic effect of HMGA and R3V6, we performed various physical characterization and transfection assays.

Materials and Methods

Synthesis of R3V6 Peptides. The peptides, with three arginines and six valines, were synthesized chemically and were purified using C18 reverse-phase chromatography (Peptron Co., Daejeon, South Korea). The peptides were then dissolved in water at 4 mg/mL and stored at −70 °C.

Expression of HMGA. First, pET21a-HMGA was transformed into *Escherichia coli* (*E. coli*) BL21 for expression. A single colony of *E. coli* was selected and cultured overnight in 10 mL Luria-Bertani (LB) medium containing 50 mg/mL ampicillin in a shaking incubator at 37 °C and 250 rpm. This bacterial culture was transferred to 4 L LB containing ampicillin and incubated under the same conditions until it reached an optical density of 0.6-0.8 at 600 nm. Then, IPTG was added to a final concentration of 500 mM to induce HMGA expression. The cells were incubated for an additional 6 h and harvested by centrifugation at 6,000 × g for 15 min at 4 °C. The supernatant was removed, and the bacterial pellet was resuspended in chilled lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, and 6 M urea; pH 8.0) containing 1 mM phenylmethanesulfonyl fluoride (PMSF). The resuspended bacterial cells were treated with lysozyme (0.3 mg/mL) for 20 min and then lysed by sonication (30 3-s bursts) with 5 min on ice between bursts. The lysate was cleared by centrifugation at 10,000 × g for 30 min at 4 °C, and the supernatant was used for HMGA purification.

Purification of HMGA. The C-terminal six-histidine stretch confers a high affinity for nickel ions. Nickel chelate affinity chromatography was the first purification step for HMGA. The cell extracts containing HMGA were loaded into a nickel (Probond resin, Invitrogen, Carlsbad, CA, USA) column (Glass Econo Column, BioRad, Hercules, CA, USA) pre-equilibrated with equilibration buffer (50 mM NaH_2PO_4, 300 mM NaCl, 6 M urea, and 20 mM imidazole; pH 8.0). Unbound proteins were removed by washing with equilibration buffer, and bound proteins were eluted by a step gradient of imidazole (100, 150, 200, 250, and 300 mM) in elution buffer (50 mM NaH_2PO_4, 300 mM NaCl, and 6 M urea; pH 8.0) at 1 mL/min. Each eluted fraction was assayed with a bicinchoninic acid (BCA) assay kit (Pierce, Rockford, IL, USA). The protein was collected and dialyzed against a cationic exchange buffer (16 mM NaH_2PO_4, 1 mM $NaHPO_4$, and 0.1 mM sodium azide; pH 5.7) containing 0.2 mM PMSF using a membrane with a 6,000-8,000 Da molecular weight cut-off (Spectra/Por dialysis membrane, Millipore, Billerica, MA, USA) at 4 °C overnight. After dialysis, the protein was loaded into a CM Sepharose (GE Healthcare, Waukesha, WI, USA) column pre-equilibrated with equilibration buffer (16 mM NaH_2PO_4, 1 mM Na_2HPO_4, 0.1 mM sodium azide, and 200 mM NaCl; pH 5.7). The unbound proteins were removed by washing with equilibration buffer, and HMGA was eluted using a step gradient of NaCl (350, 400, 450, 500, and 550 mM) in elution buffer (16 mM NaH_2PO_4, 1 mM Na_2HPO_4, and 0.1 mM sodium azide; pH 5.7) at 1 mL/min. Each eluted fraction was assayed with a BCA assay kit (Pierce). The purified protein fractions were collected and dialyzed as described above and were then analyzed by electrophoresis on a 15 % (w/v) sodium dodecyl sulfate-polyacrylamide gel. Finally, to remove any contaminating endotoxins such as lipopolysaccharide, HMGA was subjected to a polymyxin B column (Pierce). The molecular weight of HMGA was approximately 11 kDa.

Gel Retardation Assay. The formation of a plasmid DNA (pDNA)/HMGA/R3V6 complex was verified through a gel retardation assay. A fixed amount of pDNA (pβ-Luc, 0.5 μg) was mixed with increasing amounts of R3V6 peptides and HMGA in distilled water. The complex mixtures were electrophoresed on a 1% agarose gel in the presence of ethidium bromide (EtBr) for 30 min at 100 V in 1× TBE buffer solution. The pDNA was visualized using a UV transilluminator.

Heparin Competition Assay. A heparin competition assay was performed to evaluate the stability of the pDNA/HMGA/R3V6 complex. A fixed amount (0.5 μg) of pβ-Luc was mixed with HMGA/R3V6, HMGA, R3V6, or PLL (20 kDa, Sigma, St. Louis, MO, USA) at weight ratios of 1:4:10, 1:4, 1:20, and 1:2, respectively. The weight ratios were the optimal ratios for the highest transfection, as determined by previous studies[12] or in this study. The complexes were incubated for 30 min at room temperature. After incubation, heparin was added to the peptide/DNA complexes at DNA to heparin weight ratios of 1 to 1, 3, 5, 7, 9, 12, 15, and 20. The complexes were analyzed by electrophoresis on a 1% agarose gel in the presence of EtBr for 30 min at 100 V in 1× TBE buffer. DNA was visualized using a UV transilluminator.

Measurement of Zeta Potential and Complex Size. The pβ-Luc/HMGA/R3V6 complexes were prepared at 1:4:10 weight ratios, and polyethylenimine (25 kDa, PEI25k) was added to the complex solution at a 40:1 N (nitrogen of PEI25k)/P (phosphate of DNA) ratio. The complexes were incubated for 30 min at room temperature for complex formation. After incubation, the particle sizes and zeta potentials were determined by the Zetasizer Nano ZS system

(Malvern Instruments, UK) as described previously.[8]

Cell Culture and Transfection. Mouse neuroblastoma Neuro-2A (N2A) cells were seeded onto 12-well plates 24 h prior to transfection at a density of 1×10^5 cells/well in Dulbecco's Modified Eagle Medium (DMEM) with 10% fetal bovine serum (FBS) and antibiotics (penicillin and streptomycin) at 37 °C in a humidified 5% CO_2 atmosphere. To determine the optimal complex formation ratio for the highest transfection efficiency, the pβ-Luc/HMGA/R3V6 complexes were prepared with various amounts of HMGA and R3V6. The pβ-Luc/HMGA, pβ-Luc/R3V6, pβ-Luc/PLL, and pβ-Luc/lipofectamine complexes were prepared in 5% glucose solution at 1:4, 1:20, 1:2, and 1:2.5 weight ratios, respectively. The amount of DNA was fixed at 2 μg/well. The weight ratios were the optimal ratios for the highest rates of transfection, as determined in previous studies[12] or in this study. The cell culture medium was replaced with 1 mL serum-free DMEM before transfection. The DNA/carrier complexes were added to the cells and incubated for 4 h at 37 °C. After the incubation, the transfection mixtures were removed, and 1 ml fresh DMEM with 10% FBS was added to the cells. The cells were incubated for an additional 20 h at 37 °C in a humidified 5% CO_2 atmosphere.

Luciferase Assay. After transfection, the cells were washed twice with PBS, and 150 μL of reporter lysis buffer (Promega, Madison, WI, USA) was added to the cells. After 5 min of incubation at room temperature, the cell lysates were harvested and transferred to microcentrifuge tubes. After centrifugation at 13,000 rpm for 10 min, the cell extracts were transferred to new tubes. Luciferase activity was measured in a 96-well plate luminometer (Berthold Detection System, GmbH, Pforzheim, Germany). The total protein concentration of each sample was measured using a BCA assay kit (Pierce). Luciferase activity was expressed as relative light units (RLU)/mg protein.

Cytotoxicity Assay. N2A cells were seeded onto 24-well plates at a density of 5×10^4 cells/well and incubated for 24 h before transfection. Transfection with pβ-Luc (0.5 μg/well) was performed at a pDNA/HMGA/R3V6 weight ratio of 1:4:10. The pβ-Luc/R3V6 (weight ratio, 1:20), pβ-Luc/PLL (weight ratio, 1:1), and pβ-Luc/lipofectamine (weight ratio, 1:2.5) complexes were prepared in 5% glucose solution. The cell culture medium was replaced with serum-free DMEM just before transfection. The complexes were added to the cells and incubated for 4 h at 37 °C. The DMEM containing DNA/carrier complexes was removed, and fresh DMEM with 10% FBS was added to the cells. The cells were incubated for an additional 20 h at 37 °C in a humidified 5% CO_2 atmosphere. Forty microliters of 5 mg/mL 3-(4,5-dimethylthiazol-2-yl)-2,5-dephenyl-2*H*-tetrazolium bromide (MTT) reagent were added to each well and incubated for 4 h at 37 °C. Then, the MTT-containing medium was removed, and cells were dissolved with 700 μL of dimethylsulfoxide. The samples were incubated for 10 min at 50 °C, and the absorbance of the samples was measured at 570 nm.

Results and Discussion

Complex Formation and Physical Characterization. The ternary complex of HMGA, R3V6, and pDNA was prepared by a two-step method (Fig. 1). First, HMGA was mixed with pDNA to form the pDNA/HMGA complex with negative surface charges. Then, R3V6 was added to the pDNA/HMGA complex to coat the negative charges of the pDNA/HMGA complex.

A gel retardation assay was performed to confirm the formation of the HMGA/R3V6 complex with pDNA. First, various amounts of HMGA were mixed with 0.5 μg of pβ-Luc. The pDNA was significantly retarded at a 1:5 weight ratio (DNA:HMGA), but additional HMGA did not completely retard DNA even at a 1:20 weight ratio (Fig. 2). This phenomenon was also observed in a previous study that was performed with siRNA.[13] Then, the pDNA/HMGA weight ratio was fixed at 1:4, and increasing amounts of R3V6 were added to the pDNA/HMGA complex. The results showed that the pDNA was completely retarded at a 1:4:10 weight ratio (pDNA:HMGA:R3V6), suggesting that the pDNA/HMGA/R3V6 complex was formed at this ratio.

A heparin competition assay was performed to evaluate the stability of the pDNA/HMGA/R3V6 complex (Fig. 3).

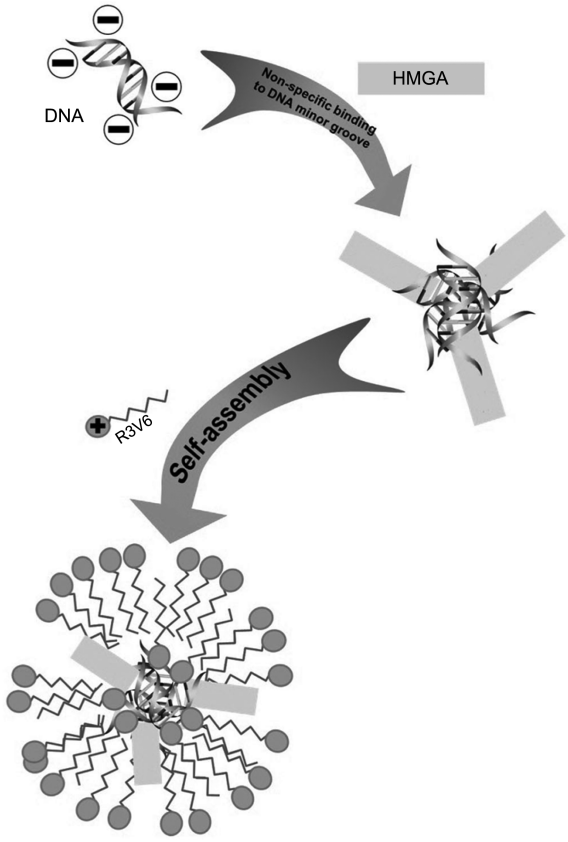

Figure 1. Schematic representation of the formation of the pDNA/HMGA/R3V6 complex. The pDNA and HMGA were mixed and incubated at room temperature for 15 min. Then, the R3V6 peptide was added to the mixture and incubated at room temperature for an additional 30 min.

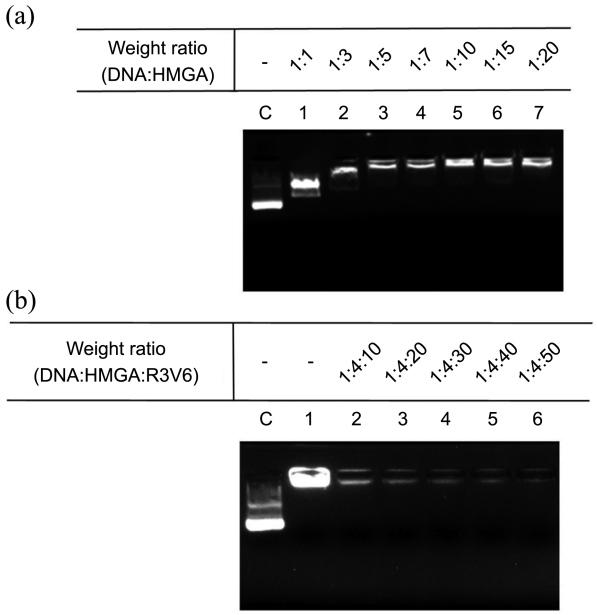

Figure 2. Gel retardation assay. The formation of the pDNA/HMGA-RV complexes was verified *via* gel retardation assay. A fixed amount of pβ-Luc pDNA (0.5 μg) was mixed with increasing amounts of HMGA and R3V6 in 5% glucose. (a) DNA/HMGA complex. (b) DNA/HMGA/R3V6 complex, lane 1 is the DNA/HMGA complex without R3V6.

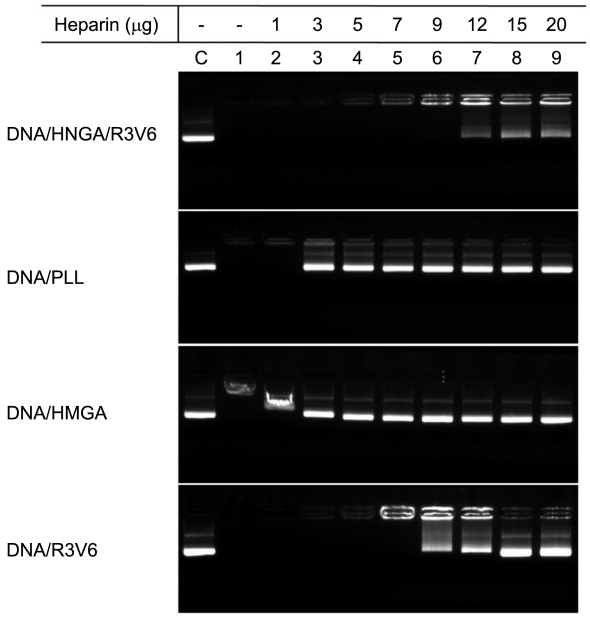

Figure 3. Heparin competition assay. The stabilities of the pDNA/HMGA/RV complexes were evaluated using a heparin competition assay. Increasing amounts of heparin were added to the complexes. After incubation, the samples were analyzed in 1% agarose gel containing ethidium bromide.

The pDNA/HMGA, pDNA/R3V6 and pDNA/PLL complexes were used as controls. Increasing amounts of heparin were added to the complexes to dissociate pDNA from the complexes. The results showed that the pDNA was released from the pDNA/HMGA/R3V6 complex by the addition of 12 μg heparin. However, pDNA quantities from the pDNA/HMGA, pDNA/R3V6, and pDNA/PLL complexes were released with 1, 9, and 3 μg of heparin, respectively. This suggests that HMGA/R3V6 formed a more stable complex with pDNA than did HMGA, R3V6, or PLL. The particle sizes of the DNA/HMGA/R3V6 complexes were measured by dynamic light scattering, and the particle size of the pDNA/HMGA/R3V6 complex was around 217 nm (Table 1). This was similar to the size of the pDNA/PLL complex (246 nm).

Table 1. Particle size and zeta-potential

Group	Avg. Size ± standard error (d.nm)	Avg. Zeta potential (mV)
R3V6	4260 ± 714	17.15 ± 1.35
HMGA	232 ± 14.3	26.15 ± 3.48
HMGA/R3V6	217 ± 8.14	38.85 ± 1.09
PLL	246 ± 18.6	36.38 ± 1.59

***In vitro* Transfection Efficiency of the pDNA/HMGA/R3V6 Complex.** *In vitro* transfection assays using the pDNA/HMGA/R3V6 complexes were performed to evaluate the transfection efficiency of the pDNA/HMGA/R3V6 complex. First, the weight ratio of pDNA and HMGA was fixed at 1:4, and various weight ratios between pDNA/HMGA and R3V6 were prepared to optimize the ratio for the highest transfection efficiency. The highest transfection efficiency of the pDNA/HMGA/R3V6 complex was obtained at the pDNA/R3V6 weight ratio of 1:10 (Fig. 4(a)). Second, the pDNA/R3V6 complex ratio was fixed at a 1:10 weight ratio, and the weight ratio between pDNA/R3V6 and HMGA was optimized. *In vitro* transfection assays showed that the pDNA/HMGA/R3V6 complex had the highest transfection efficiency at a weight ratio of 1:4:10 (Fig. 4(b)).

The transfection efficiency of the pDNA/HMGA/R3V6 complex was compared with that of other carriers such as pDNA/HMGA, pDNA/R3V6, pDNA/PLL, and pDNA/lipofectamine (Fig. 4(c)). The results showed that the pDNA/HMGA/R3V6 complex had a higher transfection efficiency than the pDNA/HMGA and pDNA/R3V6 complexes. These results suggest that the combination of HMGA and R3V6 increased the transfection efficiency compared with HMGA only or R3V6 only, due to the synergistic effect. HMGA has the NLS sequence, which can facilitate the nuclear translocation of the pDNA after cellular uptake. However, HMGA had low positive density, and pDNA was easily released from the pDNA/HMGA complex. In contrast, R3V6 had high positive density, forming a tight complex with pDNA. However, R3V6 does not have NLS for the nuclear translocation of pDNA. Therefore, the combination of R3V6 and HMGA increased the stability of the complex due to the high charge density of R3V6 and increased the transfection efficiency due to the NLS effect of HMGA. The pDNA/HMGA/R3V6 complex had a higher transfection efficiency than the pDNA/PLL complex and a similar transfection efficiency to the pDNA/lipofectamine complex.

The pDNA/HMGA/R3V6 complex should be prepared by

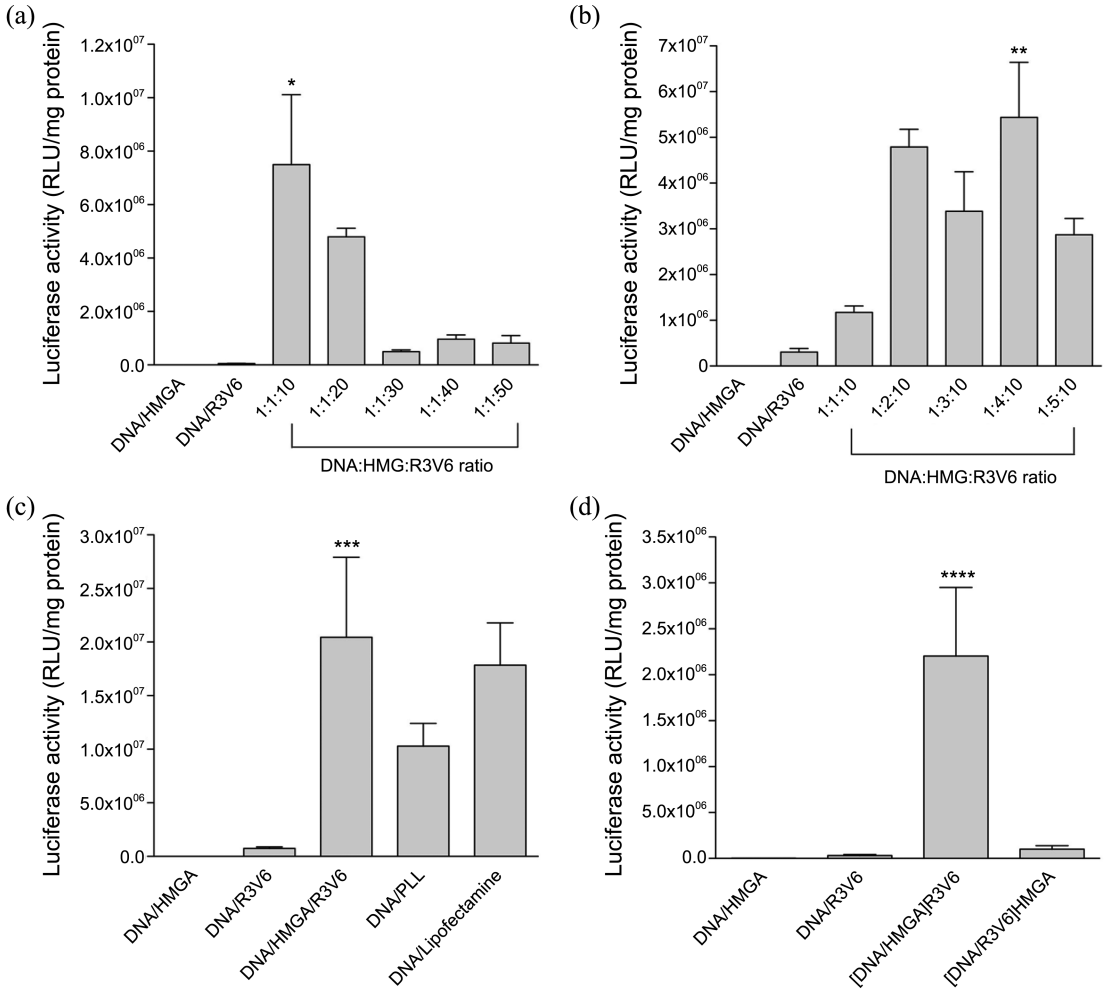

Figure 4. Optimization and comparison of transfection efficiencies of DNA/HMGA/R3V6 complexes. The DNA/HMGA/R3V6 complexes were prepared at various weight ratios of (a) R3V6 and (b) HMGA. Complexes were transfected into N2A cells and measured by luciferase assay. (c) After the optimization of R3V6 and HMGA quantities, the pDNA/HMGA/R3V6 complexes were compared with other carriers in N2A. (d) The complex formation sequence affected the transfection efficiency. $*P < 0.05$ as compared with DNA/HMGA, DNA/R3V6, 1:1:20, 1:1:30, 1:1:40, and 1:1:50. $**P < 0.05$ as compared with DNA/HMGA, DNA/R3V6, 1:1:10, and 1:5:10. $***P < 0.05$ as compared with DNA/HMGA, DNA/R3V6, and DNA/PLL. $****P < 0.01$ as compared with DNA/HMGA, DNA/R3V6, and [DNA/R3V6]HMGA.

the two-step method described in Figure 1. The mixing sequence should be pDNA → HMGA → R3V6. If R3V6 is added to pDNA prior to the addition of HMGA, the HMGA cannot bind to the pDNA/R3V6 complex. Due to the high positive charge density of R3V6, the negative surface charge of pDNA may be completely shielded by R3V6. To confirm this, we prepared the complex in two ways. The pDNA, HMGA, and R3V6 were mixed in the following sequences: DNA → HMGA → R3V6 or DNA → R3V6 → HMGA. Each complex was transfected into N2A cells. The results showed that the complex of DNA → HMGA → R3V6 had a higher transfection efficiency than the complex of DNA → R3V6 → HMGA (Fig. 4(d)).

Cytotoxicity of the pDNA/HMGA/R3V6 Complex. The cytotoxicity of the DNA/HMGA/R3V6 complex was evaluated by MTT assay. PLL and lipofectamine were used as controls. All complexes were prepared at their optimal weight ratios and transfected into N2A cells. The results showed that the DNA/HMGA/R3V6 complex did not

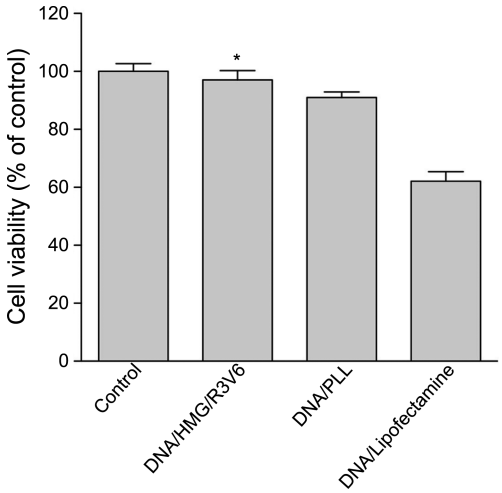

Figure 5. Cytotoxicity of the DNA/HMGA/R3V6 complex. The pDNA/HMGA/R3V6, pDNA/PLL, and pDNA/lipofectamine complexes were transfected into N2A cells. After transfection, cell viability was measured by MTT assay. $*P < 0.01$ as compared with DNA/PLL and DNA/Lipofectamine.

exhibit toxicity. However, PLL and lipofectamine had significant cytotoxicity. The results confirm that the DNA/HMGA/R3V6 complex is not toxic to cells (Fig. 5).

In conclusion, the combination of HMGA and R3V6 increased the stability and transfection efficiency of the pDNA/HMGA or pDNA/R3V6 complex. Furthermore, the pDNA/HMGA/R3V6 complex did not show cytotoxicity. Therefore, the pDNA/HMGA/R3V6 complex may be useful for the therapeutic delivery of pDNA.

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