Nonviral Gene Delivery by a Novel Protein Transduction Domain

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Gene therapy using nonviral gene delivery carriers has focused on the development and modification of synthetic carriers such as liposomes and polymers. Most polymers that are commercially used are taking advantage of their polycationic character which allows not only strong ligand-DNA affinity but also competent cell penetration. Despite the relatively high transfection efficiencies, high cytotoxicity is continuously pointed out as one of the major shortcomings of polycationic polymers such as PEI. Studies on the utilization of peptides have therefore been carried out recently to overcome these problems. For these reasons, the human transcription factor Hph-1, which is currently known as a protein transduction domain (PTD), was investigated in this study to evaluate its potential as a gene delivery carrier. Although its transfection efficiency was about 10-fold lower than PEI, it displayed almost no cytotoxicity even at concentrations as high as 100 μM. Hph-1 was oxidatively polymerized to yield poly-Hph-1. The cell viability of poly-Hph-1 transfected U87MG and NIH-3T3 cells was almost as high as the control (untreated) groups, and the transfection efficiency was about 10-fold higher than PEI. This study serves as a preliminary evaluation of Hph-1 and encourages further investigation.

Key Words : Nonviral gene delivery, Protein transduction domain, Hph-1, Polycationic polymer

Introduction

Gene therapy has made great advancements over almost half a decade and is now at a stage where several gene therapy methods are now further investigated in clinical trials. Most of the gene delivery carriers, however, are viral and bear concerns in their actual clinical application of having side effects and the uncertainty of complete safety from lethal viral characteristics.[1] Therefore, research to develop non-viral gene delivery carriers has become a relatively interesting field, since this alternative could overcome the shortcomings of viral vectors. As a result, various polymers have been developed and studied to determine their values as gene delivery carriers. Nowadays, polycationic vectors such as polythyleneimine (PEI)[2] and poly(amido amine)-arginine generation four (PAM-RG4)[3,4] are widely used since their ability to bind, condense and efficiently deliver plasmid DNA show good results *in vitro*. Substantial improvement resulting in modified non-viral gene delivery carriers with low cytotoxicity but high transfection efficiency has been achieved in the past decade, but although there have been successful transfection results *in vivo* utilizing PAM-RG4,[4] the efficacy of DNA transfer of most of such polymer/pDNA complexes (*i.e.* polyplexes) is often poor. This problem has mainly been pointed out as a major disadvantage of non-viral gene delivery carriers. The utilization of peptides has also recently been in the spotlight. Oligoarginine (R6, R8),[5] oligolysine and TAT (YGRKKRRQRRR),[6,7] for instance, have been grafted to DNA and delivered to various cell lines by taking advantage of the peptides' protein transduction abilities.[7] Other protein transduction domains (PTDs) such as antennapedia (Antp) of *Drosophila* or VP22 of herpes simplex virus seem to be less successful. A more recent study has shown that the human transcriptional factor Hph-1 has a cell-permeable domain (YARVRRRGPRR) which can also be used as a gene delivery carrier.[8] Like most of the PTDs that consist of 11-34 amino acids, Hph-1 has multiple arginine and lysine residues which presumably may be involved in contact with the negatively charged lipids in cell membrane penetration.[9]

One of the merits of this PTD is that it is derived from human, and therefore less likely to exhibit immunogenicity or other safety issues such as those of TAT, Antp *etc*. This is why Hph-1 has become an interesting research topic in the past years for its application as a gene delivery carrier. Most studies added a GAL4/UAS system to the PTD in order to enhance complex formation[10,11] and targeted stem cell transfection, however, a simpler delivery system to common cell lines is still lacking. For these reasons, this study has examined several peptides with a focus on Hph-1, in their native forms and compared their transfection efficiency and potential as gene delivery carriers. In addition, poly-Hph-1 was prepared by oxidative polymerization, which was reported to be a method to enhance PTD's transfection efficiency. The polypeptide was then evaluated for its potential as a gene delivery carrier.

Experimental

Cell Cultures and Preparations of Plamids and Gene Delivering PTDs. Mouse NIH-3T3 embryonic fibroblasts and human glioblastoma U87MG cells were grown in DMEM supplemented with 10% heat-inactivated FBS at 37 °C in a humidified atmosphere containing 5% CO_2. The

cells were maintained in T75 cell culture flasks and serially subcultured every 4 days by using trypsin/EDTA.

The firefly luciferase gene, pCN-Luci was constructed and used as reported before.[4,12] The plasmid was purified using the NucleoBond® Xtra Maxi Plasmid Purification Kit (Macherey-Nagel GmbH & Co., Duren, Germany) and spectrophotometrically quantified. A_{260}/A_{280} values of the plasmids were approximately 1.95.

Hph-1 (YARVRRRGPRR), TAT (YGRKKRRQRRR) and Hexaarginine (R6; RRRRRR) were from Peptron (Daejeon, Korea) and PEI was purchased from and Sigma-Aldrich (St. Louis, MO, USA).

Preparation of Poly-Hph-1. Poly-Hph-1 was prepared by oxidative polymerization as reported in previous reports [7,13]. In brief, C-Hph-1-C(CYARVRRRGPRRC) was dissolved in 30% DMSO at a concentration of 30 mM. The solution was incubated at room temperature for 96 h and subsequently was purified using an Amicon® centrifugal membrane filter (Merck Millipore, Darmstadt, Germany) with a molecular weight cut-off of 10,000 to remove low molecular impurities.

Ethidium Bromide Exclusion Assay. The binding of the peptides to DNA was monitored by ethidium bromide (EtBr) exclusion assay. A 2-mL solution of DNA (20 μg) and the respective peptides was prepared in ethidium bromide (EtBr) to result in N/P ratios of 0, 0.1, 0.5, 0.8, 1, 1.5, 2, 5, 10, 20 and 40, and then incubated for 30 minutes at room temperature (RT) for polyplex formation. Emission fluorescence was measured at 590 nm (544 nm excitation) by a FP-8300 spectrofluorometer (JASCO Inc., Easton, MD, USA). The resulting light intensity for a DNA-only solution served as a control and was taken as 100%. The relative light intensity of the mixture was expressed as a percentage of the light intensity relative to that of the DNA-only-EtBr mixture.[14]

Transfection Efficiency Test by Luciferase Assay. NIH-3T3 and U87MG cells were each seeded in 6-well tissue culture plates at a density of 3.0×10^4 cells/well in 2 mL of DMEM containing 10% FBS and incubated in a humidified 5% CO₂ incubator for 24 h before transfection. The cells were rinsed with PBS to remove any unattached cells and given 1.8 mL of serum-containing medium. To each well 0.2 mL of the carrier/pCN-Luci complex was added and the plates were further incubated for 24 or 48 h at 37 °C in a humidified 5% CO₂ incubator. After the growth medium was removed, the cells were washed with PBS and lysed for 30 minutes at RT with 900 μL of 1 × Reporter Lysis Buffer (Promega, Madison, WI, USA). The expression of the luciferase gene in the transfected cells was evaluated using luminescence measurements made with an LB 9507 luminometer (Berthold Technologies, Bad Wildbad, Germany). Protein contents were measured by using a Micro BCA assay reagent kit (Pierce, Rockford, IL).

Assessment of Cytotoxicity by Using MTT Assay. U87MG and NIH-3T3 cells were seeded in 6-well culture plates at 3.0×10^4 cells/well in 2 mL of medium and grown overnight to 70%-80% confluence. After rinsing with PBS, fresh serum-containing medium was added. The cells were treated with 120 μL solutions of naked DNA (pCN-Luci,
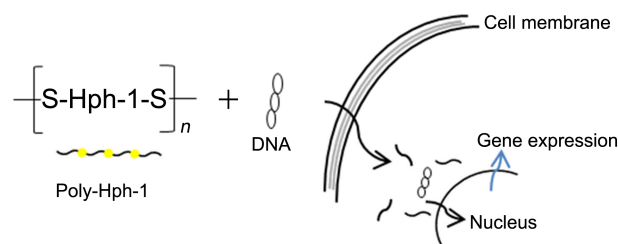
pEGFP-C2), PEI, R6, TAT, Hph-1, poly-Hph-1 and each of their polyplexes. After further incubation for 48 h, cells were exposed to 300 μL of filtered MTT stock solution (2 mg/mL in PBS). After incubation for 4 h at 37 °C, each MTT-containing medium was removed and 500 μL of dimethyl sulfoxide (DMSO) were added to dissolve the formazan crystals formed by the living cells. The samples were then transferred to 96-well plates to measure their absorbance at 570 nm with a microplate reader (Molecular Devices Co., Menlo Park, CA, USA) and SoftMax Pro v5 (Molecular Devices, Sunnyvale, CA, USA) software.

Results and Discussion

The potential of Hph-1 as a gene delivery carrier has been examined from various aspects. Previous studies have utilized this PTD by linking it with an additional DNA binding domain,[11] however, the transfection efficiency and DNA-complexing ability in its native form or as a polymer hasn't been reported yet. The present study has shown that native Hph-1 could actually complex with DNA and was stable at physiological salt conditions. The potential as a gene delivery carrier was even higher for poly-Hph-1, which was prepared by oxidative polymerization (Scheme 1). The molecular weight can be easily controlled by regulating the polymerization time in this method and according to western blot analysis, the molecular weight distribution of poly-Hph-1 ranged from 72 kDa to about 120 kDa with an estimated average of about 80 kDa (data not shown), which was used in the following studies.

A similar study on TAT and its polymerized form, poly-TAT has been conducted previously,[7] however, since safety concerns on the HIV-virus-derived TAT limit its application, we suggest that Hph-1 is a more likely candidate for clinical use.

Ethidium Bromide Exclusion Assay. Ethidium bromide exclusion assay was performed to confirm whether peptide/DNA complex has formed. It showed a significant decrease (extinction) of UV visibility at N/P ratio 1.5 for poly-Hph-1, 5 for Hph-1 and TAT, 2 for R6, and 7.5 for PEI (Fig. 1). Based on these results, further transfection assays and *in vivo* studies were performed at this particular N/P ratio. The reason why gel retardation assay results haven't been shown here is because one limitation of that assay method is that polyplexes with low-affinity ligands (polymers) dissociate in the electric field which leads to an underestimation of carrier-DNA binding. Therefore a ethidium bromide exclu-

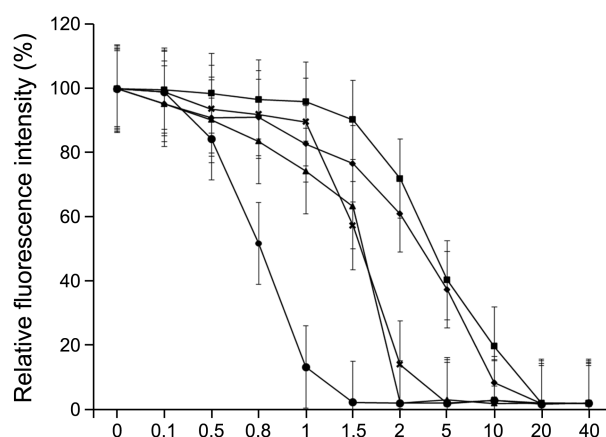**Scheme 1.** Graphic representation of gene delivery by poly-Hph-1.

Figure 1. Ethidium bromide exclusion assay. Hph-1 (◆), TAT (■), R6 (▲), PEI (×), and poly-Hph-1 (●) have been evaluated for their optimum N/P ratio to completely bind to DNA.

sion assay results are shown in the present study to independently measure the binding of DNA to each of the tested carriers. Peptide binding to DNA leads to exclusion of EtBr intercalation and decrease in UV visibility as seen at the carriers' various N/P ratios. These results show that Hph-1 binds stronger to pDNA in comparison with PEI and TAT which confirmed previous studies.[5,15] The stronger binding of DNA to Hph-1 than to TAT may be due to the aliphatic amino acid residues that allow the peptide to be relatively more flexible. Another explanation would be that because of those particular residues, Hph-1 is slightly more amphipathic than TAT[16,17] and therefore more likely to successfully deliver DNA to the cells.[18] The stronger binding of poly-Hph-1 to DNA should be due to the increase of cationic charge compared to its monomeric form.

Cell Viability *in vitro*. Non-viral gene delivery carriers that are of polymeric and polycationic nature have exhibited cytotoxicity at high concentrations[19] and are therefore still limited in clinical use.[20] Therefore, the cell viability was firstly assessed by MTT at carrier only conditions. As shown in Figure 2, among all tested carriers, PEI was the most cytotoxic one on U87MG (Fig. 2(a)) and NIH-3T3 (Fig. 2(b)) compared to R6, TAT, Hph-1 and poly-Hph-1. By increasing the amount of R6, its cell viability at an N/P ratio of 10 decreased to a similar degree of that of PEI which was consistent with previous reports.[5,15]

The carriers' respective polyplexes showed a similar pattern in cytotoxicity being highest for PEI polyplexes and lowest for TAT, Hph-1 and poly-Hph-1 polyplexes. But the cell vialbility was slightly higher for PEI and R8 polyplexes than when only the carriers had been delivered. TAT, Hph-1 and poly-Hph-1 polyplexes were also of similar cell viabilities as the control group, up to N/P ratios of 20. The results presented here have shown that PEI and R6, cell viability was higher for the polyplexes than the carriers alone. This might be due to the overall charge reduction when polyplex is formed. We therefore inferred that an excessive positive charge would affect the viability of cells. Indeed, as reported by Fisher *et al*.[21] the toxicity is a function of interactions of
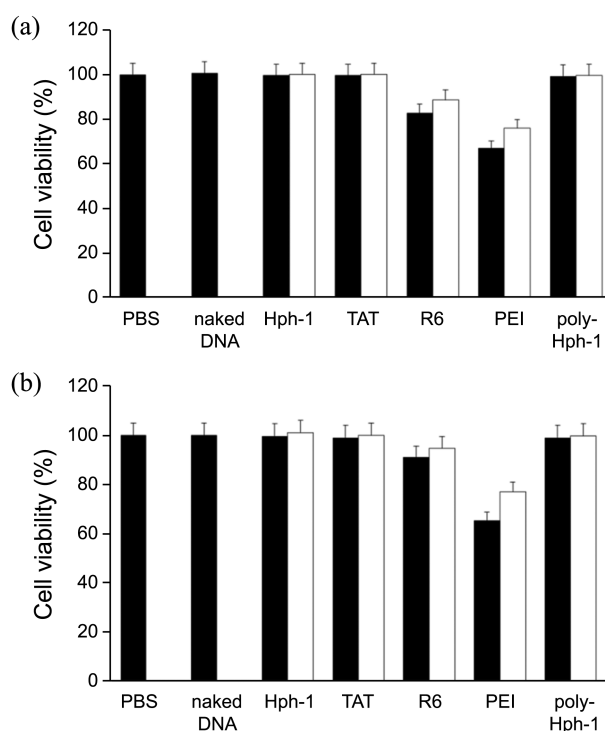

Figure 2. Cell viability assessment by MTT. Each polyplex was evaluated for cytotoxicity by MTT assay in (a) U87MG and (b) NIH-3T3 cells. Black bars are for carrier only, white bars are for polyplex treated groups.

the cationic polymers with cell membranes and/or of the efficiency of cellular uptake. In addition, despite cationic character, peptides tended to exhibit much less toxicity than the synthetic polymer PEI. TAT, Hph-1 and poly-Hph-1 didn't show any signs of cytotoxicity even at concentration levels of 100 μM (for poly-Hph-1, up to N/P ratio 10:1) and showed almost similar cell viabilities as the control (PBS added) group (Fig. 3).

In addition, the results demonstrate that among non-viral gene delivery carriers, the cytotoxicity is even lower for peptides than for polymers, which is presumably because peptides are more alike to biological substances. Poly-Hph-1 is made of multiple disulfide bonds which are stable in the
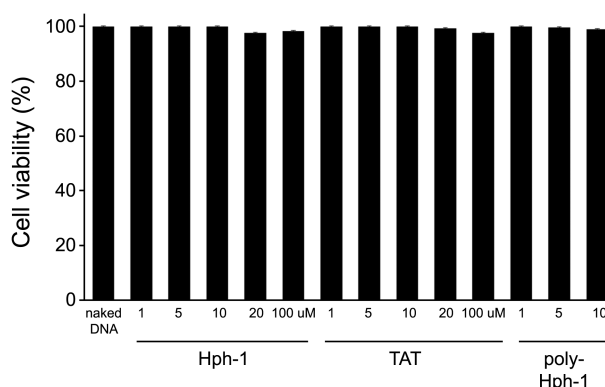

Figure 3. Different charge ratios of Hph-1, TAT and poly-Hph-1 polyplex and a 100 μM solution of Hph-1 or TAT only and their effect on cell viability is shown.
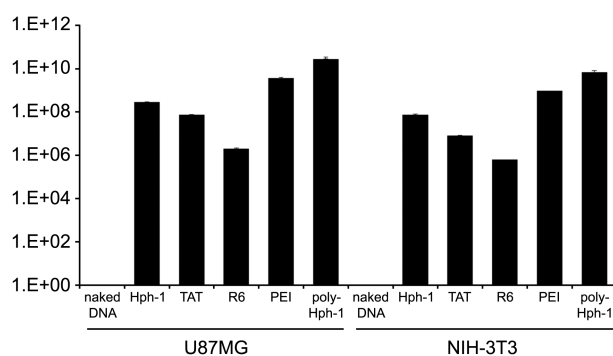
Figure 4. The transfection efficiency of each polyplex in the respective cell. The charge ratio for Hph-1, TAT, R6, PEI, and poly-Hph-1 polyplexes were 5:1, 5:1, 2:1, 7.5:1, and 1.5:1 respectively for both cell lines.

aqueous extracellular environment but easily degrade in a glutathione-rich environment such as the cytoplasm, which leads to the unpacking of DNA.

Transfection Efficiency *in vitro*. Transfection efficiency of Hph-1 was compared to that of TAT, R6 and PEI in U87MG and NIH-3T3 cell lines (Fig. 4). It has been about 10-fold higher than TAT and approximately 100-fold higher than R6 in NIH-3T3 but still lower than PEI. We initially hypothesized that since DNA condensation is an important factor in non-viral gene delivery, a higher positive charge such as in R6 would result in higher transfection efficiencies. Results, however, showed that this is actually not a dominant factor. To efficiently transfect cells with the desired gene, DNA has to form stable polyplexes until reaching and entering the target cell. This procedure doesn't only require electrostatic attraction between the DNA and the carrier, but also efficient cell-penetrating features. TAT and Hph-1 are known for their cell-penetrating, transducing characters, but the nonpolar, aliphatic residues in Hph-1 (alanine, valine and glycine) seem to facilitate the peptide a lot more to deliver the polyplex into the cell than TAT, which has a polar, uncharged residue (glutamine) in its sequence. R6, which constitutes of only the positively charged amino acid arginine, may form a strong complex with DNA as observed in the modified EtBr exclusion assay, but since it relatively lacks in aliphatic character, it doesn't seem to be as efficient in transfection. Thus, not only the net positive charge, but also the balance of positively charged residues and aliphatic residues seem to affect transfection efficiency and cytotoxicity. Although the transfection efficiency of Hph-1 was lower than PEI, we surmised that particular modifications, such as oxidative polymerization of the PTD would improve such shortcomings. Oxidative polymerization has been reported to be a useful means of polymerizing peptides which showed enhanced transfection efficiencies.[5,15] Our hypothesis was verified by the significantly higher transfection efficiency of poly-Hph-1 than PEI. Its transfection efficiency was about 10-fold higher than PEI. Due to polymerization, Hph-1 may have increased in amphipathic character and is therefore a stronger DNA condensing agent. The disulfide bonds of poly-Hph-1, however, well degrade once the polyplex

reaches the cytoplasm and efficiently releases DNA. The overall pattern in transfection efficiency was similar in U87MG cells.

Conclusion

This study shows that poly-Hph-1 can be used as an efficient gene delivery in comparison with commercial polymeric carriers. Unlike PEI, it being a polypeptide has exhibited low cytotoxicity even at high concentrations, despite its polycationic features. Transfection efficiencies of poly-Hph-1 polyplexes were significantly higher than those of PEI, R6 or TAT. It can therefore be inferred that the potential of poly-Hph-1 as a novel gene delivery carrier with minimal cytotoxicity is very high. Further studies on optimizing the polymer size and transfection conditions should be performed to complement the present research.

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