THE JOURNAL OF GENE MEDICINE
J Gene Med 2003; **5**: 700–711.
Published online 24 April 2003 in Wiley InterScience (www.interscience.wiley.com). **DOI:** 10.1002/jgm.402

RESEARCH ARTICLE

Low molecular weight protamine as an efficient and nontoxic gene carrier: *in vitro* study

Yoon Jeong Park[1]
Jun Feng Liang[1]
Kyung Soo Ko[2]
Sung Wan Kim[3]
Victor C. Yang[1]*

[1]*College of Pharmacy, The University of Michigan, Ann Arbor, MI 48109, USA*

[2]*Department of Internal Medicine, College of Medicine, Inje University, Seoul, Korea*

[3]*Center for Controlled Chemical Delivery, University of Utah, Salt Lake City, UT 84112, USA*

*Correspondence to:
Dr Victor C. Yang, Albert B. Prescott Professor of Pharmaceutical Sciences, College of Pharmacy, The University of Michigan, 428 Church Street, Ann Arbor, MI 48109-1065, USA. E-mail: vcyang@umich.edu

Received: 18 October 2002
Revised: 13 February 2003
Accepted: 7 March 2003

Abstract

Background The structural similarity between low molecular weight protamine (LMWP), prepared by enzymatic digestion of protamine, and HIV-TAT protein transduction peptide suggested the feasibility of LMWP as an efficient carrier for delivering therapeutic genes while alleviating the cytotoxicity of currently employed gene carriers.

Methods LMWP was prepared by enzymatic digestion of protamine with thermolysine. The prepared LMWP peptide and TAT peptide, as well as their complexes with plasmid DNA (pDNA), were examined for cellular uptake behaviors by using confocal microscopy and flow cytometry. The complexation of pDNA and LMWP was monitored by gel retardation test as well as size and zeta potential measurements, and was then further assessed by DNase I protection assay. The transfection efficiency of pDNA/LMWP was examined by varying the pDNA content and charge ratio in the complex, and then compared with that of pDNA/PEI. Cytotoxicity induced by pDNA/LMWP and pDNA/PEI was also examined.

Results Prepared LMWP showed similar transcellular localization behavior and kinetics to those of TAT, and efficiently transferred the pDNA into nucleus and cytoplasm in a short time period. The size and zeta potential of the pDNA/LMWP complex were 120 nm and 30 mV, respectively, which were adequately suitable for cellular uptake. After forming the complex, LMWP appeared to effectively protect pDNA against DNase I attack. The pDNA/LMWP complex showed significantly enhanced gene transfer than both naked pDNA and the pDNA/PEI complex, while exhibiting a markedly reduced cytotoxicity than that of the pDNA/PEI complex.

Conclusions The present study suggested that LMWP could be a useful and safe tool for enhancing delivery of bioactive molecules and therapeutic DNA products into cells when applied in gene therapy. Copyright © 2003 John Wiley & Sons, Ltd.

Keywords low molecular weight protamine; TAT peptide; transcellular localization; gene transfer; gene therapy

Introduction

Gene therapy offers significant opportunities to treat various kinds of life-threatening and gene-related diseases by producing, *in vivo*, the essential biological agents or stopping abnormal cell functions such as genetic failure or uncontrollable proliferation [1–4]. Therapeutic application of the gene

Copyright © 2003 John Wiley & Sons, Ltd.

products, however, has been limited due to concerns related to such gene products including their instability in body fluids, lack of cell targeting specificity, rapid degradation by nucleases, and poor transfection efficiency [5,6]. In this regard, the primary efforts in gene therapy have been focused on enhancing gene expression and reducing the cytotoxicity of the therapy [7–16]. A number of potential gene delivery carriers including viral vectors [7,8], liposomes [9,10], peptides [11,12] and cationic polymers [13–16] have been widely investigated. Viral vectors such as adenovirus and retrovirus have been proven to yield a superior gene transfection efficiency *in vivo*, yet their uses demand further exploration and careful assessments due to the adverse effects such as immunogenicity, toxicity and mutagenesis caused by the cell-infecting virus [8]. On the other hand, nonviral gene carriers such as cationic liposomes [9,10], peptides (e.g. protamine) [11,12] and synthetic polymers (e.g., poly (L-lysine) (PLL)) [13,14], polyethyleneimine (PEI) [15,16]) are shown to introduce a significantly reduced immunogenicity than the viral vectors *in vitro*, but their *in vivo* applications are hampered by the exceedingly low transfection efficiency and sometimes the poor biocompatibility, as well as by the cytotoxicity of the polymeric carriers and/or their degradation products [17]. The quest for an effective and yet nontoxic gene carrier thus remains ongoing.

Recently, we reported the development of low molecular weight protamine (LMWP) fragments as possible nontoxic substitutes of protamine in clinical heparin neutralization [18–20]. Such LMWP fragments were derived directly from native protamine sulfate by enzymatic digestion with thermolysine. In a previous investigation, these LMWP peptides, which displayed a rich arginine content in their amino acid compositions, were found to retain a nearly complete heparin-neutralizing ability and yet with a significantly less toxicity *in vivo* [18–20]. Aside from their potential in condensing DNA (Note: the use of protamine in gene delivery has been proposed elsewhere), the LMWP peptides also carried significant structural similarity to the protein transduction domain (PTD) of the HIV-TAT protein. As reported in the literature [21–23], the PTD of the TAT protein, $TAT_{(48-60)}$, comprised a highly basic and hydrophilic peptide consisting of six arginine and two lysine residues in its 13 amino acid sequence. At a concentration as low as 0.1 μM, this TAT peptide was shown to translocate through the cell membranes within a period of 5 min [21,22]. In addition, by genetic or chemical hybridization of this TAT peptide to a variety of biologically active species, including proteins, peptides, DNA, and even magnetic nanoparticles [23–25], this TAT peptide was shown to be able to facilitate a rapid internalization of all of such attached species into various cell types. Since like the highly basic TAT, LMWP also possesses 10 arginine residues in its structure, it was anticipated that LMWP would possess similar functions as TAT in translocating active agents inside cells. Indeed, in our preliminary studies, we found that LMWP was almost equally potent as TAT in mediating cell translocation of its attached species (data not shown). Based on its significantly reduced toxicity, LMWP may be used in gene therapy to overcome the current toxicity-related limitation of the gene carriers. Unlike TAT, however, the toxicity profiles of LMWP have already been fully established. Animal studies conducted in our laboratory demonstrated that LMWP was not toxic [18–20], immunogenic or antigenic [18–20]. An additional advantage of using LMWP as the gene carrier is that, unlike TAT which must be synthesized chemically, LMWP can be readily produced from native protamine in mass quantities, thereby significantly reducing the costs and the production period.

Hence, in the study reported here, we explored the possibility and potential of utilizing this nontoxic, TAT-like LMWP peptide as an effective carrier for gene delivery. The *in vitro* capability of LMWP in cell trafficking, condensing DNA, translocalizing DNA inside the cells, protecting DNA from DNase I degradation, transfecting cells, and inducing cytotoxicity of the cells was thoroughly examined.

Materials and methods

Materials

Salmin protamine, thermolysine and 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma (St. Louis, MO, USA). Psv-β-gal plasmid vector (6821 bp) and the *o*-nitrophenyl-β-D-galactopyranoside (ONPG) assay system were obtained from Promega (Madison, WI, USA). Fetal bovine serum (FBS), phosphate-buffered saline (PBS), 0.25% (w/v) trypsin-EDTA, Roswell Park Memorial Institute medium (RPMI 1640), Dulbecco's modified essential medium (DMEM), penicillin and streptomycin were purchased from Gibco-BRL (Gaithersburg, MD, USA). The BCA protein assay reagent was from Pierce (Rockford, IL, USA). The peptide containing the TAT (CYGGYGRKKRRQRRRR) sequence was synthesized by the Protein Core Facility at University of Michigan. Polyethyleneimine (PEI) was from Polyscience Co. (St. Louis, MO, USA). Hoechst 33 342 dye was purchased from Molecular Probes, Inc. (Eugene, OR, USA). Fluorescein-labeled plasmid DNA (pGeneGrip^{TM}) was obtained from Gene Therapy System Inc. (San Diego, CA, USA). All of the solvents used were analytical grade and water was distilled and deionized.

Preparation of LMWP peptide

The enzymatic method employed for the preparation of LMWP has been described in previous publications [18–20]. In brief, thermolysin and protamine were mixed and incubated for 30 min at room temperature, followed

Copyright © 2003 John Wiley & Sons, Ltd.

by quenching of the thermolysin activity with 50 mM EDTA. Thermolysin was then removed by ultrafiltration using a YM3 membrane (MWCO 3000), and the filtrate was subject to lyophilization. Since the lyophilized LMWP preparation contained a mixture of small peptide impurities, it was further purified by using heparin affinity chromatography. A total of five peptides, denoted TDSP 1–5 according to the order of elution from the heparin column, were obtained. The molecular weight and amino acid sequence of each isolated LMWP peptide were determined by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) analysis, which was performed by the Protein and Carbohydrate Research Center at the University of Michigan. Among the five fractions, TDSP 5 (VSRRRRRRGGRRRR) was utilized in all subsequent studies.

Fluorescence labeling

Peptides were labeled with fluorescein isothiocyanate (FITC) at their N-terminals. In brief, the peptide solution (pH 9.3, carbonate buffer) was reacted, in 1 : 2 molar ratio, with a FITC solution (in dimethylformamide) overnight in the dark at room temperature. Reaction was monitored by high-performance liquid chromatography (HPLC) of the absorbance change at 215 nm of the peptide peak. The labeled peptides were purified by HPLC (purity: >95%), lyophilized in the dark, and then stored at −20 °C in the dark until further use.

Cell lines

The 293T human embryonic kidney transformed cells were obtained from ATCC (Rockville, MD, USA). They were grown and maintained at 37 °C in humidified 5% CO_2 in DMEM medium containing 10% FBS, 100 units/ml penicillin-streptomycin mixture, and 2.2 mg/ml sodium bicarbonate.

Amplification and purification of the plasmid

The pSV-β-galactosidase plasmid (Promega) was amplified in the *Escherichia coli* strain DH5α (Gibco-BRL, Gaithersburg, MD, USA) and purified using the Qiagen plasmids Maxi Kits (Qiagen, Valencia, CA, USA). Purity of the plasmid DNA was confirmed by the OD_{260}/OD_{280} ratio and the intensity of corresponding DNA fragments in gel electrophoresis following treatment of the plasmid DNA with a restriction enzyme. The concentration of the plasmid DNA was determined using the ratio that 1 (OD_{260}) was equivalent to 50 µg of DNA. The plasmid DNA was stored at −20 °C until use. For visualization of plasmid DNA inside the cells, FITC-labeled β-galactosidase plasmid DNA was utilized.

Preparation of plasmid DNA/peptide complexes

The pDNA/LMWP complexes were prepared by mixing various amounts of LMWP (increasing from 1–20 µg in 10 µl water) with plasmid (1 µg in 10 µl water). The solution was allowed to stand for 30 min at 20 °C for complex formation. By mixing with various amounts of LMWP, the pDNA/LMWP complexes comprising different charge ratios (−/+) ranging from 1 : 1 to 1 : 20 were obtained. Formation of the pDNA/LMWP complexes was monitored by 1.0% agarose gel electrophoresis using DNA molecular markers. Following electrophoresis, the gels were stained with 0.5 µg/ml ethidium bromide for 45 min and analyzed on a UV illuminator to identify the locations of DNA. In addition, the pDNA/LMWP complexes were also examined for their size and zeta potential by using a Zeta-PALS (Brookhaven Instruments Corp., Holtsville, NY, USA) zetameter. All such experiments were carried out at 25 °C, pH 7.0 and 677 nm in wavelength, and a constant angle of 15°. The particle size was presented as the effective mean diameter.

FITC-labeled pDNA/LMWP and pDNA/TAT complexes were prepared by mixing LMWP or TAT with FITC-labeled pDNA (pGeneGrip™) encoding β-galactosidase at a charge ratio $(-/+) = 1:10$. The solutions were incubated at 20 °C for 30 min for complex formation.

Confocal laser scanning microscopy

Peptide internalization

Cells were plated in Lab-Tek (4-well) chambered slideglasses at 1×10^4 cells/chamber in a volume of 1 ml. After complete adhesion, the culture medium was removed. The FITC-labeled LMWP or TAT was then added to the cells at a final peptide concentration of 0.2 µM. Following 1 h incubation, cells were washed three times and fixed in 1% (w/v) paraformaldehyde in PBS for 20 min at room temperature. The fixed cells were washed again with PBS, mounted in a PBS/glycerin mixture (1 : 1, v/v) containing 2.5% DABSCO as an antifading agent, and kept at 4 °C for at least 1 h before being examined by confocal microscopy.

DNA/peptide complex internalization

FITC-labeled free pDNA, pDNA/LMWP complex, or pDNA/TAT complex was added to cells (10^4 cells) cultured in Lab-Tek chambered slideglasses. After 1 h incubation, cells were washed with PBS and further incubated with 50 ng/ml Hoechst 33 342 dye in PBS supplemented with 1% (v/v) FBS at room temperature for 15 min. Cells were then washed with PBS, and fixed with 1% paraformaldehyde for 20 min. The fixed cells were washed again and mounted with PBS/glycerol containing antifading agent. Confocal laser scanning microscopy was carried out using an inverted LSM 510 laser scanning microscope (Carl Zeiss, Gottingen, Germany) equipped

Copyright © 2003 John Wiley & Sons, Ltd.

with a Plan-Apochromat 63×1.4 N.A. or 40×1.4 N.A. lens. The laser was set at 488 (blue) and 543 (yellow) to produce the excitation wavelengths for fluorescein and rhodamine, respectively. Hoechst dye fluorescence was collected by the laser tuned to 790 nm/400 nw for two-photon excitation of the dye. Z-series were taken of a 1 to 2 micron optical section at 2-μm intervals.

Flow cytometric analyses

The 293T cells were seeded at a density of 1×10^6 cells per well in 6-well plates in 1.5 ml culture medium. One day later, the cells were washed and incubated with FITC-labeled peptides for 0.5, 1, 4, 10 and 24 h. For studies related to the complexes, FITC-labeled pDNA, pDNA/LMWP, or pDNA/TAT complex was incubated with the above 293T cells for 1 h. After incubation, the cells were washed with PBS, trypsinized, and washed. The cells were then fixed with 1% paraformaldehyde and washed with PBS. Analysis was conducted on a FACScaliber flow cytometer (Beckton Dickinson, San Jose, CA, USA) equipped with a 488 nm air-cooled argon laser. The filter settings for emission were 530/30 nm bandpass (FL1) for FITC. The fluorescence of 10 000 vital cells was acquired and data was visualized in logarithmic mode.

Stability of the LMWP/DNA complexes

The charge ratio (−/+) used in the preparation of the pDNA/LMWP complex was controlled at 1 : 2. After complex formation, DNase I (50 units, Gibco BRL) was added to the complex suspension, and the solution was incubated at 37 °C for 60 min. Naked pDNA was used as the control. At time intervals of 0, 10, 20, 40, 60, and 80 min during incubation, 50 μl of the complex suspension were withdrawn, mixed with 75 μl of the stop solution (4 M ammonium acetate, 20 mM EDTA, and 2 mg/ml glycogen), and then placed on ice. The pDNA was dissociated from LMWP by adding 37 μl 1.0% SDS to the complex suspension and then heating the mixture at 65 °C overnight. The pDNA was extracted and precipitated by treating the solution mixture with phenol/chloroform and ethanol several times. The precipitated DNA pellet was then dissolved in 10 μl of TE buffer and subjected to 1.0% agarose gel electrophoresis.

In vitro transfection

The 293T cells were seeded at a density of 2×10^6 cells/dish in 35-mm culture dishes, and incubated for 24 h before the addition of transfection complexes. Transfection mixtures were prepared separately for LMWP and PEI, whereas PEI served as a control. pDNA/LMWP (or pDNA/PEI) complexes were prepared by mixing 10 μg of pSV-β-galactosidase and various amounts of LMWP (or PEI) in 500 μl of serum-free DMEM medium, followed by incubating the mixtures for 30 min at room temperature. The molecular weight of PEI for transfection and cytotoxicity studies was either 2000 or 25 000 Da. Five hundred microliters of LMWP/DNA complex were then added to each well, and the cells were incubated for 4 h at 37 °C in a 5% CO_2 incubator. After a 6-h exposure, the transfection mixtures were replaced with 2 ml of fresh DMEM medium containing 10% FBS and 100 units/ml penicillin-streptomycin mixture. Cells were incubated for an additional 2 days at 37 °C before analysis of β-galactosidase activity. To perform the β-galactosidase assay, cells were washed, lysed with 300 μl of lysis reagent (25 mM glycylglycine, 15 mM $MgSO_4$, 4 mM EDTA, 1% Triton X-100, 1 mM DTT, 1 mM PMSF), and centrifuged at 13 000 rpm for 5 min. The activity of β-galactosidase in the supernatants was then measured using the ONPG assay described previously [26]. This ONPG assay was based on cleavage of the β-bond of ONPG by β-galactosidase, resulting in the production of a yellowish *o*-nitrophenol molecule. The reaction was quenched using 1 M Na_2CO_3. Samples were analyzed by measuring the absorbance at 420 nm with a Bio-Rad microplate reader (BioRad Laboratories, USA). Aside from β-galactosidase activity, the supernatants were measured for their protein concentrations using a BCA protein assay kit (BioRad Laboratories).

Cytotoxicity assay

Evaluation of the cytotoxicity of LMWP and pDNA/LMWP complexes was conducted by using the MTT assay. In general, the 293T cells were seeded at a density of 1.0×10^4 cells/well in a 96-well flat-bottomed microassay plate (Falcon Co., Beckton Dickinson, Franklin Lakes, NJ, USA) and incubated for 24 h. The LMWP or pDNA/LMWP complex solution was then added and the mixture was incubated for another 48 h at 37 °C. Parallel experiments were conducted using PEI and pDNA/PEI complex as the controls. At the end of the transfection experiment, the medium was replaced with 200 μl of fresh DMEM medium without serum, and 120 μl of 2 mg/ml MTT solution in PBS were then added. After incubation for an additional 4 h at 37 °C, the MTT-containing medium was removed, and 200 μl of dimethyl sulfoxide (DMSO) were added to dissolve the formazan crystals formed by live cells. Absorbance was measured at 570 nm. The cell viability (%) was calculated according to the following equation:

$$\text{Cell viability (\%)} = (OD_{570(\text{sample})})/(OD_{570(\text{control})}) \times 100$$

where $OD_{570(\text{sample})}$ and $OD_{570(\text{control})}$ represent measurements from wells treated with LMWP/DNA and PBS buffer, respectively.

Statistical analysis

All measurements were conducted in triplicate and expressed as mean ± standard deviation. Student's t-test

Copyright © 2003 John Wiley & Sons, Ltd.

was utilized to compare the statistical significance of the transfection efficiency and cytotoxicity between the LMWP and PEI complex.

Results

Synthesis of LMWP

Thermolysin-digested protamine was fractionated into five distinct fractions, which were denoted 'Thermolysine-Digested Segmented Protamine, TDSP 1–5' according to their orders of elution from the heparin column [18–20]. Table 1 shows the amino acid sequences and molecular weights of the TDSP 1–5 peptides and TAT. Among those fractions, TDSP 4 and TDSP 5 displayed the highest degree of heparin neutralization and the lowest degree of toxicity [18–20]. In the current study, TDSP 5 was utilized since it possessed almost the same molecular weight as TAT, and the two clusters of cationic amino acid sequences, similar to those seen in the structure of $TAT_{(48-60)}$.

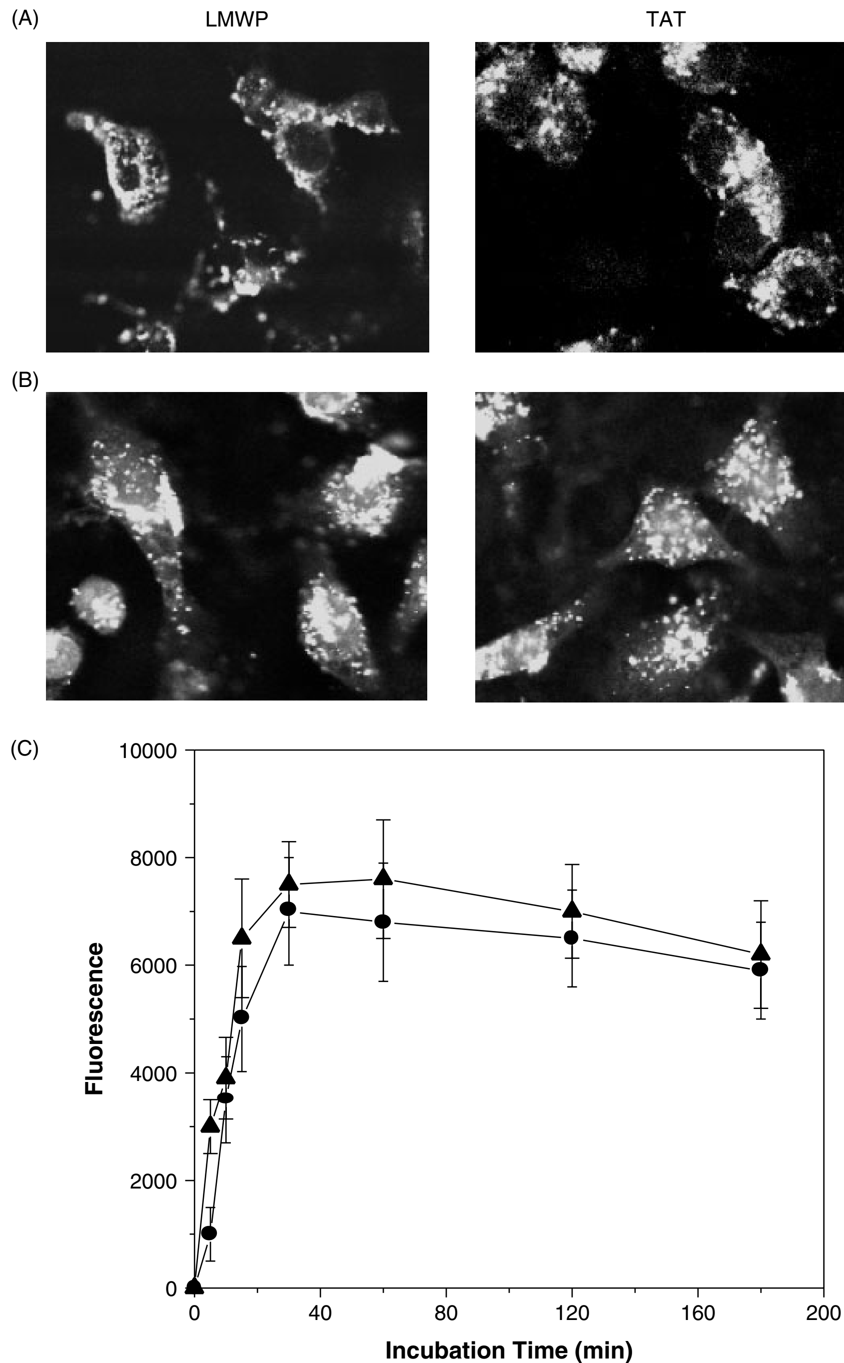

Figure 1. Confocal microscopic observation of the cells treated with either LMWP (left panel) or TAT (right panel) peptide for 15 min (A) and 1 h (B). Time course of the cellular uptake of LMWP and TAT is measured by flow cytometric analysis with cells incubated with each of the peptides for a predetermined time period (C); uptake of TAT (■) and of LMWP (●). The cellular uptake of each peptide was estimated from the mean fluorescent signal of 10 000 cells collected

Copyright © 2003 John Wiley & Sons, Ltd.

Table 1. Amino acid sequence and molecular weight of each fraction of the LMWPs

Fraction	Amino acid sequence	Molecular weight
TDSP 1	PRRRR	740.8
TDSP 2	PRRRRR	896.9
TDSP 3	PRRRRSSRRP	1325.2
TDSP 4	ASRRRRRGGRRRR	1695.5
TDSP 5	VSRRRRRRGGRRRR	1879.7
TAT	CYGGYGRKKRRQRRRR	1900.0

Cellular uptake of LMWP

Prior to investigating the capacity of LMWP as a gene carrier, we first examined the ability of LMWP to transverse into the cells, and to determine its subcellular localization. Internalization of the LMWP was monitored by confocal microscopy after 15 and 60 min incubation of the peptides with the cells. Figure 1 shows that LMWP internalized into the cell as efficiently as the TAT peptides. In less than 15 min, LMWP was found to localize mainly in the cytosol, with some existing in the perinuclear site (Figure 1A). After 1 h, both peptides were seen to translocate through cell membranes and accumulate in the cytoplasm and nucleus, with a large fraction of both internalized peptides seen in the nucleus (Figure 1B). Almost all of the cell population exhibited a high fluorescent intensity, demonstrating the unprecedented efficiency in cell internalization by both LMWP and TAT. To more precisely understand this cell internalization event mediated by these peptides, quantification of the internalized peptides was carried out. The cellular uptake of each peptide was measured by the mean fluorescent signal for 10 000 cells collected. The rate of cellular uptake of TAT turned out to be slightly faster than LMWP; however, the difference was not that significant (Figure 1C). Indeed, a virtually similar cell uptake kinetics between LMWP and TAT was observed. These results indicated that LMWP possessed a similar cell-penetration capability to TAT, rendering it feasible to serve as a carrier for effective gene delivery. In this regard, complexation of LMWP with pDNA was conducted, and the formed complex was further examined for its cell-translocating ability.

Complex formation of LMWP with DNA

A gel retardation study was performed to confirm the formation of complexes between LMWP and pDNA. One microgram of pSV-β-galactosidase plasmid was mixed with various amounts of LMWP to yield different charge ratios (−/+) between pDNA and LMWP. The pDNA/LMWP complexes were analyzed by 1.0% agarose gel electrophoresis. As seen in Figure 2A, the pDNA/LMWP complexes were completely retarded in their migration at the 1 : 1 charge ratio (−/+) between pDNA and LMWP. These results suggested that LMWP was able to effectively condense plasmid DNA into complex at the charge ratio of 1 : 1 (−/+), as the migration of the formed pDNA/LMWP complex on agarose gel was considerably retarded due to charge neutralization and the increase in the overall molecular size. These findings are in good agreement with those observed by other investigators [13,14], indicating that LMWP formed a condensed complex with pDNA as effectively as any of the currently used cationic polymeric gene carriers [13,14].

Figure 2(B) and 2(C) show the size distribution and zeta potential of the pDNA/LMWP complex,

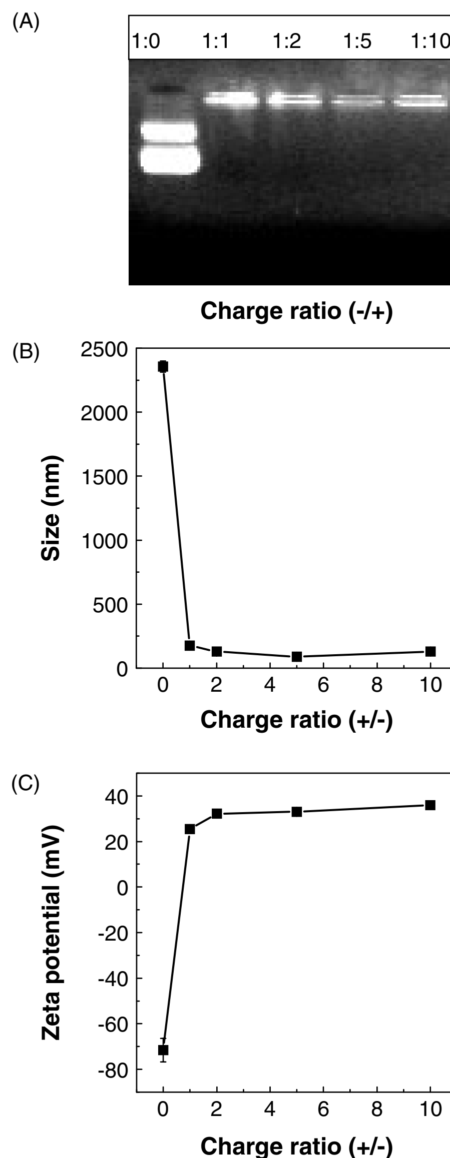

Figure 2. (A) Gel retardation assay. The pDNA/LMWP complexes were prepared at various charge ratios and incubated at room temperature and left for 20 min for complex formation. The complexes were analyzed by 1% (w/v) agarose gel electrophoresis. (B) Particle size of the complexes of plasmid DNA with LMWP as a function of the charge ratio (+/−) between LMWP and plasmid DNA. Data are presented as mean ± standard deviation. The plasmid DNA concentration was 2 μg/ml. (C) Zeta potential of complexes of plasmid DNA with LMWP as a function of the charge ratio (+/−) between LMWP and plasmid DNA. Data are presented as mean ± standard deviation. The plasmid DNA concentration was 2 μg/ml

Copyright © 2003 John Wiley & Sons, Ltd.

respectively. While the naked DNA has a size of 2300 nm, the pDNA/LMWP complex produced particles with significantly reduced size of around 120 nm at the charge ratio (−/+) 1 : 2 (Figure 2B). The charge neutralization occurred before the charge ratio (peptide/DNA) 1 : 1; therefore, particle size decreased from 2330 nm to 176 nm, 129 nm, 89 nm, as the charge ratio increased from 0 : 1 to 1 : 1, 2 : 1 and 5 : 1, respectively. The particle size was increased at a charge ratio of 10 : 1 (131 nm) due to an aggregation; however, it was still a reasonable enough size to be carried into the cells. When the charge ratio increased to over 20 : 1, however, the particle size was increased to 400 nm (data not shown) due to aggregation which resulted in a low transfection efficiency. In addition, surface zeta potential significantly increased after the formation of the plasmid DNA/LMWP complex due to charge neutralization, then it was gradually increased to +35 mV when the charge ratio reached 10 : 1. Unlike the naked plasmid DNA that showed a charge of −80 mV on its surface, complexation with LMWP rendered the surface charge positive (35 mV; Figure 2C). In this regard, results from size distribution and zeta potential measurements not only confirmed the complex formation between pDNA and LMWP, but also conferred the event of cellular uptake of the complex particles. It has been well documented that particles with a size below 200 nm and a surface charge around 20–30 mV yield the most preferable cell uptake *in vitro* [14].

Cellular uptake of the pDNA/LMWP complex

The LMWP peptide was evaluated for its ability to facilitate cellular pDNA delivery when forming the condensed complex. FITC-labeled pDNA was mixed with either LMWP or TAT with a charge ratio (−/+) = 1 : 10. As shown in Figure 3A, there was virtually no cellular uptake of free FITC-labeled pDNA. In contrast, incubation of LMWP with FITC-pDNA promoted internalization

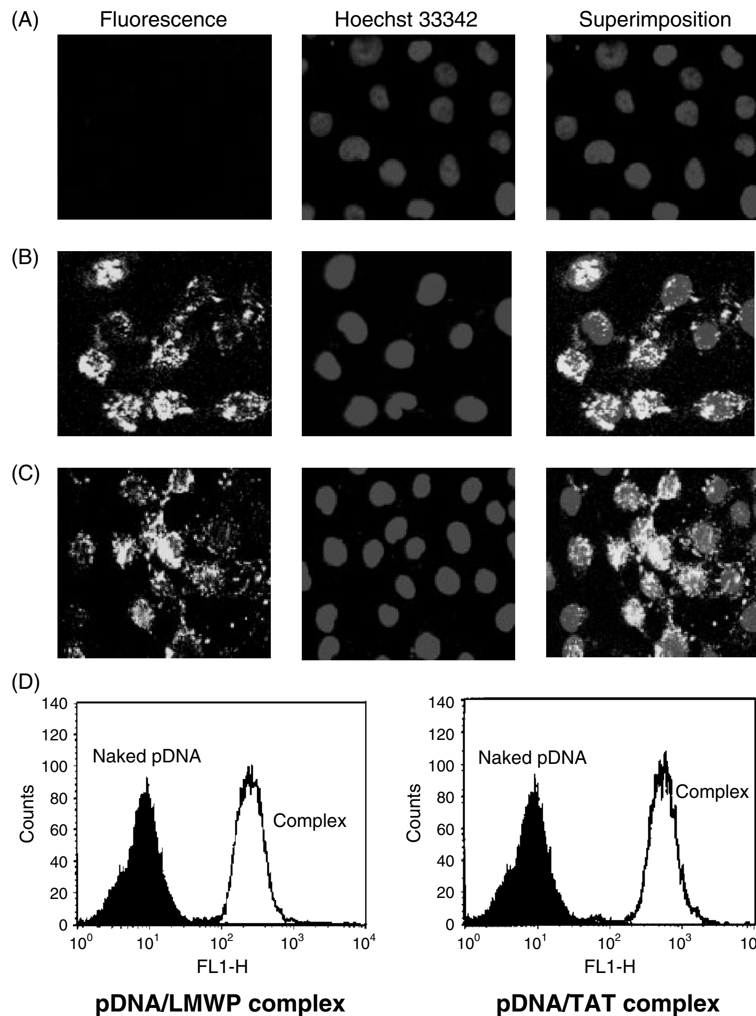

Figure 3. Cellular uptake of FITC-labeled pDNA into 293T cells. Left panels indicate confocal microscopy using a Zeiss Axiovert with 60× objective. (A) FITC-labeled pDNA with no peptide complex as a negative control; (B) FITC-labeled pDNA complex with LMWP; (C) FITC-labeled pDNA complex with TAT peptide as a positive control. (D) Flow cytometric analysis of FITC-labeled pDNA in the cells in dependence on the formation of the complexes with the peptides (ordinate indicates the number of cells and abscissa indicates fluorescent intensity). The concentration of pDNA was 5 μg/ml, and the incubation time at 37 °C was 1 h

Copyright © 2003 John Wiley & Sons, Ltd.

of FITC-labeled pDNA in the cells; with a large fraction of pDNA being seen to localize in the nucleus (Figure 3B). This enhanced cell uptake of pDNA was also seen by using the pDNA/TAT complex, which served as a control (Figure 3C). For both LMWP and TAT complexes, fluorescein was visualized in both the nucleus and cytoplasm. Superimposition of the two images by fluorescein and Hoechst 33 342 dye (for nuclear staining) clearly demonstrated a nuclear localization of the pDNA. As reported previously, TAT can facilitate the delivery of its associated molecules into cells in a short time period [21,22]. Confocal microscopy data in combination with the flow cytometric analysis (Figure 3D) confirmed that TAT mediated pDNA cell internalization within 1 h. Flow cytometric analysis also indicated that LMWP had a similar capability in promoting transcellular localization of pDNA as that of TAT (Figure 3D). Overall, results from the cell uptake studies clearly demonstrated that LMWP could effectively deliver pDNA in the cytosol by forming a complex.

DNase I protection assay

Since pDNA can be easily degraded by nucleases either in the cytosol or circulation, instability of pDNA remains one of the obstacles for the delivery of therapeutic genes *in vitro* or *in vivo* [13,14]. DNase I is known to be a major nuclease present in the serum or cytosol. Therefore, for a successful systemic gene delivery, it is essential for the carrier to protect DNA from degradation by DNase I [13,14]. To demonstrate that LMWP could protect pDNA from nuclease degradation, the DNase I protection assay was conducted by incubating the plasmid/LMWP complex with DNase I. As shown by the results from agarose gel electrophoresis (Figure 4), naked DNA was destroyed after 10 min of incubation with DNase I (Figure 4, left column). However, pDNA combined with LMWP even at a charge ratio 1 : 2 (−/+) was not destroyed after more than 80 min of incubation with DNase I (Figure 4, right column). Therefore, it was clear that LMWP could protect plasmid DNA from the degradation by DNase I.

In vitro transfection assay

The most effective charge ratio of pDNA/LMWP in cell transfection was determined by using the transfection assay of pSV-β-galactosidase into the 293T cells. As shown in Figure 5A, at a charge ratio of 1 : 2 (−/+), the transfection efficiency increased with increasing the plasmid DNA content. A further experiment was conducted by maintaining the plasmid content at 5 μg while altering the charge ratio from 1 : 2 to 1 : 30. Transfection efficiency was seen to increase to a maximum when the charge ratio of the complex was raised to 1 : 10 (−/+) (Figure 5B). Further increase in the charge ratio of the complex above 1 : 20 (−/+) resulted in no further increase in transfection efficiency. Conversely, the transfection efficiency actually decreased when the charge ratio of the complex was increased above 1 : 20 (−/+). This reduced transfection efficiency at a charge ratio above 1 : 20 (−/+) could be due to an aggregation of particles. Nevertheless, the efficiency of transfection to 293T cells in the presence of LMWP was markedly higher than that of naked DNA alone. In addition, the transfection efficiency of LMWP/DNA was compared with that of TAT/DNA. As seen in Figure 5C, transfection efficiency was almost similar for both complexes, which was in agreement with results observed by the cellular trafficking assay. In this regard, LMWP appears to be capable of delivering as much gene into the cells as TAT. To further compare the transfection efficiency, experiments were conducted by using polyethyleneimine (PEI) with a similar molecular weight to LMWP as the DNA carrier. PEI is a water-soluble and cationic gene carrier and is known to be by far the most potent carrier of pDNA internalization into cells [15,16]. To perform the experiments, various charge

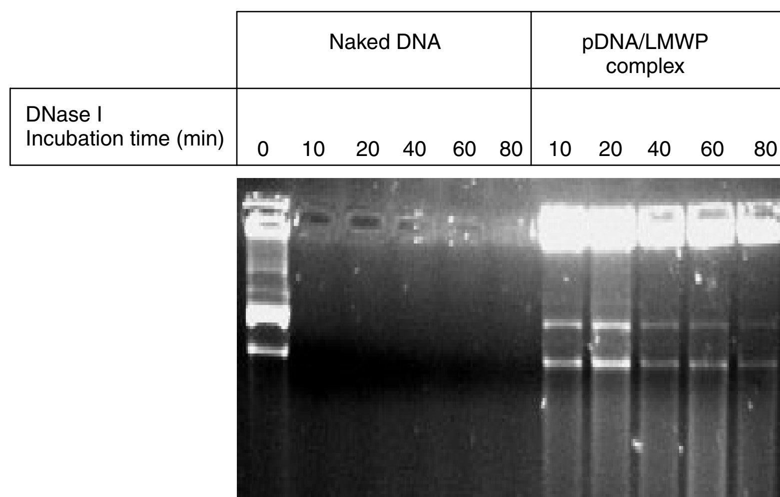

Figure 4. DNase I protection assay with the pDNA/LMWP complex. The pDNA/LMWP complex was prepared as described in 'Materials and methods' section. The complex solution was incubated with 50 units of DNase I for 10, 20, 40, 60, and 80 min. After incubation, DNA was analyzed by 1% agarose gel electrophoresis

Copyright © 2003 John Wiley & Sons, Ltd.

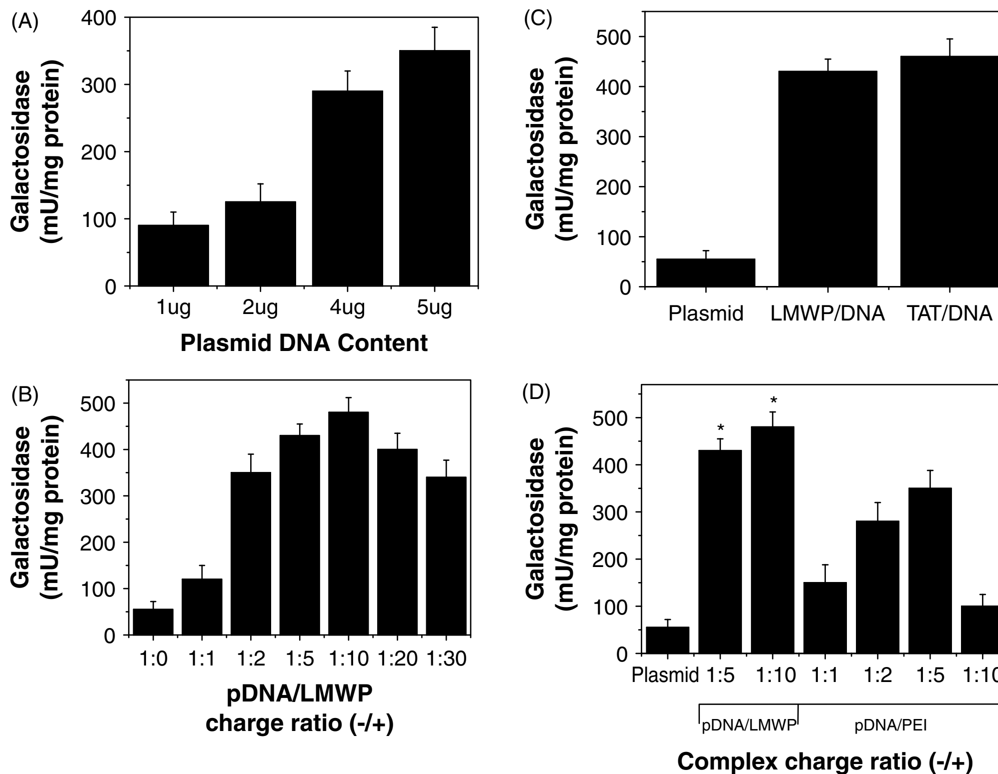

Figure 5. Transfection efficiency of pDNA/LMWP complex in 293T cells. Transfection efficiency was measured using the colorimetric β-galactosidase enzyme activity assay (ONPG assay). (A) Effects of the plasmid DNA content on the transfection efficiency. The charge ratio of the pDNA/LMWP was adjusted to (−/+) 1 : 2. (B) Effects of the charge ratio (−/+) on the transfection efficiency. Plasmid DNA content was adjusted to 5 µg. (C) Comparison of the transfection efficiency between pDNA/LMWP complexes and the pDNA/TAT complex at a charge ratio of 1 : 5 (−/+). (D) Comparison of the transfection efficiency between pDNA/LMWP complexes and various charge ratios (−/+) of pDNA/PEI complexes. Similar molecular weight of PEI (2000 Da) to LMWP (1880 Da) was used for comparison. The data are expressed as mean ± standard deviation of four experiments. *$p < 0.05$, as compared with that of plasmid DNA and that of a PEI complex at the same charge ratio

ratios of pDNA/PEI complexes were transfected into 293T cells. Figure 5D shows that at a charge ratio of 1 : 5, the pDNA/PEI complex yielded the highest transfection efficiency. At this charge ratio, however, it is clearly seen that LMWP mediates a higher transfection efficiency (i.e. by 26%) than PEI. Similarly, at a charge ratio (−/+) of 1 : 10, which is the ratio for achieving the maximum transfection efficiency for LMWP, transfection efficiency of the pDNA/LMWP complex was markedly higher than that of the pDNA/PEI complex. Since at a charge ratio (−/+) of 1 : 10 the gene carriers might induce cytotoxicity to the cells, cytotoxicity of each carrier and its associated complex was examined.

Cytotoxicity measurement of LMWP

Although the *in vivo* safety of LMWP has already been demonstrated previously [18–20], an MTT assay was still conducted to determine the cytotoxicity of LMWP. The cytotoxicity of LMWP by itself without plasmid DNA was first evaluated. Figure 6 shows that cells treated with LMWP yielded almost an identical viability in comparison to that without the treatment whereas cells treated with PEI introduced cytotoxicity to the cells by reducing the cell viability by 35%. It should be noted that the toxicity of cationic polymers has always been a concern in their use as gene carriers. In addition,

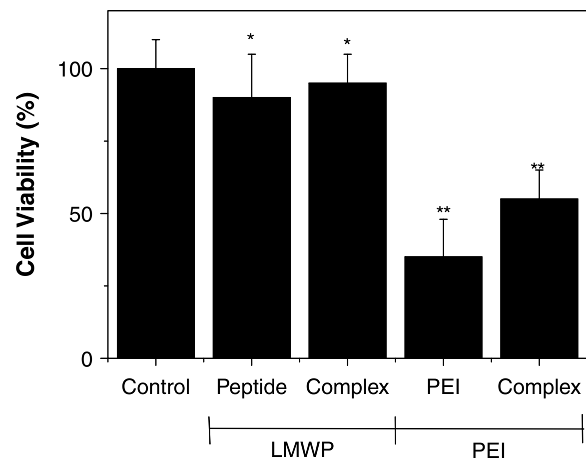

Figure 6. Cytotoxicity of either LMWP or PEI to 293T cells. Cells were plated on 96 wells and exposed to LMWP, pDNA/LMWP complex, PEI, or pDNA/PEI complex. Cytotoxicity test was conducted by MTT colorimetric assay as described in the 'Materials and methods' section. The data are expressed as mean ± standard deviation of four experiments. *$p < 0.05$, as compared with that of PEI or PEI complex, **$p < 0.05$, compared with that with control

Copyright © 2003 John Wiley & Sons, Ltd.

a greater toxicity for some polymers when applied in the absence of pDNA has been noticed [17]. When both pDNA/LMWP and pDNA/PEI complexes (5 μg of pDNA, charge ratio 1 : 10) were added to 293T cells, as seen in Figure 6, only approximately 55% of cells were viable after incubation with pDNA/PEI, whereas negligible cytotoxicity was observed after incubation with the pDNA/LMWP complex. This lack of cytotoxicity of LMWP could be very beneficial for its use as a carrier in *in vivo* gene therapy.

Discussion

Peptide carriers are one of the most promising tools for delivering biologically active molecules into cells [11,12,27–29]. In the present study, LMWP fractions were evaluated as possible carriers for gene delivery purposes. Five LMWP fractions, denoted TDSP 1–5 with different amino acid sequences and, more precisely, different arginine content, were obtained by enzymatically digesting protamine with thermolysine (Table 1). In our previous studies, these LMWP peptides were demonstrated to retain the heparin-neutralizing capability and yet be devoid of the toxic effects of protamine [18–20]. In addition, such LMWP peptides displayed a significantly reduced level of immunogenicity (i.e. the ability to induce the production of antibodies) and antigenicity (i.e. the ability to cross-react with antiprotamine antibodies), which are the two principal events in protamine-induced imunotoxicity [18–20]. This current study is therefore focused on the potential of utilizing LMWP as a carrier for delivering DNA into cells. Previous studies have demonstrated the feasibility of utilizing protamine as a possible gene carrier due to its ability to condense pDNA [12]. This finding implicates that LMWP could have a similar gene-condensing ability to protamine, since they possess identical heparin-neutralization efficiency. Recently, a number of investigators have reported the unprecedented cell-translocating activity for TAT or other arginine-rich peptides [30–32]. Since, as indicated above, LMWP is rich in arginine content and has a structural similarity to TAT, it may be used as a potent transmembrane carrier for gene delivery. Although both TDSP 4 and TDSP 5 were found to possess the highest degree of heparin affinity, the current study was focused solely on TDSP 5 because it possesses an arginine content and molecular weight that are identical to those of TAT.

Results presented in this paper indicate that cell internalization mediated by LMWP is almost as potent as that of TAT. It should be pointed out that TAT has been shown to translocate through the cell membranes via a receptor-independent pathway [21,22]. It has also been demonstrated that arginine-rich peptides possess a translocation activity similar to that of $TAT_{(48-60)}$ [21–23,30–32]. Indeed, peptides with more than six arginine sequences have been reported to follow the same pathway as TAT, suggesting that the conventional endocytosis pathway does not play a crucial role in the cell translocation for these arginine-rich peptides [30–32]. On the other hand, lysine-rich peptides [31], despite containing higher positive charges, have displayed lower degree of internalization than arginine-rich peptides under the same experimental period, suggesting that arginine residues could be responsible for this rapid and receptor-independent translocalization behavior. As shown earlier, LMWP has 10 arginine residues in its structure (VSRRRRRRGGRRRR), and is made of two arginine clusters connected through two glycine residues, which is similar to that of TAT. As a consequence, our results show that LMWP followed a similar transcellular localization to that of TAT up to 1 h. Confocal microscopic analysis of these two peptides also confirmed the cytoplasmic (in 15 min) and nuclear localization (in 1 h) of these peptides with a lower sign of adhesion to the cell membranes (Figure 1). Internalization of these peptides was not inhibited at 4 °C (data not shown), confirming the presence of a similar receptor-independent translocalization mechanism for LMWP. A maximum (80–90%) cell uptake of both TAT and LMWP occurred at the 30-min mark, which was in good agreement with data reported elsewhere for the arginine-rich peptides [21,30]. Although the exact mechanism for cellular uptake of these peptides has not been fully elucidated yet, a recognition mechanism based on several hypotheses has been suggested [30–32]. In this mechanism, it is proposed that uptake of arginine-rich peptide is mediated by the guanidine head group in the arginine, the formation of a hydrogen bond between arginine and the lipid phosphate on the membrane, or the interaction of arginine with extracellular matrices such as heparan sulfate [30–32]. The uptake of LMWP, albeit needing further investigation, could be related to these aforementioned receptor-independent uptake mechanisms.

Current methods of protein delivery based on the use of these protein transduction domain (PTD) peptides require chemical or biological cross-linking of these peptides with the target protein [29]. Delivery of gene products conducted in the current presentation, however, adopted simple complexation of the peptide with pDNA, which could offer a full retention of the biological activity of pDNA. Simple complexation of pDNA with LMWP yielded a nano-sized particle even at a low charge ratio, condensing pDNA to a favorable size for cell uptake. It has been well documented that particles within a size range between 100–200 nm can be favorably taken up by the cells [13,14]. The current study demonstrates that LMWP with a molecular weight around 2000 Da can sufficiently condense DNA to this appropriate size range. Other reports dealing with condensation of DNA with several condensing peptides were also conducted with a couple of thousand molecular weight of peptide sequence. In addition, a similar result was observed from a similar molecular weight of PEI/DNA complexation, which has an even smaller particle size at the charge ratio of 1 : 1. Gel retardation results also corroborate the formation of a stable complex between LMWP and DNA

Copyright © 2003 John Wiley & Sons, Ltd.

at a charge ratio $(-/+) = 1:1$. Aside from condensing DNA, LMWP also increases the surface potential of the pDNA complexes, thereby enhancing its interaction with the cell surface membrane. In addition, the membrane-translocating activity of LMWP could facilitate the cellular uptake of the DNA/LMWP complex. From this result, once the complex surface charge had reached 25 mV after neutralization, the addition of more peptide did not produce a significant increase in the surface zeta potential (up to 35 mV) since the peptide itself has a small molecular weight. However, in terms of transfection efficiency, this increase in the amount of surface LMWP can increase the cellular uptake of plasmid DNA since LMWP acts not only as a condensing agent, but also as a translocalizing carrier similar to TAT. A previous report also indicated that the increase in the surface molecule of TAT could induce increase of delivery of molecules into the cells.

Results presented in this paper indicate that LMWP possesses a high translocalization capability similar to that of TAT, as confocal microscopy analyses show that FITC-labeled DNA molecules are in the nucleus and cytoplasm when combined with LWMP to form a complex whereas no fluorescence is observed in the cytoplasm using the FITC-labeled naked DNA. Reports by other investigators have indicated that polyfection with PEI and with the DNA/Lipofectamine complex could induce nuclear distribution after 4 h of incubation [16,27,29]. Although the current cell uptake study did not examine such an event for that long (i.e. >4 h), it nevertheless demonstrated that LMWP could deliver pDNA into cells in an equally efficient manner within a much shorter period of time by a simple complexation with DNA. In addition, a degree of fluorescence was seen in the nucleus, implying a possible early nuclear localization of the complex. This early nuclear localization process can be explained by the fact that the pDNA/LMWP complex is delivered directly into the cytoplasm by the receptor-independent cell uptake, whereas other cationic polyplexes are delivered by the receptor-mediated (or adsorptive) endocytosis that requires a further endosomal release step inside the cell. It is not clear whether the pDNA travels inside the cell as a complex with LMWP or a dissociated form from the complex. Further experiments using fluoro-labeled LMWP to study the subcellular trafficking kinetics of the pDNA/LMWP complex and the dissociation kinetics of the DNA from the complex are currently underway in our laboratory.

The pDNA/LMWP complex showed a significantly higher transfection efficiency compared with that of the pDNA/PEI complex. As reported by other investigators, the cationic pDNA/PEI complexes were taken into the cells via an endocytotic mechanism, whereby in the endosomes the complexes were disrupted and pDNA was then released into the cytosol. On the other hand, LMWP-condensed pDNA was taken directly into the cytosol, and protected from lysosomal and DNase degradation, thereby achieving a significantly increased transfection efficiency. It should be pointed out that the transfection efficiency was almost identical to that of the pDNA/TAT complex, reflecting the similar transfecting capacity of LMWP to TAT.

Besides the advantages reported above, our previous studies also demonstrate that LMWP is nontoxic *in vitro* as well as *in vivo* [18–20]. It should be pointed out that the toxicity of cationic polymers such as PEI is always a concern in their use as gene carriers. Despite the fact that the toxicity of the formed complex could be relieved due to charge neutralization of the cationic polymer by plasmid DNA, the polymer would remain toxic after the detachment of pDNA. In this regard, the lack of toxicity for LMWP even without DNA complexation could be a strong argument for LMWP as a carrier for gene delivery purposes.

Conclusions

Overall, preliminary results presented in this paper demonstrate the feasibility of utilizing LMWP as an efficient carrier for delivery of therapeutic gene or even proteins. Data shows that LMWP can translocalize into the cells and nucleus in a high efficiency comparable to that of TAT. In addition, LMWP can form a stable complex with plasmid DNA to efficiently deliver DNA into cells and protect plasmid DNA from nuclease attack. Furthermore, LMWP displays a higher transfection efficiency and yet lower toxicity than any existing polycationic carriers including PEI in 293T cells. Detailed investigations of the cellular uptake mechanism, nuclear localization, and *in vivo* transfection efficiency of the pDNA/LMWP complex are currently in progress in our laboratory.

Acknowledgement

This study was supported in part by NIH Grants R44HL49705, R01HL55461, and R01CA103667.

References

1. Maheshwari A, Mahato RI, McGregor J, *et al*. Soluble biodegradable polymer-based cytokine gene delivery for cancer treatment. *Mol Ther* 2000; **2**: 121–130.
2. Lee M, Han S, Ko KS, Kim SW. Cell type specific and glucose responsive expression of interleukin-4 by using insulin promoter and water soluble lipopolymer. *J Control Release* 2001; **75**: 421–429.
3. Kabanov AV, Kabanov VA. DNA complexes with polycations for the delivery of genetic material into cells. *Bioconjugate Chem* 1995; **6**: 7–20.
4. Gilboa E, Eglitis MA, Kantoff PW, Anderson FW. Transfer and expression of cloned genes using retroviral vectors. *Biotechniques* 1986; **4**: 504–512.
5. Mahato RI, Rolland A, Tomlison E. Cationic lipid-based gene delivery systems: pharmaceutical perspectives. *Pharm Res* 1997; **14**: 853–859.
6. Blessing T, Remy JS, Behr JP. Monomolecular collapse of plasmid DNA into stable virus-like particles. *Proc Natl Acad Sci USA* 1998; **95**: 1427–1431.
7. Rosenfeld MA, Siegfried W, Yoshimura K, *et al*. Adenovirus-mediated transfer of a recombinant a1-antitrypsin gene to the lung epithelium in vivo. *Science* 1991; **252**: 431–434.

Copyright © 2003 John Wiley & Sons, Ltd.

8. Miller DG, Adam MA, Miller AD. Gene transfer by retrovirus vectors occurs only in cells that are actively replicating at the time of infection. *Mol Cell Biol* 1990; **10**: 4239–4242.
9. Sakurai F, Inoue R, Nishino Y, *et al*. Effect of DNA/liposome mixing ratio on the physicochemical characteristics, cellular uptake and intracellular trafficking of plasmid DNA/cationic liposome complexes and subsequent gene expression. *J Control Release* 2000; **66**: 255–269.
10. Caplen NJ, Alton EWFW, Middleton PG, *et al*. Liposome-mediated CFTR gene transfer to the nasal epithelium of patients with cystic fibrosis. *Nat Med* 1995; **1**: 39–46.
11. Sorgi FL, Bhattacharya S, Huang L. Protamine sulfate enhances lipid-mediated gene transfer. *Gene Ther* 1997; **4**: 961–968.
12. Wadhwa MS, Collard WT, Adami RC, McKenzie DL, Rice KG. Peptide-mediated gene delivery: influence of peptide structure on gene expression. *Bioconjugate Chem* 1997; **8**: 81–88.
13. Zauner W, Kichler A, Schmidt W, Sinski A, Wagner E. Glycerol enhancement of ligand-polylysine/DNA transfection. *Biotechniques* 1996; **20**: 905–913.
14. Midoux P, Monsigny M. Efficient gene transfer by hystidylated polylysine/pDNA complexes. *Bioconjugate Chem* 1999; **10**: 406–411.
15. Abdallah B, Hassan A, Benoist C, Goula D, Behr JP, Demeneix BA. A powerful nonviral vector for in vivo gene transfer into the adult mammalian brain: polyethyleneimine. *Hum Gene Ther* 1996; **7**: 1947–1954.
16. Wightman L, Kircheis R, Rossler V, *et al*. Different behavior of branched and linear polyethyleneimine for gene delivery in vitro and in vivo. *J Gene Med* 2001; **3**: 362–372.
17. Morgan DML, Laavin VL, Pearson JD. Biochemical characterization of polycation-induced cytotoxicity to human vascular endothelial cells. *J Cell Sci* 1987; **94**: 553–560.
18. Chang LC, Lee HF, Yang Z, Yang VC. Low molecular weight protamine (LMWP) as nontoxic heparin/low molecular weight heparin antidote. I. Preparation and characterization. *AAPS PharmSci* 2001; **3**(2): article 17.
19. Chang LC, Liang JF, Lee HF, Lee LM, Yang VC. Low molecular weight protamine (LMWP) as nontoxic heparin/low molecular weight heparin antidote. II. In vitro evaluation of efficacy and toxicity. *AAPS PharmSci* 2001; **3**(2): article 18.
20. Chang LC, Wrobleski S, Wakefield TW, Lee LM, Yang VC. Low molecular weight protamine as nontoxic heparin/low molecular weight heparin antidote. III. Preliminary in vivo evaluation of efficacy and toxicity using a canine model. *AAPS PharmSci* 2001; **3**(2): article 19.
21. Casellas P, Ravel S, Bourrie BJ, *et al*. T-lymphocyte killing by T101-ricin A-chain immunotoxin: pH-dependent potentiation with lysosomotropic amines. *Blood* 1988; **72**: 1197–1201.
22. Vives E, Brodin P, Lebleu B. A truncated HIV-1 TAT protein basic domain rapidly translocates through the plasma membrane and accumulates in the cell nucleus. *J Biol Chem* 1997; **272**: 16010–16017.
23. Nagahara H, Vocero-Akbani AM, Snyder EL, *et al*. Transduction of full-length TAT fusion proteins into mammalian cells: TAT-p27^{Kip1} induces cell migration. *Nat Med* 1998; **4**: 1449–1452.
24. Schwarze SR, Dowdy SF. *In vivo* protein transduction: intracellular delivery of biologically active proteins, compounds and DNA. *Trends Pharmacol Sci* 2000; **21**: 45–48.
25. Schwarze SR, Ho A, Vocero-Akbani A, Dowdy SF. In vivo protein transduction: delivery of a biologically active protein into the mouse. *Science* 1999; **285**: 1569–1572.
26. Lampela P, Raisanen J, Mannisto PT, Yla-Herttuala S, Raasmaja A. The use of low-molecular-weight PEIs as gene carriers in the monkey fibroblastoma and rabbit smooth muscle cell cultures. *J Gene Med* 2002; **4**: 205–214.
27. Tung CH, Mueller S, Weissleder R. Novel branching membrane translocational peptide as gene delivery vector. *Bioorganic Med Chem* 2002; **10**(11): 3609–3614.
28. Benimetskaya L, Guzzo-Pernell N, Liu ST, Lai JCH, Miller P, Stein CA. Protamine-fragment peptides fused to an SV40 nuclear localization signal deliver oligonucleotides that produce antisense effects in prostate and bladder carcinoma cells. *Bioconjugate Chem* 2002; **13**: 177–187.
29. Astriab-Fisher A, Sergueev D, Fisher M, Shaw BR, Juliano RL. Conjugates of antisense oligonucleotides with the Tat and Antennapedia cell-penetrating peptides: effects on cellular uptake, binding to target sequences, and biologic actions. *Pharm Res* 2002; **19**: 744–754.
30. Wender PA, Mitchell DJ, Pattabiraman K, Pelkey ET, Steinman L, Rothbard JB. The design, synthesis, and evaluation of molecules that enable or enhance cellular uptake: peptoid molecular transporters. *Proc Natl Acad Sci USA* 2000; **97**: 13003–13008.
31. Futaki S, Suzuki T, Ohashi W, *et al*. Arginine-rich peptides: an abundant source of membrane-permeable peptides having potential as carriers for intracellular protein delivery. *J Biol Chem* 2001; **276**: 5836–5840.
32. Morris MC, Depollier J, Mery J, Heitz F, Divita G. A peptide carrier for the delivery of biologically active proteins into mammalian cells. *Nat Biotechnol* 2001; **19**: 1173–1176.

Copyright © 2003 John Wiley & Sons, Ltd.