Available online at www.sciencedirect.com
SCIENCE DIRECT®
International Journal of Pharmaceutics 269 (2004) 71–80
international journal of pharmaceutics
www.elsevier.com/locate/ijpharm

Effect of oligopeptides on gene expression: comparison of DNA/peptide and DNA/peptide/liposome complexes

Masaki Tokunaga, Norio Hazemoto, Toshihisa Yotsuyanagi*

Department of Pharmaceutics, Graduate School of Pharmaceutical Sciences, Nagoya City University, Tanabe-dori, Mizuho-ku, Nagoya 467-8603, Japan

Received 21 June 2003; received in revised form 15 August 2003; accepted 30 August 2003

Abstract

Plasmid DNA is known to form complexes with a variety of cationic peptides and lipids, which have been explored as possible carriers for DNA transfection in mammalian cells. We synthesized oligopeptides consisting of nine amino acid residues including lysine (K), tryptophan (W), and cysteine (C), and also their symmetrical dimmers with a disulfide bond as possible carriers. The pDNA(pGL3)/oligopeptide complexes generally showed poor transfection efficiencies but little cytotoxicity for HeLa S3. The ternary system of pDNA/oligopeptide/liposome containing cationic liposomes formulated from the cholesterol derivative (DMB-Chol) and dioleoylphosphatidylethanolamine (DOPE) showed 10^4–10^5-fold greater effective gene expression (10^8–10^9 level, RLU/min/mg protein) than those of the corresponding pDNA/oligopeptide complexes. In the presence of 10% serum, the ternary complexes were maintained at 10^7 levels. The ethidium bromide exclusion studies showed the ternary complexes have much greater affinity to pDNA than the corresponding pDNA/oligopeptide complexes. Plasmid sensitivity against DNase I degradation showed that the ternary complexes were well protected from the digestion. Synthetic oligopeptides are active as potential enhancers for DOPE-containing cationic liposome-mediated transfection. These findings have implications for successful in vivo transfection.

Keywords: Plasmid DNA; Transfection; Synthetic oligopeptide; Cationic liposome; DNA/peptide/liposome

1. Introduction

Gene therapy requires the development of carriers that can efficiently deliver therapeutic genes to cells and express encoded proteins. It is also prerequisite that the carriers must be as safe in cytotoxicity as possible. There are mainly two gene transfer systems. Although viral vectors are very efficient for DNA transfection, they might have disadvantages like immunoreactions (Herz and Gerard, 1993), toxicity (Simon et al., 1993), and potential recombination or complementation (Ali et al., 1994). In contrast, non-viral vectors are considered less efficient but attractive due to their favorable characteristics such as little immunoreactions, lower toxicity, relative ease of large-scale production and simplicity of use. Therefore, a number of non-viral carriers have been developed in which cationic polymers such as peptide, spermine derivatives, and liposomes have been extensively examined (Behr, 1994; Gao and Huang, 1995; Vigneron et al., 1996; Felgner, 1997; Meyer et al., 1998). These cationic polymers are thought to condense DNA into a compact structure and protect it from potential enzymatic and physical

* Corresponding author. Tel.: +81-52-836-3454; fax: +81-52-836-3454.
E-mail address: yotsuyng@phar.nagoya-cu.ac.jp (T. Yotsuyanagi).

0378-5173/$ – see front matter © 2003 Elsevier B.V. All rights reserved.
doi:10.1016/j.ijpharm.2003.08.018

and chemical unfavorable degradations. They also provide a platform to link with the cell as targeting ligands. Asialoglycoprotein and transferrin were used as the ligands for receptor-mediated endocytosis (Aramaki et al., 2003; Nishikawa et al., 2000; Ogris et al., 1999). Poly-L-lysine and protamine were used as non-specific binding agents to form DNA complexes (Gao and Huang, 1996). Cationic peptides were shown to enhance cationic liposome-mediated gene transfection (Li and Huang, 1997). Thus, peptides are considered to play not only as carriers to introduce genes into mammalian cells but also as enhancers.

Previously, we reported the transfection efficiencies of polypeptides were largely depended on their amino acid composition and molecular weight (Tokunaga et al., 2003). It was especially noted that the cytotoxicity increased with increasing molecular weight, suggesting a major drawback as a carrier. Also, the hydrophilic and hydrophobic properties of amino acid components and their arrangement would have played a considerable part in the interaction of polypeptides and pDNA. We, therefore, intended to see how these properties affect the transfection efficiencies of oligopeptides.

We designed and synthesized oligopeptides consisting of nine amino acids of lysine(K), tryptophan(W), and cysteine(C) and their dimers as possible DNA carriers. The ternary complexes formulated from pDNA/oligopeptide/liposome (cholesterol derivative (DMB-Chol)/DOPE) were compared with the pDNA/oligopeptide complexes in terms of gene expression, pDNA binding, pDNA sensitivity to DNase I degradation, and effect of coexisting serum. DMB-Chol was chosen from our preliminary transfection test because it was comparable with DC-Chol and shown less cytotoxicity. The transfection efficiencies of these complexes were assayed using pGL3 (pDNA) that encoded luciferase reporter gene and HeLa S3. The optimal structure and effect of oligopeptide concentration were also explored.

2. Materials and methods

2.1. Materials

$(KWKWCWKWK)_2$ (KWK2), KKKKKKKKK (KKK), $(KKKKCKKKK)_2$ (KCK2) were synthesized by the fluoren-9-ylmethoxycarbonyl (Fmoc) procedure (Atherton et al., 1979) using a peptide synthesizer (Model 431A, Applied Biosystems, Tokyo). Monomeric amino acids were purchased from Novabiochem (Tokyo). KWKWCWKWK (KWKm) was obtained by digesting KWK2 with diethylthiothreitol. Dioleoylphosphatidylethanolamine (DOPE) was purchased from Funakoshi Co. (Tokyo). Poly-L-ornithine (PLO, average molecular weight of 140,000), cholesteryl chloroformate (Chol), and deoxyribonuclease I (DNase I) were purchased from Sigma (St. Louis, MO). Lipofectin® was obtained from Life Technologies (Gaithersburg, MD). Ethidium bromide (EtBr) was obtained from Wako Pure Chemical Industries, Ltd. (Osaka). 4-Dimethylamino-1-butanol (DMB) was obtained from Tokyokasei Co. (Tokyo). PRO-YO-1 iodide and rhodamine-labeled phosphatidylethanolamine were purchased from Funakoshi Co. (Tokyo). pGL3 (pDNA) was amplified in *Eschericha coli* and extracted by the alkali lysis. The cell line HeLa S3 was obtained from Rikagaku Institute (Tsukuba, Japan). Cell culture lysis regent (CCLR, Promega, Tokyo) was used. All other chemicals were of reagent grade.

2.2. Preparation of liposomes

A mixture of 18.2 mmol of 4-dimethylamino-1-butanol (DMB) and 5 mmol of cholesteryl chloroformate in chloroform was evaporated in vacuum and redissolved in absolute ethanol. Cholesteryl-3 β-carboxybutylene-*N*-dimethylamine (DMB-Chol) was obtained as precipitate by cooling the ethanol solution. The precipitate was dissolved in $CHCl_3$ and kept as a lipid stock solution. 3.6 μmol DMB-Chol and 2.4 μmol DOPE were added in $CHCl_3$. After organic solvent was removed under reduced pressure, 10 mM HBS (pH 7.4, 3 ml) was added to the lipid film and the whole was treated by vortexing and sonication for 20 min under nitrogen. Liposome concentration was determined by phosphorus assay (Bartlett, 1959).

2.3. Preparation of DNA/peptide complexes and DNA/peptide/liposome complexes

pDNA and peptides were combined in 10 mM HBS for 20 min at room temperature to which the DMB-Chol liposomes were added, and incubated at room

temperature for 20 min. Liposome was maintained constant at 40 nmol/ml throughout.

2.4. In vitro transfection to mammalian cells

HeLa S3 cells were seeded in 60-mm petri plates at a density of 1×10^6 cells per dish and grown in a 37 °C incubator in a 5% CO_2 atmosphere. The cells were exposed to the complexes containing 4 μg pGL3/ml and incubated for 5 h. Then the transfection medium was replaced with fresh MEM (Nissui Pharmaceutical Co., Tokyo) containing 10% calf serum. The luciferase activity was measured after 24 h.

2.5. Luciferase assay

The medium was aspirated and the cells were washed twice with PBS (pH 7.4). The cells were lysed with 250 μl CCLR per dish. The cell lysates were collected, centrifuged to pellet the cellular debris, and 20 μl of the supernatant was used for the luciferase assay. A 100 μl of the luciferase substrate was added to the cell lysate and the relative light unit (RLU) of each sample was counted for 10 s using a luminometer (Model ARVO1420, Wallalc, Turku, Finland). Protein content in the supernatant was measured by the bicinchonic acid (BCA) method (Smith et al., 1985). Luciferase activity in each sample was normalized to the relative light unit (RLU) per milligram of extracted protein.

2.6. Cytotoxicity

Cell viability was estimated by measuring the activity of lactate dehydrogenase (LDH) released from cells. After the cells were exposed to the complexes of DNA and incubated for 5 h, the cell viability was expressed on the basis that the viability in the medium without pDNA/peptide or pDNA/peptide/liposome complexes is 100%.

2.7. Particle size measurement

The particle size of complex was measured by a dynamic light scattering spectrophotometer (DLS-7000, Photal, Otuska Electronics, Osaka) with a 75 mW Ar laser and a scattering angle of 90° at 25 °C. The diameter of the complexes was determined in triplicate and estimated by Marquadt analysis. Prior to use, the cell for measurement was prewashed with filtered water to minimize particular contamination. Results are expressed as mean ± S.D.

2.8. Ethidium bromide exclusion

Peptides were added incrementally to pDNA (4 μg/ml) in the presence of EtBr (0.4 mg/ml) and the resulting fluoresecence intensity was expressed as a percentage of the initial intensity (100%) attributed to pDNA alone. The intensity was measured by a fluorescence spectrophotometer (F-4500, Hitachi, Tokyo).

2.9. DNase I protection

pDNA/peptide complexes were prepared as described above. DNase I was added to pDNA (2.4 μg) of each sample to a final concentration of 1 U DNase I/μg pDNA and the mixtures were incubated at 42 °C for 30 min. SDS was added to the samples to a final concentration of 1% to release pDNA from peptides. Samples were then analyzed by agarose gel electrophoresis and the integrity of pDNA in each formulation was compared with untreated pDNA as a control.

2.10. Agarose gel electrophoresis

Complexes prepared at varied ratios in HBS (pH 7.4) were electrophoresed on agarose gel (0.8%) for 60 min at 100 V. EtBr was loaded in the gel to detect the location of pDNA using a UV transilluminator. pDNA/PLO: 4 μg/10 μg; pDNA/oligopeptide: 4 μg/60 μg; pDNA/PLO/liposome: 4 μg/10 μg/40 nmol; pDNA/oligopeptide/liposome: 4 μg/60 μg/40 nmol.

3. Results and discussion

3.1. Transfection efficiency of complexes

Fig. 1 shows that the transfection efficiencies of pDNA/peptide complexes formed at varied amount of oligopeptides. Those of PLO and Lipofectin® were also given for comparison. The oligopeptides consisting of nine amino acid residues (K, W, and C) or their dimers were chosen from preliminary

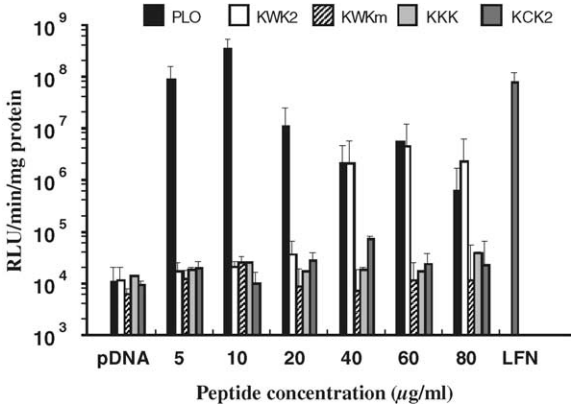

Fig. 1. Transfection efficiency of pDNA/peptide complexes. HeLa S3 cells were transfected with mixtures of pDNA (4 μg/ml) and various concentrations (0, 5, 10, 20, 40, 60, 80 μg/ml) of peptides for 5 h at 37 °C. LFN: lipofectin (20 μg/ml). Each column represents the mean ± S.D. ($n = 3$).

studies where the number of residue, the structure and the transfection efficiency were surveyed (data not shown). Although PLO showed the highest efficiency of transfection among the peptides used, the polypeptide exhibited a relatively high cytotoxicity (Tokunaga et al., 2003). This is also a background we explored the possibility of oligopeptides as effective carriers with low cytotoxicity (Figs. 4 and 5). The selected oligopeptides showed little transfection efficiencies at lower concentrations up to 20 μg/ml regardless of monomeric form or dimmer. At higher concentrations, only KWK2 exhibited about 100-fold greater efficiency compared with others. These results suggest that the substitution of the lysine for tryptophan (and the dimmer form) increased hydrophobic properties of complexes and promoted the interaction of the complexes with cell membrane or endosomal membrane as a result. Introduction of cystein at the central position also contributed to some extent to the transfection. McKenzie et al. (2000) developed a variety of low molecular weight peptides possessing cystein residues as cross-linker. They proposed that the DNA complex became small in size by condensation and gained stability which highly related to effective transfection. Our results supported their suggestion. The steric structure of pDNA complex with KWK2 due to S–S bond in addition to its hydrophobicity may be responsible for its efficient transfection.

Fig. 2 shows the transfection efficiencies of the ternary complexes consisting of pDNA/peptide/liposome. It is of interest that KKK and KCK2 exhibited strikingly higher transfection efficiencies with about 10^4–10^5-fold compared to the corresponding pDNA/peptide over the peptide concentrations examined, which were also 100-fold higher than that of DNA/liposome complexes. The results suggest that their transfection efficiencies are not simply due to the additive properties of peptide and liposomes.

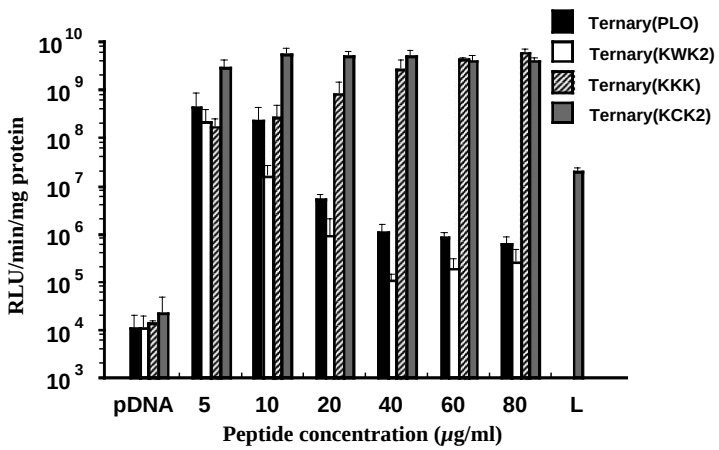

Fig. 2. Transfection efficiency of pDNA/peptide/liposome complexes. HeLa S3 cells were transfected with mixtures for 5 h at 37 °C. pDNA (4 μg/ml) was precomplexed with 0, 5, 10, 20, 40, 60, 80 μg/ml of peptides to which 40 nmol/ml of liposomes were added. L: pDNA/liposome (40 nmol/ml). Each column represents the mean ± S.D. ($n = 3$).

Table 1
Particle sizes of complexes

	Diameter (nm)	
pDNA + PLO	126.5 ± 64.6	
pDNA + KWK2	13238.6 ± 2352.7	5015.2 ± 1403.9
pDNA + KKK	981.8 ± 28.5	82.4 ± 15.9
pDNA + KCK2	1327.8 ± 238.6	84.9 ± 34.1
Ternary (PLO)	3356.7 ± 21.9	118.3 ± 33.2
Ternary (KWK2)	568.5 ± 148.6	
Ternary (KKK)	438.5 ± 86.5	
Ternary (KCK2)	406.3 ± 126.8	
pDNA + liposome	524.0 ± 112.7	152.1 ± 98.1
Liposome	197.4 ±72.0	

Mean±S.D. ($n = 3$). DNA/peptide complex (DNA, 4 μg/ml; PLO, 10 μg/ml; KWK2, KKK, and KCK2, 60 μg/ml). Ternary complex (DNA, 4 μg/ml; PLO and KWK2, 10 μg/ml; KKK and KCK2, 60 μg/ml; liposome, 40 nmol/ml). Ternary, pDNA/peptide/liposome complexes with PLO, KWK2, KKK, KCK2. Liposome:DMB-Chol/DOPE (3:2 molar ratio). Data represented particle sizes of each complex after incubation for 1 h at room temperature.

In contrast, the transfection efficiency of KWK2 was comparable to other ternary complex at 5 μg/ml peptide but decreased with increasing peptide concentration. As shown in Table 1, the ternary oligopeptide complexes were well condensed in size with added liposomes and their sizes were almost comparable with the pDNA/liposome. The ternary KWK2 (10 μg/ml peptide) was condensed to the almost same size as seen in KKK and KCK2 (60 μg/ml peptide) with added liposomes. However, pDNA/KWK2 formed extremely large aggregation at a high peptide concentration (60 μg/ml peptide) at which the ternary complex was not appropriately formed in terms of the size of complex. Structural and electrical surface properties of complexes formulated from three components must be involved.

3.2. Cytotoxicity

Fig. 3A and B represent the cell viabilities of pDNA/peptide and pDNA/peptide/liposome complexes formed at various concentrations of peptides, respectively. Oligopeptides showed little cytotoxicity to the cells against PLO, which showed a sharp concentration dependency. Also, the ternary oligopeptide/liposome complexes, KKK, and KCK2, showed little cytotoxicity over the concentration examined but KWK2 bore a slightly concentration-dependent cytotoxicity. The results indicate that the oligopeptide systems are promising carriers with liposomes in addition to the efficient transfection.

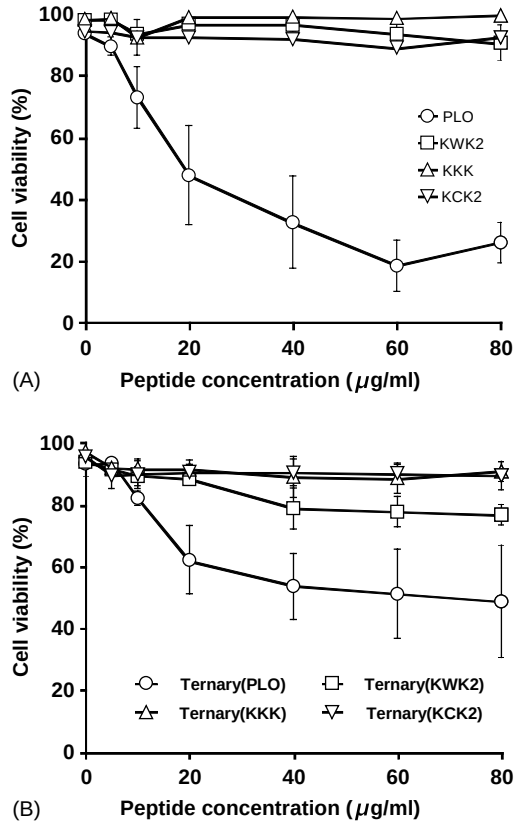

Fig. 3. Cytotoxicity of DNA/peptide and DNA/peptide/liposome complexes. (A) pDNA/peptide complexes; (B) pDNA/peptide/liposome complexes. Cell viability was represented as a percentage to the viability (100%) in the medium without complexes. Each point represents the mean ± S.D. ($n = 3$).

3.3. Ethidium bromide exclusion

The binding strength of pDNA in the complexes was assessed using the EtBr exclusion assay. Fig. 4A shows that with increasing of KKK, KCK2, and PLO of pDNA/peptide, which has relatively higher positive charges, the relative fluorescence abruptly decreased at low concentrations of peptides and reached a plateau between 20 and 50% of the initial fluorescence. The results indicate that these peptides readily brought in exclusion of EtBr from pDNA intercalation. Meanwhile, KWK2 and KWKm containing tryp-

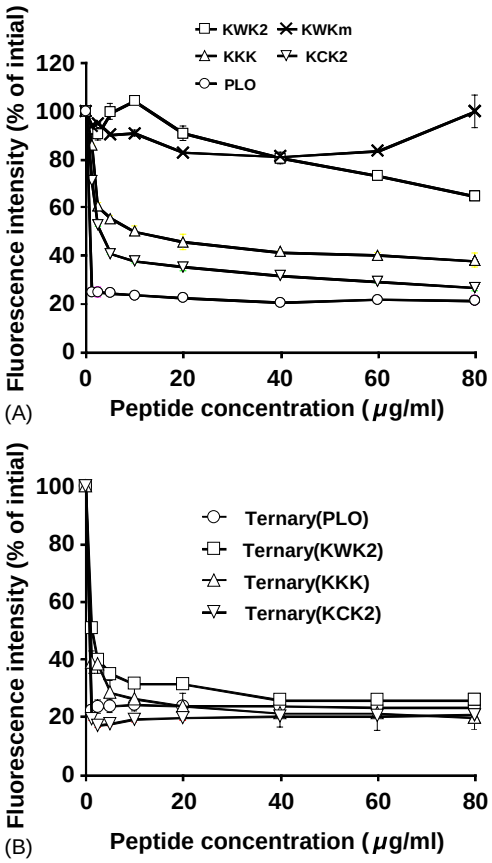

Fig. 4. Effect of peptide on ethidium bromide exclusion from pDNA/peptide and pDNA/peptide/liposome complexes. (A) pDNA/peptide complexes; (B) pDNA/peptide/liposome complexes. Peptide was added incrementally to pDNA (4 μg/ml) in the presence of ethidium bromide (0.4 μg/ml) and the resultant signal was expressed as a percentage of the initial fluorescence (100%) attributed to pDNA alone. Each point represents the mean ± S.D. ($n = 3$).

tophan residues instead of lysine residues in the sequences showed less exclusion of the EtBr and stayed in the range of 70–100%, suggesting that reduction of positive amino acid residues was responsible for their weaker affinity to pDNA. But, this does not mean that they could not form complexes with pDNA. It is because that the complex formation was confirmed at 0.625 or 1.25 μg/1 μg pDNA of the mass ratio using the agarose gel electrophoretic assay (data not shown).

Mascotti and Lohman (1993) examined the thermodynamics of the interactions of oligolysines containing one or more tryptophans with DNA and RNA. They observed an increase in the entropy of peptide binding to DNA when substituting tryptophan for lysine. However, this was compensated by a decrease in the enthalpy of binding, leading to a very little change in the association constant. Accordingly, multiple tryptophan residues were considered to little influence the magnitude of the association constant despite changes in the enthalpic and entropic contributions. However, our results from EtBr exclusion studies were not always consistent with their explanation.

Meantime, as shown in Fig. 4B, the ternary complexes demonstrated an abrupt decrease of the fluorescence intensity down to 20–30% for all the peptides examined. These results indicate that the cationic liposomes also have a strong affinity to pDNA as well as cationic peptides and further expel the intercalated marker. Because of its relatively hydrophobic nature, KWK2 was thought to be overcome by cationic liposomes in the competition of the binding with pDNA. The addition of cationic liposomes was considered to result in more compactness to the ternary forms in line with the size changes (Table 1).

3.4. Particle sizes of complexes

The pDNA/oligopeptide complexes showed large aggregation with two peaks depending on the kind of the peptides, as shown in Table 1. By contrast, pDNA/PLO complex formed one peak with about 130 nm in diameter, indicating that the complex was well condensed. The similar results are reported about PLO (Pouton et al., 1998; Ramsay et al., 2000). The difference of the binding affinity to pDNA is probably responsible for their aggregation behavior. This could be coincident with the results of EtBr exclusion (Fig. 4A). Meantime, the particle sizes of the ternary complexes of oligopeptides were all fallen within 400–500 nm in diameter, which were considered to be appropriate sizes for transfection. There was also little significant difference about the particle size of the kind of oligopeptide, suggesting that the pDNA/peptide/liposome complexes were well condensed. pDNA/liposome complex resulted in two peaks with one dominant larger aggregation than the liposomes alone, and was comparable to the ternary complexes in size.

3.5. In vitro resistance against DNase I

The resistance of pDNA complexes against the attack from a degrading enzyme was investigated using DNase I as a model enzyme in comparison with SDS treatment. The complexes which their highest expression levels exhibited were tested. The results of pDNA/peptide and pDNA/peptide/liposome were

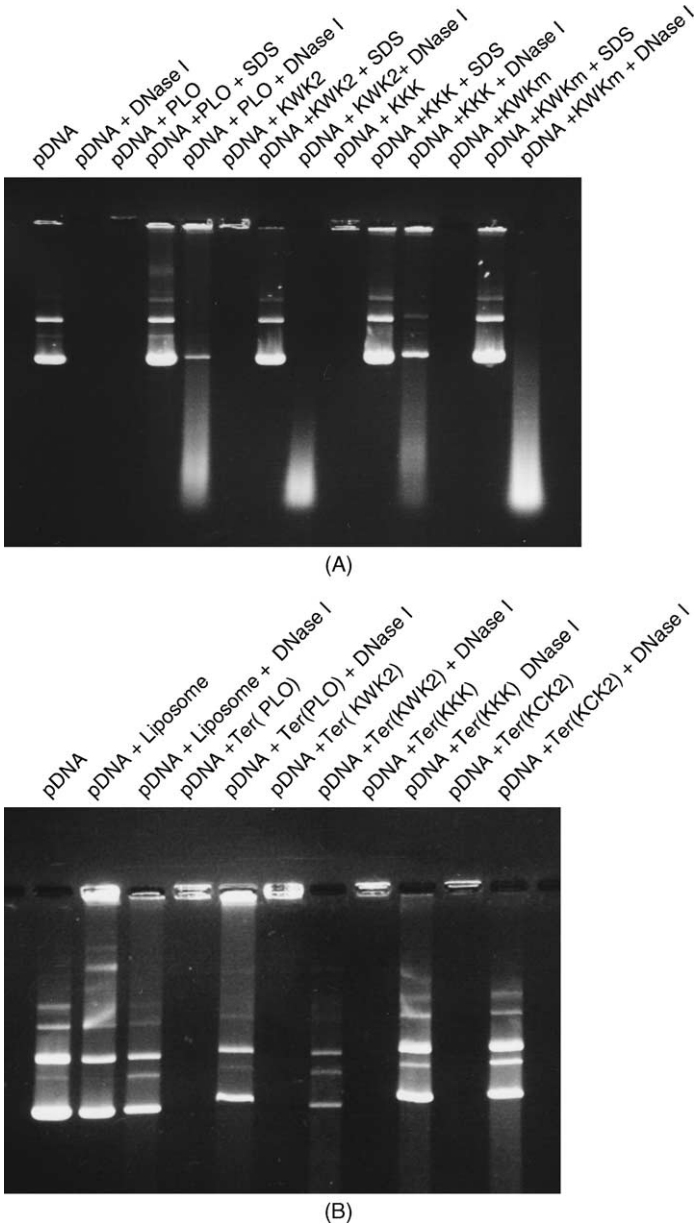

Fig. 5. Electrophoresis patterns of pDNA/peptide and DNA/peptide/liposome complexes treated with DNase I. (A) pDNA/peptide complexes; (B) pDNA/peptide/liposome complexes. pDNA was complexed with peptides at the mass ratios of the highest expression levels in the transfection experiment pDNA/PLO: 4 μg/10 μg; pDNA/oligopeptide: 4 μg/60 μg; pDNA/PLO/liposome: 4 μg/10 μg/40 nmol; pDNA/oligopeptide/liposome: 4 μg/60 μg/40 nmol.

shown in Fig. 5A and B, respectively. The first lane and the second lane are shown as references: pDNA alone and DNase I-treated pDNA. All of the pDNA/peptide complexes treated with SDS remained stable and those treated with DNase I completely degraded(Fig. 5A). Neither KWK2 nor KWKm protected the pDNA from DNase I degradation, although the dimer showed the highest transfection efficiency among oligopeptides (Fig. 1). The complexes with PLO and KKK were partially protected from the digestion. These results were consistent with those of the EtBr exclusion experiment, suggesting that the KWKWCWKWK structure containing some hydrophobic tryptophans instead of lysine has weaker binding affinity with pDNA.

As shown in Fig. 5B, the ternary complexes effectively protected the pDNA from the attack of DNase I, indicating that the cationic liposomes made pDNA/peptide complexes stable enough to resist from the enzyme attack most probably due to compactness by condensation.

3.6. Effect of serum on transfection

Fig. 6A and B shows the effect of serum on the transfection efficiencies of pDNA/peptide and pDNA/peptide/liposome complexes to cells, respectively. Serum decreased the transfection efficiency of pDNA/KWK2 complex significantly to 100th at its higher concentrations while those of other oligopeptides (KKK, KCK2) remained almost unchanged at the level of pDNA alone.

With regard to pDNA/oligopeptide/liposome complexes, their transfection efficiencies were generally affected downward as well and the similar tendencies of the transfection shown in Fig. 2 were observed. KCK2 complexes maintained about 10^7 order of the magnitude with no dependency of their concentrations. KWK2 complex were maximally maintained at 10^7 levels (RLU/min/mg protein) at around 5–10 μg/ml followed by the decreasing efficiency with increasing peptide concentration. KKK complex showed an opposite tendency to KWK2 complex. These results indicate that the liposomes or the dimmer played a very important role. The difference of charge ratios may also be responsible for maintaining high transfection. Yang and Huang (1997) reported that serum enhanced transfection

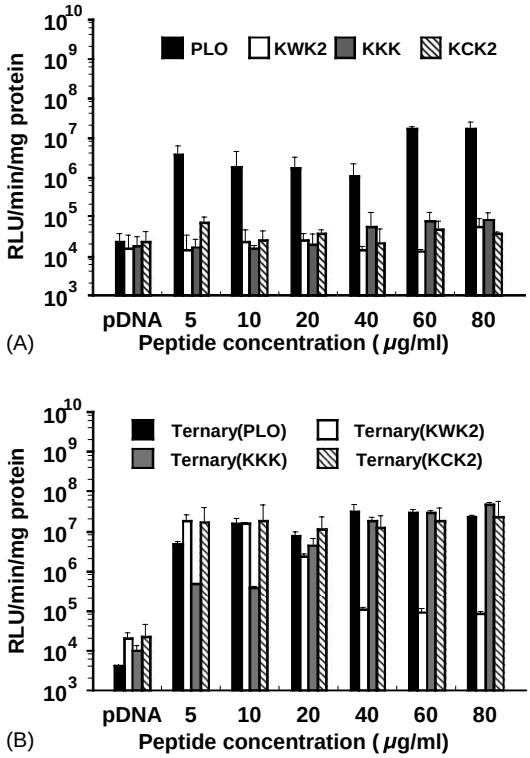

Fig. 6. Effect of serum on transfection efficiencies of pDNA/peptide and pDNA/peptide/liposome complexes. (A) pDNA/peptide complexes; (B) pDNA/peptide/liposome complexes. pDNA (4 μg/ml) was precomplexed with 0, 2.5, 5, 10, 20, 40, 60, and 80 μg/ml of peptides to which 40 nmol/ml of cationic liposomes was added. Serum: 10% throughout. Each column represents the mean ± S.D. ($n = 3$).

for Lipoplex® (DC-Chol/DOPE) with high charge ratios and inhibited transfection for those with low charge ratios. They suggested that Lipoplex® with low charge ratios might be completely neutralized by serum, resulting in less DNA binding to the cell surface and lowered the efficiency. Meantime, the excess positive charges of Lipoplex® with higher charge ratios could partially overcome the neutralization effect of serum and mediated DNA binding to the cell surface. This explanation could be applied for KKK and PLO complexes. But the decreasing tendency of KWK2 with increasing concentration may be due to hydrophobic interaction between tryptophan and serum protein in addition to charge neutralization.

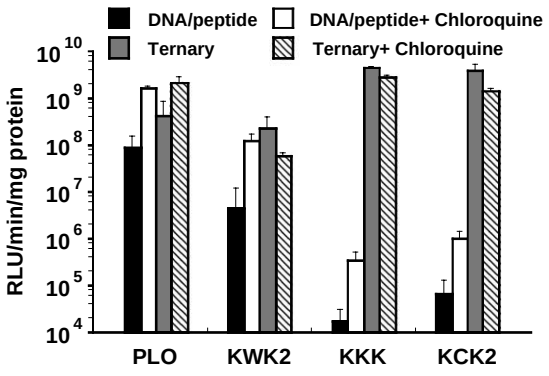

Fig. 7. Effect of chloroquine on transfection efficiencies of pDNA/peptide and pDNA/peptide/liposome complexes. pDNA/peptide/liposome complexes were formulated at the ratio provided the highest expression levels at in vitro transfection experiment (pDNA/PLO/liposome: 4 μg/10 μg/40 nmol; pDNA/oligopeptide/liposome: 4 μg/60 μg/40 nmol) in the presence or absence of chloroquine (100 μM) for 5 h at 37 °C. Each column represents the mean ± S.D. ($n = 3$).

3.7. Effect of chloroquine on the transfection efficiency

Fig. 7 shows the effect of chloroquine on the transfection efficiency was compared in the presence and absence of chloroquine which was known as a lysosomotropic agent to interfere with endocytosis by neutralizing endosomal and lysosomal pH and by inhibiting maturation of endosomes (Mellman et al., 1986). While the gene expressions of pDNA/peptide complexes increased significantly by chloroquine, the agent little affected for the corresponding pDNA/peptide/liposome complexes and rather exhibited a slightly decreasing tendency for KWK2, KKK, and KCK2. Farhood et al. (1995) showed that the helper DOPE-containing liposomes was essential for high transfection efficiency where the DOPE was considered to destabilize the endosome membrane and facilitate the release of DNA. However, the chloroquine treatment almost completely abolished the transfection, most probably due to inhibition of the maturation of endosomes.

It is interesting that chloroquine acts an entirely opposite manner between pDNA/peptide complexes and DNA/DOPE-containing liposome, suggesting that the agent is involved in one or more steps in the multiple steps from the cell surface adsorption to transcription. It should be noted that the oligopeptides in the ternary complexes compensate the negative action of DOPE-containing liposomes.

4. Conclusion

We examined the possibilities of synthetic oligopeptides of nine amino acid residues (K, W, and C) and their dimers as peptide-carriers for gene transfection. The oligopeptides alone were not effective for transfection. The ternary complex (DNA/oligopeptide/liposome) showed high transfection efficiency in addition to low cytotoxicity, higher protection from DNase I digestion and the less binding with serum proteins. The oligopeptides in the ternary formulations possess the possibility as useful enhancers of DOPE containing cationic liposomes-mediated gene delivery where oligopeptides and liposomes might have individual roles each other.

Acknowledgements

This study was supported in part by grant from the Ministry of Education, Science, Sports and Culture of Japan (10672023).

References

Ali, M., Lemoine, N.R., Ring, C.J., 1994. The use of DNA viruses as vectors for gene therapy. Gene Ther. 1, 367–384.

Aramaki, Y., Lee, I., Arima, H., Sakamoto, T., Magami, Y., Yoshimoto, T., Moriyasu, F., Mizuguchi, J., Koyanagi, Y., Nikaido, T., Tsuchiya, S., 2003. Efficient gene transfer to hepatoblastoma cells through asialoglycoprotein receptor and expression under the control of the cyclin A promoter. Biol. Pharm. Bull. 26, 357–360.

Atherton, E., Gait, M.J., Sheppard, R.C., Williams, B.J., 1979. The polyamide method of solid phase peptide and oligonucleotide synthesis. Bioorg. Chem. 8, 351–370.

Bartlett, G.R., 1959. Phosphorus assay in column chromatography. J. Biol. Chem. 234, 466–468.

Behr, J.P., 1994. Gene transfer with synthetic cationic amphiphiles: prospects for gene therapy. Bioconjug. Chem. 5, 382–389.

Farhood, H., Servina, N., Huang, L., 1995. The role of dioleoyl phosphatidylethanolamine in cationic liposome mediated gene transfer. Biochem. Biophys. Acta 1235, 289–295.

Felgner, P.L., 1997. Improvements in cationic liposomes for in vivo gene transfer. Hum. Gene Ther. 7, 1791–1793.

Gao, X., Huang, L., 1995. Cationic liposome-mediated gene transfer. Gene Ther. 2, 710–722.

Gao, X., Huang, L., 1996. Potentiation of cationic liposome-mediated gene delivery by polycations. Biochemistry 35, 1027–1036.

Herz, J., Gerard, R.D., 1993. Adenovirus-mediated transfer of low density lipoprotein receptor gene acutely accelerates cholesterol clearance in normal mice. Proc. Natl. Acad. Sci. U.S.A. 90, 2812–2816.

Li, S., Huang, L., 1997. In vivo gene transfer via intravenous administration of cationic lipid-protamine-DNA (LPD) complexes. Gene Ther. 4, 891–900.

Mascotti, D.P., Lohman, T.M., 1993. Thermodynamics of single-stranded RNA and DNA interactions with oligolysines containing tryptophan. Effects of base composition. Biochemistry 32, 10568–10579.

McKenzie, D.L., Smiley, E., Kwok, K.Y., Rice, K.G., 2000. Low molecular weight disulfide cross-linking peptides as nonviral gene delivery carriers. Bioconjug. Chem. 11, 901–909.

Mellman, I., Fuchs, R., Helenius, A., 1986. Acidification of the endocytic and exocytic pathways. Annu. Rev. Biochem. 55, 663–700.

Meyer, O., Kirpotin, D., Hong, K., Sternberg, B., Park, J.W., Woodle, M.C., Papahadjopoulos, D., 1998. Cationic liposomes coated with polyethylene glycol as carriers for oligonucleotides. J. Biol. Chem. 273, 15621–15627.

Nishikawa, M., Yamauchi, M., Morimoto, K., Ishida, E., Yakakura, Y., Hashida, M., 2000. Hepatocyte-targeted in vivo gene expression by intravenous injection of plasmid DNA complexed with synthetic multi-functional gene delivery system. Gene Ther. 7, 548–555.

Ogris, M., Brunner, S., Shuller, S., Kircheis, R., Wagner, E., 1999. DNA/transferring-PEI complexes: reduced interaction with blood components, extended circulation in blood and potential for systemic gene delivery. Gene Ther. 6, 595–605.

Pouton, C.W., Lucas, P., Tomas, B.J., Uduehi, A.N., Miloy, D.A., Moss, S.H., 1998. Polycation-DNA complexes for gene delivery: a comparison of the biopharmaceutical properties of cationic polypeptides and cationic lipids. J. Control. Release 53, 289–299.

Ramsay, E., Hadgraft, J., Birchall, J., Gumbleton, M., 2000. Examination of the biophysical interaction between plasmid DNA and the polycations, polylysine and polyornithine as a basis for their differential gene transfection in vitro. Int. J. Pharm. 210, 97–107.

Simon, R.H., Engelhardt, J.F., Yang, Y., Zepeda, M., Weber-Pendleton, S., Grossman, M., Wilson, J.M., 1993. Adenovirus-mediated transfer of the CFTR gene to lung of nonhuman primates: toxicity study. Hum. Gene Ther. 4, 771–780.

Smith, P.K., Krohn, R.I., Hermanson, G.T., Mallia, A.K., Gartner, F.H., Provenzano, M.D., Fujimoto, E.K., Goeke, N.M., Olson, B.J., Klenk, D.C., 1985. Measurement of protein using bicinchoninic acid. Anal. Biochem. 150, 76–85.

Tokunaga, M., Nagao, M., Nagata, M., Hazemoto, N., Yotsuyanagi, T., 2003. DNA transfection mediated by synthetic polycationic peptides. J. Pharm. Sci. Technol. Jpn. 63, 71–78.

Vigneron, J.P., Oudrhiri, N., Fauquet, M., Vergely, L., Bradley, J.C., Basseville, M., Lehn, P., Lehn, J.M., 1996. Guanidinium-cholesterol cationic lipids: efficient vectors for the transfection of eukaryotic cells. Proc. Natl. Acad. Sci. U.S.A. 93, 9682–9686.

Yang, J.P., Huang, L., 1997. Overcoming the inhibitory effect of serum on lipofection by increasing the charge ratio of cationic liposome to DNA. Gene Ther. 4, 950–960.