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Polycations enhance emulsion-mediated in vitro and in vivo transfection

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Abstract

To enhance the in vitro and in vivo transfection activity of the cationic lipid emulsion (LE), three natural polycations, protamine sulfate (PS), poly-L-lysine and spermine, were selected as DNA condensing active agents. Formation of the LE/polycation/DNA ternary complexes was identified by using agarose gel retardation study. The structure of these complexes was characterized by measuring the complex size and the decrease of the DNA fluorescence in the presence of ethidium bromide (EtBr). By adding a polycation, the particle size of the complex decreased, and DNA in the complex became highly condensed and resistant to intercalation of EtBr. Among the polycations, PS yielded the most highly compacted ternary complex. In vitro and in vivo transfection activities of the complexes were determined using various cell lines and Balb/c mouse intravenously and intranasally, respectively. The transfection activity of the ternary complex increases by at least 2.5–5-fold in vitro cell culture system in the presence of 80% serum as well as in vivo mouse system, as compared with LE/DNA binary complexes. More importantly, after intravenous and intranasal administrations, the in vivo transfection efficiency of the LE/PS/DNA complex was ca. 30 and 50 times higher than that of the liposome (LP)/DNA complex in spleen and lung, respectively. On the other hand, cell toxicity of the ternary complex is lower than that of binary complex. Thus, we conclude that the pre-condensation of DNA with polycations can be a promising approach to further increase in vitro and in vivo transfection efficiency of cationic lipid emulsion.

Keywords: Gene transfer; Emulsion; Protamine sulfate; Condensation

1. Introduction

Cationic lipids have been widely formulated as a liposome (LP) to deliver genes into cells (Gao and

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Huang, 1991; Felgner et al., 1994). Transfection using a LP is convenient and highly efficient in vitro cell culture system when compared with other non-viral gene carriers (Felgner et al., 1994; Zabner, 1997; Kim et al., 2001b). This LP system, however, is not efficient in transfecting cells in the presence of body fluid such as serum and mucosal secretion. Thus, much effort has been devoted to finding cationic lipids or lipid formulations that increase the transfection efficiency under in vivo conditions.

Recently, the cationic lipid emulsion (LE) was developed to deliver DNA efficiently in vitro and in vivo (Liu et al., 1996a,b; Yi et al., 2000; Kim et al., 2000, 2001a,b, 2002, 2003). LE was formulated with squalene and 1,2-dioleoyl-*sn*-glycero-3-trimethylammonium-propane (DOTAP) as major components. This cationic emulsion maintained the physical integrity with DNA and successfully delivered a reporter gene into cells in vitro in the presence of up to 80% serum. It is well known that a small amount of serum (∼10%) can dramatically reduce the transfection activity of LP/DNA complexes because it contains anionic materials (Hofland et al., 1998; Kim et al., 2000, 2001a, 2002). This physically stable emulsion was more efficient than the commercialized LPs, such as lipofectamine® and lipofectin®, for in vivo gene transfer when administered via tail vain route or intranasal route (Kim et al., 2000, 2001a, 2002). In a recent paper, we enhanced its in vitro and in vivo transfection activity further by optimizing lipid compositions of the LE formulations (Kim et al., 2001a).

There have been many reports demonstrating that LP-mediated gene transfer could be augmented by the addition of natural polycations such as protamine sulfate (PS), poly-L-lysine (PLL) and spermine (Gao and Huang, 1996; Li and Huang, 1997). These polycations are known to form a complex with DNA and condense DNA from extended conformation to highly compact structure into 30–100 nm in size. Although these polycations by itself mediate DNA delivery, they exhibit a synergistic effect when combined with several different types of cationic LPs in delivering plasmid DNA into several different types of cells (Gao and Huang, 1996). The polycations condense DNA into ternary complexes such as LP/polycation/DNA complexes. These particles showed an enhanced gene expression over that seen with LP/DNA binary complexes. This enhancement is considered to be caused by a highly compacted complex to assist in an efficient cellular uptake and to protect DNA against enzymatic digestion. Also, PS has a peptide functional moiety that can act as a nuclear localization signal (NLS) that can help DNA translocation to the nucleus (Li and Huang, 1997). In case of spermine, once these particles enter nucleus, it may dissociate more readily and help DNA to bind with transcription machinery (Ibanez et al., 1996). For these numbers of reasons, it would be worth investigating the effect of these cations on emulsion-mediated transfection.

PS, PLL and spermine were selected to form a ternary complex with LE and DNA to enhance the transfection activity of emulsion system. The counterpart DOTAP LPs was also used in formation of ternary complexes for comparison with emulsions. We have investigated the physicochemical parameters, in vitro transfection activity and cytotoxicity of the ternary complexes. The level of in vivo transgene expression was also measured after the intravenous (i.v.) administration of the complexes to the tail vein of mouse or intranasal instillation via nares.

2. Materials and methods

2.1. Materials

Squalene, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT), ethidium bromide (EtBr), protamine sulfate (molecular weight (M.W.) 5.1 kDa), poly-L-lysine (M.W. 15–30 kDa), spermine (M.W. 0.2 kDa) and Tween 80 were purchased from Sigma Chemical Company (St. Louis, MO, USA). DOTAP and 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE) were purchased from Avanti Polar Lipids (Alabaster, AL, USA) and used without further purification. Dulbecco's Modification of Eagle Medium (DMEM), Roswell Park Medical Institute (RPMI) 1640 medium and fetal bovine serum (FBS) were purchased from Gibco BRL/Life Technologies (New York, NY, USA). All other chemicals and reagents were of tissue culture grade.

2.2. Plasmid DNAs

The pCMV-beta encoding *Escherichia coli* (*E. coli*) lacZ (β-galactosidase) gene expression plasmid driven

by the human cytomegalovirus immediate-early promoter was purchased from Clontech Laboratories (Palo Alto, CA, USA). The pCMV-Luc+ encoding a cytosolic form of Phontinus pyralis luciferase cDNA was constructed in our laboratory (Kim et al., 2001a). The plasmids were amplified in the *E. coli* DH5-α strain and purified by using a Qiagen mega-kit (Qiagen Inc., Chatsworth, CA, USA) according to the manufacturer's instruction. DNA purity was determined by agarose gel electrophoresis and by measuring optical density (OD). DNA having $OD_{260}/OD_{280} \geq 1.8$ was used.

2.3. Preparation of lipid carriers

The emulsions containing 100 μl/ml squalene and DOTAP/DOPE at 20/4 mg/ml (denoted LE3 hereafter) or DOTAP/DOPE/Tween 80 at 20/4/4.5 mg/ml (LE4) were prepared as described previously (Kim et al., 2001a). Briefly, lipid emulsifiers were weighed and dispersed in water. The emulsifier/water mixture was sonicated until clear in an ice/water bath by using a probe type sonicator (high intensity ultrasonic processor, 600 W model, Sonics and Materials, Danbury, CT, USA). The lipid solution was added to oil and sonicated further in an ice/water bath for ca. 4 min to form the emulsions. Also, a liposome system (LP4) comprising DOTAP/DOPE/Tween 80 weight ratio of 20/4/4.5 was prepared by sonicating of lipid dispersion for 4 min as a counterpart LP system to LE4, which made with an identical lipid composition of emulsion. To prepare liposomal carriers, the lipid solutions were further sonicated for 4 min after the solution became clear. The emulsions and the LP were stored at 4 °C until further used. The lipid compositions of prepared lipid carriers were optimized for transfection in our previous study (Kim et al., 2001a). In this paper, to simplify the quantification of cationic lipid formulations, we note that the amount of emulsion or LP was quantified as the weight of DOTAP in each formulation. For instance, 4 mg of DOTAP in an emulsion having 20 mg/ml DOTAP and 4 mg/ml DOPE as an emulsifier corresponds to 0.2 ml of emulsion.

2.4. Preparation of ternary complex

The preparation of emulsion/DNA complexes was described previously (Kim et al., 2001a,b, 2002). In this paper, the weight ratio between DOTAP in the lipid formulation (LE or LP), polycation (P) and DNA (D) will denote the composition. While LE or LP/D and P/D denote the weight ratio of two components in binary complex, L/P/D denotes that of ternary complex system. Also, P/D_{opt}, L/D_{opt} and $L/P/D_{opt}$ denote the weight ratio of P/D, L/D and L/P/D that shows the highest in vitro transfection activity, respectively. $L/P/D_{opt}$ was obtained by varying the amount of polycation with the amount of DNA and lipid formulations fixed, where the amount of emulsion was half in binary complex at L/D_{opt}. For the formation of ternary complexes, DNA was mixed with a polycation, and subsequently the lipid carriers were added to the polycation/DNA complexes.

2.5. Agarose gel electrophoresis

The complex formation between DNA and the carrier was visualized by using gel electrophoresis. The pCMV-beta was diluted to a concentration of 1 μg per 10 μl of phosphate buffered saline (PBS). Ten microliters of the DNA solution was mixed with 10 μl of carrier solution containing different amounts of LE4 and/or polycations. In binary complex systems, LE4/DNA and polycation/DNA complexes were formed at L/D = 8 and P/D = 4, respectively. Formation of the ternary complexes was observed by mixing 1 μg of the DNA in 10 μl with an increasing amount of polycations diluted in 5 μl of PBS (P/D = 1–5) and by adding 4 μg of LE4 subsequently. After 10 min at room temperature, these samples were applied to an agarose gel electrophoresis.

2.6. Physical characterization of ternary complexes

The ternary complexes were further characterized by measuring the size and the EtBr fluorescence. The complexes for size measurement were prepared as described above except that the final concentration of DNA used was 5 μg in 1 ml of PBS. The average droplet sizes of the complexes were measured by using photon correlation spectroscopy (Malvern Zetasizer, Malvern Instruments Ltd., England) as described previously (Kim et al., 2001b). The mean value represents the average of three measurements on a single sample. Fluorescence measurement of EtBr was performed by using the ISS K2 fluorometer (ISS, Champaign, IL, USA) at excitation wavelength of 488 nm (slit width 2.0 mm) and

emission wavelength of 594 nm (slit width 1.0 mm). One milliliter of 2 μM EtBr solution was added to 1 ml of the complex solutions. Fluorescence intensity was measured after 10 min incubation.

2.7. In vitro gene transfer

A simian kidney cell line (CV-1), its derivative (COS-1), a mouse embryo fibroblast cell (NIH3T3) and a human large cell lung carcinoma (H1299) were cultured as described previously (Kim et al., 2001a). Cells were seeded at 2×10^4 cells per well on to 96-well plates 12 h before transfection. Cells were ca. 70–80% confluent at the time of transfection. For a single well, 500 ng of pCMV-beta were mixed with an appropriate amount of carriers to form a complex in 40 μl of serum free DMEM as described above. After washing the cells with serum free media, 160 μl of serum free medium (DMEM for CV-1, COS-1 and NIH3T3, RPMI1649 for H1299) were added. To test effect of serum, 160 μl of FBS was added instead of 160 μl of serum free media. After 1 h incubation, the cells were washed with serum free media to remove the remaining carrier/DNA complexes. The cells were fed again with culture media containing 10% (v/v) FBS and cultured for 24 h after transfection. The transfected cells were assayed for β-galactosidase activity using a photometric assay as described (Kim et al., 2001a). The cell viability was tested using MTT (Kim et al., 2001a). After in vitro transfection assay, 200 μl of DMEM containing 10% FBS and 50 μl of 0.5% (w/v) MTT in PBS was added to each well, and then incubated for 4 h to allow the production of the formazan crystal. The formazan crystal was dissolved by adding 200 μl of dimethylsulfoxide (DMSO) and in turn stabilized by adding 25 μl of Sorensen's glycine buffer. The quantity of formazan products was measured at 570 nm. The 100% value was obtained from the value measured in naive cells.

2.8. In vivo gene transfer and luciferase assay

To prepare carrier/DNA complexes, DNA solution containing 10 μg of pCMV-Luc+ and the carrier, whose amount corresponds to L/D_{opt} or $L/P/D_{opt}$ each diluted with 100 μl of serum free DMEM, respectively, were mixed by inversion. The complex solutions were incubated at room temperature for 10 min and were injected into female Balb/c mice (20–25 g) via the tail vein. For intranasal administration, the mice were anesthetized by Ketamine and administered topically to the nares of mouse. Twenty-four hours later, mice were sacrificed, and organs such as heart, lung, liver, kidney and spleen, for intravenous injection, and lung and nose, for intranasal inhalation, were removed and homogenized in lysis buffer. The volume-to-weight ratio of lysis buffer was 5 μl/mg for each collected organ. After two freeze/thaw cycles, the homogenized organ extracts were centrifuged at 4 °C for 10 min at 12,000 rpm. A portion of the supernatants was assayed for protein concentration by using a Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA). Luciferase activity was expressed as picogram per milligram tissue protein. Background of luciferase activity in each organ was measured from the organs of untreated mice and was negligible.

3. Results

3.1. Formation of emulsion/polycation/DNA complex

The formation of LE/polycation/DNA complex was examined by using an agarose gel assay (Fig. 1). The electrophoretic mobility of DNA (1 μg) was completely retarded by LE4 at L/D = 8 (lane 2 in Fig. 1A–C) or by PS and PLL at P/D = 4 (lane 3 in Fig. 1A and B). Spermine, however, could not completely retard the DNA mobility at P/D = 4 (lane 3 in Fig. 1C). To form the ternary complexes, 1 μg of DNA was mixed in increasing amounts of polycations corresponding to P/D values of 1–5, diluted in 5 μl of PBS (P/D = 1–5), and in turn 4 μg of LE4 was added. The mobility of DNA decreased gradually with increasing amounts of polycations. In both PS and PLL, the mobility stopped completely at L/P/D = 4/2/1 indicating that the plasmid DNA can completely form the ternary complexes at this ratio. On the other hand, the spermine could not completely retard the mobility of DNA in the range studied.

One of the main roles of the polycation might be its DNA condensing activity (Gao and Huang, 1996; Li and Huang, 1997), which can protect DNA from degradation and help cellular uptake. It could be assumed that the complex size could become small upon DNA condensation. Also, it has been reported that EtBr

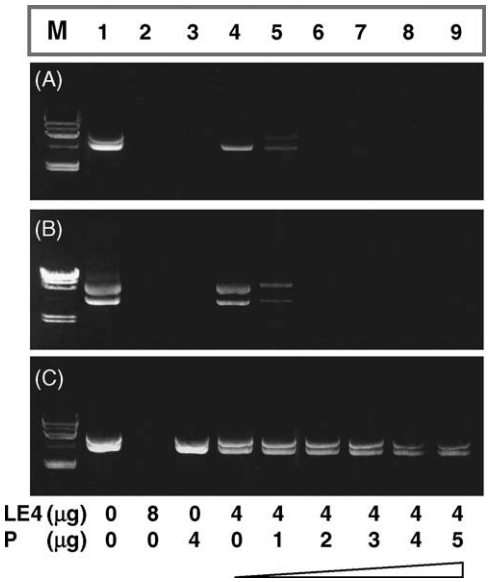

Fig. 1. Changes in mobility of DNA in emulsion/DNA complex as a function of added (A) protamine sulfate, (B) poly-L-lysine and (C) spermine amount. The plasmid DNA and their complexes with the LE and the polycations were incubated for 10 min at room temperature, and then were run on 1% agarose gel including 0.5 μg/ml EtBr. LE4 and P represent DOTAP/DOPE/Tween 80 (20/4/4.5 mg/ml) emulsion and polycation, respectively. Lane M: molecular weight marker (λ *Hind* III). Lane 1: 1 μg of pCMV-beta only. Lanes 2–9: 1 μg of the DNA complexed with the indicated amounts of the LE or the polycations.

could not intercalate into condensed DNA and, consequently, EtBr fluorescence intensity decreased as DNA condensed (Kim et al., 2001b). Therefore, we determined the particle size and the fluorescence intensity of DNA in the presence of EtBr after ternary complex formation as described in Section 2. At this time, size and polydispersity of LE4 were ca. 148 and 0.185, respectively. The ratio between LE4 and DNA was fixed to 4:1. The addition of the polycations resulted in a decline in mean particle size of the complexes (Fig. 2A). The decrease in particle size of the complexes represents the compaction of the ternary complexes by a polycation-induced DNA condensation (Gao and Huang, 1996; Li and Huang, 1997). Among those, PS reduced the particle size most efficiently. At L/P/D = 4/2/1, size and polydispersity of the complex were ca. 165 nm and 0.226, respectively. PLL was also efficient, but was slightly less efficient than PS. The decrease of particle size was smallest when spermine was used. Actually,

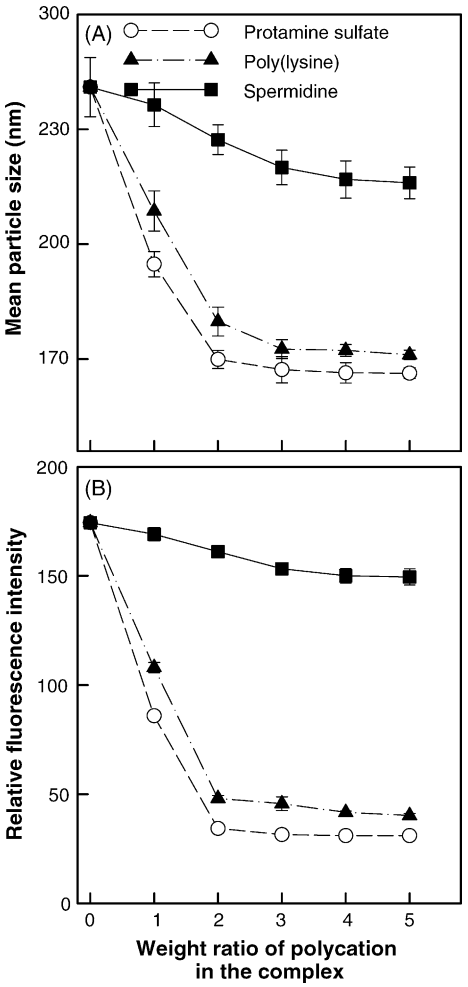

Fig. 2. Changes in (A) average particle size and (B) fluorescence intensity of the LE4/P/DNA complexes as a function of polycation amount. The ternary complexes were prepared as described in Fig. 1 except that the final concentration of DNA used in this experiment was 5 μg in 1 ml of PBS. The decrease in fluorescence by condensation of DNA was measured in the presence of 1 μM EtBr. The mean value in the graphs represents the average of three measurements.

spermine could not significantly decrease the particle size of the complexes in our condition. The degree of DNA condensation was determined more directly by determining the decrease in EtBr fluorescence intensity of DNA in the complexes having various L/P/D ratios (Fig. 2B). PS showed also the sharpest decrease in fluorescence intensity among the three cations. In contrast, spermine was ineffective. It is interesting to note the correlation between particle size and fluorescence

decrease of the ternary complexes containing different polycations.

3.2. Enhancement of emulsion-meditated transfection by polycations

We next checked the expression level of a transgene to evaluate in vitro transfection activity of the ternary complexes. The complexes were prepared at the same L/P/D ratio in Fig. 1 except for DNA concentration that was 0.5 μg in 40 μl of media per well. The complexes were applied to COS-1 cells. Transfection activities were measured by the level of expression of the exogenous gene encoding the β-galactosidase of *E. coli* (Fig. 3). By adding the polycations into the LE/D complexes, the galactosidase activity was significantly enhanced. PS and PLL yielded their maximal transfection activities at L/P/D = 4/2/1. In the case of spermine, however, only slight enhancement was observed. Among the three cations, PS was the most effective. The peak activity of LE4/PS/DNA complex in transfecting cells was ca. 10-fold higher than that of LE4/DNA (L/D = 4/1). Thus, PS was selected for further in vitro and in vivo emulsion-mediated transfection.

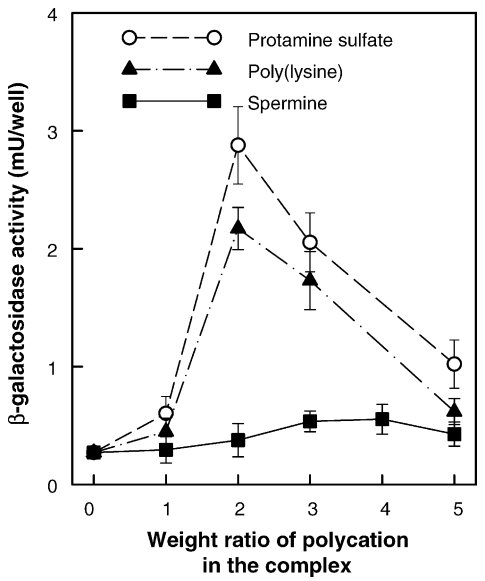

Fig. 3. Changes in in vitro transfection activity of the emulsion/polycation/DNA as a function of polycation amount in COS-1 cells. The ternary complexes were prepared as described in Fig. 1 except that the used DNA amount was 0.5 μg in 40 μl of DMEM per well. The transfected cells were assayed for β-galactosidase activity using a photometric assay.

3.3. Protamine sulfate potentiates in vitro transfection activity of emulsions as well as liposomes

The effect of PS on in vitro transfection was examined further. In addition to LE4, LE3 and LP4 were also used for in vitro transfection. LE3, LP4 and LE4 were complexed with DNA at L/D_{opt} values of 4, 6 and 8, respectively, as previously reported (Kim et al., 2001a). For LE3 and LP4, $L/P/D_{opt}$ ratios were determined as described in Fig. 3, and were 2/2/1 and 3/2/1, respectively. The P/D_{opt} of PS was also determined and was 4. The prepared complexes were applied to cells with or without 80% of serum.

The transfection activity of the PS/DNA complexes at P/D_{opt} was very low when lipid carriers were not added (Fig. 4A). However, PS enhanced the transfection activity when mixed with the LP or the emulsions. The transfection activity of all of the ternary complexes was ca. 2–2.5-fold higher than that of corresponding binary complexes achieved without PS. However, the addition of PS could not provide LP4 a serum resistance as shown in Fig. 4. The transfection activity of LP4/PS/DNA complexes was completely compromised in the presence of 80% serum. In contrast, the ternary complexes comprising the emulsions retained ca. 60–80% of their transfection activities, which was achieved in serum free condition. This result agrees well with our previous papers (Kim et al., 2000, 2001a, 2002, 2003). The cytotoxicity of the complexes was also evaluated using MTT (Fig. 4B). The PS/DNA complex did not exhibit any noticeable toxicity. In contrast, the lipid formulations were more toxic than PS. In every case, the ternary complexes displayed a low cytotoxicity as compared with the binary lipid carrier/DNA complexes.

We further performed in vitro transfection with four different cell lines using LE4/DNA or LE4/PS/DNA complex to investigate whether the addition of PS in the LE/DNA complex could increase the transfection activity in cells of different origins in the absence of serum (Fig. 5). Although the magnitude of the effect varied among the four cell lines, it was evident that the addition of PS significantly increased the transfection activity of LE4 by 1.5–3.1-fold.

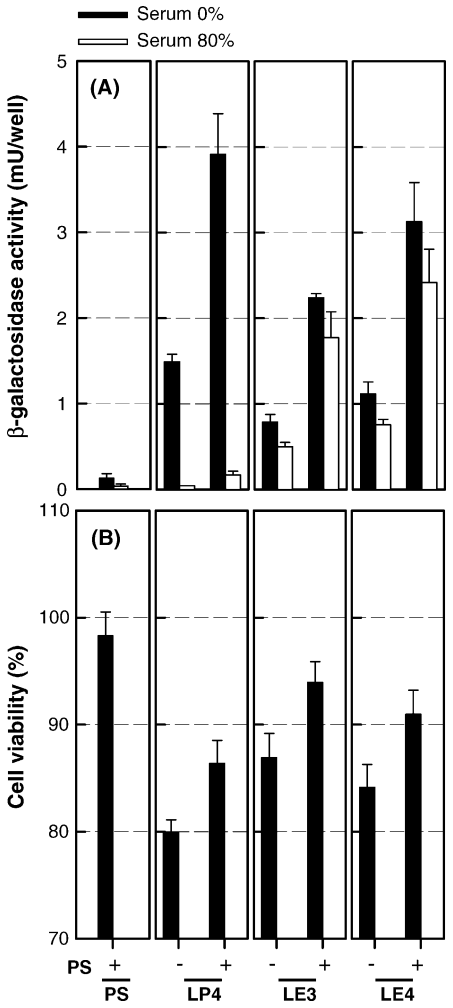

Fig. 4. (A) In vitro transfection activity and (B) cytotoxicity of the DNA complexed protamine sulfate and liposome or emulsions in COS-1 cells. The liposome (LP) or emulsion (LE) formulations contained either DOTAP/DOPE (20/4 mg/ml) (LE3) or DOTAP/DOPE/Tween 80 (20/4/4.5 mg/ml) (LE4 and LP4). For maximum gene expressions, LP4, LE3 and LE4 were complexed with DNA at different L/D ratios, which were 4, 4 and 8, respectively. In the case of the ternary complexes, the L/D ratio of LP4, LE3 and LE4 added to the PS/DNA (2/1, w/w) complex were 2, 2 and 4, respectively. The transfected cells were assayed for β-galactosidase activity using a photometric assay.

3.4. In vivo gene delivery via intravenous or intranasal administration

To evaluate the capacity of polycations in enhancing emulsion-mediated in vivo transfection activities,

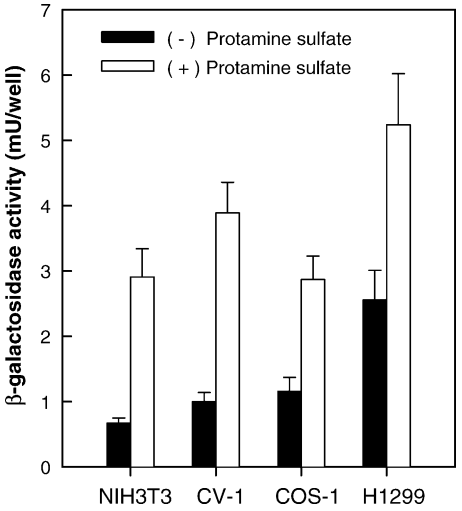

Fig. 5. Enhancement in in vitro transfection activity of an emulsion-mediated transfection by protamine sulfate in COS-1, CV-1, NIH3T3 and H1299. The LE3/PS/DNA complex was prepared at the weight ratio of 1:4:2, and then was applied to the cells. The transfected cells were assayed for β-galactosidase activity using a photometric assay.

10 μg of pCMV-Luc+ and LE4 were complexed with or without PS at their L/D_{opt} or $L/P/D_{opt}$, respectively, determined as described in Fig. 3, and administered intravenously or intranasally into Balb/c mice. The counterpart liposome, LP4, was used for comparison. Twenty-four hours after administration of the complexes, luciferase activity in different organs was analyzed. We determined the levels of gene expression in various tissues from lung, heart, spleen, liver and kidney, for intravenous injection, and nose and lung, for intranasal inhalation, respectively.

In the case of intravenous injection, the maximum expression level was observed in lung lysates for LP4 and spleen for LE4, respectively (Fig. 6A and B). LP4/PS/DNA complexes showed at least 2.5–3 times higher luciferase activities than the corresponding LP4/DNA complexes in lung. In contrast to LP4/PS/DNA complexes, LE4/PS/DNA complexes showed a maximum transfection activity in spleen, consistent with previous results (Kim et al., 2003). The transfection activity of LE4/PS/DNA was ca. 3.7 times higher than that of LE4/DNA complex in spleen.

In the case of intranasal inhalation, the maximal luciferase activity was found in lung extracts between the organs in all complexes (Fig. 6C and D). Compared with LP4, the emulsion system showed a robust

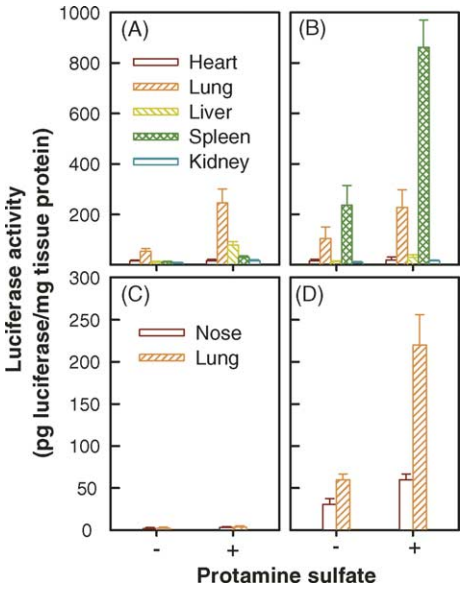

Fig. 6. Enhancement in in vivo transfection activity of LP4 (A and C) and LE4 (B and D) by protamine sulfate after intravenous (A and B) and intranasal (C and D) administration. Each complex contains 10 μg pCMV-Luc+ with different emulsion formulation in 100 μl of serum free PBS. The complex was prepared at the ratio in Fig. 5. Luciferase values were expressed as picogram per milligram of total protein extract. Background level of luciferase in each organ was measured from the organs of mice without any treatment and was negligible ($n = 3$).

luciferase expression in extracts of both nasal cavity and lung (Fig. 6D). The highest transfection activity of LE4 was obtained in lung. The addition of PS led to further increase of emulsion-mediated transfection activity in lung significantly (ca. 1.8 times; $p < 0.03$). The transfection activity, however, was negligible in both tissue lysates in the LP complex regardless of the addition of PS (<4 pg luciferase/mg tissue protein). Notably, the transfection activity of LE4/PS/DNA was at least 50 times higher than that of LP4/PS/DNA in lung.

4. Discussion

In this paper, we developed a strategy to prepare a small-sized and highly active LE/polycation/DNA complex in transfecting cells as an effort to improve the transfection activity of the emulsion formulations. Among natural polycations we tested, the addition of PS has a dramatic effect on the compaction and the transfection activity of LE/DNA complexes. These ternary complexes have more potent in vitro and in vivo transfection activity and, importantly, lower cytotoxicity than the corresponding binary complexes not having a polycation.

In the case of PS and PLL, we confirmed the formation of ternary complexes in the agarose gel retardation study. Unfortunately, however, we failed to form a spermine complex in PBS even at P/D = 20 (data not shown). Also, the pretreatment of DNA with spermine could neither induce a significant condensation of the DNA/LE complex nor reduce its particle size (Fig. 2). Although spermine is known to condense DNA, it was reported that the DNA/spermine complex is not stable at physiological ionic strength (Ibanez et al., 1996). These observations suggest that low molecular weight of polycations such as spermine doses not seem to significantly contribute to condense negatively charged DNA. This inherent low binding capacity of spermine may be responsible for the weak enhancement in an emulsion-mediated transfection (Fig. 3). In contrast, the high molecular weight polycations such as PS and PLL could form a high-condensed ternary complex and enhance the transfection activity of the LEs as well as the LP (Figs. 2 and 3). Interestingly, these data suggest that there may be a plausible relationship between structural stability and transfection activity of ternary complexes induced by pretreatment using polycations with a different molecular weight.

Between PS and PLL, PS was slightly more effective. It has been reported that PS as a condensation agent was found to be superior to PLL as well as to various other types of protamines (Gao and Huang, 1996; Li and Huang, 1997). This is likely the reason that the ternary particles precondensed by PS are smallest. On the basis of endocytosis model, these condensed particles may have a higher uptake tendency into the cells via an endocytosis pathway (Gao and Huang, 1996). There is another advantage of PS in the ternary complex. A report shows that PLL dose not have NLS motifs on its sequence, which direct the complex to the nucleus. In contrast, there exist four apparent NLS domains within PS (Gao and Huang, 1996). Therefore, the DNA in the cytosol has a better chance to enter nucleus when combined with PS. The explanation may account for the superior effect in enhancement of in vitro emulsion-mediated transfection by PS to by PLL (Figs. 5 and 6).

The pretreatment of a condensing agent could also provide a benefit in maintaining the structural integrity of emulsion during a formation of the LE/DNA complexes. In our previous report, we reported that the stability of emulsion is relevant to in vitro and in vivo transfection activity (Kim et al., 2000, 2001a,b, 2003). In one of our previous reports, we have investigated the relationship between structural stability and in vivo transfection activity of LE/DNA complexes with stability modulated emulsion by changing core oil (Kim et al., 2003). In that study, most stable emulsion-having squalene as core oil had most potent transfection activity after intravenous administration. Thus, transfection activity of emulsion was primarily associated with the stability. Plasmid DNAs are huge macromolecules. For instance, 5 kilobase (kb) of plasmid DNA is ca. 3300 kDa. These highly negative charged macromolecules could act as an emulsion destabilizer in the process of LE/DNA complexes formation. It has been noticed that DNA induces a collapse of LP structure, and then rearrange the lipid components in the LP into a lamellar or a hexagonal phase (Sternberg et al., 1994; Radler et al., 1997) during LP/DNA complex formation. This structural breaking effect of DNA, however, could be reduced in the process of ternary complex by charge reducing and condensing DNA with a polycation. Thus, pretreatment of polycation may be helpful in keep emulsion from loss of its transfection activity due to structural instability caused by passenger DNA.

The transfection activity of the LE/PS/DNA ternary complexes was not rapidly decreased in high serum condition, as shown in Fig. 4A. In contrast, that of LP/PS/DNA was. PS/DNA complexes also failed to display the detectable transgene expression in the presence of 80% serum. These data suggest that the insensitivity of LE/PS/DNA complexes to serum may come from emulsion component in the ternary complex. As mentioned in Section 1, our cationic emulsion system successfully delivered a reporter gene into cells in vitro in the presence of up to 90% serum (Yi et al., 2000; Kim et al., 2001a). Unlike liposomal carriers, the cationic emulsion that we developed retained its physical integrity at high concentrations of serum when complexed with DNA. In vitro DNA release tests showed that the physical integrity of our emulsion/DNA complex is strong, and is kept even in the presence of high concentration of model anionic polymeric destabilizers, such as poly-L-aspartic acid and heparin. In distinct contrast, for the counterpart liposome/DNA complex, the plasmid DNA was easily dissociated by adding small amount of them (Kim et al., 2000). In the presence of 0.5% serum in PBS, wherein various proteins and other small molecules reside, the size of liposome/DNA complexes increased dramatically. However, the size of emulsion/DNA complexes increased slightly (Kim et al., 2003). In the case of the ternary complexes, this pattern in size was maintained. The size of LE4/PS/DNA at $L/P/D_{opt}$ increased from 165 to 220 nm after adding it into 0.5% serum in PBS. In comparison, the size of LP4/PS/DNA increased from 322 to 1517 nm. This size difference could be one of plausible explanation for different levels of transgene expression in various tissues after systemic administration of the ternary complexes in Fig. 6A and B (Kim et al., 2003). Thus, the ternary complex containing emulsions is extremely stable and, consequently, could be protected form serum-induced inactivation of in vitro and in vivo transfection activity. Moreover, recently, we found that our emulsion/DNA complex was also extremely stable in the presence of a natural lung surfactant (unpublished data). From these observations, we strongly believe that the highly stable LE/PS/DNA complex could provide a necessary protection against inactivation by in vivo destabilizers.

It has not been reported clearly that the stability of complexes are relevant to transfection activity under in vivo condition. Sternberg et al. have investigated the morphology and transfection activity both in vitro and in vivo. In that report, they could obtain stable complexes by substituting cholesterol for DOPE as a help lipid, by adding $PEG_{2000}PE$ as steric stabilizer after the complex formation, and by pre-condensing DNA with spermidine. These stability increased complexes showed a high expression of transgene in the lung after intravenous administration. We also have investigated the relationship between structural stability and in vivo transfection activity of complexes with stability modulated emulsion by changing oil component in the emulsion (Kim et al., 2003). In that study, most stable emulsion-having squalene as the oil phase most potent transfection activity after intravenous administration. From above observations, we conclude that in vivo transfection activity may be primarily associated with the stability of the complexes.

Despite the fact that many cationic lipid formulations showed good gene transfer activity, their application has been still limited in vitro and, especially, in vivo systems due to its intrinsic toxicity mainly caused by cationic lipids (Filion and Phillips, 1997; Tousignant et al., 2000). We also observed similar phenomena, in the cytotoxicity test of the complexes at P/D_{opt}, L/D_{opt} or $L/P/D_{opt}$, the PS/DNA complexes did not show any severe toxicity. In contrast, the lipid carrier/DNA complexes were more toxic than the PS/DNA complexes as shown in Fig. 4B. Since the addition of polycations reduces the number of the lipid carriers to form transfectively active DNA/carrier complexes, the amount of the cationic lipid carrier in the ternary complex system decreases. Especially, at $L/P/D_{opt}$ we have optimized in this study, the amount of the lipid carriers is just half of the lipid carrier/DNA complex at L/D_{opt}. Consequently, the cytotoxicity caused by the lipid carriers was significantly reduced by forming the ternary complexes. Thus, the ternary complex has not only a higher transfection activity but also a lower cytotoxicity than the binary one.

In summary, we have selected PS from polycations as an enhancing agent of transfection and demonstrated that the pretreatment of PS could yield a highly compacted, active, and safe ternary complex in transfecting cells in vitro cell culture system. In the form of the ternary complex, the emulsion system surpassed the LP in gene expression level of transfected tissues when administered intravenously or intranasally using mouse in vivo system. Thus, this ternary complex has a plausible possibility as a new class of non-viral gene delivery system that might be useful for in vivo application such as gene therapy and genetic immunization.

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