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The effects of serum on the stability and the transfection activity of the cationic lipid emulsion with various oils

Young Jin Kim [a], Tae Woo Kim [a,b], Hesson Chung [a], Ick Chan Kwon [a], Ha Chin Sung [b], Seo Young Jeong [a,*]

[a] *Biomedical Research Center, Korea Institute of Science and Technology, 39-1, Hawolkok-dong, Sungbuk-ku, Seoul 136-791, South Korea*
[b] *Graduate School of Biotechnology, Korea University, 1, 5-ka, Anam-dong, Sungbuk-ku, Seoul 136-701, South Korea*

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Abstract

The emulsions with various oils such as linseed oil, soybean oil and squalene were prepared to obtain the relationship between the stability and the transfection activity of the emulsions. 1,2-Dioleoyl-*sn*-glycero-3-trimethylammonium-propane (DOTAP) was used as a single cationic lipid emulsifier. The droplet sizes and size distributions of DOTAP emulsions were dependent on oils which had different interfacial tensions. The droplet sizes followed the order of squalene emulsion < soybean oil emulsion < linseed oil emulsion. The squalene emulsion was the most stable carrier since it kept its integrity in serum and PBS solution. For in vitro gene transfer, the transfection activities of the lipid carriers in the presence of serum followed the order of squalene emulsion > soybean oil emulsion > linseed oil emulsion > DOTAP liposome. The squalene emulsion showed the least cytotoxicity with or without serum. For in vivo gene transfer, the squalene emulsion also had the most potent transfection activity in the mouse after intravenous administration. Squalene as the oil component can enhance the stability of cationic emulsion more effectively that could be useful for the transfer of genes in vitro and in vivo.

Keywords: Emulsion; Gene transfer; DOTAP; Squalene; Serum

1. Introduction

Lipid system has been widely used in pharmaceutical and medical fields as drug carriers. Cationic lipids have shown to be potent candidates for liposome-mediated gene transfer (Gao and Huang, 1991; Felgner et al., 1994). The cationic lipid emulsion was developed to deliver plasmid DNA efficiently in vitro and in vivo (Liu et al., 1996a,b; Yi et al., 2000; Kim et al., 2000). Many cationic lipids were investigated to make the emulsion system with high transfection activity (Kim et al., 2001a,b; Chung et al., 2001).

In our previous study, we have shown that the cationic o/w emulsion system and its complex with DNA could maintain the physical integrity and could successfully facilitate the transfer of genes in the presence of up to 90% (v/v) serum in COS-1 and CV-1 cells (Yi et al., 2000). The cationic emulsion was formulated with soybean oil and 1,2-dioleoyl-*sn*-glycero-3-trimethylammonium-propane (DOTAP), 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE),

* Corresponding author. Tel.: +82-2-958-5902; fax: +82-2-958-5909.
E-mail address: syjeong@kist.re.kr (S.Y. Jeong).

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and 1-palmitoyl-2oleoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[poly(ethylene glycol)$_{2000}$] ($PEG_{2000}PE$) as emulsifiers. This physically stable emulsion was more efficient than the commercialized liposomes, such as Lipofectamine® and Lipofectin®, for in vivo gene transfer to endothelial cells of the mouse nasal cavity (Kim et al., 2000).

We have also demonstrated that the transfection activity depends greatly on the compositions of cationic emulsifiers and co-emulsifiers. We adopted the principles that were used to develop liposomal transfection agents since liposomes and emulsions were similar in many respects. While lipids form bilayer leaflets that separate inner and outer aqueous phases in liposomes, they are used as emulsifiers and distribute mainly at the interface between the inner oil and the outer aqueous phases in o/w emulsions. Various emulsion formulations with different cationic lipids as emulsifiers, and additional helper lipids as co-emulsifiers, were prepared as gene carriers. We tried to produce a stable emulsion that has small particles using natural oils. In an oil-in-water (o/w) emulsion, the oil constitutes the dispersed phase (Kim et al., 2001a,b). Oil is an essential part in constituting the lipid emulsion. It is evident by a number of studies that the physicochemical properties, such as the stability and the viscosity, of the emulsions are determined by changing the core oils (Chung et al., 2001). Our earlier studies revealed that oils showing higher o/w interfacial tension formed more small and stable emulsion. The emulsions made with different oils could influence the in vitro release properties of lipophilic drugs and the transfection activity (Chung et al., 2001).

In this report, we further investigated the stability of the emulsion with various oils. Linseed oil, soybean oil and squalene were selected as the core oils because they formed the emulsions with different degree of stability (Chung et al., 2001). To avoid the complications in interpreting the results, only DOTAP was used as a single cationic lipid emulsifier. The DOTAP liposome was also prepared for the comparison with emulsion. We have investigated the physicochemical parameters of lipid carriers and their complexes with DNA, the in vitro transfection activity, and cytotoxicity in various serum concentrations. The level of gene expression was also investigated after the intravenous (i.v.) administration to the tail vein of mouse.

2. Materials and methods

2.1. Materials

Soybean oil, linseed oil, squalene, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) and poly-L-aspartic acid (PLAA, molecular weight 1.1×10^4) were purchased from Sigma (St. Louis, MO). 1,2-dioleoyl-*sn*-glycero-3-trimethylammonium-propane (DOTAP) was purchased from Avanti Polar Lipids (Alabaster, AL) and used without further purification. DMEM (Cat. No. 31600-034) and fetal bovine serum (FBS) were purchased from Gibco BRL/Life Technologies (New York, NY). β-Galactosidase standard and chlorophenol red galactopyranoside (CPRG) were obtained from Boehringer Mannheim GmbH (Germany). Deionized-distilled water (DDW) was produced by using a Milli-Q Plus water purification system (Millipore Corporation, Bedford, MA). All other chemicals and reagents were of tissue culture grade.

The plasmid pCMV-beta encoding *Escherichia coli* (*E. coli*) *lac*Z (β-galactosidase) gene expression plasmid driven by the human cytomegalovirus immediate-early promoter was purchased from Clontech Laboratories (Palo Alto, CA, USA). The plasmid pCMV-luc consists of cytosolic form of *Phontinus pyralis* luciferase cDNA, which was obtained from the plasmid pGL3 (Promega) using *Xba*I and *Hin*dIII and subcloned into the plasmid pcDNA3.1 (Invitrogen) in this laboratory (Kim et al., 2000).

2.2. Preparation of lipid carriers

The emulsions contained 100 μl/ml of core oils (linseed oil, soybean oil or squalene) and 24 mg/ml of a cationic lipid emulsifier, DOTAP. The cationic lipid was weighed and mixed with water. The cationic lipid/water mixture was sonicated until clear in an ice/water bath by using a probe type sonicator (Sigma, High intensity ultrasonic processor, 600 W model). The cationic lipid solution was added to oil and sonicated further in an ice/water bath for ca. 4 min to form emulsions. To prepare liposome carrier, the cationic lipid solution was further sonicated for ca. 4 min after the solution became clear. The prepared lipid carriers were kept at 4 °C until further experiments.

2.3. Size and surface potential measurement

The average droplet size, the surface potential of the lipid carriers (liposome and emulsions), and the DNA/carrier complexes were measured by using photon correlation spectroscopy (Malvern Zetasizer, Malvern Instruments Ltd., UK). The emulsion and liposomes were diluted 300 and 3 times, respectively, for the measurement. To quantify the magnitude of size changes induced by serum and phosphate buffered saline (PBS; 10 mM sodium phosphate buffer containing 150 mM NaCl, pH 7.4), the lipid carriers were diluted with PBS or PBS containing 0.5% of FBS before the size measurement. The mean value reported represents the average of three measurements on a single sample. The size distribution follows a log-normal distribution (Chung et al., 2001). The potential of the lipid carriers at the surface of spheres, called the zeta potential (ζ), which is derived from mobility of particles in electric field by applying the Smoluchowsky relationship, is measured at least three times and averaged at appropriate concentrations of lipid carriers.

2.4. In vitro β-galactosidase gene expression assay

COS-1 cell is a derivative of the simian kidney cell line CV1 and was cultured in DMEM supplement with 10% FBS at 37 °C in a humidified 5% carbon dioxide incubator. Cells were seed at 1×10^4 cells per well on to 96-well plates 12 h before transfection. The complexes were formed at weight ratio of 1:4 (DNA:DOTAP of carriers) in 40 μl of DMEM per well. DNA amount was 500 ng of pCMV-beta. After washing the COS-1 cells with serum free DMEM, 160 μl serum free DMEM was added to each well. Complex solutions (40 μl) were added in them. To test the effect of serum on the transfection efficiency, 160 μl of FBS was added in the place of serum free media. Forty microliters of the lipid/DNA complex solutions were prepared and added to the cells. 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 DMEM containing 10% (v/v) FBS, cultured for 24 h after transfection. The transfected cells were assayed for β-galactosidase activity using a photometric assay. Cells were disrupted by adding 50 μl of lysis buffer (0.1% Triton X-100, 250 mM Tris, pH 8.0) per well. The plates were followed by a cycle of freeze and thaw and then, 50 μl of PBS containing 0.5% bovine serum albumin (BSA) was added to each well. The substrate solution (150 μl), which contained 1 mg/ml of CPRG in β-galactosidase buffer (60 mM sodium dibasic phosphate buffer, 1 mM magnesium sulfate, 10 mM KCl, 50 mM β-mercaptoethanol, pH 8.0) was added to each well. After the color developed (ca. 1–12 h), the plates were read at 580 nm in a micro plate reader (SOFTmax® PRO, Molecular Devices corporation, CA). To quantify the activity of β-galactosidase, the standard curve was prepared by two-fold serial dilutions of β-galactosidase standard.

2.5. Cell viability assay

The cell viability was tested using MTT. After incubation of DNA/lipid complex solutions for 24 h, 200 μl of DMEM containing 10% FBS and 50 μl of 0.5% (w/v) MTT in PBS solution were added to each well and then incubated for 4 h to allow producing formazan crystal. The formazan crystal was dissolved by adding 200 μl of dimethylsulfoxide (DMSO) and further by adding 25 μl of Sorensen's glycine buffer. The quantity of formazan products was measured at 570 nm. The 100% value is obtained from the o.d. value measured in non-transfected cells.

2.6. In vivo luciferase gene expression assay

To prepare DNA–carrier complexes, DNA solution containing 10 μg of pCMV-luc and lipid solution containing 40 mg of DOTAP in formulation each diluted with 100 μl of PBS were mixed by inversion. The complex solution was allowed to incubate at room temperature for 20 min and was administrated into female Balb/c weighing approximately 20–25 g (6–8 week ages) by intravenous route. Twenty-two hours later, the mice were sacrificed, and each organ were removed and homogenized in lysis buffer (0.1 M Tris–HCl, 2 mM EDTA, and 0.1% Triton X-100 pH 7.8), which added at a volume-to-weight ratio of 5 μl/mg of the collected organs. After two freeze/thaw cycles, the homogenized organ extracts centrifuged at 4 °C for 10 min at 12,000 rpm. One portion of the supernatants was assayed for protein concentration by using a Bio-Rad protein assay kit (Bio-Rad

Laboratories, Hercules, CA) based on the Bradford method. Luciferase activity was assayed by using a kit purchased from Promega (Madison, WI, USA) with a Turner Designs Luminometer Model TD-20/20 (Promega). Peak light emission was measured for 20 s at room temperature. The luciferase contents of samples were calculated from relative light units using a standard curve of purified firefly luciferase (Sigma). 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.

3. Results

3.1. Selection of cationic lipid

As a non-viral gene carrier, cationic lipid emulsions must be stable and have high positive surface charge to bind with negatively charged DNA (Zabner, 1997). It is well known that emulsion stability depends strongly on emulsifier concentration (Ishii et al., 1989). It is also known that the stability is related strongly to the average particle size of the emulsion: the emulsion with smaller particles is more stable (Yi et al., 2000). To find the optimum DOTAP concentration, emulsions containing squalene 10% (v/v) and various concentrations of DOTAP (1–30 mg/ml) were prepared. The zeta potential and the mean particle size of emulsions having various concentrations of DOTAP were measured (Fig. 1). The zeta potential increased from ca. +30 to +75 mV as DOTAP concentration increased from 1 to 24 mg/ml and also leveled off above 24 mg/ml. The mean particle size decreased from ca. 260 to 130 nm as DOTAP concentration increased from 1 to 24 mg/ml and leveled off above 24 mg/ml. The plateau regions appeared at the saturation stage of the adsorption of DOTAP on the squalene droplets (Ishii et al., 1989). At low concentration of DOTAP (0–9 mg/ml), the mean particle sizes of droplets of the emulsions were relatively larger, and zeta potentials were relatively lower than those with higher concentrations of DOTAP. These emulsions with low DOTAP concentrations were very unstable and phase-separated within a few days in phosphate buffered saline solution (PBS). Meanwhile, at 24 mg/ml, the DOTAP emulsion was stable and maintained its structural integrity for 20 days (data not shown).

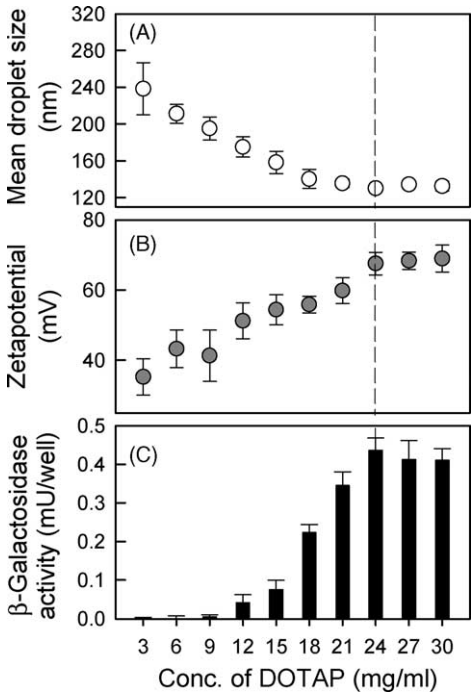

Fig. 1. Changes of (A) mean droplet size, (B) zeta potential, and (C) in vitro transfection activity of cationic lipid emulsion as a function of DOTAP concentration ($n = 3$).

In vitro transfection was performed with the emulsions having different DOTAP concentrations (Fig. 1C). The incubation time was 20 min to form complexes between emulsions and plasmid DNA before adding the complexes to COS-1 cell. The ratio between DOTAP in the emulsion and DNA was set to 4 (w/w, DOTAP/DNA) in the complex. The transfection activity of DOTAP emulsion was hard to detect at low concentrations of DOTAP (0–9 mg/ml). It was apparent that the transfection activity was increased as DOTAP concentration in the emulsion increased above 12 mg/ml. It reached a maximum value at 24 mg/ml, indicating that the optimal DOTAP concentration of DOTAP is 24 mg/ml (35 mM). At this optimum concentration, the DOTAP emulsion had the smallest size, the highest positive surface charge and the highest transfection efficiency. It means that the emulsion stability and the high surface positive charge are the important factors for the emulsion-mediated transfection.

3.2. Salt effect on stability of cationic emulsion

In our previous work, the particle size of the cationic emulsions made with different oils and their complex with DNA was investigated. In this study, the particle sizes of the emulsions themselves were determined in various conditions. The average droplet size of the emulsions containing linseed oil, soybean oil, and squalene were ca. 220.4, 204.1, and 157.4 nm, respectively (Fig. 2). The polydispersity of the emulsions containing linseed oil, soybean oil, and squalene was ca. 0.12, 0.28, and 0.13, respectively. The size of DOTAP liposome was ca. 108 nm. The long-term stabilities of the emulsions and the liposome were investigated by measuring the size changes of carriers with various time intervals. The size of the liposome was increased three to four times within 48 h, on the other hand, the sizes of emulsions with soybean oil or squalene did not show any statically significant changes for 1 year. The size of the linseed oil emulsion was increased slightly.

In our previous work, we measured the size changes of the emulsions in the presence of serum. In the presence of serum, wherein various proteins and other small molecules reside, the droplet size of all three emulsions increased slightly. However, the size of liposome became twice its size in water. The stabilities of the emulsions having different oils were further investigated in the presence of 0.5% serum in PBS. It is more harsh condition for cationic lipid carriers because the excess of negatively charged counter ions were present in PBS. The droplet sizes of the emulsions containing soybean or linseed oils were dramatically increased ca. 9 and 10 times, respectively (Fig. 2). At this condition, emulsions with linseed oils even formed large aggregates. On the other hand, the emulsions with squalene as core oil did not show any significant changes in droplet sizes as compared to the others. In our previous works, we studied the effect of oil on the emulsion particle size and stability. The o/w interfacial tension of the oils was one of the key physical properties to control the emulsion particle size. The interfacial tension of linseed oil, soybean oil, and squalene was 1.8, 14.0, 33.9 dyn/cm, respectively. In that experiment, when the o/w interfacial tension was the highest, as in squalene, the size of the emulsion was the smallest. Size stability of the emulsion depended on the initial particle size. Squalene is a colorless unsaturated aliphatic hydrocarbon, $C_{30}H_{50}$, found especially in human sebum and in the liver of sharks. It is single component oil. Soybean oil is the world's most widely used edible oil with 85% unsaturated fat profile. It mainly contains oleic, linoleic, linosinic acid. Linseed oil is extracted from the seeds of the flax plant. It contains glycerides of linolenic, linoleic, oleic, stearic, palmitic and myristic acids. Squalene is single component oil while linseed and soybean oil has many ingredients. Soybean or linseed oil contains many components that partition into the water or at the interface when water was added. These components are emulsifiers that may lower the interfacial tension of linseed oil in particular. Therefore, interfacial tension value alone may not explain the emulsion stability. Size stability of the emulsion depended on the initial particle size. Emulsions with smaller particles stayed stable for a long time. From the above results, squalene with higher o/w interfacial tension formed more stable emulsion with a small average particle size, which was resistant to various proteins and small molecules in serum and PBS.

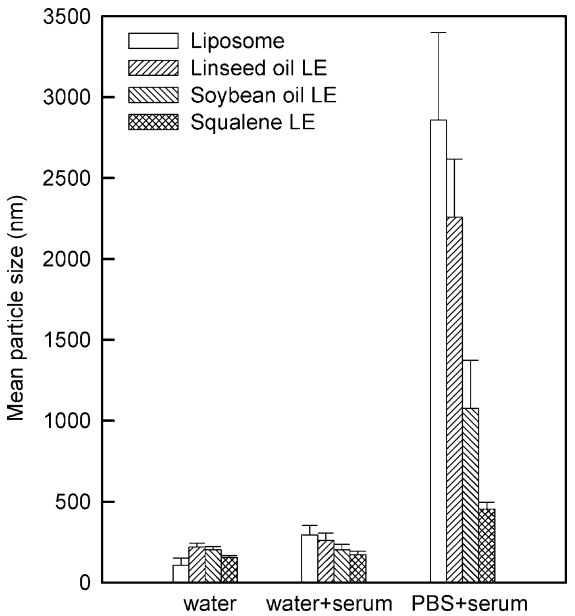

Fig. 2. Size changes of DOTAP liposome and DOTAP emulsions having different oils as a core in the presence of water, PBS, and serum ($n = 3$).

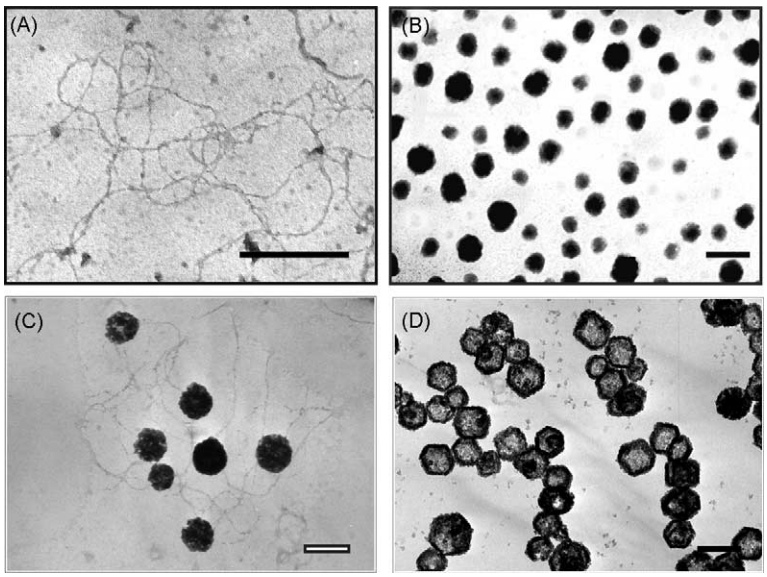

Fig. 3. Transmission electron micrographs of pCMV-beta and their complexes with trilaurin-cored emulsion at different weight ratio. (A) pCMV-beta, (B) trilaurin-cored emulsion, (C) DNA/emulsion (1/1), (D) DNA/emulsion (1/4). Bar in figure = 100 nm.

3.3. Observation of emulsion/DNA complex using TEM

The morphology of the emulsion/DNA complex was investigated using TEM. To form the emulsion/DNA complex, 0.3 μg of pCMV-beta was mixed with the emulsion at DOTAP/DNA C/D = 1 or 4 in 100 μl of TEM buffer. Trilaurin was selected as the oil phase since it is solid during the experiment due to a high melting temperature (ca. 46.5 °C). After negative staining with uranyl acetate, DNA, the emulsion and the emulsion/DNA complexes were observed using TEM. The trilaurin emulsion had a size range of 100–250 nm, which are of the same size with the measurement by photon correlation spectroscopy (Fig. 3B). At C/D = 1, a few emulsion particles was found on the string of DNA (Fig. 3C). At C/D = 4 where the emulsion/DNA complexes showed the maximal transgenic expression, the emulsion and DNA appeared to be fully combined and formed a chromatin-like structure (Fig. 3D). The structure was quite different from that of the liposome/DNA complex in which DNA was aligned between lamellar lipid sheets or formed a so-called hexagonal phase (Radler et al., 1997). Various proteins of serum possibly destabilized the structure of liposome/DNA complexes. The complexes of cationic lipid emulsion and DNA formed the stable complex, which showed the higher transfection activity than liposome complexes.

3.4. In vitro gene delivery

The in vitro transfection activities of the emulsions were evaluated in the cell culture system. The effect of serum on the in vitro transfection activities was investigated by comparing the expression level of β-galactosidase in the various concentrations of serum (Fig. 4). The complexes between DOTAP and DNA (500 ng of pCMV-beta per well) were formed with the weight ratios of 4 of DNA to DOTAP (in liposome and emulsion). In the absence of serum, the liposome showed higher transfection activity than the emulsion systems. The squalene emulsion showed the most potent transfection activity among the emulsions. Even, in the presence of 20% serum, the transfection activity of liposome/pCMV-beta complex dramatically decreased as ca. 17.5 times, from 0.7 to 0.04 mU/well. On the contrary to liposome, all of emulsions appeared to maintain significant transfection activities in the presence of serum. Among the emulsions, the squalene emulsion showed the higher transfection activity with increasing serum content and maintained

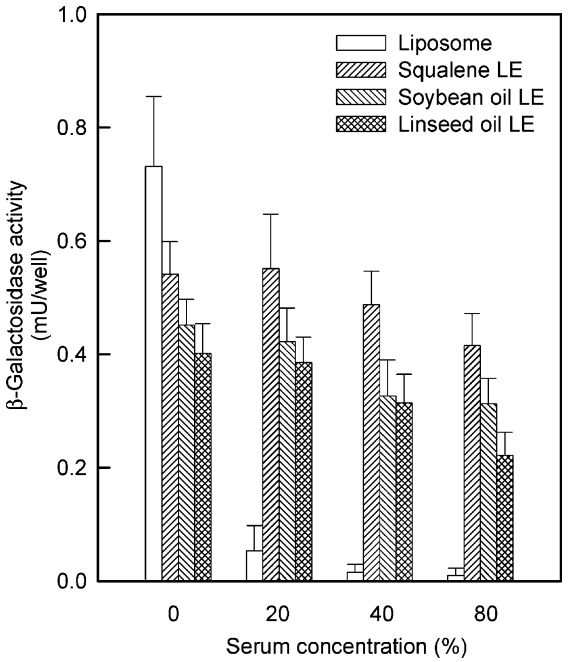

Fig. 4. In vitro transfection activity of DOTAP liposome and DOTAP emulsions having different oil as a function of serum content. The lipid carrier/DNA complexes containing 500 ng of pCMV-beta were formed at C/D = 4, and then applied to COS-1 cells ($n = 6$).

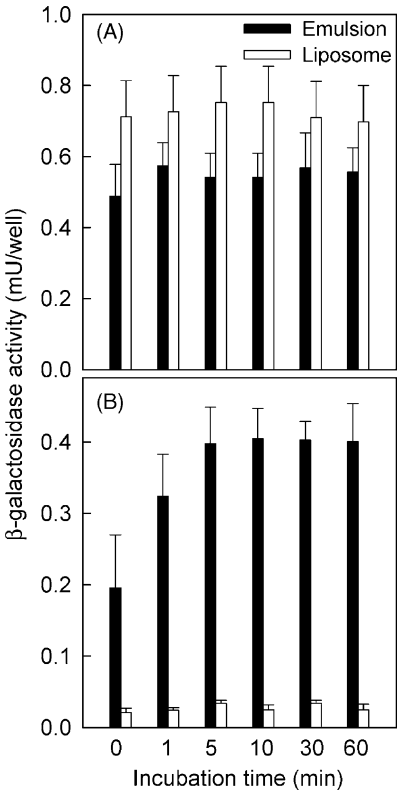

Fig. 5. In vitro transfection activity of DOTAP liposome and squalene emulsion as a function of complex formation time in (A) no serum and (B) serum condition. The lipid/DNA complexes containing 500 ng of pCMV-beta were formed at C/D = 4, and then applied to COS-1 cells ($n = 6$).

above ca. 70% of the transfection activity acquired in the absence of serum. As a result, the squalene emulsion had the highest in vitro transfection activity in the presence of 80% serum.

From cell proliferation assay using MTT, the cytotoxicity of carrier/DNA complexes was measured. Cell viabilities (%) of liposome, squalene emulsion, soybean oil emulsion, and linseed oil emulsion were 81.4 ± 4.8, 92.6 ± 8.9, 84.3 ± 6.4, and 80.1 ± 7.6, respectively. In this test, squalene emulsion/DNA complex were less toxic to the cell than the liposome and the other emulsions complexes. The squalene emulsion/DNA complex has the higher stability and the less toxicity than the liposome and the other emulsions complexes.

We investigated the complex incubation time-dependent transgenic expression in serum to confirm whether the serum resistance of emulsion resulted from the structural change in the complex with time. The lipid carriers were mixed with 500 ng of pCMV-beta per well at the same weight ratio, 4 and then incubated for different lengths of time at room temperature. At the end of incubation, complexes were added to cells in the presence of serum (final concentration 80%). In the case of the liposome, the freshly prepared complexes at zero time, which showed the high level of transfection activity in the absence of serum, lost their transfection activities in serum condition (Fig. 5). Though the serum effects seemed to decrease a little as the incubation time increased, the complex of liposome did not obtain the obvious resistance to serum inactivation. It showed the low expression level until 2 h of incubation time. However, in the case of emulsion, the freshly prepared complexes at zero time also lost their transfection activities in serum condition. Besides, its transfection activity was greatly inhibited by serum more than five times as compared to its transfection activity acquired in the absence of

serum. As the incubation time increased, complexes of the emulsions had become resistant to serum inactivation and all of the complexes showed the high transfection activities in the presence of serum after 10 min of incubation time. This result means that the complexes of emulsion and DNA undergo the maturation processes that give them the resistance to serum inactivation.

The transfection activity with the emulsions was not rapidly decreased in high serum condition. The physicochemical property was different among the lipid carrier/DNA complexes in the serum condition. There are many factors affecting the physicochemical properties of complexes. The interaction between the serum components and complexes, such as electrostatic or hydrophobic interaction, could increase the size of complexes and change the properties of complexes. The size of complexes was measured in the presence of serum in DMEM. DNA/lipid complexes were prepared by mixing the DNA solution and carrier solutions of each 1.5 ml in DMEM at a weight ratio of 1–4. After forming complexes, 15 ml of FBS was added to 3 ml of diluted complex solution, which was further incubated for 10 min. In the absence of serum, different size of complexes were formed in serum-free DMEM media. Generally, it appeared that the complexes of the emulsions were smaller than that of the liposome (Fig. 2). Among the emulsions, squalene emulsion formed the smallest complex with DNA. In the presence of serum, this pattern in size was maintained, although serum brought out the slight size change of complexes. The complex of liposome increased from 290 to 345 nm in size and was larger than those of the emulsions. Among the emulsions, squalene emulsion complex did not show any obvious size changes in the serum condition. This fact means that squalene emulsion forms the stable complex with DNA, which has the small size and the resistance to

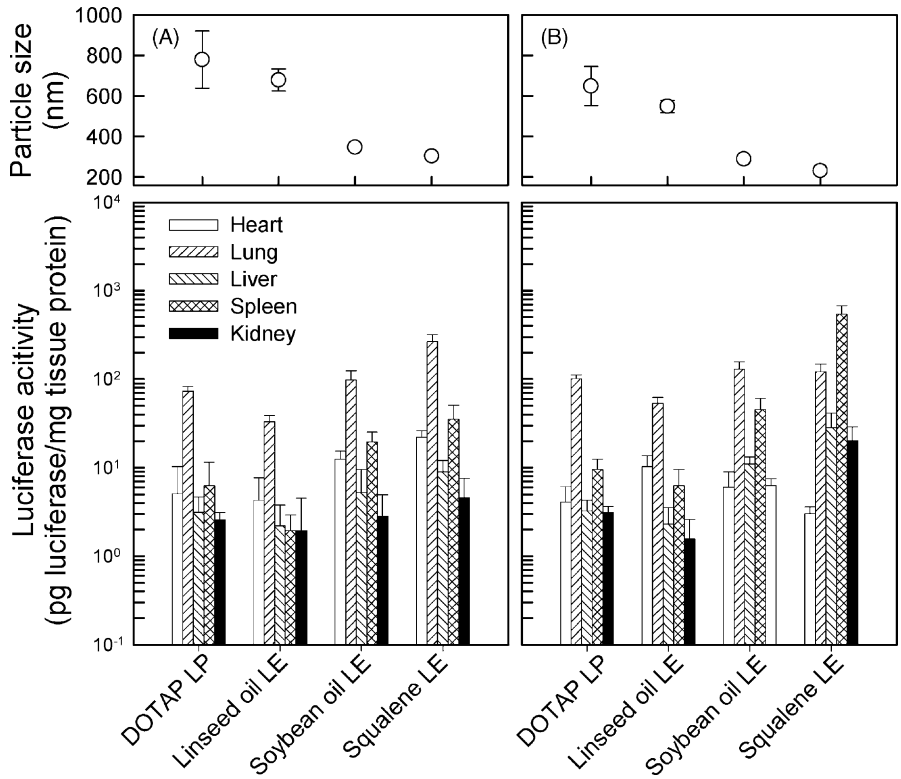

Fig. 6. In vivo gene expression of complexes between pCMV-luc and cationic lipid carriers in (A) PBS and (B) DMEM media. pCMV-luc (10 μg) were complexed with lipid carriers at C/D = 4 and administered intravenously into BALB/c mice via tail vein ($n = 5$).

the size change in serum. On the contrary, the liposome forms the unstable complex, which has the large size and is apt to interact with serum components.

3.5. In vivo gene delivery via intravenous administration

To evaluate the transfection efficiency of complexes in vivo according to lipid formulations, 10 mg of pCMV-luc containing firefly luciferase cDNA driven by cytomegalovirus (CMV) promoter were complexed with different lipid formulations and administered intravenously into Balb/c mice via tail vein. As shown in Fig. 6, we have found that detectable luciferase activity in all organs was examined including the lung, spleen, heart, liver and kidney. The maximal luciferase activity was found in lung extracts in all lipid carriers. The transfection efficiency of four DOTAP formulations followed the order of squalene emulsion > soybean emulsion > liposome > linseed oil emulsion. The linseed oil emulsion and liposome became unstable and tended to form insoluble large aggregates when they formed the complexes with 10 μg DNA in 200 μl PBS. The linseed oil emulsion and the liposome showed the low luciferase activities in tissue lysates as compared to the squalene emulsion and the soybean emulsion, which formed the stable complexes. Among the DOTAP carriers, squalene emulsion showed the most potent transfection activity in vivo. The particle sizes of lipid carrier/DNA complexes were changed with the complex medium that had different salts in it. All lipid complexes in DMEM were smaller than that in PBS from the experimental results. Especially, squalene emulsion formed the smallest complex in DMEM that was less than 200 nm. However, the salt effects of media were not clearly defined yet in the literature. It is necessary to study further in the future. PBS mainly consists of sodium and potassium phosphate but DMEM consists of various salts and amino acids. Divalent or trivalent anion in DMEM might change the stability of cationic emulsion (Liu et al., 1996a; Kim et al., 2001a,b). The organ distribution of lipid complex was mainly depended on its sizes. In case of squalene emulsion in DMEM, gene expression was more appeared in spleen than lung because the small and stable complex could escape the lung capillaries and circulate the whole body to reach to the spleen. However,

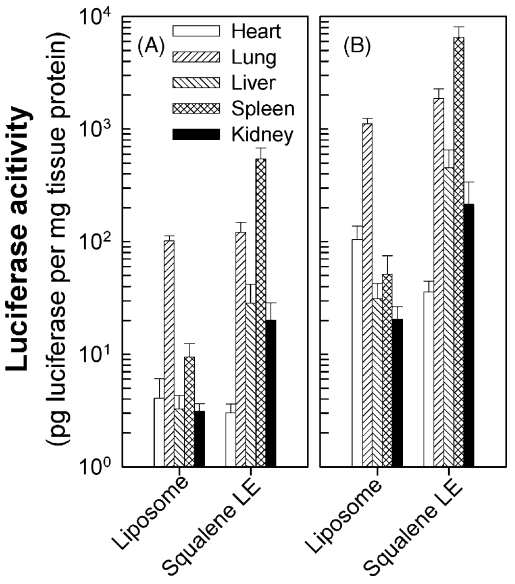

Fig. 7. In vivo gene expression of pCMV-luc which were complexed with liposome and squalene emulsion. (A) 10 μg and (B) 40 μg of pCMV-luc were complexed with lipid carriers at C/D = 4 and administered intravenously into BALB/c mice via tail vein ($n = 5$).

the squalene emulsion in PBS showed the maximum gene expression in lung since it was not stable complex so that it formed aggregates in the blood. Most of the complexes were entrapped in the pulmonary capillaries of lung. The transfection activities of liposome and squalene emulsion were increased as the DNA dose increased (Fig. 7). The increase of transfection activity was bigger in squalene emulsion. The maximal luciferase activity was also found in spleen extracts since the size of the complex was smaller than 200 nm and could circulate the whole body.

4. Discussion

Fig. 1 showed the mean droplet sizes of the emulsions determined by the concentration of DOTAP. In our previous report, the droplet sizes of the emulsions followed the order of squalene emulsion < soybean oil emulsion < linseed oil emulsion (Chung et al., 2001). In that report, we confirmed that small particles in the emulsion had good stability during the long-term storage. In our results, the emulsions having

DOTAP at more than 24 mg/ml had the smaller size and showed the most potent resistance against the size changes due to the presence of serum and PBS. The zeta potential of emulsions having linseed oil, soybean oil, and squalene ranged between 50 and 65 mV (data not shown), and that of the liposome was ca. 45 mV, which meant that the emulsions had sufficient positive surface charge to complex with anionic DNA so that they had the same DNA binding capacity as the liposome.

In general, the transfection by cationic liposomes is sensitive to the presence of serum (Yi et al., 2000). This inhibition of gene transfer by serum is considered to be one of the limitations to their in vivo applications. However, the emulsion system might resist to serum inactivation (Yi et al., 2000; Kim et al., 2001a,b). As an attempt to mimic the in vivo situation, we have carried out in vitro transfection experiment in the presence of 80% serum. It is of great significance for in vivo application that the squalene emulsion yielded high transfection levels even in the medium containing 80% serum, which was ca. 70% of the level obtained in the serum-free medium. On the other hand, at such a high serum concentration, the liposome lost the ability to transfer DNA.

The squalene emulsion formed a stable complex with DNA, which could maintain its small size in the medium containing serum. On the other hand, the liposome and the linseed oil emulsion formed unstable complexes with DNA, which could not maintain their size in the presence of serum. This result suggests that the stability of emulsion may determine the stability of its complex with DNA. The size of complexes increased in serum since the components in serum might interact with the complexes in many ways. Serum contains multitude of macromolecules and nucleases. In addition to the interference of delivery due to the complexes coated with serum components, there is another possibility that endogenous negatively charged serum components can dissociate the DNA from cationic carriers (Oku et al., 1996). The resistance of complexes against anionic polymers is important for gene transfer in the presence of serum and could be considered as an indicator for the strength of the interaction between DNA and the lipid carriers. In the exchange reaction of complexes by poly(L-aspartic acid) (PLAA), emulsion/DNA complexes did not release plasmid DNA even at 320 times higher charge concentration of PLAA than liposome/DNA complex, which suggests that emulsion/DNA complexes have an extremely strong interaction between emulsion and DNA (data not shown). This high strength of the interaction might have provided a necessary resistance against serum inactivation. This hypothesis could also be supported by the complex time-dependent serum stability of emulsion complexes in Fig. 5. The freshly prepared complexes of the emulsions were very sensitive to serum like liposome, and their transfection activities were inhibited extremely by the presence of serum in the medium. An appropriate time for complex formation is necessary to obtain the resistance against the endogenous destabilizers in serum.

In attempt to elucidate the relationship between structural stability and in vivo transfection activity of complexes, various complexes with different lipid formulations were administered intravenously into Balb/c mice via tail vein. After systemic administration of complexes, we observed different levels of gene expression in various tissues such as lung, heart, spleen, liver, and kidney. Among these, the expression level in lung lysates appeared to be 10–1000-fold higher than that in other tissue lysates. All lipid complexes showed the highest luciferase activity in the lung as reported for in vivo transfection experiment after the tail vein injection with liposome/DNA complex (Mahato et al., 1995; Liu et al., 1997; Song et al., 1997; Thierry et al., 1997). Though it is possible that lung endothelia are easier to transfect than other organ tissues, it has been reported that in vivo gene expression level of tissues is associated with the biodistribution of complexes after the systemic administration (Liu et al., 1997; Song et al., 1997). Lung accumulation may be the result of entrapment of complexes in lung capillaries by the first-pass effect, or may be due to electrostatic interaction between complexes and the large surface area of the lung endothelia. This interaction may be dependent on the zeta potential and the size of lipid/plasmid complexes (Mahato et al., 1995; Thierry et al., 1997).

For in vivo transfection assay, squalene emulsion also showed the most potent luciferase activity in tissue lysates, especially in lung lysates. On the contrary to squalene emulsion, linseed oil emulsion showed the lowest transfection activity. It is an interesting observation that in vivo transfection activity had a similar pattern to in vitro transfection in the presence

of serum. This fact suggests that in vitro cell culture system containing 80% serum is well mimicking the in vivo situation. In addition, we observed that the stability of the lipid carriers was also important in maintaining their transfection activity during the complex formation with DNA. The decrease of transfection activity of liposome/DNA and linseed oil emulsion/DNA complexes occurred as DNA concentration varied from 10 to 40 μg in 200 μl PBS due to the instability of linseed oil emulsion. At high DNA concentrations, the linseed oil emulsion and the liposome formed insoluble large aggregates with DNA. This structural instability of complexes seems to be related with the loss of transfection activity. The stable squalene emulsion did not form any large aggregates with DNA and showed a dose-dependent transfection activity (data not shown). Therefore, we suggested that the stability of a carrier was a necessary requirement to form the stable complexes with DNA, and the stability of a complex seems to correlate with in vivo transfection activity (Chung et al., 2001).

It has not been reported clearly that the stability and in vitro transfection activity of complexes are relevant to transfection activities in vivo condition. A stable lipid/DNA complex was not affected by the serum inactivation for in vitro transfection experiment. It was able to yield significant levels of transfection activity in various tissue lysates after intravenous administration (Hofland et al., 1998). Sternberg et al. (1994) have investigated the morphology and transfection activity of liposome/DNA complexes both under in vitro and in vivo conditions. In that report, they stabilized the complexes by substituting cholesterol for DOPE as helper lipid, by adding $PEG_{2000}PE$ as steric stabilizer after the complex formation, and by pre-condensing DNA with spermidine. These stability-enhanced lipid/DNA complexes showed a high expression level of a reporter gene in the lung after intravenous administration. Above observations confirmed that in vivo transfection activity was primarily associated with the stability of complexes. The oils affected the stability of complexes since the formation of physically stable and biologically active complexes with DNA could be formed when a stable emulsion was used. The emulsion/DNA complex was more stable when the complex formed the small particle, which was stable with respect to serum and time at high concentration of lipid and DNA. Therefore, the stability enhanced emulsion-based gene carrier is a good carrier for the preparation of stable complexes for in vivo applications.

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