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Free liposomes enhance the transfection activity of DNA/lipid complexes in vivo by intravenous administration

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Abstract

Factors that regulate the transfection efficiency of cationic lipid-based carriers are still largely unknown. We have shown in a previous report [F. Liu, H.W. Qi, L. Huang, D. Liu, Factors controlling the efficiency of cationic lipid-mediated transfection in vivo via intravenous administration, Gene Ther., 4 (1997) 517–523.] that the transfection efficiency, to the lung, of a lipid formulation composed of *N*-[1-(2,3-dioleoyloxy)propyl-*N*,*N*,*N*-trimethylammonium chloride (DOTMA) and Tween 80 is directly proportional to the ratio of DOTMA to DNA. In this study, we investigated the mechanism underlying the high cationic lipid to DNA ratio dependent transfection activity. Specifically, we have examined the role of free cationic liposomes in affecting the transfection efficiency of the DNA/lipid complexes in vivo by intravenous administration. The data show that greater transfection activity of DNA/lipid complexes in the lung at a higher cationic lipid to DNA ratio is due to the function of free liposomes present in the DNA/lipid mixture. Free liposomes enhance the transfection activity of DNA/lipid complexes by increasing the retention time of DNA and decreasing transgene degradation in different organs. In addition to DOTMA liposomes, liposomes composed of 1,2-dioleoyl-3-trimethylammonium propane chloride (DOTAP) and 3β[*N*-(*N*′, *N*′-dimethylaminoethane) carbamoyl] cholesterol (DC-Chol) also enhance the level of gene expression in animals transfected by DNA/DOTMA complexes. These results suggest that inclusion of free liposomes into the DNA/lipid complexes may be important in achieving an optimal transfection activity in vivo.

Keywords: Gene therapy; Cationic liposome; Gene transfer; Transfection

1. Introduction

Cationic lipid-based delivery systems have been studied for their use in gene therapy [1–5]. Of primary concern for these systems is the gene delivery efficiency and the underlying mechanisms by which the delivery efficiency is determined. The former often limits the usefulness of the lipid carrier, and the latter determines the potential for future development. In the last 10 years, many studies have shown that lipid-based gene delivery systems are capable of delivering genes to cells, both in vitro [6–13] and in vivo [11,13–19]. It is generally believed that transfection activity of the lipid systems is determined by the structure formed between the cationic liposomes and DNA. The formation of such DNA/lipid complexes

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is dictated by the structure of the cationic lipid, the excipients included in the lipid formulation, and the ratio of the cationic lipid to DNA. Although some evidences have been presented to support the existence of specific DNA/lipid complex structures [20–22], the structure–function relationship of the DNA/lipid complexes is yet to be established. Moreover, recent results from our laboratory indicate that, in addition to the importance of the DNA/lipid structure, free liposomes may play an important role in determining the transfection activity of the lipid carrier in vivo following intravenous administration [18].

We have demonstrated that the level of gene expression in the lung, spleen, heart, liver and kidney increases with increasing amount of cationic lipid used in formulating the DNA [18]. The ratio of cationic lipid to DNA resulting in the maximal level of gene expression was 36:1 (cationic lipid:DNA = nmol:μg) or greater. Theoretical calculation of the stoichiometry of the cationic lipid to DNA at such high ratio suggests the possible existence of free lipid particles in the DNA/lipid mixture. It also suggests that the higher transfection efficiency observed at high cationic lipid to DNA ratios is caused by the free lipid particles. The study described in this report was designed to examine this hypothesis. We provide direct evidence that free cationic liposomes play important role in determining the level of gene expression in vivo. These results are helpful in understanding the mechanisms involved in lipid-based transfection in vivo and are useful in developing new strategies that will further increase the gene transfer efficiency of lipid-based delivery systems.

2. Materials and methods

2.1. Materials

1,2-Dioleoyl-3-trimethylammonium propane chloride (DOTAP) was purchased from Avanti (Atlanta, GA, USA). 3β[*N*-(*N*′, *N*′-dimethylaminoethane) carbamoyl] cholesterol (DC-Chol) was purchased from Sigma (St. Louis, MO, USA). *N*-[1-(2,3-dioleoyloxy)propyl-*N*,*N*,*N*-trimethylammonium chloride (DOTMA) was kindly provided by Roche Bioscience (Palo Alto, CA, USA). Synthesis and radiolabeling of diethylenetriaminepentaacetic acid stearylamide (DTPA-SA) was performed according to Kabalka et al. [23]. A CMV driven expression system containing firefly luciferase cDNA (pCMV-Luc) was constructed in the laboratory of Dr. Leaf Huang (Department of Pharmacology, School of Medicine, University of Pittsburgh) by cloning the genes into the backbone of a pcDNA3 plasmid (Invitrogen, Carlsbad, CA, USA). Plasmid DNA was purified by the method of CsCl-ethidium bromide gradient centrifugation [24]. The purity of the plasmid DNA was determined by absorbency at 260 and 280 nm and by 1% agarose gel electrophoresis. CD 1 mice (male, 18–20 g) were obtained from Charles River, (Wilmington, MA, USA).

2.2. Preparation of DNA / lipid complexes

Lipid formulation was prepared as described previously [18]. In brief, appropriate amounts of cationic lipids in chloroform with or without mixing with Tween 80 in chloroform were placed in a glass tube and the chloroform was evaporated under a stream of N_2 gas. The lipid films were vacuum desiccated for a minimum of 2 h to remove residual amount of organic solvent. The dried lipid films were then hydrated in PBS (pH 7.4) at cationic lipid concentration of 15 mM for 30 min at room temperature. The lipid suspension was homogenized for 2–3 min using a Tissue Tearor (Biospec Products, Bartlesville, OK, USA) at the maximal speed (20,000 rpm). The average size of lipid particles as measured by light scattering using a submicron particle analyzer (Particle Sizing Systems, Santa Barbara, CA, USA) was about 200–300 nm. To prepare DNA/lipid complexes at 6:1 (cationic lipid:DNA, nmol:μg), plasmid DNA was first diluted with PBS (pH 7.4) to a final concentration of 131.6 μg/ml and lipid formulation ([DOTMA] = 15 mM) composed of cationic lipid and Tween 80 in a weight ratio of 6:2 was added dropwise to the DNA solution with gentle vortexing. The final concentration of plasmid DNA in the mixture was 125 μg/ml for all preparations used in this study. The DNA/lipid mixture was kept at room temperature for about 10 min before being administered to the animals.

2.3. Iodination of plasmid DNA

Iodination of plasmid DNA was performed as described previously [25]. In brief, 2 mCi carrier-free $Na^{125}I$ (5 μl) mixed with 10 μl acidifying solution (0.075 M HNO_3, 42-mM sodium acetate, pH 4.7) was added to 22 μl of TE buffer (10-mM Tris–HCl, 1-mM EDTA, pH 8.0) containing 20 μg of plasmid DNA (pCMV-Luc). The mixture was incubated at 60°C for 30 min after addition of Tl (III) ion (final concentration, 0.27 mM). Five hundred microliters of TNE buffer (50-mM Tris–HCl, 0.1-M NaCl, 1-mM EDTA, pH 7.5) was added and the mixture was incubated for an additional 30 min. The reaction mixture, after cooling on ice, was loaded on a pre-equilibrated Bio-Gel P30 spin column with TE buffer to remove free ^{125}I. The DNA fraction from the spin column was dialyzed at 4°C against 2×2.5 l of TE buffer for 24 h to further remove the residual ^{125}I contamination. ^{125}I-DNA was mixed with cold DNA of the same construct to the final specific activity of 10^4 cpm/μg.

2.4. Luciferase activity assay

Luciferase activity was assayed with a kit purchased from Promega (Madison, WI, USA) using a luminometer (Autolumat LB953, EG & G, Berthhold, Germany). Animals were injected through the tail vein with DNA/lipid complexes (25 μg total DNA in 200 μl/mouse) with or without pre-injection of free liposomes which were made of cationic lipid alone without Tween 80. Different organs including lung, liver, spleen, heart and kidneys were dissected 8 h after the injection of the DNA/lipid complexes. Lysis buffer (0.1-M Tris–HCl, 2-mM EDTA and 0.1% Triton X-100, pH 7.8) was added at a volume to weight ratio of 4 μl/mg of the collected organs except for liver to which 5 μl of lysis buffer/mg of liver was used. Each organ was homogenized for 15–20 s with the tissue tearor ($\sim$20,000 rpm) to make a tissue homogenate, which was then centrifuged in a microcentrifuge for 10 min at 12,000 rpm at 4°C. A 10-μl aliquot of the supernatant (containing about 200-μg total protein) was used for luciferase activity assay. Protein concentration of each tissue extract was determined by a standard protein assay (Bradford reagent from BioRad). Luciferase activity in each sample was normalized to represent relative light units (RLU)/mg of extracted protein.

2.5. Tissue distribution

^{111}In-DTPA labeled free liposomes or ^{125}I-labeled DNA/lipid complexes were injected into animals by tail vein injection. To test the effect of free liposomes on tissue distribution of DNA in the complexed form with DOTMA/Tween 80, each animal received 750 nmol of free DOTMA liposomes in 100 μl 1 min before the administration of DNA/lipid complexes containing ^{125}I-labeled plasmid DNA. Control animals received the same volume of PBS for pre-injection. Animals were sacrificed at different time points and the amount of radioactivity in each organ was measured using a Gamma counter. The radioactivity in the blood was estimated with the assumption that the total blood in mouse equals 7.4% of total body weight [26]. The blood contamination in each organ was corrected using a previously described method [27]. Results were expressed as percentage of the total injected dose/organ.

2.6. Southern analysis

Animals pre-injected with either PBS or DOTMA liposomes (750 nmol/mouse in 100 μl) were injected with 25 μg of plasmid DNA complexed with DOTMA/Tween 80 (6:2, weight ratio) at the cationic lipid to DNA ratio of 6 to 1 (nmol: μg). Animals injected with PBS (first injection) and 25 μg of free plasmid DNA (second injection) were used as a control. Animals were sacrificed 30 min after DNA administration and organs including the lung, spleen, heart, liver (about 1/5 of the whole liver) and kidney were collected and immediately frozen on dry ice. Two-milliliter lysis buffer (10-mM Tris–HCl, 0.1-M EDTA, 0.5% SDS, 20 μg/ml RNase A, pH 8.0) was added to each frozen tissue, and the tissue was homogenized for 20 s and incubated at 37°C for 1 h. Proteinase K was added at a final concentration of 150 μg/ml and the tissue homogenate was incubated for additional 5 h at 60°C. Following the extraction with phenol, phenol:chloroform (1:1) and chloroform, DNA was precipitated with 0.1 volume of 3-M sodium acetate and 2 volume of ethanol, washed with 70% ethanol and resuspended in TE buffer. Ten micro-

grams of DNA from each sample were digested with 10 units of *Hin*dIII for 1 h at 37°C and then run on a 1.0% agarose gel. DNA bands were transferred to a GeneScreen Plus membrane (DuPont NEN) overnight and the membrane was soaked for 2 h at 42°C in prehybridization buffer (50% deionized formamide, 250-mM sodium phosphate, 250-mM NaCl, 1-mM EDTA, 100 μg/ml sonicated salmon sperm DNA and 7% SDS). For hybridization, the membrane was incubated at 42°C for 24 h with ^{32}P-labeled probes recognizing the full luciferase gene of the pCMV-Luc plasmid. The hybridized bands were visualized in an X-ray film by autoradiography.

3. Results

3.1. Effect of free liposomes in liposome-mediated transfection

To test the effect of free liposomes on the transfection activity of DNA/lipid complexes, DNA/lipid complexes were prepared at a lower cationic lipid to DNA ratio with our previously used DOTMA/Tween 80 (6:2, weight ratio) formulation and pCMV-Luc plasmid. Different amounts of free DOTMA liposomes were intravenously injected into mice 1 min before the administration of DNA/lipid complexes. A 6 to 1 ratio of cationic lipid to DNA (nmol:μg) was chosen for the complex preparation because it gives a calculated charge ratio of 2:1(+/−) which is optimal for transfection in vitro [6–12]. Eight hours after the administration of DNA/lipid complexes, animals were sacrificed and the level of gene expression was examined in different organs including the lung, spleen, heart, liver, and kidney. Fig. 1 shows that, except for spleen, the level of luciferase activity in all examined organs increased with increasing amount of free DOTMA liposomes pre-injected. For example, the level of luciferase activity in animals that were pre-injected with 450 nmol of free DOTMA liposomes was about 100-fold higher than that in control animals. A dose higher than 450 nmol did not produce any further increase in the level of gene expression, suggesting a saturation at 450 nmol under the experimental conditions.

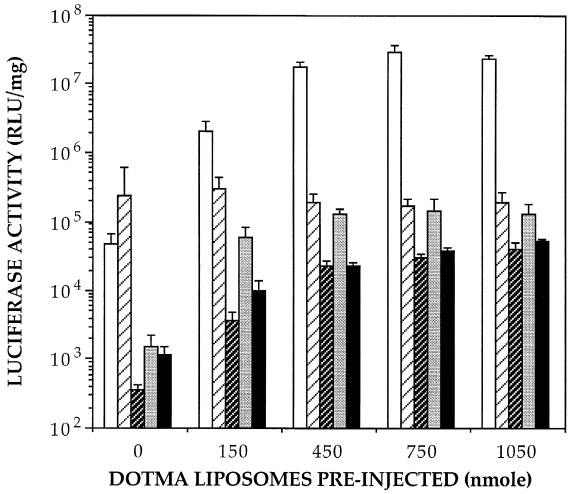

Fig. 1. Dose dependent effects of pre-injected free liposomes on the level of gene expression. DNA/lipid complexes containing 25-μg pCMV-Luc plasmid DNA were injected 1 min after the animals received different amounts of DOTMA liposomes. Luciferase activity in different tissues was assayed 8 h after the injection of DNA/lipid complexes. Results represent mean ± SEM of values obtained from 3–6 mice. (□) Lung, (square filled with diagonal zigzag lines) spleen, (solid square filled with white diagonal dots) heart, (gray) liver and (■) kidney.

3.2. Effect of the cationic lipid to DNA ratio in DNA / lipid complexes

The ratio of cationic lipid to DNA in the DNA/lipid complexes used in the above experiments was 6:1 (nmol:μg). To examine whether the potentiating effect of free liposomes was dependent on the cationic lipid to DNA ratio in the complexes, DNA/lipid complexes were prepared at different ratios and injected intravenously into animals that had been pre-injected with different amounts of free liposomes. In these experiments, the total amount of cationic liposomes and plasmid DNA each animal received was the same (25-μg plasmid DNA and 900 nmol cationic lipid). As shown in Fig. 2, except for DNA/lipid complexes at a ratio of 3:1 (nmol:μg), the ratio of cationic lipid to DNA in the complexes did not result in any significant difference in the level of luciferase activity, suggesting that the structure of DNA/lipid complexes formed at either low (6:1, nmol:μg) or high (36:1, nmol:μg) cationic lipid to DNA ratios was equally active. This suggests that the dominant factor determining the transfection activity

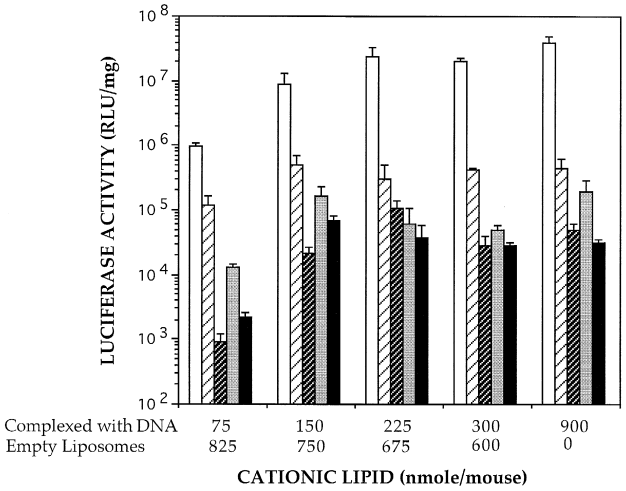

Fig. 2. Dependence of gene expression on the total cationic lipid to DNA ratio. DNA/lipid complexes with different cationic lipid to DNA ratios were administered to animals pre-injected with different amounts of DOTMA liposomes. The total amount of cationic lipid and plasmid DNA each mouse received was 900 nmol and 25 μg, respectively. Data represent mean ± SEM of values from 3–6 animals. The luciferase activity in each tissue was assayed 8 h post-injection of DNA/lipid complexes. (□) Lung, (square filled with diagonal zigzag lines) spleen, (solid square filled with white diagonal dots) heart, (gray) liver and (■) kidney.

of the DNA/lipid complexes is the overall amount of cationic lipid and DNA injected.

3.3. Potentiation activity of different types of cationic liposomes

To examine whether the ability of pre-injected free liposomes for potentiating effect depends on the structure of cationic lipids, two additional types of cationic lipids were used. DC-Chol is a cholesterol derivative with a tertiary amine as the charged head group. DOTAP has a quaternary amine as the charged head group that is linked to two hydrophobic fatty acyl chains by ester bonds. For this purpose, two sets of experiments were performed. In the first set, animals were pre-injected with either DC-Chol or DOTAP liposomes and then injected with plasmid DNA mixed with DOTMA/Tween 80 lipid formulation (DOTMA/Tween 80 = 6:2, weight ratio). In the second set, animals were first injected with free DOTMA liposomes before injection of plasmid DNA complexed with DC-Chol/Tween 80 or DOTAP/Tween 80 lipid formulation (cationic lipid:Tween 80 = 6:2, weight ratio). The total free liposomes each animal received was 750 nmol and the amount of DNA in the form of DNA/lipid complexes (cationic lipid:DNA = 6:1, nmol:μg) was 25 μg/mouse. As shown in Table 1, the control animals pre-injected with buffer showed a relatively low level of luciferase activity for all three types of DNA/lipid complexes. However, in all three cases, pre-injection of animals with 750 nmol of free liposomes significantly enhanced the level of gene expression. The greatest increase was obtained in the lung when animals received free DOTAP liposomes and DNA/DOTMA complexes.

3.4. Time course of free liposome effect

The time course for the effect of free liposomes on transfection efficiency was determined using DOTMA

Table 1
Potentiating effect of different types of liposomes on the transfection activity of DNA/lipid complexes[a]

Lipid formulation in DNA/lipid complex (cationic lipid:DNA = 6:1, nmol:μg)	Pre-injected material	Luciferase activity (RLU/mg × 10^{-3})				
		Lung	Spleen	Heart	Liver	Kidney
DOTMA/Tween 80 (6:2, weight ratio)	PBS	39.0 ± 3.0	225.6 ± 174.2	52.1 ± 33.3	9.7 ± 6.4	6.7 ± 3.7
	DC-Chol[b]	2586.1 ± 1122.3	131.2 ± 12.5	50.6 ± 9.9	10.1 ± 2.1	2.5 ± 0.6
	DOTAP[b]	9865.3 ± 756.5	964.8 ± 508.1	1261.5 ± 213.7	125.9 ± 36.3	52.4 ± 2.4
DOTAP/Tween 80 (6:2, weight ratio)	PBS	50.7 ± 25.6	36.5 ± 15.3	0.4 ± 0.1	2.0 ± 0.9	1.1 ± 0.6
	DOTMA[b]	1113.0 ± 149.5	34.3 ± 7.1	22.3 ± 5.6	15.4 ± 2.7	3.2 ± 0.4
DC-Chol/Tween 80 (6:2, weight ratio)	PBS	6.7 ± 4.4	0.5 ± 0.1	0.4 ± 0.1	0.2 ± 0.1	0.3 ± 0.0
	DOTMA[b]	773.1 ± 166.3	13.9 ± 3.2	4.9 ± 0.9	18.9 ± 3.6	3.2 ± 0.6

[a]Each animal received either PBS or 750 nmol of free liposomes 1 min before being injected with 25-μg plasmid DNA complexed with different lipid formulations. Data represent mean ± SEM of values from 3–6 animals.
[b]The cationic lipids injected were in the form of free liposomes.

liposomes. In these experiments, 750 nmol of DOTMA liposomes were injected into each animal at time zero and the DNA/lipid complexes (DNA/DOTMA-Tween 80) were subsequently injected at different times. Results show that the effect of pre-injection of free liposomes decreased with increasing time between the two injections (Fig. 3A). For example, the luciferase activity in the lung was approximately $2–5 \times 10^7$ RLU/mg of extracted protein in animals that were injected with DNA/lipid complexes within 1 h after injection of free liposomes. This activity dropped to a minimum when the second injection was performed 24 h after administration of the free liposomes, suggesting a complete loss of the potentiating activity of the pre-injected free liposomes.

The effect of post-injection of free liposomes on transfection activity of DNA/lipid complexes was also examined. Results in Fig. 3B show that post-injection of free liposomes also increased the level of gene expression. However, the degree of enhancement, compared to pre-injection, was much lower and shorter in duration. For example, the luciferase activity obtained from animals which received free liposomes 1 min after receiving the DNA/lipid complexes was 2×10^6 RLU/mg of extracted lung protein, 40-fold higher than that in the control group, but 15-fold lower than that in animals that received the same amount of free liposomes 0.5 min prior to administration of the DNA/lipid complexes (Fig. 3A). No increase in transfection efficiency was observed when free liposomes were injected 2 h after administering the DNA/lipid complexes. This indicates that pre-injection is much more effective than post-injection of free liposomes in enhancing transfection activity of DNA/lipid complexes.

3.5. Time dependent tissue distribution of free DOTMA liposomes

The long lasting effect of pre-injected free liposomes on the level of gene expression prompted us to study whether the potentiation of gene expression is directly related to the level of liposome accumulation in each organ. Table 2 shows the tissue distribution of DOTMA liposomes as a function of time. Among the internal organs examined, the lung, blood and liver are the three major organs that take up most of the injected liposomes. The amount of liposome in spleen, heart and kidney was low at less than 3% of the injected dose within our testing time period. Five minutes after liposome administration, the highest level of liposome uptake was observed in the lung (49%). Approximately equal level of liposomes were

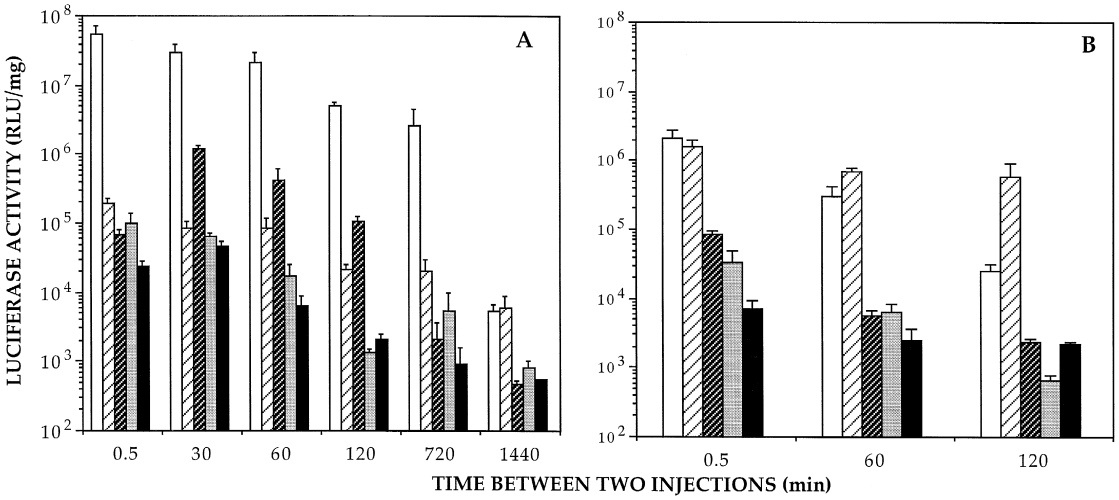

Fig. 3. Time dependent effects of pre-injecting free liposomes on gene expression. Free DOTMA liposomes (750 nmol) were injected into animals at different times either before (A) or after (B) the injection of DNA/lipid complexes. Luciferase activity in different tissues was assayed 8 h post-injection of DNA/lipid complexes. The total dose each animal received was 900 nmol for DOTMA and 25 μg for pCMV-Luc plasmid DNA. Data represent mean $\pm$ SEM ($n = 3–6$). (□) Lung, (square filled with diagonal zigzag lines) spleen, (solid square filled with white diagonal dots) heart, (■) liver and (■) kidney.

Table 2
Time dependent tissue distribution of free DOTMA liposomes[a]

Time (min)	% Injected dose						
	Blood	Lung	Spleen	Heart	Liver	Kidney	Total recovery[b]
5	16.9 ± 1.9	49.3 ± 4.2	1.1 ± 0.2	0.1 ± 0.0	17.4 ± 1.3	1.6 ± 0.1	86.2 ± 3.3
30	16.5 ± 1.9	36.7 ± 4.4	1.1 ± 0.2	0.1 ± 0.0	21.1 ± 2.1	2.4 ± 0.5	77.7 ± 1.8
120	12.6 ± 1.0	4.2 ± 0.4	2.6 ± 0.3	0.1 ± 0.0	53.4 ± 1.2	2.3 ± 0.1	75.0 ± 1.8
720	1.6 ± 0.1	4.4 ± 0.1	2.6 ± 0.2	0.2 ± 0.1	60.6 ± 0.9	2.5 ± 0.3	71.8 ± 1.1

[a]^{111}In-labeled DOTMA liposomes were injected into mice i.v. at a dose of 750 nmol/mouse. The radioactivity in each organ at specific times was calculated as percentage of total injected. Data represent mean ± SEM ($n = 3$).
[b]Total recovery represents the sum of the radioactivity from the organs listed.

found in the blood and liver (~17% injected dose). The level of liposome uptake by these organs changed over time. Levels in the lung and blood decreased with time while the level in the liver increased. For example, 12 h post-injection, the amount of liposomes in the lung dropped from the initial 49% to about 4% of the injected dose, while the amount of liposomes in liver increased from 17% to about 60% in the same period. A 10-fold decrease in liposome accumulation in blood (from 17% to 1.6%) was also observed.

3.6. Effect of pre-injection of free liposomes on the tissue distribution of DNA / lipid complexes

Results from our previous study showed that the level of gene expression in different organs, especially in the lung, was influenced by the level of DNA binding and retention time [18]. To examine whether the potentiating effect of free liposomes is related to an alteration in tissue distribution of DNA plasmid, a biodistribution study was conducted using ^{125}I-labeled plasmid DNA as a marker. The tissue distribution of DNA in DNA/lipid complexes was compared between control and test animals that were pre-injected with either buffer or 750 nmol of free DOTMA liposomes, respectively. As shown in Fig. 4, the overall tissue distribution of DNA marker between control and test animals appeared similar at the earlier time points. Five minutes after injection of DNA/lipid complexes, about 25–30% of injected dose was found in the lung and approximately 40% in the liver. Although the level of DNA in the liver remained about the same level at later time points, the level of DNA in the lung was 4–5-fold higher in test animals. These results suggest that free liposomes increase the retention time of plasmid DNA in the lung.

3.7. Effect of pre-injection of free liposomes on the rate of DNA degradation

One of the phenomena observed in our biodistribution study using ^{125}I-labeled plasmid DNA is that the

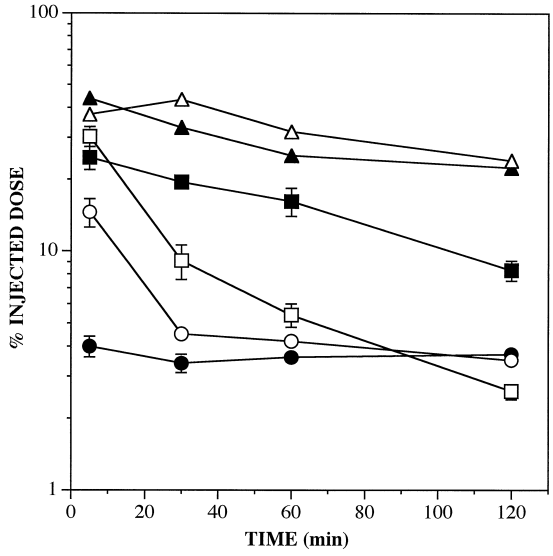

Fig. 4. Effects of pre-injecting free liposomes on the tissue distribution of DNA in DNA/lipid complexes. DNA/lipid complexes (cationic lipid:DNA = 6:1, nmol:μg) containing a trace amount of ^{125}I-labeled pCMV-Luc plasmid DNA were injected to animals 1 min after each animal received either PBS or 750 nmol DOTMA liposomes. The level of ^{125}I radioactivity in each organ was analyzed at 5, 30, 60 and 120 min post-injection of the DNA/lipid complexes. Open symbols represent the amount of ^{125}I radioactivity in tissues from animals that received PBS. Filled symbols represent ^{125}I radioactivity in tissues from animals that received free liposomes before the injection of DNA/lipid complexes. (○, ●) Blood, (□, ■) lung, and (△, ▲) liver.

total recovery of ^{125}I count is significantly lower (data not shown) than that of ^{111}In marker used in liposome studies (Table 2), suggesting that a significant level of DNA was degraded after DNA administration. To examine the possible role of pre-injected liposomes in determining the rate of DNA degradation in different organs, a Southern analysis was performed in animals with or without pre-injection of free liposomes. DNA extract from different organs was prepared and the relative level of transgene in each sample was analyzed using ^{32}P-labeled full length luciferase gene as a probe. The rate of DNA degradation was judged by the size and density of the DNA band corresponding to the full length luciferase gene and the size and density of the smear bands with smaller fragments of the luciferase gene. As shown in Fig. 5, transgene degradation occurred in all organs 30 min after DNA administration. Compared to the control group (marked as 'Complex only') pre-injected with PBS, the DNA band corresponding to the full length plasmid (control lane) is bigger and denser in organs from animals (marked as 'Free Liposome + Complex') pre-injected with free liposomes. Denser bands showing smaller DNA fragments and longer stretches of the smear bands were also evident for animals injected with a combination of PBS and DNA/lipid complexes. The same conclusion was made for other organs, especially the liver. With exception of a light spot seen in the lane of liver extract at the bottom of the gel, no DNA bands stained positive with the same luciferase gene probe in any tissues of the animals injected with free plasmid, suggesting a much faster rate of gene degradation. These results suggest that cationic liposomes decrease the rate of gene degradation.

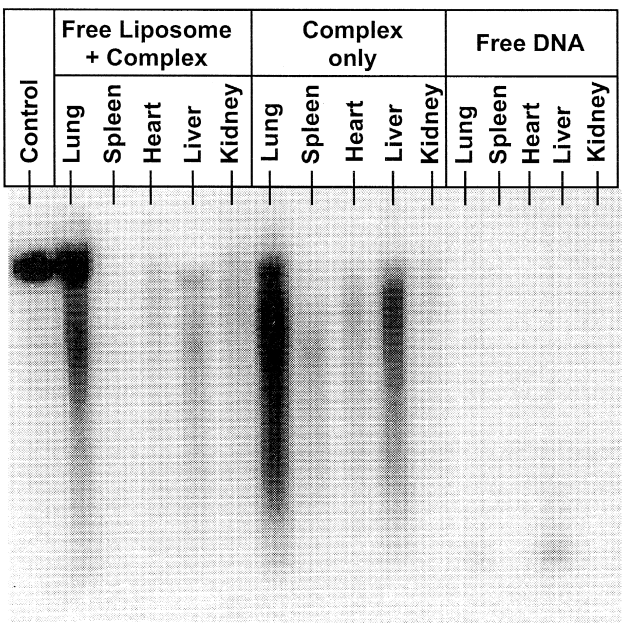

Fig. 5. Effect of pre-injecting free liposomes on DNA degradation. Southern analysis was performed with DNA extracted from different organs of animals injected with either free DNA (right panel) or DNA/lipid complexes. For animals that received DNA/lipid complexes, 750-nmol DOTMA liposomes (left panel) or PBS buffer (middle panel) were injected 5 min before the injection of DNA/lipid complexes. DNA extraction was performed 30 min after the DNA administration. Each lane contains 10 μg of total extracted DNA. DNA fragments were probed with ^{32}P-labeled full length luciferase gene. The positive control (far left lane) used was 150 ng of pCMV-Luc plasmid DNA. The amount of pCMV-Luc plasmid DNA injected to each mouse was 25 μg.

4. Discussion

Cationic lipid-mediated delivery systems have attracted increasing attention as a potential carrier for gene therapy. One of the major concerns of this carrier system is its relatively low efficiency compared to viral vector systems [1]. Two primary factors have been shown to affect the transfection efficiency of lipid carriers; the lipid composition and cationic lipid to DNA ratio. Although the detailed mechanism of how lipid composition determines the transfection activity of DNA/lipid complexes is unclear at the present time, results from our laboratory show that a higher cationic lipid to DNA ratio in the formulation increases the transfection activity in vivo by intravenous administration [18]. The results, as summarized in this report, suggest that a higher transfection activity obtained in vivo at a higher cationic lipid to DNA ratio is largely due to the potentiating activity of free liposomes (Figs. 1 and 2 and Table 1).

While it may still be premature to suggest exactly how free liposomes enhance the transfection activity of DNA/lipid complexes in different organs, data in Fig. 4 suggest that the potentiating activity of free liposomes in transfecting cells in the lung is through their ability to increase the retention time of DNA molecules in this organ. Considering the fact that

cationic liposomes form aggregates when mixed with blood components such as serum proteins, it is possible that free liposomes, upon entering the blood stream, form aggregates with blood components which are then trapped in the lung endothelial bed. Theoretically, these liposomes bound to the endothelial cells in lung could significantly slow down the flow of the DNA/lipid complexes through the lung capillary bed. A slower flow lengthens the exposure time of DNA to the endothelial cells lining the vascular wall, resulting in a higher level of gene expression. An increase in the level of gene expression resulted from pre-injection of different types of cationic liposomes (Table 1) suggests that free liposomes in the lung may enhance transfection efficiency in a non-specific manner and is not dependent on the specific structure of the cationic lipids. Interestingly, not all types of liposomes that are capable of being trapped in the lung are able to increase the retention time of subsequently injected DNA/lipid complexes. For example, we found that multilamellar liposomes ($d > 1$ μm) composed of PC/Chol/PS (10:5:1, molar ratio) were readily trapped in the lung at an injected dose of 750 nmol/mouse, the same liposome dose as that used for cationic liposomes. Approximately 36% of the injected dose was found in the lung 5 min after liposome administration, compared to 29% at 90 min. This level of liposome uptake did not enhance the retention time of subsequently injected DNA/DOTMA/Tween 80 complexes, nor the level of gene expression (data not shown). Non-cationic liposomes do not seem to potentiate transfection efficiency.

Alternatively, free cationic liposomes could act as a sensitizer that makes vascular endothelial cells more transfectable by the DNA/lipid complexes. A tissue distribution study using ^{111}In-DTPA labeled free liposomes showed that these free liposomes stayed in the lung for a relatively long time. As shown in Table 2, 12 h after the injection of 750 nmol lipid/mouse, about 4% of the injected dose still remained in the lung. This level represents a total of 30 nmol of liposomes in a mouse lung weighing about 150 mg. It is possible that this level of liposomes in the lung is sufficient to produce an enhanced level of gene expression.

Another interesting phenomenon observed in these experiments was a relatively long residence time of liposomes in the blood. Two hours post-injection, approximately 13% of the injected dose was still circulating in the blood. Such a high blood concentration is likely due to the high dose injected. It is unclear whether liposomes in the blood also contribute directly to the potentiating activity of free liposomes. It is possible that these circulating liposomes may remove inhibitors from blood which otherwise inhibit the transfection activity of DNA/lipid complexes. Result in Fig. 5 suggests that the potentiating effect of free liposomes is through their activity to decrease the rate of gene degradation. Such activity is likely the cause for a long lasting effect of pre-injected liposomes (Fig. 3). Additional experiments are needed to elucidate the detailed mechanisms involved.

In summary, the data show that free liposomes play an important role in affecting the transfection activity of DNA/lipid complexes following intravenous injection. Whether an excess amount of cationic liposomes should be included in the injectable DNA formulations used in many clinical trials remains to be tested. However, it is possible that the inclusion of free liposomes into intravenous DNA formulations may significantly improve transfection activity and result in increased therapeutic effect.

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