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Efficient Gene Transfer into Mammalian Cells Using Fusogenic Liposome

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Fusogenic liposome (FL) based on Sendai virus constitutes a unique system that delivers the content efficiently into animal cells *in vitro* and *in vivo.* In this study we characterized unilamellar FL as a gene transfer vector in comparison with cationic lipid (CL)-DNA complex. FL transferred genes efficiently into cultured cells even when incubated for as little as 10 min, while CL-DNA complex required at least 30 min to reach the same level of gene expression. FL was also much less cytotoxic than CL-DNA complex under the conditions that resulted in the same level of gene expression. In addition, FL maintained 70% of the transfection activity even in the presence of 40% fetal calf serum (FCS), while CL-DNA complex almost completely lost their activity in the presence of 5% FCS. Furthermore, we found that FL could introduce and express luciferase gene into mouse ascites tumor cells *in vivo,* but CL-DNA complex could not even at higher concentrations of DNA. We conclude that unilamellar FL is a unique and efficient nonviral vector for gene transfer *in vitro* and *in vivo.*

Delivery of foreign genes into animal cells plays an important role not only in *in vitro* research but also in clinical studies for gene therapy. Many viral and nonviral vectors for gene transfer have been developed for these purposes, and they have both advantages and disadvantages (1,2,3). Most viral vectors can transfer genes efficiently and some of them can be used for gene transfer into tissue cells *in vivo.* However, the structure and stability of transferred genes are restricted by those of the virus genome. In contrast, nonviral vectors such as cationic lipid (CL)-DNA complex can transfer genes with little restriction in their structure but with lower efficiency than viral vectors.

Previously, Okada and colleagues developed fusogenic liposomes (FL) by fusing Sendai virus with multilamellar liposomes at 37°C (4,5,6). FL constituted a unique delivery system that could introduce the materials encapsulated in the liposomes directly and efficiently into cultured animal cells (4,5,6) as well as into tissue cells of living animals (5) *in vivo* through the fusion with the cell membrane. In this system the efficacy of delivery of various macromolecules depended on the nature of the simple liposomes used for encapsulating them. For example, we found previously that the nucleic acid had to be encapsulated in multilamellar liposomes prepared by mechanical vibration (5) because of the difficulty in encapsulating huge macromolecules in unilamellar liposomes prepared by ultrasonic emulsification (7). However, it is difficult to estimate the efficiency of gene transfer mediated by the multilamellar FL because of the heterogeneity in the size and characteristics of these liposomes. The multilamellar FL also had a disadvantage that more than 85% of the particles in the final preparation were not fusogenic (5).

Recently we succeeded in developing homogeneous unilamellar FL (average diameter of 379 nm) by fusing Sendai virus with unilamellar liposomes prepared by a modified reverse phase evaporation method with filter emulsification and examined their characteristics in detail (7). Due

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Abbreviations: FL, fusogenic liposome; CL, cationic lipid; DTA, fragment A of diphtheria toxin; S-180, Sarcoma-180.

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to the improvement in preparation of unilamellar simple liposomes, the unilamellar FL could efficiently encapsulate and deliver various macromolecules including nucleic acids. In addition, we purified unilamellar FL based on the difference of the density of the particles. Purified FL was almost free of nonfusogenic simple liposomes and Sendai virus (7). Using these improved FL, we found that the mouse carrying Sarcoma-180 (S-180) ascites tumor cells were cured efficiently by i.p. injection of FL containing fragment A of diphtheria toxin (DTA) without obvious side effect (8). This suggested that the improved FL also could deliver the contents into tumor cells *in vivo.*

In this study, we examined the characteristics of the unilamellar FL as gene transfer vectors in comparison with CL-DNA complex, a common nonviral gene transfer vector.

MATERIALS AND METHODS

Construction of Plasmid

The HindIII-SmaI fragment of pRSVL (9) was cloned into the HindIII / BamHI site of pBV1 after filling the BamHI end with the Klenow fragment of DNA polymerase, resulting in pCAL1. Plasmid pBV1 is a derivative of pBY1 (10), and contains the cytomegalovirus enhancer / chicken β-actin hybrid promoter and SV40 early gene poly(A) signal. Site-directed mutagenesis to remove the 5′ non-coding sequence was performed according to Kunkel et al. (11) using the oligonucleotide (5′TTCCTCCGCAGCCAGCCATGGAAGACGGCAAAAA3′) as a mutation primer and pCAL1 as a template, resulting in pCAL2. The 5′ non-coding sequence was confirmed by nucleotide sequencing with chain termination method (12). Plasmid pCAL2 expressed 20- and 40-fold more luciferase activity in L cells than pCAL1 and pRSVL, respectively (data not shown).

Preparation of Fusogenic Liposome Containing Plasmid DNA

Unilamellar liposomes were prepared by the reverse phase evaporation method (13) with modification by 46 μmol of lipids (egg phosphatidylcholine : L-α-dimyristoryl phosphatidic acid : cholesterol = 5 : 1 : 4, molar ratio) in 1 ml of a mixture of dichloromethane and diethyl ether (53 : 47 (w/w)) and 300 μl of an aqueous solution containing pCAL2 (10 mg/ml) in 150 mM NaCl / 10 mM EDTA / 10 mM Tris, pH 7.6 (7,8). After sizing by extrusion through a 0.4 μm polycarbonate membrane (Nucleopore; Costar, Cambridge, USA), the liposomes were separated from unencapsulated DNA by stepwise sucrose gradient centrifugation (15–10%). FL were prepared by fusing the liposomes with UV (2,000 J/cm^2)-inactivated Sendai virus as described (8).

The amount of DNA encapsulated in FL was determined by means of a fluorometric assay using 3,5-diaminobenzoic acid (14) after DNA in the SDS (0.5%)-treated liposome suspension was extracted with phenol and phenol / chloroform. One milliliter of FL suspension at OD_{540} of 1.0 contained 2.0 μg DNA.

Fusogenic Liposome-Mediated Gene Transfer into Cultured Cells

Cells (1.0×10^5) were seeded in 35-mm dishes and cultured with MEM supplemented with 10% FCS. On the following day, the cells were washed once with BSS (150 mM NaCl, 2 mM $CaCl_2$, 10 mM Tris, pH 7.6) and incubated at 37°C with FL containing pCAL2 suspended in BSS for various periods. The cells were washed twice with chilled medium, then given fresh medium. After 48 h, luciferase activity in the cells was determined.

Cationic Lipid-Mediated Gene Transfer into Cultured Cells

A mixture of N-[1-(2,3-dioleyloxy)propyl]-N,N,N-trimcthylammoniumchloride and dioleoyl phosphatidylethanolamine (1 : 1 (w)) (Lipofectin; GIBCO BRL, Gaithersburg, USA) was used as CL for the transfection. Plasmid pCAL2 (3.0 μg) and CL (15.0 μg) were suspended separately in 100 μl of serum-free MEM. They were then combined, mixed gently, and incubated at room temperature for 15 min. After the mixture was diluted with serum-free MEM, the cells were processed using the same protocol with FL. A DNA : CL ratio of 1 : 5 (w) was optimal for gene transfer (data not shown), as recommended by the manufacture.

Gene Transfer into Sarcoma-180 (S-180) Cell in Vivo

S-180 cells (1×10^6 cells) were i.p. injected into male ddY mice at day 0. At 5 days, 1 ml of FL containing 1.5 μg of pCAL2 in BSS or CL-DNA complex containing 1.5 or 40.0 μg of pCAL2 (DNA : CL = 1 : 5 (w)) in serum free MEM were given i.p. At 7 days, S-180 cells were recovered from the abdomen of the mouse and luciferase activity was determined.

Luciferase Assay

Luciferase activity was measured using a luciferasc assay system (PicaGene, Toyo Inki Co. Ltd, Tokyo, Japan) and a luminometer (Lumat LB9501, EG & G Berthold, Bad Wildbad, Germany). The activity was indicated as relative light units (RLU) per μg protein or RLU per 35-mm dish.

Cytotoxicity Assay

The amount of extractable protein from the treated cells was measured as an index of cytotoxicity (15). These amounts correlated well with the cell viability, which was measured by using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) (16) (data not shown).

RESULTS AND DISCUSSION

One of the important characteristics of macromoleculc transfer into the cells by FL is the active and rapid absorption onto the cell membrane by HN glycoprotein and the succeeding efficient membrane fusion triggered by F glycoprotein (7,17). In contrast, the gene transfer activity of the CL-DNA complex depends on passive binding to the cell surface and on uptake through non-specific endocytosis by target cells (18). To investigate the effects of these different characteristics on the efficiency of gene transfer, we examined the expression of the firefly luciferase gene transferred by these vectors under various conditions.

First, we examined the effect of the length of the exposure of the vectors to the cells on the gene transfer (Fig. 1). L and HeLa cells were treated either with FL or CL-DNA complex containing 0.5 μg of pCAL2 for 1, 10, or 30 min. Neither FL or CL-DNA complex had any apparent cytotoxicity under these conditions. The cells treated with FL had significant luciferase activity even when they were incubated with the vector for 1–10 min, while they had to be incubated with CL-DNA complex for at least 30 min to have the same level of luciferase activity. When the cells were incubated with these vectors for 10 min, those treated with FL had 4 to 13-fold more luciferase activity than those treated with CL-DNA complex.

We then examined the concentration of the gene transfer vectors required to achieve gene expression (Fig. 2). L cells were treated with various amounts of FL or CL-DNA complex for 90 min. Both FL and CL-DNA complex transferred the luciferase gene in a dose dependent manner and the almost same amount of luciferase was expressed when they were incubated with the cells

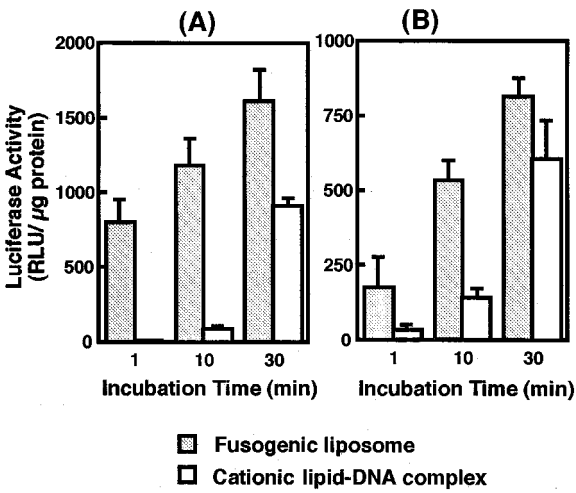

FIG. 1. Effect of exposure on gene transfer activity. L (A) and HeLa (B) cells were incubated with FL containing pCAL2 (OD_{540} of 0.25, 0.5 μg DNA/ml) or complexes of CL and pCAL2 (CL 2.5 μg/ml; DNA 0.5 μg/ml) at 37°C for 1, 10, 30 min. After 48 h in culture, the cells were harvested and luciferase activity was determined as described in Materials and Methods. Each point represents the mean ± S.D. of three experiments for FL and of four experiments for CL-DNA complex.

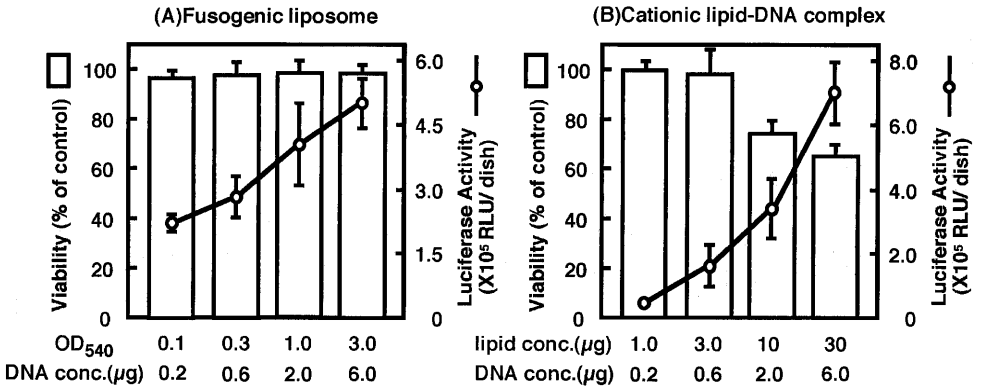

FIG. 2. Effect of the concentration of vectors on gene transfer activity and toxicity. L cells seeded on 35-mm dishes were incubated with various concentrations of FL containing pCAL2 (A) and complexes of CL and pCAL2 (B) at 37°C for 90 min as described in Materials and Methods. After 48 h in culture, the cells were harvested and the luciferase activity and the amount of extractable protein were determined as described in Materials and Methods. Each point represents the mean ± S.D. of three experiments for FL and of four experiments for the CL-DNA complex.

at the highest concentration examined (FL (OD_{540} of 3.0) containing 6.0 μg of DNA per ml; 30 μg of CL containing 6.0 μg of DNA per ml). Gene transfer by FL became saturated under the condition described in Fig. 2 (30 fold increse of DNA gave 2.2 fold increase of luciferase activity), whereas gene transfer by CL-DNA complex did not (30 fold increse of DNA gave 15 fold increase of luciferase activity). In other words, FL transferred the gene more efficiently than CL-DNA complex at a low concentration of DNA. Moreover, FL had no apparent cytotoxicity at the highest concentration examined, whereas CL was acutely cytotoxic at the same concentration of DNA as FL. The cytotoxicity induced by CL-DNA was more obvious 1 week after transfection (data not shown).

We also examined the effect of serum proteins in the incubation medium on the gene transfer (Fig. 3). FL efficiently transferred DNA in the presence of serum proteins. The cells incubated with FL in the presence of 40% FCS still had 70% of the luciferase activity of those incubated with the vector in the absence of serum proteins. On the contrary, serum proteins strongly inhibited the gene

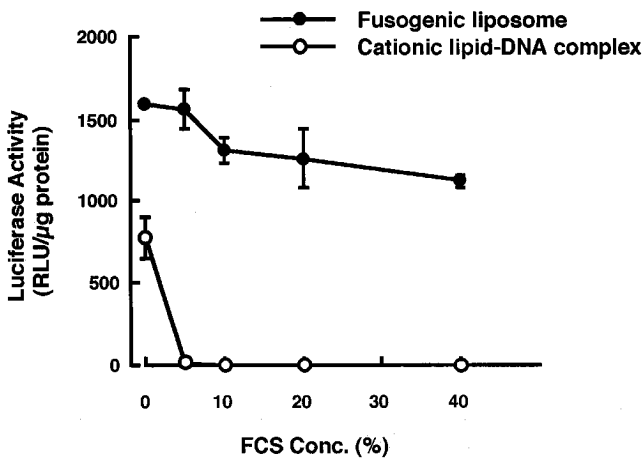

FIG. 3. Effect of serum proteins in the medium on gene transfer activity. L cells were incubated at 37°C for 30 min with a mixture of 0.5 ml of MEM supplemented with 10, 20, 40, 80% FCS, and 0.5 ml of serum-free suspensions of FL containing pCAL2 (OD_{540} of 0.5; 1.0 μg DNA/ml) or complexes of CL and pCAL2 (CL 5.0 μg/ml; DNA 1.0 μg/ml). After culture with fresh medium for 48 h, the cells were harvested and luciferase activity was determined as described in Materials and Methods. Each point represents the mean ± S.D. of four experiments.

TABLE I
Comparison of *in Vivo* Gene Transfer Activity of Fusogenic Liposome and Cationic Lipid–DNA Complex

Vectors	DNA Conc. (μg/mouse)	Luciferase Activity (RLU/10^7 cells)[a)]
Fusogenic liposome	1.5	8013 ± 809
Cationic lipid-DNA complex (DNA:CL = 1:5, w/w)	1.5	4.9 ± 0.7
	40.0	92.2 ± 18.5

S-180 (1×10^6) cells were injected i.p. into male ddY mice at day 0. At day 5, FL containing pCAL2 or CL-DNA complex were given i.p. as described in material and method. At day 7, S-180 cells was recovered and luciferase activity was determined as described in material and method.

[a)]; Means ± S.D.; n = 3.

transfer mediated by CL-DNA complex. The cells incubated with CL-DNA complex in the presence of 5% FCS showed no obvious luciferase activity compared with those incubated with the vector in the absence of serum proteins (Table I).

The sensitivity of CL-DNA complex-mediated gene transfer to serum proteins may be due to the degradation of DNA by nucleases contained in the serum, because DNA formed an unstable complex with CL but was not encapsulated in the CL membrane. In contrast, DNA in FL was encapsulated in the membranous structure and protected from nucleases (5). Another possible explanation is that the absorption to the cell surface and/or the fusogenic activity of CL may be interfered by serum lipoproteins. The resistance of FL-mediated gene transfer to FCS was surprising in another aspect, because serum contains a high concentration of sialic acid, which is a putative Sendai virus receptor, whereas saturation of gene transfer mediated by FL at a higher concentration (Fig. 2) suggested the limited availability of the receptors on cell surface. These data showed that the components containing sialic acid in serum would not compete with cell surface receptors for the binding of FL to the cell membrane.

The rapid and efficient gene transfer mediated by FL shown in Figs. 1 and 2 was clearly due to the active binding and the fusion of FL with the cell membrane. In contrast, CL-DNA complex required a longer incubation period and a higher DNA concentration to facilitate gene transfer. However, the higher the concentration of CL-DNA complex was, the more the cells became damaged. These characteristics of FL-mediated gene transfer, together with resistance to serum components, suggested that FL would be superior to CL-DNA complex, especially for gene transfer into tissue cells *in vivo.*

Therefore, we examined whether unilamellar FL could transfer genes into the cells in living animals. We examined the efficiency of gene transfer into S-180 ascites cells because we found that FL could deliver their contents into these cells in the mouse abdomen (8). When FL containing 1.5 μg of pCAL2 was i.p. injected, a high level of luciferase activity was detected in S-180 cells. In contrast, little luciferase activity was detected when CL-DNA complex containing 1.5 μg of pCAL2 was administered. Even when CL-DNA complex contained 40 μg of pCAL2, only a low level of luciferase activity was detected. The mice administered with FL or CL-DNA complex did not show apparent side effects such as a loss of weight. Taking account of both the level of gene expression and the injected dose, FL mediated the *in vivo* gene transfer over 1000-fold efficiently than CL-DNA complex. These data clearly demonstrated that FL is a unique and efficient non-viral vector for gene transfer *in vitro* and *in vivo.*

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