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A new cationic liposome for efficient gene delivery with serum into cultured human cells: a quantitative analysis using two independent fluorescent probes

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Abstract

Cationic liposomes are useful to transfer genes into eukaryotic cells in vitro and in vivo. However, liposomes with good transfection efficiency are often cytotoxic, and also require serum-free conditions for optimal activity. In this report, we describe a new formulation of cationic liposome containing DC-6–14, *O*,*O*′-ditetradecanoyl-*N*-(α-trimethylammonioacetyl)diethanolamine chloride, dioleoylphosphatidylethanolamine and cholesterol for gene delivery into cultured human cells. This liposome, dispersed in 5% serum-containing growth medium, efficiently delivered a plasmid DNA for GFP (green fluorescent protein) into more than 80% of the cultured human cell hybrids derived from HeLa cells and normal fibroblasts. Flow cytometric analysis revealed that the efficiency of the GFP gene expression was 40–50% in a tumor-suppressed cell hybrid, while it was greatly reduced in the tumorigenic counterpart. The enhanced GFP expression in tumor-suppressed cell hybrids was quantitatively well correlated with a prolonged presence of the plasmid DNA, which had been labeled with another fluorescent probe, ethidium monoazide, within the cells. These results suggest that a newly developed cationic liposome is useful for gene delivery in serum-containing medium into human cells and the stability of the plasmid DNA inside the cell is a crucial step in this liposome-mediated gene expression. The mechanisms by which cationic liposome mediates gene transfer into eukaryotic cells are also discussed.

Keywords: Cationic liposome; Gene delivery; Serum; Human cell

1. Introduction

Since gene delivery has possible applications in gene therapy for various human diseases such as genetic disorders and cancer, attention has been mainly focused on developing efficient and adequate gene delivery systems as well as on elucidating the mechanisms involved in the transfer of genes into cells [1–5]. Much work has been done on delivery systems which utilize a viral or cationic liposomal vector. As vectors, attenuated or defective viruses provide for efficient gene delivery into many cells and tissues

Abbreviations: GFP, green fluorescent protein; EMA, ethidium monoazide; DOPE, L-α-dioleoylphosphatidylethanolamine; DMEM, Dulbecco's modified Eagle's medium

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[4,5]. However, they have serious drawbacks including risk of recombination and strong immunogenicity. Cationic liposomes, in contrast, possess many physical characteristics that make them attractive as candidates for gene delivery vectors [1–3,6]. For example, cationic liposomes have proven safe on repeated in vivo use. In addition, they are suitable for ex vivo protocols. A major limitation with cationic liposomes however, is their low efficiency in transgene expression and their toxicity, although many improvements in cationic lipids and liposome preparations containing DNA have been made [1,3,7]. The presence of serum often reduces the transfection efficiency of liposomal vectors. Several new cationic liposomes useful for serum-containing media have recently been developed [1,3,8]. However, some of them are provided by commercial companies, thus their use in mechanism analysis as well as the modulation of formulations is limited.

Previously, Kikuchi, et al. [9] have reported a series of new cationic liposomes containing *O*,*O*′-ditetradecanoyl-*N*-(α-trimethylammonioacetyl)-diethanolamine chloride (DC-6–14), which have transgene activities in serum-containing medium for human peritoneal disseminated tumors both in vitro and in vivo. However, most of the results are based on qualitative analysis and the activities are varied in their formulations. Thus, factors which control the transgene activity by these liposomes remain largely unknown. In the present report, we further describe that one of these new liposomes composed of DC-6–14, DOPE and cholesterol in molar ratio of 4:3:3, efficiently promotes gene delivery with serum into HeLa-derived human cell hybrids, whose in vivo tumorigenicity may be controlled by a putative tumor suppressor gene on chromosome 11, and the paired combinations of non-tumorigenic and tumorigenic HeLa-derived cell hybrids allow the characterization of cellular changes genetically linked to this putative tumor suppressor function [10–12]. Quantitative analysis of the transgene expression induced by this cationic liposome using this cell hybrid system with two independent fluorescent probes, green fluorescent protein (GFP) and ethidium monoazide (EMA), suggested that difference in the efficiency of the GFP gene expression between human cell hybrids through this liposome vector is well consistent with the stability of the plasmid DNA within the cells. Characteristics of this new cationic liposome are discussed in comparison with other cationic liposomal vectors for gene delivery in vitro and in vivo.

2. Materials and methods

2.1. Chemicals

A humanized (Ser-65 to Thr converted) GFP plasmid, pGreenLantern-1 (pGL-1), with cytomegalovirus promoter was purchased from Gibco-BRL. The plasmids grown in *Escherichia coli* were purified with a Qiagen kit (Qiagen, Hilden, Germany), diluted in distilled water at about 1–2 μg/ml, and stored at −20°C. A fluorescent probe, ethidium monoazide (EMA, Molecular Probes, Eugene, OR) can be photolysed in the presence of nucleotides to yield fluorescently labeled nucleic acids. After its photo-cross-linking to nucleic acids, EMA sends out red fluorescence (600 nm) at a similar wavelength (464 nm) as GFP [13,14]. An approximately 4.76 mM EMA solution and pGL-1 plasmid were used for photolysis. Samples with a 50:1 molar ratio of nucleotide to EMA were individually prepared. After 15 min incubation on ice, photolysis was performed with Sunlamp (Funakoshi, Tokyo, Japan) equipped with a mercury lamp, which had spikes of high emission in its output including a spike at about 370 nm. Samples were then irradiated for 30 min by Sunlamp. To remove intercalated but not covalently bound EMA after photolysis, the ethanol precipitation and washing were repeated three times [15]. The fluorescently labeled plasmid in the bottom layer was dissolved in 100 μl of distilled water and stored at −20°C. The GFP protein with 238 amino acids stably emits green fluorescence at 511 nm when the excitation wavelength is 488 nm [16].

A cationic lipid, *O*,*O*′-ditetradecanoyl-*N*-(α-trimethylammonioacetyl)diethanolamine (DC-6–14) was purchased from Sogo (Tokyo, Japan). L-α-Dioleoylphosphatidylethanolamine (DOPE) and cholesterol were purchased from NOF (Tokyo, Japan) and Sigma (St. Louis, MO, USA), respectively. All other chemicals were of commercial analytical grade.

2.2. Cell culture

The human cell hybrids, CGL1 (non-tumorigenic) and CGL4 (tumorigenic), were derived from the fusion of a HeLa cell line D98/AH2 with a diploid normal human fibroblast line GM77, which had been established by Stanbridge et al. [10]. These cells were grown in Dulbecco's modified Eagle's medium (DMEM, Flow Laboratories, MA) supplemented with 5% (v/v) fetal bovine serum (FBS, Flow Laboratories), penicillin (100 U/ml) and streptomycin (100 μg/ml). Cultures were maintained in 100-mm plastic dishes (Corning, NY) at 37°C in a 5% CO_2/95% air incubator, as described previously [12]. These cells were free from *Mycoplasma* contamination.

2.3. Cationic liposome preparation and transfection

Liposomes were prepared by the method of freeze-dried empty liposomes (FDELs), as described previously [9]. Briefly, DC-6–14, DOPE and cholesterol were dissolved in a chloroform–methanol mixture (4:1, v/v), and the solvent was removed in a rotary evaporator. After mixing of the lipids with 9% sucrose aqueous solution, they were hydrated at 60–70°C and homogenized. To obtain a homogeneous vesicle size distribution, the dispersion was extruded twice at 60–70°C through a polyvinylidenedifluoride membrane filter with 0.22 μm pore size by using Liponizer LP-90 (Nomura Micro Science, Kanagawa, Japan). The dispersion was pipetted into glass vials (0.5 or 2 ml portion each), frozen at −50°C for 4 h and then lyophilized in a freeze-drier (Virtis, New York, USA). The dried liposomes were then reconstituted with 0.5 or 2 ml of distilled water by a gentle mixing prior to transfection.

To investigate the gene expression or transfer efficiency, the cells were seeded at a density of 1×10^5 on 6-well plates (Corning-Coaster, Tokyo, Japan) or 35 mm glass-base dishes (Iwaki Scitech, Japan) with 2 ml of DMEM containing 5% FBS and incubated for 18–24 h at 37°C in 5% CO_2. One hundred microliters of serum-free DMEM or Opti-MEM (Gibco-BRL) containing an appropriate amount of pGL-1 were mixed with 100 μl of serum-free DMEM containing appropriate amount of TFL-08 or 100 μl of Opti-MEM containing 6 μg of Lipofectamine (Gibco-BRL) with mild agitation in polystyrene tubes and incubated for 15 min at room temperature. Prior to transfection, cells were washed with serum-free DMEM and added to 800 μl of DMEM containing 6.25% FBS, serum-free DMEM or Opti-MEM. Then 200 μl of complex solution (a final volume of 1 ml and final concentration of 5% FBS or free of serum) was added. After 5 h incubation at 37°C, the old medium was removed, the cells were washed twice with serum-free DMEM, 2 ml of DMEM containing 5% FBS was added and the culture was continued for 19 h.

2.4. Electron microscopy of cationic liposome–DNA complex

A negative staining technique was used for electron microscopic analysis of the liposome–DNA complex. A drop of complex indicated in the text was placed on a 300 mesh copper grid, and the excess solution was absorbed with a filter paper. A drop of 2% phosphotungstic acid solution was then placed on the grid. This grid was then dried for 5 min under ambient conditions before microscopic analysis. The micrographs of complex were recorded using a transmission electron microscope (Hitachi H-500).

2.5. Analysis by fluorescence microscopy and FACS

To evaluate gene expression efficiency, we used fluorescent microscopy (Diaphoto-TMD, Nikon). The GFP-expressing cells were visualized under the fluorescent microscope without fixation. The green-colored cells in a culture were enumerated and the percentage of the total cell population was calculated. We also used a fluorescein activated cell sorter (FACS, Becton-Dickinson) equipped for fluorescein isothiocyanate detection at an excitation wavelength of 488 nm (an argon laser). The fluorescence intensity of individual cells was measured as relative fluorescence units (RFU). Transfected cells were harvested by incubation with 0.3 ml of 0.25% trypsin and 0.02% EDTA. After incubation, 1 ml of DMEM containing 5% FBS was added and cells were fixed with 1 ml of 4% paraformaldehyde (PFA, Wako, Osaka, Japan) for 30 min at 4°C. After centrifugation at 1000 rpm for 5 min, the supernatant was discarded and the fixed cells were then rinsed with 2 ml of phosphate-buffered solution

(PBS, Gibco-BRL) by centrifugation at 1000 rpm for 5 min. The final pellet was resuspended in 1 ml of PBS containing 0.05% sodium azide and 2 mM EDTA/2Na and delivered to 12×75-mm glass tubes. The fluorescence from approximately 10 000 individual cells collected as list-mode data, which consisted of forward and side scatter, was analyzed (Lysis software, Becton-Dickinson). The percentage of GFP-positive cells was calculated by determining the percentage of highly fluorescent cells with fluorescence emission centered at 530 nm (FL1) and subtracting the fluorescence of control cells that were exposed to the liposome only with a standard gating technique. No electric compensation was performed in these experiments.

2.6. Confocal laser scanning microscopy of the plasmid DNA labeled with a fluorescent probe

To determine the percentages of cells in which fluorescently labeled plasmid had entered, we used a confocal laser scanning microscope (Micro Systems LSM 410, Carl Zeiss, Germany). The plasmid labeled with EMA was most efficiently excited using 488 nm illumination and detected by an LP590 filter. Transfected cells were fixed immediately after transfection at the indicated times (0.5, 1, 2, 4, 8, 12 and 24 h) to retain fluorochrome within the cells. At 0.5, 1, 2 and 4 h after transfection, the cells were thoroughly washed with PBS three times to remove extracellular or membrane-bound plasmid and fixed with 1 ml of 4% PFA for 30 min at 4°C. Then the cells were rinsed with PBS three times for 10 min at 4°C and enclosed with 1 ml of 80% glycerol (Merck, Germany). At 5 h after transfection, the old medium was removed, the cells were washed twice with serum-free DMEM, 2 ml of DMEM containing 5% FBS was added and the mixture was cultured further at 37°C. At 8, 12 and 24 h after transfection, the cells were fixed and enclosed as described above. In each experiment, samples were prepared in triplicate for this determination.

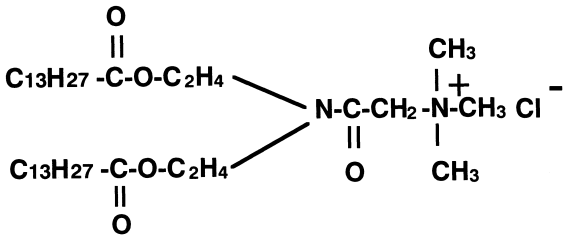

Fig. 1. Chemical structure of DC-6–14, *O*,*O*′-ditetradecanoyl-*N*-(α-trimethylammonioacetyl)diethanolamine chloride.

3. Results

3.1. Flow cytometric analysis of gene transfer mediated by a new cationic liposome containing DC-6–14, DOPE and cholesterol

Since one of the most obvious problems with using cationic liposomes in a gene-delivery system is a low transfection efficiency in serum-containing medium, we have synthesized and tested a series of cationic liposomes which display good lipofection efficiency for cultured cells in the presence of serum. As reported previously [9], most of these initial screenings were evaluated qualitatively based on the transfection efficiency of the transgene product in the whole cell lysate as units/protein amount. From these studies, we have selected a potential formulation composed of DC-6–14, DOPE and cholesterol in the molar ratio 4:3:3, which is now termed TFL-08. As shown in Fig. 1, DC-6–14 is an *N*-α-trimethyl(ammonioacetyl)diethanolamine chloride having two acyl chains. It is an analogue of a cationic liposomal component, TMAG, which is *N*-(α-trimethylammonoacetyl)didodecyl-D-glutamate chloride [6].

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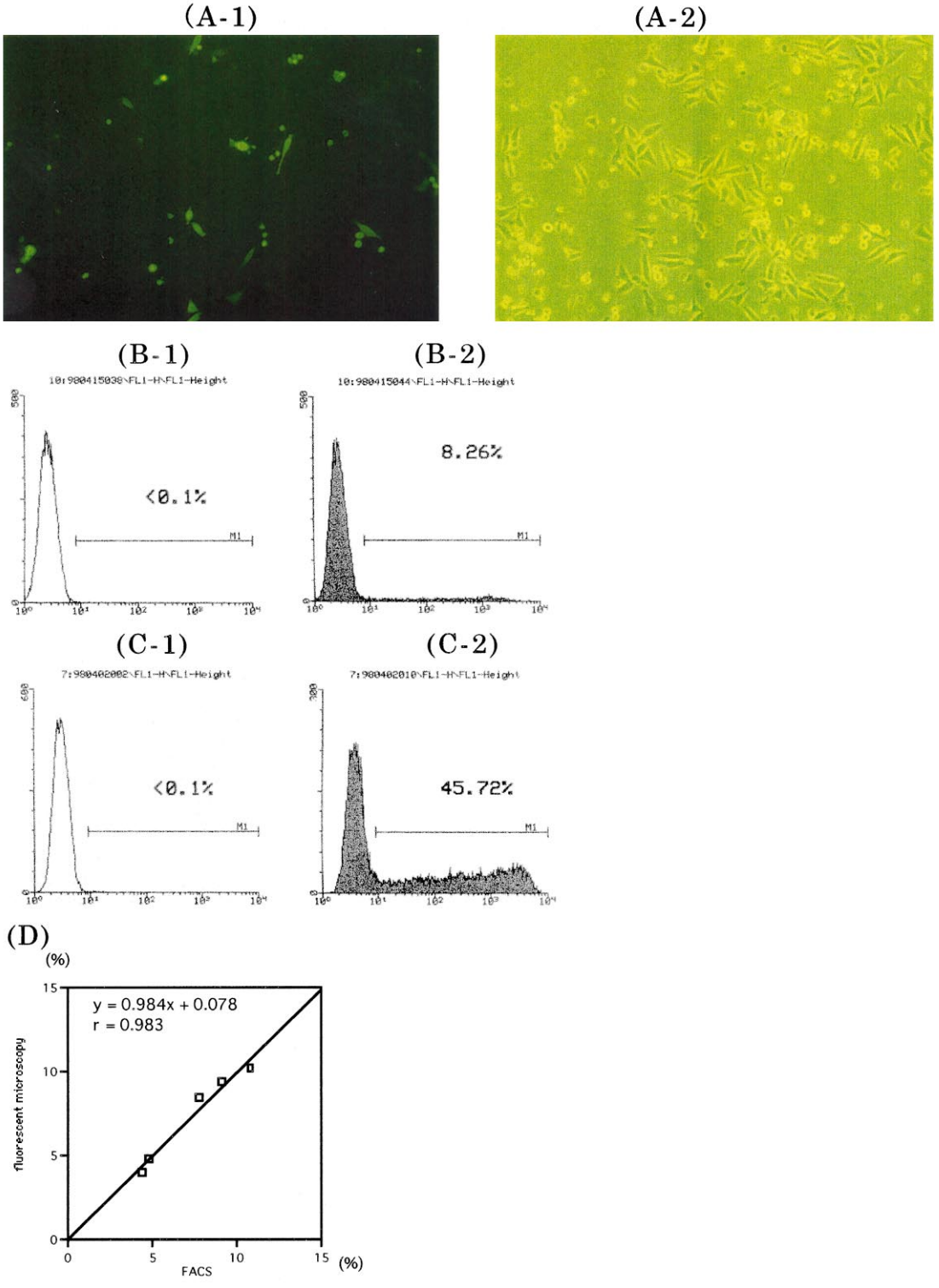
Fig. 2. GFP expression induced by DC-6–14 liposome (TFL-08). A tumorigenic HeLa cell hybrid CGL4 was transfected by the GFP plasmid (pGL-1, 1 μg) complexed with a cationic liposome TFL-08 (8 nmol as the total amount of lipids) in 5% serum-containing medium and the expression was monitored by fluorescent microscopy (A-1). The same field was also photographed by phase-contrast microscopy (A-2). Efficiencies of the transgene expression through TFL-08 liposome were analyzed by FACS as follows: (B-1) liposome alone in CGL4 cells; (B-2) liposome+pGL-1 in CGL4 cells; (C-1) liposome alone in CGL1 cells; (C-2) liposome+pGL-1 in CGL1 cells. In D, a correlation between FACS and fluorescent microscopic analyses is shown. Transfection of pGL-1 into CGL4 cells was performed with various amounts of TFL-08 (2.5–25 nmol) in 5% serum-containing medium and efficiencies of GFP expression were analyzed by FACS and fluorescent microscopy respectively, then were scatterplotted. *r*, value of correlation coefficient.

Unlike TMAG-liposomes or other commercially available liposomes, such as Lipofectamine (Gibco-BRL), TFL-08 contains cholesterol.

To examine the transfection efficiency quantitatively on a cellular basis, we have analyzed TFL-08-mediated gene transfer of the GFP plasmid into a tumorigenic HeLa-derived cell hybrid, CGL4, as a target. Fluorescent microscopy revealed that GFP



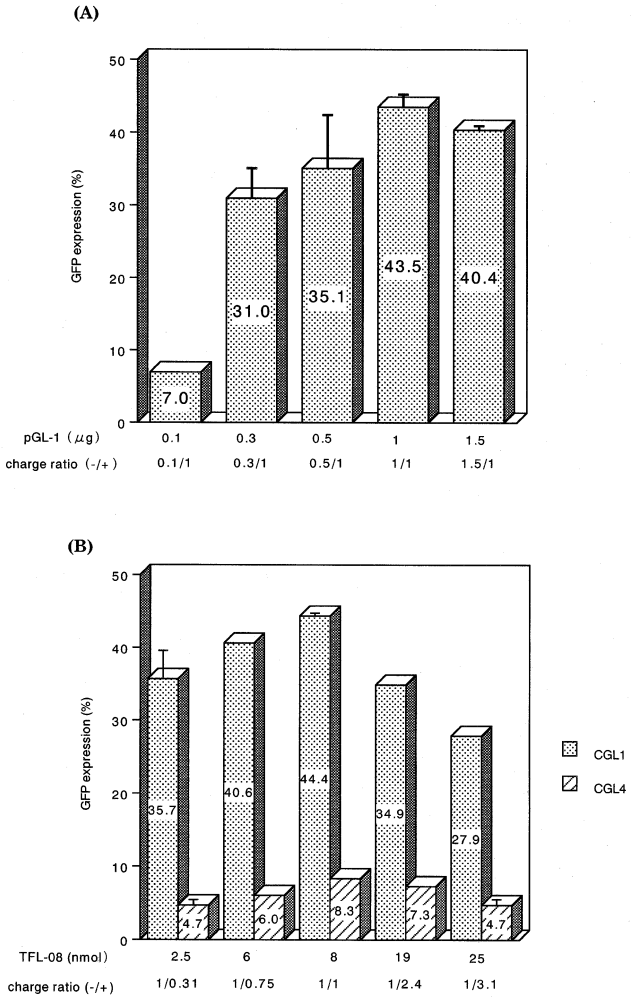


Fig. 3. Optimal conditions for gene transfer by TFL-08 liposome. (A) CGL1 cells were transfected with various amounts of pGL-1 and 8 nmol of TFL-08 in serum-containing medium and GFP expression was analyzed at 24 h after transfection by FACS. (B) Both CGL1 cells and CGL4 cells were similarly transfected with 1 μg of pGL-1 in the presence of the indicated amount of TFL-08 and the efficiencies of the GFP gene expression were determined as described in A. The estimated charge ratios of pGL-1 to TFL-08 liposome used in transfection were given as (−/+).

was expressed in these cells when transfected with TFL-08, but not in the absence of this liposome (Fig. 2A). When this GFP expression in CGL4 cells was monitored by FACS, as little as 10% of the cells were shown to be positive for GFP expression (Fig. 2B). In contrast, a more efficient GFP expression with TFL-08, as much as 45% of the cell population, was observed in the tumor-suppressed HeLa-cell hybrid, CGL1 (Fig. 2C). The transfection efficiencies of the GFP gene with varied amounts of TFL-08 monitored by FACS were quantitatively well correlated with those by fluorescent microscopy (Fig. 2D).

3.2. Optimal conditions for liposome-mediated GFP expression

Since a relatively high transgene expression of GFP through TFL-08 liposome was obtained with CGL1 cells, optimal conditions were further examined by FACS. As shown in Fig. 3, expression of the GFP gene in CGL1 cells was dependent on the plasmid DNA as well as cationic liposome TFL-08 added to the medium containing 5% fetal bovine serum. Similar results were obtained when 10% fetal bovine serum was added to the medium (data not shown). The cationic liposome at 8 nmol (3.2 nmol as a cationic DC-6–14) efficiently delivered 0.5–1.5 μg of the plasmid DNA into the cells (Fig. 3A). The molecular weight of a nucleotide is about 330 Da and hence 1 μg of pGL-1 plasmid should have about 3 nmol of negatively charged molecules. Based on this estimation, charge ratio (−/+) of the plasmid DNA to cationic lipid was indicated in Fig. 3, demonstrating that the charge ratios around neutral range were most effective.

The transgene expression was evident within 5 h

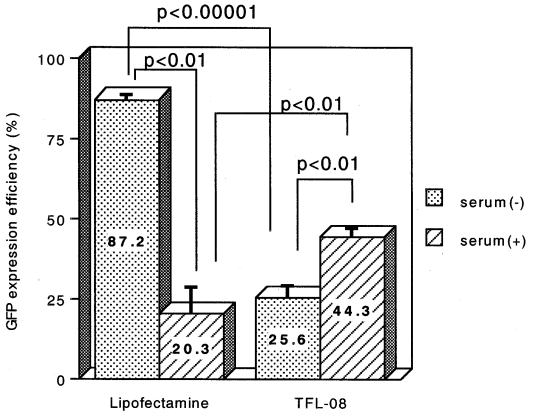


Fig. 4. Effect of serum on transgene expression by cationic liposomes. The expression of pGL-1 gene in CGL1 cells through either Lipofectamine (6 μg) or TFL-08 (8 nmol) in the absence (dotted bars) or presence of 5% fetal bovine serum (hatched bars) was determined by FACS. Results are the mean+S.D. from three or six separate experiments for Lipofectamine and TFL-08, respectively.

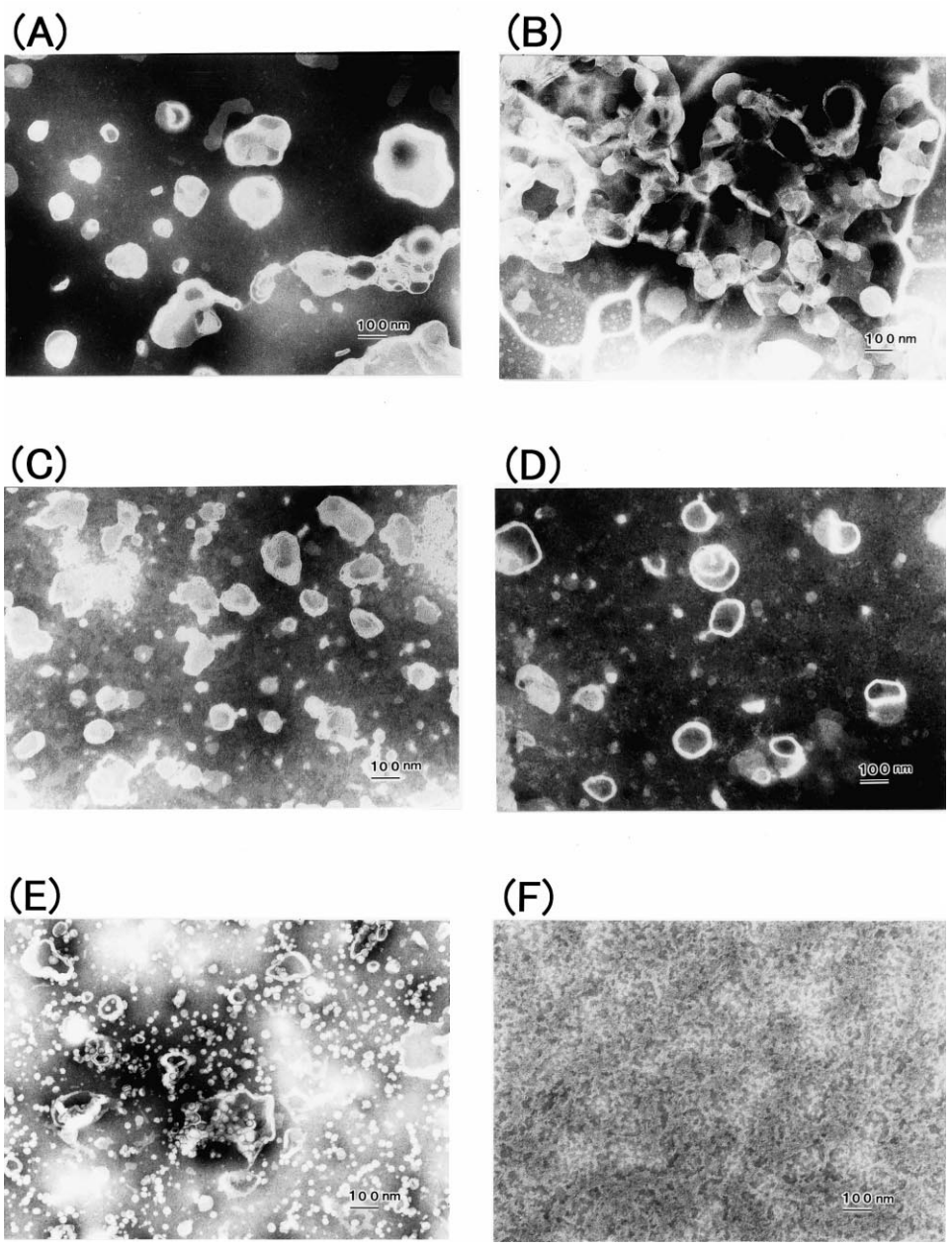


Fig. 5. Electron photomicrographs of cationic liposome–plasmid complex. TFL-08 liposome (8 nmol) was mixed with pGL-1 (1 μg/ml) in DMEM in the presence (C, 15 min; D, 1 h) or absence (B, 15 min) of 5% fetal bovine serum. Then, electron photomicrographs were taken as described in Section 2. An electron photomicrograph of TFL-08 liposome (8 nmol) alone in DMEM for 15 min is shown in (A). Electron photomicrographs of Lipofectamine (6 μg) complexed with pGL-1 (1 μg/ml) in DMEM were taken after incubation for 15 min in the presence (F) or absence (E) of 5% fetal bovine serum.

after lipofection in both CGL1 and CGL4 cells, leading to a maximum expression after transfection for 20–24 h, followed by a gradual decline (data not shown). For 1 μg of plasmid DNA, as low as 2.5 nmol TFL-08 in a charge ratio (−/+) of 1/0.31 was able to deliver the GFP gene into cells with a peak of expression observed at 8 nmol, and a decrease at higher amounts of the liposome (Fig. 3B). This may be partly due to cell damage by this liposome even in the presence of 5% serum, since cell viability

determined by [^{3}H]thymidine uptake at more than 25 nmol TFL-08 was 30–50% of the untreated cell value, while at 5–10 nmol, the viability was kept at more than 80%.

3.3. Effect of serum on TFL-08-mediated gene transfer

Serum often reduces cationic liposome-mediated gene expression in eukaryotic cells [1–3]. As shown in Fig. 4, the transfection activity of the cationic liposome, Lipofectamine, in CGL1 cells was markedly reduced in 5% serum-containing medium. In contrast, the TFL-08-induced GFP expression in CGL1 cells was higher in 5% serum-containing medium than in serum-free medium. This serum effect on cationic lipofection was not obvious in tumorigenic CGL4 cells probably due to the reduced efficiency of the transgene expression (8–10%) in the presence of serum.

3.4. Electron microscopy of TFL-08 liposome

One characteristic of TFL-08 liposome possessing enhanced gene delivery activity in the presence of serum may be its spherical structure in the medium, since preliminary studies by electron microscopy found that the typical spherical structure of several cationic liposomes was often lost in serum-containing medium. Vesicular structures of TFL-08 liposome dispersed in serum-free medium was shown in Fig. 5A. When this liposome was mixed with the GFP plasmid in serum-free medium at the concentrations used for transfection, large aggregates in spherical and granular form were observed (Fig. 5B). In 5% serum-containing medium as used for transfection, this cationic liposome–DNA complex formed rather smaller and uniform vesicular structures with a diameter of 100–200 nm (Fig. 5C), and these vesicular structures were stably maintained for 1 h after the formation of complex (Fig. 5D) and were seen for at least 6 h (data not shown). In contrast, the spherical structure of Lipofectamine seen in serum-free DMEM (Fig. 5E) was completely lost in the serum-containing medium (Fig. 5F).

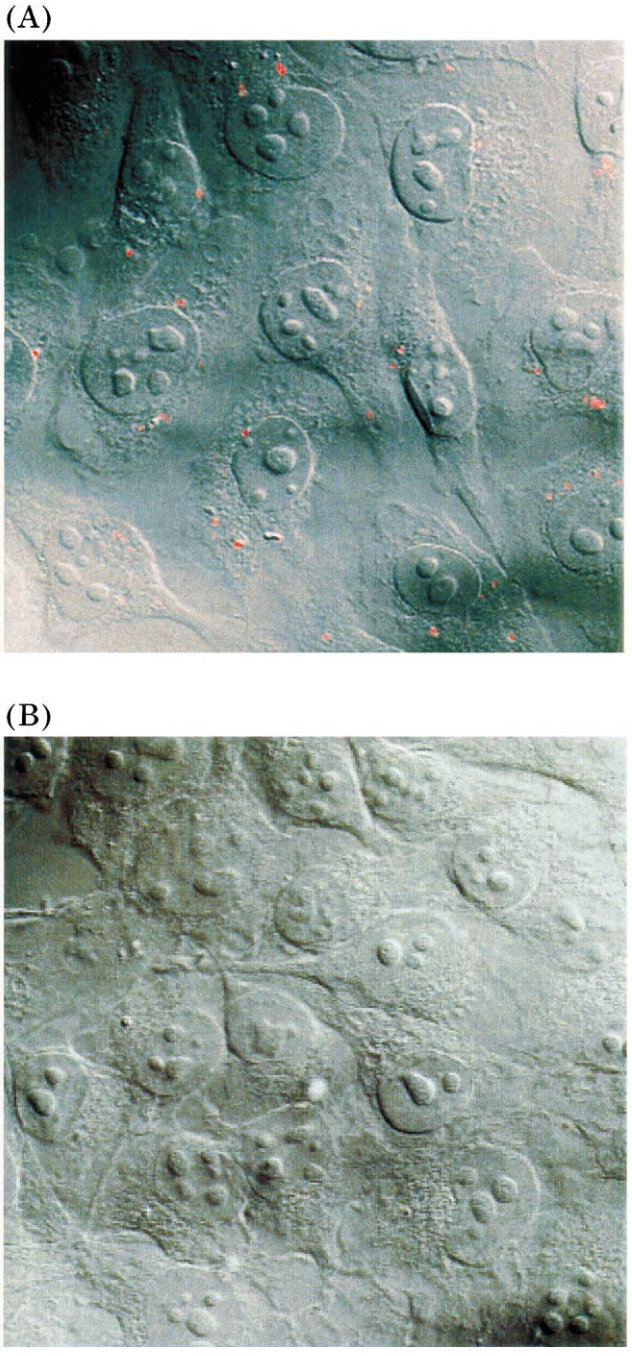


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Fig. 6. Detection of intracellular plasmid DNA labeled with ethidium monoazide (EMA). A HeLa cell hybrid, CGL1, was transfected with EMA-labeled pGL-1 (1 μg/ml) in 5% serum-containing DMEM in the presence (A) or absence (B) of TFL-08 liposome. Intracellular plasmid DNA labeled with EMA was detected by confocal laser scanning microscopy.

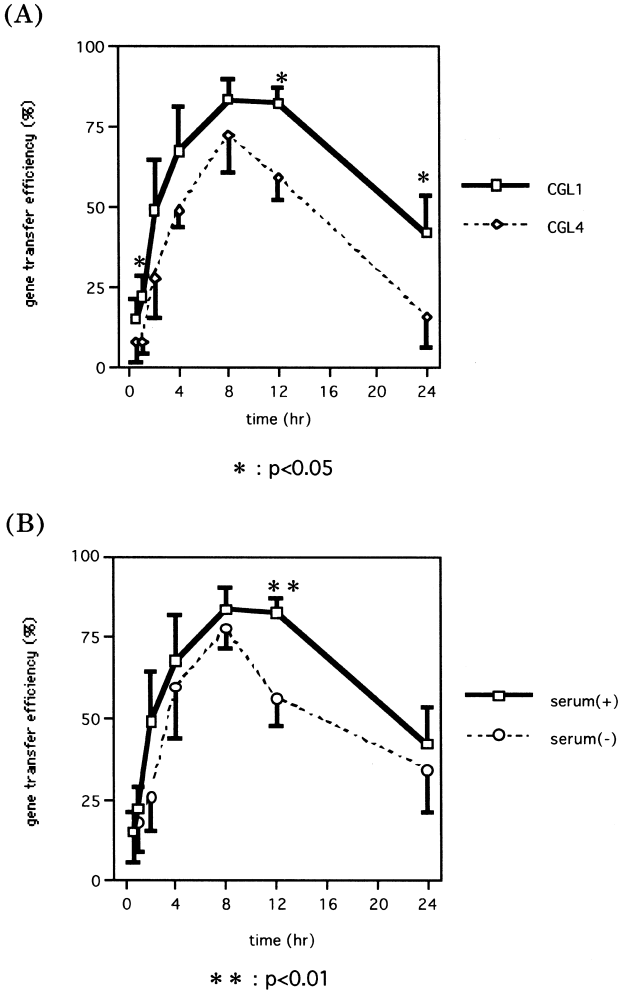

Fig. 7. Changes in intracellular plasmid DNA. (A) CGL1 cells were transfected with EMA-labeled plasmid+TFL-08 liposome as described in Fig. 6 and the level of intracellular plasmid DNA was determined at the indicated times after transfection and was expressed as a percentage of the total cells: 200–300 CGL1 cells (continuous line) or CGL4 cells (broken line). (B) A similar transfection experiment with CGL1 cells was performed in the presence (continuous line) or absence (broken line) of 5% serum.

3.5. *Relationship between the delivered plasmid and transgene expression: detection of a fluorescent-labeled DNA by confocal laser microscopy*

In the present study, the question arose as to how efficiently the TFL-08-mediated transgene expression is controlled; the efficiency was higher in a non-tumorigenic HeLa-cell hybrid CGL1 than in the tumorigenic counterpart, CGL4, and the transgene expression in CGL1 cells was stronger in serum-containing medium than in serum-free medium. These differences could be partly ascribed to the process of encapturation of the liposome–plasmid complex by the cell. To test this, EMA, a fluorescent intercalating probe, was used to label the GFP plasmid DNA covalently by photoactivation. This EMA-labeled plasmid was detected within CGL1 cells by confocal laser scanning microscopy under the conditions in which TFL-08 mediated GFP expression, but not the uninduced condition (Fig. 6). This detection also eliminated the EMA-labeled plasmid adsorbed to the cell surface.

When this lipofection-dependent delivery of the EMA-labeled plasmid into CGL1 cells was examined quantitatively at given times during transfection, the number of cells containing the labeled plasmid linearly increased up to 5–10 h, at which time about 80% of the cell were labeled (Fig. 7A). It was also noted that the labeled plasmid was mostly detectable in the cytoplasm near the nucleus. These labeled plasmids in CGL1 cells gradually disappeared with further incubation, but it remained detectable in about 40% of the cells at 24 h after transfection at which time the level of the transgene expression was highest. In tumorigenic CGL4 cells, a similar incorporation of the EMA-labeled plasmid was observed; the uptake process was not impaired in these cells. However, the labeled plasmid disappeared more rapidly and was detectable only in 20% of CGL4 cells at 24 h after transfection (Fig. 7A). A relatively rapid disappearance of the labeled plasmid was also evident in CGL1 cells when transfection was performed in serum-free medium (Fig. 7B).

4. Discussion

Cationic liposome has proved to be an effective tool for gene delivery both in vitro and in vivo [1–3,6]. Several clinical trials for gene therapy using cationic liposomes are ongoing with human genetic diseases and cancer [17,18]. However, the factors that control cationic liposome–DNA complex (CLDC)-mediated gene transfer remain poorly understood, and results have varied depending not only on the formulation of the cationic liposome, but also on the cell type. In addition, little is known about the mech-

anism of this process, including the uptake as well as targeting and stability of the complex within the cells. Thus, clarification of these factors as well as the molecular events involved in CLDC-based gene delivery are particularly important for regulating and improving the efficiency of gene therapy especially in human diseases.

One of the major shortcomings of this non-viral method of gene transfer is an inhibitory effect of serum [3,19–21]. Since serum contains various types of charged molecules, their binding to CLDC may modify its structural and physical characteristics, rendering it sensitive to nucleases. In some formulations containing a cationic lipid with spermine headgroups [22], the presence of serum makes the CLDC smaller with a reduced transgene activity. These facts led us to search for an ideal formulation of liposome in the complex form with good transgene expression in serum-containing medium [9].

In the present study, on flow cytometry of the GFP gene transfer, we observed that a cationic liposome termed TFL-08, whose formulation is DC-6–14, DOPE and cholesterol in molar ratio 4:3:3, showed fairly good efficiency to transfer the GFP gene into cultured human cell hybrids derived from a human cervical carcinoma HeLa line in the presence of 5–10% fetal bovine serum. In contrast, in a control experiment on transgene expression through Lipofectamine, a well-known cationic liposome, the transfection efficiency was greatly reduced by 5–10% serum (Fig. 4). As previously suggested, one of the reasons for the lower transgene expression in serum-containing medium may be the instability of the liposomal structures [22,23]. In another report, CLDC is often heterogeneous with respect to size and shape, which may influence transfection efficiencies [24,25]. Studies on electron microscopy of TFL-08 liposome demonstrated that the shape and size was homogeneous and the spherical structure was maintained even in serum-containing medium (Fig. 5). In contrast, the globular structures of Lipofectamine rapidly disappeared in serum-containing medium. These facts support that the stability of the spherical/globular structure of the liposome–DNA complex in serum-containing medium is particularly important to the transfection activity of cationic liposomes, which would be potentially useful for in vivo human gene therapy. Just how cholesterol relates to the structure and function of this DC-6–14-liposome is unknown, however, the efficiency of gene expression through DC-6–14 liposomes with different formulations varies among cell types [9]. This issue is further investigated quantitatively.

The physicochemical properties, such as particle sizes and surface charges of the liposome–DNA complex may be also important factors to obtain a higher transfection efficiency of the liposomal vectors. For 1 μg of plasmid DNA which has about 3 nmol of negative charged molecules, 6–8 nmol TFL-08 liposome containing 2.4–3.2 nmol of cationic lipid (DC-6–14) was effective to deliver the GFP gene into cells (Fig. 3). The estimated charge ratio of DNA–liposome complex might be within the ranges of 1:1 under the optimal conditions, suggesting that a neutralized surface charge of the liposome–DNA complex is mostly useful for the in vitro gene transfer, although the charged complex both positively and negatively would also be effective, as described with other cationic liposomes [26,27]. Further studies on the physicochemical properties of TFL-08 liposome–DNA complex and its interaction with cell membranes in serum-containing medium are in progress.

In a non-tumorigenic HeLa cell hybrid, CGL1, the transfection efficiency of TFL-08 liposome was about 50% in serum-containing medium, while in a tumorigenic cell hybrid, CGL4, it was less than 10% in the presence and absence of serum (Fig. 3). This decrease in efficiency may not simply reflect a lower transfection ability of this liposome for tumorigenic human cells, since TFL-08 could transfer genes into several other human tumor cells (unpublished results). Thus, the lower TFL-08-mediated gene expression in tumorigenic HeLa cell hybrids may be associated with the characteristics controlled by a putative tumor suppressor gene [10–12].

To examine the difference in transfection efficiency of TFL-08 between HeLa cell hybrids, the plasmid, photolabeled with EMA and transferred along with liposome into the cells, was quantitatively analyzed by confocal laser microscopy. The percentage of cells containing the labeled plasmid linearly increased with the incubation period, reaching about 80% both for CGL1 and CGL4 cells at 8–12 h after transfection (Fig. 7A). Therefore the transfer of the labeled plasmid into these cell hybrids were similarly enhanced by TFL-08 liposome. The labeled plasmid

was detected in about 40% of cells even after 24 h in the CGL1 cells, whereas in CGL4, which showed a transgene expression as low as 10%, the percentage of EMA-positive cells more rapidly decreased from the peak level and was about 20% at 24 h after transfection. A weaker transgene expression in CGL1 cells in serum-free medium seemed to be consistent with this rapid diminution of the EMA-labeled plasmid (Fig. 7B). These results suggest that the stability of the transferred plasmid within the cells is crucial to the strength and duration of transgene expression and the difference in transfection efficiency between these HeLa cell hybrids with TFL-08 liposome may be at least partly ascribed to the stability of the plasmid in the cells. Recently, the importance of stability in liposome-mediated transgene expression in vivo has also been documented [27].

The transgene seems to degrade in lysosomes or endosomes and this is a common problem when cationic liposomes are used [2,28]. DOPE, a component of TFL-08 liposome, has acted as a helper lipid in various types of liposomes for gene transfection [23,24], although none contained cholesterol. DOPE has been reported to show a strong tendency to form an inverted hexagonal structure at acidic pH and this formation induced by DOPE probably destabilizes endosomes/lysosomes, resulting in escape from lysosomal degradation and cytoplasmic release of the transgene [7,23]. Although it remains unknown whether the efficiency of transgene expression by TFL-08 with serum is directly linked to its stability and the formation of inverted hexagons in lysosomes or endosomes, the present results suggest that a strong, stable expression of the transgene in the target cells is obtained with this new formulation of cationic liposome. Further studies on molecular events underlying transgene expression with these novel cationic liposomes should improve an attractive vehicle for gene delivery to human cells.

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