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DNA transfection mediated by cationic liposomes containing lipopolylysine: characterization and mechanism of action

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Abstract

The ability of a polycationic lipid, lipopoly(L-lysine) (LPLL), to mediate efficient DNA transfection depended on scraping of the treated cells (Zhou et al. (1991) Biochim. Biophys. Acta 1065, 8–14). It was found that the mechanical treatment could be avoided by including a helper lipid to the liposome composition. Among the helper lipids tested, a hexagonal phase forming lipid, dioleoylphosphatidylethanolamine (DOPE), gave rise to the highest activity. The transfection efficiency was further optimized by varying the lipophilicity of the LPLL and the ratio of the cationic liposome to DNA. Transfection activity of the optimal DNA-liposome complexes was enhanced by up to 6-fold if cells were pretreated with agents interfering with the process of endocytosis. Meanwhile, pretreatment of cells with a peptide which inhibits membrane fusion decreased the activity by about 60%. These results indicated that DNA-liposome complexes are taken up by an endocytosis mechanism and that cytoplasmic delivery of DNA involves a fusion-related event probably in the endosome compartment. The transfection process was visualized by thin-section electron microscopy. It was found that the complexes entered the cytoplasm mainly by destabilizing endosomes and occasionally by penetrating through the plasma membrane. Therefore, our findings differ from a previous hypothesis which suggests that transfection is mediated by fusion of the liposomes with the plasma membrane of the treated cells.

Key words: Cationic liposome; Polylysine; DNA transfection; Drug delivery; Gene therapy

1. Introduction

Recent years have seen a rapid development of cationic liposomes [1–9]. This type of reagent provides a simple and efficient means of introducing nucleic acids into living cells. Cationic liposomes are usually used only for in vitro studies because of their sensitivity to the serum components. However, a recent study showed that i.v. injected DNA complexed with lipofectin, a cationic liposome preparation, was capable of mediating DNA transfection in mouse [10]. It is also reported that exogenous DNA can be detected in organs of mice injected with DNA/cationic liposome complexes [11]. It thus appears that cationic liposomes can be used for DNA delivery in animals.

Target-specific DNA carriers are desirable for studies of gene transfer. This is especially the case for human gene therapy. We have previously reasoned [7] that the cationic liposome system can be incorporated with a target-specific ligand for the construction of a targetable and injectable DNA vehicle. To develop such a carrier, we first synthesized a cationic lipid, LPLL [7], which contains multiple primary amino groups for a convenient conjugation of the targeting

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Abbreviations: CAT, chloramphenicol acetyltransferase; DEPE, dielaidoylphosphatidylethanolamine; DMDOPE, *N*-dimethyldioleoylphosphatidylethanolamine; DOPC, dioleoylphosphatidylcholine; DOPE, dioleoylphosphatidylethanolamine; DOPS, dioleoylphosphatidylserine; DOTMA, *N*-1-(2,3-dioleyloxy) propyl]-*N,N,N*-trimethylammonium chloride; DPSG, dipalmitoylsuccinylglycerol; EM, electron microscopy; LPDL, lipopoly(D-lysine); LPLL, lipopoly(L-lysine); MGDG, monogalactosyldiacylglycerol; MMDOPE, *N*-monomethyldioleoylphosphatidylethanolamine; NGPE, *N*-glutaryl-1,2-dioleoylphosphatidylethanolamine; PDL, poly(D-lysine); PLL, poly(L-lysine); Z-Gly-Phe, carbobenzoxyglycyl-L-phenylalanine; Z-Phe-Phe-Gly, carbobenzoxy-D-phenyl-L-phenylglycine.

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ligands. LPLL mediates efficient transfection in mouse L929 cells. Its activity is about 3-fold higher than that of lipofectin under the optimal conditions. Moreover, LPLL is more resistant to the neutralizing effect of serum than lipofectin. However, the transfection activity depends on the mechanical scraping of the treated cells. This requirement severely limits its application in vivo. We have now discovered that this undesirable treatment can be avoided by including a helper lipid to the liposome composition. The transfection condition for this new reagent has been optimized with respect to the transfection activity in mouse L929 cells.

Although it has been repeatedly demonstrated that cationic liposomes mediate efficient DNA transfection in mammalian cells, the mechanism of action remains obscure. It has been hypothesized that plasmid DNA interacts with cationic liposomes such that one single plasmid molecule is trapped in the interior of a complex consisting of four intact liposomes [12]. The DNA-liposome complexes then fuse with the plasma membrane of the treated cells, resulting in the delivery of the exogenous DNA into the cytoplasm [12]. The fusion hypothesis is based on the observations that free and DNA-associated cationic liposomes fuse with negatively charged liposomes and plasma membrane of the treated cells, respectively [12]. However, the fusion activity of cationic liposomes can be abolished by preincubation with DNA [5]. Fusion activities of several different cationic liposomes in the absence or presence of DNA do not correlate with their transfection activities [5]. Thus, the fusion hypothesis has not gained a broad spectrum of experimental support. Moreover, the fusion hypothesis has been seriously questioned in concept. Fusion between a liposome and a cell would lead to joining of their lipid membranes and mixing of their encapsulated aqueous compartment. Thus, DNA adsorbed to the outer surface of a liposome could not be translocated across the membrane, i.e., into the cytoplasm of a cell as the result of a fusion event. To explain the mechanism of transfection, it has been proposed that DNA-liposome complexes after endocytosis destabilize the endocytic vacuole membrane resulting in the release of the exogenous DNA into the cytoplasm [2,5,13].

Using our cationic liposome preparation, we have studied the interaction of DNA-liposome complexes with mouse L929 cells. The results have provided some insight about the mode of entry of DNA into the cytoplasm of the treated cells.

2. Materials and methods

2.1. Materials

Dipalmitoylsuccinylglycerol (DPSG), DOPE, monomethyl DOPE (MMDOPE), dimethyl DOPE (DMDOPE), dielaidoylphosphatidylethanolamine (DEPE), dioleoylphosphatidylcholine (DOPC), dioleoylphosphatidylserine (DOPS), and monogalactosyldiacylglycerol(MGDG) were purchased from Avanti Polar Lipids. Poly(L-lysine) (PLL, M_r 3300), poly(D-lysine) (PDL, M_r 3700), dimethyl sulfoxide, acetyl-CoA, cholesterol, dipalmitoyl glycerol, cytochalasin B, chloroquine, Z-Phe-Phe-Gly, and Z-Gly-Phe were purchased from Sigma. *N*-hydroxysuccinimide, 1,3-dicyclohexylcarbodiimide and triethylamine were from Aldrich. D-*threo*-[1,2-^{14}C]Chloramphenicol (57 mCi/mmol) and [^{14}C]succinic anhydride were from ICN Biomedicals. Ethyl acetate was obtained from Fluka. [^{14}C]DPSG was synthesized from dipalmitoyl glycerol and [^{14}C]succinic anhydride as described [14]. Plasmid pUCSV2CAT (4.8 kb, constructed by Dr. Mark Magnuson of Vanderbilt University) was amplified in *Escherichia coli* and purified by CsCl gradient centrifugation method.

2.2. Cell culture

L929 cells were grown in McCoy's medium supplemented with 10% calf serum. For transfection experiments, cells were seeded one day earlier in 100 mm plates to reach approx. 60% confluency at time of treatment.

2.3. Synthesis of lipopolylysines

LPLL and LPDL were synthesized according to a protocol described previously [7] with modifications: DPSG was activated to the *N*-hydroxysuccinimide ester with 1 molar equivalent each of 1,3-dicyclohexylcarbodiimide and *N*-hydroxysuccinimide in ethyl acetate. The reaction proceeded overnight at room temperature. Crystals of dicyclohexylurea, a reaction by-product, were removed by filtration through glass wool. The filtrate was dried under a stream of nitrogen gas and desiccated. *N*-Hydroxysuccinimide ester of DPSG was recrystallized from methanol/ ethanol/ chloroform (1 : 2 : 2, v/v) at 0°C. LPLL and LPDL were synthesized by mixing PLL or PDL with two molar equivalents of *N*-hydroxysuccinimide ester of DPSG in dimethyl sulfoxide. The reaction proceeded at 50°C for 10 min in the presence of triethylamine which was added at equal weight with the polylysines. Triethylamine was removed by evaporation under a stream of nitrogen gas and desiccation. LPLL and LPDL were dissolved in chloroform and stored at −20°C.

2.4. Transfection

LPLL/DOPE mixture in $CHCl_3$ was dried under a stream of nitrogen gas and desiccated. The lipid film was hydrated briefly in 100 μl sterile distilled water and sonicated in a bath sonicator to clarity. The lipo-

some suspension was combined with pUCSV2CAT DNA diluted to 100 μl with sterile distilled water. The mixture was incubated at room temperature for 15 min before added to cells in 5 ml serum-free medium. In some transfection experiments, cells were also treated with 10 mM NH_4Cl, 25 μg/ml cytochalasin B, 100 μM chloroquine, 100 μM carbobenzoxyglycyl-L-phenylalanine (Z-Gly-Phe) or carbobenzoxy-D-phenyl-L-phenylglycine (Z-Phe-Phe-Gly) 30 min before the addition of liposome/DNA complexes. After 3 h incubation at 37°C, cells were washed and cultured for 2 days. CAT activity in treated cells was assayed as described [7]. 1 U of CAT activity is defined as the conversion of 1 nmol chloramphenicol to its acetylated derivatives per min. Most of the data are expressed as relative activity with 100% activity being that of the cells treated with 285 μg of LPLL/DOPE (1:8) liposomes and 10 (Figs. 1–2) or 15 (Figs. 3–4) μg of DNA for 3 h at 37°C. Under these conditions, the cells usually showed a CAT activity of 26 to 36 mU/mg protein.

2.5. Electron microscopy

L929 cells treated with the DNA-liposome complexes were processed for thin-section EM following protocols described elsewhere [15]. All EM examinations were carried out in a Hitachi H-600 TEM/STEM microscope.

3. Results

Previously, LPLL was synthesized by conjugating *N*-glutaryl-1,2-dioleoylphosphatidylethanolamine (NGPE) to PLL [7]. The negatively charged phosphate group on NGPE was later found to interact non-specifically with PLL which is positively charged. This non-specific interaction interfered with the characterization of the reaction product, LPLL. It also contributed to the problem of aggregation during the formation of DNA/cationic liposome complex. Therefore, NGPE was replaced by DPSG. Like NGPE, DPSG has a terminal carboxylic group which can be activated to react with the amino groups of PLL. Unlike NGPE, DPSG does not carry any other charged groups on the molecule. LPLL and LPDL used in the following studies were synthesized from DPSG and PLL or PDL. The new lipopolylysines still required scraping for efficient DNA delivery. L929 cells receiving scraping or not after incubation with LPLL-DNA complexes expressed 217% and 5% CAT activities, respectively.

3.1. Dependence of the transfection activity of LPLL on the helper lipids

LPLL mediated efficient DNA transfection. However, its activity depended on scraping of the treated cells [7]. It had been shown that other cationic lipids, such as *N*-1-(2,3-dioleyloxy) propyl]-*N,N,N*-trimethylammonium chloride (DOTMA) [1] and 3β-[*N*-(*N'*,*N'*-dimethylaminoethane)carbamoyl] cholesterol [8], required a helper lipid, DOPE, for transfection activity. Helper lipids required for optimal results varied from one cationic lipid to another [5]. To investigate whether the requirement of scraping for the transfection activity of LPLL could be substituted by inclusion of another lipid to the liposome composition, a group of lipids was screened for the helper lipid activity. Liposomes composed of LPLL and various helper lipids (1:8 molar ratio) were tested for their abilities to delivery a marker plasmid, pUCSV2CAT, to the cultured mouse L929 cells. Transfection activity was measured by the level of expression of the exogenous gene coding for the chloramphenicol acetyltransferase (CAT). Fig. 1 shows that combination of DOPE with LPLL gave the highest activity. Interestingly, the increasing extent of methylation on the amino group of DOPE, i.e., MMDOPE, DMDOPE and DOPC progressively decreased the activity. This result suggests that the head group structure of the helper lipid is important for the efficient delivery of DNA. DEPE, which is a trans isomer of DOPE, also gave a reduced activity, indicating that the structure of lipid tail of the helper lipid also plays a role in the determination of transfection activity. Other neutral lipids, MGDG and cholesterol, and negatively charged lipids, DOPS and DPSG, in combination with LPLL were all inactive for transfection.

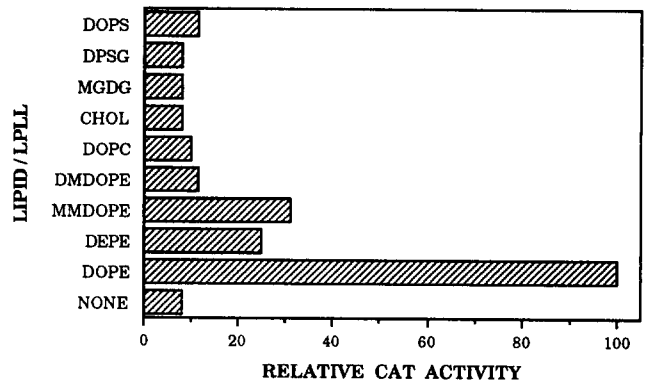

Fig. 1. Dependence of the transfection activity of LPLL on helper lipids. Lipid dispersions were prepared from 122 μg LPLL alone or mixed with eight molar excesses of DOPE, DEPE, MMDOPE, DMDOPE, DOPC, cholesterol, MGDG, DPSG or DOPS. Each of the dispersions was mixed with 10 μg of pUCSV2CAT DNA. The mixtures were used to transfect L929 cells. CAT activity in the treated cells was assayed as described in Materials and methods.

3.2. Effect of the lipophilicity of LPLL on transfection activity

While structure of the helper lipid affected transfection activity of the cationic liposomes, the structure of

LPLL might also be a determining factor. We had speculated that the lipophilicity of LPLL would influence the efficiency of DNA delivery [7]. Thus, a series of LPLL containing up to about three DPSG units per PLL moiety was synthesized and tested for their activities. Fig. 2 shows that conjugates containing an average of about two DPSG molecules per molecule of PLL had the highest activity. Fewer or more lipid units on each PLL chain had resulted in a reduced activity. This result suggests that the conformation of the PLL chain on the liposome surface may affect the transfection efficiency. Unconjugated PLL alone was inactive for transfection (Fig. 2), demonstrating the importance of the lipophilic moiety on PLL for the translocation of plasmid DNA into the cytoplasm of the treated cells. It should be noted that PLL used in this study contained an average of 16 lysine residues and 17 amino groups (16ϵ- and 1 α-amino groups). The optimal conjugate contained about two lipid groups per PLL chain, thus leaving 15 free amino groups for binding with the anionic DNA. Also shown in Fig. 2 is the lack of transfection activity of LPLL of various coupling ratios in the absence of DOPE. Thus, the requirement of a helper lipid is independent of the conjugate structure.

3.3. Optimization of the DNA / liposome ratio

We had previously found that there was an optimal ratio of DNA to free LPLL for transfection [7]. Change of this ratio dramatically decreased the transfection efficiency. Since the DNA carrier used here was LPLL/DOPE liposomes instead of free LPLL, it was important to redetermine the optimal ratio of DNA to the cationic liposome.

A fixed amount of 15 μg pUCSV2CAT DNA was used to complex with various amounts (up to 342 μg)

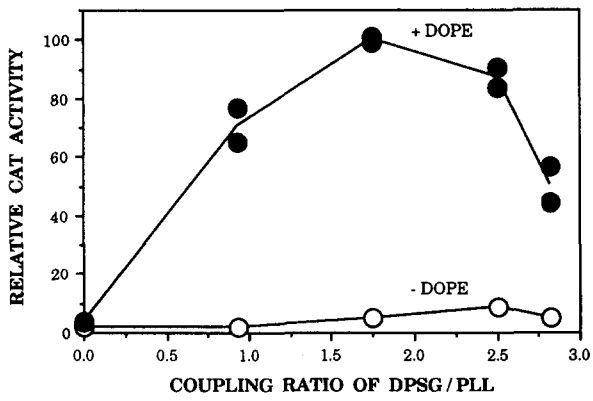

Fig. 2. Effect of coupling ratio of DPSG/PLL on transfection activity. PLL was derivatized with DPSG to different degrees. For the purpose of quantitation, PLL was trace labeled with fluorescamine, DPSG with ^{14}C. Liposomes composed of LPLL alone or mixed with eight molar excess of DOPE were assayed for their transfection activities.

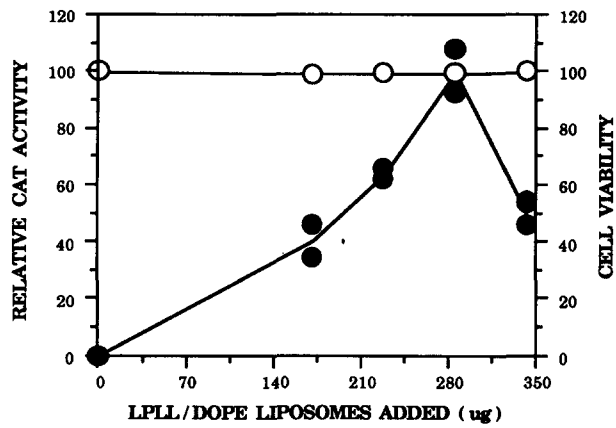

Fig. 3. Effect of liposome dose on transfection activity. Various amounts of liposomes were mixed with a fixed amount of 15 μg pUCSV2CAT DNA. Transfection activity of the complexes (cirf;) was determined. Cell viability (○) was analyzed at the end of transfection procedure by the standard Trypan blue exclusion test.

of liposomes. As shown in Fig. 3, a bell-shaped dose–response curve was obtained for the transfection efficiency of the complexes. The optimal ratio equaled to 15 μg DNA/285 μg liposome. This DNA/liposome ratio was confirmed by a similar experiment in which a fixed amount of 285 μg liposomes was complexed with various amounts of pUCSV2CAT DNA (not shown). The decline of activity at high liposome doses was not due to cytotoxicity to the treated cells; all complexes tested had no detectable cytotoxicity as measured by the Trypan blue exclusion test (Fig. 3).

Under the same conditions cationic liposomes composed of LPDL/DOPE mediated transfection with a similar activity to that of LPLL/DOPE liposomes (data not shown), indicating that no degradation of the polylysine chain is required for the delivery and the subsequent expression of the exogenous DNA. LPDL is not degradable due to the D-isomeric form of the lysine residues.

3.4. Effect of drugs on transfection mediated by DNA-liposome complexes

To investigate the cell uptake and the fate of DNA-liposome complexes, L929 cells were treated with chemical agents interfering with the endocytosis process before exposed to the transfection reagent. Data in Table 1 show that treatment with cytochalasin B enhanced the transfection activity by about 4-fold. NH_4Cl and chloroquine, which increase the intravesicular pH of endosomes, also enhanced the transfection activity by 1.6- and 6-fold, respectively. These results demonstrated that interference of the endocytosis pathway increased the efficiency of cytoplasmic DNA delivery.

Table 1
Effect of cytochalasin B, chloroquine and $NH_{44}Cl$ on transfection activity [a]

Inhibitor	Relative CAT activity
None	94, 106
Cytochalasin B	410, 445
Chloroquine	565, 670
NH_4Cl	160, 165

[a] L929 cells were pretreated with the drugs for 30 min and exposed to mixtures of 10 μg DNA/190 μg liposome for 3 h. At the end of incubation, cells were washed and cultured for 2 days then harvested and assayed for CAT activity.

The DNA-liposome complexes might be translocated into the cytoplasm via a membrane fusion or fusion-related process. Accordingly, an experiment was performed in which cells were exposed to a fusion inhibiting peptide, Z-Phe-Phe-Gly [17], before or after the addition of DNA-liposome complexes. Fig. 4 shows that this peptide inhibited the transfection activity in a time-dependent manner. Pretreatment of cells with Z-Phe-Phe-Gly for 15 min or longer reduced the activity of the complexes by about 60%. Peptide added together with the complexes yielded only about 40% inhibition of the activity; while no significant inhibition was observed if the peptide was added at 15 min or later. A control peptide, Z-Gly-Phe which has no inhibition activity [16], had no effect on transfection at all time points tested. These results indicate that the action of Z-Phe-Phe-Gly was not instantaneous; it required at least approx. 15 min preincubation with cells. Furthermore, the step inhibited by the peptide occurred soon after the addition of the DNA-liposome complex since delayed addition of peptide showed no inhibitory effect.

3.5. Electron microscopic study of complex–cell interaction

To further define the mechanism of DNA delivery, the transfection process in cells treated with the DNA-liposome complexes was visualized by thin-section EM. The DNA-liposome complexes were endocytosed (Fig. 5A) into endosomes (Fig. 5B). Interaction of complexes with cells was also seen at the coated pits (not shown). Thus, internalization of the complexes was mediated by both the receptor and non-receptor-mediated endocytosis. The complexes were identified with their high electron density and doubled-lined structures (arrowhead) as seen in samples containing the complexes alone (not shown). Figs. 5C and D show that the complexes destabilized endosomes. Arrowheads in the micrographs indicate the part of the complex which had intruded into the cytoplasm and was not surrounded by the endosome membrane, while the remaining portion of the complex stayed in the endosome. In 23% of the cells bearing complex-containing endosomes, 15% of the organelles were disrupted. It is interesting to note that most endosomes containing the disrupted morphology located near the cell surface, suggesting that these are early endosomes. Occasionally, complexes were seen penetrating directly through the plasma membrane (Fig. 5E). This event occurred much less frequently (only observed in 0.7% of the cells) compared to the endosome disruption. Complexes were also found to be free and not membrane bound in the cytoplasm (Fig. 5F). These results suggested that the DNA-liposome complexes enter the cytoplasm by destabilizing either the endosome or plasma membrane and that disruption of endosome is the major pathway of entry. Control complexes of DNA with LPLL/DOPC (1:8, molar ratio) liposomes which showed no transfection activity (Fig. 1) were only found associated with the coated pits (Fig. 5G). Thus, they were likely taken up via the receptor-mediated endocytosis pathway. These control complexes mostly remained confined in the endosomes (Fig. 5H); less than 1% of the complex-containing endosomes were destabilized. Pretreatment of cells with chloroquine enhanced the transfection activity of DNA-liposome complexes (Table 1). The drug was assumed to disable the degradative function of the lysosomes or to prevent the fusion of endosomes with the lysosomes [17]. Therefore, it should increase the possibility of the DNA-liposome complexes to escape the endocytic vacuoles. To test this hypothesis, cells were pretreated with chloroquine before being exposed to the complexes. Three representative micrographs of the sample (Figs. 5I–K) showed the process of discharge of the complexes from the swollen endocytic vacuoles into the cytoplasm. In Fig. 5I, the complex appeared to be forced to one side of the endosome probably due to osmotic swelling of the organelle resulting from the

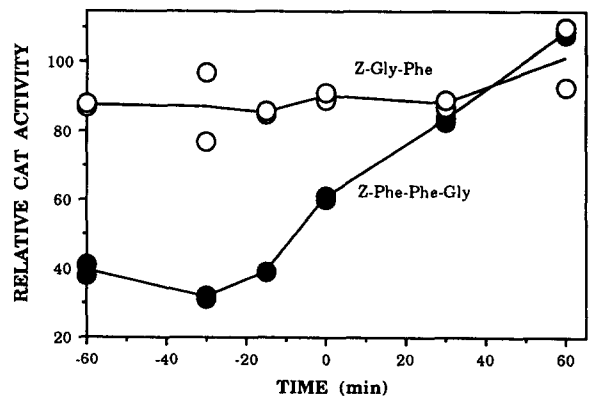

Fig. 4. Effect of a fusion-inhibiting peptide on transfection activity. L929 cells were exposed at time 0 to mixtures of 10 μg DNA/190 μg liposome for 3 h. 100 μM peptides were added at various time points. At the end of incubation, cells were washed and cultured for 2 days then harvested and assayed for CAT activity.

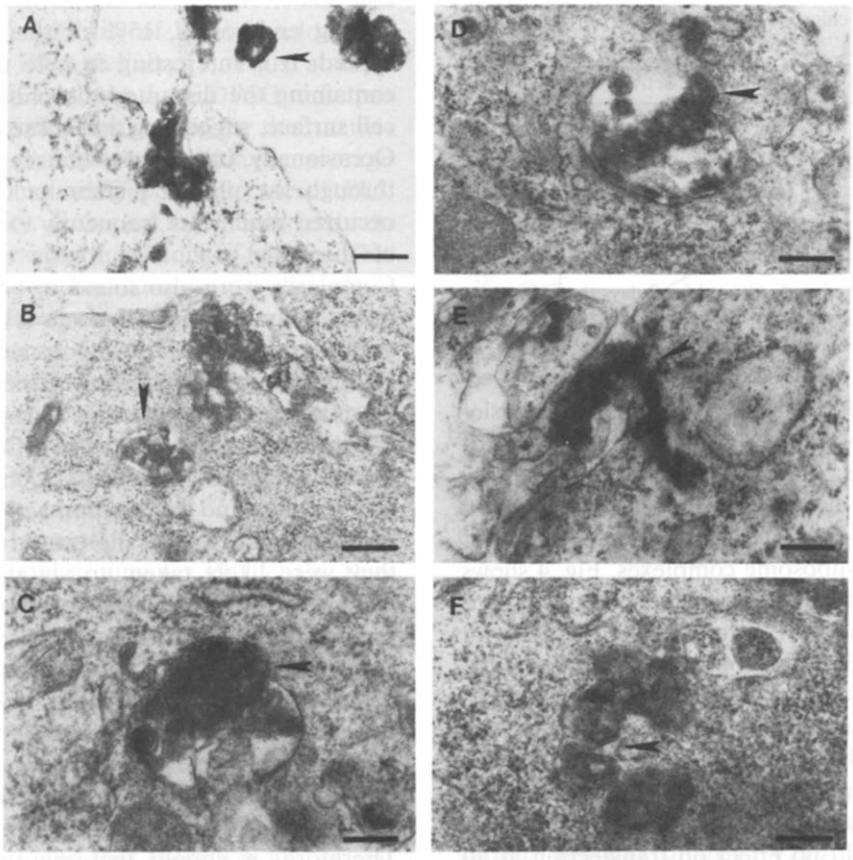

Fig. 5. Thin-section electron micrographs of the L929 cells treated with various DNA-liposome complexes. L929 cells were incubated with DNA-liposome complexes in serum-free medium for 60 min then harvested and prepared for electron microscopy. (A–F) Cells were exposed to the optimal complexes of DNA with LPLL/DOPE (1:8, molar ratio) liposomes. (A) Endocytosis of the complexes. Arrowhead highlights the doubled-line structure of the complex. (B) Internalization of the complexes into endosomes. (C,D) Complex-induced destabilization of endosomes. Arrowheads highlight the part of the complex which had intruded into the cytoplasm and was not surrounded by the endosome membrane. (E) Direct penetration of the complexes through the plasma membrane. (F) Localization of the complexes in the cytoplasm. Arrowhead highlights the doubled-line structure of the complex. (G,H) Cells were exposed to the control complexes of DNA with LPLL/DOPC (1:8, molar ratio) liposomes. (G) Interaction of the control complexes with the coated pits. (H) Confinement of the control complexes in the endosomes. (I–K) Cells were pretreated with chloroquine for 30 min before exposed to the optimal complexes of DNA with LPLL/DOPE (1:8, molar ratio) liposomes. Micrographs showed the process of complex-induced destabilization of endosomes. Arrowhead highlights the intact membrane of the endosome (panel I) and the part of the complex not surrounded by the endosome membrane (panel J,K). Bar represents 0.2 (C,F), 0.25 (A,D,E) and 0.33 μm (B,G–K), respectively.

chloroquine treatment. Endosome membrane in close contact with the complex could be distinguished although not as visible as the remaining portion. Disruption of the membrane would release the complex into the cytoplasm. The complexes shown in Fig. 5J and K (arrowheads) had obviously destabilized the endosome and had partially and almost completely entered the cytoplasm, respectively. 57% of the endosomes observed had been destabilized. This is to be compared with only 15% of endosome destabilization observed in cells not pretreated with chloroquine. Cells pretreated with cytochalasin B were also examined. Only about 2% of the cells showed direct penetration of the complexes through the plasma membrane.

4. Discussion

In an effort to develop a targetable and injectable DNA carrier, we had synthesized a polycationic lipid, LPLL [7]. Free LPLL required scraping of the treated cells for its transfection activity. The mechanical treatment was thought to produce transient membrane damage to the cells to facilitate the entry of the LPLL-DNA complexes into the cytoplasm. In comparison to the free LPLL, some LPLL containing liposomes by themselves mediated efficient transfection. In other words, these liposomes translocated the plasmid DNA into the cytoplasm without the use of mechanical scraping. LPLL mixed with DOPE gave rise to the

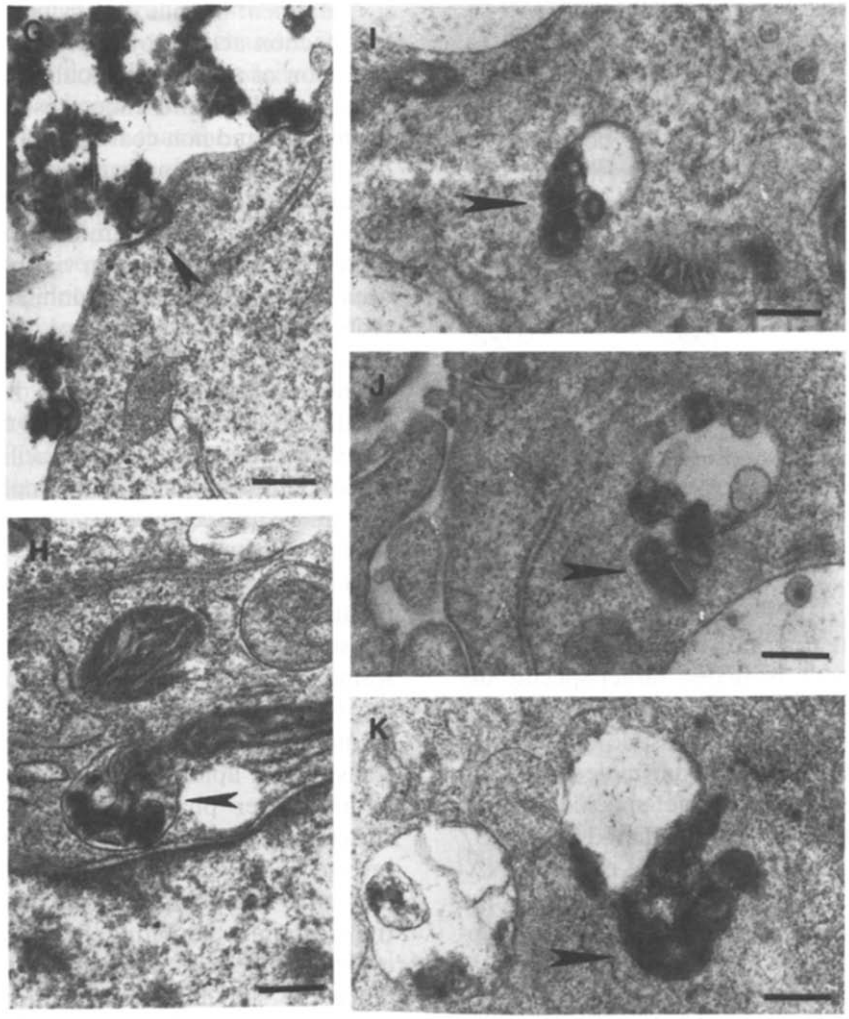

Fig. 5 (continued).

optimal activity (Fig. 1). Interestingly, a number of other cationic lipids when combined with DOPE also transfected cells efficiently [1,2,4–8]. This suggests that various cationic liposome formulations may share a similar mechanism of action.

It was known that DOPE is a hexagonal phase-forming lipid under the physiological conditions. Thus, the process of transfection mediated by the LPLL/DOPE liposomes may involve an event of hexagonal phase formation. This notion is consistent with the observation that helper lipids having less potential to promote the hexagonal phase transition, e.g., DEPE and MMDOPE, etc., had weaker transfection activities when combined with LPLL (Fig. 1).

At the optimal ratio of liposome to DNA (Fig. 3), the complexes had a positive-to-negative charge ratio of about 9. This net positive charge possibly provides the high affinity of the complexes to cell surface which is negatively charged. In this view, it is not difficult to understand why serum [7] and purified polyvalent anions such as salmon sperm DNA and sulfated proteoglycans [12] are potent inhibitors of transfection mediated by cationic liposomes. In comparison to the current reagent, complexes of DNA with other cationic liposomes such as lipofectin [1] and DC-chol/DOPE liposomes [8] only require positive-to-negative charge ratios of approx. 3 to 4 to obtain optimal results. This is probably because the charge density on LPLL/DOPE liposomes is higher than those of other cationic liposome formulations. For example, LPLL/DOPE liposomes and Lipofectin contain 1.7 and 0.5 μmol positive charges per μmol lipid, respectively. Consequently, LPLL/DOPE liposomes are less efficient in neutralizing the negative charges of DNA molecules than other cationic liposomes reported in the literature.

The current LPLL reagent differs from other cationic liposomes in that it carries multiple primary amino groups which would facilitate chemical modifications such as conjugation with carbohydrates, proteins, and other targeting ligands. This is especially important if targeted gene transfer is desired. PLL

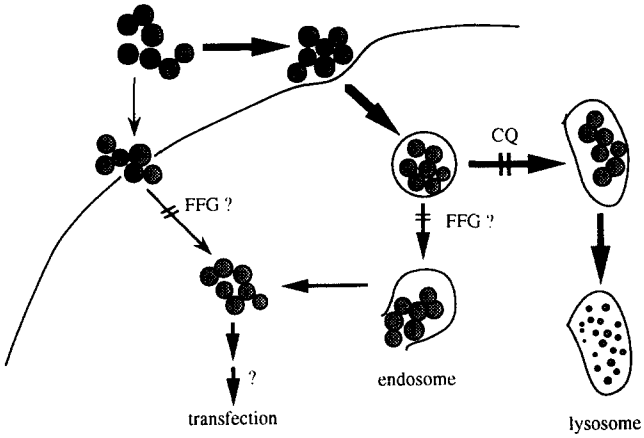

Fig. 6. A model for the DNA delivery mediated by LPLL/DOPE liposomes. Steps with heavy arrows represent the major pathway. CB, cytochalasin B; CQ, chloroquine; FFG, Z-Phe-Phe-Gly. Arrows with double bars indicate the probable step inhibited by various drugs.

derivatized with antibody mediated target-specific delivery of plasmid DNA in both cultured cells and in a mouse model [18]. It is therefore possible that LPLL/DOPE liposomes coated with targeting molecules could mediate DNA transfection in a target-specific manner.

A model for the DNA delivery mediated by LPLL/DOPE liposomes is summarized in Fig. 6. The DNA-liposome complexes either directly penetrate into the cytoplasm through the plasma membrane or are endocytosed into the endosomes. Delivery from the endocytotic vacuoles to the lysosomes results in the degradation of the complexes. Alternatively, the complexes can escape into the cytoplasm by destabilizing the endosomal membrane. Disruption of endosomes is probably the major mechanism by which the complexes enter the cytoplasm, because this event was observed much more frequently than the penetration of the complexes through the plasma membrane in the EM observations.

Data in Table 1 and Fig. 5A clearly show that DNA-liposome complexes are taken up via an endocytosis pathway. Delivery of the complexes from endosomes to lysosomes is not expected to promote transfection. Thus, inhibition of this pathway by chloroquine or NH_4Cl improves the transfection activity (Table 1). We had speculated previously that the complexes destabilized endosome membrane to enter the cytoplasm [7,14]. We have now presented direct evidence to support our speculation (Figs. 5C,D,J,K). The complexes also destabilize plasma membrane to enter the cytoplasm (Fig. 5E). Although in our EM observation it takes place only infrequently, the actual frequency could be higher. This is because direct penetration of the complex could be a faster process than the endosome disruption. Thus, one would not observe as many plasma membrane events.

Treatment of cells with cytochalasin B enhances the transfection activity by approx. 4-fold (Table 1). Interpretation of this result should be cautious. DNA-liposome complexes are endocytosed by cells via both the coated pits and non-coated pits (Fig. 5). Cytochalasin B only blocks the non-coated pits mediated endocytosis which requires intact microfilaments (Refs. 19, 20 and references therein). Therefore, the drug does not completely abolish transfection via the endosomal pathway. Meanwhile cytochalasin B inhibits the assembly of microfilaments in the peripheral cytoplasm of cells, i.e., it disrupts the microfilament network beneath the plasma membrane [21]. The drug also increases the diffusion coefficient in the cytoplasm of the treated cells [22]. Thus, cytochalasin B would facilitate the direct penetration and diffusion of the complex into the cytoplasm and nuclei, respectively, thereby increasing the transfection activity.

It is interesting to observe that a fusion-inhibiting peptide decreases the transfection activity of DNA-liposome complexes (Fig. 4). This peptide inhibits the formation of the inverted hexagonal phase by stabilizing the bilayer phase of membrane lipids [23]. Both membrane fusion and membrane destabilization, a transition of lipid molecules from the bilayer phase to a non-bilayer phase, share a common intermediate structure of inverted micelle which is a precursor of the inverted hexagonal phase [24]. Thus, it is understandable that the peptide shows an inhibitory activity of transfection by inhibiting the membrane destabilization events in the endosome and/or plasma membrane. Since the step inhibited by the peptide occurs soon (within 15 min) after the addition of the DNA-liposome complex (Fig. 4), it is consistent with an inhibitory event at the plasma and/or endosome compartment.

Our current model for the mechanism of transfection mediated by the LPLL/DOPE liposomes is in contrast to a previous hypothesis which suggested membrane fusion is responsible for the cytoplasmic DNA delivery [12]. We have incubated cells with DNA-liposome complexes containing FITC-labeled LPLL and have failed to detect any transfer of FITC-LPLL to the plasma membrane, indicating that liposome-cell fusion was not a major event. Additional evidences had also suggested that membrane fusion is not the common mechanism by which cationic liposomes mediated transfection [5]. For example, preincubation with DNA abolishes the fusion activity of cationic liposomes and that the fusion activities of various cationic liposomes do not correlate with their transfection activities [5]. However, our data support the minor mechanism by which the DNA-liposome complex adsorbs to the cell surface and induces a local destabilization of the plasma membrane, resulting in the translocation of the complex across the membrane

(Fig. 5E). Such localized membrane destabilization is different from the proposed fusion mechanism in that the destabilization event does not necessarily involve the addition of liposomal lipids into the plasma membrane. It should be emphasized that the plasma membrane destabilization mechanism, although only a minor mechanism in the studies described here, could play a more significant role in other cationic liposome systems. For example, a recent comprehensive study comparing pH-sensitive liposomes and a cationic liposome formulation which is commercially available has concluded that a substantial fraction of the DNA entry mechanism mediated by cationic liposomes is endocytosis independent, whereas endocytosis plays a major role for the entry of pH-sensitive liposome mediated DNA delivery [25]. It is interesting to note that similar to LPLL/DOPE liposomes, anionic pH-sensitive liposomes also promoted cytoplasmic delivery of DNA via the destabilization of the endosome membrane [26]. Substitution of DOPE with DOPC, a phospholipid which does not form hexagonal phase, in either the cationic or the anionic liposomes diminished the transfection activity (Fig. 1 and Ref. 26). Both types of liposomes, although carry opposite type of charge, share a similar mechanism of action.

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