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Characterization of the inhibitory effect of PEG-lipid conjugates on the intracellular delivery of plasmid and antisense DNA mediated by cationic lipid liposomes

L.Y. Song, Q.F. Ahkong, Q. Rong, Z. Wang, S. Ansell, M.J. Hope, B. Mui *

Inex Pharmaceuticals Corp., 100-8900 Glenlyon Parkway, Glenlyon Business Park, Burnaby, BC, Canada V5J 5J8

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Abstract

Poly(ethylene glycol)-lipid (PEG-lipid) conjugates are widely used in the field of liposomal drug delivery to provide a polymer coat that can confer favorable pharmacokinetic characteristics on particles in the circulation. More recently these lipids have been employed as an essential component in the self-assembly of cationic and neutral lipids with polynucleic acids to form small, stable lipid/DNA complexes that exhibit long circulation times in vivo and accumulate at sites of disease. However, the presence of a steric barrier lipid might be expected to inhibit the transfection activity of lipid/DNA complexes by reducing particle-membrane contact. In this study we examine what effect varying the size of the hydrophobic anchor and hydrophilic head group of PEG-lipids has on both gene and antisense delivery into cells in culture. Lipid/DNA complexes were made using unilamellar vesicles composed of 5 mole% PEG-lipids in combination with equimolar dioleoylphosphatidylethanolamine and the cationic lipid dioleyldimethylammonium chloride. Using HeLa and HepG2 cells we show that under the conditions employed PEG-lipids had a minimal effect on the binding and subsequent endocytosis of lipid/DNA complexes but they severely inhibited active gene transfer and the endosomal release of antisense oligodeoxynucleotides into the cytoplasm. Decreasing the size of the hydrophobic anchor or the size of the grafted hydrophilic PEG moiety enhanced DNA transfer by the complexes.

Keywords: Poly(ethylene glycol); Cationic liposome; Gene transfer; Antisense; Oligonucleotide

1. Introduction

Poly(ethylene glycol)-lipid (PEG-lipid) conjugates are now a common component of lipid-based drug delivery systems. When incorporated into conventional liposomes, they provide a steric barrier at

Abbreviations: DOPE, dioleoylphosphatidylethanolamine; DSPE, distearoylphosphatidylethanolamine; DODAC, dioleyldimethylammonium chloride; RhoPE, 1,2-dioleoyl-glycero-3-phosphoethanolamine-*N*-(lissamine rhodamine β sulfonyl); PBS, phosphate buffered saline; DMEM, Dulbecco's modified Eagle's medium; MEM, minimum essential medium; DNA, deoxyribonucleic acid; ODN, phosphorothioate oligonucleotide; FITC-ODN, ODN labeled with fluorescein at the 5′ end; PEG, poly(ethylene glycol); Gal-PEG(*n*)-DSPE, galactosylcerebroside-PEG(*n*)-DSPE, where *n* = M.Wt. of PEG; PEG(2000)-CerC_8, 1-*O*-[2′-(ω-methoxypolyethyleneglycol)succinoyl]-2-*N*-octanoylsphingosine; PEG(2000)-CerC_{14}, 1-*O*-[2′-(ω-methoxypolyethyleneglycol)succinoyl]-2-*N*-myristoylsphingosine; PEG(2000)-CerC_{20}, 1-*O*-[2′-(ω-methoxypolyethyleneglycol)succinoyl]-2-*N*-arachidoylsphingosine

* Corresponding author. Fax: 604-419-3201. *E-mail address:* bmui@inexpharm.com (B. Mui).

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the liposomal surface that inhibits opsonization, therefore extending the residence time of liposomes in the blood [1,40]. For liposomes containing drug this maximizes drug delivery to sites of disease where pores and gaps in the endothelium allow carriers to undergo passive extravasation and accumulation in both tumor and inflamed tissues [12,14]. PEG-lipids have also been utilized in the development of targeted liposomes. Low molecular weight ligands such as galactose [32] and folic acid [15] have been conjugated to the end of the polymer to increase binding of PEG-coated drug delivery systems to cell surface receptors. Antibodies and antibody fragments have also been covalently linked to liposomes via PEG-lipids to form immunoliposomes that bind to cells displaying the appropriate antigens [17,11].

Yet another application for PEG-lipids is in the formulation of DNA with cationic lipid vesicles to form lipid-based antisense and gene delivery systems suitable for systemic administration [31,41]. One of the most common lipid-based carriers of polynucleotides are lipid/DNA complexes, sometimes referred to as lipoplexes [5], formed by mixing preformed cationic vesicles with DNA at precise +/− charge ratios. These systems are used extensively as tools for the intracellular delivery of gene constructs, antisense oligodeoxynucleotides (ODN) and ribozymes to cells in culture [23]. Lipid/DNA complexes can also mediate gene transfer in vivo [23,24,37]; however, their inherent instability, rapid clearance, large particle size and toxicity limit their use as a clinically viable intravenous delivery system for DNA. More recently, lipid-based formulations with greatly improved pharmacokinetic characteristics have been developed for plasmids [6,41,46], antisense ODN and ribozymes [31] in which PEG-lipids play an active role in the formulation process by inhibiting excessive aggregation and fusion during the critical self-assembly phase when cationic lipids associate with anionic DNA. These delivery systems are stable, homogeneous particles with diameters on the order of 100 nm that completely protect their polynucleic acid payloads from nuclease degradation in plasma. Furthermore, they exhibit circulation lifetimes that enable intact plasmid, antisense and ribozyme molecules to accumulate at distal sites following systemic administration.

However, in order for lipid-based DNA delivery systems to successfully perturb endosomal membranes and release nucleic acid into the cytoplasm, exogenous lipid must be introduced into the host membrane [39,23]. This occurs through lipid mixing or fusion between the target membrane and lipid/DNA complexes when the latter possesses the appropriate lipid composition and is in sufficiently close apposition to the cell membrane. By definition a steric barrier would be expected to inhibit these processes and therefore reduce transfection activity. In this study we have employed a series of PEG-lipid conjugates in which the size of the polymer head group and amide chain length of the hydrophobic anchors have been varied to determine what effect this has on DNA transfer into cultured cells.

2. Methods

2.1. Materials

HepG2 cells were obtained from ATCC (Rockville, MD, USA) and HeLa cells were a generous gift from Dr. M. Hayden at the University of British Columbia (Vancouver, BC, Canada). Minimal essential medium (MEM) and Dulbecco's modified Eagle's medium (DMEM) were purchased from Stem Cell Technologies (Vancouver, BC, Canada), fetal bovine serum (FBS) and the antibiotic G418 were from Gibco (Burlington, ON, Canada). The plasmid used to generate the stably transfected HeLa cell line contains the luciferase gene cloned into the tIRESNeo plasmid (Clontech, Palo Alto, CA, USA) while the luciferase expression plasmid used for transfection studies in HepG2 cells was a 5650 bp plasmid coding for the luciferase gene under the control of the human CMV immediate early promoter–enhancer element. Both plasmid constructs were provided by Dr. P. Tam (Inex Pharmaceuticals, Burnaby, BC, Canada). Phosphorothioate ODNs were synthesized at the Nucleic Acid and Protein Synthesis unit at the University of British Columbia (Vancouver, BC, Canada) with the following sequences: luciferase antisense ODN (5′-GTCTTCCATGGTGGCTTTACCAACAG-3′), control luciferase (reverse sequence) ODN (5′-GACAACCATTTCGGTGGTACCTTCTG-3′), ODN and 5′-fluorescein-

labeled ODN used for uptake and microscopy studies (5′-CCGTGGTCATGCTCC-3′). The lipids dioleoylphosphatidylethanolamine (DOPE) and 1,2-dioleoyl-glycero-3-phosphoethanolamine-*N*-(lissamine rhodamine β sulfonyl) (RhoPE) were obtained from Northern Lipids (Vancouver, BC, Canada) and Avanti Polar Lipids (Alabaster, AL, USA), respectively.

2.2. Cationic and PEG-lipids

Dioleyldimethylammonium chloride (DODAC) and PEG-lipids were synthesized by Dr. S. Ansell and Z. Wang, respectively, at Inex Pharmaceuticals.

2.3. Cell culture

HepG2 cells were cultured in MEM containing 10% FBS. HeLa cells were grown in DMEM containing 10% FBS, 100 units/ml penicillin, 0.1 mg/ml streptomycin and 200 μg/ml G418.

2.4. HeLa-luciferase (HeLa-Luc) stable cell line

HeLa cells were stably transfected with the tIRESNeo plasmid containing the luciferase reporter gene using complexes made with DODAC/DOPE vesicles. Cells were exposed to vesicle/DNA complexes for 48 h, at which time the medium was replaced with normal growth medium containing 500 μg/ml G418 (Gibco). Antibiotic resistant colonies were expanded and selected for high luciferase protein expression after 2 weeks.

2.5. Vesicles

Lipids were mixed in chloroform and dried under a stream of nitrogen gas. The thin lipid film was lyophilized under vacuum for >2 h to remove residual solvent before hydration with distilled water to form multilamellar vesicles. The vesicles were freeze-thawed five times utilizing liquid nitrogen and warm water cycles and then extruded through 0.1 μm pore size polycarbonate filters using an Extruder (Lipex Biomembranes, Vancouver, BC, Canada) to generate large unilamellar vesicles [9]. Vesicles were made composed of DODAC/DOPE (1:1, mole ratio), DODAC/DOPE/PEG-lipid (47.5:47.5:5, mole ratio), DODAC/DOPE/RhoPE (49.5:49.5:1, mole ratio) and DODAC/DOPE/PEG-lipid/RhoPE (47:47:5:1, mole ratio).

2.6. Formulation of lipid/DNA complexes

Vesicle/plasmid or vesicle/ODN complexes were prepared in polystyrene tubes 10–20 min before use by quickly mixing and vortexing an equal volume of the DODAC-containing vesicles with an equal volume of plasmid DNA or ODN in distilled water. The relative proportions of lipid and nucleic acid are expressed in terms of a +/− charge ratio calculated by assuming a mean molecular weight for a phosphodiester (DNA) and phosphorothioate (ODN) nucleotide sodium salt of 325 and 357, respectively. One nucleotide base carries one negative charge while DODAC has one positive charge. An optimum +/− charge ratio of 1 and 1.5 was used for complexes made with DNA and ODN, respectively.

2.7. Luciferase transfection of HepG2 cells

HepG2 cells, plated overnight in 96-well plates at 2×10^4 cells per well, were incubated with 50 μl of lipid/DNA complexes (0.5 μg DNA/well) and 150 μl of normal growth medium. The cells were incubated at 37°C with 5% CO_2 for 24 h and then washed twice with 150 μl phosphate buffered saline (PBS). After removing the PBS, the plate was stored at −70°C for up to 2 weeks before being assayed.

2.8. Luciferase assay

The cells were thawed and solubilized by addition of 40 μl of lysis buffer (0.1% Triton X-100) and the plate was shaken at room temperature for 15 min. An aliquot of the cell lysate (20 μl) was removed and assayed for luciferase expression using a commercial kit (Promega, Madison, WI, USA). Fluorescent signal was quantified using a ML 3000 microtiter plate luminometer. The remainder of the cell lysate in the well was used for assaying cellular content using a BCA protein assay (Sigma, Oakville, ON, Canada).

2.9. Uptake of RhoPE-labeled vesicles and FITC-ODN by HepG2 cells incubated with lipid/DNA complexes

HepG2 cells, plated overnight in 96-well plates at 5×10^4 cells per well, were incubated with 40 μl of the lipid/DNA complexes in 120 μl of medium (final ODN concentration of 0.02 ng/μl or 280 nM). After 24 h, the cells were washed with PBS and lysed with 0.6% Triton X-100. Uptake of the fluorescent labels was quantified using a Biolumin 960 fluorescence plate reader (Molecular Dynamics, Sunnyvale, CA, USA) at excitation and emission wavelengths of 485 and 520 nm, respectively, for FITC-ODN (ODN labeled with fluorescein at the 5′ end) and 520 and 595 nm for RhoPE. Vesicle and ODN uptake were determined separately using RhoPE-labeled complexes or lipid/FITC-ODN complexes to minimize fluorescence overlap of the fluorophores. The results are presented as a percentage of what was added to each well taking into account background light scattering from untreated cells.

2.10. Antisense activity as measured by luciferase expression in HeLa-Luc cells

A 50 μl aliquot (3.6 ng/μl or 500 nM ODN final) was added to 150 μl of fresh medium and incubated with HeLa-Luc cells (plated overnight in 96-well plates at 1.5×10^4 cells per well). After 24 h, luciferase protein expression was assayed as outlined above.

2.11. Fluorescent microscopy

HepG2 cells, plated overnight on glass coverslips in 24-well plates at 2×10^5 cells per well, were exposed to RhoPE-labeled lipid/FITC-ODN complexes (final concentration of 2 ng/μl ODN). At various times, unbound complexes were removed, cells washed with PBS and fixed in PBS, pH 7.4, containing 3% paraformaldehyde. The coverslips were mounted on glass slides and visualized either using a Bio-Rad 600 confocal microscope or a Nikon Diaphot 300 epifluorescence microscope.

3. Results

3.1. Effect of PEG-lipids on DODAC/DOPE mediated transfection of HepG2 cells

Lipid/DNA complexes prepared by mixing unilamellar DODAC/DOPE vesicles with plasmid DNA transfect a wide variety of cells in vitro [10,22,23]. For this study HepG2 cells were transfected with a luciferase reporter gene using lipid/DNA complexes prepared at a +/− charge ratio of 1, which was previously determined to be optimal for these cells and plasmid batch (data not shown). After 24 h extensive luciferase expression was measured in cells treated with PEG-free lipid/DNA complexes (Fig. 1). However, incorporating 5 mole% PEG-lipid into the complexes profoundly inhibited transfection. In one set of experiments distearoylphosphatidylethanolamine (DSPE) PEG-lipids were employed in which a terminal galactose group was attached to the PEG (Gal-PEG-DSPE) in an attempt to enhance the cellular uptake of complexes by endocytosis via galactose receptors [32,13,43]. The presence of a large PEG moiety (M.Wt. 3400) completely blocked transfection (Fig. 1). Reducing the size of the PEG head group to

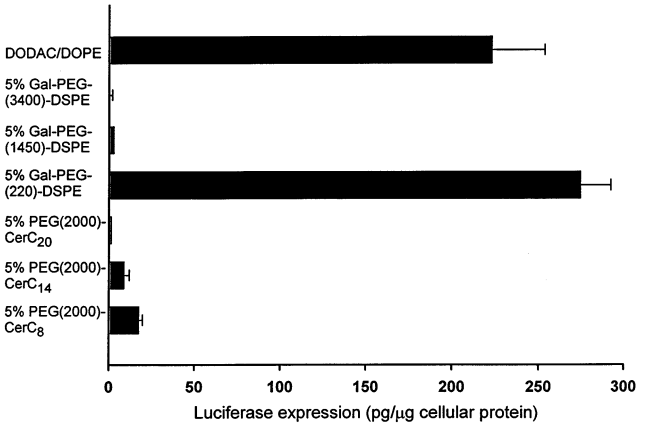

Fig. 1. Effect of PEG-lipid conjugates on luciferase transfection mediated by DODAC/DOPE/PEG vesicles in HepG2 cells. DODAC/DOPE (1:1, mole ratio) or DODAC/DOPE/PEG-lipid (47.5:47.5:5, mole ratio) vesicles were mixed with 0.5 μg of pINEXL002 and the resulting lipid/DNA complexes (50 μl) were incubated with the HepG2 cells, plated in 96-well plates at 2×10^4 cells/well in the presence of 0.2 ml medium. After 24 h, the cells were assayed for luciferase protein activity as outlined in Section 2. Each data point represents an average of four samples ± S.D.

1450 molecular weight resulted in a small but measurable level of luciferase expression while a further reduction in PEG molecular weight to 220 restored transfection to the same levels achieved using PEG-free lipid/DNA complexes. Another series of PEG-lipids (but minus galactose) were synthesized using ceramide rather than DSPE as the lipid core (PEG-Cer). Both DSPE and ceramide based PEG-lipids have been shown previously to exhibit similar steric barrier properties when associated with liposomes [40]. Galactose was not incorporated because we did not find a significant enhancement in uptake by targeting the galactose receptor (data not shown). For this series of PEG-lipids the PEG head group was kept constant at a M.Wt. of 2000, a size known to act as an effective steric barrier [40], but the hydrophobicity of the anchor was varied by changing the length of the amide chain linked to the 2-position of the sphingosine backbone. Complete inhibition of active gene transfer was observed for 1-*O*-[2′-(ω-methoxypolyethyleneglycol)succinoyl]-2-*N*-myristoyl-sphingosine (PEG(2000)-CerC_{20}), in which the amide chain was 20 carbons long, whereas a small but significant increase in gene expression was measurable when the length of the chain was progressively decreased to C_{14} and C_8 (Fig. 1).

3.2. *Effect of PEG-lipids in DODAC/DOPE mediated delivery of antisense into HeLa cells*

HeLa cells stably transfected with the luciferase gene (HeLa-Luc cells) were also used to measure the effect of PEG-lipids on the activity of an antisense oligonucleotide delivered by cationic lipid vesicles. Two antisense ODN were employed, a phosphorothioate (PS) 26-mer ODN complementary to the 5′ start region of the luciferase gene and a control ODN with the same base composition but in reverse sequence. The mechanism of action for phosphorothioate antisense ODN is mainly through the activation of RNase H, which recognizes PS ODN/RNA heterodimers as a substrate and cleaves the RNA strand [2]. Therefore successful delivery of active ODN to the cytoplasm and/or nucleus and subsequent binding to target mRNA should reduce the concentration of luciferase mRNA and subsequently luciferase protein compared to controls.

Lipid/ODN complexes, free ODN or cationic vesicles alone were incubated with HeLa-Luc cells in the presence of 10% serum. Under these conditions range-finding studies with lipid/ODN complexes indicated that the maximum antisense effect occurs over a broad range of +/− charge ratios, from 1.0 to 2.5 (data not shown). Consequently, these experiments were conducted using complexes

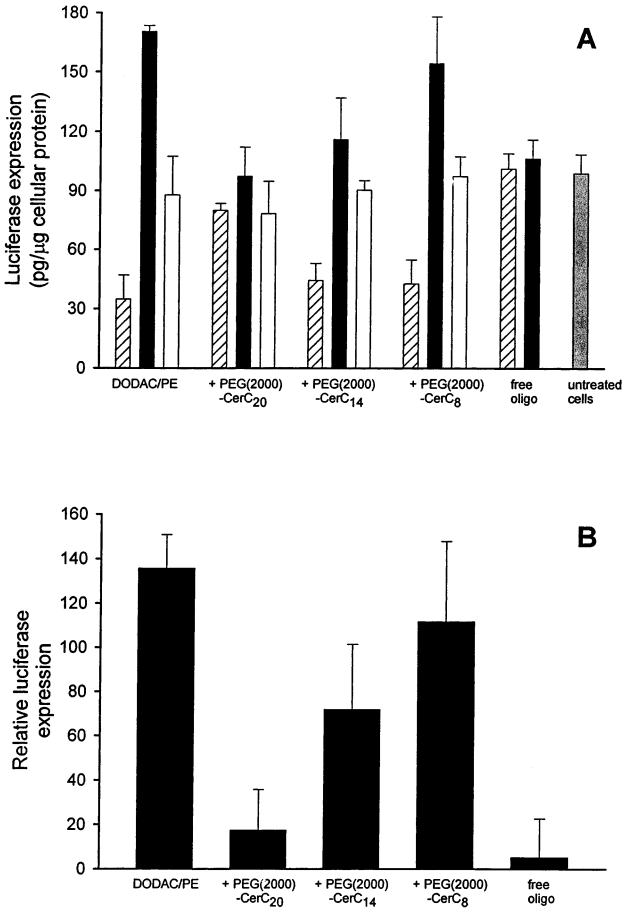

Fig. 2. Antisense activity mediated by cationic vesicle/ODN complexes in HeLa cells stably transfected with a luciferase gene. (A) ODNs either complementary to the 5′ start sequence of the luciferase gene (hatched white bars) or containing a reverse sequence (black bars) were mixed with DODAC/DOPE (1:1, mole ratio) or DODAC/DOPE/PEG-lipid (47.5:47.5:5, mole ratio) vesicles. An aliquot of the vesicle/ODN complexes (50 μl, 350 nM ODN) was incubated with HeLa-Luc cells (plated in 96-well plates at 1.5×10^4 cells/well) in 0.2 ml of medium. After 24 h, luciferase activity was assayed as outlined in Section 2. The white bars represent cells which were exposed to the vesicles alone while the gray bar are untreated cells. (B) The difference in luciferase expression between HeLa cells exposed to the active luciferase antisense ODN and the reverse ODN was calculated from the data shown in A. Each data point represents an average of four samples ± S.D.

made at a +/− charge ratio of 1.5. After 24 h, the cells were assayed for luciferase activity as outlined in Section 2. Neither cationic vesicles alone nor free ODN had a significant effect on luciferase activity compared to untreated cells (Fig. 2A). However, exposure of the cells to lipid/ODN complexes made with the active antisense ODN resulted in a significant reduction in luciferase expression relative to untreated cells. Interestingly, successful delivery of the control ODN sequence had a stimulatory effect on luciferase expression. Cytoplasmic delivery of PS ODN has been shown to non-specifically stimulate gene expression for a variety of target proteins [4] and increased mRNA levels are commonly observed when using antisense targeting the luciferase gene (T. Woolf, personal communication). The effect is thought to arise through the non-specific binding of transcription factors with the PS backbone [2].

In Fig. 2B the data are expressed as the difference in luciferase expression between cells exposed to the antisense and control ODN. When plotted this way a pattern of activity is seen more clearly as being similar to that observed for plasmid transfection (Fig. 1). Incorporating 5 mole% PEG(2000)-CerC_{20} reduced the relative expression of luciferase by $>80\%$. However, this level of inhibition was significantly reduced as the amide chain length at the 2-position of the ceramide backbone was decreased from C_{20} through C_{14} to C_8 (Fig. 2B).

3.3. Binding of lipid/ODN complexes to cells and endocytosis

One explanation for the inhibitory effect of PEG-lipids on DNA transfer could be that cellular uptake of the complexes is reduced through steric hindrance of binding to the cell surface. In order to address this a fluorescently labeled ODN was used to determine if nucleic acid uptake (binding and endocytosis) mediated by DODAC/DOPE complexes is affected by the presence of PEG-lipids. Complexes were prepared with FITC-ODN and incubated with HepG2 cells. At various times, unbound lipid/ODN complexes were removed, the cells washed with PBS and cellular fluorescence measured after lysis with Triton X-100. Over a 21 h time course only minor differences in cellular uptake of FITC-ODN mediated by PEG-

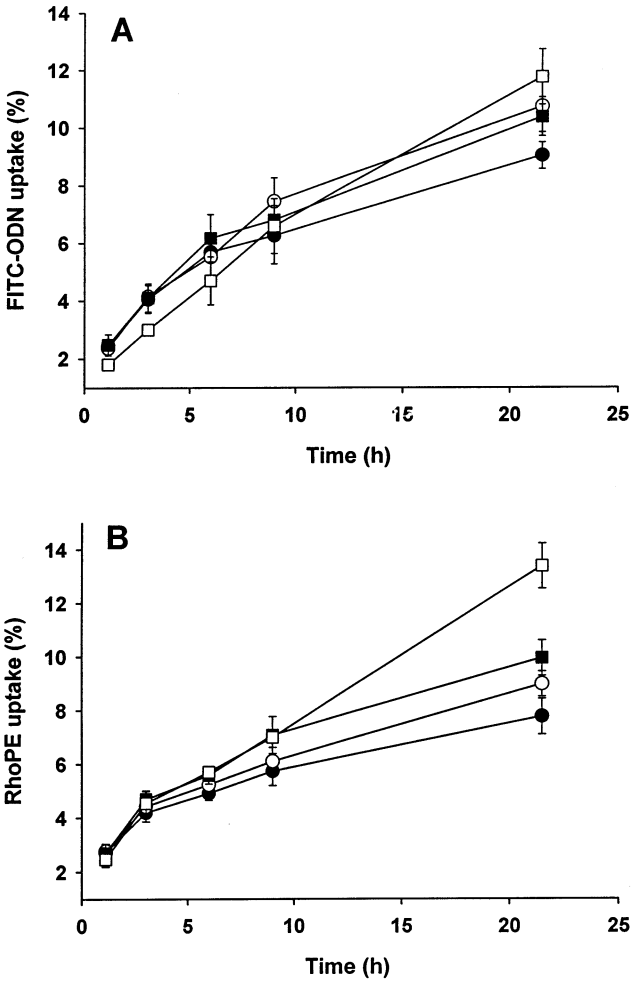

Fig. 3. Uptake of RhoPE-labeled vesicles and FITC-ODN by HepG2 cells incubated with vesicle/ODN complexes. (A) DODAC/DOPE (●), DODAC/DOPE/PEG(2000)-CerC_8 (○), DODAC/DOPE/PEG(2000)-CerC_{14} (■) or DODAC/DOPE/PEG(2000)-CerC_{20} (□) was mixed with plasmid DNA as in Fig. 1. An aliquot (40 μl) of the lipid/ODN complexes was incubated with the HepG2 cells, plated overnight in 96-well plates at 5×10^4 cells/well, in the presence of 120 μl of MEM containing 10% FBS. After 24 h, the cells were washed with PBS and lysed with PBS containing 0.6% Triton X-100. Uptake of the fluorescent labels was quantified using a fluorescence plate reader (Biolumin 960, Molecular Dynamics) at excitation and emission wavelengths of 485 and 520 nm, respectively. (B) In a parallel experiment, vesicle uptake by HepG2 cells incubated with the lipid/ODN complexes was determined using vesicles labeled with 1 mole% RhoPE. Each data point represents an average of four samples ±S.D.

free complexes or complexes containing 5 mole% 1-*O*-[2′-(ω-methoxypolyethyleneglycol)succinoyl]-2-*N*-octanoylsphingosine (PEG(2000)-CerC_8), 1-*O*-[2′-(ω-

methoxypolyethyleneglycol)succinoyl]-2-*N*-myristoylsphingosine (PEG(2000)-CerC_{14}), or PEG(2000)-CerC_{20} were measured (Fig. 3A). Similar results were obtained in a parallel experiment in which cellular uptake of the lipid carrier was measured using RhoPE as a fluorescent lipid marker. Vesicles containing 1 mole% RhoPE were employed to prepare complexes with ODN and incubated with HepG2 cells as above (Fig. 3B). As with the previous study only relatively minor differences between samples could be detected over the 21 h time course.

3.4. Endocytosis of lipid/FITC-ODN complexes visualized by confocal microscopy

The uptake levels measured in the previous section are a combination of the amount bound to the plasma membrane as well as that endocytosed, therefore confocal microscopy was used to assess whether or not PEG-lipids inhibit internalization. HepG2 cells were exposed for 6 h to lipid/ODN complexes containing FITC-ODN and RhoPE then fixed in paraformaldehyde and visualized by confocal microscopy (Fig. 4). The distribution pattern of the FITC signal indicates that the ODN has been internalized, as FITC fluorescence is observed throughout the cell with the majority of the signal concentrated in the nucleus (Fig. 4A, arrows). The outline of the nucleus is more clearly seen using the rhodamine filter set (Fig. 4B). Here the lipid label is also intracellular but remains outside the nucleus, which is a characteristic of cationic lipid mediated delivery of ODN [18,23]. When the endosome is successfully disrupted by lipids the ODN subsequently released into the cytoplasm rapidly accumulate in the nucleus, whereas the lipids appear to remain associated with the endosome/lysosome compartment. When PEG(2000)-CerC_{20} is incorporated into the labeled complexes both the FITC (Fig. 4C) and RhoPE (Fig. 4D) are internalized as before but now both signals remain perinuclear and nuclear fluorescence is not observed at any time. This is consistent with the earlier results in HeLa-Luc cells showing that PEG(2000)-CerC_{20} severely inhibits antisense activity.

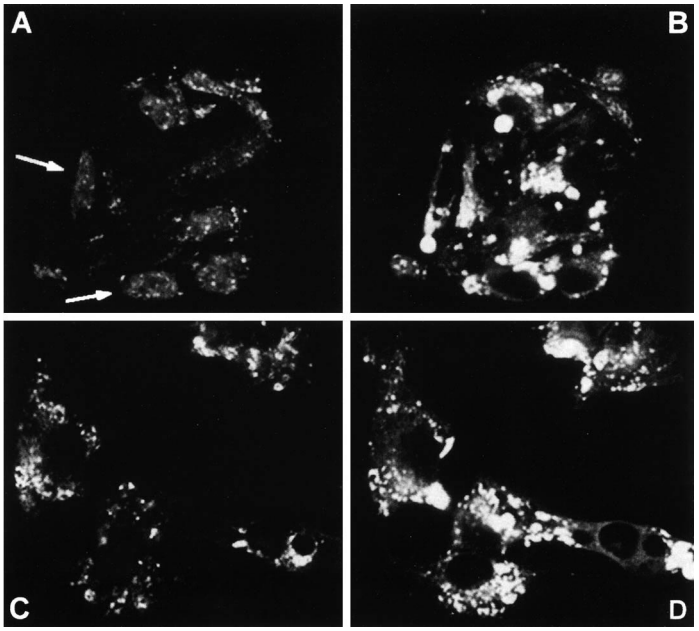

Fig. 4. Internalization of DODAC/DOPE/ODN and DODAC/DOPE/PEG(2000)-CerC_{20}/ODN complexes in HepG2 cells as visualized by confocal microscopy. DODAC/DOPE/RhoPE/FITC-ODN complexes (500 nM ODN final) were incubated with HepG2 cells in the presence of 10% FBS-MEM. After 6 h, the cells were fixed in 3% paraformaldehyde and visualized by confocal microscopy. FITC-ODN fluorescence is localized intracellularly and is concentrated in what appears to be the cell nucleus (A, arrows). The RhoPE-labeled vesicles are also internalized but, in contrast to the ODN, remain perinuclear (B). Cells incubated with DODAC/DOPE/RhoPE/PEG(2000)-CerC_{20}/FITC-ODN complexes also display internalization of the FITC-ODN (C) and RhoPE-labeled vesicles (D) but both labels remain perinuclear.

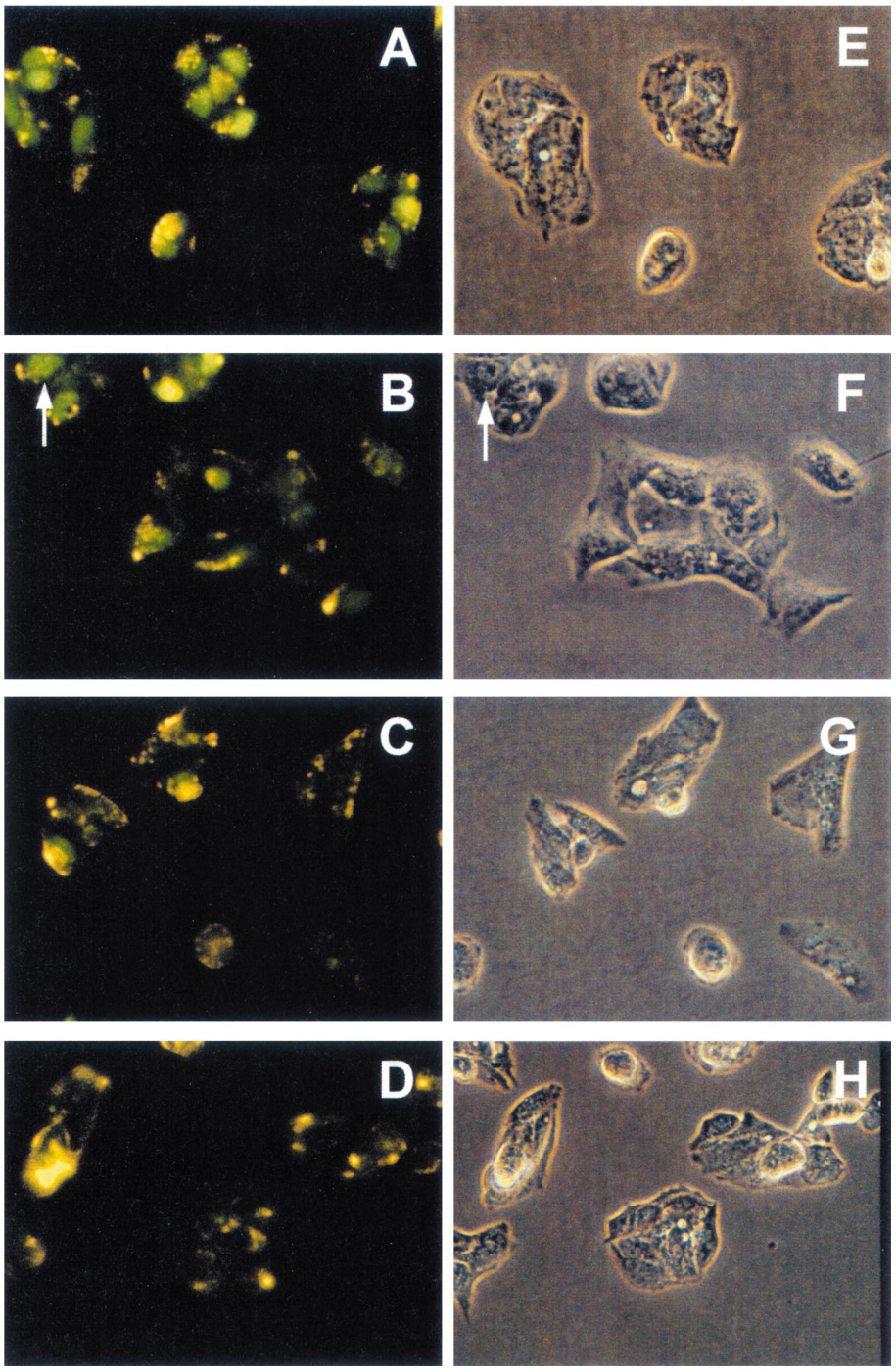

Fig. 5. Nuclear delivery of FITC-ODN mediated by DODAC/DOPE/RhoPE/PEG vesicles in HepG2 cells after 1.5 h incubation. A fluorescein-labeled ODN was mixed with various cationic vesicles containing 1% RhoPE and an aliquot of the lipid/ODN complexes (400 nM ODN final) was incubated with HepG2 cells in MEM containing 10% FBS. After 1.5 h, the lipid/ODN complexes were removed and replaced with fresh medium before being visualized either through a fluorescein filter set (left panels) or by phase contrast (right panel). The vesicles used were: (A,E) DODAC/DOPE/RhoPE (49.5:49.5:1, mole ratio); (B,F) DODAC/DOPE/RhoPE/PEG(2000)-CerC_8 (47:47:1:5, mole ratio); (C,G) DODAC/DOPE/RhoPE/PEG(2000)-CerC_{14} (47:47:1:5, mole ratio); (D,H) DODAC/DOPE/RhoPE/PEG(2000)-CerC_{20} (47:47:1:5, mole ratio). Arrows indicate FITC signal that is localized in the cell nucleus.

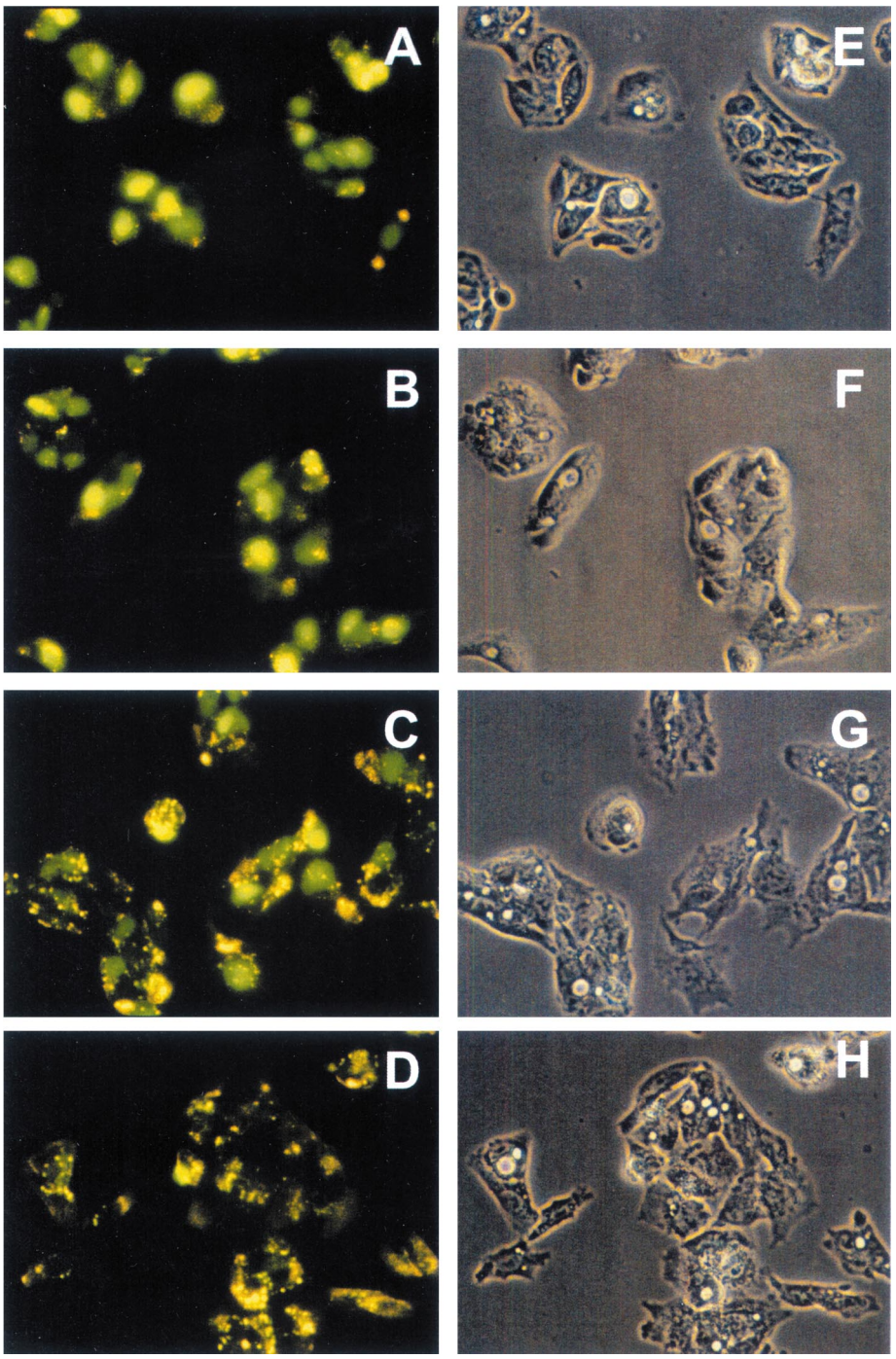

Fig. 6. Nuclear delivery of FITC-ODN mediated by DODAC/DOPE/RhoPE/PEG vesicles in HepG2 cells after 6 h incubation. Cells exposed to lipid/ODN complexes as indicated in Fig. 5 were visualized either through a fluorescein filter set (left panels) or by phase contrast (right panel) after 6 h. (A,E) DODAC/DOPE/RhoPE (49.5:49.5:1, mole ratio); (B,F) DODAC/DOPE/RhoPE/PEG(2000)-$CerC_8$ (47:47:1:5, mole ratio); (C,G) DODAC/DOPE/RhoPE/PEG(2000)-$CerC_{14}$; (D,H) DODAC/DOPE/RhoPE/PEG(2000)-$CerC_{20}$ (47:47:1:5, mole ratio).

3.5. Inhibition of endosome disruption by PEG-lipids

Our data suggest that incorporating PEG-lipids into lipid/DNA complexes does not prevent their internalization by endocytosis but interferes with disruption of the endosome and therefore release of ODN into the cytoplasm. The appearance of FITC-labeled nuclei within 2–4 h of lipid-based delivery is an excellent indicator of successful cytoplasmic delivery and can be used to compare the delivery efficiencies of different formulations [23]. Furthermore, there is a good correlation between the appearance of nuclear fluorescence and antisense specific activity as measured by decreasing levels of target mRNA [18]. In the following experiment, nuclear delivery of FITC-ODN to HepG2 cells was used to determine whether reducing the size of the hydrophobic anchor associated with PEG(2000)-Cer lipids correlated with the antisense activity data presented for HeLa-Luc cells (see Fig. 2).

Lipid/DNA complexes, labeled with FITC-ODN and RhoPE, were incubated with HepG2 cells in the presence of 10% serum and after 1.5 h the cells were washed and examined by fluorescence microscopy. Even after this relatively short incubation period, the nuclei of cells incubated with PEG-free complexes emit an intense green fluorescence resulting from the accumulation of FITC-ODN (Fig. 5A). Localization to the nuclei was confirmed by examining the same field under phase contrast (Fig. 5E). The outline of the cell nucleus is more clearly seen in Fig. 5F (arrow), correlating to the shape of the green cellular fluorescence (Fig. 5B, arrow). The yellow punctuate fluorescence seen shining through the FITC fluorescence filter set is due to intense signal from rhodamine. The location of the RhoPE label is consistent with the lipid dissociating from the ODN and remaining localized in the endosomal pathway. Incorporation of PEG(2000)-$CerC_8$ appears to slightly reduce the number of cells with nuclear fluorescence (Fig. 5B), while PEG(2000)-$CerC_{14}$ (Fig. 5C) and PEG(2000)-$CerC_{20}$ (Fig. 5D) are clearly inhibitory. FITC fluorescence is not observed in these latter samples as the ODN remains localized within endosomes with RhoPE-labeled lipid and thus FITC fluorescence is quenched through resonance energy transfer and low pH environment [44,23]. After 6 h cells incubated with formulations containing PEG(2000)-$CerC_8$ (Fig. 6B) and PEG(2000)-$CerC_{14}$ (Fig. 6C) exhibit similar nuclear fluorescence to cells incubated with PEG-free complexes. However, nuclear fluorescence was not visible in cells incubated with lipid/DNA complexes containing PEG(2000)-$CerC_{20}$ (Fig. 6D). A similar trend was seen when the size of the PEG head group was varied. Vesicles containing 5 mole% Gal-PEG(220)-DSPE mediated nuclear delivery of FITC-ODN similar to that observed for PEG-free DODAC/DOPE vesicles but inclusion of Gal-PEG(3400)-DSPE prevented endosomal release even after a 48 h incubation (data not shown).

4. Discussion

In this study we show that the presence of 5 mole% PEG-lipid conjugates, a typical concentration used in vivo [20,31,32], can interfere with the ability of DODAC/DOPE vesicles to mediate the intracellular delivery of DNA. Interestingly, PEG-lipid did not substantially inhibit uptake of the lipid/DNA complexes (Fig. 3). Instead it interfered with the ability of the lipid complexes to disrupt the endosomal membrane in a manner that was dependent on both the acyl chain length of the lipid anchor and the molecular weight of the PEG moiety.

In general, lipid-based particles that exhibit a net surface charge (positive or negative) bind more avidly to cells in culture than neutral particles, presumably via electrostatic interactions with charged proteins and carbohydrates at the cell surface [27,28]. The studies described here were all conducted in the presence of plasma proteins, which are also known to coat charged lipid membranes with more protein-to-lipid (w/mol) than neutral lipid membranes [30]. Consequently, we assume the lipid/DNA complexes employed here bind to the cell surface through electrostatic forces and/or plasma protein mediated interactions with receptors.

PEG-lipids are commonly employed in liposome and other lipid-based drug delivery systems to provide a steric barrier at the particle surface which inhibits protein binding and therefore opsonization in vivo [38,30]. PEG is a flexible hydrophilic polymer that extends approx. 3–5 nm from the membrane surface, depending on the number of PEG monomers

and membrane concentration of PEG-lipid [38,26]. The hydrophilic coating provided by PEG also inhibits the close apposition and fusion of liposomes with other membranes [8,3]. The fact that 5 mole% PEG had no significant effect on the binding and subsequent endocytosis of lipid/DNA complexes by HepG2 cells in this study (Fig. 3) was therefore surprising. It is interesting to note, however, that Semple et al. [30] found that the presence of 10% PEG(2000)-CerC_{20} in DODAC/DOPE (1:1, mole ratio) vesicles reduced the level of protein binding significantly compared to PEG-free DODAC/DOPE vesicles, but bound serum protein levels were still about 4-fold more than measured for equivalent neutral vesicles. Furthermore, Harvie et al. [6] recently concluded that PEG-induced inhibition of transfection in their lipid-based gene transfer system did not correlate with a reduction in endocytosis. These results indicate that the steric barrier does not necessarily prevent electrostatic interactions taking place with the vesicle surface.

After binding and uptake, the next phase in the DNA delivery process is to induce sufficient perturbation of the endosomal membrane to enable the release of DNA into the cytoplasm [45]. This requires lipid mixing to occur between lipid/DNA complexes and the intracellular membrane [39,42,23]. The intermembrane diffusion of long chain phospholipids or glycolipids through an aqueous space is generally negligible as a result of the large increase in hydrophobic free energy associated with forming the desorption transition state [34,33]. However, the electrostatic interaction between cationic lipid vesicles and anionic membranes is strong enough to bring the oppositely charged membrane surfaces into sufficiently close apposition to make desorption energetically more favorable [35,16,36,28]. This phenomenon, in combination with the membrane disruptive properties of DOPE, is in part why cationic lipid/DNA complexes have proven to be so effective at delivering polynucleic acids into the cytoplasm. Our data show that PEG-lipids inhibit this process (Figs. 4–6), consistent with studies of Mori et al. which indicate that incorporation of PEG-lipids in cationic vesicles will inhibit their lipid mixing with anionic membranes [21]. Furthermore, if the PEG-moiety is kept constant the extent of the inhibition correlates with the rate at which the PEG-lipid can desorb and diffuse away from the complex [41,40,33,8]. On the other hand, if the hydrophobic anchor is kept constant inhibition correlates with the capacity of the hydrophilic PEG group to act as a steric barrier [25].

These data are consistent with the following interpretations. In the presence of a stable and effective steric barrier imposed by PEG-lipids with acyl chains >14 carbons long or PEG head groups >220 M.Wt. (Figs. 1 and 2) the contact between complexes and endosomal membrane is not sufficient to enable lipid exchange and subsequent membrane disruption. Alternatively, lipid exchange may occur but the PEG-lipid stabilizes the membrane structure by preventing the induction of non-bilayer structures [7]. However, the latter is not consistent with the report of Holland et al. demonstrating that a stable steric barrier prevents vesicles–vesicle fusion as measured by a lipid mixing assay [8]. PEG-lipids with short acyl chains are known to rapidly diffuse away from liposomal membranes in the presence of a suitable 'sink' such as other membranes or lipoproteins [34,33,8,41]. Therefore complexes containing these lipids may have reduced PEG concentrations by the time they become endocytosed. It is also possible that the diffusible PEGs exchange into the endosomal membrane after endocytosis, therefore rendering the lipid/DNA particle more capable of membrane perturbation in a time dependent manner. Such a mechanism would be consistent with the data discussed below.

The inhibitory effect of PEG(2000)-CerC_{14} and -CerC_8 on gene transfection by complexes appears to be more significant than for antisense transfer (Figs. 1 and 2), although both show the same trend. This may be related to the relative stabilities of phosphodiester DNA compared to phosphorothioate DNA [2]. As the residence time for DNA inside the endosome/lysosome pathway increases, due to the inhibitory effects of PEG-lipid on its release into the cytoplasm, it will become more vulnerable to nuclease attack, especially as cationic lipid complexes only partially protect DNA from nucleases [41]. The natural phosphodiester backbone of the luciferase plasmid will be rapidly degraded compared to the relatively nuclease resistant phosphorothioate backbone of the antisense. The PEG-induced delay in DNA release is clearly seen for the FITC-labeled antisense in Fig. 5. In the absence of PEG or in

the presence of PEG(2000)-CerC_8, fluorescent nuclei are observed after 1.5 h. At this time point very little nuclear fluorescence is observed for complexes containing PEG(2000)-CerC_{14} or -CerC_{20}. However, the same level of nuclear fluorescence is achieved by complexes containing PEG(2000)-CerC_{14} after 6 h (Fig. 6), whereas even after 2 days (data not shown) complexes containing PEG(2000)-CerC_{20} were unable to deliver sufficient concentrations of FITC-ODN into the cell to produce visibly fluorescent nuclei. These data indicate that the complexes are undergoing a time dependent change in the endosome, possibly involving diffusion of exchangeable PEG-lipid into the organelle membrane. Subsequent or concurrent events, such as displacement of the cationic lipid from the ODN through interaction with anionic endosomal membrane lipids, have also been postulated to be required for endosomal release of the ODN [44].

Recently, Meyer et al. [19] demonstrated that even 2 mole% PEG-DSPE was sufficient to block the cytoplasmic delivery of fluorescent antisense into SK-BR-3 cells in vitro. The presence of intracellular (punctate) fluorescence indicates that uptake of lipid/ODN complexes occurred but that endosomal release was inhibited. What was most interesting about this study, however, was that the addition of 1% lipid-conjugated anti-HER2 F(ab′) to the complexes resulted in nuclear fluorescence [19], despite only a modest 2-fold increase in uptake due to targeting cell surface HER2. The authors suggest that in the presence of antibody, the lipid/ODN complexes are trafficked differently and enter a pathway in which the endosomes are more permissive to the escape of ODN into the cytoplasm. Similar observations have been made for liposomes containing protein antigens [29].

In conclusion, the results presented here demonstrate that a stable steric barrier firmly anchored in the membrane of cationic lipid/DNA particles inhibits its DNA transfer out of the endosome/lysosome pathway probably by reducing the insertion of perturbing lipids into the organelle membrane. This highlights the difficulties associated with using steric barrier lipids either to control aggregation during self-assembly of lipid/DNA particles or for reducing their rapid clearance and aggregation following intravenous administration. Optimizing the desorption rate of PEG-lipids may be one approach that can be used to overcome their inhibitory effects on intracellular delivery of plasmids and other polynucleic acids such as antisense, ribozymes and immunostimulatory oligonucleotides.

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