LIPID MIXING BETWEEN LIPOPLEXES AND PLASMA LIPOPROTEINS IS A MAJOR BARRIER FOR INTRAVENOUS TRANSFECTION MEDIATED BY CATIONIC LIPIDS

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It has been previously shown that transfection activity of cationic liposome/DNA lipoplexes delivered systemically is drastically inhibited by lipoproteins (Tandia, BM., Vandenbranden, M., Wattiez, R., Lakhdar, Z., Ruysschaert, JM., and Elouahabi, A. (2003) Mol Ther. 8, 264-273). In the present work, we have compared the binding/uptake and transfection activities of DOTAP and diC14-amidine-containing lipoplexes in the presence or absence of purified lipoproteins LDL and HDL. Binding/uptake of both lipoplexes, by the endothelial cell line MLE, was inhibited to a similar extent in the presence of lipoproteins. In contrast, transfection activity of diC14-amidine-containing lipoplexes was almost completely inhibited (approximately by 95%) whereas approximately 40 % of the transfection activity of DOTAP-containing lipoplexes was preserved in the presence of lipoproteins. Interestingly, the ability of lipoproteins to inhibit the transfection efficiency of lipoplexes was well correlated with their ability to undergo lipid mixing with the cationic lipid bilayer as revealed by FRET. Incubation of lipoplexes with increased doses of lipoproteins resulted in enhanced lipid mixing and reduced transfection activity of the lipoplexes in MLE cells. The role of lipid mixing in transfection was further demonstrated using lipid mixing inhibitor (lyso-PC) or activator (DOPE). Incorporation of lyso-PC into diC14-amidine-containing lipoplexes completely abolished their capacity to undergo lipid mixing with lipoproteins and allowed to reach a high transfection efficiency in the presence of lipoproteins. On the other hand, incorporation of DOPE into DOTAP/DNA lipoplex activated lipid mixing with the lipoproteins and was shown to be detrimental towards the transfection activity of this lipoplexes. Taken together, these results indicate that fusion of lipoplexes with lipoproteins is a limiting factor for in vivo transfection.

INTRODUCTION

Cationic lipids have been used for in vivo gene delivery into animal models via several routes of administration including intravenous (1-3), intratracheal (4) and intracerebral (5). The intravenous route of administration is attractive because it potentially allows the transport of the vector to various tissues in the body. It involves, however, interaction of the lipoplexes with the blood components before they reach the target cells. Intravenously injected cationic lipid/DNA lipoplexes accumulate rapidly (within 5-10 min.) in major organs including lung, liver and spleen (6, 7). Endothelial cells lying the blood vessels of these organs are the main target cells for the lipoplexes (8-10). Parts of the lung-associated lipoplexes do not enter the cells and presumably accumulate in the lumen of small blood vessels of this organ. One-two hours after injection, these lung-associated lipoplexes redistribute partially into the liver and the spleen (6, 7). This particular biodistribution has been attributed to interactions between lipoplexes and plasma components resulting in rapid aggregation of lipoplexes (11, 12). Aggregated lipoplexes are retained in the small blood vessels of the lung in a first step and are subsequently slowly removed by the blood flow. Such interactions between lipoplexes and plasma are still poorly characterized at the molecular level.

Generally the lung shows the highest transfection efficiency when lipoplexes are administered via the intravenous route. The choice of the helper lipid is crucial however in determining the final transfection efficiency.
Several studies have shown that the use of DOPE as helper lipid resulted in a decrease of the intravenous transfection efficiency of the lipoplexes in several organs (2, 12-15). Why DOPE causes a decrease in the in vivo transfection efficiency whereas it generally enhances this efficiency in vitro is still an open question. It has been suggested that interaction of the lipoplexes with plasma components may be modulated by the helper lipid and would explain differences in transfection efficiency (12). In an effort to characterize these interactions, we have previously isolated and identified the plasma proteins and lipoproteins that bind to diC14-amidine/DNA lipoplexes. Lipoprotein particles (including low and high density lipoproteins, i.e. LDL and HDL) are among the plasma components that bind to the lipoplexes. Such a binding was shown to inhibit the transfection efficiency in cultured cells (11). The reason behind this strong inhibitory effect remains however unclear.

In this study, we compared the influence of the lipoprotein particles HDL and LDL on the uptake and transfection efficiencies of DOTAP/DNA and diC14-amidine/DNA in cultured endothelial cells. Although binding/uptake by the cells of both lipoplexes was inhibited to the same extent, the transfection efficiency was affected differently depending on the cationic lipid used. This suggested that other factors were involved. Since lipoplexes and plasma lipoproteins interact together, we were wondering whether such an interaction was limited to a contact between both particles or did it involve additional reorganisation like lipid mixing. We demonstrated that efficient lipid mixing occurred between lipoproteins and diC14-amidine/DNA but not DOTAP/DNA lipoplexes. Comparison with transfection data shows a good inverse correlation between lipid mixing and transfection suggesting that this process is a limiting step for cationic lipid mediated intravenous gene transfer and expression.

**EXPERIMENTAL PROCEDURES**

**Reagents** - DiC14-amidine (3-tetradecylamino-N-tetra-decylpropionamide) was synthesized as described (16). N-[1-(2,3-dioleoyloxypropyl)-N,N,N-trimethylammonium chloride (DOTAP) and dioleoylphosphatidyl-ethanolamine (DOPE) were from Avanti Polar Lipids (Albaster, Alabama). N-(Lissamine rhodamine B Sulfonyl) Phosphatidyl-ethanolamine (Rh-PE ) and N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-1,2-dihexadecanoyl-snglycerol-3-phosphoethanolamine, triethylammonium salt (NBD-PE) were from Molecular Probes (Eugene, Oregon, USA). Trypsin was from Sigma Aldrich. Human plasma was prepared from blood collected from healthy volunteers. Ten ml of fresh blood were mixed with 5 µl of heparin (5000 IU/ml) immediately after collection and centrifuged at 5000 g, for 15 min at 4°C.

**Purification and labeling with 35S of plasmid DNA** - pCMV-luc plasmid (approximately 5.4 kb) was constructed by ligating an EcoRI-NheI fragment (corresponding to an improved sequence of the luciferase gene) of the pSP-luc+ plasmid (Promega Benelux, The Netherlands) into the pCI expression vector (Clontech, Palo Alto, CA, USA) which was cut with EcoRI and NheI. The resulting plasmid contained the luciferase gene under the control of the immediate early CMV promoter-enhancer and was purified using a Qiagen Kit (Giga plasmid prep.) according to manufacturer’s instructions and resuspended in sterile water. The purified plasmid was further treated with a polymyxin resin (Affi-prep™, BioRad) to remove endotoxins. Briefly, 200 µl of the affi-Prep resin was first rendered pyrogen-free by treatment with 600 µl of 0.5 N NaOH then washed twice with 1.5 ml of pyrogen-free water. The resin was then pelleted by centrifugation and resuspended in 400 µl of the DNA solution and mixed overnight in a rotary mixer at 4°C. The DNA was separated from the resin by centrifugation. Plasmid DNA was labeled with 35S using a modified nick-translation technique as described (11).

**Isolation of plasma lipoproteins and their treatment with trypsin** - Lipoproteins were isolated from plasma by KBr density gradient ultracentrifugation as described (17). Briefly, a discontinuous KBr density gradient was prepared in ultracentrifugation tubes using KBr solutions as follow (from bottom to top): 2 ml of d = 1.24 g/ml, 6 ml of d =1.21 g/ml containing plasma, 6 ml of d = 1.125 g/ml, 6 ml of d = 1.063 g/ml, 2 ml of d= 1.019 g/ml, 2 ml of d =1.006 g/ml and 2 ml of pure water. Samples were then centrifuged at 35,000 rpm for 16 h at 4°C. Three fractions corresponding to the very low density lipoproteins (VLDL, d = 1.0013 g/ml), low density lipoproteins (LDL, d = 1.013 – 1.046 g/ml), and high-density lipoproteins (HDL, d = 1.046 – 1.130 g/ml) were sampled.
Lipoproteins were purified from KBr using gel filtration chromatography on PD-10 columns (Pharmacia). Total protein, cholesterol, and phospholipid contents were determined in each sample using commercially available BCA (Pierce), CHOL (Roche) and PL (Roche) assay kits respectively.

For trypsinization, the lipoproteins were incubated with trypsin at a trypsin/lipoprotein ratio of 1/4 (w:w). The mixture was then incubated at 37°C for 45 min and the reaction was stopped by immersing the tubes on ice and addition of the trypsin inhibitor (Nɑ-Tosyl-L-lysine chloromethyl ketone hydrochloride) (Sigma-Aldrich). The samples were then ultracentrifuged at 35,000 rpm (in a SW60 Beckmann rotor) at 4°C for 15 min and then washed twice with HBS-20.

Preparation of liposomes and liposome/DNA complexes - DiC14-amidine or DOTAP liposomes were prepared as described previously (3). Rh-PE/NBD-PE-labeled diC14-amidine or DOTAP liposomes were prepared as described (3) except that 1.5% (molar) Rh-PE and 1.5% (molar) NBD-PE were added to the lipid solution in chloroform before film formation. The liposome suspensions were stored at 4°C and heated at 60°C for 15 min. with intermittent brief vortexing before use. For lipoplex preparation, all buffers were autoclave-sterilized and warmed at 37°C immediately before used. The pCMV-luc plasmid DNA was diluted in 150 mM NaCl or 5% dextrose (for diC14-amidine or DOTAP lipoplexes respectively) to a final concentration of 0.5 mg/ml. The diC14-amidine or DOTAP liposomes were diluted in HBS-20 (20 mM Hepes, 150 mM NaCl, pH 7.3) or HBD-20 (20 mM Hepes, 5% dextrose, pH 7.3) respectively, to a concentration of 4 mg/ml in a 4 ml polystyrene tube (Falcon) and protamine sulfate was added to a final concentration of 0.3 mg/ml. One volume of the DNA solution was added to an equal volume of the liposome-protamine mixture while gently shaking the tube (generally we prepare 100 to 400 µl of lipoplexes in a tube). The liposome/protamine/DNA mixture was allowed to stand for 15 min at room temperature. Under these conditions, the lipoplexes have a (+/-) charge ratio of 5:1 and the protamine/DNA weight ratio is 0.6:1. The preparation was stable for several hours.

In vivo transfection and luciferase expression analysis - Mice were injected through the lateral tail vein with 200 µl of the lipoplex solution at the indicated concentrations. Animals were killed by cervical dislocation 24 hours after injection and the organs were removed and tissue extracts were prepared as described (3). Luciferase activity (expressed as relative light units: RLU) was measured using 20 µl of cell lysis and 100 µl of the luciferase assay reagent (Promega) by integrating light emission over 10 sec in a TD-20/20 luminometer (Turner Designs, Sunnyvale, CA).

In vitro transfection and effect of plasma components on the transfection efficiency - To investigate the effect of plasma components on transfection, the mouse lung endothelial cells line (MLE) was chosen because lung endothelial cells are the main transfected cells after intravenous injection of cationic lipid/DNA complexes. This cell line was a gift from Dr. Nicolson (Institute for Molecular Medicine, Huntington Beach, CA) and was previously confirmed as endothelial cells (18, 19). MLE cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10 % FBS, 1 mM sodium pyruvate, 1 mM glutamine and antibiotics (complete medium) (Invitrogen). For transfection, cells were seeded in the 24-wells culture plates at 7 × 10⁴ cells/well and transfected 24 h later. Transfection was carried out in quadruplicate as follows: 100 µl of lipoplexes (prepared as described above) were diluted in 800 µl of HBS-20 (or HBD-20 for DOTAP-containing lipoplexes) and aliquots of 20 µl of the diluted lipoplex (containing 0.125 µg of DNA) were mixed with equal volumes of buffer or purified lipoprotein particles HDL and LDL at the indicated lipoprotein/lipoplex ratio (w:w). The samples were incubated at 37 ºC for 15 min and diluted with 1 ml of DMEM containing 20 mM Hepes (transfection medium). 200 µl (corresponding to 0.125 µg of DNA) were incubated in each well. After 2 h, the transfection medium was replaced with the complete medium and the cells were further incubated. Twenty-four hours post-transfection, the cells were washed twice with PBS, and lysed in 100 µl of reporter lysis buffer (Promega). The cells were scraped with disposable scrapers, transferred in Eppendorf tubes and centrifuged at 12,000 rpm at 4 ºC for 2 min to remove residual cell fragments. Luciferase activity was measured as described above.

Cell uptake of lipoplexes: role of lipoprotein particles LDL and HDL - Cells were plated and transfected as described in the in vitro
transfection section (see above) with some modifications. DNA was mixed with trace amounts of 35S-DNA before use to prepare the lipoplexes. An aliquot of the lipoplexes was kept at 4°C to quantify the total radioactivity. The remaining lipoplexes were incubated with purified lipoproteins particles (using a lipoprotein/lipoplex ratio of 9/1 w:w) and incubated with MLE cells in quadruplates for 2 h. Lipoplexes incubated with the buffer were used as controls. At the end of incubation, cells were washed three times with DMEM and twice with PBS and lysed in 100 µl of lysis buffer containing 10 mM Tris-HCl, pH 8.0, 100 mM NaCl, and 0.5% SDS. Cell lysates were then mixed with 5 ml of Ecolite scintillation liquid (ICN) and radioactivity was measured in a scintillation counter. The percentage of associated material was calculated after subtracting background radioactivity and dividing the radioactivity associated with the cells by the total radioactivity added to the well.

**Fusion assay** - Fusion between lipoplexes and lipoproteins was monitored using fluorescence resonance energy transfer assay (FRET) as described (20). Cationic liposomes containing NBD-PE and Rh-PE at 1.5% (mole) each were used to prepare lipoplexes as described above. Unless otherwise indicated, 162 µg of the lipoproteins (LDL or HDL) were added to lipoplexes (18 µg of cationic lipid) (resulting in a lipoprotein/cationic lipid weight ratio of 9:1) and loaded in a quartz fluorescence cell thermostatted at 37 °C. The fluorescence was monitored using an SLM-8000 spectrofluometer with excitation and emission slits of 4 nm. Generally, samples were excited at 470 nm and emission of both probes was recorded by scanning fluorescence between 500 and 650 nm. The initial fluorescence of the labeled lipoplex suspension was recorded as 0% fluorescence and the 100% fluorescence was determined after adding Triton X-100 at 1% (v/v) final concentration. Percentage of fusion was estimated from the fluorescence of NBD:

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\% F = \frac{(F_{NBD} - F_0)}{(F_{100} - F_0)}
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Where \(F_0\) is the maximum fluorescence of NBD (contained in the lipoplexes) before addition of lipoproteins. \(F_{NBD}\) is the maximum fluorescence of NBD (contained in the lipoplexes) in the presence of lipoproteins. \(F_{100}\) is the maximum fluorescence of NBD after addition of TX-100.

**Differential Scanning Calorimetry Measurements (DSC)** - DSC measurements were performed on a DSC-7 differential scanning calorimeter (Perkin Elmer Instruments) using twin aluminium pans. The samples were scanned from 5°C to 45°C at 2°C/min. All samples were degased before measurements. The experimental data were processed using Origin™ Software from MicroCal.

**RESULTS**

**Plasma lipoprotein particles, LDL and HDL, inhibit the in vivo transfection efficiency of lipoplexes** - Previous reports have shown that lipoproteins, including LDL and HDL, inhibit the *in vitro* transfection efficiency of diC14-amidine/DNA lipoplexes in mouse lung endothelial cells (MLE) (11). In order to evaluate the role of lipoproteins in the *in vivo* transfection process, diC14-amidine/pCMV-luc lipoplexes were prepared, incubated with purified LDL or HDL and injected intravenously into mice. Luciferase activity was measured in lung homogenates 24 h after injection. Lipoplexes preincubated with lipoproteins showed a diminished transfection efficiency in the lung as compared to controls (Fig. 1). This result confirms the role of lipoprotein particles in limiting the intravenous transfection efficiency using lipoplexes.

**Comparison of lipoprotein-mediated inhibition of binding/uptake and transfection efficiencies associated to cationic lipids diC14-amidine and DOTAP** - Inhibition of transfection efficiency by lipoproteins may be interpreted in terms of an inhibition of binding/uptake of lipoplexes. To investigate this possibility, diC14-amidine/DNA and DOTAP/DNA lipoplexes were preincubated with LDL or HDL and transfection efficiency in MLE (Mouse Lung Endothelial) cells was compared to that of control lipoplexes. In parallel, lipoplexes containing trace amount of radioactive DNA were preincubated with LDL or HDL, incubated with the cells and cell-associated radioactivity was measured. LDL and HDL inhibited drastically the binding/uptake by the cells of both diC14-amidine/DNA and DOTAP/DNA lipoplexes (Fig. 2). However, the transfection was differently affected depending on the cationic lipid used. 95% of transfection efficiency of diC14-amidine-containing lipoplexes was inhibited in the presence of the lipoproteins whereas that of DOTAP-containing lipoplex was inhibited only by approximately 60%. This suggests that the decrease of transfection efficiency cannot be interpreted only
in term of binding/uptake inhibition. Clearly other factors were involved.

**Lipoplex-plasma lipoprotein interaction leads to mixing of lipids of both components -**

Lipoplexes and plasma lipoproteins interact together (11). Since lipoproteins and lipoplexes both contain a lipid component, we were wondering whether such an interaction was limited to a contact between both particles or involved a reorganization like lipid mixing. To verify this hypothesis, we monitored lipid mixing between lipoplexes and lipoproteins by fluorescence resonance energy transfer (FRET). Two fluorescent probes NBD-PE (the donor of energy) and Rh-PE (the acceptor of energy) were inserted into the bilayer of lipoplexes at 1.5% (molar) probe concentration. Under these conditions, the fluorescence of the donor is transferred to the acceptor. Lipoplexes were prepared, then incubated with lipoproteins from human plasma and the fluorescence of the probes measured in a fluorimeter as described under experimental procedures. Excitation at 470 nm (maximum excitation of the donor fluorophore NBD-PE) of control diC14-amidine or DOTAP-containing lipoplexes, doubly labeled with NBD-PE and Rh-PE, resulted in a weak fluorescence of NBD-PE (at 525 nm) whereas Rh-PE fluorescence was quite intense (at 585 nm) (Fig. 3-A). This means that in the labeled diC14-amidine/DNA and DOTAP/DNA lipoplexes, NBD-PE and Rh-PE probes are close enough to allow a direct transfer of the fluorescence energy from the donor to the acceptor. After incubation of the labeled diC14-amidine/DNA lipoplexes with the lipoprotein HDL or LDL, fluorescence of NBD-PE strongly increased whereas that of Rh-PE decreased (Fig. 3-A). This demonstrates the mixing of lipids from the lipoprotein particles and the diC14-amidine bilayer. Interestingly, fluorescence of NBD-PE or Rh-PE was not modified after incubation of labeled DOTAP/DNA lipoplexes with the lipoproteins (Fig. 3-B). This suggested that interactions between DOTAP/DNA lipoplexes and lipoproteins did not result in lipid mixing. To rule out a direct influence of the lipoproteins on the fluorescence of the probes, lipoplexes labeled with only NBD-PE or Rh-PE were incubated with the lipoproteins HDL or LDL. No modification of the excitation or emission spectra of both probes could be detected (data not shown).

For both lipoprotein types, the lipid mixing process was slow (Fig. 4) as compared to what has been observed in other lipid mixing processes (21). Lipid mixing of different liposome populations usually takes place in few minutes whereas that observed in this study took about 40 min to reach a plateau. A possible explanation could be that fusion between lipoproteins and lipoplexes is slowed down by the presence of the apoproteins which stabilize the particles. To test this hypothesis, the LDL and HDL particles were first trypsinized to remove the accessible parts of the apoproteins and incubated with the NBD-PE/Rh-PE-doubly labeled diC14-amidine/DNA lipoplexes. For trypsinized LDL (Fig. 4-A) and HDL (Fig. 4-B), maximum lipid mixing was reached in less than 5 min. This result clearly shows that the apoproteins slow down the fusion process presumably by stabilizing the lipoprotein particles.

**Correlation between the ability of lipoproteins to fuse with the lipoplexes and to inhibit their transfection activity -**

In order to study a possible correlation between the ability of the lipoproteins to fuse with lipoplexes and their ability to inhibit the transfection efficiency, two sets of experiments were carried out in parallel. In one set, diC14-amidine/pCMV-luc or DOTAP/pCMV-luc lipoplexes were doubly labeled with NBD-PE and Rh-PE, and incubated with lipoproteins LDL or HDL as described above. Percentages of lipid mixing were calculated as described under experimental procedures. In parallel, unlabeled lipoplexes were incubated with lipoproteins and used to transfect cultured MLE (mouse lung endothelial) cells. After 24 h, the cells were lysed and the luciferase activity detected in cell lysates.

Lipid mixing between diC14-amidine/DNA lipoplex and HDL or LDL reached about 25% and 50% respectively (Fig. 5-A). In contrast, DOTAP/DNA lipoplex showed no significant fusion with either lipoproteins (Fig. 5-A). On the other hand, diC14-amidine/DNA lipoplex transfection efficiency was almost completely abolished in the presence of the lipoprotein whereas about 40% of the DOTAP/DNA lipoplex transfection efficiency was still observed in the presence of the lipoproteins (Fig. 5-B). This inverse correlation between lipid mixing and transfection efficiency suggested that lipid mixing represented an important barrier to the transfection process.

To demonstrate that lipid mixing was directly responsible for the inhibition of
transfection, the effect of inhibitors and activators of lipid mixing on the behavior of diC14-amidine/DNA and DOTAP/DNA respectively was investigated. Lyso-phosphatidylcholine (lysoPC) has been previously described as an inhibitor of lipid mixing between liposomes (22). DiC14-amidine liposomes containing an equimolar ratio of lysoPC were used to prepare lipoplexes (diC14-amidine/DNA ratio of 8:1 (w:w)). NBD-PE and Rh-PE were incorporated into the liposomes as described above. The lipoplexes were incubated with lipoproteins and the fluorescence of NBD-PE was monitored. In contrast to plain diC14-amidine-containing lipoplexes, lysoPC/diC14-amidine-containing lipoplexes were completely refractory to fusion with LDL or HDL (Fig. 6). Interestingly, the transfection activity of the lysoPC/diC14-amidine-containing lipoplexes was not inhibited by incubation with HDL or LDL (Fig. 6). On the other hand, it is widely accepted that lipid-forming inverted micelle structures, such as DOPE, are required for lipid mixing. Incorporation of DOPE into DOTAP/DNA lipoplex resulted in an activation of the fusion with the lipoproteins and was shown to be detrimental to the transfection activity of this lipoplex (Fig. 6). These results unambiguously demonstrate that fusion of lipoplexes with lipoproteins is a limiting factor for transfection.

Lipoproteins fuse with and inhibit the transfection activity of the diC14-amidine/DNA lipoplex in a dose dependent manner - The next experiment was carried out in order to determine whether fusion between lipoproteins and lipoplexes is dependent on the lipoprotein concentration. DiC14-amidine/DNA lipoplex was incubated with purified HDL or LDL at different lipoprotein/lipoplex ratios. At a low lipoprotein/lipoplex weight ratio (0.5:1), the percentage of lipid mixing was relatively low, about 7% and 15 % for HDL and LDL respectively (Fig. 7-A). At a high lipoprotein/lipoplex ratio (9:1; w:w), the percentage of fusion reached about 30% and 50% for HDL and LDL respectively (Fig. 7-A). Excess of lipoprotein was thus required for efficient lipid mixing to take place between lipoplexes and lipoproteins. Figure 7-B shows that at low lipoprotein/lipoplex ratio, the transfection activity of diC14-amidine/DNA lipoplexes was not significantly affected. When this ratio was increased, the transfection activity of diC14-amidine/DNA lipoplexes decreased significantly and was completely inhibited at 7:1 ratio (w:w) for both LDL and HDL (Fig. 7-B). This brings additional support to the conclusion that lipid mixing between lipoplexes and lipoproteins is a limiting factor for the transfection efficiency.

**DISCUSSION**

We describe here molecular interactions between lipoplexes and plasma lipoproteins that lead to inhibition of the transfection activity of lipoplexes. Adsorption of lipoproteins onto lipoplexes resulted in an inhibition of diC14-amidine/DNA and DOTAP/DNA lipoplex binding/uptake on/by the cells. Neutralization of cationic charges would cause a decrease in the binding of the lipoplexes to the cell surface and subsequent internalization. The new point is that diC14-amidine/DNA lipoplexes underwent an efficient lipid mixing with the lipoproteins and lost almost completely their transfection efficiency. DOTAP/DNA lipoplexes that did not show any significant lipid mixing with lipoproteins conserved about half of their transfection efficiency. The influence of lipid mixing between lipoprotein particles and lipoplexes on the transfection activity was further supported by two other kinds of
evidence. First, incorporation of lyso-PC, a lipid mixing inhibitor, into the bilayer blocks the fusion between the diC14-amidine/DNA lipoplexes and lipoproteins and confers resistance against inactivation of the transfection efficiency (Fig. 5). Second, incorporation of inverted conical-shaped fusogenic lipid like DOPE into DOTAP/DNA lipoplexes stimulated fusion with the lipoproteins and concomitantly inhibited the transfection efficiency.

Several groups have reported previously that DOPE is inhibitory for transfection efficiency after intravenous injection of DOTAP and DOTMA-containing lipoplexes (2, 12-15). Addition of cholesterol as a helper lipid instead of DOPE resulted in higher transfection efficiency. The reasons lying behind these observations are poorly understood. Li and colleagues have shown, using FRET, that interaction of DOTAP-DOPE/DNA lipoplexes with serum lead to an increase of the distance between the two probes inserted into the cationic lipid bilayer (12). They suggested that this was due to "disruption of vectors bilayers" after interaction with serum. Here we demonstrate that this effect is the consequence of the lipid mixing between the vector bilayer and lipoproteins and is mediated by the presence of DOPE (lipoplexes containing only DOTAP did not fuse). Thus liposome formulations that may undergo lipid mixing should be avoided for intravenous gene delivery. Fusion between lipoplexes and lipoproteins could also explain, at least in part, the high variability in intravenous transfection efficiency generally observed in the same experiment. Changes in the level of circulating lipoproteins in individual mice could be responsible for a more or less pronounced inhibition of the injected lipoplexes. Further experiments are needed however to verify this hypothesis.

Lipoprotein particles are composed of a hydrophobic core of cholesterol esters and triglycerides, and an outer monolayer composed of cholesterol and phospholipids. Aproteins stabilize the particle structure and allows tissues to recognize and take up the particle. Why is fusion between lipoplexes and lipoproteins a slow phenomenon? (Fig. 5). A possible explanation could be that the different aproteins which stabilize the LDL and HDL particles slow down the lipid mixing process. This hypothesis is supported by the fact that when the lipoproteins were treated with trypsin in order to remove the accessible parts of the aproteins, the fusion process becomes much more faster. This slow kinetics of interaction between lipoproteins and lipoplexes may help explaining why transfection of the lung with cationic liposome vectors is generally the most efficient as compared to the other organs. A possibility could be that lipoplexes, which rapidly accumulate in the lung after injection, enter the cells before they fuse with lipoproteins and therefore result in efficient transfection. Lipoplexes taken up by the other organs such as liver and spleen have a contact time with the plasma that is sufficient to allow for lipid mixing with lipoproteins and therefore inactivation of their transfection activity. This conclusion needs, however, further systematic studies comparing the ability of lipoplexes to escape fusion with lipoproteins and their capability to transfect efficiently several organs. Of course, other factors including the difference in intracellular trafficking of lipoplexes in cells of different organs and the activity of the promoter in these cells may not be neglected.

DiC14-amidine containing lipoplexes underwent an efficient lipid mixing with lipoprotein in the absence of DOPE. It is highly probable that, even in the absence of DOPE, the structure of diC14-amidine is more favorable for lipid mixing than other cationic lipids. In support to this hypothesis, diC14-amidine, in contrast to most other cationic lipids such as DOTAP or DOTMA, did not require DOPE for efficient transfection of cells in vitro (16). DiC14-amidine-containing lipoplexes were also shown to efficiently destabilize the endosomal membrane in the absence of DOPE (24).

What are the consequence of lipid mixing between lipoplexes and lipoproteins on the subsequent cellular steps that lead to the drastic inhibition of the transfection efficiency? While we can not at present accurately answer this question, hypothesis can be made based on experimental data. We (24) and others (25, 26) have previously shown that transfection activity of different types of lipoplexes depended moderately on uptake and mainly on subsequent steps of transfection, i.e. escape from endosomes. Zelphati and colleagues have shown that uptake of ODN/cationic liposomes complexes was moderately affected whereas nuclear localization of ODN could be strongly inhibited in the presence of serum (27). It could be that fusion between lipoproteins and lipoplexes decrease the ability of the later to destabilize the endosomal membrane and to
promote efficient escape of the DNA into the cytosol. The mechanism of escape of lipoplexes involves interactions between the cationic lipids and the phospholipids of the endosomal membrane (28). The properties of the cationic lipid bilayer that promote efficient destabilization of the endosomal membrane would be profoundly altered following fusion with lipoproteins. The intermixing of lipids would affect the density of charge at the cationic bilayer surface as well as its ability to adopt non-bilayer structure. Phospholipids of the lipoprotein particles mostly comprise phosphatidylcholine (in average approximately 70% of total phospholipids), sphingomyelin (between 13 and 25 %) and negatively charged phospholipids such as phosphatidylserine and phosphatidylinositol (generally about 5%). All these phospholipids would be transferred into the cationic lipid bilayer. Our DSC data support the idea that the cationic lipid bilayer is profoundly altered following fusion with lipoproteins since it lost its ability to undergo phase transition. In contrast to a simple binding of lipoproteins at the lipoplex surface, fusion between both structures is irreversible. In the absence of fusion, bound lipoproteins could dissociate from the lipoplexes after internalization.

In summary, we show here that lipid mixing between plasma lipoproteins and lipoplexes is an important barrier to the intravenous gene transfer efficiency mediated by cationic lipids. Therefore, inclusion of helper lipids that inhibit this lipid mixing into lipoplexes should improve their intravenous transfection efficiency. Assay for lipid mixing between lipoplexes and lipoproteins could also be used to predict their in vivo transfection efficiency.

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FIGURE LEGENDS

Fig. 1. Influence of preincubation of lipoplexes with lipoproteins on the intravenous transfection efficiency. DiC14-amidine/protamine/pCMV-luc lipoplexes were prepared as described under experimental procedures and incubated with the buffer or purified LDL or HDL. Lipoplexes were then injected via the lateral tail vein into mice at a dose of 25 µg DNA/mouse. 24 h later, mice were killed and luciferase activity was measured in lung homogenates. Data represent means ± SD of five mice.

Fig. 2. Influence of lipoproteins on the lipoplex binding/uptake (A) and transfection (B) efficiencies in vitro. (A) Cells were plated on 24-wells plates at 7x10^4 cells/well. 24 hours after, cells were incubated with diC14-amidine/DNA or DOTAP/DNA lipoplexes (0.125 µg DNA/well) lipoplexes containing trace amounts of ^35S-DNA in the presence or absence of lipoproteins. After 2h, cells were washed three times with DMEM, twice with PBS and lysed in lysis buffer as described under experimental procedures. Cell lysates were mixed with scintillation liquid and radioactivity counted in a β-scintillation counter. Results are expressed as percentage of cell-associated radioactivity relatively to the total radioactivity in each well. Data represent means ± SD (n = 4). (B) MLE cells were plated and transfected using lipoplexes with or without preincubation with plasma lipoproteins as described in (A) (except that no radioactivity was used). After two hours, lipoplex-containing medium was replaced with complete medium and cells were harvested 24 h post-transfection and luciferase activity was measured in cell lysates. Data represent means ± SD (n = 4).

Fig. 3. Analysis of interactions between lipoplexes and lipoproteins using FRET. DiC14-amidine or DOTAP cationic liposomes were labeled with 1.5% (molar ratio) NBD-PE and Rh-PE and lipoplexes were prepared as described under experimental procedures. The lipoplexes were incubated for 1 h at 37°C with lipoproteins at a 9:1 lipoprotein/cationic lipid weight ratio. Lipoplexes were incubated in parallel with the buffer as a background control or with TX-100 to record the maximum fluorescence. Samples were then transferred into a quartz cell and fluorescence emission spectra were recorded between 500 and 650 nm with excitation at 470 nm. (A) and (B) shows analysis of diC14-amidine and DOTAP-containing lipoplexes respectively.
**Fig. 4.** Time course of lipid mixing between diC14-amidine lipoplexes and lipoproteins. Labeled diC14-amidine-containing lipoplexes were prepared as described in the legend of figure 3 and incubated with plain (squares) or trypsinized (triangles) lipoproteins. Fluorescence of NBD-PE was recorded (excitation: 470 nm; emission: 525 nm) at different times as a measure of the extent of lipid mixing.

**Fig. 5.** Correlation between lipid mixing and transfection activities of diC14-amidine- and DOTAP-containing lipoplexes in the presence of lipoproteins. (A) Lipoplexes were labeled and incubated with lipoproteins as in the legend of figure 3. After one hour, percentage of lipid mixing was estimated as described under experimental procedures. (B) Non labeled lipoplexes were used to transfect MLE cells in the presence or absence of lipoproteins as described in the legend of figure 2. Transfection activity in the presence of lipoproteins was normalized to that of control lipoplexes preincubated with the buffer. Results represent means ± SD (n = 4).

**Fig. 6.** Influence of inhibitors or activators of fusion on lipid mixing and transfection activities of lipoplexes. Lyso-PC or DOPE were incorporated into diC14-amidine- and DOTAP-containing lipoplexes respectively (at 50% molar ratio) as described under experimental procedures. Lipid mixing and transfection activities in the presence or absence of lipoproteins were measured as in figure 5.

**Fig. 7.** Influence of the dose of lipoprotein on lipid mixing and transfection activities of lipoplexes. (A) Labeled diC14-amidine lipoplexes were prepared and incubated with lipoproteins at various lipoprotein/cationic lipid weight ratio. Percentage of lipid mixing was measured as described under experimental procedures. Results are means ± SD (n = 4). (B) Non-labeled lipoplexes were incubated with lipoproteins at various lipoprotein/cationic lipid weight ratios and used to transfect cells as described in the legend of figure 2. Bars represent means ± SD (n = 4).

**Fig. 8.** Influence of lipoproteins rigid to fluid phase transition of diC14-amidine bilayer. DiC14-amidine liposomes or diC14-amidine-containing lipoplexes were prepared and incubated with lipoproteins. Samples were analyzed by differential scanning calorimetry as described under experimental procedures. Excess molar heat capacity was plotted as a function of temperature.
Fig. 1

Luciferase activity (RLU)

- Lipoplex
- Lipoplex + LDL
- Lipoplex + HDL
Fig. 2

A: Uptake

Radioactivity (CPM/100 µl)

B: Transfection

Luciferase activity (R.L.U)

DiC14-amidine/DNA lipoplex

DOTAP/DNA lipoplex
Fig. 3

A: DiC14-amidine

B: DOTAP

Fluorescence intensity (arbitrary units)

Wavelength (nm)

- Lipoplex + buffer.
- Lipoplex + LDL.
- Lipoplex + HDL.
- Lipoplex + TX-100.
Fig. 4

A: LDL

B: HDL

NBD fluorescence intensity (arbitrary units)

Incubation time (min.)

- Lipoplex + buffer
- Lipoplex + trypsinized lipoprotein
- Lipoplex + non trypsinized lipoprotein
Fig. 5

A: Fusion

Percentage of fusion

B: Transfection

Luciferase activity (percentage of control)

- DiC14-amidine/DNA lipoplex.
- DOTAP/DNA lipoplex.
A: Fusion

Percentage of fusion

Fig. 6

B: Transfection

Luciferase activity (percentage of control).

DiC14-amidine/Lyso-PC/DNA lipoplex.

DOTAP/DOPE/DNA Lipoplex

Lipoplex + buffer
Lipoplex + LDL
Lipoplex + HDL
Lipoplex + buffer
Lipoplex + LDL
Lipoplex + HDL
**Fig. 7**

A: Fusion

- Lipoprotein/lipoplex ratio (w:w)

- Percentage of fusion

B: Transfection

- Lipoprotein/lipoplex ratio (w:w)

- Luciferase activity (percentage of control)

- LDL
- HDL
Fig. 8

A: HDL.

B: LDL.

Temperature (°C).

Kcal/mole.K

- Liposomes
- Liposomes + Lipoprotein
- Lipoplex
- Lipoplex + Lipoprotein
- Lipoprotein alone

Kcal/mole.K

10 15 20 25 30
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