Sphingosine-Based Liposome as DNA Vector for Intramuscular Gene Delivery

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Purpose. The aim of this study was to develop a labile sphingosine-based liposome for intramuscular gene delivery.

Methods. Sphingosine-based liposomes were formulated in a range of solutions with phosphatidylcholine, then were associated to DNA. The physico-chemical characteristics of the sphingosine/EPC liposomes and sphingosine/EPC/DNA lipoplexes were determined. DNA stability within sphingosine-based liposomes was evaluated in the presence of a nuclease and mouse serum. *In vivo* gene transfer was studied by intramuscular injection with and without the electrotransfer technique.

Results. By increasing the charge ratios, colloidally stable sphingosine/DNA particles with a 170 nm average diameter and a positive ζ potential were obtained. Ethidium bromide was still able to insert into plasmid DNA within the lipoplexes, even though plasmid DNA was demonstrated to be complexed to the lipid by gel electrophoresis. Additionally, DNA was shown to be accessible to DNase I, but significantly resistant to serum enzymatic digestion. Upon intramuscular injection, lipoplexes induced an inhibition of gene expression as compared with naked DNA.

Conclusions. The cationic sphingosine/EPC/DNA complexes form weakly compacted structure, potentially labile *in vivo*, which might be useful for *in vivo* gene transfer.

KEY WORDS: sphingosine; non viral gene transfer; cationic liposomes; colloidal stability; serum resistance; gene therapy

INTRODUCTION

Nonviral gene therapy aims at developing synthetic systems able to associate to DNA and to deliver it into the cell. In muscular tissues, transfection has been shown to occur by injecting naked, vector-free DNA. However, injection of naked DNA into the muscle leads to low and variable levels. The anionic nature of DNA dictates the cationic character of numerous entities that have been developed so far as transfecting agents: cationic lipids and polymers (1,2), which are very efficient to compact DNA and deliver it to cells *in vitro*. However, these cationic vectors have mostly failed *in vivo* because of their toxicity, lack of specificity, and inhibition by anionic or extra-cellular serum components. For instance, any formulation with cationic lipids has been shown to inhibit intramuscular transfection. The fact that injection of naked DNA leads to better transfection efficiency than the injection of cationic lipid/DNA could be attributed to a limiting DNA release from the vector. Thus, the production of potentially more labile lipoplexes could present a way to enhancing DNA release from the complexes, while still maintaining the transfection capacity of delivery vectors *in vivo*.

Sphingosine is a natural cationic lipid (Fig. 1), which has been shown to interact with DNA when incorporated into dimyristoylphosphatidylcholine (DMPC) liposomes (3). Scanning calorimetry experiments showed that while DMPC liposomes were unable to interact with DNA, sphingosine insertion favored this association and modified the liposome thermal phase transition behavior. This DNA/liposome interaction depended on the cationic charge of the sphingosine, as non protonated sphingosine derivatives did not associate to DNA (4). Despite the cationic charge, sphingosine associated with colipids like dioleoylphosphatidylethanolamine or dimyristoylphosphatidylcholine did not exhibit any cytotoxicity. The *in vitro* transfection efficiency was however reported to be moderate and dependent on the cell line (5). More important, sphingosine is able to increase DNA adsorption on to lipid bilayers and to enhance permeability of these bilayers, as demonstrated by measuring channel-like current pulses on liposome models (6).

We thought that the particular properties of sphingosine could be of interest for intramuscular gene delivery. Based on its monocatenar structure, a less stable sphingosine/DNA complex would be expected than with conventional lipopolyamine transfecting agents. This could increase intramuscular gene delivery in the muscle. Secondly, it has been shown in our group that electrotransfer enhances membrane permeabilization and improves transfection efficiency (7). We hypothesized that sphingosine permeabilizing effect could also improve electroporation delivery of plasmid DNA in the muscle.

MATERIAL AND METHODS

Chemicals

Egg phosphatidylcholine (EPC) was purchased from Avanti Lipid (Alabaster, AL, USA). Luciferase assay kit was obtained from Promega (Madison, WI, USA). Synthetic D-*Erythro*-Sphingosine and Deoxyribonuclease I (DNase I) were purchased from Sigma Chemical Company (St. Louis, MO). All other chemicals were of reagent grade.

Plasmids and DNA Preparation

Plasmid pXL 3031 (pCMV-Luc+) contains the cytomegalovirus promoter (nucleotides 229–890 of pcDNA3, InVitroGen) inserted upstream of the *Photinus pyralis* modified cytosolic luciferase coding sequence.

Preparation of Cationic Liposomes

Sphingosine (150 μl, 10 g/l) was mixed with EPC (304 μl, 25 g/l) in a 1:1.5 molar stoichiometry in 500 μl of $CHCl_3$. The lipid mixture was evaporated to dryness *in vacuo* to a thin film layer. The lipid film was hydrated overnight in 650 μl H_2O (ppi) to give a 10-mM final sphingosine concentration. Liposomes were gently vortexed then sonicated. Subsequently, the sample was extruded twice through a 0.4-μm pore size polycarbonate membrane (Nalgene) at 37°C. Typically, this pro-

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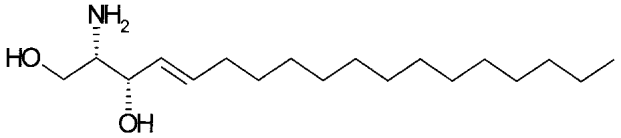

Fig. 1. Sphingosine structure: the hydrophobic moiety of sphingosine consists of 18-carbon acyl chain, with the polar headgroup bearing an amine group.

cedure generated liposomes with mean diameters of 80–100 nm. These were stored at 4°C.

Calculation of the Lipoplex Charge Ratios

The charge ratios were calculated stoichiometrically as the mole ratio of sphingosine (one positive charge per molecule) to DNA nucleotide residue (MW 330).

Lipoplex Formation

Typically, lipoplexes of different charge ratios were obtained by mixing an equal volume of various concentrations of cationic lipid with plasmid DNA at the desired concentration. For instance: to obtain a charge ratio = 4, sphingosine/EPC (97 μl, 1 mM) was filled to 400 μl H_2Oppi, then DNA (400μl, 0.02 g/l in 10% glucose / 40mM NaCl) was added dropwise.

Dynamic Light Scattering

The size distribution of the liposomes and lipoplexes were determined by dynamic light scattering using a Coulter N4 Plus particle size analyzer (Coulter, Margency, France). Size distribution analysis yielded intensity and weight mean particle diameter distributions. Samples were prepared as described above, except that lipoplexes containing 0.1 g/l DNA were diluted ten-fold to obtain a valuable signal in light scattering.

Fluorescence Studies

A Jobin–Yvon Spex Fluoromax-2 spectrofluorometer (Longjumeau, France) was used to measure ethidium bromide fluorescence (Excitation 260 nm, emission 590 nm). EtBr (3 μl, 10mg/ml) was added to the lipoplex solution (800 μl, 10 μg/ml DNA). Values are expressed as a fluorescence percentage of ethidium bromide with free plasmid DNA, taken as a 100%.

Agarose Gel Electrophoresis

Samples were prepared as described above (20 μl) and 5 μl of 20% glucose was added. The mixtures were loaded into 0.8% (w/v) agarose gel at 70V/cm in TBE buffer (1 M Tris, 0.9 M Boric acid, 0.01 M EDTA). DNA was revealed with EtBr and visualized under UV light.

Zeta Potential Analysis

The zeta potential was determined using a ZetaSizer 4 (Malvern Instruments, Southborough, MA, USA). The system was calibrated using a −50 ±5 mV standard (DTS 50/50 Standard; Malvern Instruments). Lipoplex samples prepared as described above in 20 mM NaCl subjected to 3 × 30 s (1000 Hz, no field correction) measurement.

DNA Electrotransfer and Reporter Gene Analysis

Female C57Bl/6 mice age 6 weeks were purchased from Iffa Credo (L'Isle d'Arbesle, France) and were housed in accordance with institutional guidelines. Unless otherwise stated, 10 tibial cranial muscles were included in each experimental group. Individually anesthetized mice in groups of five were injected using a Hamilton syringe into tibial cranial muscle with 3 μg of plasmid DNA that was either precomplexed with liposomes or was injected as naked DNA, in a total volume of 30 μl in 5% glucose, 20 mM NaCl, final concentration. Electrotransfer was performed as in ref 8, by using stainless steel plate electrodes. Experiments were conducted following the NIH recommendation for animal experimentation and Aventis local ethic committee on animal care and experimentation.

Mice were killed 7 days after DNA injection. Muscles were removed and assayed for luciferase reporter gene activity as described by Mir *et al* (7).

RESULTS

Formation and Colloidal Stability of Sphingosine-Based Cationic Lipoplexes

Cationic lipid-based DNA complexes form as a consequence of a self-assembly process due to electrostatic interactions between preformed cationic micelles or liposomes and the phosphates of DNA. In this study, liposomes were prepared using a chloroform evaporation procedure resulting in the formation of a film, which was then hydrated. A more homogeneous film could be obtained when EPC was used as a colipid, instead of dioleoylphosphatidylethanolamine (DOPE). Lipoplexes were then obtained by mixing DNA with these liposomes at different lipid/DNA charge ratio, considering that sphingosine only bears one positive charge, and was the only protonated lipid present at physiological pH. When the lipid/DNA charge ratio was increased, the amount of DNA being maintained constant, three zones of colloidal stability were identified by dynamic light scattering (Fig. 2), as previously described for lipopolyamines (8).

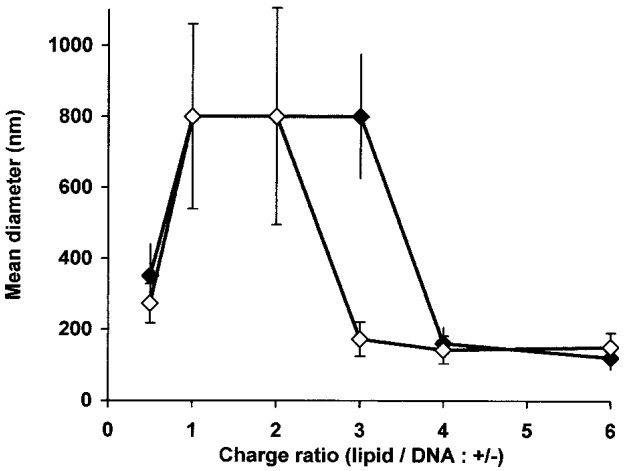

Fig. 2. Colloidal stability of sphingosine/EPC (1/1.5)/DNA lipoplexes as a function of the lipid/DNA ratio in 5% glucose (empty lozenge) and 5% glucose, 20 mM NaCl (full lozenge). Particle diameter was estimated by dynamic light scattering at 90° for 15 min.

For lipid/DNA ratios <1, small diameter particles were obtained. At a lipid/DNA ratio near neutrality or low cationic density, lipoplex aggregation was observed. Finally, an excess of lipid stabilized cationic structures, thanks to electrostatic repulsion. Under these conditions, lipoplex diameter was 170 ± 35 nm when sphingosine/EPC/DNA lipoplexes (R > 4) were formed in either 5% glucose or 5% glucose/ 20 mM NaCl (Fig. 2). Conversely, aggregated complexes (>1 μm) were obtained in higher ionic concentrations (75 to 150 mM NaCl). As described previously by Turek *et al.*, increasing the NaCl concentration extended the aggregated zone (9). This is due to an electrolyte charge shielding effect, in which the negative chloride ions surround the positively charged lipids, favoring lipoplex aggregation. Electron microscopy of uranyl acetate stained complexes (R > 4) revealed a homogeneous population of individual particles, round in shape, displaying an internal lamellar organization (data not shown).

Evidence for the complexation of DNA within sphingosine-based lipoplexes was demonstrated by agarose gel electrophoresis, as shown on Fig. 3. With an excess of DNA (charge ratio = 0.3, lane 1), only part of the DNA was retained in the well and no DNA migration was observed with higher charge ratios (lane 2 to 6). Colloidal stability and gel electrophoresis analyses of the lipoplexes at DNA concentration increasing from 10 to 100 μg/ml showed that DNA was complexed, and that the smaller lipoplexes were stable in 5% glucose /20 mM NaCl at the highest DNA concentration, thus making these complexes suitable for *in vivo* applications.

DNA Compaction State in Sphingosine-Based Lipoplexes

Ethidium bromide fluorescence experiments were performed for the determination of DNA compactation in sphingosine/EPC/DNA complexes. Ethidium bromide fluorescence intensity raises upon intercalation between DNA base pairs. The fluorescence signal is significantly reduced when DNA is compacted by cationic lipids, indicating lack of DNA accessibility within the lipoplexes, as observed in previous studies (8). As shown in Fig. 4, only limited DNA compaction was observed with the sphingosine/EPC/DNA lipoplexes, as compared with complexes obtained with the lipopolyamine

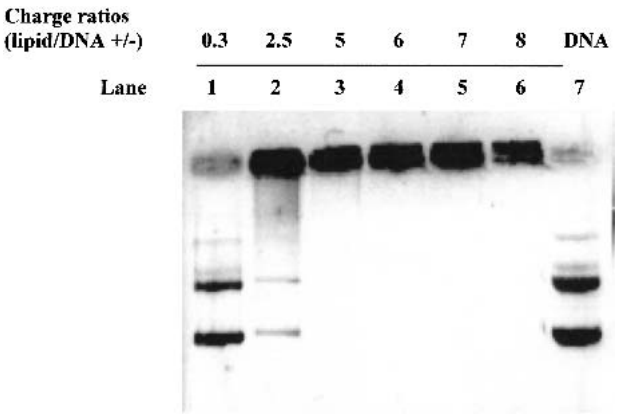

Fig. 3. Electrophoresis on a 0.8% agarose gel of sphingosine/EPC/DNA complexes at different charge ratios. DNA was stained with ethidium bromide and visualized under UV light. Naked DNA was loaded as a negative control. We observed a migration pattern differing for the three individual colloidal stability zones, as described in Fig. 2.

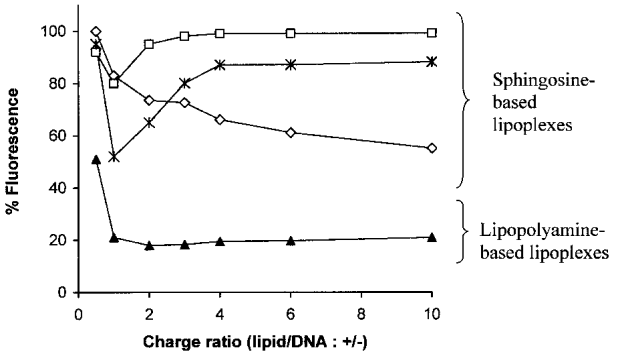

Fig. 4. Ethidium bromide intercalation assay at different lipid/DNA charge ratios. Fluorescence experiments were performed to investigate DNA condensation. Fluorescence intensity decreased as a function of the lipid/DNA charge ratio. DNA was complexed with different sphingosine-based formulation: Sph/EPC/DOPE (empty square), Sph /EPC: 1/4 (cross), Sph /EPC: 1/1.5 (empty lozenge), RPR 120535 (full triangle).

RPR120535 bearing 3 positive charges. Addition of DOPE or more EPC to the sphingosine/EPC formulation induced a further decrease of the DNA compaction, reflecting the low binding affinity of sphingosine for the DNA. The initial fluorescence drop observed in the Fig. 4 experiment may be due to the solution turbidity of the aggregated complexes that may interfere with the signal intensity. All the fluorescence data were independent of the excitation wavelength (260 or 480 nm) as described by other groups (10).

Surface Charge of the Lipoplexes

Because DNA was complexed in sphingosine-based lipoplexes, but ethidium bromide appeared still able to insert between DNA base pairs, we envisaged that DNA might interact within the liposome particle bilayer by binding to the outer particle surface. To test this hypothesis, we measured the particle ζ potentials. The counter ion distribution around the particles in aqueous media reflects surface charge, and can be assessed from the electrophoretic mobility of the particle. For cationic sphingosine liposome alone (no DNA added), a positive ζ potential value was obtained (+69 mV), as expected. Addition of DNA in excess led to an inversion of the ζ potential to negative values (–9.9 mV). Then, by increasing the lipid/DNA charge ratio, the lipoplexes exhibited progressively increasing ζ potential values to reach a plateau at 45 ± 5 mV. As a reference, the lipopolyamine RPR120535 (bearing three positive charges) used as a control in the same conditions exhibited a ζ potential plateau of +60 ± 5 mV. Thus, the ζ potential of sphingosine-based lipoplexes is positive, but lower than the lipopolyamine potential for the same lipid/DNA charge ratio. This suggests that a part of the DNA associated to the particles might be located at the surface of these particles. As a consequence, DNA stability towards nuclease mediated hydrolysis might be endangered in such structures.

Effect of Nuclease DNase I and Mouse Serum on Plasmid Integrity within Sphingosine-Based Lipoplexes

The resistance of plasmid DNA to nuclease degradation in sphingosine-based lipoplexes was analyzed. Free plasmid

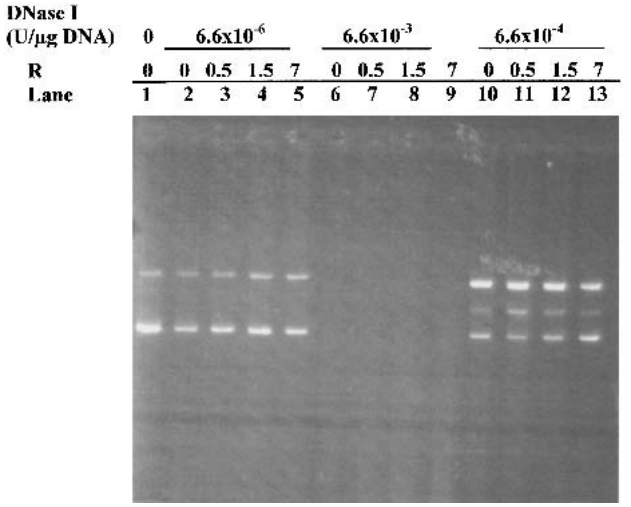

Fig. 5. Agarose gel electrophoresis for assessment of DNA integrity after DNase I incubation with lipoplexes at 37°C for 30 min, and subsequent extraction with QIAamp blood kit (QIAGEN, Valencia, CA, USA).

DNA was not degraded by $6{,}6.10^{-6}$ U/μg DNase I, partially degraded by $6{,}6.10^{-4}$ U/μg DNase I, becoming relaxed instead of supercoiled, and fully degraded by $6{,}6.10^{-3}$ U/μg DNase I. Sphingosine-based complexes, at three different charge ratios, were subjected to the described DNase conditions. After DNase treatment, DNA was extracted from the lipoplexes with a QIAamp blood kit (Qiagen, Valencia, CA, USA). As shown in Fig. 5, we found that DNA had not been protected from the effect of DNase in the sphingosine-based cationic lipid at any of the 0.5, 1.5 or 7 charge ratios tested. Under the harshest DNase conditions, DNA was totally degraded in each case. Under less stringent condition ($6{,}6.10^{-4}$ U/μg DNA), a significant amount of DNA was demonstrated to be accessible to DNase I, displaying identical ratio of respectively supercoiled, relaxed and linearized forms as with non-associated plasmid DNA.

To further characterize the sphingosine-based complexes, we evaluated their stability in the presence of mouse serum. Since our physico-chemical characterizations suggested a low affinity between the Sphingosine/EPC liposomes and DNA, it could be speculated that serum proteins would destabilize these lipoplexes, thus inducing DNA release and rendering it susceptible to serum nuclease mediated degradation. To test this hypothesis, lipoplexes were pre-incubated with different mouse serum concentrations *in vitro*. Since Li *et al.* (11) had noticed a difference in vector disruption by serum of different species, we decided to use mouse serum collected from our *in vivo* animal model (female C57Bl/6 mice). Figure 6 shows that, by incubating for 1 h at 37°C with 20% serum, most of the plasmid underwent a transition from the supercoiled to relaxed state. This transition was prevented in aggregated (R = 1.5) sphingosine-based lipoplexes or at high charge ratios (R = 7). Similarly, the plasmid was completely degraded by incubating with 100% serum, and this degradation was prevented within highly cationic lipoplexes (R = 7), although in this case the plasmid became relaxed, indicating that it could not be completely protected from nuclease attack.

Study of Sphingosine-Based Lipidic Carriers for *in Vivo* Gene Transfer

DNA transfection by sphingosine–based lipoplex injections was evaluated in the mouse leg tibial cranial skeletal muscle. Previous studies had demonstrated that polyamine-based lipoplexes drastically inhibit DNA transfection by 2 to 3 orders of magnitude, as compared to free DNA (7). Figure 7 shows the influence of the sphingosine-based lipoplexes on reporter-gene expression. An order of magnitude inhibition was observed as compared to free DNA. To investigate if the sphingosine could improve DNA transfection by modifying the membrane cells, we first injected the sphingosine-based liposomes, then plasmid DNA either immediately or after 30 m. Figure 7 shows that 30 m pre-injection of the liposomes improved the transfection level, but not significantly compared to free DNA.

Then, we evaluated if the putative membrane permeabilizing effect of sphingosine could induce a synergy with the permeabilization obtained by using the electrotransfer technique (12). Figure 8 shows that electrotransferred sphingosine-based lipoplexes inhibited the transfection compared to electrotransferred free DNA. Here again, less inhibition than with lipopolyamine-based lipoplexes was observed. When

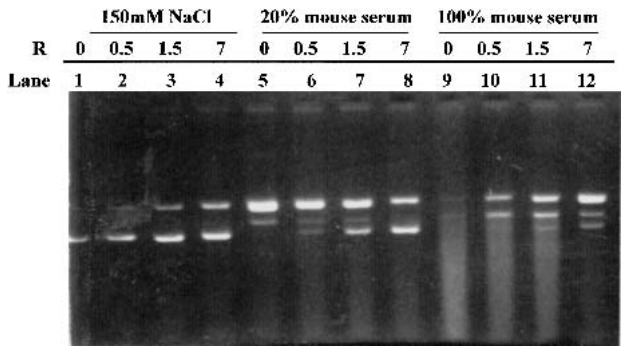

Fig. 6. Integrity of the plasmid DNA after lipoplexes exposure to mouse serum. Lipoplexes were mixed with 20% (in 150 mM NaCl) or 100% mouse serum at a ratio of 1:1 (v/v), and incubated at 37°C for 1 h. Plasmid DNA was then extracted using QIAamp Blood Kit (QIAGEN, Valencia, CA, USA) as described (11) and its integrity was analyzed on a 0.8% agarose gel.

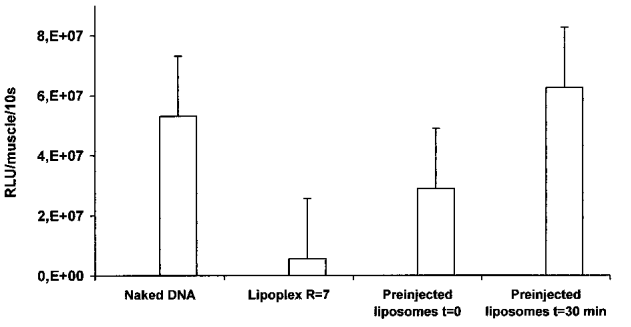

Fig. 7. Influence on reporter gene expression of sphingosine-based lipoplexes and preinjection of liposomes: first column: naked DNA, second column: Sph/EPC/DNA R = 7; third column: preinjection of Sph/EPC cationic liposome (corresponding to the amount of the lipoplex, second column), followed by immediate DNA injection; fourth column: cationic liposome preinjection followed by DNA injection 30 min later.

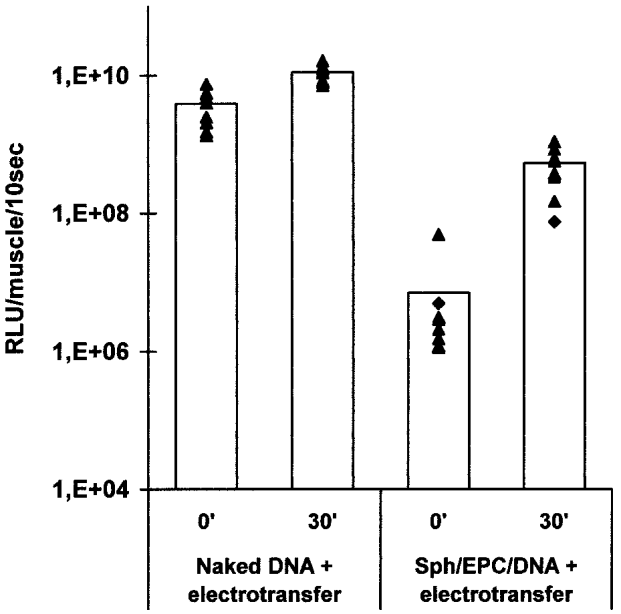

Fig. 8. Comparative effect on gene transfection efficiency of sphingosine-based lipoplexes by using electrotransfer. Columns from the left to the right: naked DNA electrotransferred immediately after i.m. injection; naked DNA electrotransferred 30 min after i.m. injection; cationic lipoplex (R = 7) electrotransferred immediately after i.m. injection; cationic lipoplex (R = 7) electrotransferred 30 min after i.m. injection.

free DNA was injected, the electric pulses could be delivered up to 30 m after plasmid injection without affecting the level of transgene expression. Conversely, when permeabilizing electric pulses were delivered 30 m (but also 60 or 120 m, data not shown) after the injection of sphingosine/EPC/DNA lipoplexes, a two logs unit increase was detected, relative to concomitant delivery of lipoplexes and electric pulses. This suggests that a 30-m delay was enough to release DNA from the complexes. This is in agreement with our physicochemical results showing that sphingosine liposomes bind with DNA with a low affinity. We however cannot exclude that sphingosine could play a role in DNA binding to cellular membrane and/or membrane permeabilization, even if we could not demonstrate this using this indirect method. Sphingosine insertion into the muscle fiber membrane might require a longer time to favor plasmid electrotransfer.

DISCUSSION

Several *in vitro* as well as *in vivo* studies have been performed on sphingosine. This cationic amphiphile is a potent inhibitor of protein kinase (13), and participates in the activation of phospholipases C and D (14). The interaction of sphingosine with nucleic acids (3,4), and particularly its participation in transferring DNA into the liposome bilayers retained our attention (6).

DNA transfer into muscular cells is still under intensive study. Injection of free DNA gives low and variable transfection efficiency. In this particular tissue, DNA complexation with most of the chemical agents tested has been shown to inhibit DNA transfection, while electrotransfer of naked DNA demonstrated high transfecting efficiency (7). With electrotransfer, inhibition of transfection was also observed as soon as DNA was associated to a cationic lipid carrier (unpublished data), suggesting that DNA was not liberated from lipoplexes, and that electric pulse delivery did not promote cell entry of lipoplexes. According to this hypothesis, less stable potentially more labile DNA/vector complexes are of interest, since potentially, they allow increased DNA stability for storage and higher DNA release after administration. In addition, an associated membrane permeabilization by the lipid, prior to DNA release, would be of particular interest to eventually enhance transfection. Thus, previously published results on sphingosine prompted us to investigate the use of sphingosine for gene delivery into the muscle.

From electrophoresis, ethidium bromide, zeta potential, nuclease and serum digestion experiments, we can conclude that DNA is indeed associated to sphingosine/EPC liposomes, but is not in an optimally compacted state within these lipoplexes, being more accessible to the exterior than classical polyamine-based lipoplexes. This property to condense DNA without compacting it makes sphingosine/EPC liposomes a novel type of labile gene vector. Moreover, our *in vivo* experiments, both in the absence and in the presence of electrotransfer suggest that sphingosine-based lipoplexes were rapidly releasing DNA after *in vivo* injection into the muscle. We have to stress however, that these experiments represent only indirect evidence of the *in vivo* release and of the putatively labile character of sphingosine-based lipoplexes.

In conclusion, we have shown that it is possible to condense DNA in weakly compacted structures. The use of a lipid bearing a single primary amine such as sphingosine is enough to mediate DNA association with liposomes. The liposomes obtained were shown to protect DNA towards seric enzyme degradation. Although no positive results have been obtained to date, this novel type of "less stable" lipoplexes might be considered as a prototype for a new generation of vectors for gene delivery. Optimization in gene transfer could be expected by conferring targeting capacity to such particles, for instance when using systemic injection.

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