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Enhanced gene delivery in vitro and in vivo by improved transferrin-lipoplexes

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Abstract

Cationic liposomes and the complexes they form with DNA (lipoplexes) constitute the most promising alternative to the use of viral vectors for gene therapy. One of the limitations to their application in vivo, however, is the inhibition of gene delivery by serum. In a previous study, we demonstrated that transferrin (Tf)-lipoplexes were superior to plain lipoplexes in transfecting HeLa cells in the presence of high concentrations of serum. With the goal of obtaining efficient gene expression in vivo, we evaluated the efficacy of Tf-lipoplexes (containing DOTAP and cholesterol) in transfecting primary hepatocytes and adipocytes in the presence of high serum concentrations. The association of transferrin with cationic liposomes increased luciferase expression compared to plain lipoplexes in primary cells as well as in HepG2 and 3T3-L1 differentiated adipocytes. The complexes were not cytotoxic and were highly effective in protecting DNA from attack by DNase I. An efficient and reliable method was developed to prepare lipoplexes containing both Tf and protamine sulfate, where the latter was mixed with transferrin, followed by the addition of cationic liposomes and DNA. The resulting protamine-Tf-lipoplexes increased significantly the levels of gene expression in cultured cells and in various tissues in mice following i.v. administration. © 2002 Elsevier Science B.V. All rights reserved.

Keywords: Gene therapy; Cationic liposome; Primary hepatocyte; Transferrin; Protamine; In vivo gene transfer; Primary adipocyte

1. Introduction

Cationic liposomes have several advantages over viral systems as gene delivery vehicles. There are no size limitations for the construction of vectors, which makes the introduction of genomic fragments, including complex transcriptional control elements, possible. They act by compacting plasmid DNA, and mediate cellular uptake primarily via endocytosis following the interaction of positively charged complexes with negatively charged biological surfaces [1–4]. One disadvantage of the use of cationic liposomes is the considerable reduction of transfection efficiency by serum components [5–8]. The inhibitory effect of serum on transfection mediated by lipoplexes has been reported for different cell lines, including hepatocytes [9,10], and represents a serious limitation for their use in vivo. Thus, during the in vitro assessment of transfection reagents, it is important to emulate in vivo conditions by using high concentration of serum, rather than serum-free medium which usually optimizes gene transfer.

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Hepatocytes and adipocytes, like many other terminally differentiated cells, are relatively resistant to transfection. It would, therefore, be of interest to develop systems that enable the efficient transfer of functional DNA molecules into such cells. Although several viral vectors including adeno- [11,12] and retroviruses [13,14], and non-viral methods such as electroporation [15], calcium phosphate [16], DEAE-dextran [15], cationic lipids [17,18] and polymer derivatives [19,20], have been developed to transfect primary hepatocytes, it is generally difficult to achieve significant levels of gene expression without causing high cytotoxicity [21]. Furthermore, none of these vectors have been utilized in the presence of high concentrations of serum. 3T3-L1 adipocytes have been transfected by adenovirus [22,23], calcium phosphate [24] and electroporation [25]. Primary adipocytes have also been transfected by electroporation [26,27] and adenovirus [28], but these techniques were also often associated with cellular toxicity. So far, transfection of DNA has not been applied successfully in primary adipocytes by non-viral vectors in the presence of high concentrations of serum.

Receptor-mediated gene delivery methods have also been explored, using receptor ligands conjugated to either polycations [29–31] or cationic lipids [32]. The expression of transferrin receptors is particularly high on proliferating malignant cells, as compared with the corresponding normal ones, making this receptor an attractive target for cancer gene therapy [33,34]. The association of transferrin (Tf) with cationic liposomes followed by complexation with DNA has been shown to generate vectors that are far more efficient in gene transfer than conventional lipoplexes [4,35–37].

When DNA is administered to the whole body it could be attacked by nucleases in the blood and/or cellular lysosomes, and finally degraded. Therefore, the gene delivery carrier should function as a protector of the DNA during in vivo administration. Protamine has been shown to cause condensation of DNA, to promote cellular entry and to potentiate gene expression by increasing the nuclear translocation of DNA [38,39]. The use of this polycation could improve the in vivo activity of lipoplexes [40,41] and has the additional advantages of being non-toxic and only weakly immunogenic in humans. It is important also to note that the way the DNA is mixed with liposomes to form lipoplexes, as well as the final size, affects greatly the efficiency of the resulting complexes [35,42].

In this report, we describe the ability of Tf-lipoplexes in enhancing gene expression in primary cells in the presence of high concentrations of serum. Additionally, we have optimized the transfection efficiency of these complexes by adding protamine and designing a new protocol to prepare them. Our results show that protamine greatly enhances the levels of transgene expression over the increase observed with Tf-lipoplexes, and that these vectors are more effective and less toxic than traditional plain complexes for in vivo systemic administration.

2. Materials and methods

2.1. Materials

The cationic lipid 1,2-dioleoyl-3-(trimethylammonium) propane (DOTAP), dioleoylphosphatidylethanolamine (DOPE) and cholesterol (CHOL) were purchased from Avanti Polar Lipids (Alabaster, AL, USA). Iron-saturated, heat-inactivated human transferrin was obtained from Collaborative Biomedical Products, Becton Dickinson (Bedford, MA, USA). The plasmids, pCMVLuc (VR-1216) encoding luciferase and pCMVLacZ (Clontech, Palo Alto, CA, USA) encoding β-galactosidase, were used for carrying out the transfection experiments. NaCl, *N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethanesulfonic acid) (HEPES) and protamine sulfate (derived from salmon) were obtained from Sigma (Madrid, Spain). Alamar blue dye was purchased from Accumed International Companies (Westlake, OH, USA), and DNase I and ethidium bromide from Gibco BRL Life Technologies (Barcelona, Spain).

2.2. Cell isolation and culture

HepG2 human hepatoblastoma cells (American Type Culture Collection, Rockville, MD, USA), were maintained at 37°C under 5% CO_2 in Dulbecco's modified Eagle's medium-high glucose (DMEM), supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS), penicillin (100 units/ml), streptomycin (100 μg/ml) and L-glutamine

(4 mM) (Gibco BRL Life Technologies). Cells were passaged 1:10 by trypsinization once a week.

3T3-L1 cells (American Type Culture Collection), are a derivative of mouse fibroblasts, which differentiate into an adipocyte-like phenotype under appropriate conditions [43]. Cells were cultured in six-well plates (35 mm well diameter, Costar, Acton, MA, USA), in DMEM, containing 10% FBS, penicillin (100 units/ml) and streptomycin (100 μg/ml). Confluent cells were induced to differentiate by incubating for 48 h with differentiation medium containing 1 μM dexamethasone, 0.5 mM isobutylmethylxanthine (IBMX), 10 μg/ml insulin and 10% FBS in DMEM. After this time, cells were maintained in postdifferentiation medium containing 10 μg/ml insulin and 10% FBS, which was changed every 2 days. Passages 3–9 were used in all studies. 3T3-L1 cells were transfected 8 days after the induction of differentiation.

Primary hepatocytes were isolated from adult male Wistar rats (250 g) by in situ perfusion of the liver with collagenase [44], following anesthesia with pentobarbital. As soon as the cells detached from the capsule of Glisson, the perfusion was stopped and the liver was removed. The hepatocytes were filtered through a 100 μm mesh in order to obtain a single-cell suspension. Hepatocytes were recovered by low speed ($50\times g$) centrifugation and resuspended in RPMI 1640 (Gibco BRL Life Technologies). When the viability of the cells, determined by trypan blue exclusion, was more than 98%, isolated hepatocytes were utilized in subsequent experiments. Hepatocytes were plated in type I collagen (Collaborative Biomedical Products, Becton Dickinson, Bedford, MA, USA)-coated six-well plates (35 mm well diameter) at a density of 10^6 viable cells per well, and cultured in RPMI 1640, supplemented with 10% (v/v) heat-inactivated FBS, penicillin (100 units/ml), streptomycin (100 μg/ml) and L-glutamine (4 mM). After attachment of the viable cells, non-viable cells were removed by changing the medium. This procedure resulted in a constant cell density at the time of transfection.

Adipocytes were isolated from epididymal fat pads of adult male Wistar rats [45]. The fat pads were minced into pieces in Krebs–Ringer HEPES buffer (pH 7.4; containing 5 mM D-glucose, 2% BSA, 135 mM NaCl, 2.2 mM $CaCl_2{\cdot}2H_2O$, 1.25 mM $MgSO_4{\cdot}7H_2O$, 0.45 mM KH_2PO_4, 2.17 mM Na_2HPO_4 and 10 mM HEPES). Adipose tissue fragments were digested in the same buffer with type I collagenase (1.25 mg/ml per gram of tissue) at 37°C with gentle shaking at 60 cycles/min for 30 min. The resulting cell suspension was diluted in HEPES-phosphate buffer and the isolated adipocytes were then separated from the undigested tissue by filtration through a 400 μm nylon mesh and washed three times. Cells were resuspended in DMEM supplemented with 1% FBS and incubated for 30 min at 37°C. Adipocytes (150 μl of 2:1 ratio of packed cells to medium) were then plated on a collagen matrix (Vitrogen 100, Cohesion, Palo Alto, CA, USA) in six-well culture plates. After a 45 min incubation at 37°C, the culture medium was added and the cells were maintained in an incubator at 37°C in 5% CO_2 for 16–24 h prior to transfection.

2.3. Liposome preparation

DOTAP/DOPE (1:1 molar ratio) liposomes were prepared by drying a chloroform solution of the lipids by rotary evaporation under reduced pressure and then by hydrating the film with 1 ml of deionized water to a concentration of 5 mg total lipid/ml. The tube was vortexed for 1 min and the suspension was diluted to a final concentration of 1 mg/ml of total lipid. The resulting multilamellar vesicles were extruded through polycarbonate membranes with 100 nm pore diameter using a Liposofast device (Avestin, Toronto, ON, Canada), to obtain a uniform size distribution and filter-sterilized (Millex 0.22 μm, Millipore, Bedford, MA, USA). DOTAP/CHOL (1:0.9 molar ratio) liposomes used for in vivo studies were hydrated with a 10 mM HEPES, 10% (w/v) glucose buffer (pH 7.4) to give a final concentration of 10 mM DOTAP/9 mM CHOL. The average diameter of the liposomes was 102 ± 10 nm. Liposomes were stored at 4°C under nitrogen and were used within 1 month after preparation.

2.4. Preparation of lipoplexes

Complexes for in vitro experiments were prepared by first mixing 400 μl of a solution of 100 mM NaCl, 20 mM HEPES, pH 7.4, with (Tf-lipoplexes) or without (plain lipoplexes) 128 μg of transferrin, with 8, 16,

32 or 64 μl of the DOTAP/DOPE liposome suspension and incubating for 15 min at room temperature. Four hundred microliters of HEPES buffer containing 3.5 μg of pCMVLuc or pCMVLacZ were then added and gently mixed. Complexes for in vivo experiments, prepared at a 5/1 (+/−) charge ratio, contained 19 mM of total lipid (DOTAP/CHOL) and 32 μg of transferrin per μg of DNA. Protamine-Tf-lipoplexes were prepared by two different protocols: one involved the precomplexation of plasmid DNA with protamine followed by the addition of pre-formed cationic liposome–transferrin complexes, prepared as described above (protocol 1). The other involved mixing of transferrin with protamine, followed by their addition to the liposomes, incubation for 15 min, addition of the plasmid and further incubation for 15 min (protocol 2). The final concentration of plasmid in the complexes was 0.3 mg/ml. The lipid to DNA charge ratio was calculated as the mole ratio of DOTAP (one charge per molecule) to nucleotide residue (average MW 330).

2.5. Cell culture transfections

For transfection, cells were seeded in 2 ml of medium in six-well culture plates (35 mm well diameter, Costar), 24 h before addition of the complexes. Cells were washed twice with DMEM or RPMI without antibiotics and then 1.2 ml of fetal bovine serum and 0.8 ml of complexes were added gently to each well. After a 4 h incubation in 60% FBS (at 37°C in 5% CO_2) the medium was replaced, and the cells were further incubated for 48 h in medium containing 10% FBS. It should be noted that in all our experiments the number of viable cells plated and the cell density at the moment of transfection were constant.

2.6. In vitro transfection activity

After 48 h, cells were washed with phosphate-buffered saline (PBS) and lysed using 250 μl of reporter lysis buffer (Promega, Madison, WI, USA) at room temperature for 10 min, followed by two alternating freeze–thaw cycles. The cell lysate was centrifuged for 2 min at $12\,000 \times g$ to pellet debris. Twenty microliters of the supernatant were assayed for total luciferase activity using the luciferase assay reagent (Promega), according to the manufacturer's protocol. A luminometer (Sirius-2, Berthold Detection Systems, Innogenetics, Diagnóstica y Terapéutica, Barcelona, Spain) was used to measure luciferase activity. The protein content of the lysates was measured by the DC Protein Assay reagent (Bio-Rad, Hercules, CA, USA) using bovine serum albumin as the standard. The data were expressed as pg or ng of luciferase (based on a standard curve for luciferase activity) per mg of protein.

2.7. Transfection efficiency

Transfection efficiency was evaluated by scoring the percentage of cells expressing β-galactosidase. Cells transfected with 3.5 μg of pCMVLacZ were washed with PBS, fixed in a solution of 2% formaldehyde and 0.2% glutaraldehyde and stained with a solution containing X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) (1 mg/ml). The cells were incubated at 37°C for 12 h and examined under a phase contrast microscope for the development of blue color. Quantification of transfected cells was performed by counting the number of stained and unstained cells in about 20 randomly selected fields.

2.8. Cell viability assay

Cell viability was quantified by a modified Alamar blue assay [46,47]. Briefly, 2 ml of 10% (v/v) Alamar blue dye in DME-HG supplemented with 10% (v/v) FBS medium were added to each well 48 h after transfection. After 2.5 h of incubation at 37°C, 200 μl of the supernatant was assayed by measuring the absorbance at 570 and 600 nm. Cell viability (as a percentage of control cells) was calculated according to the formula $(A_{570}-A_{600})$ of treated cells $\times 100/(A_{570}-A_{600})$ of control cells.

2.9. DNase I protection assays

Tf-lipoplexes at different charge ratios were prepared as described above. DNase I (1 unit per μg of DNA) was added to 2.5 μg (DNA) of each sample and the mixtures were incubated at 37°C for 30 min. Sodium dodecyl sulfate (SDS) was then included to a final concentration of 1% to release DNA from the complexes. Samples were analyzed by agarose gel

electrophoresis and the integrity of the plasmid in each formulation was compared with untreated DNA as a control. UV spectroscopic studies were performed with a reaction mixture containing 5 μg/ml of plasmid DNA. After a 15 min incubation of Tf-lipoplexes at room temperature, 1 unit of DNase I per μg of DNA was added and the absorbance was monitored at 20 s intervals for 30 min in a UV spectrophotometer (Hewlett-Packard 8452 A, Waldbronn, Germany).

2.10. Dye displacement assay

The binding of protamine to DNA inside Tf-lipoplexes was examined using a quenching method based on ethidium bromide, a monovalent DNA-intercalating agent which occupies an effective binding site of two base pairs; its fluorescence is dramatically enhanced upon binding to DNA and quenched when displaced by higher affinity compounds or by condensation of the DNA structure. Briefly, 12 μg of DNA in 2 ml of 10 mM HEPES, 10% (w/v) glucose buffer (pH 7.4) were mixed with 1.4 μg of ethidium bromide and a baseline fluorescence (F_0) was determined. Various amounts of protamine were added to the above mixture followed by cationic lipids and transferrin in order to prepare 4/1 (+/−) charge ratio Tf-lipoplexes. The fluorescence was measured ($n=3$) after each addition in an LS 50 spectrofluorimeter (Perkin-Elmer, Mountain View, CA, USA), at an excitation and emission wavelength of 520 and 600 nm, respectively, with a slit width of 6 mm. The relative fluorescence values were determined as follows: $F_r=(F_{obs}-F_e)\times 100/(F_0-F_e)$, where F_r is the relative fluorescence, F_{obs} is the measured fluorescence, F_e is the fluorescence of ethidium bromide in the absence of DNA under the given buffer conditions, and F_0 is the initial fluorescence of ethidium bromide-DNA in the absence of the lipid and the polycation.

2.11. In vivo gene expression

Female Balb-c mice (8–10 weeks of age), purchased from Harlan Ibérica Laboratories (Barcelona, Spain), and housed in accordance with institutional guidelines, were used for the experiments. Individual mice in groups of eight were injected via the tail vein with 60 μg of DNA formulated in plain, Tf- or protamine-Tf-lipoplexes in a total volume of 200 μl 5% w/v glucose. Twelve hours following i.v. injection, the mice were sacrificed with a sodium pentobarbital overdose and approx. 1 ml of blood was removed by intracardiac puncture. One milliliter of PBS was perfused through the right cardiac ventricle. The heart, lungs, liver and spleen were collected and washed with cold saline twice. The organs were homogenized with lysis buffer (Promega) using a homogenizer (Mini-Beadbeater, BioSpec Products, Bartlesville, OK, USA) and centrifuged at $12\,000\times g$ for 3 min at 4°C. Twenty microliters of the supernatant were analyzed for luciferase activity as described previously.

2.12. Particle size and ζ potential measurements

The particle size of complexes was measured by dynamic light scattering, and the overall charge by ζ potential measurements, using a particle analyzer (Zetamaster, Malvern Instruments, Spain). Samples of the prepared complexes (2.5 ml) were measured three times for 60 s at 1000 Hz and an electric current of 3 mA with zero field correction.

3. Results

3.1. Transfection activity in HepG2 and 3T3-L1 cells by plain and Tf-lipoplexes

Transfection mediated by either plain or Tf-lipoplexes at different lipid/DNA (+/−) charge ratios was examined in the presence of 60% FBS. Transfection activity in hepatic cells (HepG2) with complexes containing transferrin at 1/2, 1/1, 2/1 and 4/1 (+/−) charge ratios was respectively 160-, 500-, 130- and 6-fold higher than that obtained with plain lipoplexes. The transfection activity of Tf-lipoplexes increased as the charge ratio of complexes was increased (Fig. 1). Luciferase gene expression in 3T3-L1 differentiated adipocytes transfected with Tf-lipoplexes was at least 20-fold higher than transfections performed with plain lipoplexes. The greatest enhancement over plain lipoplexes was observed in the case of 1/2 (+/−) complexes (200-fold). The 4/1 (+/−) complexes produced the highest activity (Fig. 2). No gene expression was detected with the naked plasmid with either cell line.

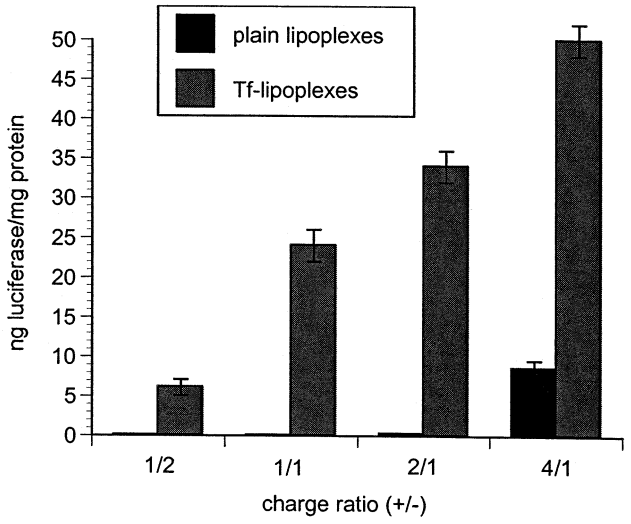

Fig. 1. Transfection of HepG2 cells by plain and Tf-DOTAP/DOPE lipoplexes. The data represent the mean ± S.D. of three wells and are representative of three independent experiments.

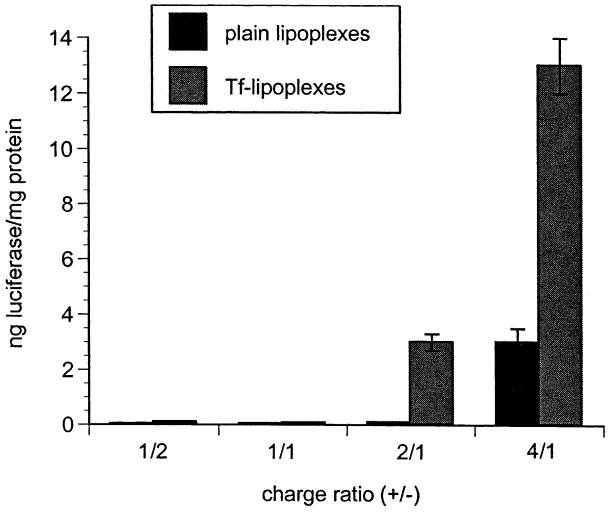

Fig. 3. Transfection of primary hepatocytes by plain and Tf-lipoplexes. The data represent the mean ± S.D. of three wells and are representative of three independent experiments.

3.2. Transfection of primary cells

Although non-dividing cells are known to be difficult to transfect, the association of transferrin with cationic liposomes also increased luciferase expression compared to plain complexes in primary culture rat hepatocytes (Fig. 3) and adipocytes (Fig. 4).

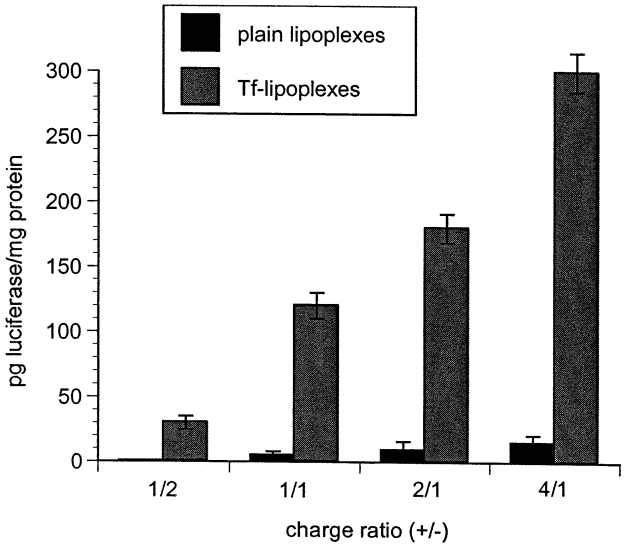

Fig. 2. Transfection activity of plain and Tf-DOTAP/DOPE lipoplexes in 3T3-L1 cells. The data represent the mean ± S.D. of three wells and are representative of three independent experiments.

Compared to plain lipoplexes, luciferase expression mediated by Tf-lipoplexes in primary hepatocytes was approx. 5–40-fold higher depending on the charge ratio used. In primary adipocytes luciferase activity was 4–5-fold higher than that obtained by plain lipoplexes. Transfection also increased with increasing charge ratio (+/−) of lipid to DNA in the complex and was much higher than that obtained with the naked plasmid.

To investigate whether the enhancement of trans-

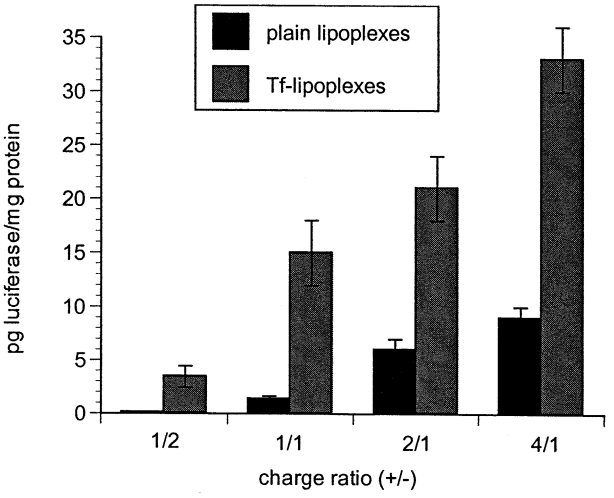

Fig. 4. Transfection of primary adipocytes with plain and Tf-lipoplexes. The data represent the mean ± S.D. of three wells and are representative of three independent experiments.

fection mediated by the association of transferrin in the presence of serum was only due to an enhancement of gene expression or also due to an increase in the number of primary cells transfected, we evaluated the transfection efficiency, defined as the percentage of cells that visibly express the β-galactosidase gene. Transfection efficiency of primary hepatocytes by Tf-lipoplexes in the presence of 60% FBS was maximal for the 4/1 (+/−) charge ratio. The number of blue cells when Tf-lipoplexes were used was always higher than that obtained with plain lipoplexes (Table 1). No blue cells were detected when naked plasmid was transfected.

3.3. Cytotoxicity

Cell viability following transfection was assessed to evaluate whether transferrin and plain complexes at various lipid/DNA (+/−) charge ratios were toxic to the cells. The Alamar blue assay showed a viability higher than 90% in HepG2 as well as in primary hepatocytes (Fig. 5). The relative cytotoxicity of lipoplexes was also assessed by the total amount of extractable cellular proteins in the cell lysate per well, and confirmed the results with the Alamar blue assay (data not shown).

3.4. Nuclease resistance of Tf-lipoplexes

To assess the role of our complexes in protecting the DNA from attack by degrading enzymes in vivo, an in vitro DNase I protection assay was performed with Tf-lipoplexes prepared at different charge ratios. The gel electrophoresis data are shown in Fig. 6A. Protection of DNA increased by increasing the charge ratio in the complex, and resulted to be, by measuring the intensity of the bands compared to untreated plasmid (100%) (lane 1), about 90% for complexes prepared at 4/1 (+/−) charge ratio (lane 6). Free DNA was completely degraded (lane 2).

Table 1
Gene transfer efficiency (% of blue cells) in primary hepatocytes by plain (DOTAP/DOPE) and Tf-lipoplexes containing pCMVLacZ in the presence of 60% serum

Charge ratio (+/−)	Plain lipoplexes	Tf-lipoplexes
0.5	1–2	2–4
1	1–2	3–5
2	1–2	6–7
4	2–3	8–10

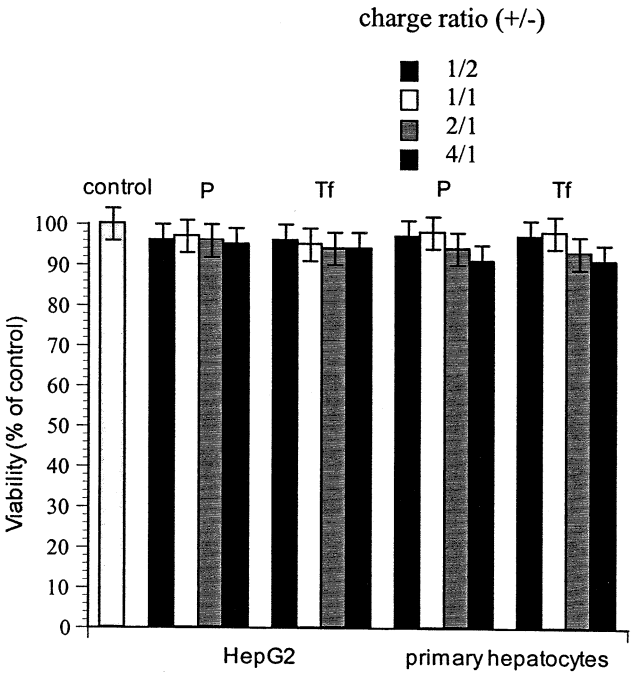

Fig. 5. Effect of plain (P) and transferrin (Tf)-lipoplexes on the viability of HepG2 cells and primary hepatocytes. Data represent the mean ± S.D. obtained from duplicate wells and are representative of two independent experiments.

Stabilization of DNA in Tf-lipoplexes was further studied by UV spectroscopy (Fig. 6B). By addition of DNase I to native DNA, absorbance of the solution was increased immediately due to the fragmentation of the plasmid. Almost 100% of the substrate was degraded within 4 min. On the other hand, DNA complexed with Tf-lipoplexes at maximal charge ratios, 2/1 and 4/1 (+/−), was completely protected, showing almost no increase in absorbance at 260 nm.

3.5. Effect of protamine sulfate on in vitro transfection by Tf-lipoplexes

Fig. 7 shows a dose-dependent gene expression by protamine included in Tf-lipoplexes compared to plain and Tf-complexes in the absence of the polycation. Maximum level of transfection was observed at the protamine concentration of 0.5 μg per μg of DNA, and was 9-fold over Tf-lipoplexes and 25-fold

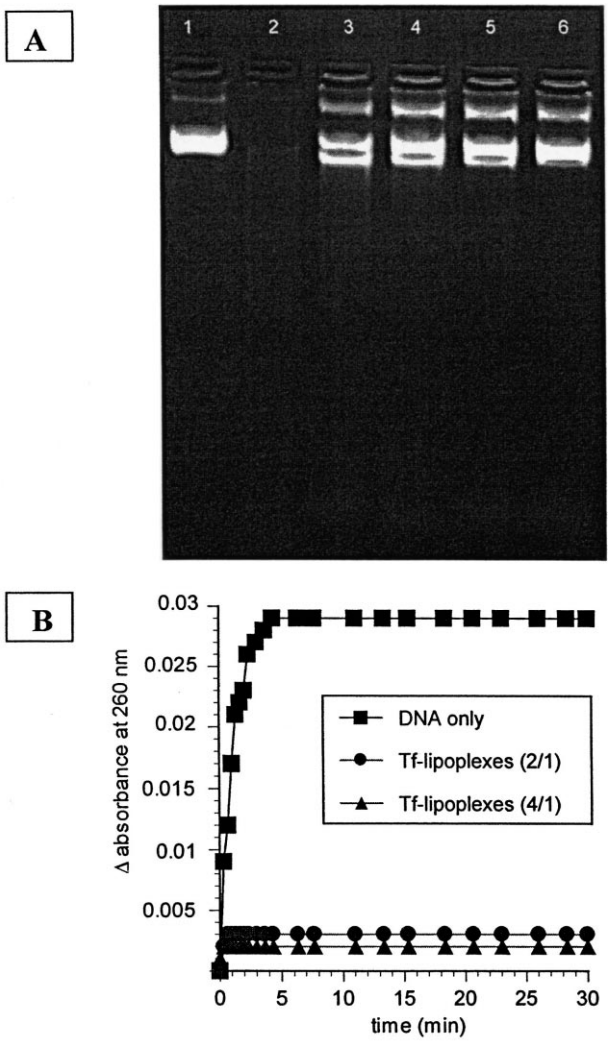

Fig. 6. (A) Susceptibility of plasmid DNA or Tf-lipid/DNA complexes to degradation by DNase I treatment. Untreated DNA (lane 1), DNA treated with DNase: naked plasmid (lane 2), DNA inside Tf-lipoplexes at 1/2 (lane 3), 1/1 (lane 4), 2/1 (lane 5) and 4/1 (lane 6) (+/−) charge ratios. (B) Degradation profile of DNA alone or Tf-lipid/DNA complexes at 2/1 and 4/1 (+/−) charge ratios with DNase I.

over the levels seen with plain complexes. The polycation itself in combination with DNA had no significant effect on gene expression. Fig. 7 also shows the influence of preparing the complexes by the newly designed method (protocol 2), by which the highest level of transfection was obtained. An 18-fold increase over Tf-lipoplexes without protamine and a 2-fold increase over protamine-Tf-lipoplexes prepared by the conventional method (protocol 1) were observed.

3.6. *Displacement of intercalated ethidium bromide by protamine inside Tf-lipoplexes: DNA binding assay*

The displacement of ethidium bromide from DNA in Tf-lipoplexes was monitored by measuring the fluorescence as protamine sulfate was added to the complexes. The addition of the polycation resulted in a rapid decrease in fluorescence. At the optimal amount of protamine obtained for in vitro studies (0.5 μg), DNA was almost completely condensed inside the Tf-lipoplexes at the 4/1 (+/−) charge ratio (Fig. 8). The residual fluorescence (F_{res}), at which the polycation can no further exclude ethidium bromide from the DNA, had a low value of 1.9.

3.7. *In vivo gene expression of protamine-Tf-lipoplexes*

Finally, to test whether Tf-lipoplexes and protamine-Tf-lipoplexes could be used for in vivo gene delivery, complexes were injected systemically into mice and their activity was compared to plain lipoplexes. Fig. 9 shows that complexes in the presence of the ligand lead to a higher level of gene expression compared to plain lipoplexes in the four

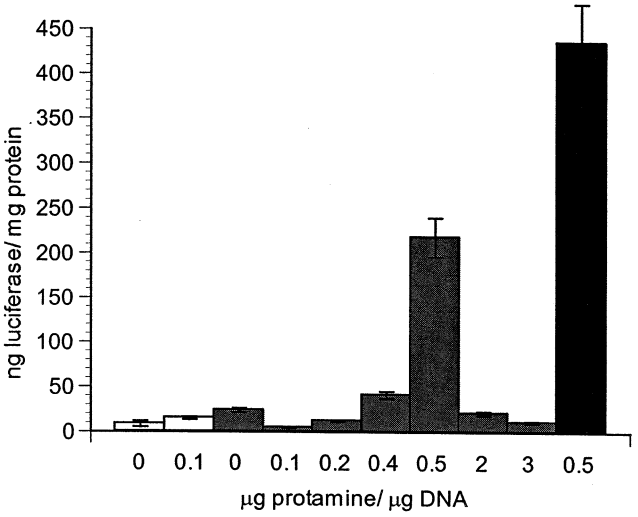

Fig. 7. Gene expression in HepG2 cells by plain (white bars) and Tf-lipoplexes (gray bars, protocol 1; black bars, protocol 2) in the absence or presence of protamine sulfate. Complexes were prepared at 4/1 (+/−) charge ratio. The data represent the mean ± S.D. of three wells and are representative of three independent experiments.

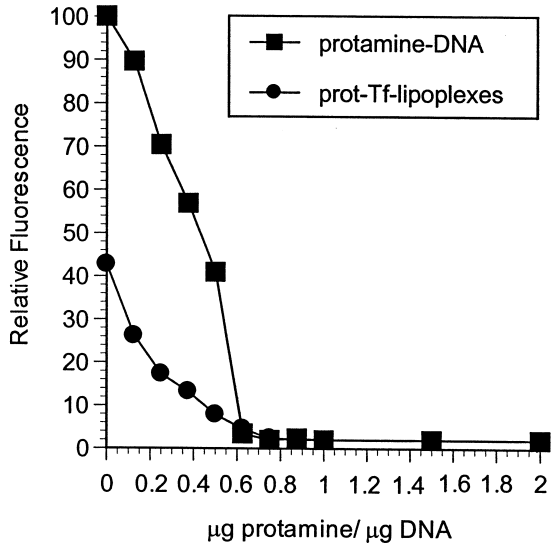

Fig. 8. Binding behavior of protamine to DNA, studied by ethidium bromide assay. Complexes were prepared at 4/1 (+/−) lipid to DNA charge ratio. Each point represents the mean of three replicate measurements.

studied organs. Protamine-Tf-lipoplexes, prepared by the newly developed protocol, resulted in an increase of 5-, 2-, 1.3- and 5-fold in the liver, lung, heart and spleen respectively, over Tf-lipoplexes.

3.8. Determination of particle size and ζ potential of complexes

The diameters of plain, Tf- and protamine-Tf-lipoplexes, used for in vivo studies, were 230, 388 and 200 nm respectively while the surface charges were 36, 16 and 20 mV respectively. Addition of protamine to the complex led to a significant decrease in the particle size compared to Tf-lipoplexes.

4. Discussion

Since lipoplex-mediated transfection of many cell types is inhibited by serum [5,48,49], most in vitro transfection studies in the literature have been carried out in the absence of serum or in the presence of at most 20% serum. It is interesting to note that in this study, all the experiments were performed in the presence of 60% FBS, following the optimal conditions reported in our previous work [7]. The results shown in Figs. 1 and 2 demonstrate that Tf-lipoplexes mediate higher gene expression in HepG2 and 3T3-L1 cells compared to plain lipoplexes. The maximal activity is observed for Tf-complexes at the 4/1 (+/−) charge ratio. One possible explanation for

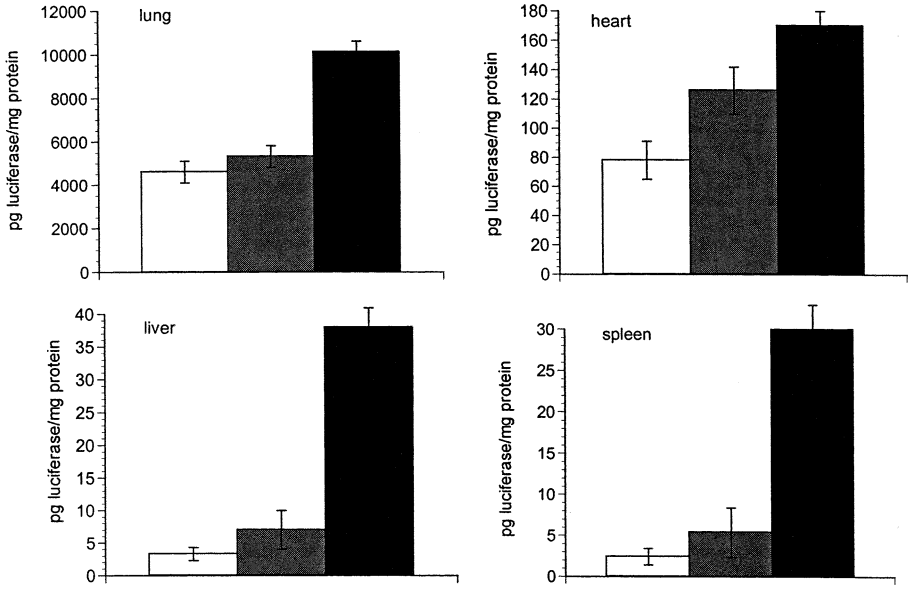

Fig. 9. In vivo transfection efficiency of plain (white bars), Tf-lipoplexes (gray bars) and protamine-Tf-lipoplexes (black bars). Complexes in the presence of the polycation were prepared with 0.5 μg of protamine per μg of DNA following protocol 2, as described in Section 2. The results are expressed as pg of luciferase per mg of protein ($n = 8$).

this enhancement is that non-specific interactions of plain lipoplexes with negatively charged macromolecules in serum most likely prevent their binding to the cells, while Tf-lipoplexes can interact with cell surface receptors. Also, the charge neutralizing effect of the negatively charged macromolecules present in serum is countered by an excess of positive charge on the Tf-lipoplexes.

Hepatocytes and adipocytes, like many other terminally differentiated cells, are relatively resistant to transfection. The development of a system that enables the efficient transfer of functional DNA molecules to such cells was one of the aims of this study. The same behavior observed in the cell lines was observed in primary hepatocytes and adipocytes, as shown in Figs. 3 and 4. Tf-lipoplexes gave always higher levels of transfection compared to plain lipoplexes. Association of Tf with the liposomes appears to facilitate the internalization of the complexes due to the ability of the ligand to stimulate endocytosis [50]. Although the highest level of gene expression was reached with positively charged complexes, it should be noted that the maximum fold increase over plain lipoplexes was achieved with neutral 1/1 and negatively charged 1/2 (+/−) lipoplexes, which remained effective despite any possible interaction with serum components. No measurable luciferase expression was observed with DNA alone either in the cell lines or in primary hepatocytes and adipocytes.

The correlation between transfection activity and efficiency, observed by the β-gal assay in primary hepatocytes (Table 1), indicated that not only is gene expression higher by using Tf-lipoplexes, but also the number of transfected cells. Conditions which enhance transfection activity also enhance transfection efficiency, although the percentage of enhancement cannot be correlated directly. It is also interesting to note that none of the complexes used in this study were toxic to the cells, concluded from the viability assay (Fig. 5).

Another possible mechanism for the enhancement in transfection by Tf-lipoplexes includes improved protection of DNA inside the complexes, as is shown in the protective ability assays (Fig. 6). Gel electrophoresis data showed the high level of nuclease resistance for DNA formulated in Tf-lipoplexes, which confirms its stable nature. At the 4/1 (+/−) charge ratio, the plasmid is almost completely protected from digestion by DNase I (Fig. 6A, lane 6). UV spectroscopy data confirmed the preceding results. When DNA itself was treated with DNase I under physiological conditions, complete degradation occurred within 4 min; however, the percentage of degraded plasmid in Tf-lipoplexes at this time, for the maximal charge ratio, was only 7% of that for native DNA (Fig. 6B). This feature of high resistance toward nuclease attack is surely an advantage of using the Tf–lipid complexes as a reservoir for DNA under physiological circumstances, when DNA degradation through nuclease attack takes place readily.

Although protamine has already been proposed to increase lipid-mediated gene transfection by condensing DNA into a compact structure, which promotes cellular entry and stability of DNA [38,39,51], one of the aims of this work was to study the role of protamine in combination with Tf to develop improved Tf-lipoplexes for in vivo gene delivery. Traditional protocols for preparing protamine-lipoplexes involves precomplexation of plasmid DNA with protamine, followed by the addition of liposomes. Under these conditions, however, large amounts of excess cationic lipids are required to achieve a maximal level of gene expression. Moreover, the charge neutralizing effect of protamine on DNA makes preparation of complexes difficult at high concentrations, especially when a high protamine/DNA ratio is required. Taking this into consideration, we hypothesized that precomplexing protamine with Tf (slightly negatively charged), before addition of the plasmid, more stable complexes could be prepared for in vivo application. The results presented in Figs. 7 and 9 confirmed our hypothesis, showing the ability of protamine to increase transfection by Tf-lipoplexes in vitro and in vivo. This enhancement was maximal when complexes were prepared with the new protocol 2, described in Section 2. On the other hand, the displacement of intercalated ethidium bromide shown in Fig. 8 demonstrates that, at the optimal amount of protamine needed for in vitro transfection, DNA is almost completely condensed. The low value of the residual fluorescence indicates the efficiency by which the polycation has condensed the DNA, which is indicative of the stability of the complex.

In vivo results showed an increase in transfection by protamine-Tf-lipoplexes over plain complexes,

which were 11-, 2-, 2- and 12-fold for the liver, lung, heart and spleen, respectively. Gene expression in the lung was significantly higher than that found in other tissues, as expected from previous studies, in which the level of transgene expression was approx. 10 ng luciferase/mg protein [40]. The capacity of transferrin to promote cellular entry, and the ability of protamine to facilitate nuclear localization of DNA, could be additional factors that cause enhanced gene expression. Complexation of Tf with the cationic liposomes could result in charge shielding, as well as provide steric hindrance to other serum components, thereby minimizing the inhibitory effects of serum.

The smaller particle size of the protamine-Tf-lipoplexes can also contribute to the higher gene expression observed. Continuous increase in the dose of protamine was not associated with a further increase in the levels of expression, as shown in Fig. 7, probably due to charge neutralization of the DNA by protamine which limits the complexation of DNA with cationic liposomes.

By using the new protocol, the competition between cationic liposomes and protamine for interaction with plasmid DNA is greatly reduced. Protamine maintains its ability to condense the DNA without reducing further interactions with the cationic lipids. Also, lipid and DNA can interact with each other inside our Tf-lipoplexes in the presence of serum, to form a new structure which is much more efficient in transfection in vivo. Moreover, although charge ratios (+/−) close to 10 are used generally for in vivo transfections, protamine-Tf-lipoplexes at 5/1 (+/−) were able to give efficient gene expression. Consequently, a reduction in lipid-induced cellular toxicity compared to plain lipoplexes was observed. Two of the eight mice died of toxicity when using plain lipoplexes; yet no signs of toxicity were noticed when protamine-Tf-lipoplexes were injected.

Our levels of transfection activity showed an improvement over the values given by transfection with other lipoplexes. For example, maximal values obtained by Li and coworkers [41] were in the range of 0.088–0.88 ng luciferase/mg protein, based on a conversion of their RLU values to ng luciferase. The lung levels obtained by Thierry et al. [52] with a comparable amount of DNA were less than 1 pg/mg. Luciferase gene expression in the lungs, following the administration of targeted adenovirus [53], was much lower than the RLU/mg values reported by Li et al. [41] and corresponding ng/mg values obtained in our experiments. Gene expression in the liver and spleen, however, was much greater with the adenoviral vector than with the protamine-Tf-lipoplexes. We should note that in this study our goal was not to achieve the highest level of transfection in vivo, but to compare the protamine-Tf-lipoplexes with plain and Tf-lipoplexes.

In summary, the novel protamine-Tf-lipoplex gene carrier developed in this study was shown to generate nanometer-sized particles with plasmid DNA and to effectively protect the DNA to be delivered to cells. The carrier had a markedly low toxicity and good transfection performance in vitro and in vivo. Moreover, it has the advantages of ease of preparation, and economy in comparison with commercial transfection reagents. Consequently, it could be used widely for gene transfection into animal cells.

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