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Efficient gene transfer by transferrin lipoplexes in the presence of serum

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Abstract

Cationic lipids are being used increasingly as reagents for gene delivery both in vitro and in vivo. One of the limitations to the application of cationic lipid–DNA complexes (lipoplexes) in vivo is the inhibition of gene delivery by serum. In this study, we have shown that transferrin (Tf)-lipoplexes, which had transferrin adsorbed at their surface via electrostatic interactions, are much more effective than plain lipoplexes in transfecting cells in the presence of relatively high concentrations (up to 60%) of fetal bovine serum (FBS). Serum even enhanced transfection by Tf-lipoplexes composed of 1,2-dioleoyl-3-(trimethylammonium) propane (DOTAP)/dioleoylphosphatidylethanolamine (DOPE)/pCMVLacZ at high lipid/DNA (+/−) charge ratios, and inhibited lipofection for those with low charge ratios when they were added to the cells immediately after the preparation of complexes. The effect of serum on lipofection was dose-dependent. Preincubation of the complexes at 20°C for 6 h led to serum resistance, even for the negatively charged transferrin-lipoplexes. A similar tendency was observed for DOTAP/cholesterol and DOTAP/DOPE/cholesterol liposomes. The percentage of cells transfected, measured by β-galactosidase expression, also increased with the serum concentration. Cell viability was not affected significantly when the cells were incubated with the complexes for 4 h at 37°C, followed by a 48-h incubation. Our findings extend the scope of previous studies where transferrin-lipoplexes were used to introduce DNA into cells, rendering these complexes and their future derivatives potential alternatives to viral vectors for gene delivery in vivo.

Keywords: Gene therapy; Serum inhibition; Cationic liposome; Gene delivery; Transferrin; Transfection

1. Introduction

At the present time, major somatic gene transfer approaches employ either viral [1–3] or non-viral vectors [4,5]. Viral vectors show high gene transfer efficiency, but are deficient in several areas. Adenoviral vectors induce host inflammatory and immune responses, rendering these vectors ineffective in repeated application [3,6–8]. Retroviral vectors require dividing cells for stable integration [9], making them unsuitable for gene therapy of terminally differentiated cells. Some of these problems can be circumvented by employing non-viral vehicles, such as cationic liposomes [10–16]. Synthetic cationic lipids and the complexes they form with DNA constitute the most promising alternative to the use of viral vectors for gene therapy. They accomplish four goals as a

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vehicle: (1) to protect partially DNA from DNase degradation; (2) to condense the DNA; (3) to mediate the adhesion to cells, overcoming the generally negative polarity of most cell surfaces; and (4) to facilitate the intracytoplasmic localization of the DNA. A neutral co-lipid, such as dioleoylphosphatidylethanolamine (DOPE), which assumes fusogenic properties and consequently increases the efficiency of transfection, is frequently added [17,18]. It has been suggested that the majority of DNA complexed to cationic liposomes is taken up through endocytosis, followed by its release from an early endosomal compartment [19–22].

In order to improve the efficiency of transfection mediated by cationic liposomes, several investigators have attempted to devise strategies to specifically target, transcribe and translate DNA. As in this study, one approach has been to introduce DNA into cells by attaching it to a ligand and exploiting the natural specificity of receptor-mediated endocytosis. This pathway has been utilized in gene transfer studies employing polycation–ligand conjugates [23–25]. One useful cell-binding ligand is transferrin (Tf), an iron-transporting serum glycoprotein, which binds to a receptor expressed on the surface of proliferating cells, with particularly high expression on erythroblasts and tumor cells [4,26].

The ability of serum to inhibit lipofection is an often described phenomenon [16,27–29]. In cell culture systems, liposome-mediated gene transfection is usually carried out in serum-free medium or in at most 10–20% serum [22,27,30–32]. The mechanism by which serum inhibits transfection is not clear. Interaction with polyanionic molecules or with complement [33], and low diffusibility through the extracellular space, are obstacles to successful in vivo gene transfer. Relatively efficient in vitro gene transfer in the presence of serum was achieved by using improved formulations [27,34–36], by precondensation of plasmid DNA with polylysine before formation of the lipoplexes [37] or by sequential addition of the lipid to plasmid DNA [36]. Although transferrin–polycation conjugates have been used to target DNA to cells [38–41], these studies were performed either in serum-free medium or in the presence of low concentrations of serum. A liposome formulation with a high transfection efficiency that is not inhibited by high serum concentrations, would provide a considerable advance toward the goal of systemic delivery.

We examined whether gene transfer efficiency in the presence of high concentrations of serum could be achieved by employing receptor-mediated endocytosis. We therefore evaluated the effect of increasing amounts of serum on the levels of transfection mediated by Tf-lipoplexes, which had transferrin adsorbed at their surface via electrostatic interactions, at different lipid/DNA (+/−) charge ratios. We also investigated whether Tf-lipoplexes with a net negative charge could overcome the inhibitory effect of serum. It has been previously shown that these complexes, particularly at charge ratios of 0.5 and 1/1 (+/−), under which conditions they are net negatively charged, increase the transfection efficiency in cell lines [42] and primary human macrophages [43]. We now report that, using transferrin in combination with DOTAP-containing liposomes, efficient gene expression can be achieved with negatively charged lipoplexes in the presence of high concentrations of serum.

2. Materials and methods

2.1. Materials

The cationic lipid 1,2-dioleoyl-3-(trimethylammonium) propane (DOTAP), dioleoylphosphatidylethanolamine (DOPE) and cholesterol 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 plasmid pCMVLacZ (Clontech, Palo Alto, CA, USA) encoding β-galactosidase was used for carrying out the transfection studies. NaCl and *N*-(2-hydroxyethyl(piperazine-*N*′-[2-ethanesulfonic acid] (HEPES) were obtained from Sigma (St. Louis, MO, USA). Alamar blue dye was purchased from Accumed International (Westlake, OH, USA).

2.2. Cell culture

HeLa cells (American Type Culture Collection, MD, USA) were maintained at 37°C under 5% CO_2 in Dulbecco's modified Eagle's medium-high

glucose (DME-HG) (Irvine Scientific, Santa Ana, CA, USA) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS) (Sigma, St. Louis, MO, USA), penicillin (100 U/ml), streptomycin (100 (g/ml) and L-glutamine (4 mM) (Irvine Scientific). Cells were passaged 1:10 by trypsinization twice a week. Human AB serum used in some transfections was purchased from Irvine Scientific. For transfection, 2×10^5 HeLa cells were seeded in 1 ml of medium in 48-well culture plates (11.3 mm well diameter, Costar, Acton, MA, USA) 24 h before addition of the complexes and used at approximately 80% confluency.

2.3. Liposome preparation

DOTAP/DOPE, DOTAP/cholesterol (1:1 molar ratio) and DOTAP/DOPE/cholesterol (1:1:1 molar ratio) liposomes were prepared by drying a chloroform solution of the lipids by rotary evaporation under reduced pressure and then 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 multilamellar vesicles were extruded through polycarbonate membranes with 100 nm pore diameter, using a Liposofast device (Avestin, Toronto, Canada) to obtain a uniform size distribution, and filter-sterilized (Millex 0.45 μm, Millipore, Bedford, MA, USA). The average diameter of the liposomes was 110 ± 30 nm. Liposomes were stored at 4°C under argon and were used within 1 month after preparation.

2.4. Preparation of lipoplexes

Complexes were prepared by sequentially mixing 100 μl of a solution of 100 mM NaCl plus 20 mM HEPES, pH 7.4, with or without 32 μg of transferrin, with 2, 4, 8 or 16 μl of the liposome suspension and incubated for 15 min at room temperature. One hundred microliters of HEPES buffer containing 1 μg of pCMVLacZ plasmid encoding β-galactosidase was then added and gently mixed. Complexes were then incubated for the time described in each experiment. 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. Transfection activity

Cells were washed twice with DME-HG without serum and antibiotics and then 0.3 ml of this medium and 0.2 ml of complexes were added gently to each well. After a 4-h incubation (at 37°C in 5% CO_2) the medium was replaced with DME-HG containing 10% FBS and antibiotics and the cells were further incubated for 48 h. If the lipofection was performed in the presence of serum, non-heat-inactivated FBS or human AB serum was added to the cells to a final concentration as indicated in the experiments.

Cells were washed 48 h after transfection with phosphate-buffered saline (PBS) and lysed with 100 μl of lysis buffer (0.25 M Tris, pH 8) at room temperature for 10 min, followed by alternating freeze–thaw cycles. The cell lysate was centrifuged for 5 min at $10\,000\times g$ to pellet debris. A 70-μl amount of the supernatant was assayed for total β-gal activity as described by Invitrogen (Carlsbad, CA, USA) using an Emax plate reader (Molecular Devices, Sunnyvale, CA, USA). 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 ng of β-gal (based on a standard curve for β-galactosidase activity) per milligram of protein.

2.6. Transfection efficiency

Transfection efficiency was evaluated by scoring the percentage of cells expressing β-galactosidase. Cells transfected with 1 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.

2.7. Cell viability assay

Cell viability was quantified by a modified Alamar blue assay [44,45]. Briefly, 1 ml of 10% (v/v) Alamar blue dye in DME-HG supplemented with 10% (v/v)

FBS medium was 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.

2.8. Particle size determinations

The size distribution of the lipoplexes was determined by dynamic light scattering in a Coulter N4 Plus Submicron Particle Analyzer (Coulter, Miami, FL, USA) at 90° with 2 min of calibration and 4 min of run time. SDP analysis was performed using 17 bins. When DOTAP/DOPE liposomes were mixed with the plasmid pCMVLacZ at different charge ratios (+/−) (0.5, 1, 2 and 4), the resultant plain complexes had size distributions of 193 ± 95, 328 ± 87, 190 ± 80 and 160 ± 70 nm, respectively. The size distributions of the Tf-complexes for the corresponding charge ratios were 390 ± 135, 370 ± 131, 460 ± 136 and 600 ± 194 nm, respectively. Measurements were carried out at least in duplicate immediately after preparation of the complexes.

2.9. Zeta potential measurements

The overall charge of the complexes was examined by zeta potential measurements using a Coulter Delsa 440 SX instrument (Coulter Electronics, Hialeah, FL, USA). Samples of the prepared complexes were placed in the measuring cell, whose position was adjusted to cover a previously determined stationary layer, and an electric current of 3 mA was applied. The zeta potential was calculated for each scattering angle (8.6°, 17.1°, 25.6° and 34.2°), and the values described represent the mean from the different angles of two measurements.

3. Results

3.1. Effect of serum concentration on lipofection activity

Transfection activity mediated by either plain or Tf-lipoplexes, at different lipid/DNA (+/−) charge ratios, was examined as a function of serum concentration. The association of transferrin with cationic liposomes composed of DOTAP and DOPE increased β-galactosidase expression both in the absence or presence of serum compared to lipoplexes without transferrin (Fig. 1). In the absence of serum, the effect of the ligand was more prevalent for 1/2 and 1/1 (+/−) lipid/DNA complexes, where an enhancement of 14- and 11-fold was observed, respectively, compared to plain lipoplexes. β-Galactosidase activity mediated by the positively charged complexes, 2/1 and 4/1 (+/−), was lower than that obtained with the negatively charged lipoplexes.

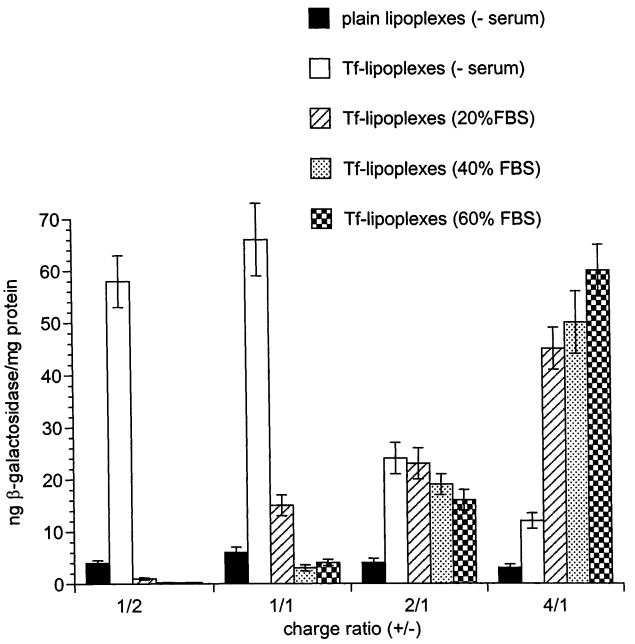

Fig. 1. Effect of serum concentration on β-galactosidase gene expression by HeLa cells transfected with plain DOTAP/DOPE or transferrin lipoplexes immediately after preparation. The data represent the mean ± S.D. of three wells and are representative of three independent experiments.

Expression of β-gal mediated by 1/2, 1/1 and 2/1 (+/−) Tf-lipoplexes was reduced as the serum concentration was increased. The inhibitory effect of serum was diminished with increasing charge ratio (+/−). For 4/1 (+/−) Tf-complexes, the inhibitory effect of serum was overcome, resulting in a similar β-galactosidase activity in 60% FBS as the maximal obtained for 1/1 (+/−) complexes in the absence of serum. Transfection activity of 4/1 (+/−) Tf-com-

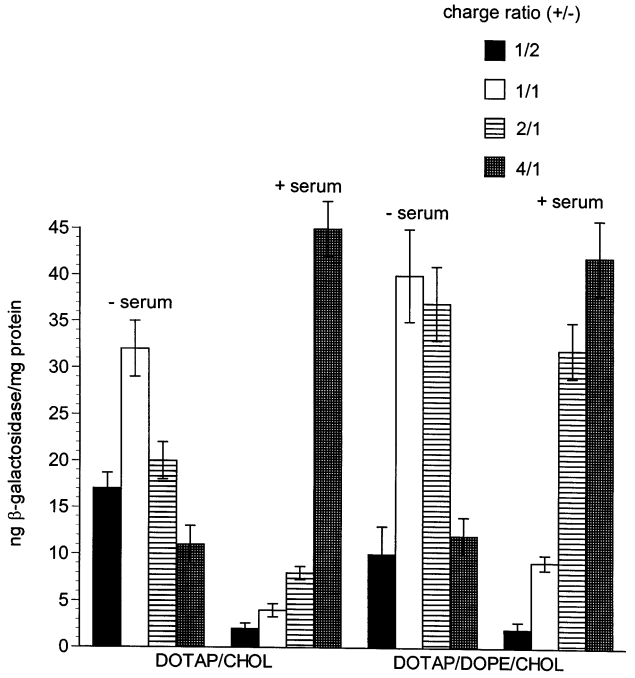

Fig. 2. Lipofection of HeLa cells by Tf–DOTAP/cholesterol and Tf–DOTAP/DOPE/cholesterol lipoplexes in the absence (−serum) or presence of 60% FBS (+serum) at different lipid/DNA (+/−) charge ratios. The data represent the mean ± S.D. of three wells and are representative of three independent experiments.

plexes in 60% FBS increased by 5-fold compared to that in the absence of serum. Heat-inactivated sera retained the potential to enhance Tf-lipoplex mediated transfection (data not shown). Gene delivery and expression by plain lipoplexes even at the lowest serum concentration used (20% FBS) was less than 0.2 ng β-galactosidase per milligram of total cell protein (data not shown). To determine whether the inhibitory effect of serum by Tf-lipoplexes at low charge ratios and enhancement at high charge ratios is limited to DOTAP/DOPE liposomes, we examined DOTAP/cholesterol and DOTAP/DOPE/cholesterol complexes for sensitivity to 60% FBS at different charge ratios (+/−) (Fig. 2). In the absence of serum the highest level of transfection was observed at a 1/1 (+/−) charge ratio for DOTAP/cholesterol complexes and at 1/1 and 2/1 charge ratios for DOTAP/DOPE/cholesterol complexes. In the presence of serum, the same effect as in DOTAP/DOPE complexes was observed, namely that 60% serum inhibited transfection by Tf-lipoplexes at low charge ratios, but the inhibitory effect of serum was overcome by Tf-complexes of 4/1 (+/−) charge ratio. Gene expression by plain lipoplexes in serum was less than 0.2 ng β-galactosidase per milligram of total cell protein (data not shown).

3.2. Effect of plain and Tf-lipoplexes on cell viability in the absence or presence of serum

To evaluate whether transferrin and plain complexes at various lipid/DNA (+/−) charge ratios were toxic to the cells, cell viability was assessed following transfection. The Alamar blue assay showed a viability higher than 90% in all transfected wells (Fig. 3). 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).

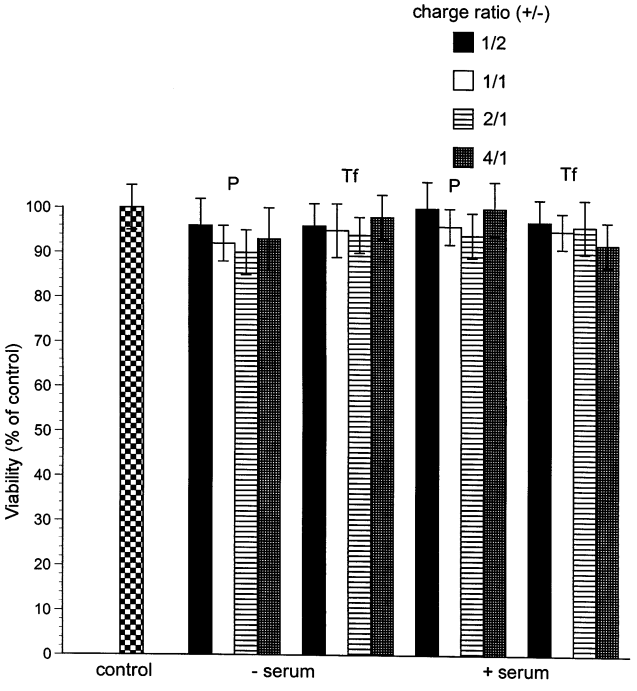

Fig. 3. Effect of plain (P) and transferrin (Tf)–DOTAP/DOPE lipoplexes on the viability of HeLa cells in the absence (−serum) or presence of 60% FBS (+serum) at different lipid/DNA (+/−) charge ratios. Cell viability was expressed as the percentage of the untreated control cells. Data represent the mean ± S.D. obtained from duplicate wells and are representative of two independent experiments.

Table 1
Gene transfer efficiency (% of blue cells) by plain (DOTAP/DOPE) and Tf-lipoplexes containing pCMVLacZ in HeLa cells as a function of the charge ratio lipid/DNA (+/−), in the absence or presence of serum

Charge ratio (+/−)	Plain lipoplexes (−serum)	Tf-lipoplexes (% serum)			
		0	20	40	60
0.5	2–4	10–12	1–2	1–2	1–2
1	2–4	12–15	3–5	2–4	2–4
2	2–4	4–6	4–6	4–6	3–5
4	2–4	3–5	6–8	8–10	10–12

3.3. Evaluation of transfection efficiency

To investigate whether the enhancement of transfection mediated by the association of transferrin in the absence or presence of serum was due to an increase in the number of cells transfected or only due to an enhancement of gene expression, we evaluated the transfection efficiency, defined as the percentage of cells that visibly express the β-galactosidase gene. In the absence of serum, 1/1 (+/−) Tf-lipoplexes showed the highest percentage of transfected cells (12–15%). Transfection efficiency by Tf-lipoplexes in the presence of 60% FBS was maximal for the 4/1 (+/−) charge ratio (10–12%) (Table 1). The number of cells expressing β-galactosidase for plain lipoplexes in the presence of serum was less than 0.1% (data not shown).

3.4. Zeta potential of plain and Tf-complexes

We examined the overall charge of the complexes at different (+/−) charge ratios by zeta potential measurements. The values were in agreement with what was expected from the theoretical calculations based on their charge and the results reported by Simões et al. [42]. The association of transferrin with DOTAP/DOPE/DNA complexes resulted in a decrease of the surface charge for all the charge ratios tested. For example the zeta potential of the 1/1 (+/−) complexes decreased from 2 to −38 mV in the presence of transferrin. This effect is most likely due to the negatively charged amino acids of the protein interacting with the cationic liposomes.

3.5. Maturation of Tf-lipoplexes enhances their serum resistance

Tf-lipoplexes at different charge ratios (+/−) were incubated for different lengths of time at 20°C and examined for β-galactosidase activity and serum sensitivity. Transfection activity in the presence or absence of serum increased by prolonging the incubation time of the complexes. This effect was even more pronounced when the complexes were incubated with the cells in 60% FBS (Fig. 4). After 6 h of incubation, the net negatively charged 1/1 (+/−) Tf-complex became serum-resistant, that is, its transfection activity was not inhibited by serum. The positively charged 2/1 (+/−) complexes overcame the inhibitory effect of serum after 30 min of incubation. Notice that Tf-complexes used immediately after prepara-

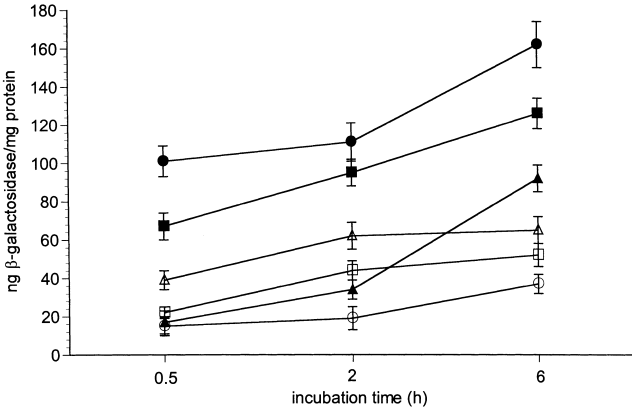

Fig. 4. Effect of the incubation time of Tf–DOTAP/DOPE lipoplexes on serum resistance. Complexes were prepared at different lipid/DNA (+/−) charge ratios (triangles, 1/1, squares, 2/1, circles, 4/1) and incubated at 20°C for the lengths of time described in the figure. Lipofection of HeLa cells was performed in the absence (open symbols) or presence of 60% FBS (filled symbols). The data represent the mean ± S.D. of three wells and are representative of three independent experiments.

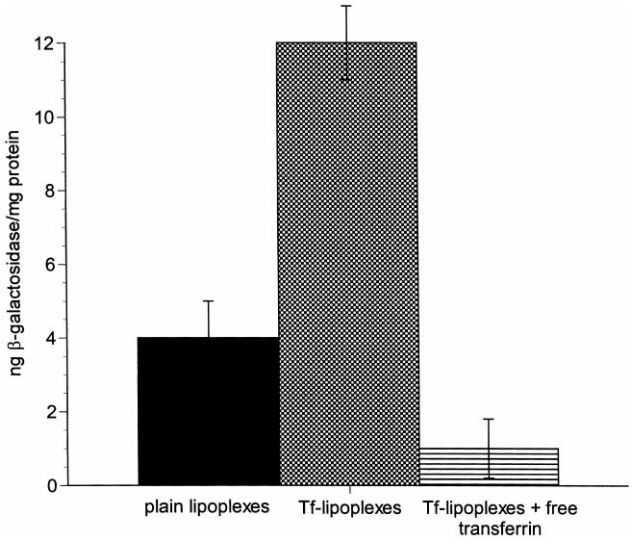

Fig. 5. Effect of specific competition for transferrin receptors on gene transfer by transferrin lipoplexes. An excess of free transferrin (10 mg/ml) was added to the cells prior the addition of the Tf-lipoplexes at the 4/1 (+/−) charge ratio. Data represent the mean ± S.D. obtained from duplicate wells and are representative of two independent experiments.

tion were serum-sensitive even at a charge ratio of 2/1 (+/−) (Fig. 1). Complexes incubated for 6 h also mediated efficient transfection in the presence of human serum (60%) (data not shown), indicating that the phenomenon is not specific to fetal bovine serum.

3.6. Specificity of targeting to the transferrin receptor

To explore whether the uptake of Tf-lipoplexes is facilitated via interaction with the transferrin-receptor, a competitive inhibition experiment was performed, using 4/1 (+/−) lipoplexes which give the highest level of transfection in serum. Treatment of the cells with a large excess of iron-saturated transferrin in the medium (10 mg/ml), representing 156 times the amount of transferrin associated with lipoplexes, led to a decrease in the level of transfection mediated by Tf-lipoplexes (Fig. 5).

4. Discussion

One of the possible reasons for the relatively low gene expression often obtained by lipoplexes is the inefficient entry of the lipid–DNA complex into the cells. Previous studies have shown that the association of a targeting ligand, transferrin, with lipoplexes enhances the transfection of cell lines [42,46] and primary cells [43]. 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 [47]. Transferrin–polylysine conjugates were shown to be targeted to cellular transferrin receptors [4,24–26,48,49]; however, these molecular conjugates have low transfection efficiency.

Our results indicate that Tf-lipoplexes always mediate higher gene expression compared to plain lipoplexes in the absence or presence of serum (Fig. 1). Conditions which enhance transfection activity also enhance transfection efficiency, although the percentage of enhancement cannot be directly correlated (Table 1). DOTAP/DOPE liposomes bind transferrin through the negatively charged groups present in the ligand at physiological pH, and the resulting structure forms a complex with DNA through a charge–charge interaction. Association of transferrin with DOTAP/DOPE/DNA complexes appears to facilitate the internalization of the complex due to the ability of the ligand to stimulate endocytosis. The competitive inhibition of transfection by excess free transferrin supports the hypothesis that the Tf-complexes are recognized by transferrin receptors on the cell surface, which in turn facilitate receptor-mediated endocytosis (Fig. 5). Further experiments with anti-Tf receptor antibodies or receptor-deficient cells will be necessary to confirm this hypothesis. Interestingly, previous experiments with 1/1 and 2/1 (+/−) Tf-lipoplexes and 8 mg free transferrin indicated no significant inhibition of transfection activity, suggesting non-specific endocytosis of these complexes [50].

The inhibitory effect of serum on transfection mediated by lipoplexes has been reported for different cell types [28,37,51] and represents a serious limitation for their use in vivo. One important element in emulating in vivo conditions is the use of high concentrations of serum for transfection experiments in vitro, rather than serum-free medium, which optimizes transfection. So far, most of the studies on the effect of serum on transfection reported have been carried out in the presence of at most 20% serum [52], a condition that is far from that found in vivo.

The lower level of gene expression by net positively

charged (2/1 and 4/1) (+/−) Tf-lipoplexes compared to the net negatively charged (1/2 and 1/1) (+/−) complexes in the absence of serum, may be related to a 'masking' of the ligand by an excess of positive charge, thus decreasing the efficiency of transferrin to recognize its receptor. The difference in transfection activity between these sets of complexes cannot be explained by different toxicities of the formulations or of the serum, since cell viability was similar in both cases (Fig. 3). It is also possible that the negatively charged Tf-lipoplexes dissociate more readily once inside the cell, as suggested by observations on plain lipoplexes [53].

For complexes with low charge ratios (1/2 and 1/1) (+/−), increasing the concentration of serum inhibited gene delivery and expression (Fig. 1). This inhibitory effect was minimal for the 2/1 (+/−) complexes. At the 4/1 (+/−) charge ratio; however, the inhibitory effect of serum on transfection was overcome. The molecular mechanism by which the inhibitory effect of serum is overcome is not known. It is possible that the charge neutralizing effect of the negatively charged macromolecules present in serum is countered by an excess of positive charge on the Tf-lipoplexes. Although the charge ratio of DC-cholesterol liposomes to DNA has been shown previously to be critical for high efficiency of lipofection [54], and it is known that the inhibitory effect of serum can be overcome by increasing the charge ratio of cationic liposome to DNA [55], it should be mentioned that the concentration of serum used in these experiments was only 20%. An excess of positive charge, however, is not sufficient to explain the superior performance of the Tf-lipoplexes compared to plain lipoplexes, since the latter do not have significant transfection activity although they are more positively charged compared to Tf-lipoplexes. In Tf-lipoplexes, the transferrin molecule, in addition to acting as a ligand, also neutralizes part of the positive charge on the liposomes, leading to diminished interactions with polyanionic molecules in serum. Consequently, complexes resistant to higher concentrations of serum can be prepared. It is also interesting to note that increasing amounts of serum increased transfection levels. The reasons for this increase are not clear at present. The enhancement of Tf-lipoplex-mediated gene transfer by high concentrations of serum was also observed for DOTAP/cholesterol and DOTAP/DOPE/cholesterol liposomes, indicating that this effect is not specific for DOTAP/DOPE (Fig. 2). The transfection activity mediated by plain lipoplexes was almost completely inhibited by serum (< 0.2 ng β-galactosidase/mg protein). Non-specific interactions of plain lipoplexes with the negatively charged macromolecules in serum most likely prevent their binding to the cells, while Tf-lipoplexes can interact with cell surface receptors.

Relatively prolonged incubation of negatively charged Tf-complexes 1/1 (+/−) before addition to the cells rendered them serum-resistant, suggesting that maturation of the complex is an essential element of serum-resistance (Fig. 4). A recent study has shown the resistance to 20% FBS of DC-cholesterol lipoplexes at 2/1 (+/−) charge ratio, after 50 min of incubation [56]. The time at which the Tf-lipoplexes turned from serum-sensitive to serum-resistant was different depending on the charge ratio. Positively charged 2/1 (+/−) Tf-lipoplexes were serum-resistant after 30 min of incubation. Note that in the case of complexes used immediately after preparation only the ones prepared at 4/1 (+/−) charge ratio were serum-resistant (Fig. 1). Although the increase in size by increasing the incubation time can have some effect on the level of transfection [57], the development of a different structure more resistant to serum in these mature Tf-lipoplexes is likely to be another reason by which the transfection activity is enhanced.

In summary, our study has established the role of the charge ratio and the time of complex maturation in serum-resistance and enhancement of transfection by Tf-lipoplexes in the presence of high concentration of serum (up to 60% FBS). Our results indicate that Tf-lipoplexes, either net negatively or positively charged, remain effective despite any possible interaction with serum components. We believe that this fact will be of benefit to gene delivery and gene therapy in vivo. Our laboratory is currently investigating how Tf-lipoplexes deliver DNA into cells and whether they can also enhance transfection in vivo.

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