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Gene delivery by negatively charged ternary complexes of DNA, cationic liposomes and transferrin or fusigenic peptides

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Potential problems with the use of viral vectors for gene therapy necessitate the development of efficient nonviral vectors. The association of transferrin, or the pH-sensitive peptide GALA, with cationic liposomes composed of 1,2-dioleoyl-3-(trimethylammonium) propane and its equimolar mixture with dioleoylphosphatidylethanolamine, under conditions where the liposome/DNA complex is negatively charged, drastically increased luciferase expression from pCMVluc. The percentage of cells transfected, measured by β-galactosidase expression, was also increased by about 10-fold. The zeta potential of the ternary complexes was lower than that of the liposome/DNA complexes. Transfection activity of positively charged complexes was also enhanced by association with transferrin, GALA or the influenza hemagglutinin N terminal peptide HA-2, but to a smaller extent compared with the negatively charged complexes. The enhancement of gene delivery by transferrin or GALA was not affected significantly by the presence of serum and did not cause significant cytotoxicity. Our results indicate that negatively charged ternary complexes of cationic liposomes, DNA and transferrin, or fusigenic peptides, can facilitate efficient transfection of cultured cells, and that they may alleviate the drawbacks of the use of highly positively charged complexes for gene delivery in vivo.

Keywords: *gene delivery; cationic liposomes; transferrin; fusigenic peptides*

Introduction

The feasibility of gene therapy for the treatment of genetic metabolic disorders, cancer and AIDS has been demonstrated in recent studies. Although various functional reporter or therapeutic genes have been introduced into cells in culture and the expression of these genes in various tissues has been shown in animal models,[1–9] human clinical trials have been disappointing, with only a few individual cases of success.[10–13] These results have prompted calls for more basic research on vectors and the cell biology of gene delivery.[12] One of the major problems of gene therapy is the effective delivery of the therapeutic gene into target cells *in vitro* and *in vivo*. Although viral vectors have certain advantages, including high levels of transfection, or efficient and stable integration of foreign DNA into a wide range of host genomes, they suffer from several problems. These include immunogenicity, toxicity, difficulty of large-scale production, size limit of the exogenous DNA, random integration into the host genome in the case of retroviruses, and the risks of inducing tumorigenic mutations and generating active viral particles through recombination.[14,15] These limitations of viral vectors have prompted investigators to try to improve methods of nonviral gene delivery.[6]

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Cationic liposomes, have been used extensively for *in vitro* and *in vivo* gene delivery, and constitute a viable alternative to viral gene delivery vehicles.[14,16,17] Using this delivery system, relatively stable expression has been achieved in a number of tissues.[18–23] Although liposomal vectors have several advantages, including lack of immunogenicity, safety, ability to package large DNA molecules and ease of preparation,[14,15,17] they have a limited efficiency of delivery and gene expression, toxicity at higher concentrations, potentially adverse interactions with biological milieux rich in negatively charged macromolecules, and inability to reach tissues beyond the vasculature unless directly injected into the tissue.[15,24–27] Genes delivered *in vivo* via cationic liposomes are expressed primarily in cells of the vascular compartment,[20,21] although transgene expression has also been noted in deep tissues.[20,22] Cationic liposome–DNA complexes ('lipoplexes'[28]) may be coated with serum proteins such as lipoproteins and albumin, or bind nonspecifically to cells such as erythrocytes, lymphocytes and endothelial cells, as well as to extracellular matrix proteins. This may limit the ability of the complexes to reach target tissues and cells.[14,16,24]

Cationic liposome–DNA complexes with a net positive charge bind electrostatically to the negatively charged cell surface. We investigated whether cationic liposome–DNA complexes with a net negative or neutral charge could be utilized for gene delivery by facilitating the binding of the complexes to cell membrane receptors and their subsequent internalization in endosomes. We

employed transferrin to target liposome–DNA complexes to cell surface transferrin receptors and examined the transfection activity and efficiency of these ternary complexes at different lipid/DNA (+/−) charge ratios. Our results indicate that net negatively charged ternary complexes of transferrin/cationic liposomes/DNA can be more effective in mediating gene delivery than positively charged complexes.

The mechanisms by which cationic liposomes deliver DNA into cells are not well understood and are being investigated. One proposed mechanism is that the complex is first endocytosed and then destabilizes the endosomal membrane, thereby microinjecting the DNA into the cytoplasm.[25,29–32] Fusion peptides of certain viral envelope proteins are known to mediate membrane fusion at the mildly acidic pH achieved in endosomes. We investigated the effect on transfection of two different synthetic fusigenic peptides, 'GALA'[33,34] and the influenza virus hemagglutinin HA2 N-terminal peptide,[35] associated with the cationic lipid/DNA complexes. Our rationale was that these peptides would facilitate the destabilization of the endosome membrane[36,37] thereby mediating the release of DNA into the cytoplasm. We have found that GALA can enhance the transfection activity of lipoplexes even under conditions where the ternary complex is net negatively charged. Some of our results have been presented previously in preliminary form.[38,39]

Results

Enhancement of transfection of HeLa cells by a targeting ligand

The limited efficiency of transfection mediated by nonviral vectors, especially when compared with that by viral vectors, is one of the main restrictions to the more frequent use of these systems in gene therapy. In an attempt to overcome this limitation, several authors have used targeting ligands to enhance the binding and internalization of nonviral vectors.[40–42] Another limitation to the use of cationic liposomes is their interaction with negatively charged macromolecules present in serum or the extracellular matrix. We examined whether complexes of cationic liposomes and DNA that have a net negative charge could mediate transfection in the presence of targeting ligands. For this purpose we prepared liposomes containing either DOTAP:DOPE or pure DOTAP complexed with transferrin and DNA at different cationic lipid/DNA charge ratios.

Figure 1 shows the levels of transfection of HeLa cells obtained with different lipid/DNA (+/−) charge ratios in the presence or absence of transferrin. The presence of transferrin enhanced transfection for all the lipid/DNA charge ratios tested. However, it is of interest to observe that this enhancement is particularly pronounced for the negatively charged complexes. For example, for the 1/2 lipid/DNA charge ratio the enhancement of transfection activity was more than 750-fold. It is also interesting to note that in the presence of transferrin the highest levels of transfection were obtained for the 1/1 (theoretically neutral) lipid/DNA charge ratio. This observation suggests that a net positively charged lipid/DNA complex is not required to obtain relatively high levels of transfection. In fact, when complexes with 2/1 and 4/1 lipid/DNA (+/−) charge ratios were tested, luciferase expression decreased compared with that of the 1/1 complex. These results also indicate that higher positive to negative charge ratios do not enhance gene transfer or expression mediated by transferrin. A similar tendency was also observed for the plain lipid/DNA complexes. Highly positively charged complexes have been proposed to be more stable in the biological milieu, since an excess of positive charge may result in a more compact or condensed complex, enabling better protection of the DNA molecule against nucleases.[43] However, for the same reasons (ie more stable or compact DNA) a decrease of transfection may also occur (as obtained with the 2/1 and 4/1 lipid/DNA complexes) due to the difficulty of dissociation of DNA from the complex and its subsequent release into the cytoplasm. In this respect, Zabner *et al*[25] have found that the transcription of positively charged lipid/DNA complexes microinjected into oocyte nuclei is much lower than that of negatively charged complexes.

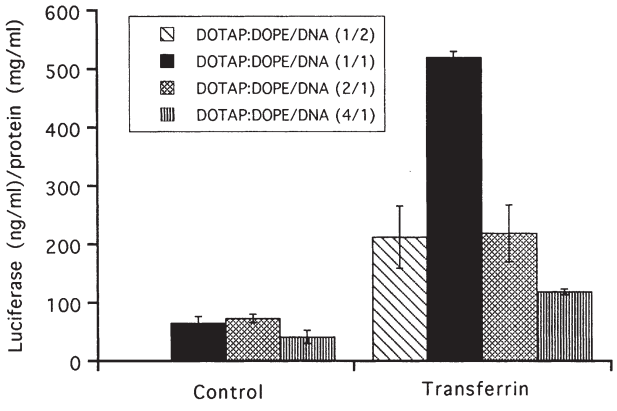

Figure 1 *The effect of transferrin complexation with DOTAP:DOPE liposomes on luciferase gene expression in HeLa cells. Cells were rinsed twice with serum-free medium and then covered with 0.3 ml of DME-HG before lipid/DNA complexes were added. The liposomes were complexed, in the presence or absence of 32 μg of transferrin, with 1 μg of pCMVluc at the indicated theoretical lipid/DNA charge ratios. After an incubation for 4 h, the medium was replaced with DME-HG containing 10% FBS and the cells were further incubated for 48 h. The level of gene expression was evaluated as described in Materials and methods. The data are expressed as ng of luciferase per mg of total cell protein (mean ± standard deviation obtained from triplicate wells) and are representative of two independent experiments.*

Similar results were obtained when pure DOTAP liposomes were complexed with pCMVluc in the presence or absence of transferrin (Figure 2). The enhancement of transfection when the DOTAP complexes were associated with transferrin was even more pronounced than that observed with DOTAP:DOPE liposomes, particularly since gene expression with the DOTAP complexes without transferrin was lower than that obtained with DOTAP:DOPE. We should note that the results in Figures 1 and 2 may be directly compared since they were obtained with the same batch of cells. Several studies have found that DOPE is the most efficient helper lipid for *in vitro* gene transfection,[31,44,45] most likely because of its tendency to form nonbilayer phases and to facilitate membrane fusion.[46,47] Our results with the control liposomes, ie in the absence of transferrin (Figures 1 and 2), also indicate that DOTAP:DOPE liposomes mediated

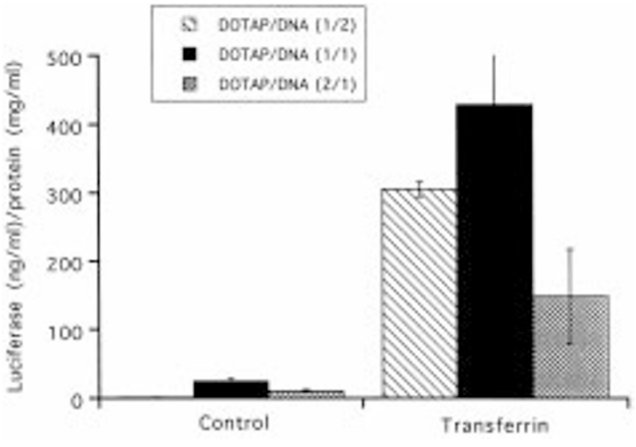

Figure 2 *The effect of transferrin complexation with DOTAP liposomes on luciferase gene expression in HeLa cells. Experiments were performed as described in the legend to Figure 1. The data, expressed as ng of luciferase per mg of total cell protein, indicate the mean ± standard deviation obtained from triplicate wells and are representative of two independent experiments.*

higher transfection activity than pure DOTAP liposomes. Nevertheless, in the presence of transferrin, this difference was not particularly apparent.

To evaluate the specificity of transferrin in enhancing gene delivery, anionized human serum albumin was complexed with DOTAP/DOPE liposomes and DNA at 1/1 and 2/1 lipid/DNA (+/−) charge ratios, in the same manner as transferrin. No enhancement of transfection was observed in HeLa cells above that obtained with the plain lipoplexes (data not shown). Furthermore, the copolymer VM-Acp, which was also net negatively charged, did not enhance transfection when complexed with lipoplexes (data not shown).

Enhancement of transfection by pH-sensitive synthetic peptides

Recent studies on the mechanism by which cationic liposomes deliver DNA into cells have pointed out endocytosis as the main process of internalization for the lipid/DNA complexes.[25,29–31] Therefore, destabilization of the endosomal membrane and consequent release of the complexes or free DNA into the cytoplasm before lysosomal degradation can occur, appear to be crucial steps for efficient gene delivery. In an attempt to surpass this limiting step, Wagner and collaborators[35] have utilized, in conjunction with transferrin-polylysine–DNA complexes, fusigenic peptides that destabilize liposomes, erythrocytes and endosomes in a low pH-dependent manner. The association of HA-2-derived peptides or the pH-sensitive 30-amino acid peptide, 'GALA', with DNA–transferrin-polylysine complexes resulted in a significant enhancement of the expression of luciferase in cells transfected with these complexes.[35,37] We tested the effect of some of these synthetic peptides on the transfection of HeLa cells with the pCMVLuc plasmid mediated by DOTAP:DOPE liposomes at different lipid/DNA charge ratios. The association of the GALA peptide with the complexes resulted in an enhancement of transfection of HeLa cells (Figure 3). The association of 0.6 μg of GALA with complexes of 2/1 (+/−) charge ratio resulted in a four-fold increase in the level of luciferase expression compared with controls. For the 1/1 and 4/1 charge ratios only a slight increase was observed. The use of a higher GALA concentration (1.2 μg per well) also resulted in larger transfection activities in the case of 2/1 and 4/1 ratios compared with those for controls. Nevertheless, doubling the peptide concentration did not result in any significant increase in the levels of luciferase expression. No effect was observed when a lower peptide concentration (0.3 μg per well) was used (data not shown).

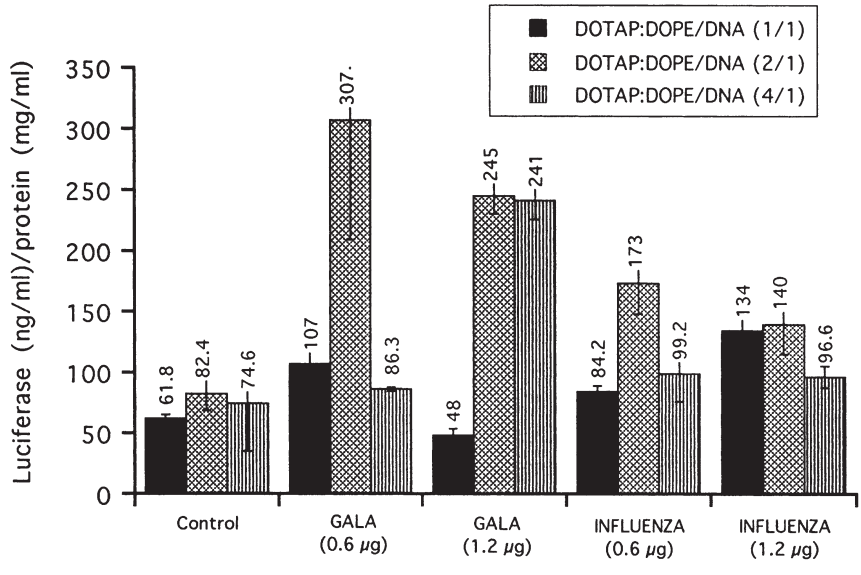

Figure 3 *The effect of association of fusigenic peptides to DOTAP:DOPE liposomes on luciferase gene expression in HeLa cells. The liposomes were complexed with 0.6 or 1.2 μg of GALA or HA-2-derived peptides and with 1 μg of pCMVluc at the indicated theoretical lipid/DNA charge ratios as described in detail in Materials and methods. The data (expressed as ng of luciferase per mg of total cell protein) are the mean ± standard deviation obtained from triplicate wells, and are representative of two independent experiments.*

To examine whether the acidification of endosomes is involved in the enhancement of transfection by GALA, we pretreated cells with bafilomycin A_1, which is a specific inhibitor of the endosomal proton-ATPase[48–50] under conditions where no cytotoxicity was observed. This treatment resulted in the reduction of transfection for both the 1/1 and the 2/1 complexes to about 50% of untreated controls (data not shown). This observation indicates that at least part of the enhancing activity of GALA is due to the acidic environment in endosomes.

A similar tendency was observed for the HA-2-derived peptide. The association of 0.6 μg peptide (per well) with complexes of 2/1 charge ratio resulted in an enhancement of transfection over controls. An increase in the amount of peptide added did not result in a proportional enhancement of transfection, even though the levels achieved with 1.2 μg peptide were higher than controls.

Effect of transferrin and GALA on transfection of COS-7 cells

Different cell lines exhibit largely varying levels of transfection activity with the same gene vector.[44,51,52] To test whether DOTAP:DOPE/DNA complexes associated with transferrin or GALA were also effective in other cell lines we performed experiments with COS-7 cells which have been used in previous transfection studies (Figure 4).[51] As observed for HeLa cells, the association of transferrin or the GALA peptide with the lipid/DNA complexes resulted in a significant enhancement of the levels of luciferase expression in COS-7 cells. It should be noted, however, that the levels of transfection for COS-7 cells were in general much higher than those obtained with HeLa cells, and that the dependence of transfection on the charge ratio was different when GALA was associated with the complexes. In fact, with COS-7 cells transfection in the presence of 0.6 μg peptide per well was optimal with the 1/1 lipid/DNA charge ratio, while with HeLa cells, the optimal ratio was 2/1. It is also of interest that a dramatic enhancement of luciferase expression was observed in the presence of transferrin at the 1/2 charge ratio (Figure 4).

Evaluation of transfection efficiency

To investigate whether the enhancement of transfection mediated by the association of transferrin or the fusigenic peptides 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 treated cells that express the transgene. For this purpose the plasmid pCMV.SPORT encoding β-galactosidase was associated with the complexes and cells transfected as described in Materials and methods. The results, presented as the percentage of cells scored for the expression of β-galactosidase (blue cells) are shown in Table 1. The association of transferrin or fusigenic peptides with the lipid/DNA complexes resulted in an increase of the number of cells that were

Table 1 Expression of β-gal in HeLa cells (% of blue cells). Effect of the association of transferrin or fusigenic peptides with DOTAP:DOPE/DNA complexes of different charge ratios

Charge ratio (lipid/DNA)	*1/2*	*1/1*	*2/1*
Control	0	1–2	1
+ Transferrin	5–10	20–25	5
+ GALA peptide		5	5–7

The liposomes were complexed, in the presence or absence of 32 μg of transferrin or 0.6 μg of the GALA peptide, with 1 μg of pCMV.SPORT-β-gal at the indicated theoretical lipid/DNA charge ratios. β-Gal expression was observed as described in Materials and methods. The percentage of cells exhibiting β-gal activity was evaluated by counting 1000 cells in duplicate wells.

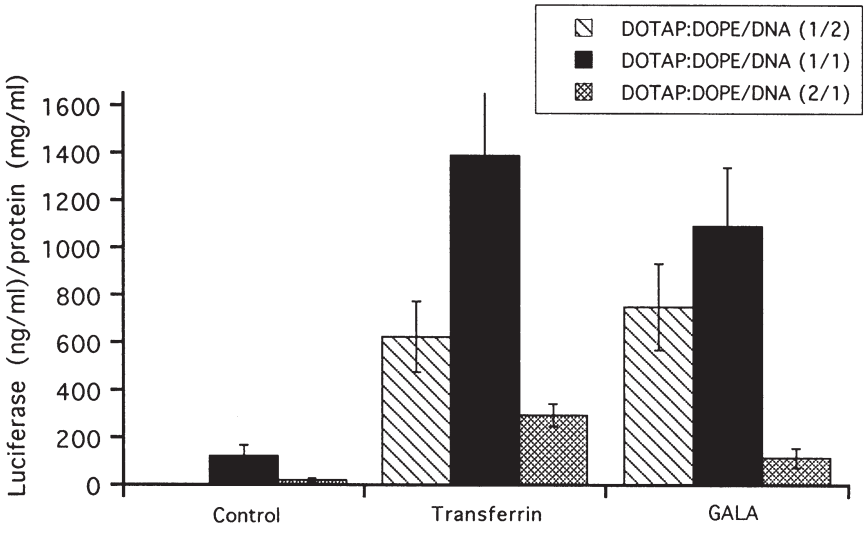

Figure 4 *Evaluation of the transfection activity in COS-7 cells mediated by DOTAP:DOPE liposomes with or without transferrin or the GALA peptide. Cells were rinsed twice with serum-free medium and then covered with 0.3 ml of DME-HG before lipid/DNA complexes were added. The preparation of the complexes and the transfection procedure were performed as described in Materials and methods. The data are expressed as ng of luciferase per mg of total cell protein (mean ± standard deviation obtained from triplicate wells) and are representative of two independent experiments.*

detectably transfected. In the case of the 1/1 charge ratio complexes, the percentage of cells scored as expressing β-galactosidase increased from 2 for controls to 25% with transferrin, and from 1 to 5%, respectively, with the GALA peptide. These results suggest that a correlation between transfection activity (level of luciferase expression) and transfection efficiency (the percentage of cells transfected) can be established in this system. It should be noted that our experiments were not designed to maximize the efficiency of transfection, but to explore the roles of transferrin, fusigenic peptides and the +/− charge ratio in transfection activity and efficiency. For example, it may be possible to enhance transfection activity and efficiency by using higher amounts of DNA. This, however, may result in cytotoxicity[26] because of the necessity to use higher quantities of cationic liposomes to obtain the same charge ratios as those used in the present study.

Effect of serum on transfection

Transfection of certain cell types by some cationic liposome compositions is sensitive to the presence of serum.[51,53,54] The inhibition of gene delivery by serum is considered to be one of the limitations to their application *in vivo*.[54] Therefore, the effect of serum on the levels of transfection mediated by the above described complexes was also examined. For this purpose, complexes were added to cells in the presence of cell culture medium containing 10% FBS and incubated for 4 h at 37°C, as described in Materials and methods. Figure 5 illustrates the level of transfection observed when control complexes, or transferrin- or GALA peptide-associated complexes at different lipid/DNA charge ratios were used under these conditions. The presence of serum did not appreciably change the levels of transfection for the different complexes tested (compare with Figures 1 and 3). These data suggest that the complexes (either net negatively or positively charged, or neutral) remain effective despite any possible interaction with serum components. The fact that transfection mediated by the DOTAP:DOPE liposomes, with or without transferrin or fusigenic peptides, is not affected by the presence of serum not only results in a simplification of the transfection procedure, since the washing steps can be eliminated, but also increases the likelihood that these complexes can be utilized for gene delivery *in vivo*.

Zeta potential measurements

In an attempt to gain some insights into the mechanisms by which the cationic liposome–DNA complexes at different (+/−) charge ratios interact with the cells we examined the overall charge of the complexes by zeta potential measurements, using a Coulter DELSA 440 instrument (Coulter Electronics, Hialeah, FL, USA). The effect on the zeta potential of the presence of transferrin or fusigenic peptides in the complex was also determined (Table 2). The values of the zeta potential of DOTAP:DOPE–DNA complexes for the different (+/−) charge ratios were in agreement with what was expected from the theoretical calculations based on their charge. Complexes containing an excess of DNA to lipid (1/2, +/− charge ratio) exhibited an overall net negative charge. Moreover, 1/1 complexes were in fact neutral, and 2/1 and 4/1 complexes were net positively charged.

The association of transferrin with the complexes resulted in a decrease of the zeta potential for all the charge ratios tested. This effect is most likely due to the negatively charged amino acids of the protein interacting with the cationic liposomes. The zeta potential of the lipid/DNA complex at a 1/1 (+/−) charge ratio decreased from 2 mV to –39 mV in the presence of transferrin. The zeta potential also decreased upon addition of 0.6 μg of GALA or HA-2-derived peptide to the 2/1 or 4/1 complexes. It should be noted that complexes presenting a net negative charge are those that exhibit higher transfection activity (Table 2, Figure 3).

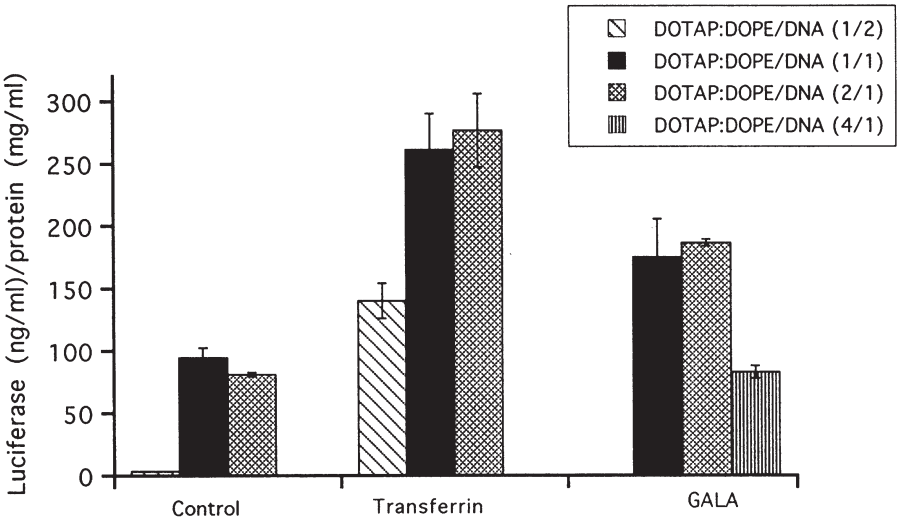

Figure 5 *The effect of the presence of serum on luciferase gene expression in HeLa cells. Cells were covered with 0.3 ml of DME-HG enriched with 10% FBS before lipid/DNA complexes were added. The liposomes were complexed, in the presence or absence of 32 μg of transferrin or 0.6 of the GALA peptide, with 1 μg of pCMVluc at the indicated theoretical lipid/DNA charge ratios. After an incubation of 4 h, the medium was replaced with 1 ml of medium containing 10% FBS and the cells were further incubated for 48 h. The level of gene expression was evaluated as described in Materials and methods. The data are expressed as ng of luciferase per mg of total cell protein. Data represent the mean ± standard deviation obtained from triplicate wells. Note that in this experiment the 4/1 lipid/DNA charge ratio was not tested for the plain lipoplexes and the complexes with transferrin, and the 1/2 charge ratio was not tested in the case of complexes containing GALA.*

Table 2 The zeta potential (mV) of liposome–DNA complexes at various charge ratios (+/–) and the effect of transferrin and fusigenic peptides

Charge ratio (+/–) (lipid/DNA)	*Zeta potential (mV)*			
	1/2	*1/1*	*2/1*	*4/1*
DOTAP:DOPE/DNA	–32.75 ± 4.8	2.44 ± 5.1	42.75 ± 0.5	48.6 ± 1.9
DOTAP:DOPE/DNA + Transferrin	–47.8 ± 4.7	–38.6 ± 5.2	25 ± 2.16	44 ± 4.3
DOTAP:DOPE/DNA + GALA 0.6 μg			–35.25 ± 3.6	32 ± 3.55
DOTAP:DOPE/DNA + Influenza 0.6 μg			–11 ± 3.5	8 ± 2.9

Zeta potential measurements of the different DOTAP:DOPE/DNA complexes were performed using a Coulter DELSA 440 instrument. Data represent the mean ± standard deviation obtained for the different angles in two measurements.

The effect of the different complexes on cell viability

Cationic liposomes can be toxic to cells, depending on the lipid concentration, duration of treatment, the cell type and the confluency of the culture.[26,51] To evaluate whether DOTAP:DOPE/DNA complexes were toxic to cells, cell viability was assessed following transfection. The colorimetric Alamar Blue assay measures the redox capacity of cells due to the production of metabolites, as a result of cell growth and allows determination of viability over the culture period without the detachment of adherent cells. Cell viability was not affected significantly when DOTAP:DOPE/DNA complexes were incubated for 4 h at 37°C with HeLa cells, followed by a 48-h incubation (Figure 6). Cell metabolic activity was unaltered even for the highest lipid/DNA charge ratio tested (2/1), where about 8 μg of total lipid were used. The addition of transferrin or the fusigenic peptides also did not result in any toxicity to the cells. These results were confirmed by both total cell protein quantification and morphological observations for treated and untreated cells (data not shown).

Discussion

Nonviral gene delivery systems appear to be very promising systems for gene therapy since they obviate some of the safety limitations associated with viral vectors. As an example of such systems, cationic liposomes, first proposed by Felgner *et al*,[51] have been used extensively *in vitro* and *in vivo*. Although a large amount of effort has been made for the development of these vectors, namely through the synthesis and evaluation of different lipid compositions, as well as of new plasmids with more efficient promoters, the results are still unsatisfactory. The heterogeneity of the complexes formed with the plasmid DNA, the lack of knowledge of the mechanisms by which these systems mediate gene delivery, toxicity under certain conditions, sensitivity to the presence of serum and especially the low levels of gene expression *in vitro* and *in vivo* (compared with viral vectors) raise doubts regarding their potential as gene delivery systems in clinical studies.

We explored two different approaches of enhancing

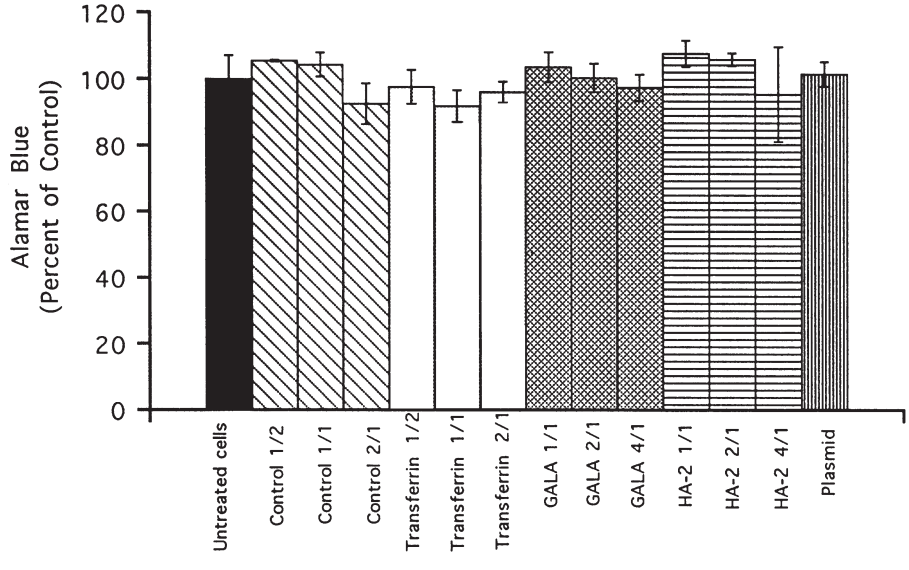

Figure 6 *Effect of DOTAP:DOPE/DNA complexes, with or without transferrin or fusigenic peptides, on the viability of HeLa cells. Cells in 48-well plates were exposed to lipid/DNA complexes at different charge ratios for 4 h at 37°C as described in Materials and methods. Cell viability was measured by the Alamar Blue assay following 48 h of incubation and was expressed as the percentage of the untreated control cells. Data represent the mean ± standard deviation obtained from duplicate wells.*

the transfection mediated by DOTAP:DOPE liposomes: (1) to promote cellular internalization of the cationic liposome–DNA complexes through receptor-mediated endocytosis; and (2) to improve the cytoplasmic release of DNA from endosomes, preventing their lysosomal degradation.

Targeting to cellular transferrin receptors had been tried successfully before by linking the ligand to polycation conjugates[40,55] or by associating the protein with cationic liposomes.[42] The latter study reported the use of iron-saturated human transferrin associated to Lipofectin reagent, composed of the cationic lipid *N*-[2,3-(dioleoyloxy)propyl]-*N*,*N*,*N*-trimethylammonium (DOTMA) and the zwitterionic lipid DOPE,[51] and showed significant differences in the levels of β-galactosidase expression depending on the amount of transferrin added to the complexes, time of transfection and methodology of preparation of the complexes. The study showed that the transerrin complexes delivered a two-fold higher amount of DNA to the cells compared with plain lipoplexes at early time-points and that the amount of DNA associated with the cells decreased after a 5-h incubation with the cells. Cheng[42] postulated a model for the formation of an efficient gene transfer vector composed of transferrin and Lipofectin. Complexation of LipofectAMINE with transferrin did not result in enhanced gene expression, while LipofectACE–transferrin complexes significantly increased transfection efficiency and activity.

Using liposomes composed of pure DOTAP or DOTAP:DOPE (1:1, weight ratio) we added iron-saturated human transferrin to the liposomes before the addition of the reporter plasmids. Our study is the first to establish the role of the +/− charge ratio in the enhancement of transfection by transferrin (Figures 1 and 2). The similarity between the structures of DOTMA and DOTAP may explain why transferrin was able to enhance gene delivery by DOTAP:DOPE liposomes as well as by Lipofectin.

The use of transferrin with the rationale to target the cationic liposome–DNA complexes to cell surface receptors and to enhance receptor-mediated uptake, drastically increases the transfection activity and efficiency of the transferrin/cationic liposome/DNA ternary complexes. Despite the highest levels of luciferase expression achieved with complexes of 1/1 (+/−) charge ratio, the most dramatic effect of transferrin is illustrated by the results obtained with the negatively charged complexes (1/2). Although, these conditions were suboptimal for condensing and complexing DNA, it was possible, by adding transferrin, to increase transfection from essentially null to relatively high values of gene expression. The effect of transferrin was not so evident for the positively charged complexes. It seems that an excess of positive charge masks the presence of the ligand, thus decreasing the efficiency of the receptor-mediated endocytotic process. Indeed, from the combination of the transfection results for both cell lines and the zeta potential values, it is clear that there is an ideal range of charge ratios where transfection is more efficient. Surprisingly, complexes that bear a net negative charge in the presence of transferrin (or the peptides) are those leading to the highest levels of gene expression. With this system, high levels of transfection could be achieved with relatively low DNA and liposome concentrations, which also resulted in lack of any significant cytotoxicity.

On the other hand, the use of low pH-activated membrane-active peptides, with the rationale to induce the destabilization of endosomes, also results in an enhancement of transfection. The lower levels of transfection activity obtained with GALA compared with those for transferrin suggest that internalization of the complexes represents the major barrier to be surpassed in order to enhance gene transfer. In our studies the synthetic GALA peptide was more effective than the influenza HA-2 peptide. Two previous studies have shown the enhancement of cationic liposome-mediated transfection by synthetic peptides derived from influenza hemagglutinin sequences.[50,56] Kamata *et al*[56] used anionic or cationic derivatives of the N-terminal peptide of the HA2 subunit of hemagglutinin and obtained between two- and seven-fold enhancement of transfection activity over that of Lipofectin alone. Kichler *et al*[50] were able to increase the levels of Transfectam (lipopolyamine)-mediated transfection by three- to 30-fold by employing various influenza-derived peptides. It has been shown that GALA (a 30-amino acid, pH-sensitive, α-helical amphipathic peptide) undergoes a transition from a random coil at pH 7.5 to an amphipathic α-helix at pH 5.0, under which conditions it strongly interacts with phospholipid membranes to induce fusion, contents leakage, and phospholipid flip-flop.[33,34,57,58] It is interesting to note that the association of GALA with the lipid/DNA complexes led to a higher enhancement of luciferase expression in COS-7 cells than in HeLa cells, the levels being similar to those observed with transferrin. Thus, in COS-7 cells internalization of the ternary complexes does not appear to be a rate-limiting step. These observations raise the possibility that the use of the GALA peptide may be an even more effective strategy to enhance gene transfer mediated by cationic liposomes in other types of cells. We have recently reported that the combined use of GALA and transferrin with lipoplexes results in successful transfection of human monocyte-derived macrophages.[59] Thus, both internalization and endosome disruption appear to be important barriers to be overcome in gene delivery to macrophages.

Synthetic peptides containing 16 to 20 amino acids of the influenza HA-2 amino terminus induce liposome fusion and cause leakage of aqueous liposomal contents at acidic pH.[35] Our use of a similar synthetic peptide (20 amino acids) as a strategy to improve the cytoplasmic delivery of DNA is based on the expectation that this peptide will adopt an α-helical conformation in the endosome, upon protonation of carboxyl groups, leading to the destabilization of the endosomal membrane. Although this peptide promoted transfection as compared with controls, the observed enhancement was not as significant as in the case of the GALA peptide or transferrin. In gene transfer experiments utilizing polylysine–transferrin complexes and BNL CL.2 hepatocytes, monomeric influenza peptides, including INF2 with an identical sequence to our HA-2 peptide, did not significantly enhance gene transfer.[37] HA-2 peptide derivatives obtained either by elongation or dimerization were shown to be more effective than the peptides themselves in gene delivery; these peptides also displayed endosome disruption activity.[37] The association of such modified

peptides with lipoplexes may be an effective method to enhance transfection.

Although the use of a targeting ligand and fusigenic peptides for enhancing gene delivery is based on a rational strategy, the actual mechanisms by which these agents enhance transfection are not known and are under investigation. The gene targeting specificity of transferrin covalently coupled to polyethylenimine has been demonstrated recently.[60] Our experiments with a negatively charged albumin used as a control nonspecific protein indicated no enhancement of gene delivery over that obtained with plain lipoplexes. In the case of influenza-derived peptides, Kichler *et al*[50] have shown that the enhancement of lipopolyamine-mediated transfection is independent of the pH sensitivity of the peptides. For example, the peptide INF6 induces the leakage of liposome contents both at acidic and neutral pH, and enhances relative light units of luciferase by more than an order of magnitude, while INF10 induces leakage in a pH-dependent manner and enhances transfection to a slightly lower extent than INF6. As would be expected from the relative pH sensitivities of the two peptides, INF10 is more sensitive to the inhibitory action of bafilomycin A_1 than INF6.[50] It should be noted that Kichler *et al*[50] added the peptides to preformed lipoplexes, whereas in our study the peptides and cationic liposomes were complexed before the addition of DNA. Gene delivery and expression mediated by the GALA lipoplexes used in our study were also inhibited by bafilomycin A_1, although to a lower extent than that observed for INF10 in the study by Kichler *et al*.[50] The latter authors found that a pH-sensitive GALA analogue, EGLA-I, also enhanced transfection. When complexed to transferrin–polylysine conjugates, influenza-derived peptides with the highest pH specificity (INF4D1, INF5 and INF7) with respect to mediating erythrocyte lysis appear to be at least an order of magnitude more active in gene delivery than peptides without the pH specificity (INF6, INF-A).[61]

A correlation between the enhancement of transfection activity (evaluated by the level of luciferase expression) and the increase in the number of cells successfully transfected (cells scored as expressing β-galactosidase) was observed. However, the enhancement of the number of cells transfected due to the presence of transferrin or the fusigenic peptides was lower than expected, especially in comparison with Cheng's results,[42] where almost 100% of HeLa cells were transfected. Even considering the subjectivity involved in scoring the assay, our results, using the same cell line, are far from these ideal values. Nevertheless, it should be pointed out that a different plasmid for β-galactosidase was used by Cheng,[42] thus making a direct comparison difficult. Evaluation of transfection efficiency by scoring cells expressing human green fluorescent protein (hGFP) also indicated that only a relatively low percentage of cells were transfected successfully (unpublished data).

The association of transferrin and fusigenic peptides to lipid/DNA complexes as an attempt to enhance transfection may overcome some of the limitations associated with the use of cationic liposomes in gene therapy. Indeed, the ternary complexes of cationic liposomes, DNA and protein or peptide, not only lead to high levels of transfection, but also have the advantage of being active in the presence of serum and being nontoxic. Moreover, such negatively charged ternary complexes are likely to alleviate the problems associated with the use of highly positively charged complexes *in vivo*, such as avid complexation with serum proteins. These complexes, and their future derivatives, may thus be potential alternatives to viral vectors for gene delivery *in vivo*.

Materials and methods

Materials

The cationic lipid 1,2-dioleoyl-3-(trimethylammonium) propane (DOTAP), and dioleoylphosphatidylethanolamine (DOPE), were purchased from Avanti Polar Lipids (Alabaster, AL, USA). Iron-saturated, heat-inactivated human transferrin was obtained from Collaborative Biomedical Products (via Becton Dickinson, Bedford, MA, USA). The GALA peptide[33,34] and the peptide named HA-2, derived from the N-terminal sequence of the influenza virus hemagglutinin subunit HA2[35] were synthesized and purified by the UCSF Biomolecular Resource Center. Succinylated human serum albumin, and a copolymer of vinylpyrrolidone and maleic anhydride modified with epsilon-aminocaproic acid (VM-Acp) were kindly provided by Dr Sergei Andreev, Institute of Immunology, Moscow. The pCMVluc plasmid (VR-1216) was a generous gift of Dr P Felgner (Vical, San Diego, CA, USA). The plasmid pCMV.SPORT-β-gal and the β-gal staining kit were obtained from Gibco BRL Life Technologies (Gaithersburg, MD, USA) and Invitrogen (San Diego, CA, USA), respectively. Alamar Blue dye was purchased from Alamar Biosciences (Sacramento, CA, USA). NaCl, 2-[N-morpholino]ethanesulfonic acid (MES), N-[2-hydroxyethyl] piperazine-N′-[2-ethanesulfonic acid] (HEPES) were obtained from Sigma (St Louis, MO, USA).

Liposome preparation

Cationic liposomes composed of DOTAP or DOTAP: DOPE (1:1 weight ratio) were prepared by first drying a film of lipid under argon and then in a vacuum oven at room temperature, and hydrating the lipid film with 1 ml deionized water at a final concentration of 5 mg/ml. The multilamellar vesicles obtained were then sonicated briefly under argon, extruded 21 times through polycarbonate filters of 50 nm pore diameter using a Liposofast device (Avestin, Toronto, Canada), diluted five times with deionized water and filter-sterilized utilizing Millex (Keene, NH, USA) 0.22 μm pore-diameter filters.

Cells

HeLa cells (American Type Culture Collection, MD, USA) were maintained at 37°C, under 5% CO_2, in Dulbecco's modified Eagles's medium-high glucose (DME-HG) (Irvine Scientific, Santa Ana, CA, USA) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS; Sigma), penicillin (100 units/ml), streptomycin (100 μg/ml) and l-glutamine (4 mm). For transfection, 0.2×10^6 HeLa cells were seeded in 1 ml of medium in 48-well culture plates and used at 80–90% confluence. COS-7 cells (UCSF Cell Culture Facility, San Francisco, CA, USA) were maintained under the same conditions described for HeLa cells. For transfection, cells were seeded in 1 ml of medium in 48-well culture plates and used at 40–60% confluence.

Preparation of the ternary complexes

Complexes were prepared by sequentially mixing 100 μl of a solution of 100 mm NaCl, 20 mm Hepes, pH 7.4, with

or without 32 μg iron-saturated human transferrin[42] with 2.5, 5, 10 or 20 μl liposomes and incubated at room temperature for 15 min. One hundred microliters of buffer containing 1 μg of pCMVluc or 1 μg pCMV.SPORT β-gal plasmid was then added and gently mixed, and the mixture was further incubated for 15 min at room temperature. Peptide complexes were prepared in a similar manner, with varying amounts of peptide, as indicated in the Figures.

Transfection activity

Cells were rinsed twice with serum-free medium and then covered with 0.3 ml of DME-HG before lipid/DNA complexes were added. Lipid/DNA complexes were added gently to cells in a volume of 0.2 ml per well. After an incubation for 4 h (in 5% CO_2 at 37°C) the medium was replaced with DME-HG containing 10% FBS, and the cells were further incubated for 48 h. The cells were then washed twice with phosphate-buffered saline (PBS) and 100 μl of lysis buffer (Promega, Madison, WI, USA) were added to each well. The level of gene expression in the lysates was evaluated by measuring light production by luciferase using a scintillation counter protocol (Promega). 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 luciferase (based on a standard curve for luciferase activity), per mg of total cell protein.

Transfection efficiency

Transfection efficiency was evaluated by scoring the percentage of cells expressing β-galactosidase. Briefly, cells transfected with 1 μg of pCMV.SPORT-β-gal were washed with PBS, fixed in a solution of 2% formaldehyde and 0.2% glutaraldehyde, and stained with an X-gal containing solution. The cells were incubated at 37°C for 24 h and then examined under a phase contrast microscope for the development of blue color. The percentage of cells exhibiting β-gal activity was evaluated by counting 1000 cells in duplicate wells.

Cell viability assay

Cell viability following transfection under the different experimental conditions was quantified by a modified Alamar Blue Assay.[62] Briefly, 1 ml of 10% (v/v) Alamar Blue dye in complete DME medium was added to each well 45 h after transfection. After 2.5–4 h of incubation at 37°C, 200 μl of the supernatant were collected from each well and transferred to 96-well plates. The absorbance at 570 nm and 600 nm was measured with a microplate reader (Molecular Devices, Menlo Park, CA, USA). 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.

Zeta potential measurements

Zeta potential measurements of the different lipid/DNA complexes, and ternary complexes with transferrin or peptides, were performed using a Coulter DELSA 440 instrument. The DELSA 440 is a laser-based multiple angle particle electrophoresis analyzer that measures the electrophoretic mobility and zeta potential distribution simultaneously with the hydrodynamic size of particles in suspension. 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 3.0 mA was applied. Measurements were recorded and the zeta potential (ζ) was calculated for each scattering angle (8.6°, 17.1°, 25.6° and 34.2°). Data represent the mean ± standard deviation obtained for the different angles of two measurements.

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