Optimization of Folate-Conjugated Liposomal Vectors for Folate Receptor-Mediated Gene Therapy

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Abstract □ A folate-targeted transfection complex that is internalized by certain cancer cells and displays several properties reminiscent of enveloped viruses has been developed. These liposomal vectors are comprised of a polycation-condensed DNA plasmid associated with a mixture of neutral and anionic lipids supplemented with folate–poly(ethylene glycol)–dioleylphosphatidylethanolamine for tumor cell-specific targeting. *N*-Citraconyl-dioleylphosphatidylethanolamine is also included for pH-dependent release of endosome-entrapped DNA into the cytoplasm, and a novel plasmid containing a 366-bp segment from SV40 DNA has been employed to facilitate transport of the plasmid into the nucleus. Because formation of the DNA core is an important step in the assembly of liposomal vectors, considerable effort was devoted to comparing the transfection efficiencies of various DNA condensing agents. It was found that complexation of plasmid DNA with high molecular weight polymers such as acylated-polylysine and cationic dendrimers leads to higher folate-mediated transfection efficiency than DNA complexed with unmodified polylysine. In contrast, compaction of plasmid DNA with small cationic molecules such as spermine, spermidine, or gramicidin S yields only weakly active folate-targeted liposomal vectors. Compared to analogous liposomal vector preparations lacking an optimally compacted DNA core, a cell-specific targeting ligand, a caged fusogenic lipid, and a nucleotide sequence that facilitates nuclear uptake, these modified liposomal vectors display greatly improved transfection efficiencies and target cell specificity.

Introduction

Gene therapy constitutes an attractive strategy for the treatment of a variety of human genetic disorders.[1−5] Although viral gene therapy can report the greatest successes in animal and human trials to date,[6−10] liposomal vectors have stimulated increased attention due to their low immunogenicity, lack of potential infectivity, and ease of assembly from chemically defined components.[11] Unfortunately, liposomal vectors also suffer from their own set of disadvantages, including generally low efficiencies of transfection and elevated toxicities toward normal cells.[9,12] Work on liposomal vectors has consequently focused on developing formulations that improve transfection efficiency without compromising host cell viability.[13,14]

One reasonable approach for addressing the deficiencies of liposomal vectors has been to identify the features of viral vectors that render them efficient vehicles for gene transfer and then mimic these features in synthetic liposomal constructs. Based on information available to date, the desirable characteristics of the more promising viral vectors would seem to include (i) their ability to efficiently compact and package the genetic material, (ii) their expression of a surface-exposed ligand for stable attachment and endocytosis/delivery of the condensed DNA into the target cell, (iii) their means of releasing their nucleic acid from the endosome into the target cell's cytoplasm, and (iv) their mechanisms for enhancing transport of their genome into the host cell's nucleus. It can be speculated that future liposomal gene therapy vectors will incorporate components that mimic many of these desirable viral functions. Indeed, some progress has already been made toward achievement of these objectives.[2,14−20]

Probably the greatest advance in optimizing liposomal vectors has stemmed from development of cationic liposomes that not only promote DNA compaction and avid cell association, but also enhance delivery of the genetic cargo into the cell's cytoplasm.[21−24] Associated with these desirable features, however, is a significant loss of cell-specific targetability, since strongly cationic complexes indiscriminately bind most cell surfaces and thereby render any ligand-specific interactions largely meaningless. While such generic cell surface affinity can be prevented by eliminating excess cationic charge, the resulting neutral DNA−lipid particles are generally poorly fusogenic, even when delivered into target cells by receptor-mediated endocytosis. Clearly, modification of cationic liposomes for cell specific targeting involves more than simple attachment of a high affinity ligand to the liposome surface.

Our laboratory has been interested in the use of folic acid as a targeting ligand to deliver attached therapeutic and imaging agents to cancer cells that overexpress the receptor for folic acid.[25−31] Because folate-linked cargo's of diameters <150 nm are efficiently bound and internalized by folate receptor (FR)-expressing cells, it seemed reasonable to explore the possibility of using folic acid to facilitate liposomal vector delivery to FR-enriched cells. In this paper we describe our efforts at integrating several desirable features of viral vectors into folate-targeted liposomal vectors. While several excellent publications describing the use of folic acid to deliver genes into cancer cells have already appeared,[29,32−34] a more comprehensive attempt to optimize components that might mimic desirable viral characteristics has not been reported.

Materials and Methods

Materials—All commercially available lipids were purchased from Avanti Polar lipids (Alabaster, AL). Citraconic anhydride, 2,6-lutidine, folic acid, poly-L-lysine hydrobromide (MW ∼ 25.6 kDa), myristoyl chloride, palmitic acid *N*-hydroxysuccinimidyl ester (NHS), oleic acid-NHS, and gramicidin S were purchased from Sigma Chemical Co. (St. Louis, MO). Spermine, spermidine, and chitosan (medium MW, 200−800 cps) were from Aldrich (Milwaukee, WI). Cationic dendrimers were obtained from Dendritech Inc. (Midland, MI) and Aldrich (St. Louis, MO). [^{3}H]-

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Cholesteryl hexadecyl ester and $Na^{125}I$ were purchased from DuPont. Folate deficient modified Eagle's medium (FDMEM) and other additives were purchased from Life Technologies, Inc. and fetal calf serum was from HyClone Labs (Logan, UT). β-galactosidase assay kits were purchased from Promega (Madison, WI). BCA protein assay reagents were from Pierce (Rockford, IL).

Plasmid Preparation—A 366 bp HindIII−KpnI fragment of SV40 DNA containing the origin and promoter region (SV40 nts 5171 to 294) was amplified by polymerase chain reaction and cloned into the TA cloning vector (Invitrogen, San Diego CA) to create plasmid pTA-DTS. Subsequently, a 384 bp HindIII−SalI fragment was isolated from pTA-DTS and inserted into the corresponding sites of pCMVβgal (Clontech, Palo Alto, CA), downstream of the lacZ gene. Protein-free preparations of plasmids pCMVlacZ and pDTSlacZ were purified following alkaline lysis using Qiagen megaprep columns.

Synthesis of Fatty Acylated Polylysine—Acylated polylysine was synthesized by reaction of polylysine (M_r ~25 700) with activated fatty acids at a fatty acid/lysine ratio of 1:5. Myristoyl chloride, palmitic acid-NHS, or oleic acid-NHS (0.077 mmol) in 1 mL of dry DMF was added to 15 mg of polylysine in 500 μL of dry DMSO, and the mixture was allowed to react for 24 h at room temperature. The solution was then added to a large excess of diethyl ether to precipitate the derivatized polylysine,[35] and the polymer was purified by dialysis against deionized water. The resulting polymer was quantified by trypan blue assay[36] using unmodified polylysine as the standard.

Preparation of Liposome Formulations—*N*-Citraconyl-dioleylphosphatidylethanolamine (C-DOPE) is a caged form of dioleylphosphatidylethanolamine (DOPE) that resists hexagonal phase formation and consequent liposome fusion until the citraconyl moiety is released by acidification at pHs < 6.[37,38] Assuming such low pHs are only experienced following endocytosis by target cells, incorporation of C-DOPE into liposomal vectors should allow acquisition of fusogenic properties only following uptake by transfected cells.

C-DOPE was synthesized by reacting DOPE with citraconic anhydride, and the resulting caged fusogenic lipid was purified as described earlier.[37,38] Liposomes were prepared by mixing chloroform solutions of DOPE, cholesterol, C-DOPE, and folate−poly(ethylene glycol)−dioleylphosphatidylethanolamine (FA-PEG-DOPE, a derivative of dioleylphosphatidylethanolamine linked covalently to folic acid via a poly(ethylene glycol) spacer[28]) or poly-(ethylene glycol)−dioleylphosphatidylethanolamine (PEG-DOPE, the nontargeted control for FA-PEG-DOPE) followed by removal of the chloroform under vacuum to produce a dry lipid film. Hydration of the lipid was then achieved by addition of 1.0 mL of sterile 20 mM HEPES buffer, pH 8.0, followed by vortexing for 1 min and bath sonication to obtain a clear emulsion. The total lipid content in these liposome formulations was ~5 mg/mL.

Preparation of Transfection Competent Complexes—Polylysine, acylated-polylysine, dendrimers, gramicidin S (stock solutions of 10 mg/mL in dimethyl sulfoxide), spermine, spermidine, and chitosan (stock solutions of 10 mg/mL in aqueous 1% acetic acid solution) were diluted to 1 mg/mL in sterile deionized water and stored at 4 °C until use. Polycation−DNA complexes were then generated by vortexing equal volumes (100 μL) of the appropriate polycation solution with 40 μg/mL pCMVlacZ plasmid DNA in serum free medium for 1 min, followed by incubation at room temperature for at least 15 min. Ratios of nucleic acid to polycation charge were estimated from the electrostatic charge present on each component.[32,39−43] The resulting polycation−DNA complexes (200 μL) were then combined with 200 μL of 0.24 mg/mL liposomes suspended in serum free medium by gentle vortexing. The hydrodynamic diameter of the complex was determined by dynamic light scattering (Coulter N4plus Submicron Particle Sizer, Miami, FL).

Cell Culture and Transfection of Cells with Liposomal Vectors—KB cells, a human nasopharyngeal cancer cell that expresses elevated levels of folate receptor were cultured in folate deficient Dulbecco's modified Eagles medium (FDMEM) containing 10% heat-inactivated fetal calf serum (FCS), penicillin (50 units/ml), streptomycin (50 μg/mL), 2 mM L-glutamine, and nonessential amino acids at 37 °C in a 5% CO_2 humidified atmosphere. In a typical experiment, cells were seeded 48 h before transfection in 24-well plates at 15% to 20% confluence. Immediately prior to transfection, the cells were washed with 0.5 mL of serum free FDMEM and then incubated for 4h at 37 °C with DNA/liposome complexes in 400 μL of serum free FDMEM. After incubation, the medium containing any free DNA/liposome complexes was replaced with vector-free medium (FDMEM) containing 10% fetal calf serum. Thirty-six hours after transfection, cells were analyzed for β-galactosidase expression, as described by others.[44]

Evaluation of Liposome Complexation with Condensed DNA—Plasmid DNA was labeled with ^{125}I using the published method of Prensky.[45] Labeled DNA was then separated from free iodine by gel filtration chromatography on a Sephadex G-25 column in 50 mM HEPES (pH 8.0) buffer. A discontinuous sucrose gradient containing layers of 3 mL, 5 mL, and 5 mL of 0%, 20%, and 40% sucrose dissolved in deionized water, respectively, was then constructed. Samples comprised of ^{125}I-labeled DNA/polycation complexes and/or ^{3}H-labeled anionic liposomes were layered on top of the gradient and centrifuged at 24 000 rpm (100 000*g*) in a Beckmann SW28 ultracentrifuge rotor for 45 min. One milliliter fractions were collected sequentially from the top to the bottom of the gradient and analyzed for lipid and DNA content by scintillation and gamma counting, respectively.

Results

In agreement with observations of others,[46−48] we have found that cationic lipid formulations cannot be readily targeted to receptor-bearing cells using cell-specific ligands such as folic acid (data not shown). We, therefore, undertook to integrate features into neutral liposomes that might facilitate uptake and delivery of encapsulated DNA into the nucleus. From previous studies, it was noted that C-DOPE, a caged form of DOPE that converts to the natural fusogenic lipid (i.e., DOPE) upon exposure to acidic pH, could enable pH-dependent release of encapsulated DNA from endosomal compartments.[38] However, because these earlier lipid formulations contained large amounts of unmodified DOPE, they were found to be unstable during storage and hence not viable for eventual clinical applications. Although addition of 40% cholesterol was observed to render these formulations stable for at least a month, the added sterol was simultaneously found to compromise the fusogenicity of the lipid complexes. We, therefore, undertook to reevaluate various lipid formulations for a combination that might be both stable and targetable, yet capable of facilitating efficient transfection of FR-expressing cells. We have used 0.1 mol % FA-PEG-DOPE as the targeting ligand in all such formulations, because we have previously found that this percentage was optimal for targeting vectors to cells expressing folate receptors and since cells lacking such receptors are not detectably transfected by such formulations.[38] Figure 1 shows that lipid envelopes of the composition DOPE/C-DOPE/cholesterol/FA-PEG-DOPE (45.9:10:40:0.1 mol %) are highly efficient in transfecting KB cells with a polylysine-compacted pCMVβgal vector. Importantly, when the FA-PEG-DOPE liposomes are replaced with nontargeted liposomes (FA-PEG-DOPE replaced with PEG-DOPE), the β-galactosidase expression decreases to background levels, indicating the above formulation facilitates ligand selective cell association. Further, when DOPS is substituted for C-DOPE in the targeted formulations, no transfection is observed, demonstrating that the caged lipid's pH dependent fusogenicity rather than its negative charge enables gene expression. Finally, when the optimal formulation is compared with unstable liposomes of the composition DOPE/C-DOPE/FA-PEG-DOPE (97:3:0.1 mol %), the cholesterol-stabilized liposomes are seen to exhibit only 15 to 20% lower transfection activity (data not shown). Since the nonstabilized formulations lose all transfection activity during 3 days of storage, formulations containing 40% cholesterol were employed in all remaining experiments.

Inclusion of C-DOPE in the lipid mixture was found to benefit the vector formulation by a second mechanism.

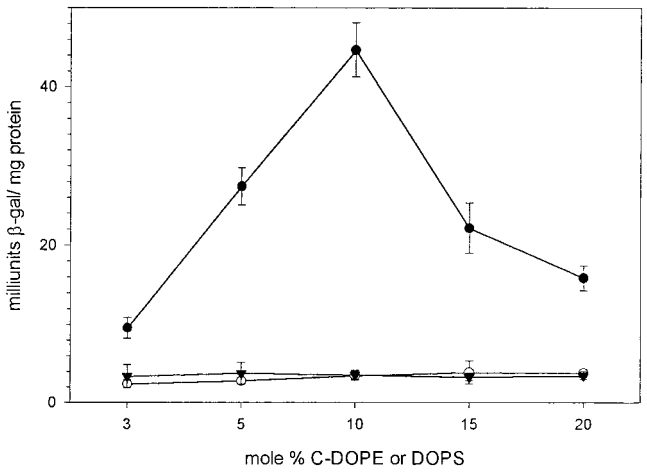

Figure 1—Optimization of C-DOPE content for maximum transfection activity. Liposomal vectors were prepared by mixing pCMVlacZ:polylysine (1:0.75 w/w) complexes with liposomes consisting of 40% cholesterol, 0.1% FA-PEG-DOPE (●,▼) or PEG-DOPE (○), plus the indicated mole % of C-DOPE (●,○) or DOPS (▼). DOPE was then added to raise the lipid total to 100%. Folate receptor bearing KB cells were transfected as described in the Methods section. Assay results are expressed as milliunits of β-galactosidase per mg cell protein. Data are presented as means ± SD where $n = 6$ from two independent experiments. Mean values were compared using one-way analysis of variance (ANOVA) followed by the Student–Newman–Keuls Multiple Comparisons Test (Sigmastat). Significance was set at $p < 0.05$. *P* values for comparison of βgal expression with FA-PEG-DOPE liposomes containing the indicated mole % of C-DOPE are 3% versus 5% < 0.001, 5% versus 10% < 0.001, 10% versus 15% = 0.003 and for 15% versus 20% = 0.002.

Because of its double negative charge at neutral pH, C-DOPE incorporation endowed the derived liposome with a strongly anionic character. Thus, upon mixing with cationic DNA/polycation complexes, a particle spontaneously formed that had a neutral to slightly negative charge (data not shown). In contrast to positively charged particles, this neutral to slightly anionic particle could be readily targeted by attachment of a cell-specific ligand (i.e. folic acid). Thus, the C-DOPE assisted in vector assembly, vector targeting, and pH-dependent vector release into the target cell's cytoplasm.

Since physical characteristics of liposomal vectors can make an important contribution to the transfection efficacy, we measured the sizes of the DNA/polylysine/liposome complexes. All vectors tested were found to have diameters of 170 ± 40 nM, and no statistical variance between the many preparations of control and C-DOPE vectors was detected. We also did not notice any trend in the size of the vectors as C-DOPE content was varied. This is probably because the primary step of DNA condensation is always performed with the same amount of polylysine, and the added C-DOPE containing liposomes simply conform to the size of this particle. Hence, we conclude that a variation in size is not the basis for the differences in transfection efficiency observed at different C-DOPE concentrations.

Effect of Incorporating a DNA Targeting Sequence into the Plasmid—Since most vectors employed for eukaryotic gene therapy must enter the nucleus to function, we next explored whether facilitated nuclear import of the plasmid DNA would enhance the efficiency of gene expression. For this purpose, a novel plasmid (pDTSβgal) was constructed (Figure 2), which is identical to the commercial plasmid (pCMVβgal) except for insertion of a 366 bp SV40 nuclear targeting sequence downstream of the *lacZ* gene and polyadenylation signal. To evaluate the possible contribution of this nuclear targeting sequence, pDTSβgal was compared with the parent pCMVβgal for expression of β-galactosidase in transfected cells. Figure 3 shows that a 1.5−2-fold increase in β-galactosidase activity was measured when pDTSβgal plasmid was substituted for pCMV-βgal in each of the three formulations tested. This nuclear-directed plasmid was therefore employed in the remainder of the studies reported here.

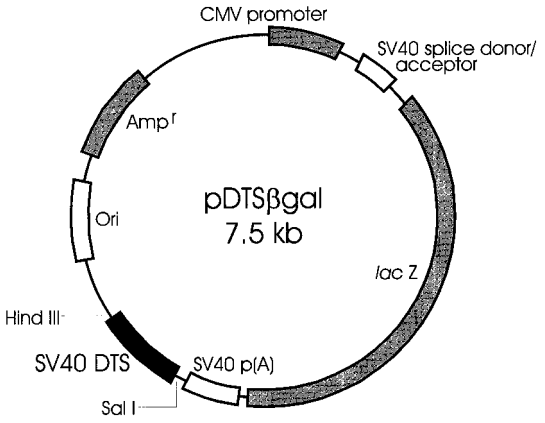

Figure 2—Map of pDTSβgal plasmid. A 384 bp HindIII–SalI fragment (SV40DTS) containing the origin and promoter region (SV40 nts 5171 to 294) from SV40 DNA was inserted into the corresponding sites of pCMVβgal, downstream of the lacZ gene, to facilitate plasmid entry into the nucleus. A cytomegalovirus immediate early gene promoter/enhancer was employed to drive expression of the reporter gene, β-galactosidase (lac Z). SV40 p(A), SV40 polyadenylation signal; Amp^r, β-lactamase gene.

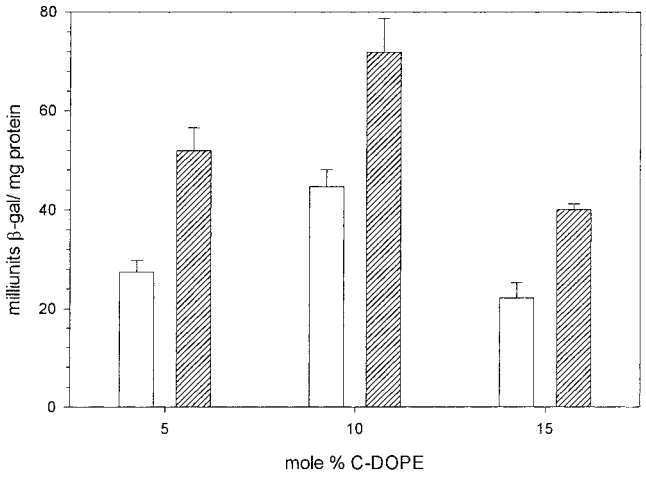

Figure 3—Effect on transfection efficiency of inserting a 366 bp SV40 nuclear targeting sequence into the pCMVlacZ plasmid. Transfection particles were prepared by mixing 4 μg of control plasmid, pCMVβgal (open bar) or 4 μg of plasmid containing a nuclear targeting sequence, pDTSβgal (slashed bar) with 3 μg polylysine, and then incubating the derived complexes with liposomes consisting of 40% cholesterol, 0.1 mol % FA-PEG-DOPE plus the indicated mole % of C-DOPE. Unmodified DOPE was added to bring the total lipid content to 100%. Transfection of KB cells was then assayed as described in the Methods section. Data are expressed as means ± SD where $n = 6$. *P* values comparing βgal expression with pCMVβgal and pDTSβgal are < 0.001 in all three cases.

Identification of Optimal DNA Compacting Polymers—Although a variety of cationic polymers have been exploited as DNA condensing agents, the resulting complexes are thought to differ in degree of compaction, compatibility with different lipid formulations, resistance to cellular nucleases, and extent of DNA unloading following entry into a cell's interior. Since ligand-mediated endocytic pathways might differ considerably from cationic lipid-promoted uptake pathways, it seemed advisable to reevaluate various DNA compaction methods for use with ligand-targeted liposomal vectors.

A panel of polycations including spermine, spermidine, gramicidin S, polyamidoamine dendrimer, polylysine, and chitosan were therefore examined for their abilities to

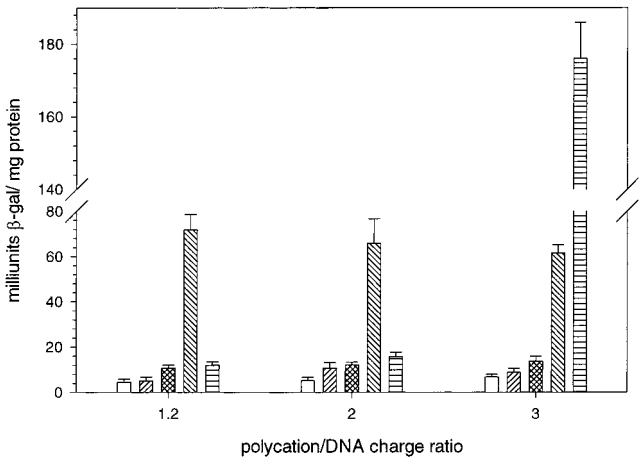

Figure 4—Effect of various oligo- and polycations on the transfection ability of folate-targeted liposomes. Polycation/DNA complexes at the indicated charge ratios were prepared by mixing pDTSlacZ with spermine (open bar), spermidine (left slashed bar), gramicidin S (double slashed bar), polylysine (right slashed bar), or 68 Å polyamidoamine cationic dendrimers (horizontal slashed bar). FA-PEG-liposomes were then prepared and transfection efficiencies compared, as described in the Methods section. The lipid composition of the complexes was 10 mol % C-DOPE, 49.9 mol % DOPE, 40% cholesterol, 0.1 mol % FA-PEG-DOPE. The polycation/DNA charge ratio refers to the charge ratio of the complex before addition of the anionic liposomes. Data are expressed as means ± SD where $n = 6$.

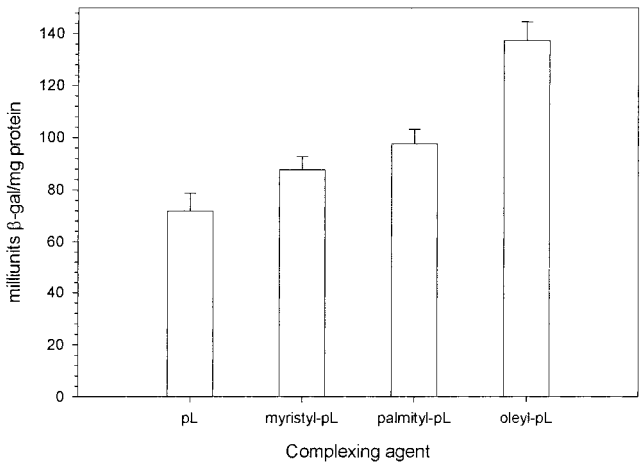

Figure 5—Effect of polylysine acylation on transfection efficiency. pDTSlacZ plasmid was mixed with polylysine (pL) or polylysine derivatized with the indicated acyl chains (see Methods) at a ± charge ratio of 1.2/1. The resulting complexes were then incubated with FA-PEG-liposomes and tested for transfection efficiency on KB cells. The lipid composition of the FA-PEG-liposomes was 10 mol % C-DOPE, 49.9 mol % DOPE, 40 mol % cholesterol, 0.1 mol % FA-PEG-DOPE. Data are expressed as means ± SD where $n = 3$. *P* values for comparison of βgal containing the indicated type of polylysine are pL versus myristyl-pL = 0.032, myristyl-pL versus palmityl-pL = 0.086 and for palmityl-pL versus oleyl-pL = 0.002.

facilitate folate-targeted gene expression. Charge ratios (±) of 1.2/1, 2/1, and 3/1 were chosen so that upon further complexation with anionic lipid mixtures the vectors would remain anionic, neutral, or even slightly cationic to ensure low levels of nontargeted gene transfection. As seen in Figure 4, only the high molecular weight polylysine and polyamidoamine dendritic polymers were highly active. Oligocations such as spermine, spermidine, and gramicidin S, in contrast, yielded low transfection efficiencies at the charge ratios tested. It was also noted that transfections with chitosan-condensed DNA (data not shown) resulted in high mortality, eliminating this complex from further consideration. However, the near absence of toxicity of the polylysine and dendritic polymers qualified them for further exploration, as described below.

Further Examination of Polylysine-Derived DNA Condensing Agents—Encouraged by the potential displayed by polylysine, and motivated by a recent report that partially acylated isoforms of the polycation might enable still better transfection efficiencies,[35] we undertook to compare various acylated polylysines in our folate-targeted KB cell transfection assay. Acylated polylysines were synthesized by N-alkylation of polylysine at a fatty acid-to-lysine ratio of 1:5 under anhydrous coupling conditions. Following purification[35] and determination of residual charge,[36] pDTSβgal plasmid DNA was successively complexed with acylated-polylysine and then DOPE/C-DOPE/cholesterol/FA-PEG-DOPE (45.9:10:40:0.1 mol %) liposomes. As shown in Figure 5, folate-targeted vectors with acylated polylysines showed higher transfection activities (~1.3 to 2-fold) than vectors with nonacylated polylysine. Furthermore, oleic acid-derivatized polylysine displayed higher transfection efficiency than either palmitic acid- or myristic acid-conjugated polylysine.

Optimization of Dendrimer/DNA Complexes for Transfection—To determine the optimal charge ratio of dendrimer/DNA complexes for ligand-targeted gene therapy, KB cells were transfected with dendrimer-compacted DNA (generation 6; 68 Å diameter) complexed with either FA-PEG-DOPE- or PEG-DOPE-containing liposomes. β-Galactosidase expression was then measured as a function of dendrimer/DNA charge ratio, which ranged from 1.2:1 to

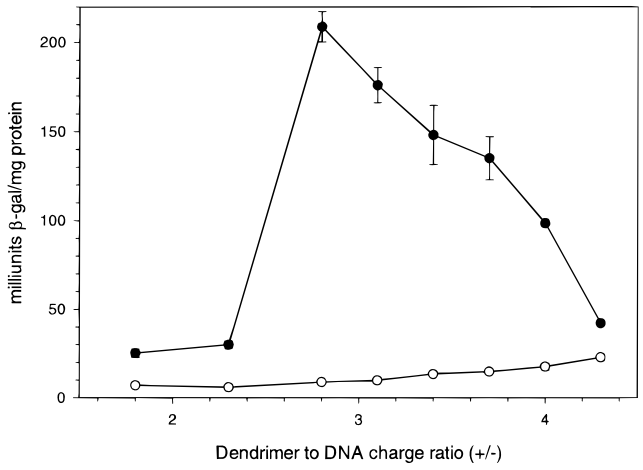

Figure 6—Effect of dendrimer/DNA charge ratio on folate-targeted liposomal vector transfection of KB cells. Complexes were prepared by mixing dendrimer/pDTSlacZ complexes at increasing charge ratios with C-DOPE liposomes consisting of 10 mol % C-DOPE, 49.9 mol % DOPE, 40% cholesterol, and 0.1 mol % FA-PEG-DOPE (●) or PEG-DOPE (○). Transfection competency was then analyzed in KB cells, as described in the Methods. The dendrimer to DNA charge ratio refers to the charge ratio of the complex before addition of the anionic liposomes. Data are expressed as means ± SD where $n = 6$.

4.5:1. As seen in Figure 6, the maximum level of β-galactosidase expression was observed at a charge ratio of 2.8:1, while at none of the examined charge ratios were nontargeted liposomes effective agents for gene delivery. A charge ratio of 2.8:1 was, therefore, used in all further dendrimer-based studies.

The heterogeneous nature of most DNA−polycation complexes makes it difficult to determine which of the diversity of complexes in any suspension is most effective in mediating transfection.[49] In contrast, dendrimers with their uniform shapes and sizes allow quantitative analysis of the influence of vector size on the efficiency of gene transfer. As shown in Figure 7, an increase in transfection efficiency was observed with increasing dendrimer size from 40 to 68 Å, followed by a decrease in activity at 84 Å. Interestingly, expression of β-galactosidase with the optimum (68 Å) dendrimer complexes was 2.5- to 3-fold higher

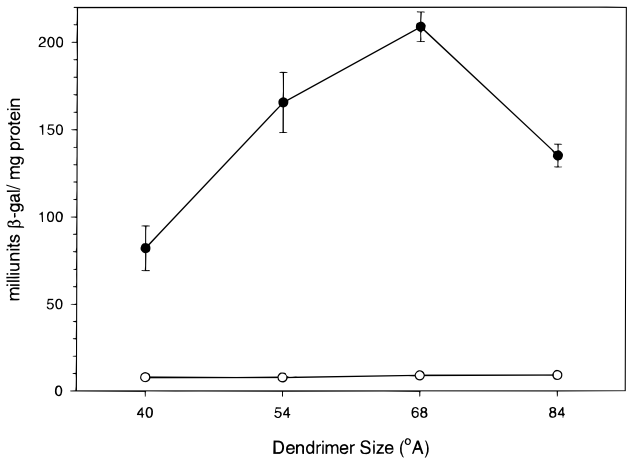

Figure 7—Influence of dendrimer size on gene transfer efficiency. pDTSlacZ plasmid vectors were formulated with dendrimers of the indicated sizes at the optimal dendrimer/DNA charge ratio of 2.8:1. The resulting complexes were then mixed with FA-PEG-liposomes (●) or PEG-liposomes (○) and tested for transfection efficiency on KB cells, as described in Figure 6. Data are expressed as means ± SD where $n = 3$.

than that seen with polylysine complexes (compare with Figure 3). As before, negligible levels of β-galactosidase expression were observed when FA-PEG-DOPE was replaced with equimolar PEG-DOPE in the lipid fraction of all dendrimer complexes tested.

Analysis of the Interaction of Polycation/DNA Complexes with Anionic Liposomes—With any ligand-targeted gene therapy formulation, it is important to determine the fraction of ligand-derivatized empty liposomes that might compete with DNA containing liposomes for cell surface receptors. For this purpose, oleyl-polylysine/DNA or cationic dendrimer (68 Å)/DNA complexes were prepared at their optimal DNA:polycation ratios, and the complexes were incubated with anionic liposomes comprised of DOPE/C-DOPE/cholesterol/FA-PEG-DOPE. The resulting folate-tethered liposomal vectors were then fractionated on a 0%/20%/40% discontinuous sucrose gradient and fractions were analyzed for their DNA and lipid contents. As noted in Figure 8 and elsewhere,[32,39] free DNA and free liposomes remained on top of the gradient, while unprotected polycation/DNA complexes migrated to the bottom (Figure 8A). In contrast, fully assembled polycation/DNA/liposome complexes were of intermediate density and sedimented as a broad band in the middle of the gradient (Figures 8B and 8C). Quantitative evaluation of the distribution of lipid and DNA radiolabels in these gradients revealed that the majority of the DNA complexes were associated with liposomes, and very few empty liposomes remained in the preparations to compete with the DNA-containing liposomes for folate receptors.

Discussion

We have shown that the transfection efficiency of folate-targeted, cholesterol-stabilized liposomal vectors can be significantly enhanced by (i) use of a pH-sensitive fusogenic lipid, (ii) incorporation of a nuclear localization sequence into the encapsulated plasmid, and (iii) selection of the optimal composition, size and charge density of the compacting polycation. Indeed, when compared to polylysine-compacted conventional plasmids, associated with folate-targeted, but pH-insensitive (lacking C-DOPE), liposomes, an increase in gene expression of more than 100-fold is observed. While further improvements in each of these features may be necessary before a clinically useful vector can be assembled, the above exploratory study constitutes

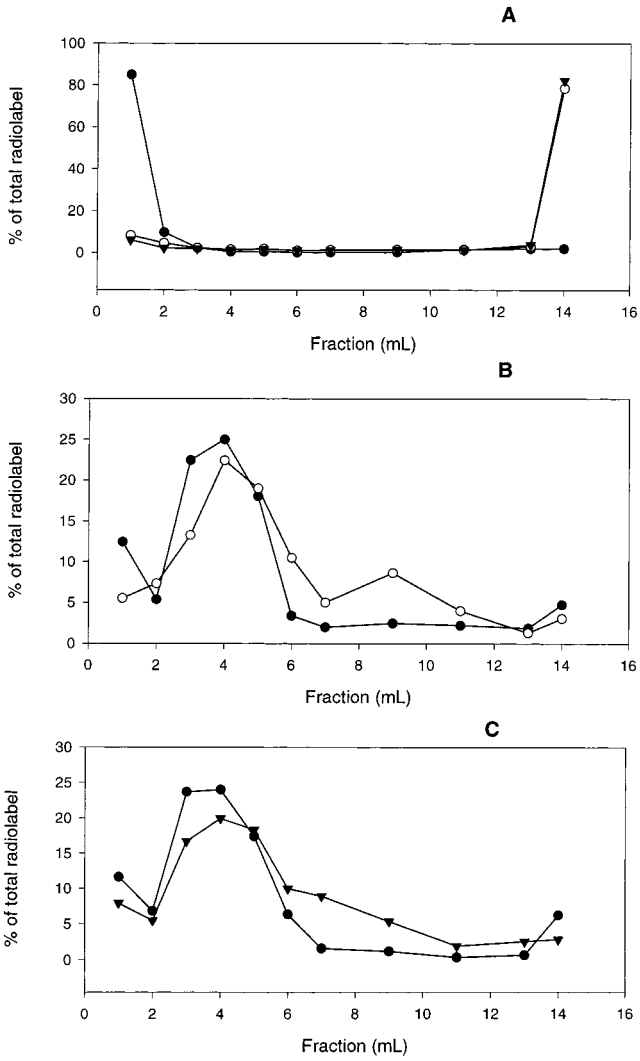

Figure 8—Analysis of polycation–DNA–liposome interactions using sucrose density gradient ultracentrifugation. (A) Oleyl-polylysine/DNA (○) or dendrimer/DNA (▼) complexes containing ^{125}I-labeled DNA (20 μg), and free liposomes containing ^{3}H-labeled lipids (240 μg) (●) were separately fractionated on a 0/20/40% (3/5/5 mL) discontinuous sucrose gradient at 100 000*g* for 45 min. One milliliter fractions were collected starting from the top of each gradient and counted for ^{125}I and ^{3}H radioactivity. After incubating the liposomes with either oleyl-polylysine/DNA (B) or dendrimer/DNA (C) particles, as described in the text, the assembled liposomal vectors were fractionated using the same gradient and counted for radioactivity. The oleyl-polylysine/DNA and the dendrimer/DNA complexes are seen to sediment to the bottom of the gradient, while the anionic liposomes and free DNA remain at the top. Mixed complexes sediment to an intermediate position in the gradient. Data are expressed as means ± SD where $n = 3$.

at least a first step toward development of a liposomal vector that mimics the desirable traits of many viral vectors.

One of the most critical limitations of standard neutral liposomal vectors appears to be their inability to dock with and enter mammalian cells.[50] Cationic liposomes obviously do not share this problem, but they are encumbered with their own set of limitations.[14] Although the advantages of ligand-mediated cell association and internalization were not emphasized in this report, it should be noted that the nontargeted vectors were generally 50 to 100-fold less effective than their folate-targeted counterparts. This differential would suggest that ligand-mediated cell association and uptake is a viral feature that should be mimicked in liposomal vectors whenever possible.

A second characteristic that contributes measurably to the usefulness of viral vectors involves their ability to

promote gene transfer across a target cell's plasma/endosomal membrane into the cell's cytoplasm. In the liposomal vector described here, this capability is accomplished in two steps. First, the cell's natural folate receptor-mediated endocytosis pathway is exploited to carry the therapeutic vector into the target cell's endosome, and then both the citriconylated and unmodified DOPE combine to facilitate escape of the DNA from the endosome into the cytoplasm. According to this strategy, the vector's full fusogenic potential is not realized until the citraconyl group of the C-DOPE is hydrolyzed at low pH. Because the required acidic conditions are not encountered prior to entry into endosomes, incorporation of C-DOPE limits vector unloading prior to intracellular uptake. With the optimized combination of C-DOPE and DOPE employed here, an increase in gene expression over vectors lacking C-DOPE of 40- to 70-fold was observed. Excess C-DOPE presumably reduced transfection efficiency, either because too much C-DOPE had to be hydrolyzed to enable fusion, or because the excess lipid assembled into folate-targeted but plasmid-free liposomes that competed with the plasmid-containing liposomes for cell surface receptors.

Probably the easiest viral trait to mimic in liposomal vector formulations lies in the ability of the viral genome to promote its own nuclear import. Because the DNA sequences involved in this process are located within the origin of replication and promoter regions, the likely mechanism for nuclear import involves binding of specific cellular proteins and their facilitation of genome transfer across the nuclear membrane.[51] While the enhancement accompanying inclusion of this viral sequence into our plasmid vector was only ~2-fold (Figure 3), the benefit to vectors targeted to less actively dividing cells could be considerably greater.[52] Thus, DNA transit across the nuclear membrane only becomes rate-limiting when the nuclear membrane remains intact over long intervals. In rapidly dividing cells such as those used in our study, this membrane is dismantled each time the cell enters M phase.

Viral capsids are able to condense their nucleic acids to a size that can be efficiently packaged and internalized by target cells. Synthetic polycations have been traditionally employed to achieve the same objective with liposomal vectors.[20,53] Indeed, for folate-targeted neutral liposomes, both polylysine and polyamidoamine dendrimers were judged to be acceptable mediators of DNA compaction (Figure 4). Further, when polylysine was partially derivatized with fatty acyl chains of varying length, an additional increase in transfection efficiency was observed. This enhancement in expression activity could conceivably be due to a decrease in electrostatic interaction between the acylated polylysine and DNA, thereby allowing the internalized plasmid to dissociate from the polylysine and enter the nucleus more easily.[35,54] Alternatively, the lipophilicity of the acylated polylysine could enhance association of the DNA/polylysine complex with the anionic liposomes or improve the stability of the folate-targeted vectors. We suggest that incorporation of acylated polycationic condensing agents warrants further examination in any quest for an optimal liposomal vector.

In contrast to the substituted and unmodified polylysines, simple oligocations such as spermine, spermidine, and the cyclic amphipathic peptide, gramicidin S, were only weakly active. Although spermine, spermidine,[40] and gramicidin S[41] are known to condense DNA and facilitate transfection, the spermine/DNA complexes are unfortunately not stable at physiological ionic strength.[39,55] This inherent instability may be responsible for the overall weak transfection activity of these targeted liposomal formulations.

The complex of plasmid DNA and cationic dendrimers was found to display the highest targeted gene expression activity of any formulation tested. Unlike the polycations of low molecular weight, cationic dendrimers have a higher surface charge density and are consequently capable of forming DNA complexes that are stable at a variety of pHs and salt concentrations.[56] With these dendrimers, transfection efficiency was found to depend on the number and size of dendrimers in each complex, with the highest expression obtained using the 68 Å dendrimer at a $\pm$ charge ratio prior to liposome addition of 2.8/1. Although stable DNA complexes could be formed at charge ratios greater than 5:1, these complexes were invariably ineffective, even though such vectors had been previously shown to be optimal in the absence of complexation with anionic lipid.[57] Why the more strongly charged complexes were less efficient in our hands is not clear, but an excess of noncomplexed dendrimer could have competed for association with the folate-linked anionic liposomes, thereby reducing the total number of dendrimer/DNA/liposome complexes capable of folate receptor binding on the cell surface.

Conclusions

Although neutral liposomal vectors generally exhibit lower transfection efficiencies than cationic liposomal vectors, they simultaneously display several advantages not shared by their cationic counterparts. Thus, vectors with low surface charge density can be readily targeted to specific cell types, commonly exhibit little or no nonspecific cytotoxicity,[20] and display reduced tendency to activate complement,[58] in contrast to most cationic liposomal vectors.[58] With the development of novel components/strategies to facilitate (i) DNA compaction, (ii) cell surface association and entry, (iii) endosome unloading, and (iv) intranuclear delivery, the prospects for improving the transfection efficiency of neutral liposomal vectors is increasingly bright.

References and Notes

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