AAPS Pharmsci 1999; 1 (4) article 19 (http://www.pharmsci.org)

Receptor-Targeted Gene Delivery ViaFolate-Conjugated Polyethylenimine

Submitted: August 3, 1999; Accepted: November 8, 1999; Published: December 10, 1999.

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ABSTRACT A novel synthetic gene transfer vector was evaluated for tumor cell-;specific targeted gene delivery. The folate receptor is a tumor marker overexpressed in more than 90% of ovarian carcinomas and large percentages of other human tumors. Folic acid is a high affinity ligand for the folate receptor that retains its binding affinity upon derivatization via its gamma carboxyl. Folate conjugation, therefore, presents a potential strategy for tumor-selective targeted gene delivery. In the current study, we investigated a series of folate conjugates of the cationic polymer polyethylenimine (PEI) for potential use in gene delivery. A plasmid containing a luciferase reporter gene (pCMV-Luc) and the folate receptor expressing human oral cancer KB cells were used to monitor gene transfer efficiency in vitro. Transfection activity of polyplexes containing unmodified polyethylenimine was highly dependent on the positive to negative charge (or the N/P) ratio. Folate directly attached to PEI did not significantly alter the transfection activity of its DNA complexes compared to unmodified PEI. Modification of PEI by polyethyleneglycol (PEG) led to a partial inhibition of gene delivery compared to unmodified PEI. Attaching folates to the distal termini of PEG-modified PEI greatly enhanced the transfection activity of the corresponding DNA complexes over the polyplexes containing PEG-modified PEI. The enhancements were observed at all N/P ratios tested and could be blocked partially by co-incubation with 200 µM free folic acid, which suggested the involvement of folate receptor in gene transfer. Targeted vectors based on the folate-PEG-PEI conjugate are potentially useful as simple tumor-specific vehicles of therapeutic genes.

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INTRODUCTION

Gene therapy is a promising approach for treatment of human diseases rooted in genetic disorders, such as cancer. An important obstacle to the successful clinical application of gene therapy is the development of effective gene transfer vectors. Both viral and synthetic vectors are currently under evaluation. Synthetic vectors, though generally not as efficient as viral vectors, have the potential advantages of being nonimmunogenic and amenable to synthetic modifications. The first-generation nonviral vectors primarily consist of naked DNA, DNA complexed to cationic liposomes (lipoplexes), or DNA complexed to cationic polymers (polyplexes). Naked DNA is highly sensitive to serum nuclease digestion; therefore, it is mostly administered via intramuscular injection. Lipoplexes and polyplexes shield the condensed DNA from nuclease digestion and rely on excess cationic charge to mediate cellular entry. They are quite efficient in gene delivery in vitro but are only slightly active when administered intravenously. The lung is usually the organ with the highest DNA deposition and reporter gene expression, indicating extensive nonspecific trapping. Because of negatively charged plasma components, cellular entry mechanism based on charge interaction is likely to be ineffective for in vivo gene transfer by DNA vectors administered intravenously. Therefore, development of targeted vectors that enter cells through tissue-specific receptors is highly desirable. Tissue-specific targeting could greatly improve gene expression in the target tissue, reducing uptake by nontarget tissues and potential systemic toxicity. Tumor targeting may be accomplished by attachment of a tumor-specific ligand, such as folic acid, to a synthetic vector.

The folate receptor is a glycosylphosphatidylinositol (GPI)-anchored, high-affinity membrane folate binding protein overexpressed in a wide variety of human tumors, including >90% of ovarian carcinomas (1,2). Meanwhile, normal tissue distribution of folate receptor is highly restricted, making it a useful marker for targeted drug delivery to tumors (2). A monoclonal antibody against the folate receptor

(MOv18) and its derivatives has been evaluated for the scintigraphy and tomography imaging, radiotherapy, and immunotherapy of ovarian cancer in clinical studies (3-;7). Folic acid, a high-affinity ligand for the folate receptor (K_d~$10^{-;10}$ M), retains its receptor binding property when covalently derivatized via its gamma-carboxyl. Folate conjugates have been shown to be taken into receptor-bearing tumor cells via folate receptor-mediated endocytosis, as illustrated in Figure 1. Folate-conjugation, therefore, presents a useful method for receptor-mediated drug delivery into receptor-positive tumor cells. Folic acid is potentially superior to antibodies as a targeting ligand because of its small size, lack of immunogenicity, ready availability, and simple and defined conjugation chemistry. Receptor-specific delivery has been shown with folate conjugates of protein toxins, anti-T-cell receptor antibodies, liposomes, radioimaging agents, chemotherapy agents, and gene transfer vectors (8-15). Folate conjugated poly-L-lysine coupled with replication-defective adenovirus has been shown to mediate receptor-specific transfection in cultured tumor cells (16). Folate conjugated F_{ab} fragment against adenoviral fiber has been shown to alter the tropism of the viruses toward receptor-bearing tumor cells (18). Folate-targeted pH-sensitive liposomes complexed to polylysine-condensed DNA have been formulated as LPDII particles for the receptor-specific cellular delivery of plasmid and antisense oligonucleotides (11,19).

In this study, we evaluated a simple system for targeted gene delivery based on folate-conjugated polyethylenimine (PEI). Linear or branched, PEI is a cationic polymer ideally suited for forming compact charge complexes with plasmid DNA. In addition to its DNA-condensing properties, this polymer also possesses significant endosomolytic activity because of its strong buffering capacity at the acidic endosomal pH (19,20). PEI-DNA polyplexes have been shown to mediate efficient transfection of cultured cells (20,21). Attachment of targeting ligands, including transferrin and galactose, has been shown to facilitate the delivery of PEI polyplexes to specific cell types (20,21). Folate conjugates of PEI, however, have not previously been evaluated for gene delivery. Therefore, a series of folate conjugates of PEI were synthesized and evaluated for their ability to mediate receptor-specific gene transfer in cultured KB cells that overexpress the folate receptor.

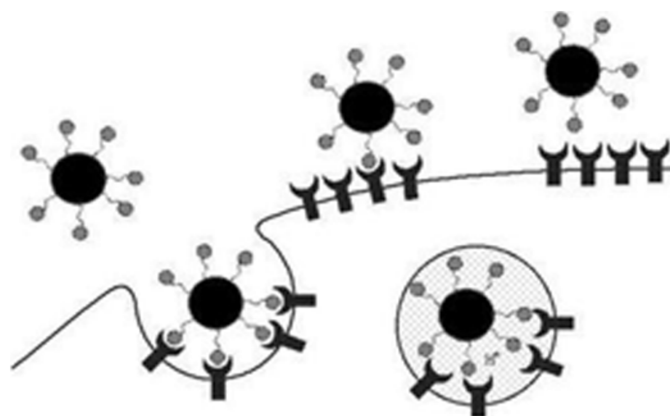

Figure 1. Receptor-mediated endocytosis of folate conjugates

MATERIALS AND METHODS

Materials

Folic acid dihydrate, polyoxyethylene-bis-amine (PEG-bis-amine, M_r. ~ 3350), and Sephadex G-100 resin were purchased from Sigma Chemical Co (St. Louis, MO). Polyethylenimine (PEI, M_r. ~ 25,000), dicyclohexylcarbodiimide (DCC), N-hydroxysuccinimide (NHS), maleiimidobenzoyl-N-hydroxysuccinimide ester (MBS), and Traut's Reagent (2-iminothiolane) were purchased from Aldrich Chemical (Milwaukee, WI). Luciferase assay kits were obtained from Promega (Madison, WI). BCA protein assay kits were purchased from Pierce Chemical (Rockford, IL). Tissue culture media were purchased from Life Technologies (Rockville, MD). All reagents were used without further purification.

Synthesis of Folate-PEI and Folate-PEG-PEI

An activated ester of folate, NHS-folate, was synthesized as described previously (22). Briefly, folic acid (1 g) was dissolved in 50 mL dimethylsufoxide (DMSO) along with a 1.1 molar excess of NHS; 1.1 molar excess of DCC was then added, and the reaction mixture was stirred overnight in darkness at room temperature. The insoluble by-product, dicyclohexylurea, was removed by filtration through glass wool. The filtrate containing the DMSO solution of the NHS-folate product was stored at -;20°C until use in further synthesis.

Folate-PEI conjugate was synthesized by reacting PEI with a 5 molar excess of the above-synthesized NHS-folate in DMSO, as shown in Figure 2A. The product was then diluted in 2 volumes of deionized water and then passed through a 10-mL Sephadex G-100 column (Pharmacia Biotech) equilibrated in phosphate-buffered saline (PBS, 136.9 mM NaCl, 2.68 mM KCl, 8.1 mM Na_2HPO_4, 1.47 mM KH_2PO_4, pH 7.4). One-mL fractions were collected. The folate-PEI conjugate, which was of high molecular weight, eluted as a faintly yellow band in fractions 4-;5, whereas the folate byproduct eluted at approximately the same rate as the buffer as a separate yellow band in fractions 10-;12. PEI concentrations in the conjugates were determined by the colorimetric ninhydrin amine assay using underivatized PEI as a reference. Folate content was determined by measuring ultraviolet absorption at 363 nm and calculating based on a molar extinction coefficient of 6500 $M^{-;1}cm^{-;1}$ in PBS. Approximately three folates were attached for each molecule of PEI.

Folate-PEG-PEI was synthesized as follows: Folate-PEG-amine was first synthesized by reacting PEG-bis-amine (M_r. ~3350) with equimolar amount of NHS-folate in DMSO. The product was purified by gel-filtration on a Sephadex G-25 column equilibrated in deionized water. The folate-PEG-amine, which was eluted as a light yellow band in the void volume, was lyophilized and then dissolved in DMSO. A 1.1 molar excess of MBS was then added to yield folate-PEG-maleiimide. Meanwhile, PEI (branched polymer, M_r. ~ 25,000) was converted to a thiol derivative by reacting with Traut's Reagent in PBS at pH 7.4. The thiol-PEI was purified by gel-filtration on a Sephadex G-100 column. The product was eluted near the void volume and was readily detected via its amino groups by ninhydrin assay. The thiol-PEI was reacted with a 1.5 molar excess of the folate-PEG-maleiimide derivative to yield folate-PEG-PEI, via a thioether linkage, as shown in Figure 2B. The product was purified by passing through a 10-mL Sephadex G-100 column equilibrated with PBS. Ninhydrin-positive fractions 4-;5 were collected as the product. Trace amounts of unreacted folate-PEG-maleiimide and other low molecular weight by-products should be eluted in later fractions, as suggested by the elution profile of free folate-PEG-amine on the same column (data not shown). PEI and folate contents were analyzed as described for folate-PEI. The folate-to-PEI ratio was determined to be ~1 to 1. As a nontargeted control, PEG-PEI conjugate was synthesized by reacting PEI with 1.5 molar excess of methoxy-SSA-PEG 5000 (Shearwater Polymers) in DMSO, followed by purification by gel filtration on Sephadex G-100. The PEI conjugates were stored as a frozen solution at -20°C until use.

Plasmid Preparation

Supercoiled plasmid containing a firefly luciferase reporter gene under the cytomegalovirus promoter pCMV-Luc was purified from alkaline lysate of DH5a *Escherichia coli* cells using the Qiagen maxi kit (Qiagen, Santa Clarita, CA). Plasmid DNA was quantified by UV absorbance at 260 nm on a Shimadzu UV-160U Spectrophotometer, analyzed by electrophoresis using a 1% agarose gel and stored at -;20°C in TE buffer (10 mM Tris, pH 7.4, 1 mM EDTA).

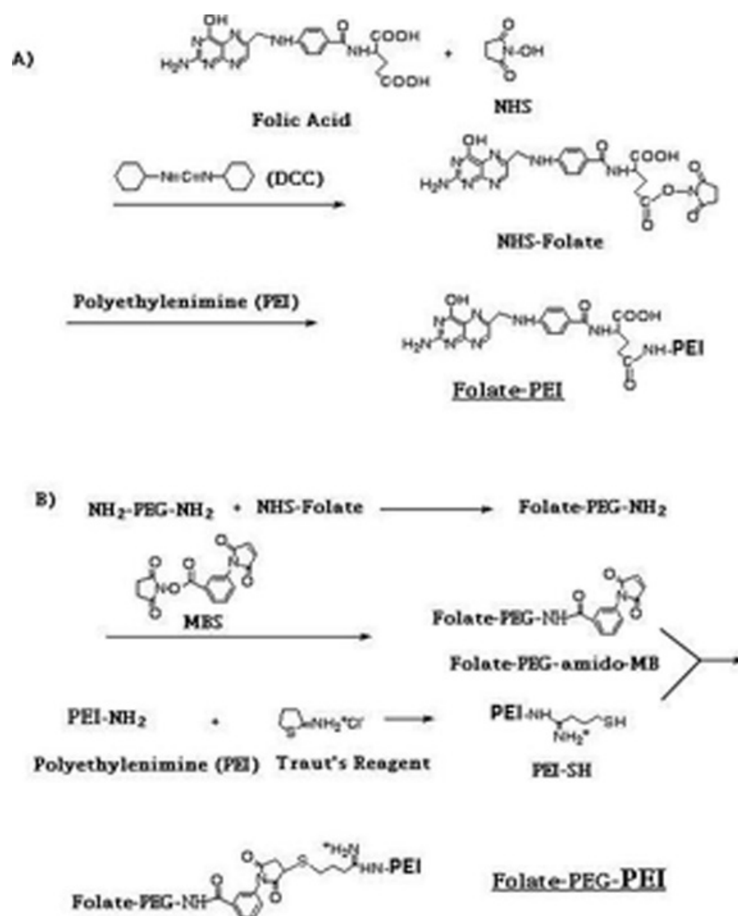

Figure 2. Synthesis of A) Folate-PEI and B) Folate-PEG-PEI

Cell Culture

Human oral cancer KB cells, which overexpress the folate receptor, were obtained as a gift from Dr. Philip Low at Purdue University (West Lafayette, IN). The cells were cultured as an attached monolayer in folate-free RPMI media supplemented with antibiotics and 10% fetal bovine serum in a humidified atmosphere containing 95% air and 5% CO_2 at 37°C. This media contained a final folate concentration similar to that of human plasma. Regular tissue culture media were not used because they contain µM levels of folate (i.e., orders of magnitudes higher than that of the physiological level) and may block receptor binding of folate conjugates.

Preparation of DNA Complexes and Transfection Protocol

DNA-polycation complexes were prepared by rapid addition of PEI or the various folate conjugates to plasmid DNA at a series of PEI nitrogen to DNA phosphate (N/P) ratios, each diluted in 50 µL of dionized water. For transfection studies, $\sim 10^5$ cells per well were plated in 24-well plates the day before the transfection experiments. At the time of transfection, the cells were ~75% confluent. During treatment, cells (in triplicates) were incubated with 1 µg of DNA in various formulations in 500 µL of serum-free media for 4 hours at 37°C. The cells were then gently washed twice with cold PBS and incubated for a further 24 hours in fresh culture medium containing 10% fetal bovine serum. The cells were lysed with a lysis buffer (0.5% Triton X-100, 100 mM Tris-HCl, 2 mM EDTA, pH 7.8), and the lysate was analyzed for protein content using a BCA protein assay kit (Pierce Chemical, Rockford, IL), and luciferase activity was measured using a kit from Promega on a Lumat LB9501-16 Luminometer (EG&G Berthold). Relative light units (RLUs) were converted to picograms luciferase using a standard curve generated with a firefly luciferase enzyme standards under identical assaying conditions.

RESULTS

Synthesis of Folate-PEI and Folate-PEG-PEI

PEI is a cationic polymer with both DNA condensing and endosomolytic activities. The latter is presumably a result of the high buffering capacity of PEI at the acidic endosomal pH. These properties make PEI ideally suited for formulating polyplexes with plasmid DNA for gene delivery. PEI contains primary amino-groups, which account for 25% of the nitrogen atoms, through which a desired targeting ligand may be attached, either directly or via a spacer. Previous studies with folate-derivatized, small unilammelar liposomes showed that a long PEG (M_r ~ 3350)-based spacer was needed to allow the liposome-linked folate to be recognized by the folate receptor (9). Folates attached to the lipid head group directly or via short linkers were shown to be ineffective in mediating liposome targeting (9). Because PEI/DNA complexes (mean diameter ~50-;100 nm) were similar in size to small unilammelar liposomes, we anticipated a similar requirement for long spacers to achieve effective receptor targeting. Therefore, folate-PEG-PEI was synthesized incorporating a PEG (M_r ~ 3350) spacer, as illustrated in Figure 2. Because the presence of PEG on PEI may interfere with its ability to complex with DNA and/or interact with cells as DNA polyplexes, a PEG-PEI conjugate without the attached folate was also synthesized to assess the effect of PEG derivatization on the transfection activity of corresponding DNA polyplexes.

Transfection Activities of Folate-PEI (No Spacer) and Folate-PEG-PEI DNA Complexes

The pCMV-Luc plasmid containing the firefly luciferase was used in the studies, and the transfection efficiency was determined by luciferase assay. A standard curve of amount of luciferase in picograms versus RLUs (Figure 3) was used to convert data obtained into picograms luciferase per mg extractable protein.

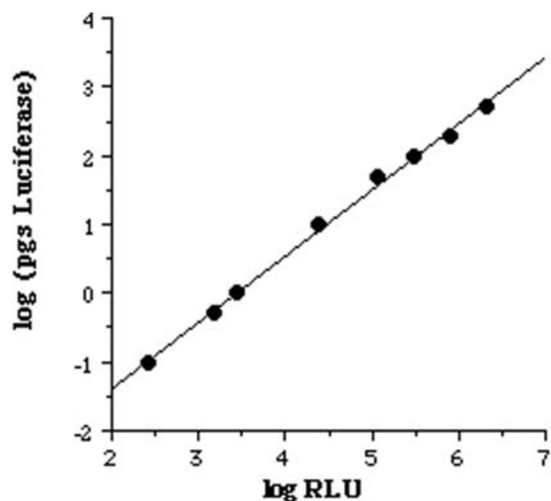

Figure 3. Standard Curve of Amount of Luciferase (in picograms) versus Relative Light Units (RLUs)

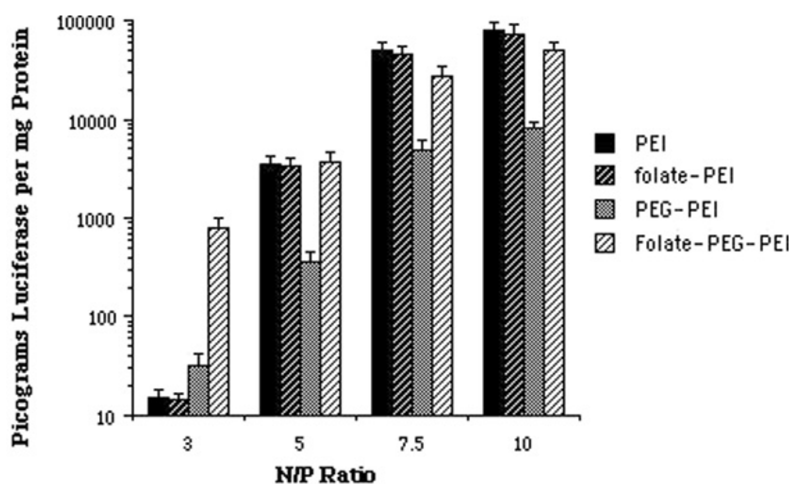

Figure 4. Transfection of KB Cells by Various PEI Derivatives

KB cells, which overexpress the folate receptor, were treated with polyplexes formulated with PEI, folate-PEI (no spacer), folate-PEG-PEI, or PEG-PEI at PEI nitrogen to DNA phosphate (N/P) ratios of 3, 5, 7.5, and 10, in serum-free RPMI culture media. The transfection results are shown in Figure 4

The N/P ratio of PEI/DNA polyplexes reflects the overall positive to negative charge balance of the DNA complex. Polyplexes formed at higher N/P ratios are more positively charged; therefore, they are likely to interact more effectively with the negatively charged cell surface via nonspecific charge interaction. For each of the PEI conjugates and the unmodified PEI, transfection activity was increased with an increase in N/P ratio. This could be due to the combination of an increase in the positive charge of the corresponding polyplexes and an increase in PEI content in the DNA polyplex, which leads to elevated cellular uptake and more efficient endosome release of the internalized polyplexes, respectively.

At an N/P ratio of 3, where close-to-neutral polyplexes were formed, vectors containing PEI, folate-PEI (no spacer), or PEG-PEI all showed very low levels of transfection activity. Meanwhile, polyplexes containing folate-PEG-PEI had much higher transfection activity compared to those containing PEG-PEI. This suggests that transfection by the folate-PEG-PEI polyplexes was facilitated by binding to the folate receptor and that a spacer is required between folic acid and the PEI for effective binding to the receptor, similar to the scenario of liposomes.

At higher N/P ratios, folate-PEG-PEI polyplexes showed transfection activities comparable to those of the underivatized PEI and the folate-PEI, and ~10 times higher than the PEG-PEI polyplexes. It is possible that, at these higher N/P ratios, charge-mediated cellular uptake was highly efficient for polyplexes formed with PEI or folate-PEI due to the presence of a net positive charge. Polyplexes formed with PEG-PEI had reduced cellular uptake due to the steric hindrance introduced by PEG, resulting in an ~10-fold reduction in transfection activity compared to PEI polyplexes. Cellular uptake was restored when folate was attached to the distal end of the PEG in folate-PEG-PEI polyplexes. In addition, the apparent dependence of transfection on N/P ratio for these polyplexes indicates that the observed transfection activities were likely a reflection of the combined effects of charge-mediated cellular uptake and folate receptor mediated endocytosis.

To further define the role of targeting in gene delivery, transfection studies were also performed in the presence of 200 μM folic acid at the N/P ratio of 5. As shown in Figure 5, 200 μM free folate reduced the transfection activity of folate-PEG-PEI/DNA complexes by ~75%. Meanwhile, free folate had little effect on the transfection activity of PEI, folate-PEI/DNA, or PEG-PEI complexes (data not shown). These data showed that receptor-mediated transfection of folate-PEG-PEI polyplexes can be competitively blocked by excess free ligand folic acid.

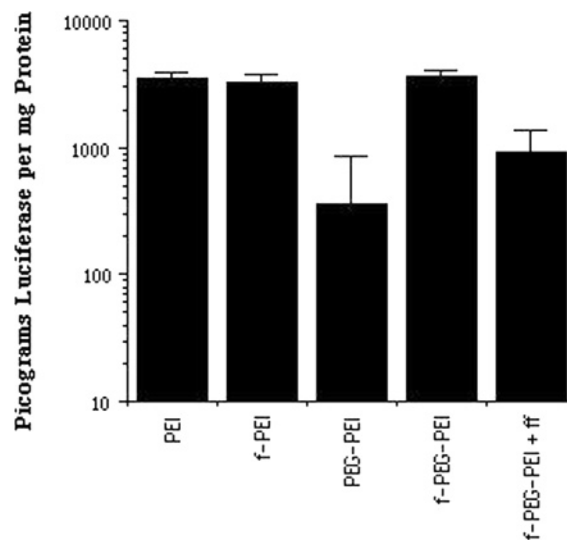

Figure 5. Effect of Free Folate on the Transfection Activity of Folate-PEG-PEI/DNA Complexes

DISCUSSION

Folate-PEG-PEI/DNA polyplexes were shown to mediate efficient transfection of cultured tumor cells overexpressing the folate receptor. Targeting effect was more evident at low N/P ratios with slightly positively charged DNA complexes. As was the case with folate-targeted liposomes, a spacer between folate and PEI was shown to be necessary to allow for efficient receptor targeting. Polyplexes containing PEG-modified PEI were much less active in transfecting cells compared to PEI due to the steric hindrance introduced by PEG, the resulting reduction in charge-mediated cellular uptake, and, perhaps, the necessary polyplex interaction with the endosomal membrane for cytosolic escape. Cellular transfection activity could be restored by the attachment of a folate at the distal end of the PEG. This targeting effect appeared to be additive to the effect of increased transfection activity of various polyplexes at higher N/P ratios. The role of folate receptor in mediating transfection is further indicated by the observation that transfection activity of folate-PEG-PEI polyplexes could be partially blocked by excess free folate. At the N/P ratio of 5, assuming that each polyplex contained only one plasmid DNA molecule and that all folate-PEG-PEI were involved in DNA complexation, each polyplex would theoretically contain ~120 folates. This observation means each polyplex was capable of multivalent interaction with the cellular folate receptor and, therefore, that each polyplex exhibits dramatically higher apparent affinity for receptor-bearing cells compared to folate, a monovalent ligand. This scenario could explain the incomplete inhibition of transfection by 200 μM free folate, which translates into a 5×10^5-fold excess compared to the number of vector particles assuming one plasmid DNA per polyplex. Because folate carries 1-;2 negative charges and is known to form oligomeric stacks, higher concentrations of folate were not used (23). High concentrations of folate, therefore, may significantly disrupt the charge complexes formed between the cationic polymers and the plasmid DNA (22). Physiological concentration of folate in human plasma is ~20 nM, primarily in the form of 5-methyltetrahydrofolate (5-MeTHF). 5-MeTHF has ~10-fold lower affinity for the folate receptor and is unlikely to affect folate receptor targeting in vivo at this concentration range.

Our data were consistent with earlier reports on vectors containing folate-polylysine (16,17). However, because polylysine has little endosome lytic activity of its own, coupling to an additional lytic component, such as inactivated adenoviruses, was required for efficient gene delivery (16,17). In contrast with folate conjugates of PEI, a long spacer was not required for receptor targeting with the folate-polylysine conjugate (16,17). The use of folate-PEG-PEI was significantly simpler and involved only one synthetic component and a single step of formulation. However, charge-mediated cellular uptake to a large extent masked the receptor-mediated uptake, which reduced the selectivity of the polyplexes for the target tissue. Future studies will investigate methods to improve the specificity of folate-PEG-PEI polyplexes.

ACKNOWLEDGMENTS

This work was partly supported by a grant from the Parenteral Drug Association (PDA) and grant RPG-99-097-01-MGO from the American Cancer Society.

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