Cancer gene delivery with folate receptor targeting lipid-based nanoparticle

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Abstract
Cancer gene therapy has been intensively developed using non-viral vectors, among which cationic liposomes and nanoparticles are the most investigated. For targeted delivery to tumors, vitamin folic acid has been utilized for folate receptor (FR)-mediated drug delivery since FR is frequently overexpressed on many types of human tumors. Liposomes conjugated to folate ligand have been used as carriers of chemotherapeutic agents and DNA to receptor-bearing tumor cells in vitro. The folate-linked lipid-based nanoparticles that we developed could deliver genes extensively to FR-negative LnCaP and PC-3 cells as well as FR-positive KB and Hela cells. Here, we outline folate-linked liposomes and nanoparticles, and show the effectiveness of folate-linked lipid-based nanoparticles as a vector for DNA transfection to human nasopharyngeal and prostate tumors.

Abbreviations
CHEMS, cholesteryl hemisuccinate; Chol, cholesterol; DC-Chol, 3 ([N-(N', N'-dimethylaminoethane)-carbamoyl] cholesterol; DOPE, dioleoyl phosphatidylethanolamine; DPEP, dipalmityl phosphatidylethanolamine; DSPE, distearoyl phosphatidylethanolamine; FR, folate receptor; GCV, ganciclovir; HSV-tk, herpes simplex virus thymidine kinase; OH-Chol, cholesteryl-3 -carboxymidoethy-lene-N-hydroxylamine; ODN, oligodeoxynucleotide; PEG, polyethylene glycol; PEI, polyethylenimine; PLL, poly-L-lysine; PEG-DSPE, polyethylene glycol-distearoylphosphatidylethanolamine; PSMA, prostate specific membrane antigen; RES, reticuloendothelial systems; RFC, reduced folate carrier.

1. Introduction
Cancer gene therapy has been intensively developed using non-viral vector [1]. Viral vectors such as retroviruses [2], adenoviruses [3], adeno-associated viruses [4] and several other viral types [5], are efficient in transfection, but pose risks to the host from the immunogenicity of viral proteins, a lack of desired tissue selectivity, the potential for oncogenesis due to chromosomal integration, and the generation of infectious viruses due to recombination, making non-viral vectors an attractive alternative. Synthetic vectors such as cationic polymers, liposomes and nanoparticles have been widely studied for DNA delivery due to their potential for tissue-specific targeting, their lack of immunogenicity, the relative safety, and relative ease of large-scale production. For targeted delivery to tumors, vitamin folic acid has been utilized for folate receptor (FR)-mediated drug delivery since the FR is frequently overexpressed on human tumors [6,7]. Liposomes conjugated to folate ligand have been reported as carriers of chemotherapeutic agents to FR-bearing tumor cells in vivo [8-14]. While much has been published on folate-drug conjugates and folate-linked carriers, relatively little is known about the targeting of gene delivery. The use of a folate ligand as a targeting ligand to deliver DNA also has been reported in vitro [15-19], but not been successful in in vivo gene therapy [20, 21]. Therefore, we describe the current understanding of folate-linked lipid-based vectors, liposomes and nanoparticles.

2. Folate Receptors
FR has been found to be overexpressed in a wide range of tumors, and is known as a high-affinity membrane folate-binding protein, which mediates uptake of the vitamin by receptor-mediated endocytosis. Therefore, it presents an attractive target for tumor-selective delivery. FR-targeting materials can continuously accumulate into cells due to receptor recycling. FR-targeting imaging agents arrived on the market in 2004. Three isoforms of FR have been identified and two, FR-α and -β, are attached to the cell by a glycosylphosphatidylinositol (GPI)-anchor, while FR-γ is secreted due to the lack of an efficient signal for GPI modification [7]. The role of FRs in the cellular transport of folate is not well understood, although a potocytosis (caveolin-coated endocytosis) model has been proposed [22]. FR-α was found to be clustered in membrane region called caveolae or rafts [22]. While

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an elevated expression of FR has frequently been observed in various types of human tumors, the receptor is generally absent in normal tissues with the exception of the choroid plexus and placenta, with low levels in the lung, thyroid and kidney [23]. FR-\(\alpha\) is frequently overexpressed in tumors, including ovarian, colorectal, breast, lung, renal cell carcinomas and brain metastases derived from epithelial cancers [6,7]. FR-\(\beta\) is frequently overexpressed in tumors of non-epithelial cell lineages such as sarcomas and acute myeloid leukemia [24], and FR-\(\gamma\) is overexpressed in malignant hemopoietic cells [25]. The causes of FR overexpression in tumors are unclear, but high levels of FR may be associated with increased biological aggressiveness of carcinomas.

3. Folate-linked Vectors

Folic acid as a targeting ligand offers many potential advantages: (1) small size of the targeting ligand, which often leads to favorable pharmacokinetic properties of the folate conjugates and reduced probability of immunogenicity; (2) convenient availability and low cost; (3) relatively simple and defined conjugation chemistry; (4) high affinity for FR and lack of FR expression in normal tissue; (5) the receptor/ligand complex can be induced to internalize via endocytosis and (6) high frequency of FR overexpression among human tumors. Therefore, folate-linked targeting systems show great potential for clinical and therapeutic application.

Several cationic polymer-folate conjugates and/or cationic liposomes, and cationic nanoparticles incorporating folate-derivatives have been developed for FR-targeted gene delivery (Table I). Folic acid retains its receptor-binding and endocytic properties when covalently linked to a wide variety of molecules. The liposomes used in recent studies have been coated with folate-PEG-lipid to facilitate tumor-targeting by an active mechanism (via FR) and a passive mechanism (prevention/reduction of RES uptake) [20, 26].

<table>
<thead>
<tr>
<th>Vector</th>
<th>Description</th>
<th>in vivo</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polymer</td>
<td>Poly-L-Lys (PLL)</td>
<td>Polylex prepared with PLL-folate or PLL-PEG(_{1800})-folate</td>
<td>-</td>
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<tr>
<td>Polymer</td>
<td>Polyethyleneimine (PEI)</td>
<td>Polylex prepared with PEI-folate or PEI-PEG(_{1800})-folate</td>
<td>-</td>
</tr>
<tr>
<td>Polymer</td>
<td>Polydimethylamino methylmethacrylate (pDMAEMA)</td>
<td>Polylex prepared with pDMAEMA-PEG(_{1800})-folate</td>
<td>-</td>
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<tr>
<td>Cationic</td>
<td>Incorporating 0.1 mol% folate-PEG(<em>{1800})-DOPE, 5 mol% folate-DOPE, 0.5-5 mol% folate-PEG(</em>{1800})-DSPE or 5 mol% folate-PEG(_{4000})-cholesterol into liposome</td>
<td>i.v.</td>
<td>[15, 17-20, 34]</td>
</tr>
<tr>
<td>Liposome</td>
<td>LPDI type</td>
<td>Polylex prepared from protamine was mixed with cationic liposome containing 0.03 mol% folate-PEG(<em>{1800})-DSPE or 2 mol% folate-PEG(</em>{5000})-DSPE</td>
<td>i.p. i.v.</td>
</tr>
<tr>
<td>Liposome</td>
<td>LPDII type</td>
<td>Polylex prepared from PLL or PEI was mixed with pH-sensitive anionic liposome containing 0.1 mol% folate-PEG(<em>{3350})-DOPE or folate-PEG(</em>{2000})-DSPE</td>
<td>-</td>
</tr>
<tr>
<td>Nanoparticle</td>
<td>NPI-F</td>
<td>Polylex prepared with cationic dithiol-detergent ((C(<em>{16})Corn)n) was mixed with 2mol % folate-PEG(</em>{1800})-DPPE</td>
<td>-</td>
</tr>
<tr>
<td>Nanoparticle</td>
<td>NPII-F</td>
<td>Incorporating 1 or 2 mol % folate-PEG(_{2000})-DSPE into cationic nanoparticle based DC-Chol.</td>
<td>i.v. i.t.</td>
</tr>
<tr>
<td>Nanoparticle</td>
<td>NPIII-F</td>
<td>Incorporating 1 mol % folate-PEG(_{2000})-DSPE into cationic nanoparticle based OH-Chol.</td>
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i.v.: intravenous injection, i.p.: intraperitoneal injection; i.t.: intratumoral injection.
**3.1 Polymers**

Polyplexes are composed of charged complexes of plasmid DNA and a cationic polymer, such as poly-L-lysine (PLL), polyethylenimine (PEI) and polyamidoamine dendrimers. Cationic polymers and plasmid DNA can form condensed particles with a net positive charge. This protects the DNA from nucleasemediated degradation and enables charge-mediated nonspecific cellular association. For FR-targeted gene delivery, PLL-folate [27-29], PLL-PEG-folate [30], PEI-folate [31], PEI-PEG-folate [31, 32], and poly (dimethylaminomethyl methacrylate) (pDMAEMA)-PEG-folate [33] have been synthesized. These folate-conjugates facilitated efficient FR-targeted gene delivery without additional vector components in vitro. It appears that the incorporation of a long PEG spacer between folate and the cationic polymer is important for efficient FR-targeted gene delivery [29].

**3.2 Liposomes**

Lipoplexes are composed of charge complexes of plasmid DNA and cationic liposome. Lipopolyplexes are composed of plasmid DNA attached to both polymers and lipids. Cationic liposomes are generally composed of a cationic lipid, such as dioleoyltrimethylammonium chloride (DOTMA), dioleoyltrimethylammonium propane (DOTAP), or dimethylaminoethanolamine carbamoyl cholesterol (DC-Chol), and a helper lipid, such as DOPE or cholesterol (Chol), which provides fusogenicity and stability to the lipoplex. Depending on the preparation, the lipoplex may not be a single aggregate, but an intricate structure in which the condensed DNA is surrounded by a lipid bilayer. FR-targeting cationic liposomes were incorporated with folate-derivatives, folate-DOPE [34], folate-PEG-DOPE [15,16], folate-PEG-DSPE [17-21,35], folate-PEG-Chol [20], etc. Hofland et al. [20] showed that both folate-PEG<sub>2000</sub>-DSPE and folate-PEG<sub>2000</sub>-Chol, when combined with a cationic lipid RPR209120 and DOPE, formed lipoplexes with greatly reduced normal tissue gene transfer and efficient in vivo tumor gene transfer.

LPDI-type lipoplexes (lipopolyplex) consist of a ternary complex of cationic liposomes, DNA-condensing polycation, and plasmid DNA. In a report by Reddy et al. [21], polyplexes prepared from protamine were mixed with cationic liposomes containing folate-PEG<sub>2000</sub>-DSPE as a targeting ligand and DOPE as a helper lipid. This vector showed superior clinical application of therapeutic genes.
transfection activity in FR-positive M109 murine lung carcinoma cells as well as in ascitic cells derived from L1210A murine lymphocytic leukemia cells. LPDII-type lipoplexes (lipopolyplex) consist of a ternary complex of anionic liposomes, DNA-condensing polycation, and plasmid DNA. Lee et al. [16] reported a formulation of LPDII-type vector, in which DNA was first attached to PLL and then mixed with pH-sensitive anionic liposomes composed of DOPE/CHEMS/folate-PEG₃₄₀-DOPE. PH-sensitive liposomes are fusogenic at acidic pH and thus can be used to facilitate the endosomal disruption and subsequent release of plasmids in the cytoplasm. Shi et al. [36] reported efficient gene delivery using an LPDII vector that incorporated PEI as a DNA-condensing agent and a cationic/anionic lipid pair, composed of dimethyldioctadecylammonium bromide (DDAB)/CHEMS/polyoxyethylene sorbitan monoolate (Tween80)/folate-PEG₃₄₀-DSPE.

3.3 Nanoparticles
Nanoplexes are composed of charged complexes of plasmid DNA and cationic nanoparticles. Here the definition of a lipid-based nanoparticle is a formula containing no bilayers like liposomes. Dauty et al. [37] reported that an FR-targeting cationic nanoparticle incorporating folate-PEG₃₄₀-DPPE and a cationic dithiol-detergent (dimerized tetradecyl-ornithinylcysteine, (C₁₄Corn)₂) showed efficient FR-dependent cellular uptake and transfection. In our laboratory, folate-linked nanoparticles, NPII, NPII-1F, NPII-2F and NPII-3F, consisting of 1 mg/ml DC-Chol as a cationic lipid, 5 mol% Tween 80, and 0, 1, 2 and 3 mol% folate-PEG₃₄₀-DSPE, respectively, were prepared by a modified ethanol injection method [38, 39]. Cholesterol derivatives are usually unable to form stable bilayers unless used in combination with DOPE or some other neutral lipid. Therefore, these particles may be nanoparticles. Folate-linked nanoparticles (NPII) are illustrated in Fig. 2. The average size of each nanoparticle was about 100-200 nm (Table II). The ζ-potential of NPII, NPII-1F and NPII-2F was about 53, 44 and 39 mV, respectively, decreasing as the amount of folate-PEG₃₄₀-DSPE added increased, except for NPII-3F [39]. Here the definition of a lipid-based nanoparticle is a formula containing no bilayers like liposomes.

4. Gene Transfection
The preferential expression of a gene in tumor cells contributes to the safety and efficacy of gene therapy. For FR-targeted gene transfection, the concentrations of folic acid and linker in vectors are important.

4.1 Folate Concentration
For drug delivery, folate-targeting liposomes contained 0.1-0.5 mol% folate-PEG₃₄₀-DSPE or folate-PEG₃₄₀-DSPE for targeting, and about 4 mol% PEG₃₄₀-DSPE for PEG-coating were used [8-13]. For gene de-
livery, FR-targeting liposomes and nanoparticles contained 0.03-5 mol% folate-PEG-lipid. Reddy et al. [21] reported that the cationic liposome formulated with less than 0.03% of folate-PEGDSPE showed the greatest cell association. The folate moieties located at the distal end of the PEG spacers would likely not interact with each other at concentrations lower than 0.03%. Bruckheimer et al. [35] reported that 2 mol% of folate-PEGDSPE conjugate increased the cellular association with tumor cells and transfection potency. Dauty et al. [37] reported that an FR-targeting cationic nanoparticle incorporating 2 mol% folate-PEGDSPE and a cationic diethanolamine (dimerized tetradecyl-ornithinyl-cysteine, (C14Corn)₂) showed efficient FR-dependent cellular uptake and transfection. Cationic liposomes with 5 mol% folate-PEG2-Chol showed high gene transfer activity into a FR-positive cell line, M109 [20]. Xu et al. [34] reported specific in vivo gene delivery to tumors with a liposome containing about 5 mol% folate-DOPE.

4.2 Linker

PEG of Mr 2,000 to 5,000 has been used as a linker (Table I). A certain distance between the folate moiety and the lipid particles is needed for FR-targeting. This is believed to be due to the need for folate to enter the binding pocket of FR on the cell surface. Lee et al. [40] were the first to describe the dependence of folate-liposome-targeting on the distance between the folate and liposome, and reported that a PEG₂₀₀₀ linker was necessary for the targeting. Leamon et al. [17] optimized the targeting activity of the liposomes by modifying the length of the PEG-linker, and found that PEGs as small as Mr 1,000 could function as effective linkers. Ward et al. [29] reported that a folate-linked PEG₂₀₀₀-polymer-modified PLL/DNA complex did not lead to a significant increase in in vitro transgene expression. A PEG spacer > Mr 1,000 might be essential for FR-targeting.

4.3 Nanoplex and Transfection Activity in vitro

Folate-linked nanoparticles (NPI), consisting of 1 mg/ml DC-Chol as a cationic lipid, 5 mol% conventional Tween 80 (about 50% pure), and 0-1 mol% folate-PEG₂₀₀₀-DSPE (f-PEG₂₀₀₀-DSPE) or folate-PEG₂₀₀₀-DSPE (f-PEG₂₀₀₀-DSPE), respectively, were prepared by a modified ethanol injection method [38, 39]. All nanoparticles showed about 100-200 nm in size and about 50 mV in ζ-potential. Nanoplexes are composed of charged complexes of plasmid DNA and cationic nanoparticles. The size of nanoplex of NPI with 0.3 mol% f-PEG₂₀₀₀-DSPE at a charge ratio (+/-) of cationic nanoparticle to DNA (3/1) in the presence of 50% serum increased up to 940 nm. The nanoplex of NPI with 1 mol% f-PEG₂₀₀₀-DSPE did not increase greatly in size in serum-containing medium, but showed lower transfection activity than that with 1 mol% f-PEG₂₀₀₀-DSPE [38].

The folate-linked nanoparticles (NPII) were composed with the same composition as NPI with 1-3 mol% f-PEG₂₀₀₀-DSPE except substituting Tween 80 (purity 99%) for conventional Tween 80. The average size of each NPII was about 100-200 nm [39] (Table II). Three mol% of f-PEG₂₀₀₀-DSPE may not be incorporated in NPII because a reduction in the ζ-potential of NPII with the addition of f-PEG₂₀₀₀-DSPE was not observed (about 55 mV) [39]. The concentration of f-PEG₂₀₀₀-DSPE in the mixture of ethanol and water to prepare NPII-2F containing 2 mol% f-PEG₂₀₀₀-DSPE and NPII-3F containing 3 mol% f-PEG₂₀₀₀-DSPE was about 40 and 60 μM, respectively. f-PEG₂₀₀₀-DSPE below the critical micelle concentration (CMC) might lead to an efficient incorporation into the particles. However, f-PEG₂₀₀₀-DSPE above the CMC may help to stabilize micellized f-PEG₂₀₀₀-DSPE and to inefficient insertion into the particle. The CMC of f-PEG₂₀₀₀-DSPE may be between 40 - 60 μM and might affect the incorporation of f-PEG₂₀₀₀-DSPE in NPII-3F with 3 mol% f-PEG₂₀₀₀-DSPE.

In the presence of 10% and 50% serum, NPII-2F with 2 mol% f-PEG₂₀₀₀-DSPE (NPII-F) formed injectable-sized nanoplexes (Table II), which showed the highest transfection efficiency among NPII with 0-3 mol% f-PEG₂₀₀₀-DSPE, at the optimal charge ratio (+/-) of (3/1) in nasopharyngeal cancer KB cells, being comparable to Tfx20, a commercially available DNA transfection reagent (Fig. 3) [39].

4.4 Selectivity of Folate-linked Nanoparticle

NPII-F showed greater transfection efficiency in human prostate cancer LNCaP cells, human prostate adenocarcinoma PC-3 cells, and human cervix carcinoma Hela cells, than in KB cells, in the comparison with Tfx20 (Fig. 4A). There were three FR isoforms, α, β and γ, each with a distinctive tissue distribution. FR-α mRNA was expressed strongly in KB and Hela cells, but not expressed in LNCaP or PC-3 cells (Fig. 4B). FR-β and -γ mRNAs were not detected in any of the cell lines using the RT-PCR method. Reduced folate carrier (RFC), a carrier-mediated folate transporter, was weakly expressed in all cell lines examined (Fig. 4B). The cellular uptake of NPII-
Fig. 3. Comparison of transfection efficiency in KB cells with luciferase expression between NPII nanoparticles and Tf x20. The NPII nanoplex and the lipoplex were prepared by mixing 2 μg of plasmid DNA with the luciferase gene under the control of the cytomegalovirus (CMV) promoter, with nanoparticles and Tf x20, respectively. The luciferase assay was carried out 24 h after the incubation of nanoplexes in medium with 10% serum. Each column represents the mean ± S.D. (n=3). **P<0.01, compared with NPII-F. NPII-F refers to formulation in Table II.

Fig. 4. Comparison of transfection efficiency between NPII-F and Tf x20 in the NPII-F nanoplexes and Tf x20 lipoplex delivered with the luciferase plasmid into various cell lines (A). Each column represents the mean ± S.D. (n=3). FR-α, RFC and β-actin mRNA expression was detected in various cell lines by RT-PCR (B).

F in Hela and KB cells was mediated via FR-α, following the induction of transfection activity. The selectivity of NPII-F to carry genes into KB cells was validated using FITC-oligooxynucleotide (FITC-ODN) from the result of a competitive experiment in the presence of folic acid by flow cytometry [39].

In LNCaP and PC-3 cells, FR mRNAs were not often observed. In the human prostate, a high-affinity folate binding protein was characterized [41] and folic acid binds to the membrane fraction that cross-reacts with the anti-prostate-specific membrane antigen (PSMA) antibody. PSMA is a transmembrane protein with a pattern of overexpression restricted to malignant human prostate tissue and LNCaP cells [42]. The physiological role of PSMA in prostate cancer remains unknown, but PSMA shows hydrolase enzymatic activity with a folate substrate [42] and is internalized via an endocytic mechanism [43]. If PSMA functions as a receptor mediating the internalization of a putative ligand similar to folic acid, this suggests that the folate-linked nanoparticle binds to PSMA and is then taken up via an endocytic mechanism by LNCaP cells as we reported [38].

In PC-3 cells, our study using RT-PCR confirmed the presence of RFC mRNA, but found no FR or PSMA mRNA [39]. A FITC-labeled folate-BSA conjugate was taken up by PC-3 cells and the cellular association was significantly decreased in the presence of 1 mM folic acid [39]. Xu et al. [34] also reported that a folate-cationic liposome system could mediate gene therapy with p53 antisense DNA in prostate cancer (DU145 cells). We found that NPII-F is a useful vector for transfection in prostate androgen-dependent and independent cancer cells as well as KB cells.

Nanoparticles composed of cholesteryl-3 β-carboxyamidoethylene-N-hydroxylamine (OH-Chol) instead of DC-Chol (NPIII) could incorporate up to 5 mol% f-PEG2000-DSPE (Fig. 2). For FR-targeted vectors, NPIII-1F (NPIII-F), -2F and -5F consisted of NPIII with 1, 2 and 5 mol% f-PEG2000-DSPE, respectively. The average size of each nanoparticle was 100-200 nm (Fig. 5A) [44]. When the NPIII nanoparticles were mixed with DNA at a charge ratio (+/-) of 3/1 in water, the size of each NPIII nanoplex increased from 250 to 300 nm (Fig. 5B) [44]. When the amount of f-PEG2000-DSPE in NPIII was increased, the association of plasmid DNA with nanoparticles was inhibited significantly (Fig. 6) and a reduction in luciferase activity was observed [44]. A large amount of f-PEG2000-DSPE in nanoparticles might prevent enough DNA being carried into the cells, and/or reduce the cellular association with the nanoplex as reported in PEG-lipid [45]. The NPIII-F based on OH-Chol exhibited...
activity was observed [44]. A large amount of f-PE GDSPE in nanoparticles might prevent enough DNA being carried into the cells, and/or reduce the cellular association with the nanoparticle as reported in PEG-lipid [45]. The NPIII-F based on OH-Chol exhibited about 40 times higher transfection efficiency than the NPII-F based on DC-Chol in KB cells [39, 44]. It was reported that the hydroxyl group of OH-Chol reduced the stability of the lipoplex, and enhanced transfection efficiency by facilitating the process by which DNA was liberated from the endosome [46].

It is reported that a negatively charged folic acid forms a charge-mediated complex with positively charged particles through its carboxyl groups and has an effect on the transfection efficiency [47]. We prepared nanoplastes in water at a charge ratio (+/-) of 1:1 to protect to form complex for co-incubation of the positive NPIII with folic acid in the medium, and then incubated them with KB cells. The cellular association of FR-targeting NPIII occurred via FR and the interaction of 1 mol% f-PEGDSPE in the NPIII with FR was higher than that of 2 and 5 mol% ones (Fig. 7). The NPIII-F with 1 mol% f-PEGDSPE nanoplastes at a charge ratio (+/-) of NP-F to DNA of 1/1 exhibited higher selectivity to FR, but lower transfection...
activity relative to that at a charge ratio (+/-) of 3/1 in vitro [44]. From competitive experiment of luciferase assay in presence of free folic acid in the medium, gene expression of the NPIII-F with 1 mol% F-PEG5000–DSPE nanoplexes was reduced [44]. For selectivity and transfection activity in FR targeted gene delivery, it is needed to optimize the charge ratio of folate-linked lipid-based nanoparticles to DNA.

4.5 Transfection Activity in vivo

Both systemic and local administration offers several biological opportunities for gene therapy. The systemic route allows non-invasive access to many target cells that are not accessible otherwise by direct administration. The folate-linked liposomes showed efficient FR-dependent cellular uptake and transfection in vitro. However, the use of a folate ligand as a targeting ligand to deliver DNA has not been successful in in vivo gene therapy [20, 21]. The major limitation of in vivo gene therapy using liposomes is the low transfection efficiency. Several factors have the potential to adversely affect FR-targeted gene transfer in vivo. The first is the presence of endogenous folate in the systemic circulation, which potentially can block FR-binding. Plasma folic acid may interfere with the binding of FR. The human serum folic acid concentration, following FDA-mandated dietary supplementation, is ~42 nM [48]. Earlier reports [40] indicated that serum folic acid at this concentration should not significantly inhibit the binding of FR mediated by liposomes. Our recent study [39] showed that the mice on a folate-deficient diet were actually able to maintain a plasma folate level within the physiologic range of humans. In contrast, the mice on a normal diet maintained a much higher serum concentration of folic acid. Therefore, mice on a folate-deficient diet should be considered relevant to humans with respect to serum folate levels. A second concern is that the size of gene transfer vectors, escaping the vasculature and intratumoral diffusion, could be limiting to targeted delivery. To address this issue, formulation parameters can potentially be optimized to improve the pharmacokinetic properties of the vectors. For example, the vector can be PEGylated to reduce plasma protein binding and RES uptake, which results in an extended systemic circulation time [49]. In addition, the size of the vector should be kept under 300 nm since this is the approximate limit for efficient tumor extravasation. Nonspecific cell uptake by the RES (for example, Kupffer cells in the liver) is expected to be reduced by incorporating PEGylated lipid within the lipid-DNA complex.

When the NPII-F nanoplex was injected via a tail vein, DNA in the blood was still detectable 4 h later by PCR (Fig. 8A and B) [50]. Free DNA is known to have an extremely short T1/2 in blood (0.5–1 h) depending on the DNA dose [51, 52]. The NPII-F seemed to keep the DNA stable in circulation by forming a nanoplex. NPII-F induced greater gene expression in liver and kidney than NP (unpublished data). FR is on the brush borders of proximal renal tubes and provides for the reabsorption of folate. Recently, Paulos et al. discovered that activated liver-derived macrophages (Kupffer cells) in mice do express the FR [53]. The Kupffer cells in the liver and the tubular cells in kidneys of mice may be responsible for capturing NPII-F by FR. Therefore, FR-targeted delivery of therapeutic genes damages normal cells in organs such as the liver and kidney and may subsequently cause death [13]. For cancer gene therapy, using a tumor-specific promoter to regulate expression transcriptionally in target cancer cells has promise. It will be essential to use a strong and tissue-specific promoter region if a therapeutic gene is to be selectively expressed in the cancer cells.

Fig. 8. PCR analysis of plasmid DNA in blood after intravenous administration (A and B). Nanoplexes of 50 mg of plasmid were injected into the tail of male BALB/c mice. At 1, 2, 4 and 24 h after the administration of nanoplexes, blood samples were taken via a tail vein, and then DNA was isolated from the blood. DNA samples were diluted with water to a final concentration of 1 ng/ml for PCR analysis. The plasmid DNA was amplified by PCR using 1 ng of DNA from the blood as a template with luciferase-specific primers. PCR products were analyzed by agarose gel electrophoresis. A, NPII; B, NPII-F; C, control (1, 10, and 100 pg of plasmid as a template for PCR).

Intraperitoneal and intratumoral injections of
Fig. 9. Comparison of transfection efficiency in KB-xenografted tumors in nude mice with luciferase activity of NPII-F and Tfx20. The nanoplex and the lipoplex were prepared by mixing 5 μg of pCMV-Luc with NPII-F and Tfx20, respectively. The luciferase assay was carried out 24 h after the incubation of nanoplexes after intratumoral injection. Each column represents the mean ± S.D. (n=3). **P<0.01, compared with NPII-F.

lipoplex and nanoplex have been applied in mice bearing tumors. Reddy et al. [21] reported that maximum in vivo transfection activity of reporter gene (lucifera-se) occurred with intraperitoneally administered folate-liposome using a disseminated intraperitoneal L1210A tumor model. When the NPII-F nanoplexes of the luciferase plasmid were injected directly into the nasopharyngeal tumor, KB, xenografts, NPII-F showed about 100-fold more luciferase activity than Tfx20, suggesting that the NPII-F nanoplex remained small enough to migrate into the timorous tissue (Fig. 9) [39].

5. Conclusions

We showed that folate-linked lipid-based nanoparticles could deliver DNA with high transfection efficiency and selectivity. These findings indicate that folate-linked lipid-based nanoparticles have potential as a clinically effective vector in cancer gene therapy. However, there is generally little correlation between in vitro and in vivo gene transfer efficacies of vector formulations, due to very different parameters. For systemic administration, a balance of long-circulating vectors and FR-targeting vectors will be key to using folate-PEG-lipid. Further efforts aimed at optimizing FR-targeting vector formulations for systemic administration should lead to the clinical evaluation of these vectors for cancer gene therapy.

参考文献