ORIGIAL ARTICLE

Potential use of folate-polyethylene glycol (PEG)-appendend dendrimer (G3) conjugate with α-cyclodextrin as DNA carriers to tumor cells

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We previously reported that polyamidoamine STARBURST dendrimer (generation 3, G3) (dendrimer) conjugate with α-cyclodextrin (α-CyD) having an average degree of substitution of 2.4 of α-CyD (α-CDE) provided remarkable aspects as novel carriers for DNA and small-interfering RNA through the electrostatic interaction and bind to glycosaminoglycans (heparan sulfate, hyaluronic acid and chondroitin sulfate) on cell surface,2-4 and have been shown to be more efficient and safer than either cationic liposomes or other cationic polymers for in vitro gene transfer.5,6 Cyclodextrins (CyDs) are cyclic (α,1,4)-linked oligosaccharides of α-D-glucopyranose containing a hydrophobic central cavity and hydrophilic outer surface, and they are known to be able to act as host molecules.7-9 CyDs have recently been applied to gene transfer and oligonucleotide delivery.10-13 We previously reported that of various dendrimer conjugates with α-CyD (α-CDE), α-CDE (generation 3, G3) with the degree of substitution (DS) of 2.4 was revealed to have the highest transfection efficiency in vitro and in vivo with low cytotoxicity.14-16 Moreover, we previously reported the potential use of α-CDEs bearing galactose (Gal-α-CDE), mannose (Man-α-CDE) or lactose (Lac-α-CDE) with the various DS values of these sugar moieties as gene delivery carriers.17-20 The targeted gene delivery using bioconjugates is exploring to increase the efficiency of drug delivery to specific tissues as well as to decrease the minimum effective dose of the drug as well as its side effects.21 Strategies to develop tumor-cell-specific bioconjugates are multimodal, but all attempts to selectively deliver therapeutics to cells use nano- and submicron-scale carriers such as dendrimers, liposomes, polymers, emulsions or viruses including active and/or passive targeting moieties.22 Folic acid (FA) has been shown to be one of the most promising ligands for targeting a range of human carcinomas, including breast, ovary, endometrium, kidney, lung, head and neck, brain and myeloid cancers, which are known to express folate receptors (FR).23,24 Moreover, FR is a relatively small molecule (MW 441 Da) that consequently has only limited effects on the dimensions of the carrier system, high stability, compatibility with both organic and aqueous solvent, low cost, non-immunogenic character and the ability to

INTRODUCTION

Polyamidoamine STARBURST dendrimers (dendrimers) are biocompatible, non-immunogenic and water soluble, and possess terminal modifyable amine functional groups for bearing various targeting or guest molecules.1 Unlike classical polymers, dendrimers have a high degree of molecular uniformity, narrow molecular weight (MW) distribution, specific size and shape characteristics, and a highly functionalized terminal surface.2 Dendrimers can form complexes with nucleic acid drugs such as plasmid DNA (pDNA), short-hairpin RNA and small-interfering RNA through the electrostatic interaction and bind to glycosaminoglycans (heparan sulfate, hyaluronic acid and chondroitin sulfate) on cell surface,3,4 and have been shown to be more efficient and safer than either cationic liposomes or other cationic polymers for in vitro gene transfer.5,6

Cyclodextrins (CyDs) are cyclic (α,1,4)-linked oligosaccharides of α-D-glucopyranose containing a hydrophobic central cavity and hydrophilic outer surface, and they are known to be able to act as host molecules.7-9 CyDs have recently been applied to gene transfer and oligonucleotide delivery.10-13 We previously reported that of various dendrimer conjugates with α-CyD (α-CDE), α-CDE (generation 3, G3) with the degree of substitution (DS) of 2.4 was revealed to have the highest transfection efficiency in vitro and in vivo with low cytotoxicity.14-16 Moreover, we previously reported the potential use of α-CDEs bearing galactose (Gal-α-CDE), mannose (Man-α-CDE) or lactose (Lac-α-CDE) with the various DS values of these sugar moieties as gene delivery carriers.17-20 The targeted gene delivery using bioconjugates is exploring to increase the efficiency of drug delivery to specific tissues as well as to decrease the minimum effective dose of the drug as well as its side effects.21 Strategies to develop tumor-cell-specific bioconjugates are multimodal, but all attempts to selectively deliver therapeutics to cells use nano- and submicron-scale carriers such as dendrimers, liposomes, polymers, emulsions or viruses including active and/or passive targeting moieties.22 Folic acid (FA) has been shown to be one of the most promising ligands for targeting a range of human carcinomas, including breast, ovary, endometrium, kidney, lung, head and neck, brain and myeloid cancers, which are known to express folate receptors (FR).23,24 Moreover, FR is a relatively small molecule (MW 441 Da) that consequently has only limited effects on the dimensions of the carrier system, high stability, compatibility with both organic and aqueous solvent, low cost, non-immunogenic character and the ability to
conjugate with a wide variety of molecules, so it has attracted wide attention as a targeting agent. So far, some papers regarding folate-appended dendrimers have been published. For example, Konda et al. reported the novel folate dendrimer magnetic resonance imaging contrast agents to the high affinity FR expressed in ovarian tumor xenografts. Shukla et al. demonstrated the FR-targeted boronated dendrimers as potential agents for neutron capture therapy. In addition, Singh et al. reported that folate-PEG-dendrimer conjugate was significantly safe and effective in tumor targeting for 5-fluorouracil, compared with a non-PEGylated formulation.

In the subsequent discussion, therefore, we prepared folate-appended α-CDEs (Fol-α-CDE) and folate-PEG-appended α-CDEs (Fol-PαC) with various degrees of substitution of folate (DSF) as novel DNA carriers to clarify the effect of PEG and the DSF values, and examined in vitro and in vivo gene transfer activity, cytotoxicity, cellular uptake and the physicochemical properties.

MATERIALS AND METHODS

Materials

α-CyD was donated by Nihon Shokuhin Kako (Tokyo, Japan). Dendrimer (G3, the terminal amino groups = 32, MW = 6909 Da) was purchased from Aldrich Chemical (Tokyo, Japan). Polyethyleneimines (PEIs, linear, 10 kDa and 25 kDa) were obtained from Wako Pure Chemical Industries (Osaka, Japan). p-Toluene sulfonfyl chloride and FA were purchased from Nacalai Tesque (Kyoto, Japan). α-Amino-α-carboxyl polyethylene glycol (PEG, MW = 3290 Da) was purchased from NOF corporation (Tokyo, Japan). Plasmid pRL-CMV-Luc vector encoding Renilla luciferase (pDNA) was obtained from Promega (Tokyo, Japan). The purification of pDNA amplified in bacteria was carried out using Qiagen (Crawley, West Sussex, UK). Endo-Free plasmid maxi kit (<0.1 endotoxin unit μg⁻¹ endotoxin). Other chemicals and solvents were of analytical reagent grade.

Preparation of Fol-α-CDEs (G3) and Fol-PαC (G3)

Figure 1 shows the schemes for the preparation of Fol-α-CDEs (G3) and Fol-PαC (G3). Fol-α-CDEs (G3) were prepared according to the method of Majoros et al. Herein, α-CDE (G3, DS of α-CyD = 2.4) was prepared as previously reported. In brief, FA in dimethylformamide (DMF)/dimethyl sulfoxide containing 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide was mixed at room temperature (r.t.) for 1 h. Then, α-CDE (G3) was added to the solution and incubated at r.t. for 48 h. In addition, Fol-PαCs (G3) were prepared as follows: FA in dimethyl sulfoxide containing N,N-dicyclohexylcarbodiimide and N-hydroxysuccinimide was mixed at r.t. for 30 min. Then, α-amino-α-carboxyl PEG (MW = 3290 Da) and pyridine were added into the solution and incubated at r.t. for 2 h. After removal of intact FA by a precipitation method with water and purification by gel filtration (TOSOH TSKgel HW-40S, Tokyo, Japan), Fol-PEG-COOH was activated with 0.2 M boric acid solution containing 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide and N-hydroxysuccinimide, and then mixed at r.t. for 2 h. Then, α-CDE (G3) was added to the solution and incubated for 48 h. Fol-α-CDEs (G3) and Fol-PαCs (G3) were purified by dialysis and/or gel filtration.

Cell culture

KB cells, a human carcinoma of the nasopharynx, were grown in RPMI-1640 culture medium (FA-free) containing penicillin (1 × 10⁵ MU ml⁻¹) and streptomycin (0.1 mg ml⁻¹) supplemented with 10% fetal calf serum at 37°C in a humidified 5% CO₂ and 95% air atmosphere. A549 cells, a human lung epithelium cell line, were cultured as reported previously.

In vitro gene transfer

In vitro transfection of the pDNA complexes with carriers was performed utilizing the Renilla luciferase gene system in the various cells as reported previously. The Tris-EDTA buffer containing pDNA (2.0 μg) was generally mixed with 200 μl of serum-free medium containing α-CDE (G3), Fol-α-CDEs (G3, DSF 1, 2 or 3) or Fol-PαCs (G3, DSF 2, 5 or 7) mildly
agitated, and then allowed to stand at r.t. for 15 min. These pDNA complexes were prepared at a charge ratio of 50/1 (carrier/pDNA), where the optimal points for the gene transfer activity were in all of the pDNA complexes. The cells (2 × 10⁶ cells per well) were seeded 6 h before transfection, and then washed twice with serum-free medium. Four hundred microliters of culture medium containing the complexes with various carriers supplemented with 10% FCS were added to each well, and then incubated at 37 °C for 24 h. After transfection, the luciferase gene expression was measured as reported previously.15,16

Cytotoxicity

The effects of pDNA complex with α-CDE (G3), Fol-PsCs (G3, DSF 5) or PEIs (10 kDa and 25 kDa) on cell viability were measured as reported previously.15,16 In brief, the transfection was performed as described in the transfection section. After washing twice with Hank’s balanced salt solution (HBSS) (pH 7.4) to remove pDNA and/or various carriers, 270 μl of fresh HBSS and 30 μl of WST-1 reagent (Wako Pure Chemical Industries, Osaka, Japan) were added to the plates and incubated at 37 °C for 30 min. The absorbance of the solution was measured at 450 nm, with referring absorbance at 655 nm, with a Bio-Rad Model 550 microplate reader (Bio-Rad Laboratories, Tokyo, Japan).

Cellular association

Cellular association of the complex was determined by a flow cytometry. Two micrograms of Alexa488-labeled pDNA (Alexa-pDNA) were mixed with α-CDE (G3) or Fol-PsCs (G3, DSF 5) at a charge ratio of 50 (carrier/pDNA). After transfection with the complexes of Alexa-pDNA/carrier for 1 h in KB cells and A549 cells, the cells were washed with phosphate-buffered saline (pH 7.4) twice and immediately scraped with 1 ml of phosphate-buffered saline (pH 7.4). The cells were collected and filtered through nylon mesh. Data were collected for 1 × 10⁵ cells on a FACSCalibur flow cytometer, using CellQuest software (Becton-Dickinson, Mountain View, CA).

Confocal laser scanning microscopy

To observe the cellular association of Alexa-pDNA complex with α-CDE (G3) or Fol-PsCs (G3, DSF 5), KB cells (2 × 10⁶ cells per dish) were incubated with the complexes of Alexa-pDNA/carrier for 3 h. After incubation, the cells were rinsed with phosphate-buffered saline (pH 7.4) twice and immediately scraped with 1 ml of phosphate-buffered saline (pH 7.4). The cells were collected and filtered through nylon mesh. Data were collected for 1 × 10⁵ cells on a FACSCalibur flow cytometer, using a CellQuest software (Becton-Dickinson, Mountain View, CA).

Interaction between pDNA and carriers

Electrophoretic mobility of the pDNA complexes with α-CDE (G3) or Fol-PsCs (G3, DSF 5) was measured using a gel electrophoresis system. Various amounts of α-CDE (G3) or Fol-PsCs (G3, DSF 5) were mixed with 0.2 μg of pDNA in HBSS (pH 7.4). Gel electrophoresis was carried out at r.t. in TBE buffer (45 mM Tris-borate, 1 mM EDTA, pH 8.0) in 1% agarose gel including 0.1 μg/ml of ethidium bromide using the Mupid system (Cosmo Bio, Tokyo, Japan) at 100 V for 40 min. The pDNA bands were visualized using an UV illuminator.

Particle size and ζ-potential

The solution containing α-CDE (G3) or Fol-PsCs (G3, DSF 5) at various charge ratios was added to Tris-HCl buffer (10 mM, pH 7.4) containing 5 μg of pDNA. Then, the solution was incubated for 15 min. The particle size and ζ-potential of the pDNA complex of α-CDE (G3) or Fol-PsCs (G3, DSF 5) were determined by dynamic light scattering using a Zetasizer Nano (Malvern Instruments, Worcestershire, UK). The dynamic light scattering was analyzed by the general-purpose mode. The measurements were carried out at least in triplicates.

DNA condensed assay

pDNA (0.5 μg) and α-CDE (G3) or Fol-PsCs (G3, DSF 5) were added to 350 μl of HBSS (pH 7.4) at the various charge ratios. The solutions were incubated at 25 °C for 15 min, and then 1.75 μl of Picogreen double stranded DNA reagent and 348.25 μl of HBSS were added to the solutions, and incubated at 25 °C for 30 min. The fluorescence (λex = 495 nm, λem = 525 nm) was measured by fluorescence spectrometer Hitachi F-4500 (Tokyo, Japan). Samples containing pDNA (0.5 μg) and Picogreen double stranded DNA reagent (1.75 μl, Life Technologies, Grand Island, NY) were used to calibrate the apparatus to 100% fluorescence against a background of Picogreen double stranded DNA reagent (1.75 μl).

Surface plasmon resonance optical biosensor

The molecular interaction of folate-binding protein (FBP) with Fol-PsCs (G3, DSF 5) was examined using an optical biosensor IAsys based on surface plasmon resonance (Affinity Sensor, Cambridge, UK). The immobilization of FBP on the sensor cuvette was carried out by the reaction of a reactive linker molecule with the cuvette surface. After activation by washing with 8% urea solution containing 10 mM MnCl₂, the interaction curves were measured at the concentrations of carriers (10⁻⁸ - 10⁻⁵ M) in 10 mM acetate buffer (pH 5.3), with 1 mM CaCl₂ and 100 mM NaCl at 25 °C. The association constant was obtained by measuring the change in the refractive index according to the usual procedure. The computational results were derived using a software FAST-fit (Affinity Sensor, Cambridge, UK) equipped in the IAsys.

In vivo gene transfer

Murine colon-26 adenocarcinoma cells (5 × 10⁵ cells per 100 μl) were incubated subcutaneously in male 4-week-old BALB/c mice (ca. 20 g). After 10 days, the tumor-bearing mice were intratumorally injected with 500 μl of a 5% mannitol solution, containing the pDNA complex of α-CDE (G3) or Fol-PsCs (G3, DSF 5) at a charge ratio of 20 (carrier/pDNA) at the amount of 20 μg of pDNA in 30 μl under anesthesia with ether. Twelve hours after intratumoral administration, the mice were killed, and tumor tissues were isolated. The tissues were washed twice with ice-cold saline and were added to 2 ml of the Promega cell lysis buffer containing the Roche protease inhibitor, Complete (Tokyo, Japan). The tissues were homogenized with a Polytron tissue grinder (Ultra-Turrax T25 Basic S1, IKA Works, Wilmington, NC). After three cycles of freezing and thawing, the homogenate was centrifuged for 10 min at 10 000 g (4 °C), and 20 μl of the supernatant was added to 100 μl of the Renilla luciferase assay buffer (Promega). Luminescence was immediately measured for 10 s (Lumat LB9506, EG&G Berthold Japan, Tokyo, Japan). Total protein content of the supernatant was determined by Bio-Rad protein assay kit. The luciferase activity in the tumor cells was determined as described above.

Data analysis

Data are given as the mean ± s.e.m. Statistical significance of mean coefficients for the studies was performed by analysis of variance, followed by Scheffe’s test. P-values for significance were set at 0.05.

RESULTS AND DISCUSSION

Preparation of Fol-α-CDEs and Fol-PsCs

The preparations of Fol-α-CDEs (G3) and Fol-PsCs (G3) were carried out according to the method of Majors et al.29 and Oh et al.30 Fol-α-CDEs (G3) were prepared by direct conjugation of FA to the α-CDE (G3, DS of α-CyD = 2.4) by using 1-ethyl-3(3-dimethylaminopropyl) carbodiimide and N-hydroxysuccinimide in DMF/dimethyl sulfoxide, and then the resulting conjugates were purified by a dialysis (molecular weight cut-off = 3500) and gel filtration (Figure 1). In the case of Fol-PsCs (G3), Fol-PEG-COOH was firstly prepared by using ω- amino-ω-carboxyl PEG (MW ≈ 3290 Da), and the preparation of Fol-PEG-COOH was confirmed by the MALDI-TOF-Mass spectrum (Supplementary Fig. S1). Next, a carboxyl group of the Fol-PEG-COOH was condensed with primary amino group of α-CDE (G3, DS of α-CyD = 2.4), and then the
resulting conjugates were purified by a dialysis (MWCO = 15,000) (Figure 1). The DSF values of Fol-α-CDEs (G3) and Fol-PsCs (G3) were controlled by adjusting the additive amounts of FA and Fol-PEG-COOH, respectively. All of the resulting conjugates included no unreacted FA or Fol-PEG-COOH. In the 1H-nuclear magnetic resonance spectra, Fol-α-CDEs (G3) and Fol-PsCs (G3) gave peaks of each component, such as dendrimer, α-CyD and FA or Fol-PEG (Supplementary Figs. S2, S3). The DSF values of these conjugates were accurately determined by measuring peak areas of the anomeric proton of α-CyD and benzoic proton of FA. The product yields of conjugates were 28% (DSF 1), 61% (DSF 2) and 54% (DSF 3) in the Fol-α-CDEs (G3) system, and 72% (DSF 2), 79% (DSF 5) and 89% (DSF 7) in the Fol-PsCs (G3) system.

In vitro gene transfer activity
To investigate whether Fol-α-CDEs (G3) and Fol-PsCs (G3) have tumor cell-specific and efficient gene transfer activity, and which carrier has the highest gene transfer activity, Renilla luciferase activity after transfection of pDNA complexes at a charge ratio of 50/1 (carrier/pDNA) in various cells was determined (Figures 2 and 3). Here, we confirmed that FR expresses in KB cells, but not in A549 cells, using a reverse transcriptase-PCR method (Supplementary Fig. S4), which is consistent with the results previously reported. In KB cells, α-CDE (G3) showed efficient gene transfer activity, compared with dendrimer (G3), possibly because of the enhancement of endosomal escape of pDNA, as reported by Arima et al. Gene transfer activity of Fol-α-CDE (G3, DSF 2) and Fol-α-CDEs (G3, DSF 1 and 3) was almost comparable to α-CDE (G3) and significantly lower than α-CDE (G3), respectively, in the KB cells (Figure 2). The insufficient gene transfer activity of Fol-α-CDEs (G3) may result from the low receptor-binding activity to FR on the KB cells. Meanwhile, the previous reports demonstrated that an introduction of PEG as a spacer between FA and carriers increases FR-dependent gene transfer activity of liposomes and poly-L-lysine. To improve the FR-binding activity of Fol-α-CDE (G3), therefore, we prepared Fol-PsCs (G3), which have a PEG spacer between dendrimer and FA (Figure 1), and examined FR-selective gene transfer activity of Fol-PsCs (G3, DSF 2, 5 or 7) in KB cells and A549 cells (Figure 3). In KB cells, Fol-PsC (G3, DSF 5) showed a higher gene transfer activity than α-CDE (G3) and Fol-PsCs (G3, DSF 2 and 7) (Figure 3a). Meanwhile, in A549 cells, all of Fol-PsCs (G3, DSF 2, 5 or 7) showed a markedly lower gene transfer activity than α-CDE (G3) (Figure 3b). The activity of Fol-PsC (G3, DSF 5), not Fol-α-CDE (G3, DSF 2), in KB cells was 3.7-fold higher than that in A549 cells, although the activity of α-CDE (G3) in KB cells was decreased 0.9-fold relative to that in A549 cells (Figure 3c). These results suggest that gene transfer activity of Fol-PsC (G3, DSF 5) in KB cells is in an FR-dependent manner. Herein, the reason why Fol-PsC (G3, DSF 5) showed a higher gene transfer activity than α-CDE (G3) and Fol-PsCs (G3, DSF 2 and 7) in KB cells (Figure 3a) may be explained by the findings that Fol-PsC (G3, DSF 5) has a superior binding ability with both FR and pDNA to α-CDE (G3) and Fol-PsCs (G3, DSF 2 and 7). However, the enhancing effect of Fol-PsC (G3, DSF 5) on in vitro gene transfer activity was not outstanding. This insufficient gene transfer activity of Fol-PsC (G3, DSF 5) could be attributed to its still low binding ability with FR and a weak interaction of pDNA with Fol-PsC (G3, DSF 5), as described below.

![Figure 2](image1.png)

**Figure 2.** Transfection efficiencies of plasmid DNA (pDNA) complexes with α-CDE (generation 3, G3) and folate-appended α-CDE (Fol-α-CDE) (G3, degrees of substitution of folate (DSF) 1, 2 or 3) in KB cells. KB cells were cultured in FA-free RPMI 1640 medium. The luciferase activity in cell lysates was determined 24 h after incubation. Culture medium was supplemented with 10% FCS. The charge ratio of carriers/pDNA was 50. Each value represents the mean ± s.e.m. of three to four experiments. *P < 0.05 compared with α-CDE (G3).

![Figure 3](image2.png)

**Figure 3.** Transfection efficiencies of plasmid DNA (pDNA) complexes with α-CDE (generation 3, G3) and folate-polyethylene glycol (PEG)-appended α-CDE (Fol-PsC) (G3, degrees of substitution of folate (DSF) 2, 5 or 7) in KB cells (a) and A549 cells (b), and their comparison (c). KB cells were cultured in FA-free RPMI 1640 medium. The luciferase activity in cell lysates was determined 24 h after incubation. Culture medium was supplemented with 10% FCS. The charge ratio of carriers/pDNA was 50. Each value represents the mean ± s.e.m. of three to four experiments. *P < 0.05 compared with α-CDE (G3).
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Cytotoxicity
Cytotoxicity is often associated with less transfection efficiency of non-viral vectors.\(^{34}\) Therefore, we evaluated cytotoxicity of Fol-P\(\alpha\)C (G3, DSF 5) by the WST-1 method (Figure 4). No cytotoxicity of pDNA complexes with Fol-P\(\alpha\)C (G3, DSF 5) was observed in KB cells (Figure 4a) and in A549 cells (Figure 4b) up to a charge ratio of 100/1 (carrier/pDNA). Meanwhile, cytotoxicity of the pDNA complexes with \(\alpha\)-CDE (G3) increased as the charge ratio increased in both cells, indicating that the pDNA complex with Fol-P\(\alpha\)C (G3, DSF 5) possesses low cytotoxicity rather than that with \(\alpha\)-CDE (G3), which has been reported as a safe carrier.\(^{36}\)

As described above, an introduction of PEG between dendrimer and FA in the Fol-P\(\alpha\)C (G3, DSF 5) molecule can improve its binding activity to FR. In addition, an introduction of a PEG chain to drug carriers is generally acknowledged to improve its half-life in blood and biocompatibility.\(^{35}\) Therefore, an introduction of PEG is useful to improve not only binding activity to FR but also low cytotoxicity. Meanwhile, the pDNA complexes with PEIs (10 and 25 kDa) markedly lowered cell viability, that is, it decreased to about 50% even at a charge ratio of 20/1 (PEI/pDNA) (Figure 4).

These results suggest that Fol-P\(\alpha\)C (G3, DSF 5) has negligible cytotoxicity under the present experimental conditions.

Cellular association and intracellular distribution
As FR, which likely increases local folate concentrations at the plasma membrane to allow its efficient uptake via folate transporters, is a glycosylphosphatidylinositol-linked protein, which is entered by the clathrin-independent carrier/glycophosphatidylinositol-anchored protein- enriched early endosomal compartment pathway,\(^{36}\) we hypothesized that pDNA complex with Fol-P\(\alpha\)C (G3, DSF 5) is entered by clathrin-independent carrier/glycophosphatidylinositol-anchored protein- enriched early endosomal compartment endocytosis pathway. To verify this hypothesis, we examined the cellular association of Alexa-\(\alpha\)-pDNA 1 h after transfection of the complexes of Alexa-\(\alpha\)-pDNA/carriers with or without FA, a competitor for FR, in KB cells by a flow cytometric analysis (Figure 5). The cellular association of Alexa-\(\alpha\)-pDNA in the \(\alpha\)-CDE (G3) system was not changed by the addition of FA (Figure 5a). Meanwhile, the competitive effect of FA on the cellular association of pDNA complex with Fol-P\(\alpha\)C (G3, DSF 5) was observed in KB cells (Figure 5b). These results suggest that cellular association of the pDNA complex with Fol-P\(\alpha\)C (G3, DSF 5) could be mediated by FR on KB cells. However, the competitive effect of FA on the cellular association of pDNA complex with Fol-P\(\alpha\)C (G3, DSF 5) was somewhat weak in KB cells, suggesting that the cellular association of the pDNA complex with \(\alpha\)-CDE (G3) entered cells by clathrin- and raft-mediated endocytosis.\(^{37}\)

Albertazzi et al.\(^{38}\) reported that dendrimers are internalized by both clathrin-dependent endocytosis and macropinocytosis. Actually, the pDNA complex with Fol-P\(\alpha\)C (G3, DSF 5) showed the similar physicochemical properties to the pDNA complex with

![Figure 4](image.png)

Figure 4. Cytotoxicity of plasmid DNA (pDNA) complexes with various carriers in KB cells (a) and A549 cells (b). Cells were incubated with carriers/pDNA complexes for 24 h. Cell viability was assayed by the WST-1 method. The amount of pDNA was 2.0 \(\mu\)g. Culture medium was supplemented with 10% FCS. Each point represents the mean ± s.e.m. of four experiments. *\(P<0.05\) compared with Fol-P\(\alpha\)C (generation 3 (G3), degrees of substitution of folate (DSF) 5). ○, folate-polyethylene glycol (PEG)-appended \(\alpha\)-CDE (Fol-P\(\alpha\)C) (G3, DSF 5); ▲, \(\alpha\)-CDE (G3); ■, polyethyleneimine (PEI) (10 kDa); ●, PEI (25 kDa).

![Figure 5](image.png)

Figure 5. Effects of FA on cellular association of Alexa-\(\alpha\)-plasmid DNA (pDNA) complexes with \(\alpha\)-CDE (generation 3, G3) (a) and folate-polyethylene glycol (PEG)-appended \(\alpha\)-CDE (Fol-P\(\alpha\)C) (G3, degrees of substitution of folate (DSF) 5) (b) into KB cells. The fluorescence intensities of Alexa-\(\alpha\)-pDNA in cells 1 h after incubation were determined by a flow cytometer. The amount of Alexa-\(\alpha\)-pDNA was 2 \(\mu\)g. The concentration of FA was 1 mM. The charge ratio of carriers/pDNA was 50.

Cancer Gene Therapy (2012), 358 - 366 © 2012 Macmillan Publishers Limited
α-CDE (G3) as described below. These lines of evidence make it tempting to speculate that pDNA complex with Fol-PaC (G3, DSF 5) could enter KB cells by both an FR-dependent and -independent endocytosis.

Next, we investigated the intracellular distribution of Alexa-pDNA after transfection of the complexes of Alexa-pDNA/carriers using a confocal laser scanning microscopy (Figure 6). The complex of Alexa-pDNA with dendrimer or α-CDE (G3) gave a moderate fluorescence in KB cells. Meanwhile, more intense fluorescence was observed in the pDNA/Fol-PaC (G3, DSF 5) complex system, suggesting FR-dependent and efficient cellular uptake of pDNA complex with Fol-PaC (G3, DSF 5) in KB cells. This cellular association and intracellular distribution of pDNA complex with Fol-PaC (G3, DSF 5) is likely to be in accordance with its gene transfer activity (Figure 2). These results suggest that FR-mediated cellular uptake of pDNA complex with Fol-PaC (G3, DSF 5) is strongly involved in its cell-specific and efficient gene transfer activity.

Physicochemical properties

To make sure whether pDNA/Fol-PaC (G3, DSF 5) complex has adequate physicochemical properties, we examined the complex formation between pDNA/α-CDE (G3) and pDNA/Fol-PaC (G3, DSF 5), using an agarose electrophoresis. As shown in Figure 7, the intensity of the band derived from pDNA decreased as the charge ratio of pDNA/α-CDE (G3) increased, and at a charge ratio of 1 (carrier/pDNA) the band disappeared. In the case of Fol-PaC (G3, DSF 5), the bands vanished at a charge ratio of 2. These results suggest that Fol-PaC (G3, DSF 5) can form the complex with pDNA at a charge ratio of >2, although the complexation ability of Fol-PaC (G3, DSF 5) with pDNA could be slightly lower than that of α-CDE (G3), owing to a decrease in the number of the positively charged primary amino groups in the molecule and/or steric hindrance by an introduction of Fol-PEG moieties to dendrimer molecule. Meanwhile, Fol-PaC (G3, DSF 5) provided the highest gene transfer activity at a charge ratio of >50 (Supplementary Fig. S5). Herein, we calculated molar ratios (carrier/pDNA) at the charge ratio (carrier/pDNA) of 50, that is, the molar ratios of pDNA complexes with Fol-PaC (G3, DSF 5) and α-CDE (G3) calculated were $1.66 \times 10^4$ and $1.43 \times 10^4$ under the optimal in vitro transfection conditions. Thus, the free extents of Fol-PaC (G3, DSF 5) and α-CDE (G3) must be raised, when the charge ratios increased, because the molar ratios were so high that these carriers may be unable to bind to pDNA. Therefore, the enhancement of gene transfer activity may be ascribed to the additional free Fol-PaC (G3, DSF 5) and α-CDE (G3) at the higher charge ratios, causing an enhanced endosomal escape of pDNA complexes with Fol-PaC (G3, DSF 5) and α-CDE (G3), as previously reported.

Next, we determined the particle sizes and ζ-potential values of the pDNA complexes with α-CDE (G3) and Fol-PaC (G3, DSF 5) (Table 1). The mean diameters of the complexes with these carriers were around 100 nm, and the ζ-potential values were negative values at a charge ratio of 1 (carrier/pDNA). Meanwhile,
the particle sizes and ζ-potential values of the pDNA complexes increased at a charge ratio of 50 in both systems. It should be noted that ζ-potential values of the pDNA complex with Fol-Pc-C (G3, DSF 5) at a charge ratio of 50 were close to neutral, compared with that of α-CDE (G3), because of an introduction of Folate-PEG moieties to dendrimer molecule. Leamon et al. reported that a neutral complex is desirable to incorporate into a ligand-targeted gene transfer carrier because it eliminates the opportunity for the non-specific adsorptive binding to non-target cells. Reddy et al. demonstrated that folate-linked carriers of diameters <150 nm are efficiently bound and internalized by FR-expressing cells. Thus, the low ζ-potential values of the pDNA complex with Fol-Pc-C (G3, DSF 5) at a charge ratio of 50 may be associated with its cell-selective gene transfer activity.

Cationic non-viral vectors such as cationic polymers and cationic lipids are acknowledged to exert pDNA compaction through electrostatic interaction, leading to the enhancing gene transfer activity. To examine the effects of α-CDE (G3) and Fol-Pc-C (G3, DSF 5) on pDNA condensation, fluorescence intensity of Picogreen double stranded DNA reagent was determined (Figure 8). The relative fluorescence intensity decreased to 15% and 20% in the α-CDE (G3) system and the Fol-Pc-C (G3, DSF 5) system at a charge ratio of 100, respectively (Figure 8). These results suggest that the compaction ability of Fol-Pc-C (G3, DSF 5) to pDNA was lower than that of α-CDE (G3). Gabrielson et al. reported that the release of pDNA from complexes with cationic polymers such as PEI in cellular nucleus is crucial for a higher gene transfer activity. However, these results indicate that Fol-Pc-C (G3, DSF 5) had a strong binding affinity with FBP, compared with α-CDE (G3).

In vivo gene transfer

We examined in vivo gene transfer activity of pDNA complexes with Fol-Pc-C (G3, DSF 5) in mice bearing Colon-26 tumor cells. Figure 9 shows gene transfer activity 12 h after intratumoral administration of the solution containing the pDNA/α-CDE (G3) complex or pDNA/Fol-Pc-C (G3, DSF 5) complex at a charge ratio of 25 (carrier/pDNA) to mice bearing tumor cells. In vivo gene transfer activity of Fol-Pc-C (G3, DSF 5) in the tumor cells was higher than that of α-CDE (G3). However, there was not statistically significant difference between the both carriers. Thus, the in vivo gene transfer activity of Fol-Pc-C (G3, DSF 5) may be still low. In our preliminary study, luciferase activity in tumor tissues 24 h after intravenous administration of pDNA complex with Fol-Pc-C (G3, DSF 5) at a dose of 20 µg pDNA per mice and a charge ratio of 20 (carrier/pDNA) was actually found to be extremely low (data not shown).
and in vitro adequate physicochemical properties. These findings may provide gene transfer carrier because of its FR-mediated gene delivery, the potentially used as an FR-overexpressing cancer cell-selective

**CONCLUSION**

The present results suggest that Fol-PzC (G3, DSF 5) could be potentially used as an FR-overexpressing cancer cell-selective gene transfer carrier because of its FR-mediated gene delivery, the extremely low cytotoxicity, endosomal-escaping ability and adequate physicochemical properties. These findings may provide useful information for design and evaluation of FR-overexpressing cancer cell-selective gene transfer carrier, using cationic polymers in vitro and in vivo.

**CONFLICT OF INTEREST**

The authors declare no conflict of interest.

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Supplementary Information accompanies the paper on Cancer Gene Therapy website (http://www.nature.com/cgt)