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Nanoparticles Electrostatically Coated with Folic Acid for Effective Gene Therapy

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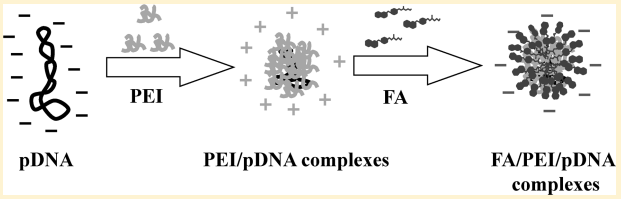
ABSTRACT: We developed a novel vector, electrostatically coated poly(ethylenimine) (PEI)/pDNA complexes with folic acid (FA). Without covalent binding, the FA molecules could coat the PEI/pDNA complexes, and stable anionic nanoparticles were formed at a charge ratio greater than 60. The addition of FA markedly decreased the cytotoxicity of the cationic PEI/pDNA complexes to the melanoma cell line, B16-F10 cells, which regularly expressed FA-specific receptor (FR). Furthermore, the anionic FA60/PEI/pDNA complexes showed high transgene efficiency via the FR-mediated pathway in B16-F10 cells. The FA60/PEI/pDNA complexes did not show agglutination with erythrocytes. After the intravenous injection of FA60/PEI/pDNA complexes into mice, a higher transgene efficiency than PEI/pDNA complexes was observed in the liver, kidney, spleen, and lung with FR. The gene expressions of FA60/PEI/pDNA complexes were significantly inhibited by preadministration of FA. Thus, the FA60/PEI/pDNA complexes were useful for effective gene therapy.



KEYWORDS: gene delivery, folic acid, poly(ethylenimine), pDNA, electrostatic interaction

1. INTRODUCTION

In the field of gene delivery, many nonviral vectors have emerged because they have advantages with their low immunogenicity, the absence of endogenous virus recombination, low production cost, and reproducibility.[1] Among nonviral vectors, poly(ethylenimine) (PEI) is reported to form cationic complexes with pDNA and show high gene expression in in vitro and in vivo experiments through specific mechanisms such as binding to the cell surface, being taken up by the endocytotic pathway, and release of pDNA from endosomes via the so-called "proton sponge mechanism".[2−6] On the other hand, those cationic complexes caused nonspecific gene expressions, high cytotoxicity, and aggregation with blood components because of their cationic surface charges.[7−11]

Therefore, a novel gene delivery vector with high transgene efficiency and low toxicity is necessary. A promising approach to improve efficiency and toxicity is the development of cell-specific gene delivery systems. For cell-specific gene delivery, receptor-mediated endocytosis systems possessed by various cell types would be useful, and a number of gene delivery vectors have been developed.[12−14] Folic acid (FA) is an essential vitamin with anionic charges composed of a pteridine ring, paraminobenzoic acid, and glutamic acid and is often used as a ligand for FA-specific receptor (FR)-mediated drug delivery systems.[15−17] We hypothesized that FA is able to coat the PEI/pDNA complexes electrostatically without covalent binding because of its anionic character, and the coated complexes will be taken up by cells via the FR-mediated pathway. This pharmaceutical modification would have several benefits, such as easy manufacturing, a simple application to various types of cationic gene delivery vectors, improving targeting efficiency, and decreased toxicity by neutralizing cationic charges.

We discovered that FA itself was able to modify PEI/pDNA complexes electrostatically. In the preliminary experiment, the complexes with FA showed little cytotoxicity and agglutination with erythrocytes by neutralizing the surface charge and had high transgene efficiency via the receptor-mediated endocytotic pathway. Therefore, in this experiment, we investigated a novel gene delivery vector, electrostatically coated PEI/pDNA complexes with FA, for its in vitro and in vivo utility.

2. MATERIALS AND METHODS

2.1. Chemicals. PEI (branched form, average molecular weight 25 000) and rhodamine B isothiocyanate were purchased from Aldrich Chemical Co. (Milwaukee, WI, USA). FA was obtained from Sigma (St. Louis, MO, USA). Heparin sodium salt was purchased from Tokyo Chemical Industry Co. Ltd. (Tokyo, Japan). Fetal bovine serum (FBS) was purchased from Biological Industries Ltd. (Kibbutz Beit Haemek, Israel). RPMI 1640, FA-free RPMI 1640, antibiotics (penicillin 100 U/mL and streptomycin 100 μg/mL), and other culture reagents were obtained from GIBCO BRL (Grand Island, NY, USA). The 2-(4-iodophenyl)-3-(4-nitrophenyl)-2*H*-tetrazolium, monosodium

Received: January 5, 2011
Accepted: May 10, 2011
Revised: May 2, 2011
Published: May 10, 2011

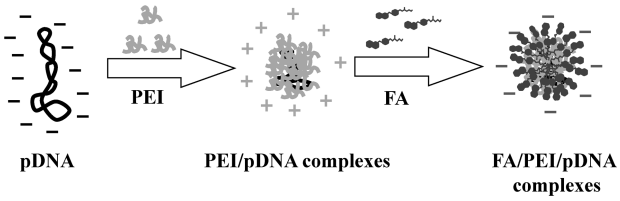


Figure 1. Formation of novel gene delivery vector electrostatically coated by folic acid.

salt (WST-1), and 1-methoxy-5-methylphenazinium methylsulfate (1-methoxy PMS) were obtained from Dojindo Laboratories (Kumamoto, Japan). Rhodamine-PEI (Rh-PEI) was prepared in our laboratory. Briefly, PEI and rhodamine B isothiocyanate were dissolved in dimethylsulfoxide and stirred overnight at room temperature in the dark. Rh-PEI was purified by gel filtration. Almost 1.5% of PEI nitrogen was labeled with rhodamine B. All other chemicals were of the highest purity available.

2.2. Construction of pDNA. pCMV-Luc was constructed by subcloning the *Hind* III/*Xba* I firefly luciferase cDNA fragment from the pGL3-control vector (Promega, Madison, WI, USA) into the polylinker of the pcDNA3 vector (Invitrogen, Carlsbad, CA, USA). Enhanced green fluorescent protein (GFP) encoding pDNA (pEGFP-C1) was purchased from Clontech (Palo Alto, CA, USA). The pDNA was amplified using an EndoFree Plasmid Giga Kit (QIAGEN GmbH, Hilden, Germany). pDNA was dissolved in 5% dextrose solution to 1 mg/mL and stored at −80 °C until analysis.

2.3. Preparation of Complexes. An appropriate amount of stock PEI solution (pH 7.4) was mixed with a diluted solution of pDNA by pipetting thoroughly to prepare PEI/pDNA complexes and was incubated for 15 min at room temperature. FA was dissolved in phosphate buffer (pH 8.0) to 10 mg/mL. To coat the PEI/pDNA complexes surface with FA, FA solution was mixed with the PEI/pDNA complexes solution by pipetting thoroughly and incubated for a further 15 min at room temperature (Figure 1). In this study, we constructed the complexes at various theoretical charge ratios, such as FA carboxylate/PEI nitrogen/pDNA phosphate = 0:8:1 (PEI/pDNA complexes), 15:8:1 (FA15/PEI/pDNA complexes), 30:8:1 (FA30/PEI/pDNA complexes), 45:8:1 (FA45/PEI/pDNA complexes), 60:8:1 (FA60/PEI/pDNA complexes), 75:8:1 (FA75/PEI/pDNA complexes), and 90:8:1 (FA90/PEI/pDNA complexes).

2.4. Physicochemical Properties of the Complexes. The ζ-potential and particle size of the complexes were measured using a Zetasizer Nano ZS (Malvern Instruments, Ltd., Malvern, UK). The number-fractioned mean diameter is shown.

To determine complex formations, 10 μL aliquots of complex solutions containing 1 μg of pDNA were mixed with 2 μL of loading buffer (30% glycerol and 0.2% bromophenol blue) and loaded onto a 0.8% agarose gel containing 0.5 μg/mL ethidium bromide. For the assessment of complex dissociation, heparin was added to PEI/pDNA complexes at a charge ratio of 90. The theoretical charge ratio of heparin to pDNA was calculated as the molar ratio of heparin sulfate to pDNA phosphate. Electrophoresis (i-Mupid J; Cosmo Bio, Tokyo, Japan) was carried out at 50 V in running buffer solution (40 mM Tris/HCl, 40 mM acetic acid, and 1 mM ethylenediaminetetraacetic acid (EDTA)) for 60 min. Retardation of the pDNA was visualized using a FluorChem Imaging System (Alpha Innotech, CA, USA).

2.5. Cell Culture. The mouse melanoma cell line, B16-F10 cells, was obtained from the Cell Resource Center for Biomedical Research Institute of Development, Aging and Cancer, Tohoku University, Japan. The cells were maintained in RPMI 1640 supplemented with 10% FBS and antibiotics (culture medium) under a humidified atmosphere of 5% CO_2 in air at 37 °C.

2.6. WST-1 Assay. Cytotoxicity tests of various complexes on B16-F10 cells were carried out using a WST-1 commercially available cell proliferation reagent. WST-1 reagent was prepared (5 mM WST-1 and 0.2 mM 1-methoxy PMS in PBS) and filtered through a 0.22 μm filter (Millex-GP; Millipore Co, Bedford, MA, USA) just before the experiments. B16-F10 cells were plated on 96 well plates (Becton-Dickinson, Franklin Lakes, NJ, USA) at a density of 3.0×10^3 cells/well in the culture medium. Each complex containing 1 μg of pDNA in 100 μL of FA free RPMI 1640 (transfection medium) was added to each well and incubated for 2 h. After incubation, the medium was replaced with 100 μL of culture medium and incubated for another 22 h. The medium was replaced with 100 μL of culture medium, and 10 μL of the WST-1 reagent was added to each well. The cells were incubated for an additional 2 h at 37 °C, and absorbance was measured at a wavelength of 450 nm with a reference wavelength of 630 nm, using a microplate reader (Multiskan Spectrum; Thermo Fisher Scientific, Inc., Waltham, MA, USA). The results are shown as a percentage of untreated cells.

2.7. In Vitro Transfection Experiments. The cells were plated on 24 well plates (Becton-Dickinson, Franklin Lakes, NJ, USA) at a density of 1.5×10^4 cells/well and cultivated in 0.5 mL of culture medium. In the transfection experiment, after 24 h preincubation, the medium was replaced with 0.5 mL of transfection medium, and each complex containing 1 μg of pDNA was added to the cells and incubated for 2 h. After transfection, the medium was replaced with culture medium, and cells were cultured for a further 22 h at 37 °C. After 22 h incubation, the cells were washed with PBS and then lysed in 100 μL lysis buffer (pH 7.8 and 0.1 M Tris/HCl buffer containing 0.05% Triton X-100 and 2 mM EDTA). Ten microliters of lysate samples were mixed with 50 μL luciferase assay buffer (Picagene; Toyo Ink, Tokyo, Japan), and the light produced was immediately measured using a luminometer (Lumat LB 9507; EG & G Berthold, Bad Wildbad, Germany). The protein content of the lysate was determined by the Bradford assay using BSA as the standard. Absorbance was measured using a microplate reader at 570 nm. Luciferase activity was indicated as relative light units (RLU) per mg protein.

2.8. Fluorescent Microscopy. To visualize the uptake of the complexes and gene expressions, the cells were transfected by the PEI/pDNA complexes and FA60/PEI/pDNA complexes constructed with pEGFP-C1, Rh-PEI, and FA, as described above. After 22 h incubation, the relative levels of Rh-PEI and GFP expressions were characterized using fluorescent microscopy (200× magnification; ECLIPSE TE 200; Nikon, Tokyo, Japan).

2.9. Inhibition Study. For the inhibition study, the cells were transfected as described above with FA60/PEI/pDNA complexes in transfection medium containing various concentrations of FA. After transfection, the medium was replaced with culture medium, cells were cultured for a further 22 h at 37 °C, and then luciferase activities were determined as described above.

For determination of the endocytotic pathway, after 23 h preincubation, the cells were treated with 0.014 mM chlorpromazine (CPZ) as an inhibitor of clathrin-mediated endocytosis, 0.2 mM genistein as an inhibitor of caveolae-mediated

endocytosis, or 1 mM amiloride as an inhibitor of macropinocytosis for 1 h.[18] After treatment, the FA60/PEI/pDNA complexes were added to the medium containing each inhibitor and incubated for 2 h. After 2 h transfection, the medium was replaced with culture medium, cells were cultured for a further 22 h at 37 °C, and then the luciferase activities were determined as described above.

2.10. Animals. Animal care and experimental procedures were performed in accordance with the Guidelines for Animal Experimentation of Nagasaki University with approval from the Institutional Animal Care and Use Committee. Male ddY mice (5−6 weeks old) were purchased from Japan SLC (Shizuoka, Japan). After shipping, mice were acclimatized to the environment for at least one week before the experiments.

2.11. Agglutination Study. Erythrocytes from mice were washed three times at 4 °C by centrifugation at 5000 rpm (Kubota 3700; Kubota, Tokyo, Japan) for 5 min and then resuspended in PBS. A 2% (v/v) stock suspension was prepared for the agglutination study. The PEI/pDNA complexes and FA60/PEI/pDNA complexes were added to the erythrocytes (complexes: stock suspension = 1:1). The suspensions were incubated for 15 min at room temperature. The 10 μL suspensions were placed on a glass plate, and agglutination was observed by microscopy (400× magnification).

2.12. In Vivo Study. The mice were injected intravenously with PEI/pDNA complexes and FA60/PEI/pDNA complexes containing 40 μg of pDNA at a volume of 300 μL per mouse. At 6 h following injection, the mice were sacrificed, and the liver, kidney, spleen, heart, and lung were dissected. The tissues were homogenized in lysis buffer. The homogenates were centrifuged at 15 000 rpm for 5 min. The supernatants were used for luciferase assays, as described above. Luciferase activity was indicated as RLU per gram of tissue.

FA was known to be eliminated from blood rapidly. So, for the inhibition study, the mice were intravenously administrated with 8 mg of FA which is much higher than the blood concentrations of FA. Five minutes after administration, FA60/PEI/pDNA complexes were injected intravenously into the mice, and luciferase activities of various organs were measured as described above.

2.13. Statistical Analysis. The statistical significance between two groups was identified by the Mann−Whitney U test. Multiple comparisons among groups were made by Dunnett's pairwise multiple comparison *t* test.

3. RESULTS

3.1. Physicochemical Property of the Complexes. The ζ-potential and particle size of various complexes are shown in Table 1. The PEI/pDNA complexes had a 43.0 ± 0.3 mV ζ-potential and 37.0 ± 7.7 nm particle size. The addition of FA decreased the ζ-potential of PEI/pDNA complexes concentration-dependently, and it reached a plateau at FA60/PEI/pDNA complexes. FA/PEI/pDNA complexes with a charge ratio lower than 30:8:1 showed aggregation and particle size could not be measured by Zetasizer Nano ZS; however, stable anionic particles were formed on the nanoscale at a charge ratio greater than 60:8:1.

Complex formations were examined by a gel retardation assay (Figure 2). Naked pDNA was detected as a band on agarose gel. On the other hand, in the lanes of PEI/pDNA complexes and FA/PEI/pDNA complexes, no band was detected. Furthermore, in the lane of PEI/pDNA complexes with heparin, one band was detected clearly.

Table 1. ζ-Potential and Particle Size of the Complexes[a]

complexes	ζ-potential (mV)	size (nm)
PEI/pDNA	43.0 ± 0.3	37.0 ± 7.7
FA15/PEI/pDNA	−0.5 ± 0.5	n.d.
FA30/PEI/pDNA	−8.3 ± 0.1	n.d.
FA45/PEI/pDNA	−17.7 ± 0.1	549.4 ± 113.7
FA60/PEI/pDNA	−30.4 ± 1.2	262.7 ± 18.9
FA75/PEI/pDNA	−30.0 ± 0.6	194.9 ± 9.0
FA90/PEI/pDNA	−32.7 ± 0.9	166.0 ± 22.8

[a] Data are the mean ± standard error (SE) of three experiments. n.d.: not detectable.

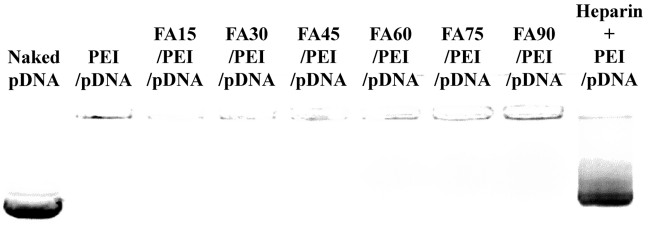


Figure 2. Effect of folic acid on electrophoretic migration of pDNA through agarose gel. Each complex was loaded onto agarose gel, and electrophoresis was carried out. Retardation of pDNA was visualized using ethidium bromide.

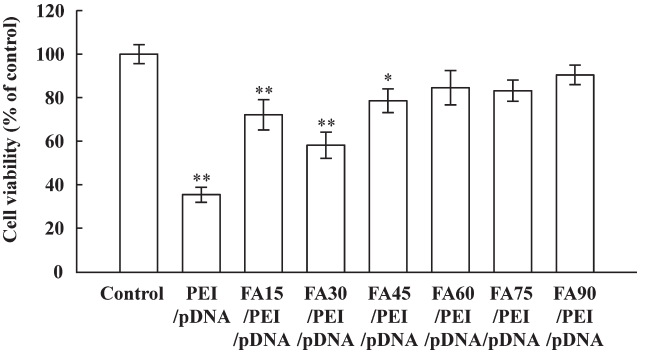


Figure 3. Cytotoxicity tests of various complexes on B16-F10 cells. Cell viabilities of cells treated with various complexes were measured by the WST-1 assay. Cells were incubated with various complexes for 2 h, and the cell viabilities were measured at 22 h after treatment. Data are the percentage to untreated cells. Each bar is the mean ± SE of eight experiments. *: $P < 0.05$, **: $P < 0.01$ vs control.

3.2. Cytotoxicities. For the evaluation of cytotoxicity, each complex was added to B16-F10 cells, and cell viability was determined by WST-1 assay (Figure 3). The PEI/pDNA complexes showed significantly lower cell viability than the control ($P < 0.01$). On the other hand, addition of FA decreased the cytotoxicity of PEI/pDNA complexes, and FA/PEI/pDNA complexes with a charge ratio greater than 60 did not substantially affect the cell viability of B16-F10 cells.

3.3. In Vitro Transfection Experiments. To determine the transgene efficiencies of the complexes, FR-positive B16-F10 cells were transfected with various complexes, and luciferase activities were evaluated (Figure 4). The PEI/pDNA complexes

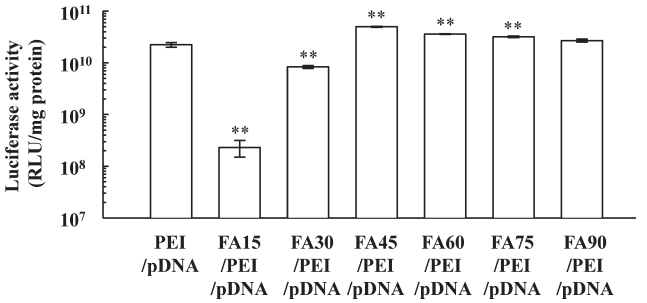


Figure 4. Transgene efficiencies of various complexes. B16-F10 cells were transfected with complexes containing pCMV-Luc. After 22 h transfection, luciferase activity was evaluated. Each bar is the mean ± SE of three experiments. **: $P < 0.01$ vs PEI/pDNA complex.

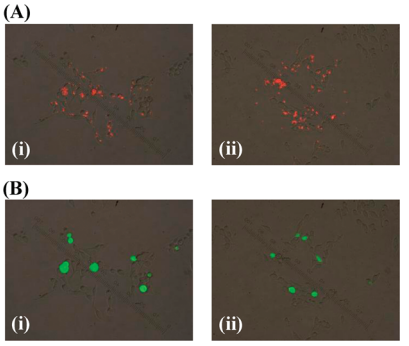


Figure 5. Fluorescent microscopy image of B16-F10 cells transfected with FA60/PEI/pDNA complexes. Cells were transfected with complexes containing pEGFP-C1 and Rh-PEI. After 24 h transfection, the uptake of Rh-PEI (A) and the expression of GFP (B) were monitored (200× magnification). (i) PEI/pDNA complexes; (ii) FA60/PEI/pDNA complexes.

had high transgene efficiency (2.24×10^{10} RLU/mg protein), whereas the FA15/PEI/pDNA complexes and FA30/PEI/pDNA complexes had a significantly lower transgene efficiency than PEI/pDNA complexes ($P < 0.01$). As the FA increased, however, the transgene efficiency of FA/PEI/pDNA complexes increased, and FA/PEI/pDNA complexes with charge ratios 45:8:1, 60:8:1, and 75:8:1 showed significantly higher transgene efficiency than PEI/pDNA complexes ($P < 0.01$).

On the basis of the results of physicochemical properties, the WST-1 assay, and in vitro transfection experiments, we performed further studies on the properties of the FA60/PEI/pDNA complexes.

3.4. Fluorescent Microscopy. To visualize the uptake of the complexes and gene expression, the cells were transfected with PEI/pDNA complexes and FA60/PEI/pDNA complexes containing Rh-PEI and pEGFP-C1 (Figure 5). Both PEI/pDNA complexes and FA60/PEI/pDNA complexes showed red dots of Rh-PEI in most cells, and bright green fluorescence of GFP was also observed.

3.5. Inhibition Study. Inhibition studies were performed with various inhibitory agents. Figure 6A shows the gene expression of the FA60/PEI/pDNA complexes in medium containing various concentrations of FA. FA concentration-dependently inhibited the transgene efficiency of the FA60/PEI/pDNA complexes.

For assessment of the endocytotic pathway, we examined the effects of endocytotic inhibitors on the transgene efficiency of the FA60/PEI/pDNA complexes (Figure 6B). The inhibition of

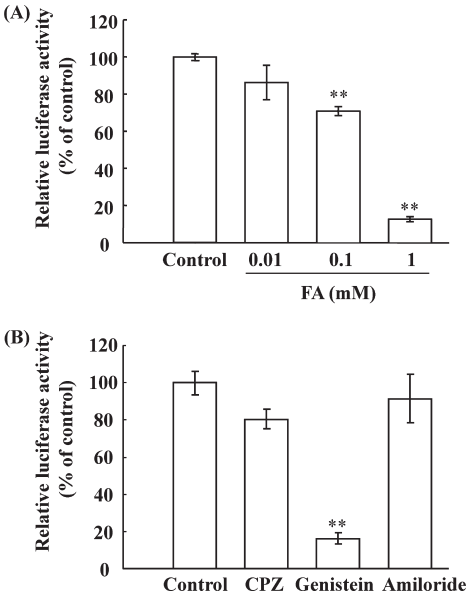


Figure 6. Influence of FA (A) and endocytotic inhibitors (B) on the transgene efficiency of FA60/PEI/pDNA complexes. (A) FA60/PEI/pDNA complexes were transfected in medium containing various concentrations of folic acid. (B) FA60/PEI/pDNA complexes were transfected in medium with various endocytotic inhibitors. After 22 h transfection, luciferase activity was evaluated. Each bar is the mean ± SE of three or six experiments. **: $P < 0.01$ vs control.

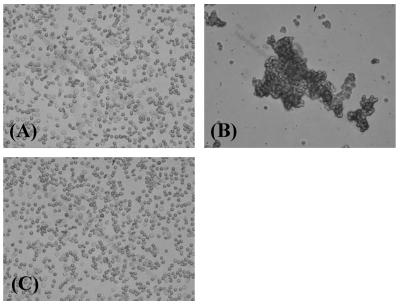


Figure 7. Agglutination of complexes with erythrocytes. Each complex was added to erythrocytes, and agglutination was observed by microscopy (400× magnification). (A) PBS, (B) PEI/pDNA complexes, and (C) FA60/PEI/pDNA complexes.

caveolae-mediated endocytosis with genistein significantly decreased the transgene efficiency of the FA60/PEI/pDNA complexes ($P < 0.01$), which was lower than 20%. On the other hand, chlorpromazine and amiloride had little effect on the transgene efficiency of the FA60/PEI/pDNA complexes.

3.6. Agglutination Study. Before the in vivo study, the agglutination activities of the PEI/pDNA complexes and FA60/PEI/pDNA complexes with erythrocytes were examined (Figure 7). The PEI/pDNA complexes showed severe agglutination, but no agglutination was observed in the FA60/PEI/pDNA complexes.

3.7. In Vivo Study. The PEI/pDNA complexes and FA60/PEI/pDNA complexes were administered to ddY mice, and their transgene efficiencies in various organs were evaluated (Figure 8A). The PEI/pDNA complexes had high gene expressions in the liver, spleen, and lung. On the other hand, FA60/PEI/pDNA complexes showed significantly higher gene expression than PEI/pDNA complexes in the liver, kidney, spleen, and lung

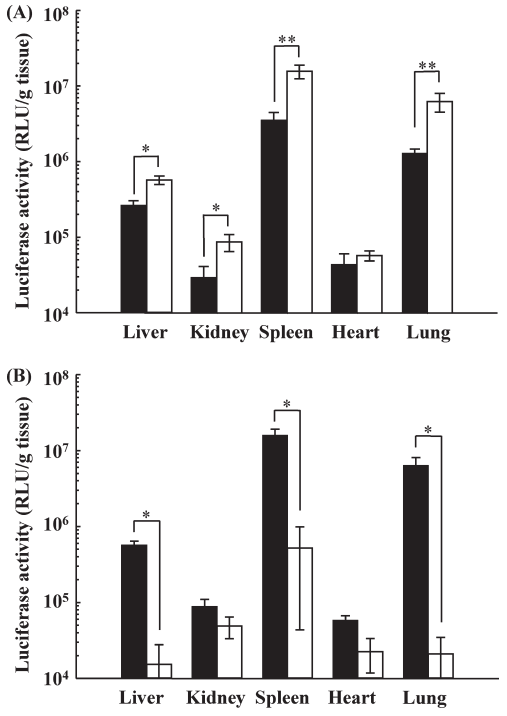


Figure 8. In vivo transgene efficiencies of the complexes (A) and effect of FA on the gene expressions of FA60/PEI/pDNA complexes (B). (A) PEI/pDNA complexes (■) and FA60/PEI/pDNA complexes (□) were administrated intravenously to mice. (B) FA60/PEI/pDNA complexes were injected intravenously to mice without (■) or with (□) preadministration of FA. After 6 h, mice were sacrificed, and the luciferase activities of the liver, kidney, spleen, heart, and lung were determined. Each bar is the mean ± SE of 3−10 experiments. *: $P < 0.05$, **: $P < 0.01$.

($P < 0.05$). At the same time, gene expressions of PEI/pDNA complexes were not increased by preadministrated FA (data not shown).

A large amount of FA was administrated to the mice before FA60/PEI/pDNA complex injections, and its effects on the gene expressions of FA60/PEI/pDNA complexes were evaluated (Figure 8B). The gene expressions of FA60/PEI/pDNA complexes were significantly inhibited by preadministration of FA in the liver, spleen, and lung ($P < 0.05$).

4. DISCUSSION

FA was isolated from spinach in 1941 and was shown to be a growth factor for *Streptococcus lactis R*. FA is composed of a pteridine ring, paraminobenzoic acid, and glutamic acid and was named pteroylglutamic acid.[19] The maintenance of the folate status is important throughout the life cycle, since this water-soluble vitamin is essential for one-carbon metabolism, including the DNA synthetic pathway.[20,21] FA is also used as a ligand in FR-mediated drug delivery systems.[22] We hypothesized that FA, a natural component, is able to coat the PEI/pDNA complexes electrostatically because of its anionic character and the coated complexes will be taken up by cells via the FR-mediated pathway. In fact, the addition of FA to the PEI/pDNA complexes concentration-dependently decreased the ζ-potential of PEI/pDNA complexes and reached a plateau at FA60/PEI/pDNA complexes, as shown in Table 1. Moreover, the addition of FA did not release pDNA from the PEI/pDNA complexes, even if heparin dissociated PEI/pDNA complexes and released pDNA from the complexes, as shown in Figure 2. These results indicated that FA molecules could coat the PEI/pDNA complexes without disrupting the conformation of the complexes, and stable anionic particles were formed on a nanoscale at a charge ratio greater than 60. Furthermore, as a result of ultracentrifugal analysis of the FA60/PEI/pDNA complexes, we confirmed that approximately 2.5 μg of FA interacted with PEI/pDNA complexes containing 1 μg of pDNA.

Therefore, we first examined the cytotoxicity of FA/PEI/pDNA complexes in a mouse melanoma cell line, B16-F10 cells. PEI/pDNA complexes showed high cytotoxicity in B16-F10 cells. In previous reports, cationic PEI/pDNA complexes were reported to bind to cellular membrane proteoglycans, destabilizing the membrane and causing severe cytotoxicity.[7,9,10] On the other hand, the addition of FA improved the cytotoxicity of PEI/pDNA complexes, as shown in Figure 3. The anionic surface, including FA molecules of FA/PEI/pDNA complexes, should prevent this destabilization.

Second, we evaluated the in vitro transgene efficiencies of FA/PEI/pDNA complexes in B16-F10 cells, which have regular FR expressions.[23,24] In cationic gene delivery vectors, strong electrostatic interaction with a negatively charged cellular membrane can contribute to high gene expression.[3,7,10] In fact, PEI/pDNA complexes had extremely high transgene efficiency (2.24×10^{10} RLU/mg protein), as shown in Figure 4. In general, anionic complexes repulse the cellular membrane and cannot be taken up well by cells. Anionic FA/PEI/pDNA complexes with charge ratios of 45, 60, and 75, however, showed significantly higher transgene efficiency than cationic PEI/pDNA complexes ($P < 0.01$). This transgene efficiency was much greater than the commercially available transfection reagent "lipofectin" (1.49×10^8 RLU/mg protein). On the basis of the results of physicochemical properties, the WST-1 assay, and in vitro transfection experiments, we performed further studies on the properties of the FA60/PEI/pDNA complexes.

We also confirmed the uptake and gene expression of PEI/pDNA complexes and FA60/PEI/pDNA complexes by fluorescent microscopy (Figure 5). High uptake of Rh-PEI and gene expressions of GFP was observed in both complexes.

For clarification of the uptake mechanism, FA60/PEI/pDNA complexes were added to B16-F10 cells in medium containing various concentrations of FA. Inhibitory concentrations of FA (0.01−1 mM) were markedly higher than FA concentrations in the FA60/PEI/pDNA complexes (approximately 11.3 μM). FA concentration-dependently inhibited the transgene efficiency of the FA60/PEI/pDNA complexes (Figure 6A). This result suggested that anionic FA60/PEI/pDNA complexes were specifically taken up by the FR-mediated pathway and showed high transgene efficiency, regardless of the anionic surface charge.

It was reported that FA and FA-mediated drug delivery vectors were taken up via the caveolae-mediated endocytosis pathway with the interposition of FR.[25−27] We also performed an inhibition study with various endocytotic inhibitors, such as chlorpromazine for clathrin-mediated endocytosis, genistein for caveolae-mediated endocytosis, and amiloride for macropinocytosis.[18] The transgene efficiency of the FA60/PEI/pDNA complexes was significantly inhibited by genistein ($P < 0.01$), as shown in Figure 6B. These results indicated that the FA60/PEI/pDNA complexes were taken up by the caveolae-mediated endocytotic pathway with the interposition of FR.

In previous reports, cationic gene vectors agglutinated the erythrocytes and caused adverse events, such as embolism and

inflammatory reactions.[28,29] The PEI/pDNA complexes showed markedly high agglutination activities; however, the FA60/PEI/pDNA complexes did not show any agglutination, as shown in Figure 7. Secure and effective gene delivery vectors such as the FA60/PEI/pDNA complexes suggest its suitability for systemic administration. We administrated the complexes intravenously to the mice, and gene expressions of the liver, kidney, spleen, heart, and lung were evaluated (Figure 8A). The PEI/pDNA complexes showed high transgene efficiency in the liver, spleen, and lung. Gene expressions of PEI/pDNA complexes were not affected by preadministrated FA (data not shown). On the other hand, the FA60/PEI/pDNA complexes had significantly higher transgene efficiency than PEI/pDNA complexes in the liver, kidney, spleen, and lung ($P < 0.05$). The FA60/PEI/pDNA complexes might be taken up by the organs via the FR-mediated pathway because these organs were reported as FR-positive.[30] Furthermore, to confirm the interposition of FR, mice were administrated a large amount of FA before FA60/PEI/pDNA complex injections. The physiological FA concentrations were reported to be very low (3 ng/mL).[31] In this experiment, the preadministration of FA significantly inhibited gene expressions of FA60/PEI/pDNA complexes in the liver, spleen, and lung, as shown in Figure 8B ($P < 0.05$). These results strongly indicated that the anionic FA60/PEI/pDNA complexes might circulate in the blood and be taken up by such organs via the FR-mediated pathway, resulting in high gene expressions.

5. CONCLUSIONS

In this experiment, we developed FA/PEI/pDNA complexes with electrostatic interactions. FA could coat PEI/pDNA complexes stably, and anionic nanoparticles were self-assembled. The addition of FA markedly decreased the toxicity of PEI/pDNA complexes with high transgene efficiency in B16-F10 cells. The FA60/PEI/pDNA complexes were taken up by cells via the caveolae-mediated endocytotic pathway with interposition of FR. After intravenous administration to mice, the FA60/PEI/pDNA complexes also showed high transgene efficiency in FR-positive organs, such as the liver, kidney, spleen, and lung, via the FR-mediated pathway. These results indicated that FA60/PEI/pDNA complexes could be useful for effective gene therapy.

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ACKNOWLEDGMENT

This study was supported in part by the Global COE Program, Nagasaki University, Japan and a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology, Japan.

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