European Journal of Pharmaceutics and Biopharmaceutics 70 (2008) 718–725

Contents lists available at ScienceDirect

European Journal of Pharmaceutics and Biopharmaceutics

journal homepage: www.elsevier.com/locate/ejpb

Research paper

Folate-linked lipid-based nanoparticles for synthetic siRNA delivery in KB tumor xenografts

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ARTICLE INFO

Article history:
Received 18 September 2007
Accepted in revised form 20 June 2008
Available online 4 July 2008

Keywords:
Folic acid
Folate receptor
Synthetic siRNA
Cationic nanoparticles
Nasopharyngeal tumor
Her-2

ABSTRACT

RNA interference (RNAi) is a sequence-specific gene-silencing mechanism triggered by synthetic small interfering RNA (siRNA), and is utilized in a wide range of fields including cancer gene therapy by down-regulating a specific target protein. In this study, for tumor-targeted siRNA delivery, we developed a folate-linked nanoparticle (NP-F), and evaluated the potential of NP-F-mediated tumor gene therapy in human nasopharyngeal KB cells, which overexpressed folate receptor (FR). NP-F was composed of cholesteryl-3β-carboxyamidoethylene-*N*-hydroxyethylamine (OH-Chol), Tween 80 and folate-poly(ethylene glycol)-distearoylphosphatidylethanolamine conjugate (f-PEG_{2000}-DSPE), and NP-P was substituted f-PEG_{2000}-DSPE in NP-F PEG_{2000}-DSPE for a non-targeting nanoparticle. The NP-F and siRNA complex (nanoplex) formed at a charge ratio (+/−) of 2/1 in the presence of 5 mM NaCl was injectable size and increased transfection efficiency in the cells. NP-F showed a significantly higher intracellular amount of siRNA and stronger localization of siRNA in the cytoplasm than NP-P. When Her-2 siRNA was transfected into cells by NP-F and NP-P, NP-F significantly inhibited tumor growth, and selectively suppressed Her-2 protein expression more than NP-P. In *in vivo* gene therapy, a NP-F nanoplex of Her-2 siRNA by intratumoral injection significantly inhibited tumor growth of KB xenografts compared with control siRNA, but a NP-P nanoplex did not. These results of the experiments have provided optimal conditions to form folate-linked nanoparticle complexes with siRNA for folate-targeted gene therapy.

1. Introduction

RNA interference (RNAi) is a post-transcriptional mechanism of gene silencing mediated by cleavage of target RNA [1]. This phenomenon occurs by small interfering RNA (siRNA) in the cytoplasm of mammalian cells [2]. In siRNA technology, two delivery mechanisms were considered; direct delivery of synthetic siRNA nucleotide, and introduction of a plasmid DNA (pDNA) encoding a short hairpin construct (shRNA) that will be enzymatically degraded into siRNA. Synthetic siRNAs, which are 21–28 bp small double-stranded RNA, are substrates for the RNA-induced silencing complex. RNAi has potential not only as a tool in biological analysis but also as an evolutional drug for cancer and other diseases. To elevate their gene inhibition effect, many vectors were used to attempt to deliver synthetic siRNA and pDNA encoding as shRNA [3].

Viral vectors like retrovirus- [4,5], adenovirus- [6–8] and lentivirus-based vectors [9–11] have been successful in obtaining efficient and long gene silencing using siRNA. Among them, adenoviral vectors are widely used both *in vitro* and *in vivo*, however; they possess high immunogenicity and proinflammatory effects. Hydrodynamic injections of naked synthetic siRNA have led to the silencing of endogenous genes in various animal tissues, which include the liver, spleen, lung, kidney and pancreas [12–14]. However, the need for a large volume limits this method of delivery *in vivo*. Alternative delivery technologies such as formulated synthetic siRNA using liposomes or atelocollagen as carriers have been reported [3,15,16]. Cationic liposomes are routinely used for gene delivery of pDNA and have recently been applied for synthetic siRNA delivery [17]; therefore, reports of synthetic siRNA delivery strategies are very limited compared with pDNA delivery.

Cancer therapy with synthetic siRNA or pDNA encoding shRNA needs a selective delivery system to the tumor. The receptor for folic acid has been identified as a marker for ovarian carcinomas [18] and has also been found to be frequently overexpressed in a wide range of tumors [18,19]; therefore, folic acid was utilized as tumor-targeting ligand for the delivery of chemotherapeutic agents, pDNA, and antisense ODN to receptor-bearing tumor cells [20–23]. However, there has been no report about folate receptor (FR)-targeting delivery of synthetic siRNA and pDNA encoding shRNA except for cationic polymer folate-PEG-PEI [24,25]. Folate-PEG-PEI of EGFP siRNA exhibited effective suppression of EFGP into EGFP stably expressed human nasopharyngeal tumor KB cells compared with PEI, however; it has not been applied for cancer therapy with antitumor siRNA.

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doi:10.1016/j.ejpb.2008.06.026

Recently, we demonstrated that folate-linked nanoparticles (NP-F) greatly increased transfection efficiency when the NP-F nanoplex of pDNA was formed in the presence of NaCl [26]. In this study, we evaluated the potential of NP-F to deliver synthetic siRNAs to tumor *via* FR and the therapeutic effect of siRNA targeted for Her-2 protein in KB tumor cells and xenografts.

2. Materials and methods

2.1. Materials

Cholesteryl-3β-carboxyamide ethylene-*N*-hydroxyethylamine (OH-Chol) was synthesized as previously described [26]. Poly(ethylene glycol)-distearoylphoshatidylethanolamine (PEG_{2000}-DSPE) and Tween 80 were obtained from NOF Co. (Tokyo, Japan). Folate-polyethyleneglycol-distearoylphoshatidylethanolamine (f-PEG_{2000}-DSPE) (mean MW of PEG: 2 kDa) was synthesized as previously described [27].

2.2. Synthetic siRNA

Duplexed stealth siRNAs targeting against human Her-2 mRNA (Her-2 siRNA), Stealth™ RNAi Negative Control Duplexes Medium GC and Low GC (control-M and control-L siRNAs, respectively) (Invitrogen, Carlsbad, CA, USA) were used for the control of Her-2 siRNA, respectively. The sequences of these siRNAs were as follows: Her-2 sense strand, 5′-AAACGUGUCUGUGUUGUAGGUGACC-3′; Her-2 antisense strand, 5′-GGUCACCUACAACACAGACACGUUU-3′.

EGFP siRNA, random siRNA, and 5′-carboxyfluorescein (FAM)-labeled GL3 siRNA were purchased from Hokkaido System Science (Hokkaido, Japan). Sequences of GL3 were designed for negative control as previously reported [3]. The sequences of EGFP and random siRNAs were as follows: EGFP siRNA sense strand, 5′-ACGGCAUCAAGGUGAACUUCAAGAUAG-3′ and EGFP siRNA antisense strand, 5′-AUCUUGAAGUUCACCUUGAUGCCGUAU-3′; random siRNA sense strand, 5′-CGAUUCGCUAGACCGGCUUCAUUGCAG-3′ and siRNA antisense strand, 5′-GCAAUGAAGCCGGUCUAGCGAAUCGAU-3′.

2.3. Cell culture

KB cells were supplied by the Cell Resource Center for Biomedical Research, Tohoku University (Miyagi, Japan). The cells were grown in folate-deficient RPMI-1640 medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Invitrogen) and kanamycin (100 μg/ml) at 37 °C in a 5% CO_2 humidified atmosphere.

2.4. Preparation of nanoparticles and nanoplexes

Nanoparticles were prepared by a modified ethanol injection method as previously described [26]. Briefly, the formulation of nanoparticle consisted of 1 mg/ml OH-Chol as a cationic lipid, and 5 mol% Tween 80 (NP). NP-F and NP-P were incorporated with 1 mol% f-PEG_{2000}-DSPE or 1 mol% PEG_{2000}-DSPE into the NP formulation. For example, in the case of NP-F, OH-Chol:Tween 80:f-PEG_{2000}-DSPE = 94:5:1 molar ratio (=10:1.3:0.65, weight) was dissolved in about 5 ml of ethanol, then the ethanol was removed with a rotary evaporator until 1–2 ml was left. Next, a constant volume of water was added to the ethanol solution. Nanoparticles formed instantly after further evaporation of the residual ethanol. The concentration of OH-Chol was adjusted to 1 mg/ml in the final nanoparticle suspension with a drop of water. Then the nanoparticle suspension was filtered through 0.45 μm Millex-HA filters (Millipore, Cork, Ireland) to sterilize it. In 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI) (Lambda Probes & Diagnostics, Graz, Austria)-labeled nanoparticles, DiI was incorporated at 0.04 mol% of total lipid.

To prepare nanoplexes of siRNA, siRNA was mixed with aliquots of each nanoparticle in water, 5 or 10 mM NaCl solution, and the mixture was kept at room temperature for 15 min. Particle size distributions and ζ-potentials were measured by ELS-Z2 (Otsuka Electronics Co., Osaka, Japan) at 25 °C after diluting the dispersion to an appropriate volume with water.

2.5. Gel retardation assay

One microgram of pDNA, double-stranded oligodeoxynucleotide (dsODN), and siRNA were mixed with nanoparticles at various charge ratios (+/−) from 1/1 to 4/1 in water. After 15 min incubation of the nanoplexes, they were analyzed by 1.5% agarose gel for pDNA or 12.5% acrylamide gel electrophoresis for siRNA and dsODN in Tris–Borate–EDTA (pH 8.0, TBE) buffer and detected by ethidium bromide staining as previously reported [26].

2.6. Transfection of siRNA nanoplex

KB cells were prepared by plating cells in a 35 mm culture dish 24 h prior to each experiment. The cells at confluences of 50% in the well were transfected with each nanoplex. Nanoplex at a charge ratio (+/−) of 2/1 or 3/1 was formed by mixing each nanoparticle and siRNA in water or in 5 mM NaCl solution and leaving at room temperature for 15 min. For transfection with lipofectamine 2000, lipofectamine 2000 lipoplex was prepared according to the manufacturer's protocol. Briefly, 5 μl of lipofectamine 2000 was diluted with 125 μl Opti-MEM I^R reduced serum medium (Invitrogen) and incubated for 5 min. The mixture was combined with 100 pmol siRNA diluted with 125 μl Opti-MEM I^R reduced serum medium and then leaving at room temperature for 20 min. The nanoplex and lipoplex were diluted with culture medium containing 10% FBS and incubated with the cells at a final concentration of 100 nM siRNA in the medium for 24 or 48 h at 37 °C.

2.7. Antiproliferative activity

KB cells were seeded in 96-well plates 24 h prior to transfection. Cells at confluences of 50% in the well were transfected with each nanoplex of Her-2 siRNA. We used EGFP, control-M and control-L siRNAs as control siRNAs. Nanoplex at a charge ratio (+/−) of 2/1 or 3/1 was formed in 5 mM NaCl solution, transfected into the cells, and incubated for 72 h. Then, cell viability was measured by WST-8 assay (Dojindo Laboratories, Kumamoto, Japan).

2.8. Western blot analysis

Cells were seeded in a 35 mm culture dish 24 h before transfection. Nanoplex of Her-2, EGFP and control-M siRNAs at a charge ratio (+/−) of 2/1 was formed in 5 mM NaCl solution. For transfection with lipofectamine 2000, lipofectamine 2000 lipoplex was prepared according to the manufacturer's protocol. After transfection of the nanoplex, cells were washed in PBS twice. Cell protein extracts were prepared with sampling buffer containing 1% Triton X-100, protease inhibitor cocktail set III (Calbiochem, Darmstadt, Germany) in PBS. After they were centrifuged at 10,000*g* for 10 min, the protein concentration of the supernatant was quantitated with the bicinchoninic acid protein assay reagent (Pierce, Rockford, IL, USA). For the detection of β-actin protein, 5 μg of protein was separated by 12.5% SDS–PAGE, and for the detection of Her-2 protein, 20 μg of protein was separated by 7.5% SDS–PAGE. Then they were transferred to a polyvinylidene difluoride (PVDF) membrane (FluoroTrans® W, PALL Gelman Laboratory, Ann Arbor,

MI, USA). Membranes were blocked in PBS containing 0.1% Tween-20 with 0.5% skimmed milk at 4 °C for 18 h. The blot of Her-2 protein was probed with rabbit anti-human Her-2 antibody (Lab Vision, Fremont, CA, USA). Blots of β-actin protein were probed with rabbit anti-β-actin antibody (Lab Vision). Subsequently, the membranes were incubated with a horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Immunoblots were detected using a SuperSignal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL, USA).

2.9. Flow cytometry

KB cells were prepared by plating cells in a 35 mm culture dish 24 h prior to each experiment. Cells at confluences of 50% in the well were transfected with each nanoplex of FAM-labeled siRNA. Twenty-four hours after transfection, the cells were detached with 0.05% trypsin and centrifuged at 1500*g*. The supernatant was removed and the cells were resuspended with 0.1% BSA and 1 mM EDTA in PBS. The suspended cells were filtrated with 42 μm nylon mesh and introduced into a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA, USA) equipped with a 488 nm argon ion laser using CELLQUEST software (PharMingen, USA). Data for 10,000 fluorescent events were obtained by recording forward scatter (FSC), side scatter (SSC), and green (530/30 nm) fluorescence.

2.10. Confocal microscopy

Nanoplex at a charge ratio (+/−) of 2/1 was formed by mixing each nanoparticle and FAM-labeled GL3 siRNA in water or in 5 mM NaCl solution, and incubating with KB cells for 24 h. The cells were washed twice with PBS and fixed with 4% formaldehyde in PBS for 15 min at room temperature. For staining the nucleus, the fixed cells were washed with PBS and incubated with 0.5 mg/ml of RNase in PBS for 20 min at 37 °C. Subsequently, the cells were washed with PBS and incubated with 2.5 mg/ml propidium iodide (PI) for 5 min at room temperature. Each sample was examined whole in a Radiance 2100 confocal laser-scanning microscope (Bio-Rad, CA, USA) as previously described [27].

2.11. Competition analysis by folic acid or folate receptor antibody

KB cell cultures were prepared by plating cells in a 35 mm culture dish 24 h prior to each experiment. Cells were pre-incubated in the presence or absence of 1 mM folic acid or 20 μg/ml monoclonal antibodies to FR α (Mov18/ZEL, Alexis Biochemicals, CA, USA) for 1 h before transfection. DiI-labeled NP-P or NP-F was mixed with 100 pmol siRNA and then diluted to 100 nM siRNA in 1 ml of folate-deficient-RPMI medium containing 10% FBS. Cells were incubated with the nanoplex for 3 h, and then were analyzed using a FACSCalibur flow cytometer as described in the above section. Data for 10,000 fluorescent events were obtained by recording forward scatter (FSC), side scatter (SSC), and red (585/42 nm) fluorescence.

2.12. Assessment of KB tumor growth

Male BALB/c nu/nu mice (5 weeks of age, CLEA Japan, Inc., Tokyo, Japan) were maintained on a folate-deficient rodent diet (Oriental Yeast Co., Ltd., Tokyo, Japan) on arrival and for the duration of the study. To generate KB tumor xenografts, 1×10^7 cells suspended in 50 μl of PBS were inoculated subcutaneously in the flank region of the mice. The tumor volume was calculated using the formula, tumor volume = $0.5 \times a \times b^2$, where a and b are the larger and smaller diameters, respectively.

When the average volume of the xenograft tumors reached 80 mm^3 (day 0), these mice were divided into two groups: group I, control-M siRNA (10 μg) as a control; group II, Her-2 siRNA (10 μg). Both experimental groups consisted of six tumors. NP-F or NP-P nanoplexes of 10 μg of siRNA per tumor were directly injected into xenografts on days 0, 2 and 4. Tumor volume was measured at days 0, 2, 4, 5, 7, 8, 9 10 and 11. Tumor volume is shown as the mean ± SE. Animal experiments were conducted with ethical approval from our institutional animal care and use committee.

2.13. Statistical analysis

Statistical differences between different groups were analyzed with one-way analysis of variance on ranks with Tukey–Kramer's post-hoc test. A p value of 0.05 or less was considered significant. For the animal study, statistical comparison was performed by Student's t-test.

3. Results and discussion

3.1. Gel retardation assay

Three different cationic nanoparticle formulations were prepared, and all formulations consisted of 1 mg/ml OH-Chol as a cationic lipid and 5 mol% Tween 80 (NP). NP-P consisted of NP with 1 mol% PEG_{2000}-DSPE. For FR-targeted vectors, NP-F consisted of NP with 1 mol% f-PEG_{2000}-DSPE. We previously reported that NP-F modified with 1 mol% f-PEG_{2000}-DSPE could efficiently deliver pDNA into KB cells, which overexpressed FR [26]. First, we evaluated the association of NP-F with siRNA, and compared with that with pDNA or dsODN. The association of siRNA with each nanoparticle was monitored by gel retardation electrophoresis (Fig. 1). The migration pattern of siRNA in the nanoplex changed when the siRNA was mixed with nanoparticles in water at charge ratios (+/−) from 1/1 to 4/1. The migration of siRNA in the nanoplex gradually ceased as the charge ratio increased. Beyond charge ratios (+/−) of 3/1 in NP, and of 4/1 in NP-P and NP-F, no migration was observed. This result suggested that the association of siRNA with the nanoparticles was inhibited by the incorporation of f-PEG_{2000}-DSPE or PEG_{2000}-DSPE into nanoparticles. When the nanoplex complexed with dsODN and pDNA, beyond charge ratios (+/−) of 3/1 in NP, NP-P and NP-F, no migration was observed. siRNA might make a weaker association with nanoparticles than pDNA and ODN.

3.2. Antiproliferative activity

Previously, we demonstrated that transfection activity was significantly increased when the nanoplex was formed in the presence of NaCl solution [26]. It was also reported that lipoplexes formed in 5 mM NaCl solution increased transfection efficiency [28,29]. Furthermore, the nanoplexes formed at charge ratios (+/−) above 4/1 exhibited cytotoxicity by increasing the lipid concentration (data not shown). Therefore, to examine the effect of the charge ratio (+/−) on the selectivity of transfection, we transfected the nanoplex formed in 5 mM NaCl solution at charge ratios (+/−) of 2/1 and 3/1, and compared cell viability among the nanoparticles. First, to confirm the effects of Her-2 siRNA transfected into KB cells by nanoparticles, cell viability was measured by WST-8 assay 72 h after incubation. Her-2 proteins are well-known proteins overexpressed in many tumors and are related to apoptosis and cell growth [30]. Commercially available lipofectamine 2000 was used as a control vector for transfection of siRNA. In the transfection of Her-2 siRNA at a charge ratio (+/−) of 2/1, cell viability was 76%, 109% and 71% in cells transfected by NP, NP-P and NP-F, respectively (Fig. 2A), whereas at a charge ratio (+/−) of 3/1, it was 83%, 88% and 80% by NP, NP-P and NP-F, respectively (Fig. 2B). NP-F and NP-P decreased cell viability at charge ratios (+/−)

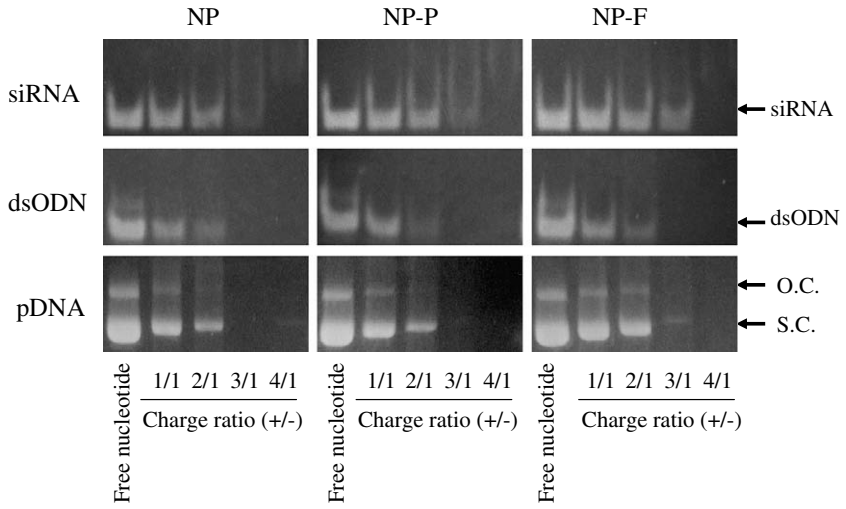

Fig. 1. Association of pDNA, dsODN or siRNA with NP, NP-P and NP-F nanoparticles. Each nanoplex was formed with pDNA, dsODN or siRNA in water at various charge ratios (+/−) from 1/1 to 4/1, and analyzed using agarose gel for pDNA or acrylamide gel electrophoresis for dsODN and siRNA. O.C. indicates open circular pDNA; S.C. indicates supercoiled pDNA.

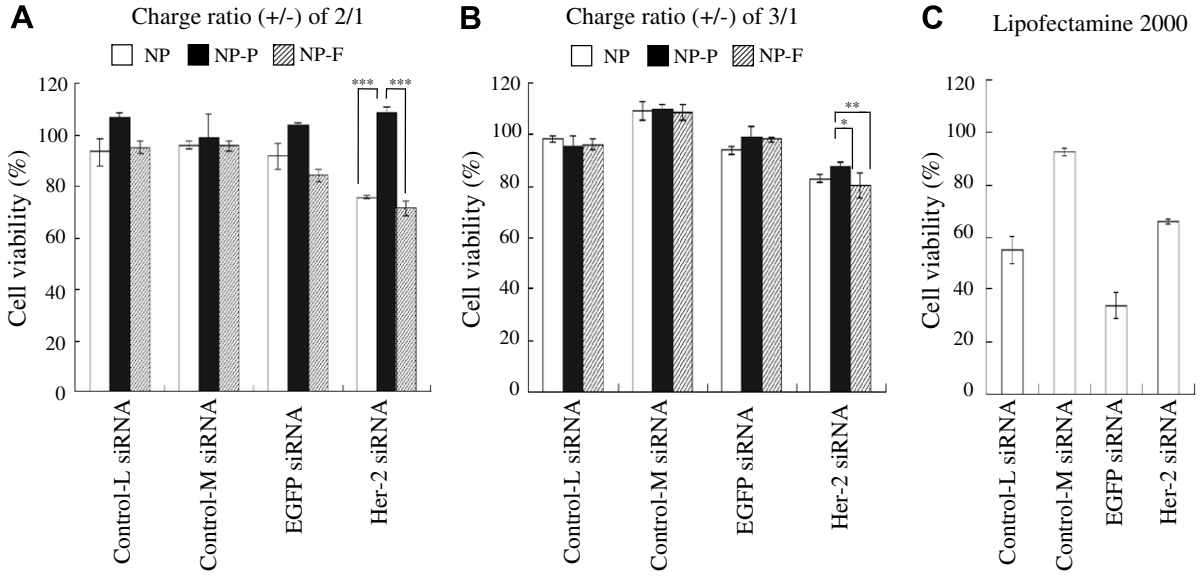

Fig. 2. Antiproliferative activity 72 h after transfection of Her-2 siRNA by nanoparticles into KB cells. Nanoplexes were prepared by mixing nanoparticles with siRNAs in 5 mM NaCl solution at a charge ratio (+/−) of 2/1 (A) or 3/1 (B). Lipofectamine 2000 lipoplex (C) was prepared according to the manufacturer's protocol. Each column represents the mean ± SD ($n = 3$). $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ by one-way analysis of variance on ranks with Tukey–Kramer's post-hoc test.

of 2/1 and 3/1, and more significantly at 2/1. Each nanoplex of control-M, control-L and EGFP siRNA had no apparent effect on cell growth. The transfection of lipofectamine 2000 lipoplex with Her-2 siRNA strongly suppressed cell growth (66%), and those with control-L siRNA and EGFP siRNA also strongly induced the inhibition of cell growth by off-target effect (55% and 34%, respectively) (Fig. 2C). These findings suggested that the NP-F nanoplex of Her-2 siRNA at a charge ratio (+/−) of 2/1 could selectively suppress the growth of tumor cells without an off-targeting effect, but lipofectamine 2000 with Her-2 siRNAs strongly suppressed cell growth with an off-targeting.

3.3. Western blot analysis

To confirm whether the nanoplexes of Her-2 siRNA at a charge ratio (+/−) of 2/1 suppressed Her-2 expression, we assessed the expression of Her-2 protein in KB cells 72 h after transfection of nanoplexes of Her-2 siRNA by Western blotting. NP and NP-F nanoplexes inhibited the expression of Her-2, while NP-P nanoplex was insufficient (Fig. 3). All nanoplexes and the lipofectamine 2000 lipoplex of control-M siRNA did not affect the expression of Her-2 protein and β-actin as a control protein. These results showed clearly that NP-F nanoplex down-regulated targeted proteins strongly without off-target effects.

3.4. Size of nanoplex

To apply nanoplex to intratumoral injection, the size of nanoplexes formed in water, 5 and 10 mM NaCl solutions were measured. The size of NP nanoplex formed in water at a charge ratio (+/−) of 3/1 increased, but NP-P and NP-F nanoplex did not, suggesting that PEGlylation of NP might stabilize its structure (Table 1). The sizes of NP, NP-P and NP-F nanoplex formed in 5 mM NaCl solution at a charge ratio (+/−) of 3/1 increased to about 300–

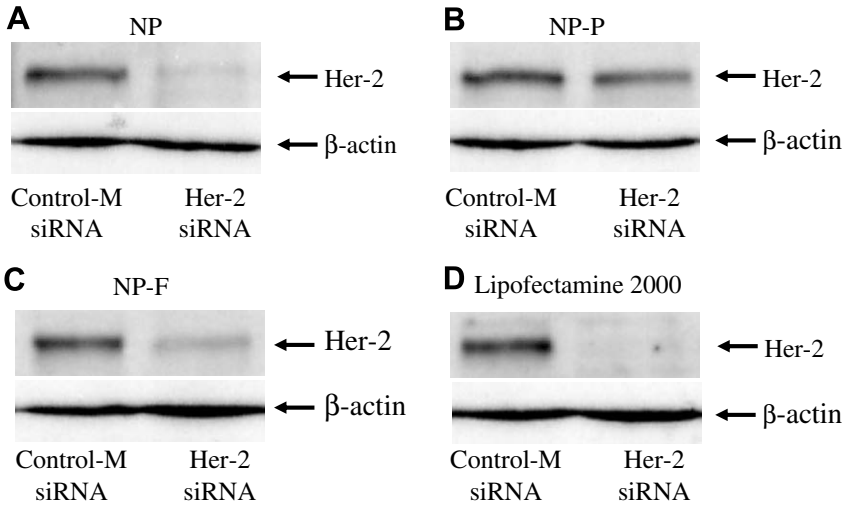

Fig. 3. Down-regulation of Her-2 protein expression by siRNA/NP nanoplexes. KB cells were treated with NP (A), NP-P (B), NP-F (C) nanoplex formed in 5 mM NaCl solution at a charge ratio (+/−) of 2/1 at a final concentration of 100 nM Her-2 or control-M siRNA. Lipofectamine 2000 lipoplex (D) was prepared according to the manufacturer's protocol. Levels of Her-2 and β-actin protein were evaluated by Western blotting 72 h after incubation.

Table 1
Size and ζ-potential of nanoplexes formed in water, 5 and 10 mM NaCl solution at various charge ratios (+/−)

	Charge ratio (+/−)	Size (nm) of nanoplex forming			ζ -potential (mV)
		In water	In 5 mM NaCl	In 10 mM NaCl	In water
NP	1/0	113.4 ± 1.1	95.4 ± 1.5	88.2 ± 6.3	54.3 ± 0.8
	1/1	104.3 ± 0.7	135.3 ± 1.1	596.1 ± 298.7	−29.0 ± 0.8
	2/1	107.2 ± 2.2	177.4 ± 4.6	382.2 ± 11.8	−19.6 ± 0.7
	3/1	322.8 ± 16.5	339.2 ± 0.7	145.2 ± 0.6	13.8 ± 2.5
NP-P	1/0	129.7 ± 0.9	116.5 ± 1.8	102.2 ± 2.0	47.7 ± 0.7
	1/1	125.2 ± 0.6	155.3 ± 0.4	139.4 ± 2.4	−24.4 ± 1.3
	2/1	139.4 ± 2.4	161.1 ± 1.5	339.4 ± 82.3	−21.6 ± 0.8
	3/1	132.8 ± 1.8	429.9 ± 26.0	311.3 ± 1.9	20.7 ± 1.3
NP-F	1/0	146.8 ± 2.3	123.3 ± 0.5	115.9 ± 1.3	49.3 ± 1.0
	1/1	151.7 ± 3.2	180.7 ± 2.4	169.8 ± 1.4	−28.8 ± 2.3
	2/1	144.4 ± 2.2	192.8 ± 2.7	183.1 ± 18.4	−9.4 ± 3.0
	3/1	144.7 ± 1.7	575.1 ± 17.8	482.6 ± 55.5	20.0 ± 1.7

Each value represents the mean ± SD (n = 3).

500 nm, but those in 10 mM NaCl solution increased at charge ratios (+/−) of above 1/1, 2/1 and 3/1, respectively. All nanoplexes remained small when formed in water or 5 mM NaCl at a charge ratio of 2/1, and became slightly larger at a charge ratio (+/−) of 3/1. This may be due to that the ζ-potential of each nanoplex was increased in parallel with the increasing charge ratio (+/−) and became positive at a charge ratio (+/−) of above 3/1 (Table 1). The cationic charge on the surface of the nanoplex may be neutralized by the presence of NaCl, resulting in instability of the nanoplex and facilitating the size increase. From the results of antiproliferative activity and injectable size of nanoplex, we used the nanoplexes formed in 5 mM NaCl at a charge ratio of 2/1 in subsequent experiments.

3.5. Association of FAM-labeled nanoplexes with KB cells

Next, to compare association with KB cells among nanoplexes formed in water or in NaCl solution, nanoplexes with FAM-labeled siRNA were studied by flow cytometry after 24 h incubation. The associations of all nanoplexes formed in 5 mM NaCl solution were found to be about 8∼9-fold higher than those in water, respectively (Fig. 4). In NP-P nanoplex, the surface modification of NP with PEG resulted in reduced non-specific uptake; therefore, NP-P nanoplex exhibited lower cellular association. NP-F nanoplex may restore the interaction between the folate moiety of NP-F and FR of KB cells, exhibiting significantly higher cellular association than NP-P.

The results were consistent with the finding that lipoplexes prepared in 5 mM NaCl solution increased transfection efficiency [28,29], but it is not clear why the nanoplex formed in 5 mM NaCl enhanced cellular association. Usually, nanoplexes are prepared in non-ionic solution such as water or glucose solution because they aggregate in the presence of ionic solutions such as saline, however; the nanoplex formed in water was exposed to ionic solution such as culture medium or blood and subsequently increased in size or aggregate. Controlling the size of the nanoplex after exposure to ionic solution is important for efficient gene transfer. The presence of NaCl during nanoplex formation can regulate the repulsion between cationic nanoplexes [28]. This stabilized effect of nanoplex in ionic solution might increase cellular association.

3.6. Localization of siRNA transfected into KB cells

The intracellular localization of siRNA after transfection is crucial for its successful function as an inductor of the RNA interference process. pDNA must be transferred into the nucleus for the expression of transgenes, but siRNA does not need to enter the cell nucleus since the targets for siRNA applications are in the cytoplasm. To investigate whether siRNA after transfection localized

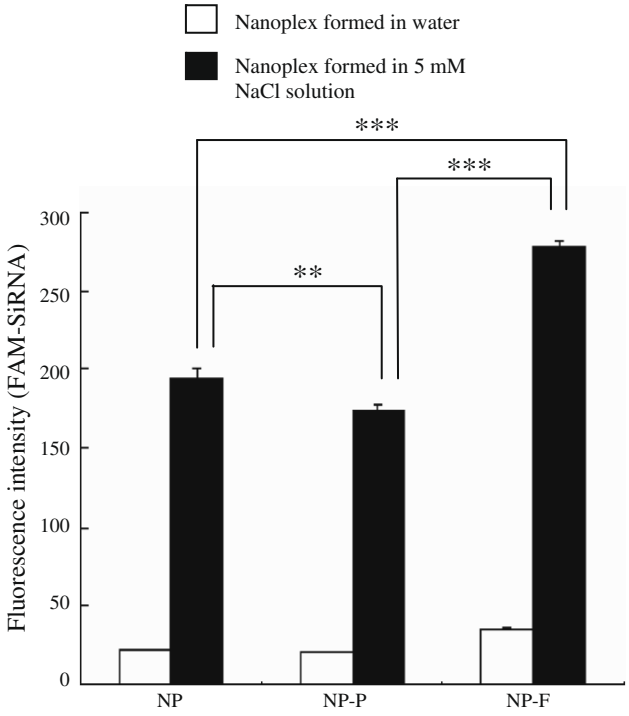

Fig. 4. Uptake of FAM-labeled siRNA by nanoparticles into KB cells 24 h after incubation by flow cytometry. NP, NP-P and NP-F nanoplexes at a charge ratio (+/−) of 2/1 were prepared by mixing FAM-labeled siRNA with each nanoparticle in the presence or absence of 5 mM NaCl solution. KB cells were incubated with these nanoplexes at a final concentration of 100 nM siRNA. Each column represents the mean ± SD ($n = 3$). $^{**}P < 0.01$ and $^{***}P < 0.001$ by one-way analysis of variance on ranks with Tukey–Kramer's post-hoc test.

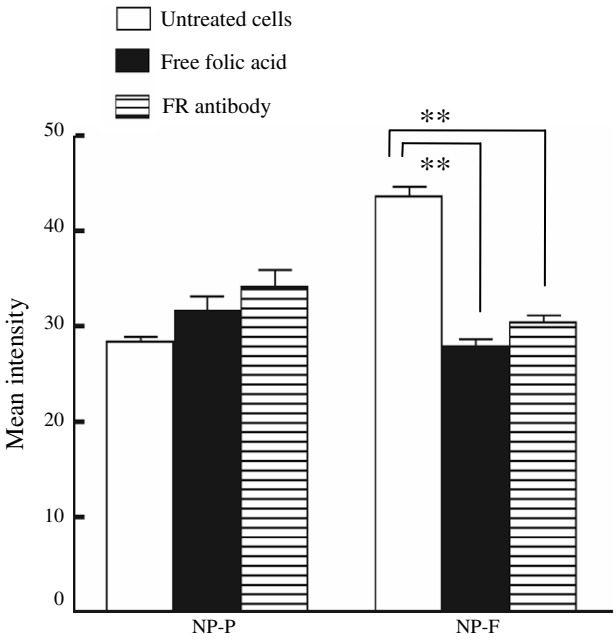

Fig. 6. Competition analysis of NP-F nanoplex with KB cells by flow cytometry. KB cells were incubated for 1 h in the absence or presence of 1 mM free folic acid or 20 μg/ml FR antibody. DiI-labeled NP-P or NP-F was mixed with siRNA at a charge ratio (+/−) of 2/1 in 5 mM NaCl. Nanoplexes were incubated with cells for another 3 h. The results indicate the mean ± SD ($n = 3$).

in cytoplasm where RNAi occurs, we examined the localization of FAM-labeled siRNA in cells 24 h after transfection. When NP and NP-F nanoplex was transfected into cells, FAM-labeled siRNA was

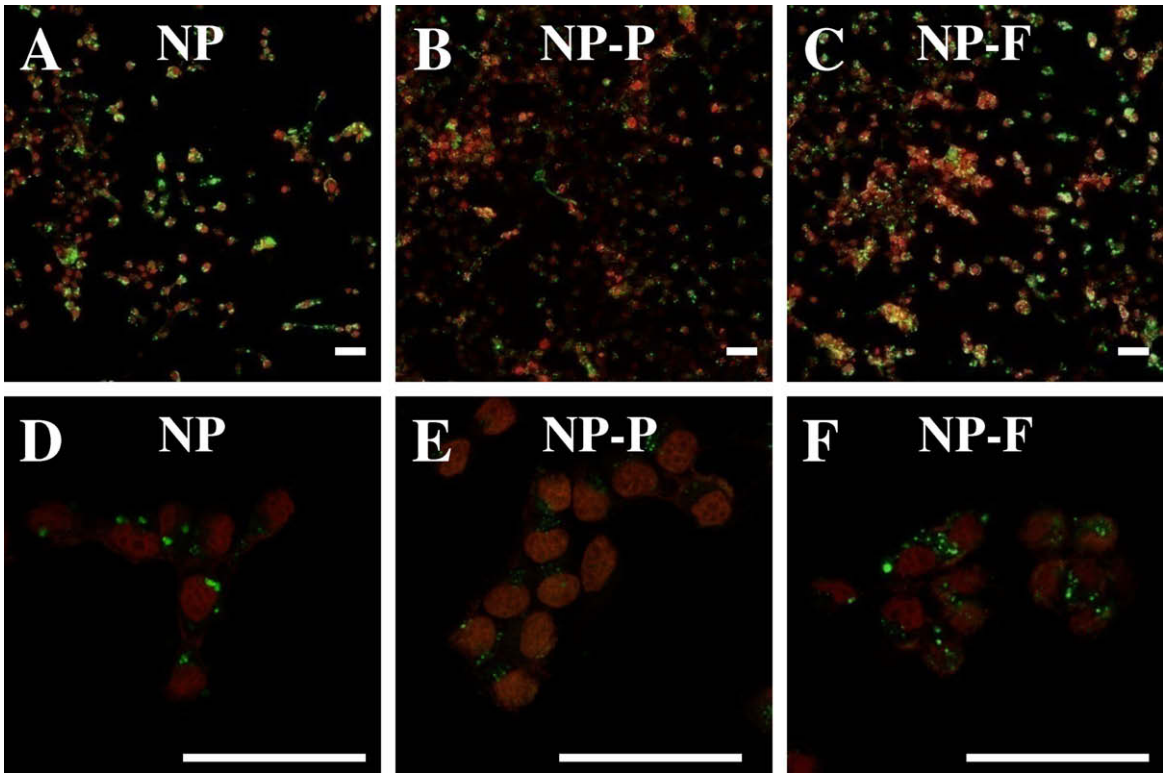

Fig. 5. Intracellular localization of FAM-labeled siRNA 24 h after incubation with KB cells by confocal laser-scanning microscope. FAM-labeled siRNA was complexed with NP, NP-P and NP-F, respectively, at a charge ratio (+/−) of 2/1 in 5 mM NaCl and then diluted to 100 nM siRNA with medium. ×200 (A–C) or ×1200 (D–F) magnification. Nuclei were stained by propidium iodide. Scale bar = 50 μm.

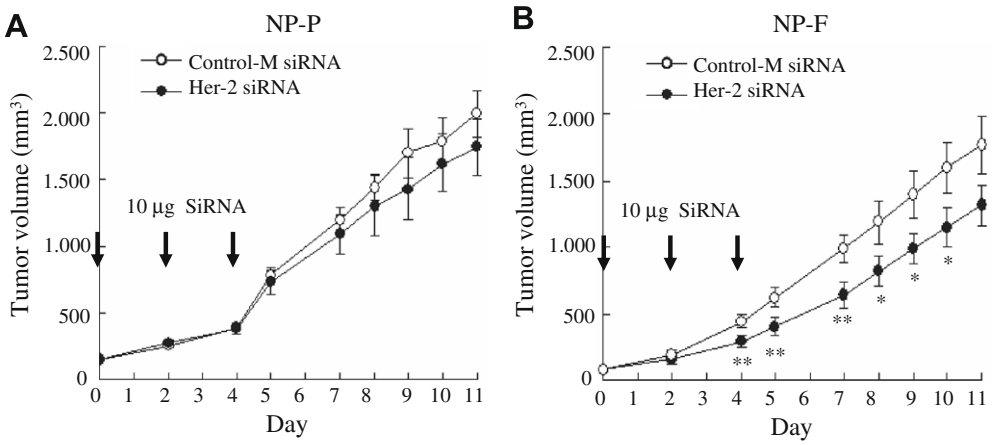

Fig. 7. *In vivo* gene therapy of KB tumor xenografts with Her-2 siRNA in mice. Mice were divided into two groups: group I, control-M siRNA (10 μg) as a control; group II, Her-2 siRNA (10 μg). NP-P (A) or NP-F nanoplexes (B) of siRNAs formed at a charge ratio (+/−) of 2/1 in 5 mM NaCl were injected directly into the tumor three times (days 0, 2 and 4). The results indicate the mean ± SE (n = 4–6). $^{*}P < 0.05$ and $^{**}P < 0.01$, compared with control-M siRNA by Student's t-test.

found in most of the cells (Fig. 5A and C) and in the cellular cytoplasm and nucleus (Fig. 5D and F), however; when NP-P was transfected, signals were hardly detected in the cells (Fig. 5E). This suggested that NP-F could efficiently transfer siRNA into the cells.

3.7. Competition analysis of NP-F nanoplex with KB cells

To further examine the selectivity of NP-F to carry genes into KB cells, we measured the association of a non-exchangeable fluorescent membrane probe, DiI-labeled NP-F complexed with siRNA, by flow cytometry. As shown in Fig. 6, competitive experiments in the presence of folic acid or FR antibody revealed a largely decreased amount of NP-F in the cells compared with NP-P. These results clearly indicated that the association of NP-F nanoplex with KB cells occurred *via* FR.

3.8. In vivo gene therapy in KB tumor xenografts

Finally, we evaluated the antitumor effect by direct injection into KB tumor xenografts with the NP-F nanoplex of Her-2 siRNA. The growth of KB tumors was not inhibited in mice treated with the NP-P nanoplex of Her-2 siRNA compared with control-M siRNA (Fig. 7A). However, the growth was significantly inhibited in mice treated with the NP-F nanoplex of Her-2 siRNA on day 4, 5, 7, 8, 9 and 10 compared with control-M siRNA (Fig. 7B).

Lipofectamine 2000 lipoplexes induced undesired inhibition in control siRNAs by an off-targeting effect in *in vitro* transfection (Fig. 2C), and could not be available for *in vivo* transfection. In contrast, the NP-F nanoplex of Her-2 siRNA could significantly inhibit *in vitro* growth of tumor cells without an off-targeting effect (Fig. 2A), and also suppress *in vivo* growth of tumor xenografts (Fig. 7B). Thus, NP-F is more appropriate for siRNA delivery than lipofectamine 2000 from the aspects of off-targeting effect and *in vivo* application. However, complete regression of tumor was not observed in the xenografts transfected with Her-2 siRNA by the NP-F nanoplex. In *in vitro* study, transfection with Her-2 siRNA could suppress expression of Her-2 protein (Fig. 3) and cell growth, but not lead to complete cell death (Fig. 2A). Her-2 activates cell survival pathways, which represents an advantage for tumor cells as they became resistant to chemotherapy-induced apoptosis [31,32]. Down-regulation of Her-2 expression in tumor cells leads to an activation of apoptosis pathway and enhances cytotoxocity by chemotherapy [33,34]. Therefore, for complete regression of tumor, a combination therapy with Her-2 siRNA and chemotherapy might be needed.

4. Conclusions

In this study, we showed that folate-linked nanoparticles delivered synthetic siRNA with high transfection efficiency and selectivity into nasopharyngeal tumor KB cells by forming a nanoplex in NaCl solution. In *in vitro* experiments, the association of NP-F nanoplex with the cells occurred *via* FR, and NP-F nanoplex of Her-2 siRNA suppressed the cell viability. In *in vivo*, the NP-F nanoplexes with Her-2 siRNA by intratumoral injection significantly suppressed the growth of KB xenografts in mice, compared with NP-P nanoplexes. These findings suggest that folate-linked nanoparticles have potential as a clinically effective vector in nasopharyngeal cancer synthetic siRNA gene therapy.

Acknowledgements

This project was supported in part by a Grant-in Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science, and Technology of Japan, and by the Open Research Center Project.

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