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Cell-penetrating peptide-conjugated lipid nanoparticles for siRNA delivery

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ABSTRACT

Lipid nanoparticles (LNP) modified with cell-penetrating peptides (CPP) were prepared for the delivery of small interfering RNA (siRNA) into cells. Lipid derivatives of CPP derived from protamine were newly synthesized and used to prepare CPP-decorated LNP (CPP-LNP). Encapsulation of siRNA into CPP-LNP improved the stability of the siRNA in serum. Fluorescence-labeled siRNA formulated in CPP-LNP was efficiently internalized into B16F10 murine melanoma cells in a time-dependent manner, although that in LNP without CPP was hardly internalized into these cells. In cells transfected with siRNA in CPP-LNP, most of the siRNA was distributed in the cytoplasm of these cells and did not localize in the lysosomes. Analysis of the endocytotic pathway indicated that CPP-LNP were mainly internalized via macropinocytosis and heparan sulfate-mediated endocytosis. CPP-LNP encapsulating siRNA effectively induced RNA interference-mediated silencing of reporter genes in B16F10 cells expressing luciferase and in HT1080 human fibrosarcoma cells expressing enhanced green fluorescent protein. These data suggest that modification of LNP with the protamine-derived CPP was effective to facilitate internalization of siRNA in the cytoplasm and thereby to enhance gene silencing.

1. Introduction

An indispensable technology to develop RNA interference (RNAi) medicines is perceived to be a drug delivery system, since naked RNA is easily degraded by RNase in the body and is hardly internalized into target cells [1]. In recent years, nanoparticle-mediated delivery of RNAi effectors such as small interfering RNA (siRNA) and microRNA has been widely studied for RNAi therapies [1]. Many studies have demonstrated that protection of these small RNAs from enzymatic degradation can be achieved by formulation of them into nanoparticles [2]. Our non-invasive pharmacokinetic studies using positron emission tomography clearly showed that the plasma half-life and biodistribution of siRNA were improved by incorporating it into nanoparticles [2,3]. For efficient delivery of RNAi effectors to target tissues, we previously developed dicetyl phosphate-tetraethylenepentamine (DCP-TEPA)-based polycation liposomes (TEPA-PCL) [4]. Lipoplexes formed from TEPA-PCL and RNAi effectors showed effective gene silencing both *in vitro* and *in vivo* [3–5]. Systemic delivery of siRNA to tumors was achieved in mice by use of TEPA-PCL modified with polyethyleneglycol and targeting peptides [3,5]. After systemic administration of siRNA formulated in our vectors, specific gene silencing in tumors and suppression of tumor growth were clearly observed [3]. Our recent studies and those of others showed that nanoparticle-mediated delivery of RNAi effectors is a promising approach to establish RNAi therapies [5–7].

In addition to the lipoplex-type vectors as described above, lipid-coated cores encapsulating RNAi effectors have also been demonstrated to be a promising formulation for RNAi therapies. For instance, a multifunctional envelope-type nano device (MEND) [8] and lipid-coated calcium phosphate (LCP) [9] were reported to induce *in vivo* gene silencing mediated by RNAi after systemic administration. In this type of vector, cores carrying siRNA were coated with phospholipids for the delivery of RNAi effectors. In addition, the surface of these vectors was often modified with certain ligands for targeted delivery of RNAi effectors [5,9]. On the other hand, cell-penetrating peptides (CPP) such as Tat peptide

Abbreviations: LNP, lipid nanoparticles; CPP, cell-penetrating peptides; CPP-LNP, CPP-decorated LNP.

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[10], oligoarginine [11], and low molecular weight protamine (LMWP) [12] have also been studied for efficient siRNA delivery. As the result of research, various CPP-decorated nanoparticles such as nanomicelles modified with Tat peptide [13], MEND modified with octaarginine [14], and complexes of LMWP and siRNA [12] have been reported as potential siRNA vectors. CPP-decorated nanoparticles are considered to be one of the promising vectors for RNAi therapies.

In this study, we used Arg-Arg-Arg-Arg-Arg-Arg-Gly-Gly-Arg-Arg-Arg-Arg-Gly (RRRRRRGGRRRRG) peptide derived from protamine as both a CPP and a siRNA carrier. Lipid derivatives of RRRRRRGGRRRRG peptide were newly synthesized and applied to the preparation of CPP-decorated lipid nanoparticles (CPP-LNP) encapsulating siRNA (CPP-LNP-siRNA). To obtain CPP-LNP, we prepared cationic cores carrying siRNA by using protamine or palmitoyl RRRRRRGGRRRRG, coated them with lipids, and modified the thus prepared LNP with DOPE-conjugated RRRRRRGGRRRRG peptide by post-insertion. For the purpose of RNAi therapy, we investigated the potential of our newly developed CPP-LNP as a vector for siRNA delivery.

2. Materials and methods

2.1. Materials

Cholesterol was kindly donated by Nippon Fine Chemical (Takasago, Hyogo, Japan). Dioleoylphosphatidylethanolamine (DOPE) and dimyristoylphosphatidylglycerol (DMPG) were purchased from NOF (Tokyo, Japan). 1,2-Dioleoyl-3-trimethylammonium-propane (DOTAP) was purchased from Avanti Polar Lipids (Alabaster, AL). All siRNAs including 6-fluorescein-6-carboxamido hexanoate (FAM)-conjugated ones were purchased from Hokkaido System Science (Hokkaido, Japan). The nucleotide sequences of siRNA for GFP (siGFP) with a 2-nucleotide overhang (underline) were 5′-GGCUACGUCCAGGAGCGCACC-3′ (sense) and 5′-UGCGCUCCUGGACGUAGCCUU-3′ (antisense); and for enhanced luciferase 2 (siLuc2), 5′-GCUAUGGGCUGAAUACAAATT-3′ (sense) and 5′-UUUGUAUUCAGCCCAUAGCTT-3′ (antisense). In the use of FAM-labeled siRNA (FAM-siRNA), FAM was conjugated to siGFP at the 3′ end of the antisense strand.

2.2. Synthesis of a CPP-DOPE conjugate

DOPE-conjugated RRRRRRGGRRRRG peptide was synthesized as follows: The synthetic peptide RRRRRRGGRRRRG, (40 mg, 0.023 mmol) was dissolved in milliQ water (0.25 mL). To this solution, $(Boc)_2O$ (15.0 mg, 0.069 mmol) dissolved in 1,4-dioxane (0.5 mL) was added along with triethylamine (10 μL). The reaction mixture was stirred for 6 h at room temperature. After solvent removal under reduced pressure, the residue was suspended in $CHCl_3$, and then washed with water. The $CHCl_3$ solution was dried with $MgSO_4$, and then the solvent was removed. The resulting Boc-peptide (100% yield) gave a ninhydrin- and iodine-active single spot on TLC at R_f 0.35 (SiO_2, $CHCl_3$/MeOH/H_2O, 13/6/1). MALDI-TOF-MS for $(C_{71}H_{140}N_{43}O_{16})^+$: calcd, 1852.1; found, 1852.3.

The Boc-peptide and DOPE (25.7 mg, 0.035 mmol) were dissolved in *N*-methylpyrrolidone/CH_2Cl_2. To this solution, BOP (30.5 mg, 0.069 mmol) followed by triethylamine (10 μL) was added; and the mixture was stirred for 6 h. The solvent was removed under reduced pressure at 37 °C. The residue was suspended in MeOH, and then the solution was filtered. After solvent removal, the residue was resuspended in acetonitrile followed by centrifugation (150*g*, 5 min). The resulting pellet was subjected to column chromatography using a Sep-Pak C18 Cartridge (Waters) to purify Boc-peptide-DOPE (46% yield). The product gave ninhydrin- and molybdenum blue-active single spot on TLC at R_f 0 (SiO_2, $CHCl_3$/MeOH/H_2O, 13/6/1). MALDI-TOF-MS for $(C_{112}H_{216}N_{44}O_{23}P)^+$: calculated molecular weight (calcd), 2578.2; found, 2578.9.

Boc-peptide-DOPE (27.3 mg, 0.011 mmol) was dissolved in TFA (1 mL) and stirred for 1.5 h. TFA removal under reduced pressure gave DOPE-peptide conjugate (25.7 mg, 98% yield). MALDI-TOF-MS for $(C_{107}H_{208}N_{44}O_{21}P)^+$: calcd, 2478.0; found, 2478.6.

2.3. Preparation of CPP-decorated LNP

Protamine sulfate (40 μg, Sigma–Aldrich, St Louis, MO) or a palmitoyl derivative of RRRRRRGGRRRRG peptide (22 μg, Operon Biotechnologies, Tokyo, Japan) was incubated with siRNA (1 nmol) in RNase-free water (1 mL, Invitrogen, Rockville, MD) for 20 min at 25 °C to obtain cationic cores. On the other hand, DOPE, cholesterol, and DMPG (9/2/2 for protamine-based cores or 6/5/2 for palmitoyl peptide-based cores as a molar ratio, total lipids: 5 μmol) dissolved in chloroform were evaporated under reduced pressure, and stored *in vacuo* for at least 1 h. LNP encapsulating siRNA (LNP-siRNA) were prepared by hydration of the thin lipid film with 1 mL of RNase-free water containing the cationic cores. LNP-siRNA was purified by using a Sepharose™ 4 Fast Flow column (GE Healthcare, Piscataway, NJ). Absorbance at 260 and 750 nm in each fraction was monitored as an indicator of siRNA and LNP, respectively. RRRRRRGGRRRRG-DOPE (6 mol% to total lipids of LNP) dissolved in RNase-free water was added to LNP-siRNA solution, which was then incubated for 30 min at 40 °C to modify LNP with CPP.

The efficiency of encapsulation of siRNA by LNP was determined by performing a RiboGreen assay. LNP-siRNA were incubated with 1% *n*-octyl-β-D-glucoside (Wako Pure Chemical, Osaka, Japan) and 1% poly-(αβ)-DL-aspartic acid sodium salt (Asp, Sigma–Aldrich) for 30 min to isolate the siRNA. Then, siRNA was incubated with RiboGreen RNA reagent for 5 min. The fluorescence intensities of samples were measured by using a Tecan Infinite M200 microplate reader (Salzburg, Austria) according to the manufacturer's instructions (ex. 490 nm, em. 530 nm).

For the preparation of DOTAP liposomes, DOTAP and cholesterol were dissolved in *t*-butyl alcohol and freeze-dried. DOTAP liposomes were produced by hydration of the lipid mixture with RNase-free water and then sized by extruding them 10 times through a 0.1-μm pore size PVDF membrane filter (Millipore, Bedford, MA, USA).

Particle size and ζ-potential of nanoparticles diluted with RNase-free water were measured by using a Zetasizer Nano ZS and MPT-2, respectively (Malvern, Worcs, UK).

2.4. Stability of siRNA in LNP exposed to serum

LNP-siRNA, cationic cores containing siRNA, or naked siRNA (0.2 nmol siGFP/100 μL) were incubated with 900 μL of fetal bovine serum (FBS; AusGeneX, QLD, Australia) for 24 h at 37 °C. Extraction of siRNA from each sample after the incubation was performed by using Trizol LS (Invitrogen). Intact siRNA was separated by electrophoresis on a 15% acrylamide sequencing gel, stained with ethidium bromide, and visualized by using a LAS 3000 chemiluminescence system (Fujifilm, Tokyo, Japan).

2.5. Cell cultures

B16F10 murine melanoma cells were obtained from the American Type Culture Collection. B16-F10-luc2 Bioware® Ultra Cell Line, a luciferase-expressing cell line stably transfected with the firefly luciferase gene (Luc2), were purchased from Caliper Life Sciences (Hopkinton, MA). These cells were cultured in DME/Ham F12 medium (Wako Pure Chemical) supplemented with 10% FBS,

100 units/mL penicillin (MP Biomedicals, Irvine, CA), and 100 μg/mL streptomycin (MP Biomedicals) in a CO_2 incubator. HT1080 human fibrosarcoma cells constitutively expressing EGFP (EGFP/HT1080 cells) had been previously established [15] and were cultured in DME/Ham F12 medium supplemented with 10% FBS, 100 units/mL penicillin, 100 μg/mL streptomycin, and 100 μg/mL geneticin (Sigma–Aldrich) in a CO_2 incubator.

2.6. Cellular uptake of siRNA

The amounts of siRNA taken into B16F10 cells were determined fluorometrically by using FAM-siRNA. B16F10 cells (4×10^4 cells/well) were seeded onto 24-well plates. After the cells had been cultured overnight, the medium was changed to a fresh one containing 10% FBS but no antibiotics. These cells were incubated for 30 min at 37 °C with a given endocytosis inhibitor, such as 0.8 M sucrose (Wako Pure Chemical), 25 μg/mL filipin complex (Sigma–Aldrich), 12.5 mM amiloride hydrochloride hydrate or 20 units/mL heparin. FAM-siRNA (60 pmol) formulated in CPP-LNP was added to each well, and incubation was carried out for 3, 6, 12 or 24 h at 37 or 4 °C. FAM-siRNA complexed with Lipofectamine™ 2000 (LFA2K, Invitrogen) was also added to each well as a control. The cells were lysed with 1% *n*-octyl-β-D-glucoside containing protease inhibitors (1 mM phenyl methyl sulfonyl fluoride, 2 μg/mL leupeptin, 2 μg/mL aprotinin, and 2 μg/mL pepstatin A). The fluorescence intensities of FAM were measured by using the Tecan Infinite M200 microplate reader (ex. 492 nm, em. 535 nm) and corrected for protein amounts, determined with a BCA Protein Assay Reagent Kit (PIERCE Biotechnology, Rockford, IL), according to the manufacturer's instructions.

2.7. Confocal microscopic study

B16F10 cells (5×10^3 cells/well) were cultured on a Lab-Tek® II Chamber Slide (Nalge Nunc International, Naperville, IL) for 24 h at 37 °C. Then, these cells were incubated with a given endocytosis inhibitor for 30 min at 37 °C. FAM-siRNA (60 pmol) formulated in CPP-LNP was added to each well, and incubation was conducted for 6 h at 37 °C. Late endosomes/lysosomes of the cells were stained with LysoTracker according to the manufacturer's instructions. After having been fixed with 4% paraformaldehyde, the cells were incubated with 10 μg/mL 4′,6-diamidino-2-phenylindole (DAPI, Invitrogen) for 15 min and mounted with Mountant Perma-Fluor (Lab Vision, Fremont, CA). Localization of FAM-siRNA was fluorescently determined under an LSM510 META confocal laser-scanning microscope (Carl Zeiss, Oberkochen, Germany).

2.8. Gene silencing induced by CPP-LNP-siRNA

B16-F10-luc2 cells were seeded onto 96-well luminunc white plates (Nalge Nunc International) at the density of 5×10^3 cells/well and precultured overnight. Before transfection with siRNA, the medium was changed to a fresh one containing 10% FBS but no antibiotics. siLuc2 (24 pmol) formulated in CPP-LNP was added to each well, and incubation then proceeded for 24 h. After the medium had been changed to a fresh one containing 10% FBS and the antibiotics, the cells were cultured for a further 24 h. The luminescence intensity and cell viability were measured by using a ONE-Glo™ Luciferase Assay System (Promega, Madison, WI) and CellTiter-Fluor™ Cell Viability Assay (Promega), respectively, according to the manufacturer's instructions. Measurement of the fluorescence (ex. 400 nm, em. 505 nm) and the luminescence was performed with the Tecan Infinite M200 microplate reader.

EGFP/HT1080 cells were seeded onto 24-well FALCON plates (BD Bioscience, San Jose, CA) at the density of 4×10^4 cells/well and precultured overnight. Before transfection with siRNA, the medium was changed to a fresh one containing 10% FBS but no antibiotics. siGFP (60 pmol) formulated in CPP-LNP was added to each well, and incubation was then carried out for 24 h. After the medium had been changed to a fresh one containing 10% FBS and the antibiotics, the cells were cultured for a further 24 h. The fluorescence intensities of EGFP were measured by using the Tecan Infinite M200 microplate reader (ex. 485 nm, em. 535 nm) and corrected for protein amounts, determined by using the BCA Protein Assay Reagent Kit, according to the manufacturer's instructions. LFA2K was used as a control vector according to the manufacturer's instructions.

2.9. Statistical analysis

Differences between groups were evaluated by analysis of variance (ANOVA) with the Tukey *post-hoc* test.

3. Results

3.1. Encapsulation of siRNA into LNP

The ζ-potential of cationic cores composed of protamine and siRNA was +29.7 mV; on the other hand, that of LNP was

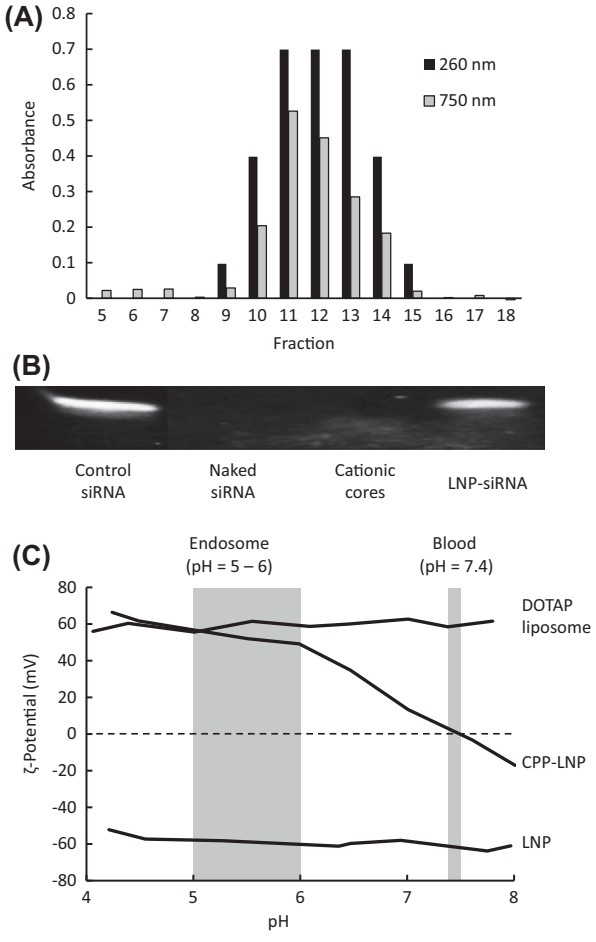

Fig. 1. Preparation and characterization of CPP-LNP-siRNA. (A) LNP were purified using a Sepharose™ 4 Fast Flow column. Closed bars represent absorbance at 260 nm (siRNA fractions); and Gray bars, that at 750 nm (lipid fractions). (B) Stability of siRNA in lipid nanoparticles exposed to serum. LNP, cationic cores, or naked siRNA were incubated in the presence of 90% FBS for 24 h at 37 °C. After the incubation, intact siRNA was extracted by Trizol treatment, separated by electrophoresis in acrylamide gel, and stained with ethidium bromide. (C) ζ-Potential of nanoparticles was measured in water (pH = approx. 4.0–8.0) by using the Zetasizer Nano ZS and MPT-2.

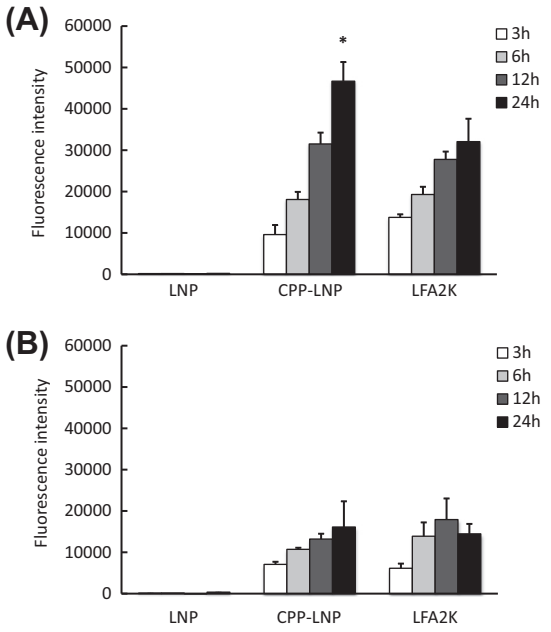

Fig. 2. Association of CPP-LNP-siRNA with B16F10 cells. The amount of FAM-siRNA associated with B16F10 cells was determined after LNP-siRNA or CPP-LNP-siRNA were allowed to interact with these cells for 3, 6, 12 or 24 h at 37 °C (A) or at 4 °C (B). FAM-siRNA complexed with LFA2K was also used as a control. At each time point, the cells were lysed with 1% *n*-octyl-β-D-glucoside containing protease inhibitors. The fluorescence intensities of FAM were measured and corrected for protein amounts. A significant difference from the LFA2K group is indicated ($^{*}p < 0.01$).

−44.5 mV, suggesting that the cores had been successfully coated with outer lipids. Dynamic light scattering measurements showed that the particle size of LNP-siRNA (462 ± 118 nm) was larger than that of the cores (208 ± 16 nm). As shown in Fig. 1A, gel filtration chromatography indicated that fractions containing siRNA (absorbance at 260 nm) were consistent with those containing LNP (absorbance at 750 nm). The presence of siRNA in these fractions was also observed by acrylamide gel electrophoresis (data not shown). The results of the RiboGreen assay showed that the encapsulation efficiency of siRNA into LNP was approximately 60%. Although naked siRNA and siRNA in the cores were completely degraded by incubation in the presence of 90% serum for 24 h, siRNA in LNP was still intact after the incubation (Fig. 1B). Thus, the stability of siRNA in serum was improved by encapsulation of siRNA into LNP. The particle size of LNP after modification with CPP was 435 ± 152 nm, which was similar to that of LNP. Thus, the process of CPP-modification hardly affected the particle size of LNP. The ζ-potential of CPP-LNP was dependent on pH. The ζ-potential of CPP-LNP under acidic conditions was higher than that under neutral conditions, whereas that of LNP and DOTAP liposomes was independent of pH over the range of pH's tested (Fig. 1C).

3.2. Delivery of siRNA into cells by use of CPP-LNP

The potential of CPP-LNP for siRNA delivery was evaluated by performing uptake experiments using FAM-siRNA. As a result, CPP-LNP-siRNA was associated (bound to the cell surface or internalized into the cells) with B16F10 cells in a time-dependent manner, although LNP without CPP hardly interacted with these cells (Fig. 2A). The amount of siRNA associated with B16F10 cells in CPP-LNP was significantly larger than that in LFA2K after a 24-h incubation. Binding of CPP-LNP-siRNA on the surface of B16F10 cells was also determined after incubation at 4 °C. As a result, binding of CPP-LNP-siRNA to B16F10 cells was slightly increased in a time-dependent manner; although the amount of CPP-LNP-siRNA bound to the cells at 4 °C was obviously smaller than the cell-associated amount found at 37 °C (Fig. 2B). Taken together, these data indicate that most of the CPP-LNP-siRNA bound to B16F10 cells was internalized into the cells at 37 °C. The binding amount was not significantly different between CPP-LNP and LFA2K.

The intracellular distribution of siRNA delivered by CPP-LNP was determined by observation under a confocal microscope. As shown in Fig. 3A, most of the siRNA was distributed in the cytoplasm of the B16F10 cells and did not become localized in lysosomes. Uptake of siRNA into B16F10 cells mediated by delivery via CPP-LNP was observed in almost all cells in the field of observation (Fig. 3B). Such uptake of siRNA was dramatically diminished by the treatment of these cells with heparin, an inhibitor of

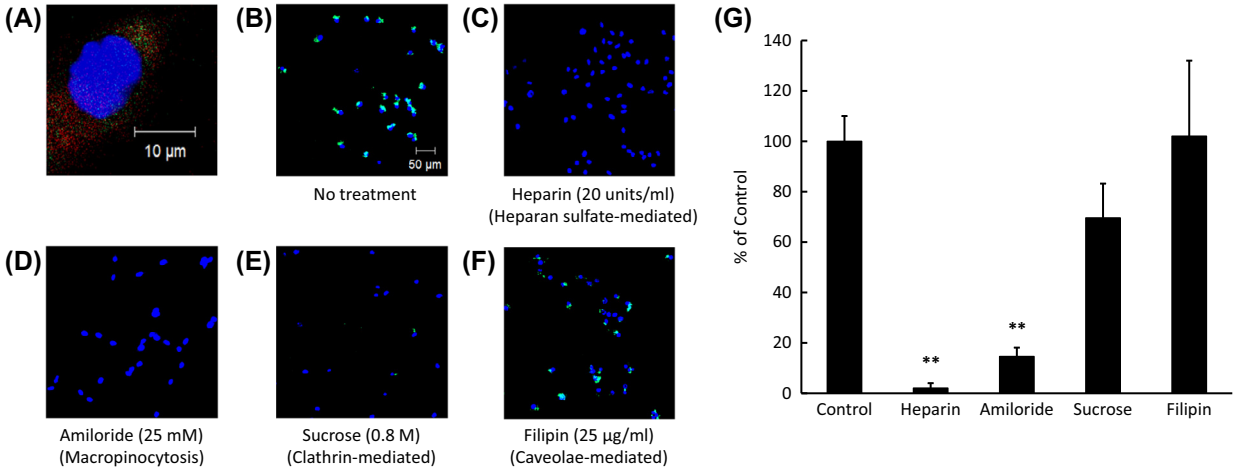

Fig. 3. Endocytosis pathway of siRNA formulated in CPP-LNP. B16F10 cells were incubated at 37 °C for 6 h with FAM-siRNA encapsulated in CPP-LNP and observed by confocal laser scan microscopy. (A) Localization of FAM-siRNA (green) was observed after lysosomes and nuclei had been stained with LysoTracker (red) and DAPI (blue), respectively. (B–F) B16F10 cells were incubated at 37 °C for 6 h with CPP-LNP-encapsulated FAM-siRNA in the absence (B) or presence of each endocytosis inhibitor indicated in the figure (C–F). Association of FAM-siRNA with these cells was observed after their nuclei had been stained with DAPI (blue). (G) B16F10 cells were incubated as described for "B"–"F," after which the fluorescence intensity of FAM in the cell lysates was measured and corrected for protein amount. Data are presented as a percent of control (untreated). Significant differences from the control group ($^{**}p < 0.001$) are indicated. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

heparan sulfate-mediated endocytosis (Fig. 3C), or with amiloride, an inhibitor of macropinocytosis (Fig. 3D); and it was partly diminished by sucrose, an inhibitor of clathrin-mediated endocytosis (Fig. 3E). On the other hand, the treatment with filipin, an inhibitor of caveolae-mediated endocytosis, did not affect the uptake of siRNA formulated in CPP-LNP (Fig. 3F). In the analysis of the endocytosis pathway(s) involved, quantitative data on the fluorescence intensities in each cell lysate were approximately consistent with the confocal microscopic images (Fig. 3G).

3.3. Knockdown of reporter genes with CPP-LNP

The luciferase activity of B16-F10-luc2 murine melanoma cells was suppressed by treatment of the cells with siLuc2 formulated in CPP-LNP (CPP-LNP-siLuc2, Fig. 4A). The knockdown efficiency of CPP-LNP-siLuc2 in these cells was approximately 70% and comparable in level to that obtained with LFA2K. In contrast, siLuc2 formulated in the cores (data not shown) or LNP did not induce gene silencing in these cells. No cytotoxicity of CPP-LNP-siLuc2 toward the transfected B16-F10-luc2 cells was observed in a cell viability assay (data not shown). CPP-LNP encapsulating siGFP (CPP-LNP-siGFP) also induced gene silencing in EGFP/HT1080 human fibrosarcoma cells (Fig. 4B). The fluorescence intensity of EGFP/HT1080 cells was decreased by the treatment with siGFP formulated in CPP-LNP but not when formulated in LNP. The amount of protein in EGFP/HT1080 cells treated with CPP-LNP-siGFP was not statistically different from that in untreated cells, suggesting that CPP-LNP was not cytotoxic in these cells, either. Taken together, these data show that CPP-LNP induced knockdown of reporter genes in both human and murine cells without causing severe cytotoxicity.

Finally, a palmitoyl derivative of RRRRRRGGRRRRG peptide was used instead of protamine to capture siRNA during CPP-LNP preparation. The particle size of LNP containing the palmitoyl peptide was approximately 138 ± 25 nm. Thus, a smaller particle size of LNP was obtained by using the palmitoyl peptide instead of protamine. The ζ-potential of CPP-LNP containing the palmitoyl peptide was similar to that of protamine-based CPP-LNP (data not shown). Stability of siRNA in serum was similarly improved by formulating siRNA in these 2 kinds of CPP-LNP (data not shown). By use of the palmitoyl peptide, knockdown efficiency of CPP-LNP-siLuc2 in B16-F10-luc2 cells was significantly increased compared with that obtained with the protamine-based formulation (Fig. 4C). The use of the palmitoyl peptide instead of protamine did not affect the type of endocytosis pathway taken by CPP-LNP (data not shown).

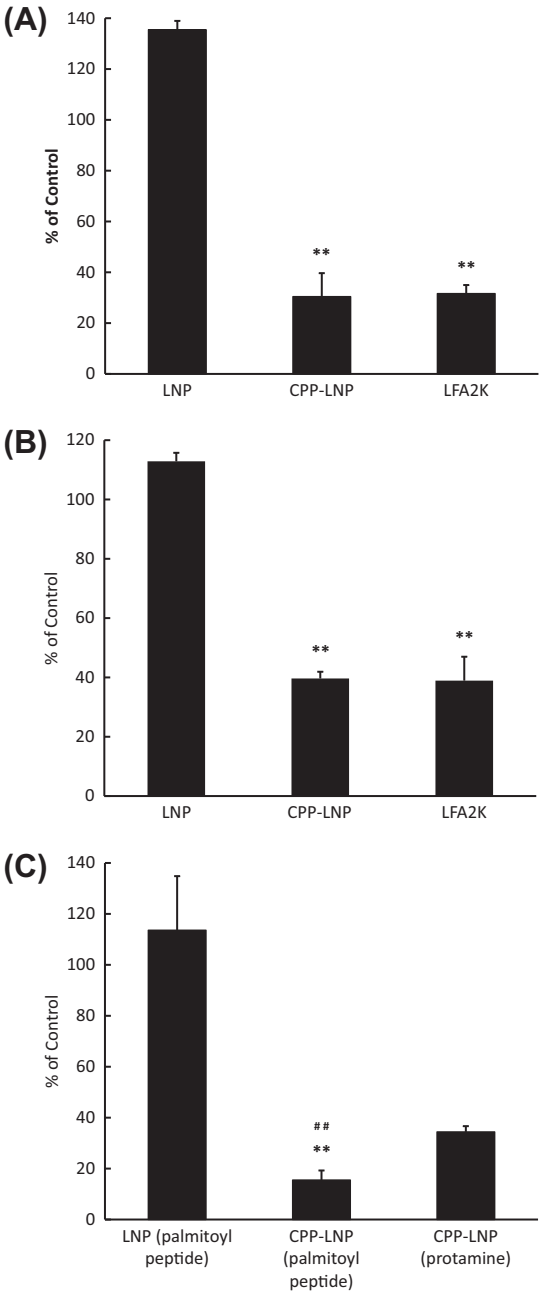

Fig. 4. Gene silencing induced by treatment of B16-F10-luc2 and EGFP/HT1080 cells with CPP-LNP-siRNA. (A) B16-F10-luc2 cells were transfected with CPP-LNP-encapsulated siLuc2 for 24 h. After the residual CPP-LNP-siRNA had been removed, these cells were cultured for an additional 24 h. The luminescence intensity of luciferase was measured and corrected for the titer of living cells. (B) EGFP/HT1080 cells were transfected with CPP-LNP-encapsulated siGFP for 24 h. After the residual CPP-LNP-siRNA had been removed, these cells were cultured for an additional 24 h. The fluorescence intensities of EGFP were measured and corrected for protein contents. (C) The palmitoyl derivative of the RRRRRRGGRRRRG peptide was used instead of protamine to capture siRNA during CPP-LNP preparation. B16-F10-luc2 cells were transfected thus prepared CPP-LNP encapsulating siLuc2 as described in (A) LFA2K was used as a control. Data are presented as a percent of the control (untreated) with SD bars. Significant differences from the LNP group ($^{**}p < 0.001$) and the CPP-LNP (protamine) group ($^{\#\#}p < 0.001$) are indicated.

4. Discussion

In the present study, RRRRRRGGRRRRG peptide derived from protamine was conjugated with DOPE and used as a CPP for efficient delivery of siRNA. Our data indicated that modification of the surface of LNP-siRNA with CPP enhanced internalization of siRNA into B16F10 cells. Uptake of siRNA was dramatically diminished by the treatment of these cells with heparin or amiloride, suggesting that CPP-LNP was mainly internalized via heparan sulfate-mediated endocytosis and macropinocytosis. In addition, the treatment of these cells with sucrose partly inhibited uptake of siRNA, suggesting that a part of CPP-LNP was internalized via clathrin-mediated endocytosis. After internalization of CPP-LNP-siRNA into B16F10 cells, siRNA was efficiently transferred into the cytoplasm from endosomes because it was not localized with the lysosomes. Although the precise mechanism of endosomal escape is not clear at present, several possibilities can be considered. One possibility is that CPP may interact with the endosomal membrane and facilitate the transfer of siRNA into the cytoplasm. Another possibility is that CPP may contribute to the proton sponge effect, because the ζ-potential of CPP-LNP became positive in response to acidic conditions. We speculate that both of these factors, namely, facilitation of siRNA transfer and the proton sponge effect, contributed to

the efficient delivery of siRNA to the cytoplasm, resulting in silencing of reporter genes.

We used protamine to prepare the inner cationic cores of CPP-LNP because it has been often used as polycation to capture nucleic acids such as plasmid DNA and siRNA [16]. As expected, protamine-based CPP-LNP delivered siRNA into the cytoplasm and had gene silencing effects. However, the particle size of protamine-based CPP-LNP is large and difficult to control. Therefore, we also tested the palmitoyl derivative of RRRRRRGGRRRRG peptide instead of protamine to prepare the inner cationic core. As a result, LNP having a particle size of approximately 100–160 nm were obtained. Hydrophobic interaction between palmitoyl moieties may contribute to compaction of the cationic cores, resulting in downsizing of LNP.

Our present data demonstrate that surface modification of LNP with RRRRRRGGRRRRG peptide as CPP is an ideal approach to siRNA delivery.

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