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TITLE:
Enhanced Intrapulmonary Delivery of Anticancer siRNA for Lung Cancer Therapy Using Cationic Ethylphosphocholine-based Nanolipoplexes

ABSTRACT:
Here, we report a cationic nanolipoplex as a pulmonary cellular delivery system for small-interfering RNA (siRNA). Six nanoliposomes differing in cationic lipids were formulated and screened in vitro and in vivo for cellular delivery functions in lung cells/tissues. Although the six nanoliposomes showed similar siRNA delivery efficiency in vitro, they exhibited significant differences in pulmonary cellular delivery functions in vivo. Among the various nanoliposomes, cationic dioleoyl-sn-glycero-3-ethylphosphocholine and cholesterol (ECL)-based nanoliposomes showed the highest pulmonary cellular delivery in vivo and the lowest cytotoxicity in vitro. The delivery efficiency of fluorescent siRNA in ECL nanoliposomes was 26.2-fold higher than that of naked siRNA in vivo. Treatment with Mcl1 (myeloid cell leukemia sequence 1)-specific siRNA (siMcl1) using ECL nanolipoplexes reduced target expression in B16F10 cell lines, whereas control, luciferase-specific siGL2 in ECL nanolipoplexes did not. In metastatic lung cancer mouse models induced by B16F10 or Lewis lung carcinoma (LLC) cells, intratracheal administration of siMcl1 in ECL nanolipoplexes significantly silenced Mcl1 mRNA and protein levels in lung tissue. Reduced formation of melanoma tumor nodules was observed in the lung. These results demonstrate the utility of ECL nanoliposomes for pulmonary delivery of therapeutic siRNA for the treatment of lung cancers and potentially for other respiratory diseases.

Introduction:
Despite the initial hope of small-interfering RNA (siRNA) as a next-generation therapeutic modality, progression of siRNA therapeutics to clinical trials has been halting.1,2 One of the biggest challenges for progress in the siRNA field is the strong dependence of siRNA efficacy on effective intracellular delivery systems.3,4,5 Since siRNA is processed and binds to specific mRNAs in the cytoplasm, the intracellular delivery of siRNA is a prerequisite for the silencing of target genes. However, the relatively large size and negative charges of siRNA makes it impossible for siRNA to diffuse through the cell membrane from extracellular spaces.
The lung has been an attractive target organ for siRNA-based therapy.6 Intravenous administration of functionalized lipopolyamine was reported to provide knockdown of a target gene in the lung tissue of normal mice.7 However, upon systemic administration, siRNA may be rapidly degraded and cleared from the bloodstream, leading to inefficient delivery to target cells.8 Compared with systemic delivery, the direct localized administration of siRNA via the pulmonary route may allow higher retention of siRNA in lung tissues and reduce systemic toxicity.9 Reflecting the several advantages of pulmonary delivery over systemic administration, two of the four siRNA drugs currently in Phase II clinical trials are delivered intranasally or by inhalation.8
Despite these advances, there remains a need for the development of effective nanocarriers of siRNA for direct pulmonary delivery. A recent study reported that delivery is a crucial barrier against the effective silencing of target genes by intratracheally administered naked siRNA.10 Several nanocarriers have been studied for localized lung delivery of siRNA. For example, a poly (ester amine) polymer has been used for aerosolized siRNA delivery in mice,11 and a fatty acid-modified polyethylenimine derivative was recently studied for intratracheal administration of siRNA in mice.12
In this study, we screened various cationic nanoliposomes for delivery efficiency in vitro and in vivo after intratracheal administration in mice. For cationic nanoliposomes, we tested 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP), 1,2-di-O-octadecenyl-3-trimethylammonium propane (DOTMA), and 1,2-dioleoyl-sn-glycero-3-ethylphosphocholine (EDOPC) as cationic lipid components. DOTAP and DOTMA have been used previously as cationic lipid components of cationic liposomes or nanoparticles for siRNA delivery. For example, DOTAP-modified cationic poly (DL-lactide-co-glycolide acid) nanoparticles have been used in an inhalable dry powder formulation of siRNA,13 and DOTAP-based cationic liposomes have been reported as a system for delivering siRNA to lung cancer cells.14 DOTMA-based cationic liposomes have been used to increase the cellular delivery of siRNA to human airway epithelial cells, and mouse neuroblastoma cells in vitro.15 Compared with DOTAP and DOTMA, EDOPC is a relatively new cationic lipid, and is less studied as a carrier of nucleic acid therapeutics. Although it has been studied for transfection of human umbilical vein endothelial cells with plasmid DNA,16 and in vivo systemic

delivery of plasmid DNA in mice,17 there are still few reports on the application of EDOPC-based nanoparticles for the delivery of siRNA.
Moreover, given the high mortality of lung cancers worldwide,18 we tested the efficacy of anticancer siRNA in metastasized lung cancer models. Here, we report that in vitro and in vivo pulmonary cellular delivery functions of various cationic nanoliposomes are uncorrelated. Using Mcl1 (myeloid cell leukemia sequence 1)-specific siRNA (siMcl1) as an anticancer siRNA, we demonstrate the potential of EDOPC-based cationic nanolipoplexes as an effective in vivo siRNA delivery system that shows target silencing and anticancer activity in mouse metastasized lung cancer models.

Characterization of cationic nanolipoplexes ::: Results:
The formation of nanolipoplexes between cationic nanoliposomes and siRNA was confirmed by gel retardation assays and size measurement. Regardless of nanoliposome compositions described in Table 1, retarded mobility of siRNA on gels was observed at N/P (nitrogen-to-phosphorus) ratios of 10:1 and above (Figure 1a). Upon complexation with siRNA, a slight increase in size was observed for all cationic nanoliposomes (Table 1). The extent of the mean size increase in nanolipoplexes relative to nanoliposomes was <25 nm. Regardless of cationic nanoliposome compositions, the zeta potential values of nanolipoplexes were >20 mV from the N/P ratio of 10:1 (Figure 1b). However, further increase of N/P ratios did not substantially enhance the zeta potential values as compared with the N/P ratio of 10:1.

In vitro cellular uptake of fluorescent double-stranded RNA in cationic nanolipoplexes ::: Results:
The in vitro cellular uptake of fluorescent double-stranded RNA (dsRNA) did not significantly differ among various nanolipoplexes (Figure 2a). All nanoliposomes formulated in this study showed cellular uptake of fluorescent RNA similar to that of the commercial transfection agent, L2K. Fluorescence-activated cell sorting analysis of B16F10 cells showed that the use of 3β-[N-(N′,N′-dimethylaminoethane)-carbamoyl]cholesterol (DC-Chol) in the nanoliposomal formulations (DTDL, DMDL, and EDL) did not significantly alter the cellular delivery functions of fluorescent dsRNA compared with nanoliposome formulations using Chol (DTCL, DMCL, and ECL). Moreover, the types of cationic lipids (i.e., DOTAP in DTCL, DOTMA in DMCL, and EDOPC in ECL) did not significantly affect the in vitro cellular uptake of fluorescent dsRNA.

In vivo pulmonary cellular uptake of fluorescent dsRNA in cationic nanolipoplexes ::: Results:
Unlike in vitro intracellular delivery, in vivo pulmonary cellular uptake of fluorescent dsRNA was significantly dependent on the lipid composition of nanolipoplexes (Figure 2b). Among DC-Chol–based nanoliposomes, EDL yielded a higher mean value of fluorescent-positive cell population compared with DTDL and DMDL. However, there was no significant difference among the DC-Chol–based cationic nanoliposomes in fluorescent dsRNA delivery functions in vivo. In contrast, Chol-based cationic nanoliposomes showed distinct differences in the in vivo pulmonary cellular delivery function of fluorescent dsRNA depending on the cationic lipid used in the nanolipoplexes. The use of EDOPC in ECL produced 8.0- and 3.2-fold higher levels of pulmonary cellular uptake of fluorescent dsRNA compared with DOTAP in DTCL and DOTMA in DMCL, respectively. Fluorescent dsRNA complexed with ECL produced 26.2-fold higher in vivo delivery than did the naked form. Representative flow cytometry data are presented in Figure 3a.

In vivo lung distribution of siRNA ::: Results:
Although Figures 2b and 3a show significantly higher pulmonary cellular uptake of fluorescent dsRNA in ECL nanolipoplexes relative to the free form, imaging of lung tissue, including extracellular spaces, did not reveal significant differences (Figure 3b). Molecular imaging analyses of whole lungs showed a similar increase in fluorescence intensity in groups treated with fluorescent dsRNA in free or ECL nanolipoplexes compared with the untreated control group (Figure 3c).

Cytotoxicity of various cationic nanolipoplexes ::: Results:
Although the in vitro cellular delivery of fluorescent dsRNA was not significantly different among cationic nanoliposome formulations, cytotoxicity was highly affected by the composition of cationic nanoliposomes (Figure 4). Cytotoxicity of luciferase-specific siGL2 complexed with various nanoliposomes was measured in B16F10 cells over 2 days. The cytotoxicity of siGL2/ECL nanolipoplexes was similar to that of siGL2/L2K complexes. After 2 days of treatment, the viability of cells treated with siGL2/ECL nanolipoplexes was 64.9 ± 7.5%, which is comparable to that of

siGL2/L2K-treated cells (65.9 ± 2.6%). Among the nanolipoplexes prepared in this study, siGL2/ECL nanolipoplexes had the least effect on cell viability, followed by siGL2/DTCL and siGL2/EDL nanolipoplexes. Compared to cells treated with siGL2/DMCL nanolipoplexes, those treated with siGL2/ECL nanolipoplexes showed 3.2-fold higher viability after 48 hours of treatment.

In vitro reduction of target gene expression ::: Results:
Since siGL2/ECL nanolipoplexes exhibited the lowest cytotoxicity (Figure 4), we tested the in vitro target gene reduction capability of siRNA using ECL nanoliposomes. As a functional siRNA, Mcl-1–specific siMcl-1 was complexed to ECL nanoliposomes. For comparison, luciferase-specific siGL2 was complexed to ECL nanoliposomes. Gel electrophoresis of reverse transcription-PCR (RT-PCR) products (Figure 5a) revealed that Mcl-1 mRNA levels were reduced after treatment of cells with siMcl-1 in ECL nanolipoplexes or L2K lipoplexes. However, no decrease in Mcl-1 mRNA was observed after treatment of B16F10 cells with siGL2 complexed to ECL nanoliposomes or L2K.
Real-time RT-PCR was used to quantify the extent of Mcl-1 mRNA downregulation after treatment with siMcl-1 in ECL nanolipoplexes (Figure 5b). Mcl-1 mRNA expression levels following treatment of cells with nonfunctional siGL2 in L2K and ECL nanolipoplexes were 100.4 ± 2.5 and 102.7 ± 3.5%, respectively. In contrast, following treatment of B16F10 cells with siMcl-1/ECL nanolipoplexes, Mcl-1 mRNA expression levels were reduced to 8.1 ± 2.4% of those observed in the group treated with siGL2/ECL nanolipoplexes ($P < 0.05$).

In vivo antitumor effects of siMcl-1 delivered in ECL nanolipoplexes ::: Results:
Intratracheal administration of siMcl-1 in ECL nanolipoplexes inhibited the growth of B16F10 and Lewis lung carcinoma (LLC) cells in lung tissues. After intravenous injection of B16F10 or LLC on day 0, siRNA, naked or in ECL complexes, was intratracheally injected on days 5, 7, 9, and 11 (Figure 6a). Lung tissue was extracted on day 14 for evaluation of the metastasis and growth of intravenously administered B16F10 or LLC. Mice injected with B16F10, but not treated with any siRNA, were used as controls. Black colonies of metastasized B16F10 were observed in lung tissues of untreated groups (Figure 6b) and in groups treated with free siMcl-1 (Figure 6c), siGL2/ECL nanolipoplexes (Figure 6d), or siMcl-1/ECL nanolipoplexes (Figure 6e). However, the extent of blackish B16F10 tumor nodules was lowest in the group treated with siMcl-1/ECL nanolipoplexes (Figure 6e).

In vivo silencing of target gene expression ::: Results:
Silencing of Mcl-1 in lung tissues was observed at both mRNA and protein levels after delivery of siMcl-1 in ECL nanolipoplexes (Figure 7). Quantitative real-time RT-PCR data (Figure 7a) revealed that relative Mcl-1 mRNA expression levels normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were reduced to 19.9 ± 7.3 and 50.7 ± 1.9% with B16F10 and LLC-bearing lung tissues, respectively after intratracheal treatment of mice with siMcl-1 in ECL nanolipoplexes compared with 86.9 ± 14.0 and 92.7 ± 4.1 % in mice treated with siGL2/ECL nanolipoplexes ($P < 0.05$). Similar to mRNA expression levels, protein expression of Mcl-1 in lung tissues was reduced upon intratracheal administration of siMcl-1/ECL nanolipoplexes. In both lung cancer animal models induced by B16F10 (Figure 7b) or LLC (Figure 7c), western blot analyses showed notable silencing of Mcl-1 protein expression in the siMcl-1/ECL nanolipoplex-treated group, but not in other groups. There was no difference in β-actin protein levels among groups.

Conclusion ::: Discussion:
In conclusion, our results suggest the importance of in vivo screening in evaluating siRNA nanocarriers and in vivo cellular level uptake studies in differentiating between extracellular and intracellular delivery of nanocarriers. Moreover, ECL nanolipoplexes might be applied in future for intrapulmonary delivery of siRNA to treat various lung diseases caused by the overexpression of pathogenic proteins.

Materials and Methods:
Preparation of cationic nanoliposomes. Cationic nanoliposomes were prepared using the lipid-film hydration method as previously described.32 Chol was purchased from the Sigma-Aldrich (St Louis, MO), and the lipids EDOPC, DOTAP, DOTMA, DC-Chol, and DOPE were obtained from Avanti Polar Lipids (Birmingham, AL, USA). These lipids, dissolved in chloroform, were mixed at different compositions and evaporated using a rotary evaporator to eliminate the organic solvent.

For nanoliposome preparation, EDOPC, Chol, and DOPE (15 μmol total lipids) were mixed at a molar ratio of 8:5:2 (Table 1). In other nanoliposome formulations, DOTAP or DOTMA were used instead of EDOPC, and DC-Chol was used in place of Chol. Thin lipid films were hydrated with 1 ml of 20 mmol/l HEPES (pH 7.4), and the resulting nanoliposomes were extruded three times through 0.2 μm polycarbonate membrane filters (Isopore; Millipore, Billerica, MA) using an Extruder (Northern Lipids, Vancouver, British Columbia, Canada). After complexation of various cationic nanoliposomes with siRNA, the sizes and zeta potentials of nanolipoplexes were determined using an ELS-8000 instrument (Photal, Osaka, Japan).

Cell culture and siRNA uptake. The murine melanoma cell line B16F10 and murine LLC cells were purchased from the American Type Culture Collection (Manassas, VA). Both cells were maintained in Dulbecco's modified Eagle's medium at 37 °C in a humidified 5% CO2 atmosphere. All media were enriched with 10% fetal bovine serum (HyClone, Logan, UT) and 1% of each penicillin and streptomycin (Sigma-Aldrich). Cellular uptake of siRNA was determined in B16F10 cells seeded onto 24-well plates 1 day before treatment. After replacing medium with fresh (300 μl/well), fluorescently labeled dsRNA (Block-iT; Invitrogen, Carlsbad, CA) at a concentration of 50 nmol/l was mixed with nanoliposomes at an N/P ratio of 20:1, or with Lipofectamine 2000 (L2K; Invitrogen), according to the manufacturer's instructions. The resulting nanolipoplexes were added to the cells and incubated at 37 °C for 24 hours. Cells were harvested, washed three times with phosphate-buffered saline (PBS), and evaluated by flow cytometry using a BD FACSCalibur system and Cell Quest Pro analysis software (BD Biosciences, San Jose, CA).

Cytotoxicity assay. The cytotoxicity of cationic nanoliposomes was monitored using 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assays. B16F10 cells were seeded onto 48-well plates at a density of 1 × 104 cells/well and allowed to attach for 1 day, after which the medium was replaced with 200 μl of fresh culture medium. Luciferase-specific GL2 siRNA (siGL2; ST Pharm., Seoul, Korea), which is nonfunctional in mammals, was complexed with various cationic nanoliposomes at an N/P ratio of 20:1, or with L2K according to the manufacturer's instruction. siGL2-containing nanolipoplexes were added to cells at an siGL2 concentration of 50 nmol/l. After incubation for various time periods, cells were treated with 20 μl of a 5 mg/ml MTT solution for 2 hours. Untreated cells were used as a control. The culture medium was then removed, and 200 μl of a 0.04 N HCl/isopropanol solution was added. The viability of cells was measured at a wavelength of 570 nm using a microplate reader (Sunrise; TECAN, Männedorf, Switzerland). The viability of cells in each group was expressed relative to that of untreated cells as a percentage.

RT-PCR and quantitative real-time RT-PCR. Knockdown of mRNA by siRNA was evaluated by RT-PCR and quantitative real-time RT-PCR. For in vitro knockdown studies, siMcl1 or siGL2 (at a concentration of 50 nmol/l) was complexed with ECL nanoliposomes at an N/P ratio of 20:1, and the resulting nanolipoplexes were applied to cells seeded onto a 24-well plate. After 24 hours, total RNA was isolated using the TRIzol reagent (Invitrogen) and reverse transcribed into cDNA using AccuPower RT PreMix (Bioneer, Daejeon, Korea). For in vivo knockdown studies, free siRNA or siRNA complexed in ECL nanolipoplexes was intratracheally administered (see below), and total RNA was isolated from the collected lung tissues and reverse transcribed into cDNA. The primers for murine Mcl1 were 5′-GCATGCTCCGGAAACTGGACATTA-3′ (sense) and 5′-CTTTGTTTGACAAGCCAGTCCCGT-3′ (antisense). RT-PCR products were electrophoresed on 1% agarose gels and visualized by ethidium bromide staining.

Quantitative real-time RT-PCR was performed in 20 μl glass capillaries using a LightCycler 2.0 instrument with LightCycler FastStart, DNA Master PLUS SYBR Green i reagents; data were analyzed using the LightCycler software program (Roche Diagnostics, Mannheim, Germany). Thermocycling parameters consisted of a hot start at 95 °C for 10 minutes followed by 45 cycles of 95 °C for 10 seconds, 57 °C for 20 seconds, and 72 °C for 20 seconds. A melting curve analysis was performed to confirm the specificity of the PCR products after the amplification step. The level of Mcl1 mRNA expression was normalized to that of the housekeeping gene, GAPDH.

Flow cytometry and molecular imaging.

In vivo uptake of siRNA by lung tissue was tested by flow cytometry and molecular imaging. Six weeks old female BALB/c mice were purchased from Daehan Biolink (Seungnam, Korea). All animals were maintained and used in accordance with the Guidelines for the Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, Seoul National University. siRNA delivery efficiency in various nanoliposomes was monitored after administering nanoliposomes complexed with Block-iT siRNA at an N/P ratio of 20:1 via the intratracheal route. After 4 hours, mice were killed and lung tissue samples were collected and homogenized in 1 ml PBS using a 40 μm pore diameter Cell Strainer (SPL Life Sciences, Pochon, Korea) followed by

centrifugation at 700g for 5 minutes. Pellets were suspended and incubated at room temperature for 5 minutes in 1 ml of red blood cell lysis buffer (0.165 mol/l NH_4Cl and 0.1266 mmol/l ethylenediaminetetraacetic acid) and centrifuged at 700g for 5 minutes. After washing three times with PBS, the suspended cells were analyzed using flow cytometry. For molecular imaging, extracted lung tissues were evaluated with a LAS 1000 image analyzer (FUJIFILM, Tokyo, Japan). Fluorescence intensity was analyzed using Image Gauge Analyzer Software (FUJIFILM).

In vivo silencing by siRNA in nanolipoplexes. The in vivo tumor model was prepared by injecting 6-week-old female BALB/c mice with 1 × 106 B16F10 or 2 × 106 murine lung carcinoma LLC cells in 200 μl PBS via the tail vein. The siRNA for specific silencing of Mcl1 (siMcl1) was purchased from Bioneer. Five days after inoculation, mice were given initial intratracheal injections of siMcl1 or siGL2 in nanocomplexes at a dose of 0.21 mg/kg using a MicroSprayer (Penn-century, Wyndmoor, PA). Mice were administered with nanolipoplexes four times every other day and killed on day 15. Lung tissue samples were collected and prepared as cell suspensions as described above. The in vivo silencing of target mRNA and protein by siMcl1 was analyzed using quantitative real-time RT-PCR (as described above) and western blot analysis, respectively.

Western blot analysis.

In vivo silencing of target protein expression by siMcl was evaluated by western blotting as previously described.45 RNA interference effects at the protein level were evaluated by first homogenizing extracted lung tissues in cell lysis buffer (0.05% Triton X-100 and 2 mmol/l ethylenediaminetetraacetic acid in 0.1 mol/l Tris-HCl) followed by centrifugation at 13,000g for 15 minutes. Extracted total proteins were quantified using the BCA protein assay kit (Thermo Fisher Scientific, Rockford, IL) according to the manufacturer's instruction, and separated by SDS-PAGE on 10% polyacrylamide gels. After transferring proteins onto polyvinylidene difluoride membranes, western blotting was performed using specific antibodies to Mcl1 (1:1,000, ab32087; Abcam, Cambridge, UK) and β-actin (1:2,500, sc-47778; Santa Cruz Biotechnology, Santa Cruz, CA). Bands were visualized using an alkaline phosphatase-conjugated anti-IgG antibody (Santa Cruz Biotechnology).

Statistics. Analysis of variance was used to analyze experimental data with application of a post hoc Student–Newman–Keuls test for pairwise comparisons. All statistical analyses were done using SigmaStat software (version 3.5, Systat Software, Richmond, CA); a P value < 0.05 was considered significant.