약학석사학위논문

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양이온성 지질 기반의 나노입자를 이용한 항암 치료용 siRNA의 국소전달

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이 논문을 약학석사학위논문으로 제출함

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최현우의 석사학위논문을 인준함

2012년 6월

위 원 장 이 봉 진 (인)

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Abstract

Enhanced intrapulmonary delivery of anticancer siRNA using cationic liposome for lung cancer therapy

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Here we report a cationic nanolipoplex for pulmonary cellular delivery system of siRNA. Six nanoliposomes differing in cationic lipids were formulated and screened at in vitro and in vivo for cellular delivery functions in lung tissues. Although the six nanoliposomes showed similar siRNA delivery efficiency in vitro, they exhibited significant differences in vivo pulmonary cellular delivery functions. Among the various nanoliposomes, cationic dioleoyl-sn-glycero-3-ethylphosphocholine- and cholesterol-based nanoliposomes (ECL) showed the highest pulmonary cellular delivery in vivo and the lowest cytotoxicity in vitro. The fluorescent siRNA delivery efficiency of ECL nanoliposomes was 26.2-fold higher than that of naked siRNA in vivo. The treatment of Mcl1-specific siMcl1 using ECL nanolipoplexes provided the reduction of target gene in B16F10 cell lines, whereas luciferase-specific siGL2 in ECL nanolipoplexes did not provide the reduction of the target gene. In B16F10 metastasized lung cancer model in mice, the intratracheal administration of siMcl1 in ECL nanolipoplexes revealed the lower formation of tumor nodules in the lung. Moreover, the intratracheal delivery of siMcl1 in ECL nanolipoplexes showed the significant silencing of Mcl1 in mRNA and protein levels at the lung tissue. These results indicate the utility of ECL nanoliposomes

for pulmonary delivery of therapeutic siRNA for the treatment of lung cancers and potentially for other respiratory diseases.

Keywords: siRNA, pulmonary delivery, cationic nanoliposomes, lung cancer

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Ⅰ. Introduction

Despite the initial hope of small interfering RNA (siRNA) as future generation therapeutics, not much progress has been achieved to the clinical trials of siRNA therapeutics [1, 2]. One of the biggest challenges for progress in the siRNA fields is the strong dependence on the effective intracellular delivery systems [3-5]. Since siRNA is processed and binding to the specific mRNA in cytoplasm, the intracellular delivery of siRNA should be a prerequisite for inducing the silencing of target gene. However, the relatively large size and negative charges of siRNA make it impossible for siRNA to diffuse through the cell membrane from extracellular spaces.

Lung has been one of attractive target organs for siRNA-based therapy [6]. Intravenous administration of functionalized lipopolyamine was reported to provide a knock-down of target gene in the lung tissue of normal mice [7]. However, upon systemic administration, siRNA may confront inefficient targeting, and the rapid degradation and clearance from the bloodstream, leading to inefficient delivery to target cells [8]. As compared to the systemic delivery, the direct localized administration of siRNA via pulmonary route may allow the higher retention of siRNA in lung tissues and reduce the systemic toxicity. Due to 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 [9].

For direct pulmonary delivery, there still exists a need for development of effective nanocarriers of siRNA. A recent study reported that the 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. A poly (ester amine) polymer was used for

aerosolized siRNA delivery in mice [11]. Fatty acid-modified polyethylenimine derivative was studied for intratracheal administration of siRNA in mice [12].

In this study, we screened the delivery efficiencies of various cationic nanoliposomes in vitro and in vivo after intratracheal administration in mice. Moreover, given the high mortality of lung cancers worldwide [13], we tested the efficacy of anticancer siRNA in metastasized lung cancer model. Here, we report that there exists a lack of correlation between in vitro and in vivo pulmonary cellular delivery functions of various cationic nanoliposomes. Using Mcl1-specific siRNA (siMcl1) as an anticancer siRNA, we report the potential of 1,2-dioleoyl-sn-glycero-3-ethylphosphocholine (EDOPC)-based cationic nanolipoplexes for effective in vivo silencing and anticancer activity against metastasized lung cancer model in mice.

Ⅱ. Materials & Methods

2. 1. Preparation of cationic nanoliposomes

Cationic nanoliposomes were prepared using the lipid-film hydration method as previously described. Cholesterol was purchased from Sigma-Aldrich Co. (St. Louis, MO) and the lipids 1,2-dioleoyl-sn-glycero-3-ethylphosphocholine (EDOPC), 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP), 1,2-di-O-octadecenyl-3-trimethylammonium propane (DOTMA), 3ß-[N-(N',N'-dimethylaminoethane)-carbamoyl]cholesterol (DC-Chol), and 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE) were obtained from Avanti Polar Lipid Inc. (Birmingham, AL). 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, cholesterol and DOPE were mixed at a molar ratio of 8:5:2containing15mmole of lipids in total. In other nanoliposome formulations, DOTAP or DOTMA were used instead of EDOPC, and DC-Chol was used replacing Chol. Thin-lipid films were hydrated with 1 ml of 20 mM HEPES (pH 7.4). The resulting nanoliposomes were extruded three times through 0.2 mm polycarbonate membrane filters (Isopore™, Millipore Corp., Billerica, MA) using an Extruder (Northern Lipids, British Columbia, Canada). The sizes of cationic nanoliposomes were determined using an ELS-8000 dynamic light scattering instrument (Photal, Osaka, Japan).

2. 2. Cell culture and siRNA uptake study

The murine melanoma cell line B16F10 was purchased from the American Type Culture Collection (Manassas, VA). B16F10 cells were maintained in Dulbecco's Modified Eagle Medium under 37°C, 5 % CO2 in a humid

atmosphere condition. Each medium was enriched with 10% fetal bovine serum (HyClone, Logan, UT) and 1% of penicillin and streptomycin each (Sigma-Aldrich Co.). To determine the cellular uptake of siRNA, B16F10 cells were seeded onto 24-well plates a day before treatment. After replaced with fresh medium (300μl/well), a 50 nM of fluorescent-labeled dsRNA, Block-iTTM (Invitrogen) was mixed with nanoliposomes at the N/P ratio of 20:1, or with LipofectamineTM 2000 (L2K, Invitrogen, Carlsbad, CA) according to the manufacturer's instruction. The resulting nanolipoplexes were added to the cells and incubated at 37 °C for 24 hr. Cells were harvested, washed three times with phosphate-buffered saline (PBS) and evaluated by flow cytometry and analyzed by a BD FACS Calibur using Cell Quest Pro software (BD Bioscience, San Jose, CA).

2. 3. Cytotoxicity assay

The cytotoxicity of cationic nanoliposomes was monitored using 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. B16F10 cells were seeded onto 48-well plate at a density of 1×104 cells/well and allowed to attach for 1 day. The medium was replaced with 200 μl of fresh culture medium. Luciferase-specific GL2 siRNA (siGL2, ST Pharm., Seoul, Korea), which is non-functional in mammals, was complexed with various cationic nanoliposomes at the N/P ratio of 20:1, or with L2K according to the manufacturer's instruction. The nanolipoplexes were added to the cells in 50 nM of siGL2 concentration. After incubation for various time periods, cells were treated with 20 μl of 5 mg/ml MTT solution for 2 hr. For comparison, untreated cells were used. The culture medium was then removed and 200 μl of 0.04 N HCl/isopropanol solution was added. The viability of cells was measured at a wavelength of 570 nm using a microplate reader (SunriseTM, TECAN,

Männedorf, Switzerland). Cell viability of each group was expressed as a percentage relative to that of untreated cells.

2. 4. Reverse transcription polymerase chain reaction (RT-PCR) and quantitative real-time PCR

Knock-down of mRNA induced by siRNA was evaluated by RT-PCR and quantitative real-time PCR. For in vitro knock-down study, siMcl1 or siGL2 was complexed with ECL nanoliposomes at the N/P ratio of 20, and resulting nanolipoplexes were treated to the cells seeded onto 24-well plate in 50 nM concentration. After 24 hr, total RNA was isolated using the TRIzolTM reagent (Invitrogen) and reverse transcribed into cDNA using AccuPower RT PreMix (Bioneer). For in vivo knock-down study, siRNA in free or ECL nanolipoplexes was intratracheally administered as described 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′ for sense and 5′-CTTTGTTTGACAAGCCAGTCCCGT-3′ for antisense. The products of RT-PCR were electrophoresed on a 1 % agarose gel, and visualized by ethidium bromide staining.

Quantitative real-time PCR was performed in 20 μl glass capillaries, using a LightCycler 2.0 instrument with LightCycler FastStart, DNA Master PLUS SYBR Green I reagents and the data were analyzed by the LightCycler® software program (Roche Diagnostics GmbH, Mannheim, Germany). Thermocycling parameters composed a hot start at 95 °C for 10 min followed by 45 cycles of 95 °C for 10 sec, 57 °C for 20 sec, and 72 °C for 20 sec. Melting curve determination was performed to confirm the specificity of the PCR products after the amplification step. The mRNA expression level of Mcl1 was normalized to those of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase

(GAPDH).

2. 5. Flow cytometry and molecular imaging

The in vivo lung tissue uptake of siRNA was tested by flow cytometry and molecular imaging. The Six-week-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. To monitor the siRNA delivery efficiencies of various nanoliposomes, nanoliposomes complexed with Block-iTTM at the N/P ratio of 20 were administered via intratracheal route. After 4 hr, mice were sacrificed 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 700 x g for 5 min. Pellets were suspended and incubated at room temperature for 5 min in 1 ml of red blood cell lysis buffer (0.165 M NH4Cl and 0.1266 mM ethylenediamine tetraacetic acid) and centrifuged at 700 x g for 5 min. After washing three times with PBS, the suspended cells were analyzed using flow cytometry. For molecular imaging, extracted lung tissues were evaluated by LAS 1000 image analyzer (FUJIFILM, Tokyo, Japan). Fluorescence intensity was analyzed by Image Gauge Analyzer Software (FUJIFILM).

2. 6. In vivo silencing study by siRNA in nanolipoplexes

For in vivo tumor model construction, six-week-old female BALB/c mice were injected with 1 x 106 B16F10 cells in 200 μl PBS via the tail vein. The siRNA for specific silencing of Mcl1 (siMcl1) was purchased from Bioneer Co. (Daejeon, Korea). Five days after inoculation, mice were given first intratracheal injections of siMcl1 or siGL2 in nanocomplexes at the dose of 0.21 mg/kg by

MicroSprayer (Penn-century Inc., Wyndmoor, PA). The nanolipoplexes were administered 4-times every other day and sacrificed at day 15. Lung tissue samples were collected and prepared into cell suspension as described above. The in vivo silencing of target protein by siMcl1 was analyzed using quantitative real time-PCR at the conditions described above and western blot analysis.

2. 7. Western blot analysis

In vivo silencing of target proteins expression by siMcl was evaluated by western blot as previously described. To evaluate the RNA interference effect at protein level, extracted lung tissues were homogenized in cell lysis buffer (0.05 % Triton X-100 and 2 mM ethylenediamine tetraacetic acid in 0.1 M Tris-HCl) followed by centrifugation at 13,000 x g for 15 min. Extracted total proteins were quantified with the BCA protein assay kit (Thermo Fisher Scientific Inc., Rockford, IL) according to the manufacturer's instruction, and separated on a 10 % SDS-PAGE gel. Proteins were transferred onto poly (vinylidene difluoride) membranes. Western blots were performed with specific antibodies to Mcl1 (1:1000, ab32087, Abcam, Cambridge, UK) and β-actin (1:2500, sc-47778, Santa Cruz Biotechnology, Santa Cruz, CA). For visualization of the bands, alkaline phosphatase-conjugated anti-IgG antibody (Santa Cruz Biotechnology) were used.

2. 8. Statistics

ANOVA was used to analyze the experimental data with Student–Newman–Keuls test for post-hoc pairwise comparison. All of statistical analysis was done using SigmaStat software (version 3.5, Systat Software, Richmond, CA) and a p value of 0.05 was considered significant.

Ⅲ. Results

3. 1. Characterization of cationic nanolipoplexes

The formation of nanolipoplexes between cationic nanoliposomes and siRNA was confirmed by gel retaradation assay and size measurement. Regardless of the nanoliposome compositions, the gel retardation of siRNA was observed from the N/P ratio of 10:1 (Fig. 1A). Upon complexation with siRNA, a slight increase in size was observed for all cationic nanoliposomes (Fig. 1B). The extent of mean size increases of nanolipoplexes as compared to nanoliposomes were less than 25 nm. Zeta potential values of ECL nanolipoplexes increased with the N/P ratios (Fig. 1C). At the N/P ratio of 20:1, the zeta potential value was 30.3 ± 6.0 mV.

3. 2. In vitro cellular uptake of fluorescent dsRNA in cationic nanolipoplexes

The in vitro cellular uptake of fluorescent dsRNA did not significantly differ among various nanolipoplexes (Fig. 2A). All the nanoliposomes formulated in this study showed cellular uptake of fluorescent RNA similar to commercial transfection agent, L2K. In B16F10 cells, FACS analysis showed that the use of DC-Chol in the nanoliposomal formulations (DTDL, DMDL, and EDL) did not show significant differences in the cellular delivery functions of fluorescent dsRNA as compared to the nanoliposome formulations using Chol (DTCL, DMCL, and ECL). Moreover, the types of cationic lipids such as DOTAP in DTCL, DOTMA in DMCL, and EDOPC in ECL did not significantly affect the in vitro cellular uptake of fluorescent dsRNA.

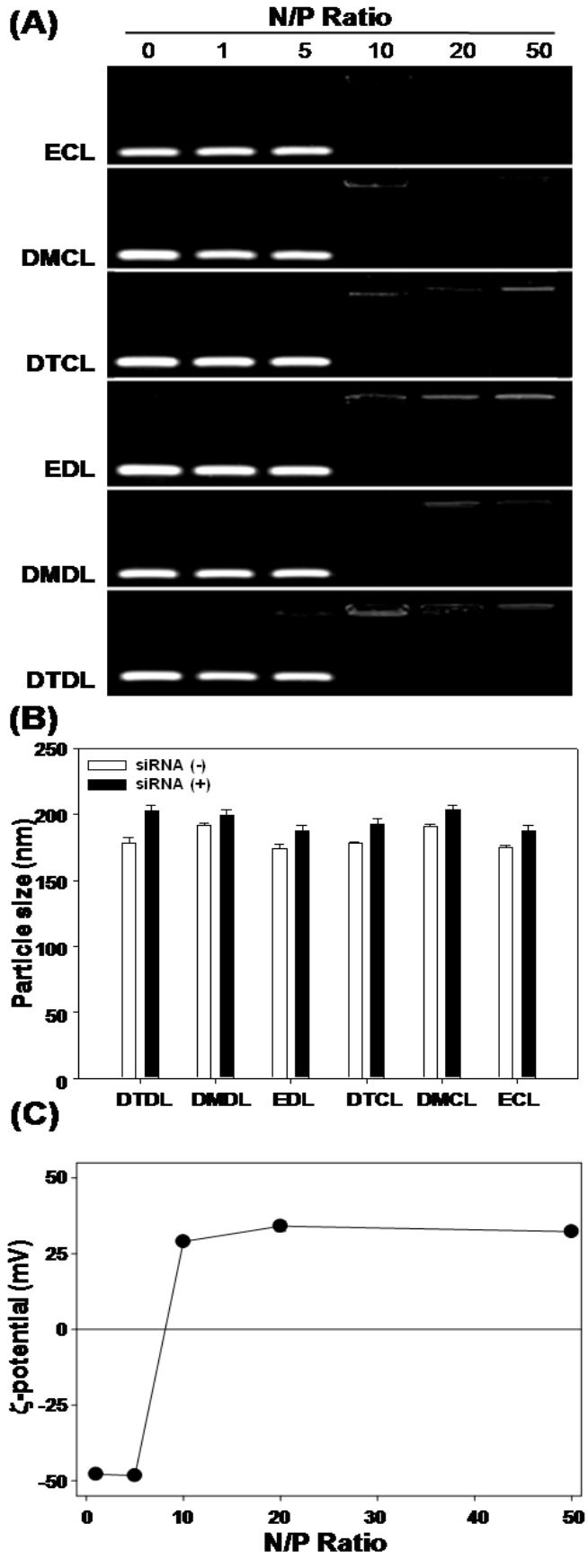
(A)
N/P Ratio
0
1
5
10
20
50
ECL
DMCL
DTCL
EDL
DMDL
DTDL
(B)
siRNA (-)
siRNA (+)
Particle size (nm)
250
200
150
100
50
0
DTDL
DMDL
EDL
DTCL
DMCL
ECL
(C)
ζ-potential (mV)
50
25
0
-25
-50
0
10
20
30
40
50
N/P Ratio

Fig. 1. Gel retardation, size and zeta potential of siRNA in nanolipoplexes.

Cationic nanoliposomes were complexed with siRNA at different N/P ratio. (A) For gel retardation, siRNA in naked form or various nanolipoplexes were run on a 1.5 % (w/v) agarose gel. The migration of siRNA was visualized by ethidium bromide staining. (B) Sizes of nanoliposomes before and after siRNA complexation were measured by light scattering method (n=4). (C) Zeta potentials of siRNA complexed with ECL at various N/P ratios were presented (n=4).

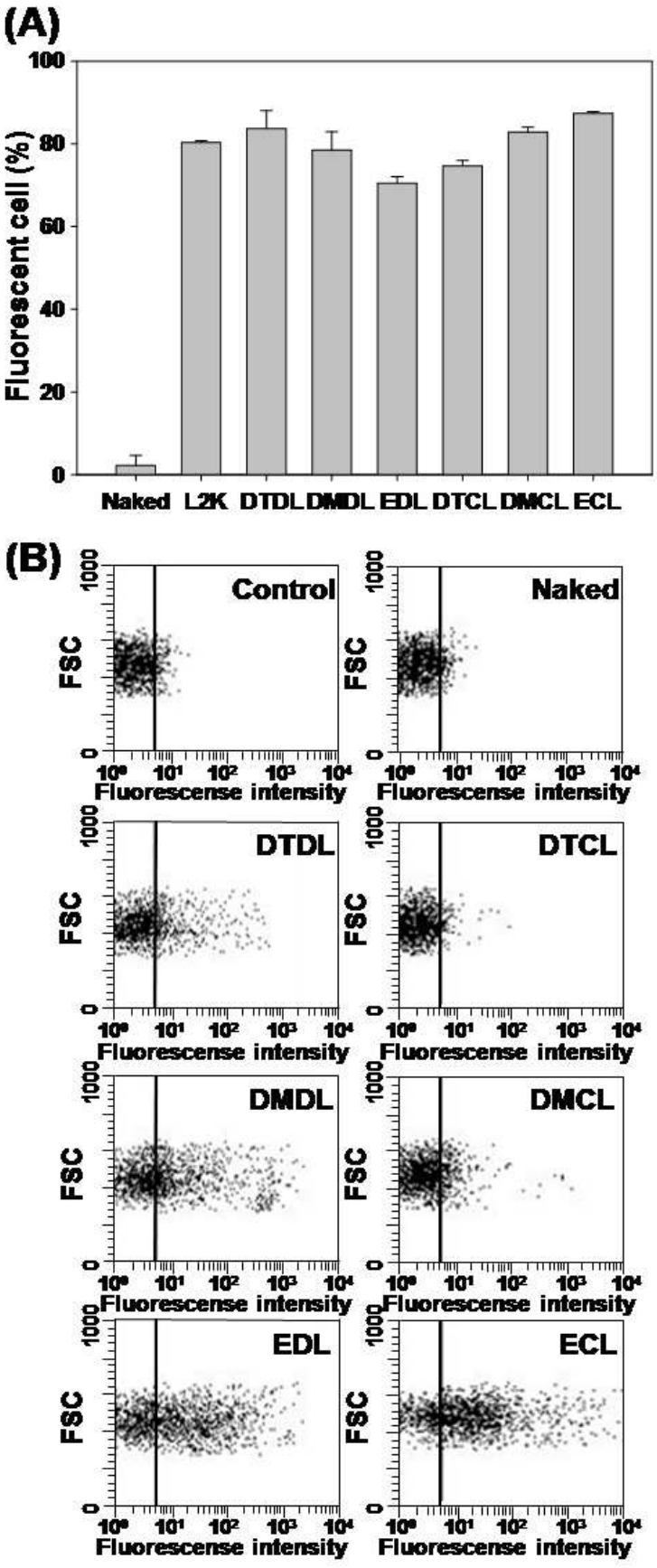
(A)
Fluorescent cell (%)
100
80
60
40
20
0
Naked
L2K
DTDL
DMDL
EDL
DTCL
DMCL
ECL
(B)
Control
Naked
DTDL
DTCL
DMDL
DMCL
EDL
ECL
FSC
1000
0
10⁰ 10¹ 10² 10³ 10⁴
Fluorescense intensity

Fig. 2. In vitro and in vivo cellular uptake of fluorescent dsRNA in nanolipoplexes.

Various cationic nanoliposomes were complexed with fluorescent marker-labeled dsRNA at the N/P ratio of 20:1. (A) B16F10 cells were treated with fluorescent dsRNA in various nanolipoplexes. After 24h, the fluorescent dsRNA uptake levels were quantified by flow cytometry (n=4). Untreated cells were used as control. (B) Fluorescent dsRNA in naked or nanolipoplexes was administered by intratracheal injection to BALB/c mice. Lung tissues were extracted at 4 hr post-dose, and the fluorescence of lung cells were measured by flow cytometry (n=3).

3. 3. In vivo pulmonary cell uptake of fluorescent dsRNA in cationic nanolipoplexes

Unlike in vitro intracellular delivery, in vivo pulmonary cellular uptake of fluorescent dsRNA was significantly dependent on the lipid composition of nanolipoplexes (Fig. 2B). In DC-Chol-based nanoliposomes, EDL showed a higher mean value of fluorescent positive cell population as compared to other DC-Chol-based nanoliposomes, DTDL and DMDL. However, there was no significant difference among the DC-Chol-based cationic nanoliposomes in the in vivo fluorescent dsRNA delivery functions. In contrast, Chol-based cationic nanoliposomes showed distinct differences 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 showed 8.0- and 3.2-fold higher fluorescent dsRNA pulmonary cell uptake as compared to DOTAP in DTCL and DOTMA in DMCL, respectively. ECL showed 26.2-fold higher in vivo fluorescent dsRNA delivery than did naked form. The representative flow cytometry data are presented in Fig. 3A.

3. 4. In vivo lung distribution of siRNA

Although Fig. 2B and 3A showed the significantly higher pulmonary cellular uptake of fluorescent dsRNA in ECL nanolipoplexes relative to free form, the lung tissue imaging including extracellular spaces did not reveal significant differences (Fig. 3B). The molecular imaging analysis of whole lung organ showed the similar intensity increase in the groups treated with fluorescent dsRNA in free or ECL nanolipoplexes as compared to untreated control group (Fig. 3C).

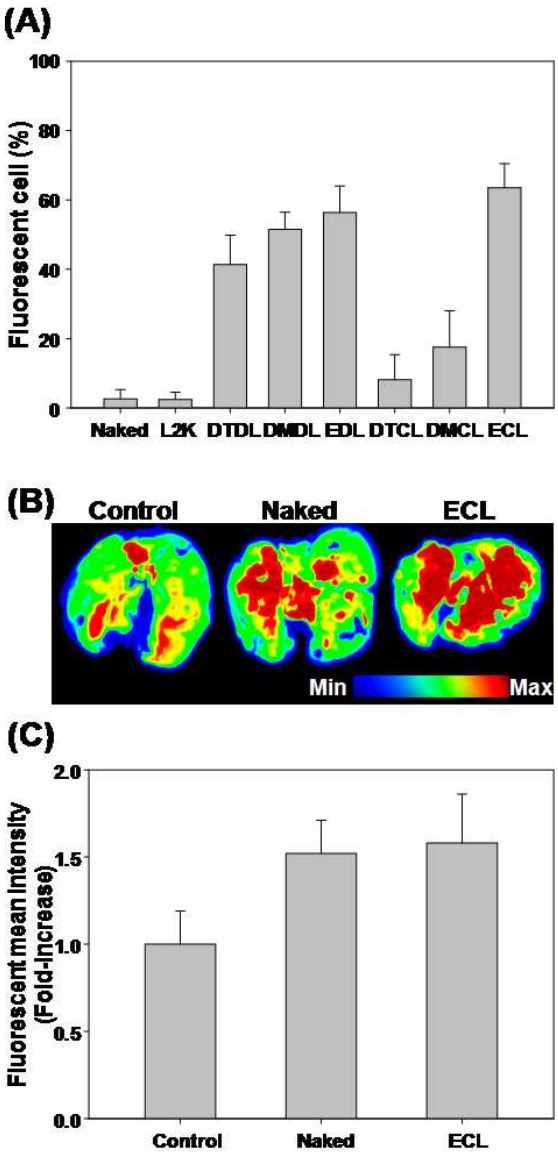

Fig. 3. In vivo lung retention and representative lung cellular uptake.

Fluorescent dsRNA in naked or nanolipoplexes was administered by intratracheal injection to BALB/c mice. (A) Representative lung cell uptake patterns of fluorescent dsRNA in naked form or various nanolipoplexes are presented. (B) Lung tissues were extracted at 4 hr after intratracheal injection, and the retention of fluorescent dsRNA in the lung was visualized by molecular imaging. (C) The mean intensity of fluorescence in lung tissues were quantified, and presented as fold-increase compared to untreated control (n=4).

3. 5. Cytotoxicity of various cationic nanolipoplexes

Although the in vitro cellular delivery of fluorescent dsRNA was not significantly different among cationic nanoliposome formulations, the cytotoxicity was highly affected by the composition of cationic nanoliposomes (Fig. 4). In B16F10 cells, the cytotoxicity was measured for luciferase-specific siGL2 complexed with various nanoliposomes over 2 days. The cytotoxicity of siGL2/ECL nanolipoplexes was similar to that of siGL2/L2K complexes. After 2 days of treatment, siGL2/ECL nanolipoplex showed 64.9 ± 7.5 % of cell viability which is comparable with siGL2/L2K-treated cells showing 65.9 ± 2.6 % viability. Among the nanolipoplexes prepared in this study, siGL2/ECL nanolipoplexes showed the highest cell viability, followed by siGL2/DTCL and siGL2/EDL nanolipoplexes. As compared to siGL2/DMCL nanolipoplex, siGL2/ECL nanolipoplex showed 3.2-fold higher cell viability at 48 hr of treatment.

3. 6. In vitro reduction of target gene expression

Since Fig. 5 revealed the lowest cytotoxicity of siGL2/ECL nanolipoplexes, the in vitro target gene reduction capability of siRNA was tested 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. The gel electrophoresis of RT-PCR products (Fig. 5A) revealed that the mRNA expression of Mcl-1 was reduced after treatment of the cells with siMcl-1 in ECL nanolipoplexes or L2K lipoplexes. However, no decrease of Mcl-1 RT-PCR products was observed after treatment of B16F10 cells with siGL2 complexed to ECL nanoliposomes or L2K.

The quantitative real time PCR provided the quantified reduction extent of Mcl-1 mRNA expression levels after treatment with siMcl-1 in ECL nanolipoplexes (Fig. 5B). The treatment of cells with nonfunctional siGL2 in

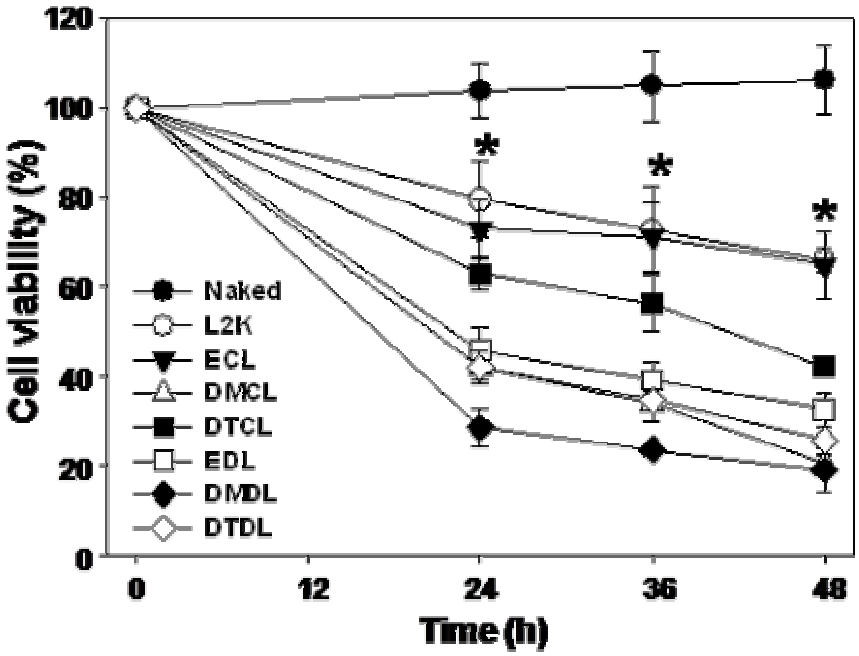

Fig. 4. Cytotoxicity of nanolipoplexes in lung cancer cell line.

B16F10 cells were treated by nanolipoplexes of siGL2 with L2K or various cationic nanoliposomes. After incubating for 24h, 36h, and 48 h, the cell viabilities were measured by MTT assay (n=4). *: significantly higher than DMCL, DTCL, EDL, DMDL, DTDL-treated groups (ANOVA and Student-Newman-Keuls).

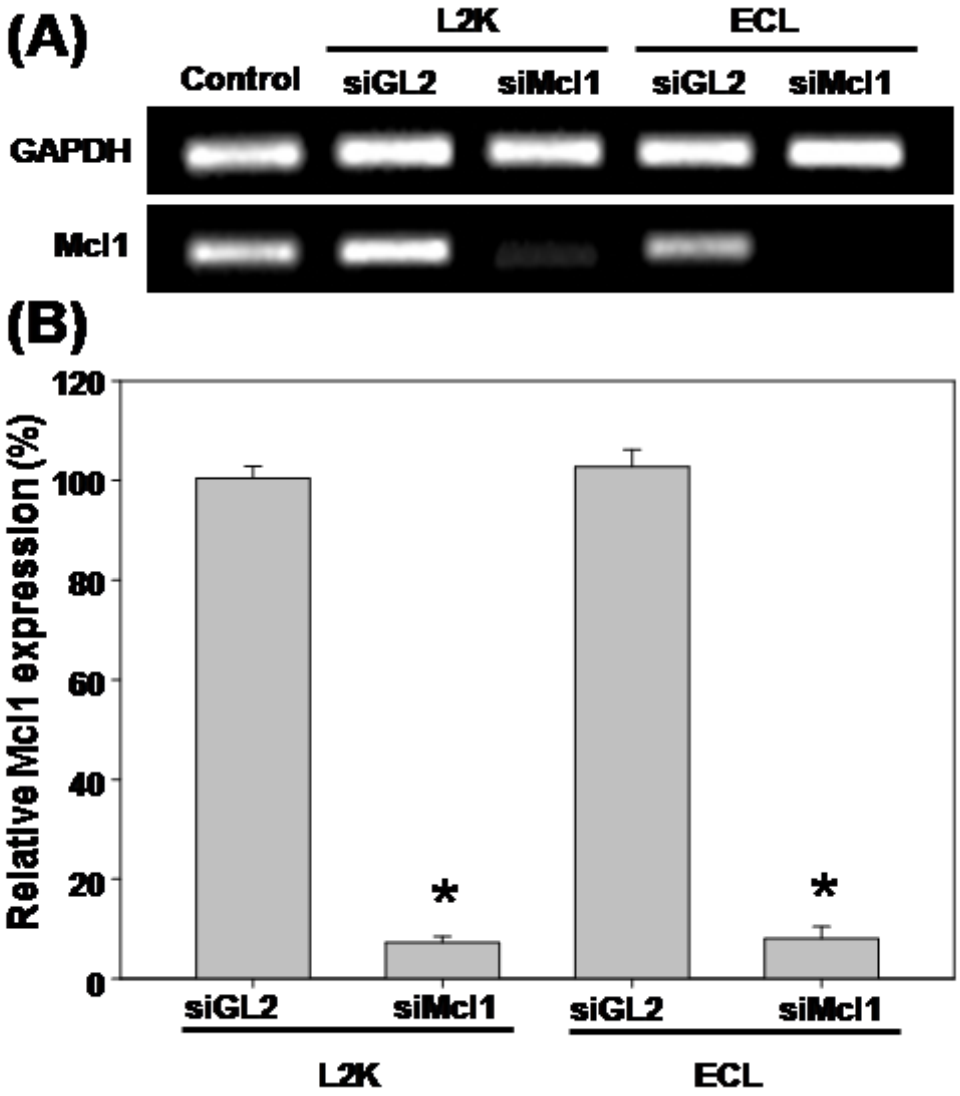

Fig. 5. In vitro reduction of target mRNA expression by ECL nanolipoplexes.

The siGL2 or siMcl-1 was complexed with L2K or ECL and treated to B16F10 cells. After 24h, RT-PCR products were electrophoresed on a 1% agarose gel (A). The mRNA expression level of target gene was quantified by quantitative real-time PCR (B) using those of GAPDH to normalize. *: significantly lower than control, siGL2-treated groups.

L2K and ECL nanolipoplexes resulted in the Mcl-1 mRNA expression levels of 100.4 ± 2.5% and 102.7 ± 3.5%, respectively. In contrast, following treatment of B16F10 cells with siMcl-1/ECL nanolipoplexes, the Mcl-1 mRNA expression level was 8.1±2.4 %, significantly lower than the mRNA levels observed in the group treated with siGL2/ECL nanolipoplexes.

3. 7. In vivo antitumor effect of siMcl-1 delivered in ECL nanolipoplexes

The intratracheal administration of siMcl-1 in ECL nanolipoplexes inhibited the growth of B16F10 cells in the lung tissues. After intravenous injection of B16F10 on day 0, siRNA in naked or ECL complexes were intratracheally injected on day 5, 7, 9, and 11 (Fig. 6A). The lung tissue was extracted on day 14 to detect the metastasis and growth of intravenously administered B16F10. As control, the mice injected with B16F10, but not treated with any siRNA were used. The black colonies of metastasized B16F10 in lung tissues were observed for the groups untreated, treated with free siMcl-1, siGL2/ECL nanolipoplexes, and siMcl-1/ECL nanolipoplexes. (Fig. 6B) The extent of blackish B16F10 tumor nodules, however, was the lowest in the group treated with siMcl-1/ECL nanolipoplexes as compared to other groups.

3. 8. In vivo silencing of target gene expression

The silencing of Mcl-1 target gene in the lung tissues was observed both in mRNA and protein levels after delivery of siMcl-1 in ECL nanolipoplexes (Fig. 7). The quantitative real time PCR data (Fig. 7A) reveal that the mRNA expression level of Mcl-1 was significantly reduced after intratracheal treatment of mice with siMcl-1 in ECL nanolipoplexes, showing 19.9 ± 7.3% of relative expression level normalized to GAPDH. The treatment of mice with siGL2/ECL

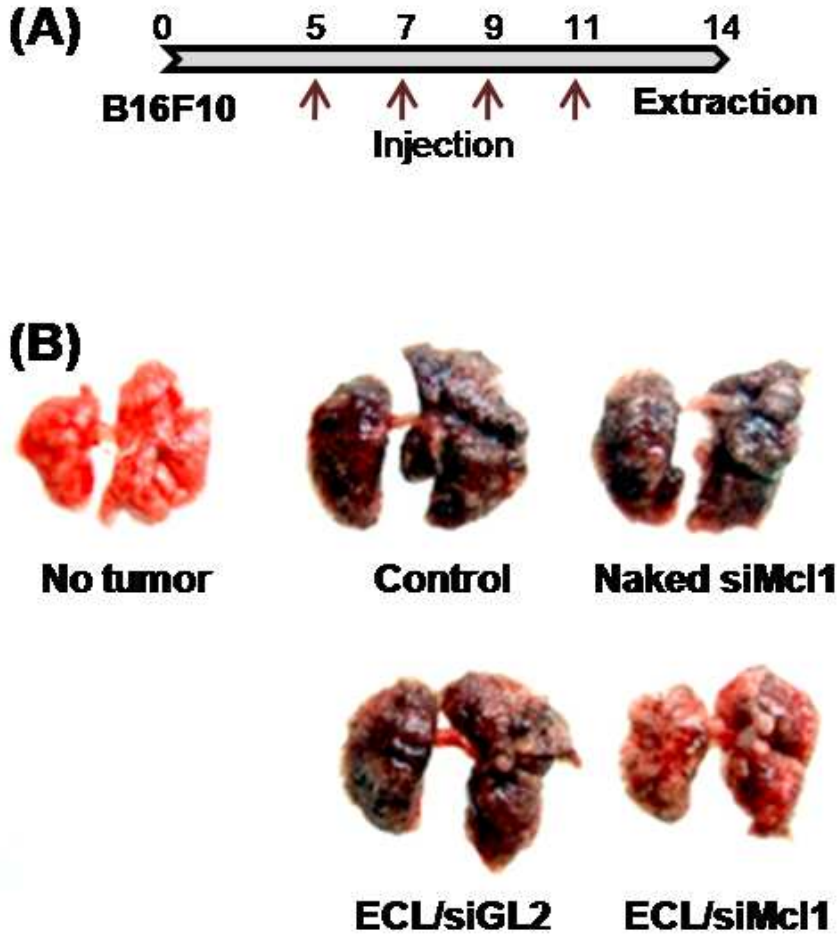

Fig. 6. Anticancer effect of siMcl-1 delivered in ECL nanolipoplexes.

The siGL2 or siMcl-1 (0.21mg/kg) in naked or ECL nanolipoplexes was sprayed into pulmonary area of B16F10-metastasised BALB/c mice. Intratracheal injection was done in every other day on four occasions beginning at day 5 after tumor inoculation (A). On day 14, mice were sacrificed and lung tissues of each group were extracted (B).

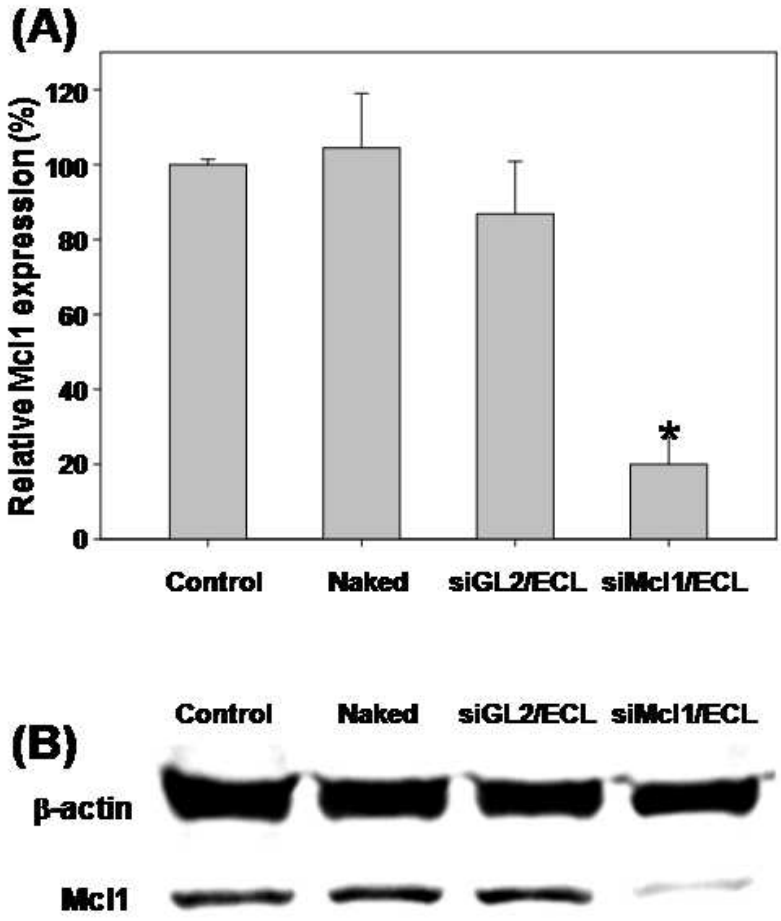

Fig. 7. In vivo silencing of Mcl-1 by ECL nanolipoplexes.

The siGL2 or siMcl-1 (0.21mg/kg) in naked or ECL nanolipoplexes was sprayed into pulmonary area of B16F10-metastasised BALB/c mice. Intratracheal injection was done in every other day on four occasions beginning at day 5 after tumor inoculation. On day 14, mRNA and protein level expression levels of Mcl-1 in the lung tissues were analyzed by quantitative real-time PCR (A, n=4) and western blotting (B), respectively. *: significantly lower than other groups.

nanolipoplexes exhibited 86.9 ± 14.0% of relative Mcl-1 mRNA expression level normalized to GAPDH. Similar to the mRNA expression levels, protein expression of Mcl-1 in the lung tissues was reduced upon intratracheal administration with siMcl-1/ECL nanolipoplexes. The western blot (Fig. 7B) shows that the silencing of Mcl-1 protein expression was notable in siMcl-1/ECL nanolipoplex-treated group, but not in other groups. There was no difference in the β-actin protein levels among the groups.

Ⅳ. Discussion

In this study, we demonstrated that in vitro siRNA delivery efficiencies do not exactly reflect in vivo pulmonary siRNA delivery efficiencies. Moreover, we showed that the in vivo efficiency of siRNA to pulmonary cells was significantly affected by the type of cationic lipids and co-lipids. The use of ECL nanolipoplexes provided the highest in vivo siRNA delivery efficiency to pulmonary cells. The intratracheal administration of siMcl1/ECL nanolipoplexes inhibited the growth of mestasized lung cancers in mice, with significant silencing of Mcl1 in mRNA and protein levels.

For cationic nanoliposomes, we used DOTAP, DOTMA and EDOPC as a cationic lipid component. DOTAP and DOTMA have been used as a cationic lipid component of cationic liposomes or nanoparticles for siRNA delivery. DOTAP-modified cationic poly (DL-lactide-co-glycolide acid) nanoparticles were used as inhalable dry powder formulation of siRNA [14]. DOTAP-based cationic liposomes were reported as a delivery system of siRNA to lung cancer cells [15]. DOTMA-based cationic liposomes were used to increase the cellular delivery of siRNA to human airway epithelial cells, and mouse neuroblastoma cells in vitro [16]. As compared to DOTAP and DOTMA, EDOPC is a relatively new cationic lipid, and less studied as a carrier of nucleic acid therapeutics. EDOPC was studied for transfection of plasmid DNA to human umbilical vein endothelial cells [17], and in vivo systemic delivery of plasmid DNA in mice [18]. However, there are few reports on the application of EDOPC-based nanoparticles for delivery of siRNA.

In addition to cationic lipids, cationic nanoliposomes were prepared using DOPE and DC-Chol or Chol as co-lipids. Regardless of the use of DC-Chol or Chol, all the nanoliposomes contained DOPE as a fusogenic lipid component [19]. Due to the fusogenic function, DOPE has been used as a helper-lipid of

DOTAPor DOTMA-based liposomes. DC-Chol, a derivative of Chol, structurally differs from Chol in that it has a cationic moiety in the structure. Recent study reported the use of DC-Chol and DOPE-based cationic liposomes for delivery of plasmid DNA and siRNA [20].

The comparison between in vitro and in vivo cellular uptake data indicates that there exists substantial discrepancy between in vitro and in vivo. Although all nanoliposomes showed similar fluorescent dsRNA delivery efficiency in vitro, they differed in pulmonary cellular dsRNA delivery functions in vivo. This result suggests a caveat that it needs caution to extrapolate the in vitro cellular uptake data for the screening of siRNA delivery nanocarriers. Currently, it remains to be understood the mechanisms underlying the higher in vivo siRNA delivery efficiency of ECL nanolipoplexes than other nanolipoplexes (Fig. 2B). Previously, the enhanced transfection of plasmid DNA was observed in EDOPC-based liposomes for human umbilical vein endothelial cells. Moreover, EDOPC was known to be serum compatible, enhancing the transfection efficacies of plasmid DNA regardless of the presence of serum [21, 22]. Based on the previous finding, we can not exclude the possibility that ECL might retain stability in bloodstream due to serum stability, and confer increased binding to the surface of pulmonary endothelial cells, facilitating the uptake of complexed siRNA into the cells.

In addition to the lack of correlation between in vitro and in vivo fluorescent dsRNA delivery efficiency, there was lack of correlation between the pulmonary cellular delivery efficiency (Fig. 3A) and whole lung tissue molecular imaging (Fig. 3C). Fluorescent dsRNA has been used as a model of a marker-labelled siRNA for cellular uptake evaluation of various siRNA nanocarriers [23]. Although we observed more than 20-fold increase in fluorescent dsRNA delivery in ECL nanolipoplexes as compared to naked form, there was no differences in

intensity of whole lung tissue imaging. The discrepancy between the in vivo cellular level and whole tissue level data might be contributed by the existence of naked fluorescent dsRNA in the extracellular spaces of lung tissues, whereas the existence of nanolipoplexed fluorescent dsRNA inside pulmonary cells. This result emphasizes the importance of measuring the cellular uptake levels of siRNA in vivo for evaluation of nanocarriers, in addition to the molecular imaging of whole tissues or body. Previously, the in vivo tumor distribution of polymeric micelles with or without folate ligand modification was studied in tumor tissue and tumor cell levels [24]. In the study, the tumor tissue distribution of polymeric micelles was found to be similar regardless of folate ligand modification. Despite the same levels of distribution in whole tissue levels, polymeric micelles with folate ligand were taken up by the cells whereas plain polymeric micelles were found in the extracellular spaces. This study also supports the possible source of discrepancy between whole tissue distribution and target cell level uptake.

ECL nanolipoplexes not only showed the highest in vivo pulmonary cellular delivery of fluorescent dsRNA, but also the lowest cytotoxicity in B16F10 cells. The cytotoxicity of cationic nanocarriers has been reported to be due to the interaction of cationic component with the mitochondrial membrane, the activation of caspase 3 pathway, inducing mitochondrially-mediated apoptosis [25, 26]. The dependence of cytotoxicity on the cationic lipids might be in part explained by the degradation kinetics of the lipids in the endosomal environments, and the toxicity of cellular degradation products of the cationic lipids. Unlike chemically synthesized cationic lipids such as DOTA and DOTAP, EDOPC is a derivative of natural lipid phosphatidylcholine with simple modification [27]. Due to the similarity to natural phosphatidylcholine, EDOPC is regarded as more readily metabolizable lipid in the body. EDOPC has been reported to be easily

hydrolyzed by phospholipase A2, and metabolized with a half-life of a few days in cells. Moreover, the higher cell viability of ECL relative to EDL (Fig. 4) may be explained by the additional existence of cationic lipid DC-Chol in EDL rather than the natural Chol as a component in ECL.

As an anticancer siRNA, siMcl1 was complexed to ECL nanoliposomes for treatment of lung cancer. Mcl1 has been reported as an anti-apoptotic protein related in the proliferation and survival of lung cancer cells [28, 29]. Moreover, overexpression of Mcl1 has been found in lung cancer tissues [30]. The silencing of the Mcl1 protein by siMcl1 may promote the apoptosis of cancer cells. Recently, siMcl1 has been reported to enhance the apoptosis of various solid tumor cells including lung cancer cells [31]. The intratumoral treatment of siMcl1 complexed to tocopherol derivative of oligochitosan-based nanoparticles was shown to inhibit the growth of KB tumors xenografted in mice. The co-delivery of siMcl1 with a histone deacetylase inhibitor using a cationic nanoliposome provided the synergistic anticancer activity after intravenous administration [32].

After intratracheal administration, siMcl1/ECL nanolipoplexes reduced the formation of B16F10 tumor nodules in lung tissue. As a lung cancer model, we used lung metastasis B16F10 after intravenous injection. In previous studies, intravenously administered B16F10 cells have been used for establishment of in vivo lung metastasis animal model [33, 34]. This lung cancer model has a potent advantage as an orthotopic disease model which may mimic the pathogenesis of lung cancer in human due to metastasis. For lung delivery of siMcl1, intratracheal nebulization was used as an administration method with a microsprayer [35]. This device may be more mimetic to clinically suitable aerosol dosage form than intranasal route.

The lowest formation of tumor nodules in siMcl1/ECL nanocomplex-treated

group could be due to the highest uptake of siMcl1 by the pulmonary cells in vivo (Fig. 2B), and to the silencing of tumor apoptosis-preventing protein Mcl1 in the lung tissue as evidenced by mRNA (Fig. 7A) and protein (Fig. 7B) levels. Previously, siRNA against SARS coronavirus was intratracheally administered to Rhesus macaque [36]. In the study, siRNA was delivered in naked form using 5% glucose in distilled water as a carrier solution. A recent study reported the uptake and efficacy of naked siRNA via intratracheal administration in mice. The study suggested that delivery remains a key obstacle to the efficacy of topically administered, naked oligonucleotide in the lung, supporting the importance of effective delivery systems for localized delivery of siRNA.

Although in this study, we presented the anticancer therapeutic effect of siMcl1 in ECL nanolipoplexes in B16F10-metastasized lung tumor model, the effective pulmonary cellular delivery efficiency of siRNA and the in vivo silencing of target protein in the lung tissue suggest the utility of ECL nanoliposomes for inhalation dosage forms of other siRNA and nucleic acid therapeutics. Inhalable siRNA is expected to be applied to various lung diseases such as cystic fibrosis, inflammatory condition, infectious diseases, and cancers. Given the pathology of various lung diseases due to the overexpression of malignant proteins, the ECL nanoliposomes may be applied to delivery such pathogenic protein-specific siRNA to lung cells via aerosol dosage forms.

Ⅴ. Conclusion

In conclusion, our results suggest the importance of in vivo screening for evaluation of siRNA nanocarriers, and the in vivo cellular level uptake study for differentiating the extracellular and intracellular delivery of nanocarriers. Moreover, ECL nanolipoplexes for intrapulmonary siRNA delivery might be applied for future treatment of various lung diseases due to the overexpression of pathogenic proteins.

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국문초록

양이온성 지질 기반의 나노입자를 이용한 항암 치료용 siRNA의 국소전달

기존 항암제가 가지는 여러 한계점과 비특이적 세포전달과 같은 단점을 극복하기 위한 대안으로 siRNA를 이용한 항암치료 연구가 주목받고 있지만 핵산의 짧은 반감기, 세포내로의 낮은 투과효율 등이 그 한계로 작용하고 있다. 이와 같은 한계를 극복하기 위해 siRNA의 효과적인 전달 시스템의 개발 필요성이 대두되고 있다.

이에 폐암치료의 새로운 모델로서 양이온성 지질 기반의 나노입자를 활용한 항암 치료용 siRNA의 폐부전달시스템을 소개하고자한다. 조성에 따른 siRNA 전달능의 차이를 확인하기 위하여 6개의 다른 조성을 가진 양이온성 나노입자를 제조하였으며 모든 입자는 in vitro에서 유사한 siRNA 전달능을 보였지만 폐부 조직으로의 in vivo 전달 효율은 유의적인 차이를 보였다. 그 중 cationic dioleoyl-sn-glycero-3-ethylphosphocholine- and cholesterol-based nanoliposomes (ECL)이 가장 높은 siRNA 전달능을 보였으며 in vitro에서의 세포독성도 가장 낮은 것으로 확인되었다. 형광 siRNA를 이용하여 폐부로의 siRNA 전달능을 정량적으로 분석한 결과, ECL을 활용한 전달 시스템이 대조군 대비 26.2배 더 높은 효율을 보였다. 또한 종양생성의 중요한 역할을 하는 Mcl1 단백질을 저해하기 위한 siMcl1을 전달하여 in vitro에서 B16F10세포의 Mcl1 mRNA가 유의적으로 감소함을 확인하였다. 치료효과를 확인하기 위하여 B16F10세포를 이용한 폐암전이모델을 확립하였다. ECL을 활용한 siMcl1의 전달시스템의 경우

종양의 성장이 효과적으로 저해되었으며 Mcl1의 mRNA와 단백질 수준이 대조군에 비해 현저히 감소하였다.

이러한 결과를 바탕으로 ECL이 호흡기 질환 치료를 위한 siRNA전달시스템 중 하나로 효과적인 수단이 될 수 있으며 핵산치료제의 잠재적 가치를 부가시킬 도구로 사용될 수 있음을 확인하였다.

Key words:
siRNA, pulmonary delivery, cationic nanoliposomes, lung cancer

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