Biochemical and Biophysical Research Communications xxx (2018) 1–7

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

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

PEGylated DC-Chol/DOPE cationic liposomes containing KSP siRNA as a systemic siRNA delivery Carrier for ovarian cancer therapy

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ARTICLE INFO

Article history:
Received 7 July 2018
Accepted 20 July 2018
Available online xxx

Keywords:
Cationic liposome
siRNA
RNAi
Systemic delivery
Cancer therapy

ABSTRACT

Although siRNA-mediated downregulation technology has been highly successful in suppressing the expression of any disease-related gene, systemic delivery of siRNA for the clinical applications remains challenging, especially in the use of cancer therapy. DC-Chol/DOPE cationic liposomes as one of the most attractive vehicles for gene delivery have been widely exploited for transfection of siRNA into cells, but complexity of systemic delivery has allowed only their direct injection into local targets due to the formation of aggregations with negatively-charged blood components. Herein, we demonstrate the effects of PEGylation on DC-Chol/DOPE cationic liposomes for systemic siRNA delivery in cancer therapy. In contrast to non-PEGylated DC-Chol/DOPE-siRNA lipoplexes, PEGylated DC-Chol/DOPE-siRNA lipoplexes reduce the excretion by kidneys and scavenging in liver, prolonging the circulation time *in vivo*, and ultimately increase their preferential tumor accumulation. Therefore, systemic injection of PEGylated DC-Chol/DOPE liposomes loaded with siRNA against kinesin spindle protein (KSP) gene exhibited a high level of target gene silencing at tumor sites and substantial suppression of tumor growth. Furthermore, systemically administered PEGylated lipoplexes did not lead to any activation of innate immune responses in the immunocompetent mice. These results suggest the potential of PEGylated DC-Chol/DOPE liposomes as a systemic delivery carrier for siRNA-mediated cancer therapy.

1. Introduction

The chemically synthesized siRNAs can easily degrade target mRNA without the need of nuclear entry and have been exploited to suppress the expression of cancer-related genes in body, including those traditionally expected to be 'undruggable' [1]. However, efficient and safe systemic delivery of siRNA into the tumor tissues remains challenging, due to the *in vivo* barriers such as short circulation lifetime, enzymatic degradation in the bloodstream and insufficient accumulation at tumor sites [2].

Considering the safety concerns in body, cationic liposomes offer several important advantages over viral vectors such as complete biodegradability and less toxicity [3]. Cationic liposomes easily form a complex with anionic siRNA *(i.e.* lipoplexes) and can enhance the cellular uptake of siRNA due to the electrostatic interactions with negative-charged plasma membrane of cells [4]. DC-Chol/DOPE cationic liposomes, composed of DC-Chol cationic lipids and dioleoylphosphatidylethanolamine (DOPE) neutral lipids, have been extensively investigated as one of the most attractive cationic liposomes for transfection of siRNA into cells [5]. Many researchers have reported the satisfactory *in vitro* applications of DC-Chol/DOPE-siRNA lipoplexes, which mainly focused on their transfection efficiency and toxicities. However, DC-Chol/DOPE liposomes readily aggregate with negatively-charged blood components including blood cells and serum proteins, and thus rapidly eliminated during blood circulation by lungs, liver and spleen [6]. Currently, there is little insight that can be used to explore the *in vivo* gene silencing effect of systemically injected DC-Chol/DOPE-siRNA lipoplexes, although it has been reported that DC-Chol/DOPE liposomes loaded with ferritin specific-siRNA could suppress the tumor growth in the human glioma U251-bearing mice *via* direct injection into the local tumor sites [7].

Grafting of poly(ethylene glycol) (PEG) on the surface of cationic liposomes was introduced to overcome the short blood circulation time and potential aggregation of cationic lipoplexes [8], but it should be noted that PEGylation may reduce the transfection efficiency by hampering the cellular uptake or endosomal escape after endocytosis [9]. Thus, this discrepancy issue, commonly referred to

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https://doi.org/10.1016/j.bbrc.2018.07.104
0006-291X/

as the PEG-dilemma, should be addressed prior to the *in vivo* use of PEGylated cationic liposomes.

Herein, we describe the effects of PEGylation on DC-Chol/DOPE liposomes in the systemic siRNA delivery and also evaluate the antitumoral effects of PEGylated DC-Chol/DOPE liposomes containing kinesin spindle protein (KSP) siRNA on the ovarian tumor-bearing mice. It is well known that inhibition of KSP function causes mitotic arrest during cell cycle and ultimately induce apoptosis, since KSP plays an important role in the formation of bipolar mitotic spindle during centrosome separation [10]. We examine tumor-specific accumulation and biodistribution of lipoplexes after systemic administration using optical imaging techniques. In addition to protection study of loaded siRNA against serum RNases in the blood, we evaluate the innate immune responses of lipoplexes during blood circulation of C57BL/6J mice. Finally, we investigate the *in vivo* gene silencing effects of lipoplexes on the tumor growth inhibition using various biological assay methods.

2. Material and methods

2.1. Preparation of PEGylated DC-Col/DOPE liposomes

PEGylated liposomes could be prepared according to a thin-film hydration and repeated extrusion method [11]. Briefly, lipids mixture including DC-Chol (487.5 nmol), DOPE (487.5 nmol) and $mPEG_{2000}$-DSPE (2.5 nmol) were dissolved in chloroform solvent. After chloroform was evaporated off, the thin lipid film was hydrated with 1 ml of 10 mM PBS buffer containing 2 nmol siRNA, leading to multilamellar vesicles (MLVs). The MLVs was sonicated for 30 s, vortexed for 30 m, and homogenized by repeated extrusion through 100 nm pore size of polycarbonate membrane filters (Nucleopore, USA) using a Mini-Extruder. According to the size-exclusion chromatography data, total 1 μmol of lipids fully complexed with 1 nmol of siRNA, and consequently, the concentrations of liposomes and loaded siRNA were determined 1 mM and 2 μM, respectively (N/P ratio = 11.9).

2.2. Characterization of lipoplexes

Hydrodynamic diameter and zeta potential of lipoplexes were measured using a Zetasizer Nano ZS (Malvern Instrument, USA). CM30 electron microscope (Philips, USA) was operated at 80 kV acceleration voltage, and all the samples were negatively stained with 2.5 wt % aqueous uranyl acetate. For size-exclusion chromatography analysis, a fixed amount of siRNA (2 nmol) were complexed with various amounts of liposomes (from 2 to 0.2 μmol), and the mixture was loaded onto Sepharose CL-4B column (Sigma Aldrich, USA). The eluted samples were collected in 1 mL of volume per tube and the amounts of siRNA were measured using Quant-it™ RiboGreen RNA assay (ThermoFisher, USA). For siRNA protection study in serum conditions, each of naked siRNA and liposomes-siRNA lipoplexes were mixed with PBS buffer containing 40% FBS and incubated at 37 °C for the given periods of time. After RNase inhibitor and 0.5% Triton-X 100 were added, each sample was electrophoresed on 2% agarose gels.

2.3. Measurement of KSP mRNA and protein levels in the lipoplexes-transfected cells

For qRT-PCR, total RNA samples were extracted from SKOV3 cells 48 h post-transfection using Trizol reagent (Invitrogen, USA). The cDNA could be generated using TOPscript™ cDNA synthesis Kit (Enzynomics, Korea). After the cDNA was added to the mixture of SYBR Premix Ex Taq II (TaKaRa, Japan), forward and reverse primers for KSP gene, or β-actin, DNA amplification was performed using a StepOnePlus real-time PCR system (Applied Biosystems, USA). For western blotting, the harvested cells were lysed with RIPA buffer and centrifuged. The supernatant samples were electrophoresed on 12% SDS-PAGE gels, and the separated proteins were electrotransferred onto polyvinylidene difluoride membranes at 70 V at 4 °C. The membranes were blocked with 5% skim milk in TBST supplemented with 0.05% Tween-20 for 2 h and stained with anti-KSP antibodies (Abcam) or anti-β-actin antibodies (Sigma Aldrich) overnight. After additional washing, the membranes were incubated with HRP-conjugated secondary antibodies for 1 h. The blots could be visualized using an Enhanced Chemiluminescence System and EZ-Capture MG (Japan).

2.4. In vivo biodistribution study of Cy5.5-labeled lipoplexes

SKOV3 cells were subcutaneously inoculated into the flanks of Balb/c nude mice (4-6 week-old, female, Orient Bio Inc.) (n = 3 per group). When tumor volumes reached 170–190 mm^3, mice were intravenously injected with Cy5.5-siKSP lipoplexes (1.5 mg/kg) or equivalent amounts of free Cy5.5-siKSP. The whole body fluorescence images were obtained 24 h post-injection using IVIS Spectrum and IVIS Living Imaging Software (Caliper Life Science Inc., USA). Mice were sacrificed to excise the major vital organs and tumors 24 h post-injection, and their *ex vivo* fluorescence images were measured. All animal experiments were performed according to the regulations of the Institutional Animal Care and Use Committee of Korea Institute of Science and Technology.

2.5. Antitumoral effects of systemically injected lipoplexes

Antitumoral efficiency of lipoplexes was examined on the aforementioned SKOV3-bearing mice. When the tumors were palpable (170–190 mm^3), tumor-bearing mice were randomly divided into three groups: saline control group (0.5 mg/kg, n = 3), LS_siSCR group (1 mg/kg, n = 3), and LS_siKSP group (1 mg/kg, n = 4). All treatments were intravenously administered *via* tail vein every other day for a total of eight injections. We assessed tumor volumes according to the previously described equation [12]. Tumor growth inhibition (TGI) was defined as the difference between Median Tumor Volume (MTV) of a treated group and control group: $TGI = (MTV_{control} - MTV_{treated})/MTV_{control}$ [13]. One day after final injection, all the mice were euthanized to obtain the tumor tissues. To measure body weights periodically as a parameter of systemic toxicity, lipoplexes containing siKSP (1 mg/kg, n = 3), siSCR (1 mg/kg, n = 3) or saline solution (0.5 mg/kg, n = 3) were intravenously injected into tumor-free ddY mice every other day for a total of eight injections.

2.6. Gene silencing effects of systemically injected lipoplexes

For a qRT-PCR analysis, tumor tissues were homogenized in lysis buffer composed of 50 mM Tris-HCl (pH 7.4), 100 mM NaCl, 0.2% sodium deoxycholate, and 0.5% NP-40. All the procedures required for qRT-PCR analysis were carried out in the same manner as described above. For an immunohistochemical staining assay, tumor tissues were fixed by ice-cold methanol and dehydrated by higher concentration of methanol prior to paraffin embedding. After paraffin-embedded tumors were sectioned at 6 μm by a microtome, samples were stained with anti-EG5 antibodies specific for KSP proteins (Abcam), hematoxylin, and Histostain-Plus kit (Invitrogen).

Please cite this article in press as: J. Lee, H.J. Ahn, PEGylated DC-Chol/DOPE cationic liposomes containing KSP siRNA as a systemic siRNA delivery Carrier for ovarian cancer therapy, Biochemical and Biophysical Research Communications (2018), https://doi.org/10.1016/j.bbrc.2018.07.104