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Neutralization of negative charges of siRNA results in improved safety and efficient gene silencing activity of lipid nanoparticles loaded with high levels of siRNA

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ABSTRACT

Lipid nanoparticles (LNPs) are one of the leading technologies for the *in vivo* delivery of short interfering RNA (siRNA). Cationic lipids are an important component for efficient endosomal escape *via* membrane fusion followed by release of siRNAs in cytosol where the site of action is located. A high cationic lipid/siRNA charge ratio is usually necessary for maximizing the gene silencing activity of the siRNA-loaded LNPs. However, high levels of cationic lipids are known to cause cytotoxicity through interactions with negatively charged biocomponents. A strategy for solving this dilemma is important, in terms of producing clinically applicable LNPs with a wide therapeutic window. We herein report on the development of LNPs with a high gene silencing activity and a low cationic lipid/siRNA charge ratio, which we refer to as low lipid core-nanoparticles (LLC-NPs). The negative charges of the siRNAs were neutralized by protamines, cationic proteins, to reduce the net dose of cationic lipid, YSK05, which was developed in our laboratory, for endosomal escape, resulting in preserved fusogenic activity and gene silencing activity, both *in vitro* and *in vivo* factor VII mouse model. In addition, the LLC-NPs showed an improved hepatotoxicity compared to conventional LNPs, which have a relatively higher cationic lipid/siRNA charge ratio. The concept of the LLC-NPs helps to realize clinically applicable LNPs with a wide therapeutic window and has the potential for use in various applications and for the delivery of different classes of nucleic acid.

1. Introduction

The use of short interfering RNA (siRNA) in the treatment of various refractory diseases is a promising strategy because of its specific and highly potent gene silencing *via* RNA interference mechanism [1]. The physicochemical properties of siRNA, which include a high molecular weight, hydrophilic properties and a polyanionic charge, represent a challenge for use in *in vivo* applications as well as clinical applications. Use of lipid nanoparticles (LNPs) is one of the most extensively investigated technologies for the delivery of siRNA *in vivo* [2]. Many of the studies of siRNA-loaded LNPs have focused on the efficiency of siRNA delivery. The recent development of sophisticated cationic lipids through rational design or combinatorial screening approaches has led to dramatic improvements in the efficiency of the LNP-mediated siRNA delivery [3–8].

The safety of the siRNA delivery system, as well as the efficiency of delivery are both important for any applications from basic research to clinical use. While cationic lipids are important for the efficient cytosolic delivery of siRNAs through their ability to enhance cellular uptake and endosomal escape, it is known that the exposure of high concentrations of cationic lipids can cause undesired side effects through multiple mechanisms, including the production of reactive oxygen species, the induction of pro-inflammatory cytokine production and apoptosis, both *in vitro* and *in vivo* [9–13]. Attempts have been made to avoid or minimize such cationic lipid-mediated cytotoxicity. These include the introduction of biodegradable linkages, including an ester bond, into the structure of the cationic lipids, which leads to their rapid elimination after delivery of the cargo [14–16]. Maier et al. reported that biodegradable cationic lipids are rapidly degraded and eliminated from the animal body, and showed a higher degree of safety compared to a counterpart with low biodegradability. Premedication with anti-inflammatory drugs is also a useful strategy for avoiding an elevation in the levels of pro-inflammatory cytokines which are induced by exposure to the cationic lipid [17, 18]. Abrams et al. reported that an intravenous injection of siRNA-loaded LNPs resulted in an elevation in serum chemistry parameters and the production of various

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inflammatory cytokines, and these reactions were significantly decreased by a co-treatment with dexamethasone, a glucocorticoid receptor agonist [17] and with a Janus kinase 2 inhibitor [18]. On the other hand, an elevation in pro-inflammatory cytokine levels was reported in a phase I clinical trial of ALN-VSP in spite of such a pretreatment, indicating that the efficacy of the pretreatment continues to be a controversial subject [19]. We recently found that the accumulation of the LNPs in liver sinusoidal endothelial cells causes their activation, cytokine production and subsequent neutrophilic inflammation followed by elevated blood chemistry levels including alanine transaminase (ALT) and aspartate transaminase (AST) [20]. It is also noteworthy that this toxicity can be completely avoided by the actively targeted delivery of LNPs to hepatocytes through the strategic modification of their surface with both polyethyleneglycol (PEG) and a hepatocyte-specific ligand, *N*-acetyl-D-galactosamine (GalNAc) [20].

In addition to the above-mentioned strategies for reducing cationic lipid-mediated cytotoxicity, decreasing the cationic lipid/siRNA charge ratio, which is consistent with reducing cationic lipid mass per siRNA or increasing the drug/lipid ratio, would be an alternative and straightforward strategy. However, it has been reported that reducing the cationic lipid/siRNA charge ratio of LNPs results in a decrease in the efficiency of siRNA delivery [21]. This issue can be attributed to a decrease in the fusogenicity of the LNPs with endosomal membranes due to a significant consumption of active cationic lipids for membrane fusion [22]. Because negatively charged siRNAs are encapsulated by electrostatic interactions with cationic lipids, most cationic lipids are consumed in binding with siRNAs and are not available for interacting with endosomal membranes in cases of a low cationic lipid/siRNA charge ratio. Therefore, excess molar ratio of cationic lipid against siRNA is typically used in most formulations.

We herein report on the use of the protein protamine, which carries a net positive charge at physiological pH, to reduce the net dose of cationic lipids, YSK05, which was developed in our laboratory, for endosomal escape, resulting in preserved fusogenic activity and gene silencing activity, both *in vitro* and *in vivo* factor VII mouse model. Protamine sulfate is one of the widely used and well characterized cationic proteins in the field of drug delivery system [23, 24] and is an FDA-approved compound which can be injected intravenously with a documented safety profile. Therefore, we decided to use protamines for neutralizing the negative charges of the siRNA as a proof of concept. In addition, the newly developed LNPs showed superior safety properties compared to the conventional LNPs with relatively higher cationic lipid/siRNA charge ratios. The current concept of the design for potent and safe LNPs advances our efforts to create clinically applicable LNPs with a wide therapeutic window and has the potential for use in a wide variety of applications as well as for the delivery of different classes of nucleic acids.

2. Materials and methods

2.1. Materials

Cholesterol (chol), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphatidylethanolamine (POPE), 1,2-dimyristoyl-*sn*-glycerol methoxypolyethylene glycol 2000 ($_{m}PEG_{2k}$-DMG), 1,2-dioleoyl-*sn*-glycero-3-phosphatidylserine (DOPS) and 1,2-dioleoyl-*sn*-glycero-3-phosphatidylcholine (DOPC) were purchased from the NOF Corporation (Tokyo, Japan). 1,2-Dioleoyl-*sn*-glycero-3-phosphoethanolamine-*N*-(7-nitro-2-1,3-benzoxadiazol-4-yl) (NBD-DOPE) and 1,2-dioleolyl-*sn*-glycero-3-phosphoethanolamine-*N*-(lissamine rhodamine B sulfonyl) (Rho-DOPE) were obtained from Avanti Polar Lipids (alabaster, AL). YSK05 was synthesized in our laboratory as described previously [25] (please refer to Fig. 6C for the structure). EverFluor TMR-labeled YSK05 was synthesized in our laboratory. Protamine was purchased from Calbiochem (San Diego, CA, USA). EverFluor TMR-X was purchased from Setareh Biotech (Eugene, OR, USA). 6-(*p*-Toluidino)-2-naphthalenesulfonic acid (TNS) was obtained from Wako Chemicals (Osaka, Japan). Ribogreen, 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI) and 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindodicarbocyanine perchlorate (DiD) were obtained from Molecular Probes (Eugene, OR, USA). TRIzol reagent was obtained from Invitrogen (Carlsbad, CA, USA). Dual-Luciferase Reporter Assay Reagent was purchased from Promega Corporation (Madison, WI, USA). FITC-conjugated Isolectin B4 was purchased from Vector Laboratories (Burlingame, CA). HeLa human cervical carcinoma cells was purchased from RIKEN Cell Bank (Tsukuba, Japan). HCT116 human colorectal carcinoma cells were purchased from ATCC (Manassas, VA, USA). All siRNAs ware obtained from Hokkaido System Science Co. Ltd. (Sapporo, Japan). The sequences for the sense and antisense strands of the siRNAs used in this study are listed in Supplementary Table.

2.2. Animals

Female ICR mice, 4 weeks of age, were purchased from Japan SLC (Shizuoka, Japan). The experimental protocols were reviewed and approved by the Hokkaido University Animal Care Committee in accordance with the guidelines for the care and use of laboratory animals.

2.3. Preparation of LNPs

A 400 μL of 90% *t*-BuOH solution containing YSK05/POPE/chol/$_{m}PEG_{2k}$-DMG at a molar ratio of 50/25/25/3 (for *in vitro* experiments) or 50/0/50/1.5 (for *in vivo* experiments) were prepared. DiI or DiD from 0.1 to 1 mol% of total lipid was added to the above solution for labeling. These lipid solutions were mixed with 300 μL of an siRNA in 1 mM citrate buffer (pH 4.5) to give the indicated lipid/siRNA charge ratio. Concerning the LLC-NPs, a negatively charged siRNA core with the indicated nitrogen/phosphate (N/P) ratio was prepared by adding 138.5 μL of a protamine in 1 mM citrate buffer (pH 4.5) to 171.5 μL of an siRNA in 1 mM citrate buffer (pH 4.5) under vigorous mixing, and the siRNA core solution was diluted to become 400 μL by 1 mM citrate buffer (pH 4.5) and then added to the above lipid solutions under vigorous mixing. LNPs were prepared by rapidly diluting this mixture with 1 mM citrate buffer (pH 4.5). The resulting LNP solution was diluted with Dulbecco's PBS(−) (D-PBS(−)) and subjected to two ultrafiltrations to remove the *t*-BuOH, replacing external buffer with D-PBS(−) and concentrating the LNPs using amicon ultra centrifugal filters. The size of the LNP in D-PBS(−) was measured by means of dynamic light scattering using a Zetasizer Nano ZS ZEN3600 instrument (Malvern Instruments, Worcestershire, UK). Number-weighted average sizes are expressed. The encapsulation efficiency and total concentration of siRNA were measured by a Ribogreen assay as described previously [25]. Apparent acid dissociation constant (pKa) of the LNPs was measured by TNS assay as described previously [25].

2.4. Cell culture and transfection

HeLa cells stably expressing Firefly and Renilla luciferase (HeLa-dluc) were cultured in growth medium, DMEM supplemented with 10% fetal bovine albumin, penicillin (100 U/mL), streptomycin (100 mg/mL) and G418 (0.4 mg/mL) at 37 °C in 5% CO_2. For luciferase gene silencing, HeLa-dluc cells were seeded at a density of 5×10^3 cells per well in 96-well plates in growth medium 24 h prior to transfection and incubated overnight at 37 °C in an atmosphere of 5% CO_2. For transfection, growth media containing LNPs at the indicated concentration of siGL4 were added to cells after aspiration of the spent media. The LNPs were allowed to incubate with cells for 24 h prior to analysis for luciferase expression.

HCT116 cells were cultured in growth media, DMEM supplemented with 10% FBS, penicillin (100 U/mL) and streptomycin (100 μg/mL) at 37 °C in 5% CO_2. For polo-like kinase 1 (PLK1) silencing, HCT116 cells were seeded at a density of 1.5×10^5 cells per well in 6-well plates in