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Structure, activity and uptake mechanism of siRNA-lipid nanoparticles with an asymmetric ionizable lipid

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ABSTRACT

Lipid nanoparticles (LNPs) represent the most advanced platform for the systemic delivery of siRNA. We have previously reported the discovery of novel ionizable lipids with asymmetric lipid tails, enabling potent gene-silencing activity in hepatocytes in vivo; however, the structure and delivery mechanism had not been elucidated. Here, we report the structure, activity and uptake mechanism of LNPs with an asymmetric ionizable lipid. Zeta potential and hemolytic activity of LNPs showed that LNPs were neutral at the pH of the blood compartment but become increasingly charged and fusogenic in the acidic endosomal compartment. ^{31}P NMR experiments indicated that the siRNA was less mobile inside particles, presumably because of an electrostatic interaction with an ionizable lipid. The role of Apolipoprotein E (apoE) was studied using recombinant human apoE both in vitro and in vivo. A comparative study in wild-type and apoE-deficient mice revealed that apoE significantly influenced the in vivo biodistribution of LNPs and enhanced the cellular uptake. Pretreatment of mice with siRNA targeting low-density lipoprotein receptor (LDLR) impaired gene-silencing of the following siRNA treatment, demonstrating that in vivo activity of LNPs is dependent on LDLR. Our studies on the detailed mechanism should lead to the creation of more sophisticated LNP-based RNAi therapeutics.

1. Background

The specific downregulation of gene expression by RNAi has the potential to create next-generation therapeutics for treating disorders for which small molecule or antibody treatments are ineffective (Elbashir et al., 2001). The key to enabling RNAi therapy is the development of clinically viable delivery materials (Whitehead et al., 2009). To date, considerable efforts have been directed toward the creation of delivery materials, including viral vectors(Waehler et al., 2007); self-delivered RNA with increased chemical and biological stability (Hickerson et al., 2011); targeting RNA with ligands composed of sugar (Nair et al., 2014; Rozema et al., 2007; Rozema et al., 2015; Prakash et al., 2014), and nanoparticles developed from lipid-like materials (Akinc et al., 2008; Love et al., 2010; Dong et al., 2014; Dahlman et al., 2014; Whitehead et al., 2014), and ionizable lipids (Semple et al., 2010; Jayaraman et al., 2012; Maier et al., 2013; Sato et al., 2012). Currently, lipid nanoparticles (LNPs) – an emerging category of nanomedicines – represent the most advanced platform for the systemic delivery of siRNA and have been tested in several clinical trials (Coelho et al., 2013; Fitzgerald et al., 2014). More recently, we have reported the discovery of novel ionizable lipids with an asymmetric lipid tail (Suzuki et al., 2015). The developed LNPs show potent hepatic gene-silencing activity in mice following intravenous injection ($ED_{50} \sim 0.02$ mg/kg), as well as long-term storage stability over 1.5 years. Because we have focused on screening viable materials, the detailed properties of developed LNPs have remained unclear. To date, several groups have reported the structure and uptake mechanism of LNPs (Akinc et al., 2010; Leung et al., 2012; Sahay et al., 2013; Gilleron et al., 2013; Wittrup et al., 2015), revealing that (a) LNPs have an electron-dense core and do not have an internal aqueous phase, as do conventional liposomal formulations (Leung et al., 2012), and (b) apolipoprotein E is an important endogenous protein that efficiently recruits LNPs to hepatocytes (Akinc et al., 2010). However, previously reported LNPs utilize an ionizable lipid with a symmetric lipid tail, which is distinct in chemical structure from our developed lipid (Suzuki et al., 2015). In nanomedicines, lipid components have a significant impact on the structure, activity and biodistribution (Allen and Cullis, 2013; Gindy et al., 2014; Leung et al., 2015; Mui et al., 2013). Therefore, clarifying the properties of novel LNPs with an asymmetric ionizable lipid is of great importance.

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In this work, we investigated the structure, activity and uptake mechanism of LNPs with an asymmetric ionizable lipid. First, the structure of LNPs was estimated using dynamic light scattering and ^{31}P NMR techniques. Second, to assume the course of endosomal escape, the relationship between membrane disruption and pH value was studied using a hemolysis assay. Finally, the in vivo uptake mechanism of LNPs was investigated using exogenous apoE and apoE-deficient mice. The role of apoE in the activity of LNP was studied to determine whether apoE plays a significant role in the delivery of siRNA to hepatocytes. Understanding the detailed uptake mechanism would provide novel strategies for the development of optimized LNPs for future RNAi therapeutics.

2. Materials and methods

2.1. Materials

The materials used in this study were purchased from the following companies: 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC) and cholesterol from Nippon Fine Chemical (Osaka, Japan); 1,2-dimyristoyl-sn-glycerol, methoxypolyethylene glycol 2000 (mPEG2000-DMG, Cat. No. GM-020) from NOF (Tokyo, Japan); sodium acetate, phosphate-buffered saline powder, Triton X-100, ethanol, 4% paraformaldehyde phosphate buffer solution and Dulbecco's modified Eagle's medium from Wako (Tokyo, Japan); penicillin-streptomycin and fetal bovine serum from ThermoFisher (Massachusetts, USA); Polyacrylamide precast gels (Cat. No. 2331750) from ATTO (Tokyo, Japan); xylene cyanol FF (Cat. No. 366-21) from Nacalai Tesque (Kyoto, Japan); SYBR Green II (Cat. No. 5771A) from Takara-bio (Shiga, Tokyo); Hoechst 33342 (Cat. No. H342) from Dojindo (Kumamoto, Japan); recombinant human apoE3 (Cat. No. SRP4696) from Sigma-Aldrich (Missouri, USA); siRNA from Gene Design (Osaka, Japan); Biophen FVII assay kit (Cat No. 221304) from Aniara (Ohio, USA); Quant-iT ribogreen RNA assay kit (Cat No. R11491) from Invitrogen (Massachusetts, USA); sensitized sheep red blood cells, which is a component of the CH50 assay kit (Cat No. 400017) from Denka Seiken (Tokyo, Japan); 100 kD Float-A-Lyzer G2 (Cat. No. G235035) from Spectrum Laboratories (California, USA); HeLa and HuH-7 cells from ATCC (Virginia, USA); female BALB/cCr Slc, C.KOR/StmSlc-*Apoe*^{shl}, and female ICR:Slc mice from Japan SLC (Shizuoka, Japan); Zetasizer from Malvern (Worcestershire, UK); IVIS imaging system Lumina II from PerkinElmer (Massachusetts, USA); NMR Spectrometer from Bruker (Massachusetts, USA); fluorescence microscope BZ-X710 from Keyence (Tokyo, Japan); and microplate reader SpectraMAX M5e from Molecular Devices (Washington, USA); imaging analyzer LAS-4000 from Fujifilm (Tokyo, Japan).

2.2. Ionizable lipid synthesis

The ionizable lipid 1-(2-octylcyclopropyl)heptadecan-8-yl-methylpiperidine-4-carboxylate was synthesized and purified by Sogo Pharmaceuticals as previously described (Suzuki et al., 2015).

2.3. Formulation of LNPs

The siRNA was dissolved in 25 mM sodium acetate at pH 4.0 to produce a 0.45 mg/mL siRNA solution. An ionizable lipid, DSPC, cholesterol and mPEG2000-DMG (60/8.5/30/1.5, mol/mol) were dissolved in ethanol to produce 40 mM total lipids. The siRNA/total lipid ratio was 0.06 (wt/wt). With two syringe pumps, the siRNA solutions and the lipid solutions were mixed at flow rates of 6 mL/min and 2 mL/min, respectively. The resultant solution was dialyzed with PBS at pH 7.4 overnight using 100 kD dialysis tubes and then filtered by a 0.22-μm membrane filter to produce LNPs, which were then used for further experiments.

2.4. Particle size, zeta potential and encapsulation efficiency

Zetasizer was used to determine the particle size and zeta potential. Zeta potential was measured on particles after suspending LNPs in deionized water at pH = 3.5–7.5. The free and total siRNA concentrations in LNPs were determined using the Quant-iT Ribogreen RNA assay kit as previously described(Walsh et al., 2014). The encapsulation efficiency (EE, %) was calculated as follows: EE (%) = (1 − free siRNA concentration/total siRNA concentration) × 100.

2.5. Stability of siRNA in LNP exposed to serum

In a plastic tube, 5 μL of naked siRNA (150 μM) or siRNA encapsulated in LNPs were mixed with 95 μL of freshly prepared mouse serum at 37 °C. The final concentration was 7.5 μM siRNA and 95% mouse serum. Aliquots was then mixed with loading buffer containing glycerol (10%), Triton X-100 (0.5%) and xylene cyanol (0.02%) in water, which were subjected to native PAGE (15% gel, 20 mA) using Tris-Glycine buffer for 40 min. Gels were stained with SYBR Green II and analyzed with an LAS-4000 imaging analyzer.

2.6. Hemolysis assay

Using a component of the CH50 assay kit, a fresh red blood cell (RBC) suspension was prepared according to the manufacturer's instruction. To a 96-well round-bottom plate, 100 μL of RBC suspension was mixed with 100 μL of 0.6, 0.06 and 0.006 mM LNPs in 10 mM HEPES and 130 mM NaCl buffer at pH 5.0–7.5. As a positive control, 100 μL of RBCs was mixed with 100 μL of 1% Triton X-100 to produce complete hemolysis (= 100% hemolysis). As a negative control, 100 μL of RBCs was mixed with 100 μL of saline to produce no hemolysis (= 0% hemolysis). After incubation at 37 °C for 2 h, the plates were centrifuged (4 °C, 400*g*, 5 min), and the supernatant was transferred to a 96-well clear-bottom plate. The absorbance of the supernatant at 405 nm was measured. The percentage hemolysis was calculated as follows: [1–(sample O.D. – negative control O.D.)/(positive control O.D. – negative control O.D.)] × 100.

2.7. ^{31}P NMR study

To avoid a ^{31}P signal from DSPC, LNPs containing FVII siRNA were formulated using an ionizable lipid, cholesterol and mPEG2000-DMG (60/38.5/1.5, mol/mol). Proton-decoupled ^{31}P NMR spectra were obtained using a Bruker AV 400 spectrometer operating at 162 MHz. Free induction decays (FID) corresponding to 5×10^4 scans were obtained with a 6.6 μs, 30° pulse with a 1 s interpulse delay and a spectral width of 65 kHz. An exponential multiplication corresponding to 10 Hz line broadening was applied to the FID prior to Fourier transformation. The sample temperature was regulated using a Bruker BCU 05 temperature unit. Measurements were performed at 25 °C.

2.8. Cellular uptake

HeLa and HuH-7 cells were maintained in Dulbecco's modified Eagle's medium with penicillin, streptomycin, and fetal bovine serum. For cellular uptake, 1×10^4 cells were seeded in 96-well clear-bottom plates overnight, and LNPs containing Alexa647-labeled siRNA were added at 20 nM for 1 h. In apoE preassociation experiments, LNPs containing Alexa647-labeled siRNA were premixed with 1 μg/mL recombinant human apoE protein for 5 min at 37 °C prior to use. Cells were fixed in 4% paraformaldehyde and counterstained with Hoechst. All of the images were acquired