Small interfering RNA delivery into the liver by cationic cholesterol derivative-based liposomes

Yoshiyuki Hattori1, Yoko Machida1, Maho Honda2, Nozomi Takeuchi1, Yuki Yoshiike3, Hiroaki Ohno2, and Hiraku Onishi1

1Department of Drug Delivery Research, Hoshi University, Tokyo, Japan and 2Graduate School of Pharmaceutical Sciences, Kyoto University, Kyoto, Japan

Abstract

Purpose: Previously, we reported that the cationic liposomes composed of a cationic cholesterol derivative, cholesteryl (2-((2-hydroxyethyl)amino)ethyl)carbamate (OH-C-Chol) and 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE) (termed LP-C), could deliver small interfering RNAs (siRNAs) with high transfection efficiency into tumor cells. In this study, to develop a liposomal vector for siRNA delivery in vivo, we prepared the poly(ethyleneglycol) (PEG)-modified cationic liposomes (LP-C-PEG) and evaluated their transfection efficiency in vitro and in vivo.

Materials and methods: We prepared LP-C-PEG/siRNA complexes (LP-C-PEG lipoplexes) formed in water or 50 mM NaCl solution, and evaluated their siRNA biodistribution and gene silencing effect in mice after intravenous injection.

Results: LP-C-PEG lipoplexes strongly exhibited in vitro gene silencing effects in human breast tumor MCF-7 cells as well as LP-C lipoplexes. In particular, formation of LP-C and LP-C-PEG lipoplexes in the NaCl solution increased the cellular association. When LP-C-PEG lipoplexes with Cy5.5-labeled siRNA formed in water or NaCl solution were injected into mice, accumulation of the siRNA was observed in the liver. Furthermore, injection of LP-C-PEG lipoplexes with ApoB siRNA could suppress ApoB mRNA levels in the liver and reduce very-low-density lipoprotein/low-density lipoprotein levels in serum compared with that after Cont siRNA transfection, although the presence of NaCl solution in forming the lipoplexes did not affect gene silencing effects in vivo.

Conclusions: LP-C-PEG may have potential as a gene vector for siRNA delivery to the liver.

Keywords

Cationic cholesterol derivative, cationic liposome, liver targeting, siRNA delivery, transfection

Introduction

Synthetic small interfering RNAs (siRNAs), which are small double-stranded RNAs, are substrates for the RNA-induced silencing complex (Kim et al., 2005). siRNAs suppress the expression of a target gene by triggering specific degradation of the complementary mRNA sequences. The liver is an important organ with a number of potential therapeutic siRNA targets, including cholesterol biosynthesis, fibrosis, hepatitis and hepatocellular carcinoma (Gonzalez-Rodriguez & Valverde, 2015). Although none of the currently available methods of siRNA delivery is optimal for liver siRNA therapy, concerted effort from researchers has provided a wide range of choices for siRNA transfer to the liver (Haynes & Huang, 2014).

For efficient siRNA delivery by cationic liposomes, many different cationic lipids have been synthesized. In particular, cholesterol- and glycerol-based cationic lipids have been used extensively for cationic liposome-mediated siRNA delivery. For gene delivery using cationic liposomes, cationic cholesterol derivatives are useful because of their high transfection efficiency and low toxicity (Hasegawa et al., 2002; Nakanishi, 2003). Cationic cholesterol derivatives are composed of three distinct parts: a cholesteryl skeleton, a cationic amino group and a linker arm between the cholesteryl skeleton and the cationic amino group. The stability and the toxicity of cationic cholesterol derivatives are determined by the linker. Cationic cholesterol derivatives with an ether linker are too stable to be biodegraded; therefore, chemically stable and biodegradable linkers such as a carbamate or amide are most commonly used.

Previously, we reported that cationic nanoparticles composed of N-(2-(2-hydroxyethylamino)ethyl)cholesteryl-3-carboxamide (OH-Chol) and Tween80 could deliver siRNA into tumor cells efficiently (Hattori et al., 2008b). OH-Chol is a cationic cholesterol derivative with a hydroxyethyl group at the amino terminus and a carboxamide-type linker (Figure 1). It has been reported that the hydroxyethyl group of OH-Chol reduced the stability of lipoplexes and enhanced transfection efficiency by facilitating the process by which nucleic acids
Therefore, in this study, to develop a liposomal vector for increase transfection efficiency of siRNA, we prepared PEGylated cationic liposomes (LP-PEG), and investigated whether LP-PEG could increase transfection efficiency in vivo, by forming lipoplexes in the presence of NaCl solution.

**Small interfering RNAs**

siRNAs targeting nucleotides of firefly pGL3 luciferase (Luc siRNA), Cy5.5-labeled Luc siRNA (Cy5.5-siRNA), nonsilencing siRNA (control [Cont] siRNA) as a negative control for Luc siRNA, cholesterol-modified apolipoprotein B siRNA (ApoB siRNA) and cholesterol-modified luciferase siRNA (Cont siRNA) as a negative control for ApoB siRNA were synthesized by Sigma Genosys (Tokyo, Japan). The siRNA sequences of the Luc siRNA were as follows: sense strand: 5′-GUGGAUUUUCAGUGUACGUCAUUAACCU-3′ and antisense strand: 5′-AAGACGAUCGAAAUCCACAUUUA-3′. In Cy5.5-siRNA, Cy5.5 dye was conjugated at the 5′-end of the sense strand. The siRNA sequences of the Cont siRNA as a negative control for Luc siRNA were as follows: sense strand: 5′-GUACCACUCUGCCUAUCGUACUC-3′ and antisense strand: 5′-UACCGAGUAGCCGUAGUCGUAC-3′. The siRNA sequences of the ApoB siRNA were as follows: sense strand: 5′-GUAUCACACUGAUUAACAUUA*Chol-3′ and antisense strand: 5′-AUUGGUAAUACUGUGUGAGAC*Chol-3′ (Soutschek et al., 2004). The siRNA sequences of the Cont siRNA as a negative control for ApoB siRNA were as follows: sense strand: 5′-GUAACUGUGUGAGCCU*Chol-3′ and antisense strand: 5′-AGGACCUCUCAACAGU*Chol-3′ (Soutschek et al., 2004). The lower-case letters represent 2′-O-methyl-modified nucleotides; asterisks represent phosphorothioate linkages. Alexa Fluor®488-labeled AllStars Negative Control siRNA (AF-siRNA) was obtained from Qiagen (Valencia, CA).

**Preparation of cationic liposomes and lipoplexes**

The cationic cholesterol derivative-based liposomes were prepared from OH-Chol/DOPE (composition designated as LP) and OH-C-Chol/DOPE (termed LP-C), LP-C lipoplexes exhibited a larger gene silencing effect than LP lipoplexes (Hattori et al., 2015). However, the systemic delivery of siRNA by the cationic liposomes may be hindered by the instability of lipoplexes in physiological conditions. The poly(ethylene glycol) (PEG)-modification of cationic liposomes by incorporating PEG-lipid could protect the lipoplexes from aggregation and macrophage capture, reduce protein absorption and consequently prolong the blood circulation (Zhang et al., 2012). Furthermore, it has been reported that cationic lipoplexes with plasmid DNA (pDNA) prepared in NaCl solution could increase transfection efficiency in vivo (Fumoto et al., 2004; Kawakami et al., 2005). Therefore, in this study, to develop a liposomal vector for siRNA delivery in vivo, we prepared PEGylated LP-C (termed LP-C-PEG), and investigated whether LP-C-PEG could increase transfection efficiency in vitro and in vivo by forming lipoplexes in the presence of NaCl solution.

**Materials and methods**

**Materials**

N-(2-(2-Hydroxyethylamino)ethyl)cholesteryl-3-carboxamide (OH-Chol) and cholesteryl (2-(2-hydroxyethyl)amino) ethyl)carbamate (OH-C-Chol) (Figure 1) were synthesized as described previously (Hattori et al., 2005, 2015). 1,2-Dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE) and methoxy-poly(ethylene glycol)-distearylphosphatidylethanolamine (PEG2000-DSPE, PEG mean molecular weight, 2000) were obtained from NOF Co. Ltd. (Tokyo, Japan). All other chemicals available were of the finest grade.

Figure 1. The structure of cationic cholesterol derivatives with a hydroxyethyl group at the amino terminus.

were liberated from endosomes (Hasegawa et al., 2002). Furthermore, cationic nanoparticles composed of OH-Chol/ Tween 80 resulted in efficient siRNA transfer into cells when positively charged nanoplexes was prepared in the presence of 50 mM NaCl solution (Hattori et al., 2008a,b). However, carboxamide-type cholesterol derivatives such as OH-Chol have a main drawback in their synthesis, especially for large-scale synthesis. Therefore, we designed a new carbamate-type cholesterol derivative, cholesteryl (2-((2-hydroxyethyl)amino)ethyl)carbamate (OH-C-Chol) (Figure 1). OH-C-Chol can be prepared easily from cholesteryl chloroformate (Hattori et al., 2008a,b) and is easily available from several companies.

Upon comparing the transfection efficiency of siRNA in cells between cationic liposomes composed of OH-Chol/1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE) (composition designated as LP) and OH-C-Chol/DOPE (termed LP-C), LP-C lipoplexes exhibited a larger gene silencing effect than LP lipoplexes (Hattori et al., 2015). However, the systemic delivery of siRNA by the cationic liposomes may be hindered by the instability of lipoplexes in physiological conditions. The poly(ethylene glycol) (PEG)-modification of cationic liposomes by incorporating PEG-lipid could protect the lipoplexes from aggregation and macrophage capture, reduce protein absorption and consequently prolong the blood circulation (Zhang et al., 2012). Furthermore, it has been reported that cationic lipoplexes with plasmid DNA (pDNA) prepared in NaCl solution could increase transfection efficiency in vivo (Fumoto et al., 2004; Kawakami et al., 2005). Therefore, in this study, to develop a liposomal vector for siRNA delivery in vivo, we prepared PEGylated LP-C (termed LP-C-PEG), and investigated whether LP-C-PEG could increase transfection efficiency in vitro and in vivo by forming lipoplexes in the presence of NaCl solution.

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**Small interfering RNAs**

siRNAs targeting nucleotides of firefly pGL3 luciferase (Luc siRNA), Cy5.5-labeled Luc siRNA (Cy5.5-siRNA), nonsilencing siRNA (control [Cont] siRNA) as a negative control for Luc siRNA, cholesterol-modified apolipoprotein B siRNA (ApoB siRNA) and cholesterol-modified luciferase siRNA (Cont siRNA) as a negative control for ApoB siRNA were synthesized by Sigma Genosys (Tokyo, Japan). The siRNA sequences of the Luc siRNA were as follows: sense strand: 5′-GUGGAUUUUCAGUGUACGUCAUUAACCU-3′ and antisense strand: 5′-AAGACGAUCGAAAUCCACAUUUA-3′. In Cy5.5-siRNA, Cy5.5 dye was conjugated at the 5′-end of the sense strand. The siRNA sequences of the Cont siRNA as a negative control for Luc siRNA were as follows: sense strand: 5′-GUACCACUCUGCCUAUCGUACUC-3′ and antisense strand: 5′-UACCGAGUAGCCGUAGUCGUAC-3′. The siRNA sequences of the ApoB siRNA were as follows: sense strand: 5′-GUAUCACACUGAUUAACAUUA*Chol-3′ and antisense strand: 5′-AUUGGUAAUACUGUGUGAGAC*Chol-3′ (Soutschek et al., 2004). The siRNA sequences of the Cont siRNA as a negative control for ApoB siRNA were as follows: sense strand: 5′-GUAACUGUGUGAGCCU*Chol-3′ and antisense strand: 5′-AGGACCUCUCAACAGU*Chol-3′ (Soutschek et al., 2004). The lower-case letters represent 2′-O-methyl-modified nucleotides; asterisks represent phosphorothioate linkages. Alexa Fluor®488-labeled AllStars Negative Control siRNA (AF-siRNA) was obtained from Qiagen (Valencia, CA).

**Preparation of cationic liposomes and lipoplexes**

The cationic cholesterol derivative-based liposomes were prepared from OH-Chol/DOPE (composition designated as LP) and OH-C-Chol/DOPE (composition designated as LP-C), at a molar ratio of 3:2 by a thin-film hydration method (Hattori et al., 2015). For PEGylated cationic liposomes, 1 mol% PEG2000-DSPE was added to the formulation of LP and LP-C (LP-PEG and LP-C-PEG, respectively). The thin film was hydrated with water at 60°C by vortex mixing. The liposomes were sonicated in a bath-type sonicator for 1 min at room temperature, and then filtered 10 times through 100 nm polycarbonate membrane filters (Whatman, Brentford, UK).

To prepare the cationic liposome/siRNA complexes (lipoplexes), each liposome preparation was added to 50 pmol siRNA in water (directly mixing of liposome suspension and siRNA solution dissolved in water) or 50 μL of 50 mM NaCl solution at a charge ratio (+:−) of 7:1 of cationic lipid to siRNA with gentle shaking and left at room temperature for 15 min. The charge ratio (+:−) of liposomes:siRNA is expressed as the molar ratio of cationic lipid to siRNA phosphate, since one cationic lipid molecule has only one positive charge.
Size and ζ-potential of cationic liposomes and lipoplexes

The particle size distributions of cationic liposomes and lipoplexes were measured by the cumulant method using a light-scattering photometer (ELS-Z2, Otsuka Electronics Co. Ltd., Osaka, Japan) at 25 °C after diluting the dispersion with an appropriate volume with water. The ζ-potentials were measured using the ELS-Z2 at 25 °C after diluting the dispersion with an appropriate volume of water.

Cell culture

Human breast cancer MCF-7-Luc (TamR-Luc#1) cells stably expressing firefly luciferase (pGL3) were donated by Dr. Kazuhiro Ikeda (Division of Gene Regulation and Signal Transduction, Research Center for Genomic Medicine, Saitama Medical University, Saitama, Japan). MCF-7-Luc cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM), supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 μg/mL kanamycin and 0.5 mg/mL G418 at 37 °C in a 5% CO₂ humidified atmosphere.

Luciferase activity

MCF-7-Luc cells were plated into 6-well culture dishes at a density of 1.5 × 10⁵ cells per well. For transfection, each lipoplex of 50 pmol Luc siRNA or Cont siRNA forming in water or 50 μL 50 mM NaCl at a charge ratio (+:−) of 7:1 was diluted in 1 mL of medium supplemented with 10% FBS, and then the mixture was added to the cells. Forty-eight hours after transfection, luciferase activity was measured as counts per second (cps)/μg protein using a luciferase assay system (Pica Gene, Toyo Ink Mfg. Co. Ltd., Tokyo, Japan) and BCA method (Berthold Technologies). The tissues after fluorescence imaging, the excitation and emission wavelengths were set at 630/20 and 680/30 nm, respectively. The exposure time for fluorescence was 5 s. A grayscale body-surface reference image was collected using a NightOWL LB981 CCD camera. The images were analyzed using the IndiGo2 software provided with the in vivo imaging system (Berthold Technologies). The tissues after fluorescence imaging were frozen on dry ice and sliced at 16 μm thicknesses. The localization of Cy5.5-siRNA was examined using an Eclipse TS100-F microscope.

ApoB mRNA level in the liver and low-density lipoprotein/very-low-density lipoprotein levels in serum

All animal experiments were performed with the approval from the Institutional Animal Care and Use Committee of Hoshi University. LP-PEG and LP-C-PEG lipoplexes with 25 μg Cy5.5-siRNA formed at a charge ratio (+:−) of 7:1 in water or 100 μL 50 mM NaCl solution were administered intravenously via the lateral tail veins into the female BALB/c mice (8 weeks of age; Sankyo Lab Service Corp., Tokyo, Japan). One hour after the injection of cationic lipoplexes, the mice were sacrificed, and Cy5.5 fluorescence imaging of the tissues were obtained using a NightOWL LB981 NC100 system (Berthold Technologies, Bad Wildbad, Germany). In Cy5.5 fluorescence imaging, the excitation and emission filters were set at 630/20 and 680/30 nm, respectively. The exposure time for fluorescence was 5 s. A grayscale body-surface reference image was collected using a NightOWL LB981 CCD camera. The images were analyzed using the IndiGo2 software provided with the in vivo imaging system (Berthold Technologies). The tissues after fluorescence imaging were frozen on dry ice and sliced at 16 μm thicknesses. The localization of Cy5.5-siRNA was examined using an Eclipse TS100-F microscope.

Agglutination assay

Erythrocytes were collected from mouse blood at 4 °C by centrifugation at 300 g for 3 min and resuspended in phosphate-buffered saline (PBS) as a 2% (v/v) stock suspension of erythrocytes. Each liposome preparation was mixed with 2 μg of siRNA at a charge ratio (+:−) of 7:1 in water or 50 μL 50 mM NaCl solution. The lipoplexes were added to 100 μL erythrocyte suspension. After incubation for 15 min at 37 °C, the sample was placed on a glass plate and agglutination was observed by microscopy.
Determination of transaminase activities in serum

LP-C-PEG lipoplexes with 25 µg Cont siRNA or ApoB siRNA formed in water or 100 µL 50 mM NaCl solution was administered intravenously via the lateral tail veins into the female BALB/c mice once a day for three consecutive days. For the measurement of aspartate aminotransferase (AST/GOT) and alanine aminotransferase (ALT/GPT) activities, serum was prepared by the separation of coagulated whole blood from mice at 24 h after the final injection of the lipoplexes. GOT and GPT levels in the serum were determined using commercially available reagent kits (Transaminase CII-test kit, Wako Pure Chemicals, Osaka, Japan). Normal values were determined using blood obtained from the age-matched untreated mice.

Statistical analysis

Data were compared using analysis of variance and evaluated with Student’s t test. A p values of 0.05 or less was considered significant.

Results

Characterization of cationic lipoplexes

In this study, we prepared PEGylated LP-C (LP-C-PEG), and evaluated in vitro and in vivo gene silencing effects. First, we examined the formulation of cationic cholesterol-based liposomes for siRNA delivery. Here, we used OH-Chol and OH-C-Chol as cationic cholesterol derivatives for the preparation of cationic liposomes. LP and LP-C were prepared from OH-Chol/DOPE and OH-C-Chol/DOPE, respectively, at a molar ratio of 3:2. For PEGylated liposomes, LP-PEG and LP-C-PEG included 1 mol% PEG2000-DSPE into the formulations of LP and LP-C, respectively. The sizes of LP, LP-PEG, LP-C, and LP-C-PEG were 164, 164, 153, and 138 nm, respectively, and their ζ-potentials were 51 ± 51, 28 ± 28, 53 ± 53, and 49 ± 49 mV, respectively (Table 1). PEGylation of LP and LP-C reduced their ζ-potentials.

We reported previously that LP and LP-C lipoplexes forming at a charge ratio (+/-) of 7:1 exhibited high suppression of a target gene in cells (Hattori et al., 2015). Therefore, we examined the physicochemical properties of lipoplexes formed at a charge ratio (+/-) of 7:1 in water or NaCl solution. Regarding the concentration of NaCl, 50 mM was used from a previous report (Hattori et al., 2008b). The sizes of LP and LP-PEG lipoplexes formed in water were ~208 and 383 nm, and their ζ-potentials were ~50 and 39 mV, respectively (Table 1). Furthermore, the sizes of LP and LP-PEG lipoplexes formed in NaCl solution were ~290 and 303 nm, respectively, and their ζ-potentials were ~50 and 40 mV, respectively. In contrast, the sizes of LP-C and LP-C-PEG lipoplexes formed in water were ~215 and 180 nm, respectively, and their ζ-potentials were ~55 and 45 mV, respectively. The presence of NaCl solution during the formation of LP-C and LP-C-PEG lipoplexes did not greatly affect the size of their lipoplexes. Among the lipoplexes, LP-C-PEG lipoplexes were smallest in size (about 180 nm) even when they were formed in NaCl solution.

Effect of formation of lipoplexes in NaCl solution on gene knockdown efficacy

We examined the effect of the presence of NaCl during the formation of lipoplexes on gene knockdown. Lipoplexes with 50 nM siRNA were added into human breast tumor MCF-7-Luc cells. LP lipoplexes with Luc siRNA formed in NaCl solution exhibited moderate suppression (Figure 2A). Generally, PEGylation abolishes the effect of gene suppression by siRNA owing to the high stability of the lipoplexes; however, PEGylation of LP did not greatly affect the suppression of luciferase activity (Figure 2A). In transfection of siRNA with LP-C and LP-C-PEG, the lipoplexes with Luc siRNA formed in water or NaCl solution strongly suppressed luciferase activities, similar to a commercially available reagent, Lipofectamine RNAiMax (Figure 2B). In contrast, large reductions in luciferase activity were not observed after transfection of Cont siRNA using LP, LP-PEG, LP-C, or LP-C-PEG (Figure 2A and B).

Association of nanoplexes with cells

Next, to examine the effect of PEG-modification of cationic liposomes on cellular uptake, we observed the cellular uptake...
of siRNA 3 h after transfection with LP, LP-PEG, LP-C, and LP-C-PEG lipoplexes by means of fluorescence microscopy (Figure 3A). siRNAs transfected using LP-C lipoplexes were strongly detected in the cells compared with those involving LP lipoplexes; however, PEGylation of LP-C and LP lipoplexes decreased the amount of siRNA taken up by the cells. Furthermore, we observed the cellular uptake of siRNA 24 h after transfection with those lipoplexes. siRNAs transfected using LP-C lipoplexes were strongly detected in the cells compared with those involving LP-C-PEG lipoplexes (Supplemental Figure 1). These results indicated that PEG coating of the liposomes reduced cellular association although it did not fully abolish the effect of gene suppression by siRNA.

To clarify the mechanism of increased gene silencing by forming the lipoplexes in NaCl solution, we examined the cellular association by flow cytometric analysis. Cellular association with LP, LP-PEG, LP-C and LP-C-PEG lipoplexes was increased when the lipoplexes were formed in NaCl solution (Figure 3B–E). These results suggested that the formation of LP or LP-PEG lipoplexes in NaCl solution increased gene knockdown efficacy by means of an increase of cellular association.

Interaction with erythrocytes

Electrostatic interactions between positively charged lipoplexes and negatively charged erythrocytes can cause agglutination. The agglutinates contribute to high entrapment of the lipoplex in the highly extended lung capillaries (Eliyahu et al., 2002). Therefore, we evaluated the agglutination with erythrocytes by cationic lipoplexes. As a result, LP and LP-C lipoplexes formed in water or NaCl solution exhibited agglutination with erythrocytes (Figure 4). In contrast, LP-PEG and LP-C-PEG lipoplexes formed in water or NaCl solution did not exhibit agglutination with erythrocytes, indicating that PEG coating on the surface sterically hindered the interaction of erythrocyte with the lipoplexes.

Biodistribution of small interfering RNA after injection

We examined the biodistribution of siRNA after intravenous injection of LP-PEG and LP-C-PEG lipoplexes formed in water or NaCl solution. For in vivo experiments, we used PEGylated liposomes, because PEGylation could protect liposomes from agglutination with blood components (Figure 4). When LP-PEG lipoplexes formed in water was injected intravenously into mice, siRNA largely accumulated in the liver (Figure 5A and B); however, the lipoplexes formed in NaCl solution accumulated in both the lungs and liver. In contrast, intravenous injection of LP-C-PEG lipoplex formed in water or NaCl solution accumulated siRNA in the liver. These results suggested that LP-PEG lipoplexes might be destabilized in blood circulation by forming lipoplexes in NaCl solution.

Gene knockdown in liver

To investigate whether LP-C-PEG lipoplexes could suppress the expression of a target gene in the liver, we chose ApoB gene, a hepatocyte-expressed gene involved in cholesterol transport. We evaluated gene knockdown of ApoB mRNA using LP-C-PEG lipoplexes formed in water or NaCl solution, because their lipoplexes could efficiently suppress gene expression in the cells (Figure 2) and deliver siRNA efficiently in the liver by intravenous injection (Figure 5). We reported previously that the injection of naked ApoB siRNA did not affect ApoB mRNA levels in the liver compared with Cont siRNA (Hattori et al., 2014). As a result, both LP-C-PEG lipoplexes formed in water and NaCl solution could significantly suppress the ApoB mRNA levels in the liver, compared with Cont siRNA (~59 and 62% knockdown, respectively), but the presence of NaCl during the formation
of LP-C-PEG lipoplexes did not increase the gene silencing effect on the ApoB mRNA in the liver (Figure 6A).

ApoB is an essential protein in the formation of LDL and VLDL in the metabolism of dietary and endogenous cholesterol. Therefore, we measured the LDL and VLDL (LDL/VLDL) cholesterol level in serum after injection of LP-C-PEG lipoplexes for three consecutive days. Injections of LP-C-PEG lipoplexes with ApoB siRNA formed in water or NaCl solution resulted in \( C24 \) \( 54\% \) and \( 60\% \) reductions (0.019 ± 0.003 mg/ml and 0.021 ± 0.005 mg/ml) compared with Cont siRNA (0.040 ± 0.007 mg/ml and 0.053 ± 0.007 mg/ml), respectively (Figure 6B). This result indicated that the reduction in ApoB level in the liver induced a decrease in LDL/VLDL cholesterol level in serum. These data suggested that LP-C-PEG lipoplexes could deliver siRNA into the liver by intravenous injection and suppress the expression of a target gene in hepatocytes.

**Side-effects for the liver**

For the clinical application of cationic lipoplexes, toxicity is an important factor. Therefore, in order to evaluate liver toxicity to mice, we assessed GOT and GPT levels in serum after intravenous injections of LP-C-PEG lipoplexes with Cont or ApoB siRNA once a day for three consecutive days. As a result, injections of LP-C-PEG lipoplexes formed in water or NaCl solution did not elevate GOT and GPT levels in serum at 24 h after the final injection (Figure 7A and B). These results suggested that LP-C-PEG lipoplexes had no side-effects with regard to hepatotoxicity by intravenous injection.

**Discussion**

Previously, we reported that cationic liposomes composed of OH-C-Chol (Figure 1) and DOPE (LP-C) could deliver siRNA into tumor cells and suppress the expression of a target gene (Hattori et al., 2015). DOPE is thought to improve transfection efficiency by destabilizing the endosomal membrane, thereby facilitating the release of nucleic acids into the cytoplasm (Liu & Huang, 2002; Wasungu & Hoekstra, 2006). Furthermore, we demonstrated that cationic cholesterol-based nanoparticles composed of OH-Chol/Tween 80 resulted in efficient siRNA transfer into cells when positively charged nanoplexes were prepared in the presence of 50 mM NaCl (Hattori et al., 2008b). Therefore, in this study, to develop a liposomal vector for siRNA delivery in vivo, we prepared PEGylated LP-C (LP-C-PEG), and investigated whether LP-C-PEG could increase the transfection efficiency in vitro and in vivo by forming lipoplexes in the presence of NaCl solution.

For the ideal design of siRNA lipoplexes, cationic lipoplexes needs to be stabilized without any loss knockdown ability. In in vitro transfection analyses, LP-C-PEG lipoplexes
strongly exhibited gene silencing effects in tumor cells as well as LP-C lipoplexes (Figure 2). Generally, PEG coating of liposomes inhibits cellular association and/or fusion with endosomal membranes, thus decreasing silencing or transfection efficiency. However, PEGylation of LP-C with 1 mol% PEG$_{2000}$-DSPE did not reduce the knockdown efficiency (Figure 2). Daniels et al. reported that incorporation of 2 mol% or 5 mol% PEG$_{2000}$-DSPE into liposomes composed of cationic cholesterol derivatives (Chol-T or MS09)/DOPE only marginally reduced knockdown efficiency.
In contrast, Zhang et al. reported that cationic liposomes composed of 3\([N-(N',N'-\text{dimethylaminoethane})-\text{carbamoyl}]\) cholesterol (DC-Chol)/DOPE did not show any siRNA silencing efficiencies by PEGylation with 1\(\sim 5\) mol% PEG2000-DSPE (Zhang et al., 2010). In our preliminary study, PEGylated LP-C with 1 mol% PEG2000-DSPE exhibited gene silencing effects, but PEGylation with 2 mol% or 3 mol% PEG2000-DSPE abolished gene silencing effects by LP-C (data not shown). These findings indicated that the effect of PEGylation of cationic liposomes on silencing effect by siRNA might be dependent on the type of cationic cholesterol derivatives. As a result, in OH-C-Chol- and OH-Chol-based liposomes, incorporation of 1 mol% PEG2000-DSPE into the formulation of OH-C-Chol/DOPE or OH-Chol/DOPE did not largely affect knockdown efficiency, but it was not clear why the reduction in cellular uptake by the PEGylated lipoplexes (Figure 3A) did not reduce the gene silencing effect (Figure 2).

LP-C-PEG lipoplexes exhibited higher cellular association and knockdown efficiency than LP-PEG lipoplexes (Figures 2 and 3). The difference between OH-C-Chol and OH-Chol in terms of structure (Figure 1) is the linker between the cholesteryl skeleton and the cationic amino group. The linker group of cationic cholesterol derivatives controls the conformational flexibility, degree of stability, biodegradability and gene transfection efficacy (Ghosh et al., 2000;}

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the liver. The formation of LP-C-PEG lipoplexes for the siRNA delivery to the liver. The results provided optimal conditions for the cationic lipid for efficient siRNA delivery.

In \textit{in vivo} transfection, injection of LP-C-PEG lipoplexes formed in water or NaCl solution produced an accumulation of siRNA in the liver (Figure 5), but the suppression of ApoB mRNA levels by LP-C-PEG lipoplexes with ApoB siRNA formed in NaCl solution was similar to that by LP-C-PEG lipoplexes formed in water (Figure 6A). Kawakami et al. reported that 1,2-di-octadecenyl-3-trimethylammonium propane chloride (DOTMA)/Chol lipoplexes (about 120 nm in size) with pDNA formed in 10 mM NaCl solution could induce high transfection efficiency in the lungs when injected intravenously into mice (Kawakami et al., 2005). Furthermore, Fumoto et al. reported that galactosylated DOTMA/Chol lipoplexes (about 120 nm in size) formed in 5 mM NaCl solution increased the transfection activity of pDNA into hepatocytes when injected \textit{via} the portal vein of mice (Fumoto et al., 2004). They suggested that the presence of NaCl during lipoplex formation can regulate the repulsion between cationic lipoplexes. Moderate neutralization of the positive charge on the surface of cationic lipoplexes with a suitable concentration of NaCl can ensure sufficient repulsion of the lipoplexes and prevent the aggregation of lipoplexes in ionic solution. The stabilized effect of lipoplexes in ionic solution might increase cell association and knockdown efficiency. In our study, we used 50 mM NaCl in the formation of cationic lipoplexes in accordance with our previous report (Hattori et al., 2008b). The optimal concentration of NaCl might be different for cationic lipids of cationic liposomers. However, it has been reported that the penetration of cationic lipoplexes through the hepatic fenestrated endothelium into parenchymal cells was restricted by the size. The average diameter of fenestrae is 141 nm in C57BL/6 mice and 107 nm in humans with a healthy liver (Jacobs et al., 2010). From these findings, the reason that formation of LP-C-PEG lipoplexes in NaCl solution could not increase transfection efficiency \textit{in vivo} might be due to size of the lipoplexes (about 180 nm). The size of LP-C-PEG lipoplexes formed in NaCl solution must be reduced for efficient \textit{in vivo} transfection into hepatocytes \textit{via} the fenestrated endothelium.

In conclusion, in this study, we applied OH-C-Chol-based cationic liposomes for synthetic siRNA delivery vector and optimized a lipoplex-forming solution for efficient transfection. Formation of LP-C-PEG lipoplexes in NaCl solution could increase transfection efficiency \textit{in vitro} but did not largely affect the gene silencing effect \textit{in vivo}. Cationic liposomes composed of OH-C-Chol, DOPE and PEG-DSPE may have potential as a safe gene vector for siRNA delivery to the liver. The results provided optimal conditions for the formation of LP-C-PEG lipoplexes for the siRNA delivery to the liver.

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Declaration of interest
The authors declare no conflicts of interest.

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Supplementary material available online
Supplemental Figure S1