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Development of a 2′,4′-BNA/LNA-based siRNA for Dyslipidemia and Assessment of the Effects of Its Chemical Modifications *In Vivo*

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Recent advances in RNA interference (RNAi)-based drug development have partially allowed systemic administration of these agents *in vivo* with promising therapeutic effects. However, before chemically modified small-interfering RNAs (siRNAs) can be applied clinically, their *in vivo* effects should be thoroughly assessed. And while many studies have assessed the effects of chemically modified siRNAs *in vitro*, there has been no comprehensive assessment of their effects *in vivo*. Here, we aimed to elucidate the effects of administering chemically modified siRNAs *in vivo* and to propose a 2′,4′-bridged nucleic acid (BNA)/locked nucleic acid (LNA)-based siRNA candidate for dyslipidemia. A potentially therapeutic siRNA, siL2PT-1M, was modified with phosphorothioate (PS) and 2′,4′-BNA/LNA in its sense strand and with 2′-methoxy (2′-OMe) nucleotides in its immunostimulatory motif; administration of siL2PT-1M resulted in sustained reductions in serum total cholesterol (TC) (24 days) and a concomitant apolipoprotein B (apoB) mRNA reduction in liver without adverse effects. The 2′,4′-BNA/LNA modification in the sense strand was greatly augmented the duration of the RNAi effect, whereas cholesterol conjugation shortened the duration. Cholesterol-conjugated immunostimulatory siRNA (isRNA) induced higher serum interferon-α (IFN-α) levels than did nonmodified isRNA, indicating that the immune reaction was facilitated by cholesterol conjugation. Our results indicated that modification of the adenosine residues complementary to the immunostimulatory motif and of central 5′-UG-3′ in the sense strand would ameliorate the negative immune response.

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Introduction

RNA interference (RNAi) is an emerging therapeutic strategy for chronic or genetic disorders.[1,2] However, RNAi agents such as small-interfering RNA (siRNA) have some unfavorable properties including low stability toward nucleases, inadequate biodistribution and nonspecific off-target effects (OTEs).[3–5] Many attempts have been made to develop siRNA delivery vehicles and artificial nucleic acids to achieve RNAi effects for *in vivo*. Indeed, a number of studies have succeeded in delivering siRNA to specific organs–including the liver, kidney, lung, or brain–or to several types of tumors.[6–8] Notably, stable nucleic acid lipid particle formulations[9] and lipidoids[10] are representative delivery vehicles that efficiently deliver the siRNA to the liver.

In nucleic acid chemistry, many different artificial nucleic acids have been developed to alter the behavior of siRNAs under physiological conditions. In particular, phosphorothioate (PS), 2′-methoxy (2′-OMe), and 2′-fluoro nucleic acid have often been used to modify the siRNA.[4,11,12] Our group has developed a novel class of conformationally restricted artificial nucleic acids [bridged nucleic acids (BNAs)] and characterized their basic properties.[13] The bridged structure between 2′-*O* and 4′-*C* fixes the furanose ring to the *N*-type and 2′,4′-BNA gives its nucleotide analog high nuclease resistance and affinity to complementary RNA.[14,15] The concept of introducing a bulky group at the 2′-position of the furanose ring to achieve high nuclease resistance has little impact on sugar fluctuation, whereas in the BNAs, the bicyclic structure provides steric hindrance at the phosphodiester backbone and the reduction of entropic loss by the sugar fluctuation, and thus both high nuclease resistance and stabilization of the RNA duplex are realized. Many studies related to usage of 2′,4′-BNA/locked nucleic acid (LNA) modification in siRNAs have been conducted *in vitro*.[15–19] Elmén *et al*. showed the RNAi effect by kind of 2′,4′-BNA/LNA-modified siRNAs targeting luciferase *in vitro* and elucidated the alteration of strand bias, elongation of half-life of siRNA and influences of modification site by 2′,4′-BNA/LNA modification in canonical siRNA.[15] Kjems *et al.* suggested a novel structural siRNA variant, the small internally segmented interfering RNA, and showed that the 2′,4′-BNA/

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LNA-modified small internally segmented interfering RNA improved the RNAi effect and abolished unintended mRNA degradation by RNA-induced silencing complex (RISC) containing the sense strand.[16] They also demonstrated combination effect on RNAi effect *in vitro* by using 2′,4′-BNA/LNA, unlocked nucleic acid or other artificial nucleic acids in canonical siRNA targeting enhanced green fluorescent protein.[18] However, to the best of our knowledge, no study has attempted to comprehensively assess the effects caused by systemic administration of 2′,4′-BNA/LNA-modified siRNAs combined with other artificial nucleic acids *in vivo*. Many unclear functions remain on the 2′,4′-BNA/LNA with or without other artificial nucleic acids in siRNA *in vivo*. Here, we assessed the effects caused by 2′,4′-BNA/LNA modification in combination with other modifications (PS, 2′-OMe, and 3′-cholesterol conjugation) in siRNA using an *in vivo* model of dyslipidemia (**Figure 1a**).

Most siRNAs designed for a given target with phosphorodiester backbone can stimulate cytokine production, because most siRNAs contain immunostimulatory motifs such as GU-rich, AU-rich, 5′-UCA-3′.[20] Because chemical modification of an antisense strand with artificial nucleic acids is generally not tolerable in the RNAi machinery, except in the case of 2′-fluoro nucleotide,[21] efforts to reduce OTEs on immune responses have focused on chemical modification of the sense strand of siRNA, if possible, when the antisense strand of a potential siRNA contains an immunostimulatory motif. However, there are no universal rules regarding modification of siRNAs with the intent to abrogate the innate immune responses while maintaining their RNAi activity, and thus is necessary to identify the relevant properties of each modification empirically.[22] In the present work, therefore, we attempted to elucidate the impacts of four types of chemical modification on several important biological parameters–pharmacological effects, innate immune response and other toxicities–in chronological order for *in vivo*.

Familial hypercholesterolemia is one of the best-known of the genetic and metabolic disorders, and is caused by mutations on genes related to the low-density lipoprotein (LDL) receptor pathway. In particular, in homozygous familial hypercholesterolemia patients, LDL cholesterol levels do not respond to pharmaceutical treatment including statins, ezetimibe, and resin enough to reduce coronary risk.[23,24] There is thus an enormous need to develop novel drugs for the efficient and continuous reduction of LDL cholesterol levels in these patients. Several studies have attempted to use siRNAs that target apolipoprotein B (apoB) as a means of lowering LDL cholesterol, and one of the reported potential sequences has an immunostimulatory motif in the antisense strand.[25] Here, we have used this siRNA sequence to generate chemically modified variants (*e.g.*, PS, 2′-OMe, 2′,4′-BNA/LNA, and cholesteryl-triethyleneglycol-conjugated variants), and these siRNAs were administered to C57BL/6J mice that were fed an atherogenic diet to assess the effects of each or combined modification on pharmacological parameters, innate immune response, and toxicities and to identify the candidate modification for development of a therapeutic siRNA-targeting apoB mRNA.

Results

2′,4′-BNA/LNA-modified siRNAs showed similar levels of RNAi efficacy as well as siApoB-1

We designed two types of 2′,4′-BNA/LNA-modified siRNA, called siLNA-1 and siLNA-2, according to our previous study in addition to nonmodified siApoB-1 (**Table 1**).[19] There are five consecutive 2′,4′-BNA/LNA modifications at the 5′-end of the sense strand of siLNA-1 that were designed to prevent the OTE related to incorporation of an unexpected strand into RISC.[15,26,27] In contrast, siLNA-2 has three 2′,4′-BNA/LNA modifications around the 5′-end of the sense strand and two near the 3′-end of the sense strand. The 3′-end modifications were designed to enhance the nuclease resistance, because

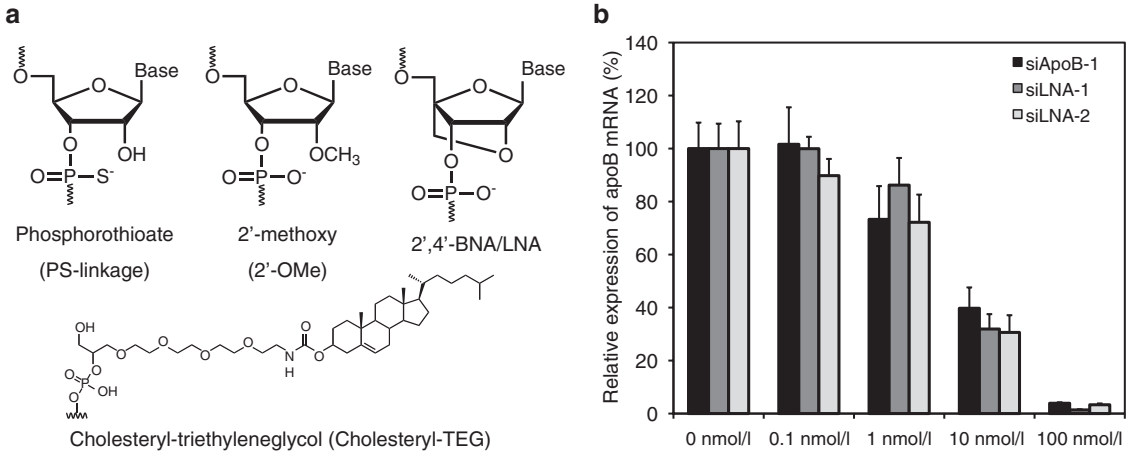

Figure 1 Structures of chemical modifications used in the study and concentration-dependent RNAi effect of nonmodified or 2′,4′-BNA/LNA modified siRNAs. (**a**) Structures of the artificial nucleic acid and cholesterol conjugates used in this study. (**b**) RNAi effect of 2′,4′-BNA/LNA modified siRNAs on apolipoprotein B (apoB) mRNA in NMuLi, mouse hepatic cells. ApoB mRNA expression was normalized to GAPDH expression. Error bar indicates SD values in this experiment.

Table 1 Sequence and T_m value (°C) of modified and nonmodified siRNAs used in this study

siRNA	*Strand*	*Sequence*	*Tm (°C)*
siApoB-1	Sense	5′-gucaucacacugaauaccaauu(dT)(dT)-3′	70.6
	Antisense	3′-(dT)(dT)caguagugugacuuaugguuaa-5′	
siLNA-1	Sense	5′-GTCATcacacugaauaccaauu(dT)(dT)-3′	80.8
	Antisense	3′-(dT)(dT)caguagugugacuuaugguuaa-5′	
siLNA-2	Sense	5′-GTcaTcacacugaaTacCaauu(dT)(dT)-3′	83.3
	Antisense	3′-(dT)(dT)caguagugugacuuaugguuaa-5′	
siL2PT-1	Sense	5′-GTcsasTcsascacugaaTascsCasasusus(dT)(dT)-3′	82.9
	Antisense	3′-(dT)(dT)caguagugugacuuaugguuaa-5′	
siL2PTC-1	Sense	5′-GTcsasTcsascacugaaTascsCasasusus(dT)(dT)_Chol-3′	83.0
	Antisense	3′-(dT)(dT)caguagugugacuuaugguuaa-5′	
siL2PTC-1M	Sense	5′-GTcsasTcsascacugaaTascsCasasusus(dT)(dT)_Chol-3′	84.9
	Antisense	3′-(dT)(dT)caguagu(M)gu(M)gacuuaugguuaa-5′	
siL2PTC-1L	Sense	5′-GTcsasTcsascacugaaTascsCasasusus(dT)(dT)_Chol-3′	85.8
	Antisense	3′-(dT)(dT)caguagTgTgacuuaugguuaa-5′	
siApoB-1C	Sense	5′-gucaucacacugaauaccaauu(dT)(dT)_Chol-3′	70.0
	Antisense	3′-(dT)(dT)caguagugugacuuaugguuaa-5′	
siL2PT-1M	Sense	5′-GTcsasTcsascacugaaTascsCasasusus(dT)(dT)-3′	85.6
	Antisense	3′-(dT)(dT)caguagu(M)gu(M)gacuuaugguuaa-5′	
siNTCtrl	Sense	5′-guaucucuucauagccuua(dT)(dT)-3′	N.D.
	Antisense	3′-(dT)(dT)cauagagaaguaucggaau-5′	

Abbreviations: apoB, apolipoprotein B; BNA, bridged nucleic acid; siRNA, small-interfering RNA; LNA, locked nucleic acid.
The following characters were used in this table to express each modification, upper cases: 2′,4′-BNA/LNA; lower cases: natural RNA; small "s": phosphorothioate modification; "Chol": cholesteryl-TEG conjugation; "(M)": 2′-Methoxy modified nucleotide; and "dT": thymine deoxyribonucleotide which was introduced at two overhang nucleotides. As the duplex stability, T_m values were analyzed with a UV experiment in a subset of siRNAs. The T_m value of siNTCtrl was not determined, and marked ND.

siRNAs are mainly degraded by 3′-exonuclease.[12,28,29] siLNA-1 and -2 have the same number of 2′,4′-BNA/LNA modifications, and each has a higher T_m value than does siApoB-1 (**Table 1**).

Using mouse hepatic cells (NMuLi), the RNAi effect of each siLNA was measured and compared to that of siApoB-1. Treatment with either siLNA-1 or -2 resulted in a concentration-dependent reduction in apoB mRNA expression, and the maximum reduction by siLNA-1 or -2 was by up to >95% at 100 nmol/l concentration of siRNA as did siApoB-1 (**Figure 1b**). The IC_{50} values of siApoB-1, siLNA-1, and siLNA-2 were 4.4, 4.4, and 2.9 nmol/l, respectively. There was no loss of RNAi effect by 2′,4′-BNA/LNA modification in these siLNAs.

2′,4′-BNA/LNA modification extends the half-life of siRNA

Previously, the bicyclic structure of nucleotides was shown to substantially enhance the nuclease resistance of siRNA.[15,19,30] We assessed the effects of 2′,4′-BNA/LNA modifications on half-life of siLNAs compared with that of nonmodified siApoB-1. Each siRNA (siApoB-1, siLNA-1, and siLNA-2) was incubated in mouse serum at 37 °C, and samples of the siRNA-serum mixtures were taken at several time points and analyzed using polyacrylamide gel electrophoresis. Nonmodified siApoB-1 was rapidly degraded within 2 hours, whereas each siLNA showed significant elongation of the stability against enzymatic degradation (**Figure 2a**). Notably, the intact duplex band of siLNA-2 was still detectable until 48 hours of incubation (**Figure 2a**). The half-life of siApoB-1 was ~138 minutes, whereas those of siLNA-1 and siLNA-2 were 268 minutes and 1,106 minutes, respectively (**Figure 2b**). Chemical modification of the sense strand alone resulted in a substantial increase in the half-life of the siRNAs. The half-life of siLNA-2 was longer than that of either of the other siRNAs (siApoB-1 or siLNA-1), and it was approximately eightfold longer than that of siApoB-1. The extremely long half-life of siLNA-2 was attributed to 2′,4′-BNA/LNA-mediated protection of the region around the 3′- and 5′-ends of the sense strand, which appeared to change the environment around 3′-end of both the sense and antisense strands of siRNA.

Design of further modified siRNAs based on siLNA-2 and their RNAi effect *in vitro*

Because siLNA-2 had a substantial RNAi effect and was highly resistant to nuclease, four other siRNAs with additional chemical modifications were designed based on siLNA-2 (**Table 1**). PS modifications were added to the 5′- and 3′-ends of the sense strand of siLNA-2 to generate siL2PT-1. These

additional modifications were designed to achieve further enhanced stability toward nucleases and to assess the alterations on the immune response by partially overlapping the complementary motif of the immunostimulatory motif (the underlined motif in **Table 1**). Based on the work reported by Davies *et al*, 3′-end conjugation on a canonical siRNA might disturb the interaction between 3′-conjugated siRNA and Toll-like receptor 3 (TLR3) by its steric hindrance.[31] We hypothesized that conjugation of a cholesterol moiety to the sense strand of siRNA would affect its immunostimulatory properties and, therefore, that the cholesterol-conjugated siRNA would induce a smaller immune response than its nonmodified counterparts, or possibly no immune response at all. Thus, siL2PTC-1 was designed to have an additional cholesterol conjugation at the 3′-end of the sense strand of siL2PT-1. Previously, the 2′-OMe[32] and 2′,4′-BNA/LNA[33] modifications were demonstrated to eliminate the immune response of the siRNA if these modifications were in the immunostimulatory motif. Therefore, siL2PTC-1M and siL2PTC-1L, which were designed based on siL2PTC-1, have two additional 2′-OMe or 2′,4′-BNA/LNA modifications, respectively, in immunostimulatory motif of the antisense strand. These additional modifications were intended to completely abrogate the innate immune response caused by the siRNA. First, we measured the T_m value of each siRNA, and despite the PS, 2′-OMe modifications and cholesterol conjugation, each siRNA had a T_m value comparable to that of siLNA-2 (**Table 1**).

We evaluated the RNAi effect of each siRNA in the siLNA-2 series using mouse hepatic cells (NMuLi), and two siRNA concentrations, 10 or 100 nmol/l, were used in this assay. Regardless of the concentrations (10 or 100 nmol/l), each of the newly designed siRNAs was less potent as an RNAi agent than siLNA-2 or siApoB-1. At concentrations of 100 nmol/l, siL2PT-1, siL2PTC-1 and siL2PTC-1M showed reduction of apoB mRNA by approximately up to 80%, whereas siL2PTC-1L reduced apoB mRNA by just 60% (**Figure 3a**). Although

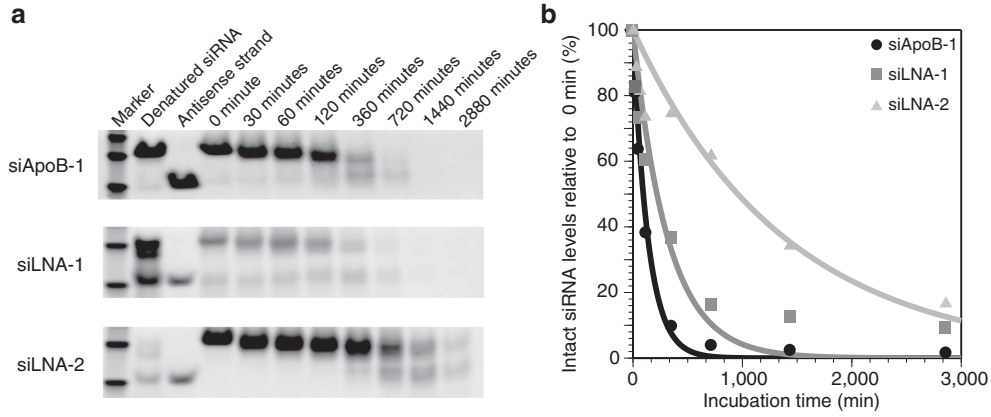

Figure 2 2′ A′-BNA/ LNA modification contributed to the nuclease resistance of the siRNAs. Serum stability of siLNAs and nonmodified siApoB-1 in mouse serum was analyzed using incubation times of 30, 60, 120, 360, 720, 1,440, and 2,880 minutes. (**a**) Samples taken at each time were subjected to native polyacrylamide gel electrophoresis in a 20% TBE gel. Images of polyacrylamide gels stained with SYBR gold are shown. (**b**) The density of bands representing duplex siRNAs was quantified using Image J software, which is freely available on the Internet. Band intensities were normalized using the band intensity of the first "0 minute" time point.

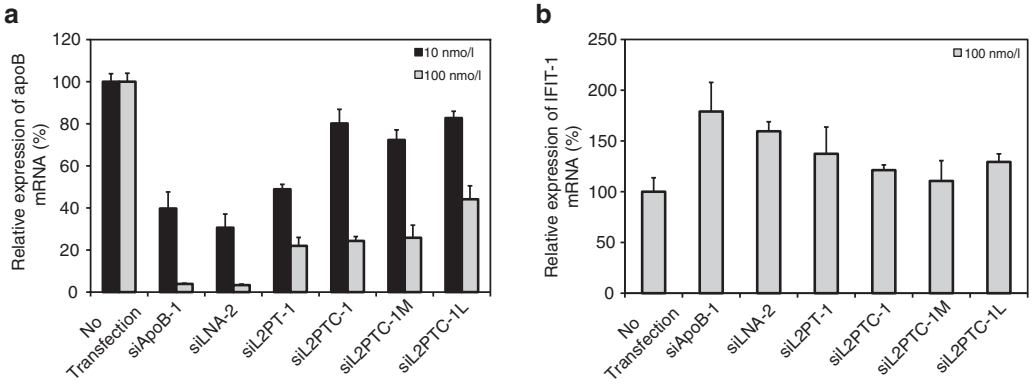

Figure 3 RNAi effects on apoB mRNA of modified siRNAs based on siLNA-2 and the downstream effects on IFIT-1 mRNA levels. (**a**) All siRNAs were transfected into NMuLi cells at concentrations of 10 or 100 nmol/l using Lipofectamine RNAiMAX. ApoB mRNA expression was normalized to GAPDH expression, and relative values were calculated using the no-transfection group. (**b**) Expression of interferon-induced tetratricopeptide repeats 1 (IFIT-1) mRNA was measured as the inflammatory response of the cell; IFIT-1 expression was normalized to GAPDH expression. Error bars indicate SD.

siL2PTC-1M and siL2PTC-1L had modifications at the same positions in their antisense strand, siL2PTC-1L showed a less effective RNAi effect than siL2PTC-1M, indicating that the 2′,4′-BNA/LNA markedly affected the RNAi machinery. At concentrations of 10 nmol/l, each cholesterol-conjugated siRNA had a less pronounced RNAi effect than did the nonconjugated siRNAs (**Figure 3a**). We also evaluated the expression of IFIT-1 (interferon-induced protein with tetratricopeptide repeats 1) mRNA, which was used as an indicator of immune responses and was related to the production of interferon-α (IFN-α).[34] At concentrations of 100 nmol/l, IFIT-1 mRNA expression was significantly higher (1.8-fold higher; $P < 0.01$) in siApoB-1-treated cells than in phosphate-buffered saline (PBS)-treated cells (**Figure 3b**). On the other hand, the elevation of IFIT-1 mRNA was gradually inhibited by each additional chemical modification (**Figure 3b**).

Therapeutic experiment with siLNA-2 series and their effects *in vivo*

To evaluate the pharmacological and toxic effects of each siRNA in this collection, we used atherogenic diet-fed C57BL/6J mice as a mouse model of dyslipidemia. We used Invivofectamice 2.0 reagent (Invitrogen, Carlsbad, CA) as a delivery vehicle of the siRNA. Mice were treated with a single tail vein injection of 5 mg/kg of one siRNA formulated with Invivofectamine 2.0 and adjusted to a final volume of 200 µl with PBS. Mice in the control PBS-treated group were injected with 200 µl of PBS via the tail vein.

Mice receiving siApoB-1 exhibited overt body weight loss, shedding up to 9% of their initial body weight by day 1 ($P < 0.01$) (**Figure 4a**). Among chemically modified siRNAs, siLNA-2, and siL2PTC-1, in particular, seemed to cause overt body weight loss of up to >6% of their initial body weight by day 1. Previous studies have indicated that overt body weight loss is often caused by a strong innate immune response to the siRNA.[32] On the other hand, the average body weight change in each of the other siRNA-treated groups was <3%.

Serum total cholesterol (TC) levels were measured at several time points (day 2, 7, 14, and 24). All siRNAs achieved a similar serum TC reduction of ~55–60% at 2 days after injection (**Figure 4b**). The cholesterol-conjugated siRNAs (siL2PTC-1, siL2PTC-1M, and siL2PTC-1L) showed similar or shorter duration of serum TC reduction than those

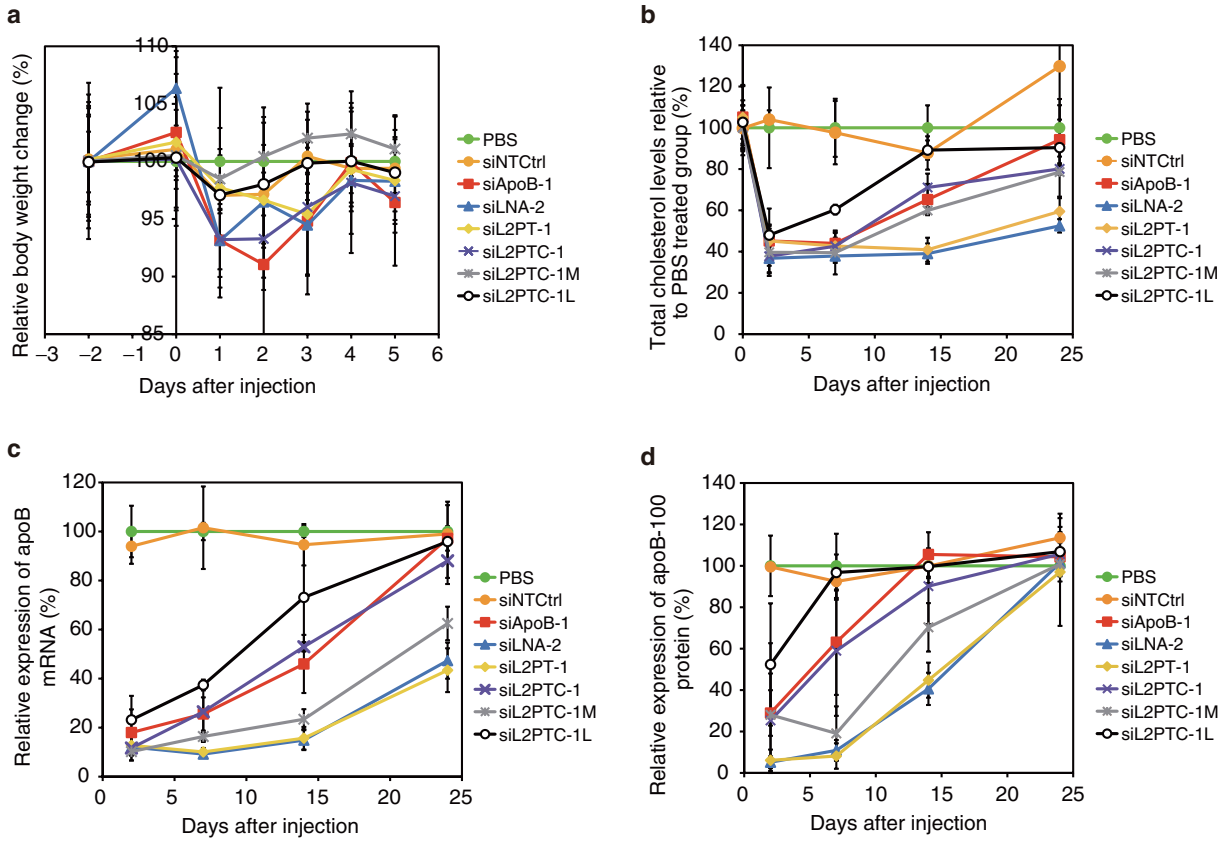

Figure 4 Therapeutic effects and body weight changes in mice receiving an siRNAs. (**a**) Body appearance, including body weight, of each mouse was observed for a week prior to the 2 days before the injection. Body weight changes in mice treated with an siRNA were indicated as ratio relative to that in the PBS-treated group. (**b**) Serum total cholesterol levels were indicated as ratio relative to that in the PBS-treated group. (**c**) ApoB mRNA expression in the liver tissues was normalized to GAPDH expression and was indicated as ratio relative to that in the PBS-treated group. (**d**) ApoB-100 protein levels in liver tissues were quantified by determining the intensity of the band on a western blot membrane, and these intensities are presented as a ratio relative to the band intensities of samples from the PBS-treated group. All values are the means ±SD of three to five animals.

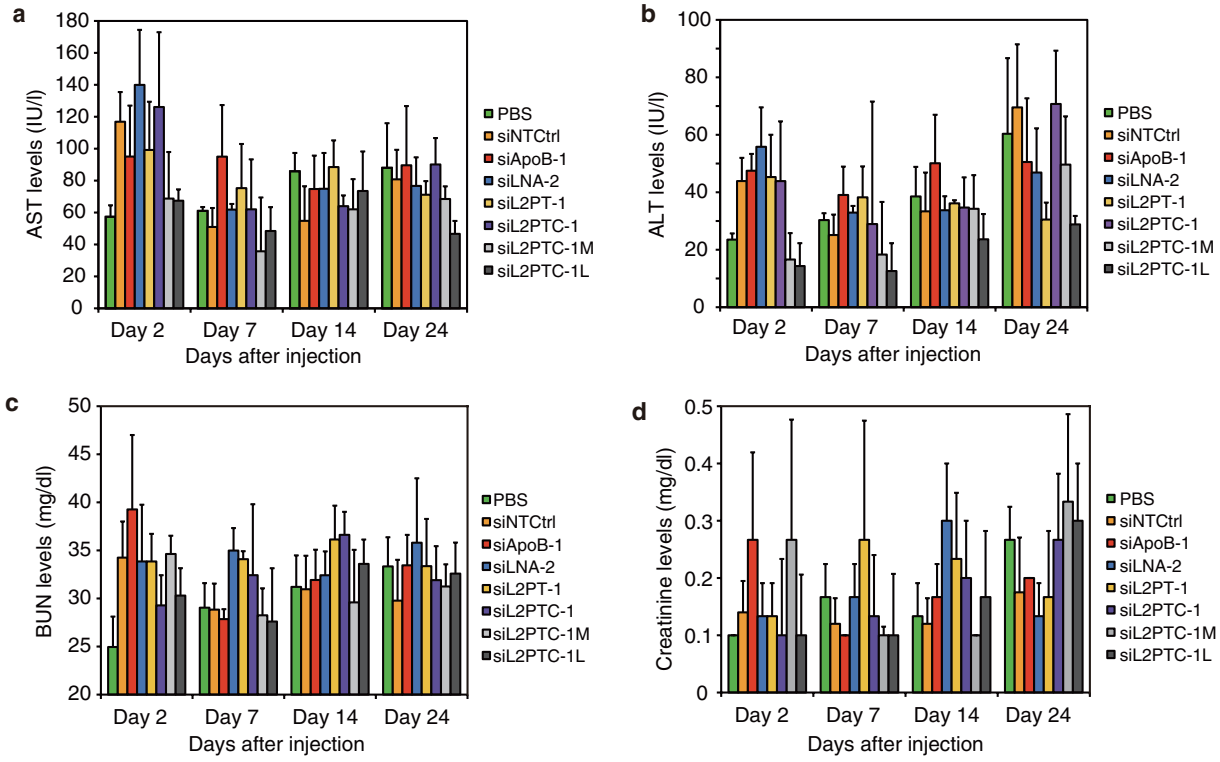

Figure 5 Changes in serum chemistry after the injection of an siRNAs. Aspartate aminotransaminase (AST), alanine aminotransferase (ALT), blood urea nitrogen (BUN), and creatinine levels were measured at each time point as indicators of hepatotoxicity and nephrotoxicity. Raw data on (**a**) AST, (**b**) ALT, (**c**) BUN, and (**d**) Creatinine levels are indicated. All values are the means ±SD of values from three to five animals.

of siApoB-1, whereas siLNA-2 and siL2PT-1 showed significantly longer duration of lipid-lowering effect than that of siApoB-1. In mice treated with siLNA-2 or siL2PT-1, reductions in serum TC of up to >50% lasted until approximately day 24. Serum TC levels of siLNA-2- or siL2PT-1-treated mice did not recover to the baseline levels by day 40 (**Supplementary Figure S1**). Injection of a Nontargeting control siRNA, siNTCtrl, did not cause any reduction in serum TC levels compared with the PBS-treated group (**Figure 4b**).

ApoB mRNA levels in the liver samples were also measured at the same time points. All siRNAs achieved significant inhibitions in the apoB mRNA levels of up to 90% compared to the PBS-treated group on day 2 (**Figure 4c**). A similar tendency in recovery curve was observed between apoB mRNA and serum TC levels. In contrast, siNTCtrl did not cause a significant reduction in apoB mRNA expression. In mice receiving siL2PTC-1L, a slight loss of RNAi effect was observed and the recovery of apoB mRNA expression was fastest. Significant RNAi effects (>50% inhibition of apoB mRNA) were sustained for 24 days after injection of siLNA-2 or siL2PT-1. In contrast, the RNAi effects were no longer significant at 24 days after injection of any other siRNAs (**Figure 4c**). The time required for apoB mRNA expression to recover by 50% (hereafter referred to as RT_{50}) in the siLNA-2 and siL2PT-1 treatment groups was 25 and 26 days, respectively. The RT_{50} values for the siL2PTC-1M, siL2PTC-1, siApoB-1, and siL2PTC-1L treatment groups were 22, 16, 15, and 12 days, respectively.

For each group, relative apoB-100 protein levels in the liver samples were measured by western blotting. While the recovery curves for apoB mRNA expression and serum TC levels were similar, levels of apoB-100 protein recovered faster than did apoB mRNA levels in all siRNA-treated groups (**Figure 4d**). In the siLNA-2- or siL2PT-1-treated group, up to >50% inhibition of apoB-100 was still observed on day 14, whereas, in mice receiving any other siRNAs, full expression of apoB-100 protein levels was observed on day 14. In contrast, siL2PTC-1L resulted in the least inhibition of apoB-100 production (up to 50% on day 2), and apoB-100 levels in these mice had recovered by day 7. In all pharmacological parameters (TC levels, mRNA and protein expression), the cholesterol-conjugated siRNAs (siL2PTC-1, siL2PTC-1M and siL2PTC-1L) shortened the duration, even though two of these siRNAs (siL2PTC-1 and siL2PTC-1M) showed a level of RNAi effects comparable to that of siL2PT-1 *in vitro* at 100 nmol/l. No remarkable reductions of apoB-100 protein level were observed in the siNTCtrl-treated group.

In translation, there was a high correlation between apoB mRNA recovery and apoB-100 protein recovery ($R^2 = 0.929$) (**Supplementary Figure S2**). When apoB mRNA expression recovered to ~40% of the initial levels, apoB-100 protein expression had already recovered to the initial level, indicating that target knockdown by siRNA facilitates the translation of target mRNA to protein. Despite the high correlation between apoB mRNA and apoB-100 protein, the correlation

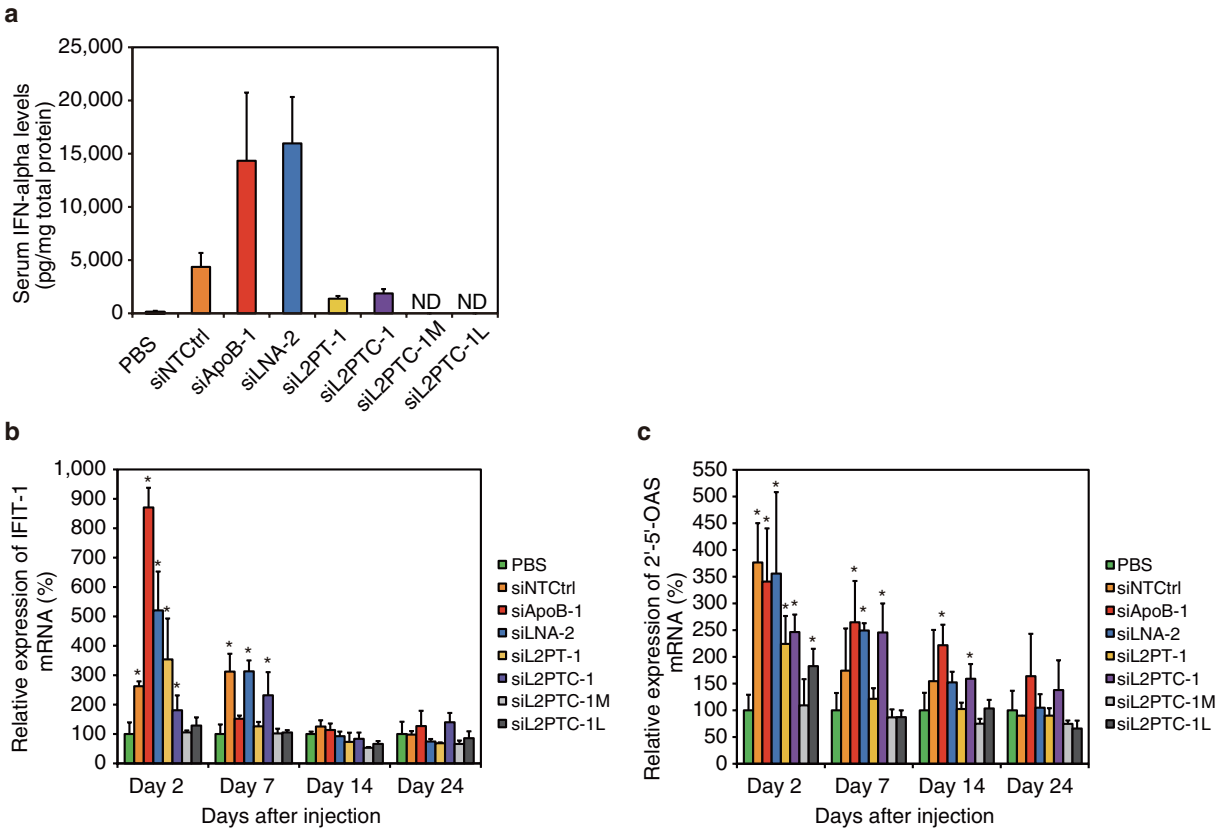

Figure 6 Serum interferon-α (IFN-α) levels and IFN-α-related gene expression. (**a**) Serum IFN-α levels were measured 6 hours after the injection of each siRNAs using enzyme-linked immunosorbent assay. (**b**) Interferon-induced tetratricopeptide repeats 1 (IFIT-1) and (**c**) 2′-5′-oligoadenylate synthetase 1 (OAS-1) mRNA expression in the liver was measured at each time point. IFIT-1 and OAS-1 mRNA expression was normalized to GAPDH expression. All values are the means ±SD of four to five animals.

between serum TC levels and apoB-100 protein levels was less pronounced (R^2 = 0.710).

Hepatotoxicity and nephrotoxicity in each siRNA-treated group

Serum levels of liver aminotransferase [aspartate aminotransaminase (AST) and alanine aminotransferase (ALT)] activities were measured as indicators of hepatotoxicity, and blood urea nitrogen (BUN) and creatinine levels were measured as indicators of nephrotoxicity. Although, on day 2, the mice injected with siRNA that had no modifications to the immunostimulatory motif had higher AST activities, ALT activities and BUN levels than did PBS-treated mice, the elevations of ALT activities and of BUN levels were within the normal range (**Figure 5a–c**). Gradual increases in ALT levels were observed in all groups throughout the experiment, indicating the influence of the atherogenic diet. No notable changes in creatinine levels were observed in any of the siRNA-treated groups (**Figure 5d**).

IFN-α concentrations in serum and IFN-α-related gene expression in liver

IFN-α levels in serum collected 6 hours after injection were measured using enzyme-linked immunosorbent assay. Overt increases in IFN-α levels were observed in mice receiving siApoB-1 or siLNA-2, which showed overt body weight loss on day 1, whereas the elevation of IFN-α levels was significantly attenuated in mice receiving siL2PT-1, which contained additional PS modification (**Figure 6a**). However, additional inhibition of the overt increases in IFN-α levels was not observed in mice receiving siL2PTC-1. The IFN-α concentrations were especially similar in mice receiving siL2PTC-1 or siL2PT-1. In contrast, no IFN-α production was observed in mice receiving siL2PTC-1M or siL2PTC-1L, the immunostimulatory motifs of which were modified with 2′-OMe or 2′,4′-BNA/LNA, respectively. The nontargeting control siRNA, siNTCtrl, also had a different immunostimulatory RNA motif in its sense strand. Mice receiving siNTCtrl had much higher serum IFN-α levels than did the PBS-treated group, but significantly lower serum IFN-α levels than those mice receiving siLNA-2 or siApoB-1 ($P < 0.05$), indicating that the different immunostimulatory motifs induced different levels of innate immune response (**Figure 6a**).

We also confirmed the changes in the expression of two IFN-α–related gene, IFIT-1 and 2′-5′-oligoadenylate synthetase 1 (OAS-1), using quantitative PCR. OAS-1 is induced by IFN-α and synthesizes 2′,5′-oligoadenylates that activate the RNase L. On day 2, mice receiving siApoB-1 exhibited approximately tenfold higher expression of IFIT-1 mRNA than did PBS-treated mice, and the overexpression diminished gradually with each additional chemical modification (**Figure 6b**). On day 2, mice receiving siL2PTC-1 exhibited

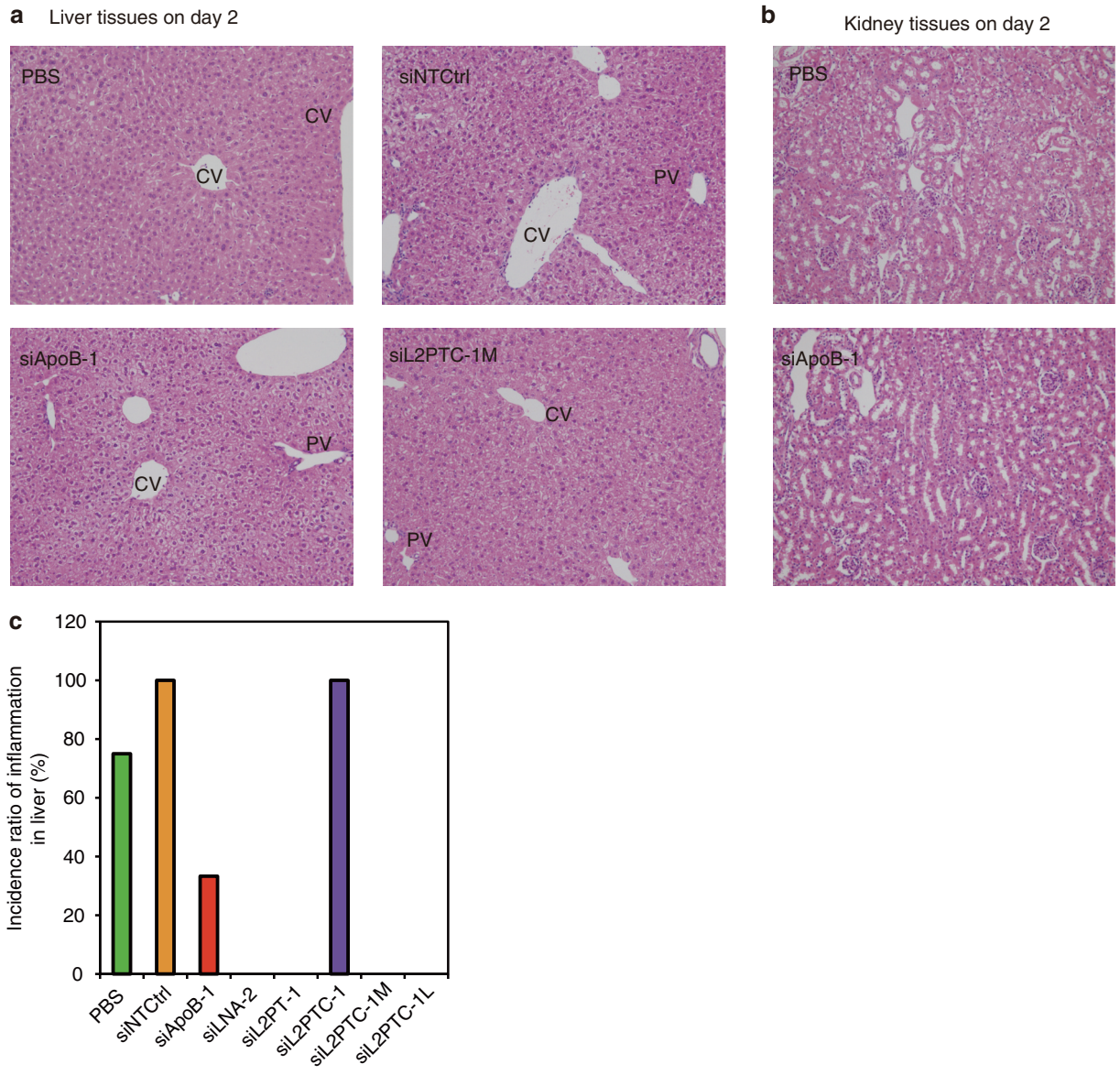

Figure 7 Liver and kidney tissue damage was assessed histopathologically using H&E staining. (**a**) Day 2 liver tissues of siNTCtrl, siApoB-1, siL2PT-1, and siL2PTC-1M were indicated as representative. (**b**) Day 2 kidney tissues of PBS and siApoB-1 were indicated as representative. (**c**) The number of mice with inflammation in the liver tissue was counted in each group, and the incidence ratio was indicated.

increases in IFIT-1 mRNA levels that were similar to those in mice receiving siL2PT-1. On day 7, continuous up-regulation of IFIT-1 expression was observed in the siLNA-2- and siL2PTC-1-treated groups. On day 2, OAS-1 expression, like IFIT-1 expression, was clearly higher in each group, except for the siL2PTC-1M- and siL2PTC-1L-treated groups, than in the PBS-treated group (**Figure 6c**). Mice receiving siApoB-1, siLNA-2, and siL2PTC-1 expressed high levels of OAS-1 mRNA on day 7 and on day 14. In contrast, neither siL2PTC-1M nor siL2PTC-1L showed significant upregulation of either gene.

Histopathological analysis for the liver and kidney

Liver and kidney tissue samples from each group were examined histopathologically. The livers of mice treated with any siRNA and PBS showed cytoplasmic vacuolation throughout the experiment. Furthermore, centrilobular hypertrophy of the liver was evident in a majority of the groups with the exception of the PBS- and siL2PTC-1M-treated groups on day 2 (**Figure 7a**). However, these lesions were seen in samples from every group on day 14. On day 14, inflammatory lesions were observed in mice treated with PBS, siNTCtrl or siApoB-1, and also in mice treated with siL2PTC-1 on day 24. In addition, slight granuloma or focal necrosis was sporadically seen. In contrast, no evidence of any toxicological effect was found in kidneys from any of the mice at any point in the experiment (**Figure 7b**). On day 24, we counted the mice in each group that exhibited liver inflammation (**Figure 7c**). Some mice in the PBS-, siNTCtrl-, siApoB-1-, and siL2PTC-1-treated groups showed inflammation in the liver, whereas

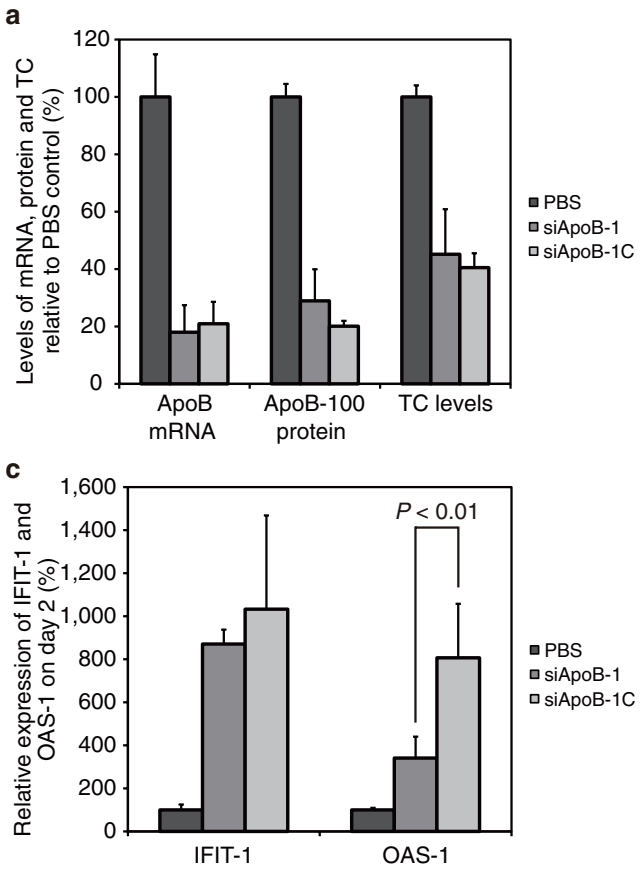

Figure 8 Pharmacological parameters and innate immune responses 2 days after the injection of siApoB-1C or siApoB-1. (**a**) ApoB mRNA inhibition, apoB-100 protein levels in the liver, and serum TC levels were measured 2 days after the injections. (**b**) IFN-α levels in serum 6 hours after injection of siApoB-1 and siApoB-1C. (**c**) Comparison of the effects of siApoB-1 and siApoB-1C on IFN-α-related gene expression. IFIT-1 and OAS-1 mRNA expression was normalized to GAPDH expression. All values are the means ± SD of three to four animals.

none of the mice in any other groups showed evidence of liver inflammation.

Cholesterol-conjugated siApoB-1 induced slightly high levels of IFN-α and its related gene expression compared with siApoB-1

Cholesterol-conjugated siL2PTC-1 not only abbreviated the duration of pharmacological parameters but also induced sustained high expression of IFN-α-related genes such as *IFIT-1* and *OAS-1* (**Figures 4c** and **6b,c**). Only mice receiving siL2PTC-1 exhibited inflammation in the liver on day 24; there was no inflammation in any mouse receiving other modified siRNAs. We hypothesized that cholesterol conjugation may have facilitated the interaction between the siRNA and some proteins that play an important role in evoking the innate immune response. We designed cholesterol-conjugated siApoB-1 (siApoB-1C; **Table 1**) to assess the impact of this modification on immune response, and used siApoB-1 as a control. Mice were injected with 5 mg/kg of siApoB-1C formulated with Invivofectamine 2.0 reagent via the tail vein and sacrificed 2 days after injection. Blood samples were collected from the tail vein at 6 hours after injection to measure the serum IFN-α concentration. Mice treated with siApoB-1C exhibited overt body weight loss of up to 11% on days 1 and 2. On day 2, the values of three pharmacological parameters (apoB mRNA, apoB-100 protein levels and serum TC levels) were similar in animals receiving siApoB-1C and those receiving siApoB-1 (**Figure 8a**). IFN-α induction in mice receiving siApoB-1C was significantly higher than that in mice receiving siApoB-1 ($P < 0.02$) (**Figure 8b**). Interestingly, IFIT-1 mRNA levels were similar in the siApoB-1C-treated group and the siApoB-1-treated group, but OAS-1 mRNA levels were twofold higher in the siApoB-1C-treated group than in the siApoB-1-treated group ($P < 0.01$) (**Figure 8c**).

The siL2PT-1M induced an efficient reduction of pharmacological parameters without any induction of IFN-α or upregulation of IFN-α-related gene expression

Based on these *in vivo* analyses and previous work by Judge *et al*., we designed siL2PT-1M, which had additional 2′-OMe modifications in the immunostimulatory motif, as well as siL2PTC-1M, to completely eliminate the immune response;[32] siL2PT-1M, like siL2PT-1, was designed to be effective for apoB lowering. Mice were injected with 5 mg/kg of siL2PT-1M formulated with Invivofectamine 2.0 reagent via the tail vein and sacrificed on day 2 or 24. No remarkable reduction of body weight was observed in the siL2PT-1M-treated group compared with the PBS-treated group. The RNAi effect

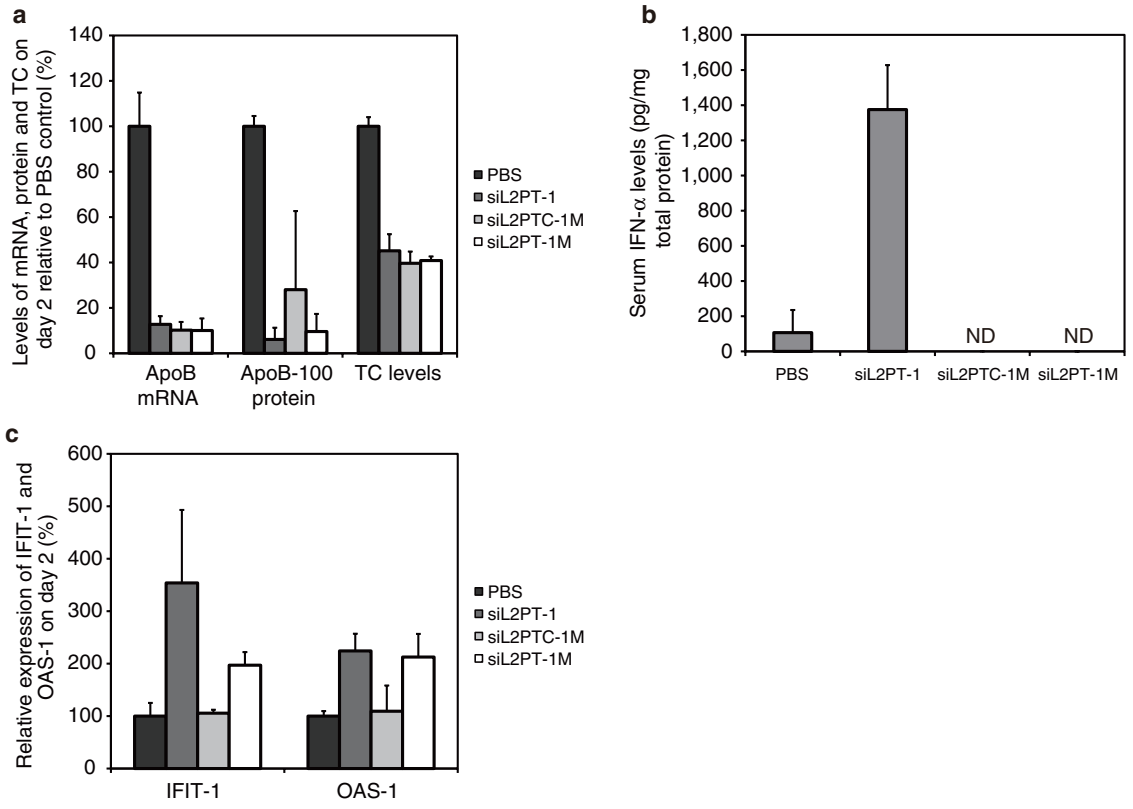

Figure 9 Comparison between siL2PT-1-, siL2PTC-1M-, and siL2PT-1M-mediated effects on pharmacological parameters and immune responses on day 2. (**a**) ApoB mRNA inhibition, apoB-100 protein levels in the liver, and serum TC levels were all measured 2 days after the injection of the siRNAs. (**b**) IFN-α levels in serum 6 hours after injection of each siRNA. (**c**) Comparison of the effects of siL2PT-1, siL2PTC-1M and siL2PT-1M on IFN-α-related gene expression. IFIT-1 and OAS-1 mRNA expression was normalized to GAPDH expression. All values are the means ± SD of three to four animals.

of siL2PT-1M was up to >90% on day 2, and thus was similar to that of siL2PT-1 (**Figure 9a**). On day 2, the serum TC levels of the siL2PT-1M-treated group showed a significant reduction of up to 60% as well as that of the siL2PT-1-treated group. This effective serum TC lowering lasted until day 24, and then, the serum TC levels were similar to those in mice receiving siL2PTC-1M. Although the IFIT-1 and OAS-1 mRNA expressions were slightly higher in the siL2PT-1M-treated group than in the PBS-treated group, serum IFN-α was not detected in the siL2PT-1M-treated group as well as in the siL2PTC-1M-treated group (**Figure 9b,c**).

Discussion

First, we designed two types of 2′,4′-BNA/LNA-modified siRNAs to avoid the undesired incorporation of sense strand with RISC and to give it nuclease resistance. Although siLNA-1 has been hypothesized to be more active than siLNA-2 *in vitro*, since the key property in strand bias was proven to be thermodynamic stability of different region (5′ or 3′-side) of duplex, siLNA-2 had a lower IC_{50} value and a much higher nuclease resistance than siLNA-1 (**Figures 1b** and **2b**).[26,35–37] However, incorporation of 2′,4′-BNA/LNA in the immunostimulatory motif (as represented by siL2PTC-1L) abolished not only the immune reaction but also the RNAi effect due to unrecognizable nucleic acids in the position related to the RNAi machinery (**Figure 4c**). Our results crucially suggested that 2′,4′-BNA/LNA modifications should be used in the sense strand as nuclease resistance and regulation of strand bias, but not in the site related to RISC formation including pre-cleavage of sense strand and RISC-mediated cleavage of target RNA.

To the best of our knowledge, no study has attempted to comprehensively assess the effects caused by 2′,4′-BNA/LNA-modified siRNAs combined with other artificial nucleic acids *in vivo*. Our siRNAs that were based on siLNA-2 and had additional modifications exhibited various effects both *in vitro* and *in vivo*. The RNAi effects of siL2PT-1, which had additional PS modifications, persisted longest with slightly inhibited IFN-α production compared with that of siLNA-2 (**Figures 4c** and **6a**). These findings indicated that it is possible to eliminate the innate immune response when the PS modifications are in appropriate positions within siRNA. Previously, Judge *et al*. reported that although modification of all cytidine residues with 2′-OMe in the sense strand of an siRNA that had the same sequence in our study evoked immune response, modification of all adenosine residues in the sense strand almost inhibited the IFN-α induction. And modification of all uridine or all guanosin in the sense strand of siRNA completely abrogated the immune response. These findings indicated that modification of the sense strand alone

could eliminate the immune reactions in any siRNAs and that uridine and guanosine were directly related to the induction of an innate immune response, and partial adenosine also related to immune response.[32] In our study, the slightly diminished IFN-α levels associated with siL2PT-1 (**Figure 6a**) were attributed to partial adenosine modification in the 5′-CACAC-3′ motif, which is complementary to the immunostimulatory motif. Taken together, our results and those of Judge *et al*. indicated that modifying the adenosine residues complementary to the immunostimulatory motif and 5′-UG-3′ (tenth from the 5′-end) in a sense strand results in elimination of innate immune response with only sense strand chemical modification in this sequence. Although, in this sequence, 5′-UG-3′ is in the position related to pre-cleavage of the sense strand, 2′-OMe modification at this site was proven to be well tolerated by the RNAi mechanism.[38,39]

Cholesterol conjugation has been widely used as a delivery vehicle for naked siRNA and nuclease resistance.[25,40,41] We hypothesized that cholesterol conjugation of siRNA would lower the recognition of nucleases and alleviate the recognition of innate immune systems due to its slight bulky structure at the 3′-end.[31] Unexpectedly, cholesterol conjugation resulted in a markedly reduced RT_{50} of mRNA inhibition and slightly enhanced immune reactions (**Figures 4a,c** and **6b,c**). Although a similar RNAi effect was observed on day 2 between the siL2PTC-1- and siL2PT-1-treated groups in the *in vivo* experiment (**Figure 4c**), these effects were attributed to saturation of the RISC complex due to excessive concentrations of siRNA in the liver cell.[42] The factors that affect the duration of pharmacological effects are the disappearance rate of siRNA and turnover rate of active RISC in the cell. Although the cholesterol conjugation in the sense strand wields an influence on the RNAi effect via RISC formation under the conditions of a low concentration of siRNA (**Figure 3a**), the stability of siRNA against enzymatic degradation has an impact on the sustained RNAi effect when the siRNA is in excess in the cell, as was the case in our *in vivo* experiment. In the RNase T experiment, the stability of siL2PTC-1 was comparable to that of siL2PT-1, although the image of siL2PTC-1 indicated partial degradation of siL-2PTC-1 (**Supplementary Figure S3 and Supplementary Materials and Methods**). This partially degraded moiety was considered to be cholesteryl-TEG, because the bands after 0 hour showed a molecular weight similar to those of siL2PT-1. According to previous work conducted by Haley *et al*., the turnover rate of active RISC complexes containing the antisense strand that was the same sequence between siL2PT-1 and siL2PTC-1 would be comparable.[42] Namely, it was suggested that cholesterol conjugation might slightly alter the disappearance rate of siRNA in the cell, except for consumption by RISC formation. IFN-α levels of siL2PT-1 and siL2PTC-1 were comparable and cholesterol-conjugation did not alter the innate immune response (**Figure 6a–c**). However, interestingly, cholesterol-conjugated siApoB-1 enhanced IFN-α production compared with nonmodified siApoB-1 (**Figure 8b**). Therefore, the mechanism by which cholesterol-conjugation provokes an immune reaction may have an impact on the disappearance rate of siRNA. Indeed, siL2PTC-1M having a 2′-OMe-modified immunostimulatory motif showed slightly better duration of pharmacological parameters than those of siL2PTC-1 (**Figure 4b–d**). Primary recognition of siRNAs is evoked by TLR7, which is located in the endosomal compartment.[33] In the cytosol, retinoic acid-inducible gene-I (RIG-I) acts as a sensor of viral RNA via binding and was activated by single-strand RNA or double-strand RNA containing 5′-triphosphates or blunt-ended siRNA.[43,44] Although RIG-I induces type-I IFNs by recognizing viral RNA, their mRNA expression is affected by IFN-α from other signal pathways.[44] RIG-I mRNA expression in the mice receiving siL2PT-1 or siL2PTC-1 was slightly higher than that in mice receiving PBS (**Supplementary Figure S4**), and in each group, RIG-I mRNA expression was slightly correlated with serum IFN-α levels, indicating that slightly elevated levels of RIG-I mRNA were attributed to the response to IFN-α. Therefore, the facilitation of the immune response by cholesterol conjugation might happen in the endosome. There are three possible hypotheses. (i) Cholesterol-conjugated siRNA binds more strongly to proteins in serum or cytosol than does normal siRNA.[25,45] The cholesterol moiety may hydrophobically interact with some sort of protein (proteins on an endosomal membrane, such as TLRs, which have a hydrophobic leucine-rich repeat) or endosomal membranes, resulting in facilitating the interaction between siRNA and TLR. Then the aberrant siRNA-protein complex is metabolized from the cell, which leads to early lowering of the level of siRNA in the cell. (ii) Due to the strong affinity between cholesterol and delivery vehicle used in this study, cholesterol-conjugated siRNA cannot readily escape from delivery vehicles, resulting in generation of the aberrant complex, and the complex was metabolized. (iii) Due to the hydrophobicity of the cholesteryl-TEG group, cholesterol-conjugated siRNAs or precleavage debris of the sense strand containing a cholesteryl-TEG moiety could form micelles or be aggregated in the cell. This aberrant complex induces cytotoxic effects.

Our results indicated that using the cholesterol-conjugated siRNA with the lipid carrier resulted in not only shortened duration of pharmacological parameters, but also an enhanced immune response. The cholesteryl-TEG group of cholesterol-conjugated siRNA was immediately eliminated in the RNase T solution (**Supplementary Figure S3**). We measured mRNA levels of SREBP2 (a transcriptional activator required for lipid homeostasis) and ACAT1 (an enzyme which esterifies the free cholesterol and stores cholesteryl esters in lipid droplets) on day 2 in the siApoB-1C- and siApoB-1-, and PBS-treated groups (**Supplementary Figure S5**). There was no significant difference in these mRNA levels among the groups, indicating that the cholesterol synthesis and conservation system were not influenced by precleavage debris of the cholesteryl-TEG group of siRNA or by silencing the apoB mRNA.

Based on the histopathological analyses and serum chemistry, the siRNA-carrier complex used in this study caused almost no nephrotoxic effects. In contrast, some siRNAs (siApoB-1, siLNA-2, siL2PTC-1, siApoB-1C, and siNTCtrl) cause serious liver damage. Although siRNAs which induced IFN-α were also associated with elevation of serum AST levels on day 2, there was no correlation between IFN-α concentration and serum AST levels. However, the elevated AST levels on day 2 and centrilobular hypertrophy were highly associated findings. The siRNAs with chemical modifications in the immunostimulatory motif exhibited normal range

in centrilobular hypertrophy, claiming the needs for complete elimination of the immune response by an siRNA. Mice exhibiting the IFN-α production exhibited overt up-regulation of CYP7α1 (the first and rate-limiting enzyme in bile acid synthesis) mRNA on day 2, indicating that cholesterol was converted to bile acid at high rates in the cells (**Supplementary Figure S6**). We also found an association between the increases in ALT levels and inflammatory lesions on day 24 in PBS-, siNTCtrl- and siL2PTC-1-treated mice, but not in siApoB-1-treated mice. The inflammation and increased ALT levels in the PBS-treated group were attributed to deposition of fat in the liver caused by the atherogenic diet. Almost all mice receiving an siRNA targeting apoB mRNA showed no incidence of inflammation in the liver on day 24 but not in mice receiving siApoB-1 or siL2PTC-1 (**Figure 7c**), indicating that translational restriction of apoB may be involved in chronic liver toxicity in mice fed an atherogenic-diet.

This is the first report to show comprehensive effects by 2′,4′-BNA/LNA-modified siRNA combined with other artificial nucleic acids *in vivo*. In this article, we demonstrated that each siRNA that was designed based on siLNA-2 significantly reduced the apoB mRNA levels in liver and serum TC levels. In particular, siL2PT-1M-mediated sustained duration of the RNAi effect and other pharmacological parameters, but did not induce IFN-α. In cases of dyslipidemia, including homozygous familial hypercholesterolemia, apoB is an attractive drug target for LDL-lowering therapy because inhibition of apoB-100 directly downregulates the generation of LDL particles. Our results have much importance for the development of apoB-lowering siRNA medicines. Importantly, our findings on immunostimulation by immunostimulatory siRNA (isRNA) raise a question about the use of cholesterol-conjugated isRNA with lipidic or cationic delivery vehicles. It was also suggested that modifying the adenosine residues complementary to the immunostimulatory motif and 5′-UG-3′ (tenth from the 5′-end) in the sense strand result in elimination of the innate immune response with chemical modification only in the sense strand. If we were able to alter the nuclease resistance and to abrogate the immunostimulation of potential siRNAs by chemical modification of only the sense strand, many siRNA candidates that have been rejected without any analyses would be reassessed and analyzed, and these changes could lead to the development of more efficient siRNA drugs. Our analyses of the effects of chemical modification in siRNAs on pharmacological parameters, tissue toxicity and innate immune response may contribute in important ways to the development and design of siRNA drugs for various genetic or chronic disorders.

Materials and methods

Design of siRNAs. The siRNAs used in this study consisted of 23-nucleotide sense and antisense strands. The targeted site in apoB mRNA was chosen based on the study conducted by Soutchek *et al*.[25] We also prepared an siRNA consisting of 21-nucleotide sense and antisense strands as a control siRNA (siNTCtrl). This sequence was previously reported as a nontargeting control sequence that contains at least four mismatches to any human, mouse or rat gene.[46] All sequences and modifications of siRNA are listed in **Table 1**. All siRNAs were chemically synthesized by GeneDesign (Ibaraki, Japan) and were received as desalted. Equimolar concentrations of each sense and antisense strand were incubated at 95 °C for 5 minutes in RNase/DNase free water and then cooled slowly at room temperature for 12 hours.

UV melting experiment. We measured the T_m value of each siRNA by conducting UV melting experiments with a Beckman DU-650 spectrometer including a T_m analysis accessory. Annealed siRNAs were dissolved in 10 mmol/l sodium phosphate buffer (pH 7.2) containing 100 mmol/l NaCl to a final concentration of 1 μmol/l. The melting profiles at temperatures ranging from 25°C to 95 °C were recorded at 260 nm using a scan rate of 0.5 °C/min. The melting temperature (T_m) was calculated as the temperature at which the duplexes were half dissociated and each T_m was determined by taking the first derivative of the melting curve.

Serum stability. To determine the stability of siRNAs in serum, each siRNA (final concentration of 0.5 μmol/l) was incubated in a sample of 100% mouse serum at 37 °C. We measured the amount of intact siRNA that remained in the serum at defined time points. Specifically, we took a 5-μl sample from each serum-siRNA mixture at 0 minute, 30 minutes, 60, 120, 360, 720, 1,440, and 2,880 minutes. Each 5-μl sample was diluted with 5 μl of three times TBE buffer (final concentration: 50 mmol/l THAM (Tris(hydroxymethyl)aminomethane), 48.5 mmol/l borate, 2 mmol/l EDTA, pH 8.2) and 5 μl of distilled water and then frozen in liquid nitrogen. Denatured siRNA was prepared by incubating double-strand siRNA at 95 °C for 5 minutes and immediately cooled at 4 °C. These 15 μl of samples and 10 bp DNA step ladder (Invitrogen) were then subjected to electrophoresis on 20% TBE gel (Invitrogen) for 110 minutes at 120 V. RNA bands were visualized by using SYBR Gold (Invitrogen). Gel images were taken with LAS-4000 mini image analyzer (FUJIFILM, Tokyo, Japan) and the intensity of bands was measured by using Image J software (http://rsbweb.nih.gov/ij/) freely available on the Internet.

Cell culture and transfection of siRNAs. To evaluate the gene-silencing ability of individual siRNAs *in vitro*, we used a mouse hepatic cell line, NMuLi. The cells were seeded in 9.62 cm^2 6-well plates at 5×10^5 cells/well in DMEM (GIBCO, Grand Island, NY) containing 10% fetal bovine serum and 1% penicillin/streptomycin (Gibco) and incubated for 24 hours under 5% CO_2 at 37 °C. After incubation, the culture medium was replaced with antibiotic-free DMEM. Each siRNA, at several different concentrations, was transfected to the cells with Lipofectamine RNAiMAX (Invitrogen) reagent as directed in the manufacturer's protocol and with Opti-MEM medium (Invitrogen). After incubation for 24 hours under 5% CO_2 at 37 °C, the cells were lysed with TRIzol reagents (Invitrogen) and total RNAs were extracted in accordance with the manufacturer's protocol.

In vivo *therapeutic experiments in a mouse model of hypercholesterolemia.* All animal experiments were conducted according to the guidelines of the Animal Care Ethics Committee of the National Cerebral and Cardiovascular Center Research Institute (Osaka, Japan). Male C57BL/6J mice (Japan, SLC, Inc., Hamamatsu, Japan) aged 7 weeks were

used in this study. The mice were maintained on a 12-hour light/12-hour dark cycle and fed *ad libitum*. To induce hypercholesterolemia, wild-type C57BL/6J mice were fed an atherogenic diet, F2HFD1, containing 1.25% cholesterol (Oriental Yeast, Tokyo, Japan) from the beginning one week before administration of any siRNA and throughout the experiment.

To deliver each siRNA, we used Invivofectamine 2.0 reagent (Invitrogen) according to the manufacturer's instructions. Five mg/kg of each siRNA was formulated with Invivofectamine 2.0 reagent, and each mouse received one siRNA (siNTC-trl, siApoB-1, siLNA-2, siL2PT-1, siL2PTC-1, siL2PTC-1M, siL2PTC-1L, siL2PT-1M, and siApoB-1C) as an siRNA–Invivofectamine 2.0 complex via tail vein injection. The physical appearance and body weight of each mouse were recorded during this study. Before an animal was sacrificed, a blood sample was collected from the tail vein using BD Microtainers (BD, Franklin Lakes, NJ). Each of these blood samples was subsequently subjected to centrifugation. TC levels in serum samples were measured using Cholesterol E (Wako, Osaka, Japan) according to the manufacturer's instructions with adjustment for a 96-well microplate. Mice ($N = 3–5$) were sacrificed 2, 7, 14, or 24 days after injection of siRNA. Mice were anesthetized before being sacrificed, and samples of whole blood were collected from each inferior vena cava to measure the activity of AST and ALT and to quantify the BUN and creatinine levels. Next, the liver and kidneys of each animal were harvested to quantify apoB mRNA levels using quantitative PCR; samples of liver tissue and kidney tissue were also collected for histopathological analysis.

Six hours after injection of each siRNA, we collected a blood sample from the tail vein of each mouse. We processed each of these samples to assess serum levels of IFN-α using an IFN-α enzyme-linked immunosorbent assay kit (PBL Interferon Source, Piscataway, NJ) according to the manufacturer's directions.

Harvested liver samples were cut into 2 × 2 mm squares and immediately frozen using liquid nitrogen. For each mouse, one 2 × 2 mm square was put into 1 ml of TRIzol reagent (Invitrogen) for homogenization, and total RNA was isolated in accordance with the manufacturer's protocol accompanying the TRIzol regent.

Quantitative PCR analysis. We used a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA) to prepare cDNA from 10 µg of each sample of total RNA extracted from cells or tissues. Each cDNA sample was subjected to Real-Time PCR (StepOnePlus Real-Time PCR System; Applied Biosystems) to measure the relative quantities of APOB, GAPDH, IFIT-1, and OAS-1 expression using SYBR Green reagent (Applied Biosystems) and of SREBP2, α1 using the TaqMan Gene Expression Assay (Applied Biosystems). The expression levels of target genes were normalized using GAPDH expression as an internal control. The following primer sets were used for quantitative PCR: for mouse ApoB, forward primer 5′-TGGGCAACTT-TACCTATGACTT-3′ and reverse primer 5′-AAGGAAATG-GGCAACGATA-3′; mouse GAPDH, forward primer 5′-CA AAATGGTGAAGGTCGGTGTG-3′ and reverse primer 5′-ATTTGATGTTAGTGGG GTCTCG-3′; mouse IFIT-1, forward primer 5′- AGGCTGGAGTGTGCTGAGAT-3′ and reverse primer 5′- TCTGGATTTAACCGGACAG-3′; mouse OAS1, forward primer 5′-CTGACCTGGTGGTGTTCCTT-3′ and reverse primer 5′-CCACCATGAACTCTGGACCT-3′. TaqMan Gene Expression Assay; assay ID for SREBP2, ACAT-1 CYP7α1 were Mm01306297_g1, Mm00507463_m1 and Mm00484152_m1, respectively. After an amplification consisting of 40 cycles, relative expression of each target was analyzed with SDS analysis software (Applied Biosystems).

ApoB-100 western blot analysis. Mouse liver tissues were homogenized in Ripa Buffer (Sigma-Aldrich, St Louis, MO) containing complete mini (Roche, Indianapolis, IN). Samples were immediately subjected to centrifugation at 4 °C, 13,000*g* for 5 minutes. The supernatant of each sample was collected into an Amicon Ultra centrifugal Filters Ultracel-50k (Millipore, Billerica, MA), and the contents of the supernatant were concentrated by centrifugation at 4 °C, 20,000*g*. The final volume of each retentate was adjusted to 200 µl with PBS, and the total protein concentration of each sample was determined using a BioRad DC protein assay kit (Lowry method; BioRad, Hercules, CA).

Individual samples (50 µg) of total protein in liver homogenates were subjected to electrophoresis on a 3–8% NuPAGE Tris-Acetate Gel (Invitrogen) and transferred to a polyvinylidene-fluoride membrane (Millipore). Membranes were then incubated with Blocking One reagent (Nacalai Tesque, Kyoto, Japan) for 12 hours at 4 °C for block nonspecific antibody binding. Membranes were then incubated with primary antibody for mouse apoB-100 or -48 (Meridian Life Science, Inc., Saco, ME) for 2 hours at room temperature. After incubation with primary antibody, each membrane was washed with PBS-containing 0.1% of Tween 20, and then the membranes were incubated with goat anti-rabbit immunoglobulin G secondary antibody conjugated with peroxidase (Santa Cruz Biotechnology, Santa Cruz, CA) dissolved in PBS containing 0.1% of Tween 20-containing 0.5% blocking one reagent for 2 hours at room temperature. Protein bands were visualized using the ECL Advance Western blot detection kit (Amersham Biosciences, Buckinghamshire, UK) according to the manufacturer's directions. Images of each western blot were taken with a LAS-4000 mini image analyzer (FUJIFILM), and the intensity of each band was measured using Image J software (http://rsbweb.nih.gov/ij/), which is freely available on the Internet.

Hepatotoxicity and nephrotoxicity assay. To assess the hepatotoxicity associated with each siRNA, we measured the activities of AST and ALT using a GOT·GTP CII kit (Wako) according to the manufacturer's protocol adjusted to 96-well microplate. Results from these assays are presented as AST and ALT activity in IU/L units. As the nephrotoxicities, BUN values in the serum of each sample were measured using a Urea Nitrogen B-test Wako (Wako) kit, and creatinine values in the serum of each sample were measured using FUJI DRI-CHEM CRE-P III with a FUJI DRI-CHEM instrument (FUJIFILM). The Urea Nitrogen B-test Wako was used in accordance with the manufacturer's protocol adjusted to 96-well microplate.

Histopathological analysis. Harvested liver and kidney samples were sectioned and processed for histopathological analysis. Liver and kidney specimens were fixed in 10 %

formalin-PBS (formaldehyde was diluted with PBS, formaldehyde; Wako) for 2 days at room temperature and processed through to paraffin embedding. Routinely, we picked upper and lower lobe regions of the liver and picked kidney tissue including the renal pelvis. All paraffin-embedded tissues were sliced into 5-μm-thick sections using a microtome (Leica Microsystems, Wetzlar, Germany). These sections were stained with Carrazzi's hematoxylin and eosin (Sakura Finetek USA, Inc., Torrance, CA) solutions for histopathological examination.

Statistical analysis. Therapeutic experiments *in vivo* were performed such that for each time point (day 2, 7, 14, and 24), there were 3–5 mice in each treatment group. A Student's *t*-test was performed for comparison of two arms. Values of $P < 0.05$ or $P < 0.01$ were considered to indicate statistical significance.

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Supplementary material

Figure S1. Relative serum total cholesterol (TC) levels on day 40 in the siLNA-2 or siL2PT-1 treated group.
Figure S2. Correlation of apoB mRNA recovery and apoB-100 protein recovery.
Figure S3. Stability of siRNAs toward RNase T.
Figure S4. RIG-I mRNA expression in the liver on day 2 in the siL2PT-1-, siL2PTC-1-, siApoB-1- or siApoB-1C-treated groups.
Figure S5. SREBP2 and ACAT-1 mRNA expression in the liver on day 2 in the siApoB-1- or siApoB-1C-treated groups.
Figure S6. CYP7α1 mRNA expression in the liver on day 2.
Materials and Methods.

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Supplementary Information accompanies this paper on the Molecular Therapy–Nucleic Acids website (http://www.nature.com/mtna)