patient's *APOC2* gene, we constructed reporter constructs of the *APOC2* minigene amplified from genomic DNA of the patient and control subject; subsequently, the luciferase gene was cloned just before the stop codon of the *APOC2* gene (**Fig. 4A**). Transient transfection of these constructs to HEK293 cells or HepG2 cells revealed that transcriptional activity of the patient's minigene was significantly decreased by 39% and 51%, respectively, compared with that of the control construct (**Fig. 4B**). These results demonstrate that transcriptional repression of the patient's *APOC2* gene is at least partially due to a functional defect in the *cis*-regulatory region of the *APOC2* gene.

Sequencing of the *APOC2* and Other Genes in Plasma TG Metabolism

Next, we sought to identify if there are any sequence variants in the *APOC2* gene of the patient that account(s) for the extremely low levels of apoC-II proteins in the patient's plasma. Sequencing of *APOC2* of the patient and control subject, followed by comparison with the reference sequence (**NC_000019.9**), revealed 50 SNPs (**Table 6**). We also sequenced *APOC2* of the patient's mother and found 42 SNPs that were heterozygous for the patient's mother and homozygous for the patient (data not shown); however, all of these SNPs were common variants with allele frequencies higher than 0.35 (as reported in the dbSNP database, or as identified by direct sequencing of Japanese individuals (n=384) for the SNPs where allele frequencies were not reported in the dbSNP database). As apoC-II deficiency is less frequent than LPL deficiency, which occurs in one in a million[30], these common variants are unlikely to be the causative mutations.

Sequencing of other genes involved in plasma TG metabolism (*LPL*, *APOA5*, *APOC3*, *LMF1* and *GPIHBP1*) revealed no rare variant in the patient's gene (data not shown). These results indicate that other yet-to-be-identified genes, which regulate plasma apoC-II protein levels, are responsible for type I hyperlipidemia associated with apoC-II deficiency in this patient.

Discussion

ApoC-II deficiency has been well characterized since its first description in 1978[7]. In most cases, homozygous mutations were identified in the protein-coding region, resulting in null, truncated, or non-functional apoC-II protein[15, 16]; however, several atypical cases of apoC-II deficiency have been described, where apoC-II is severely reduced in plasma, but still

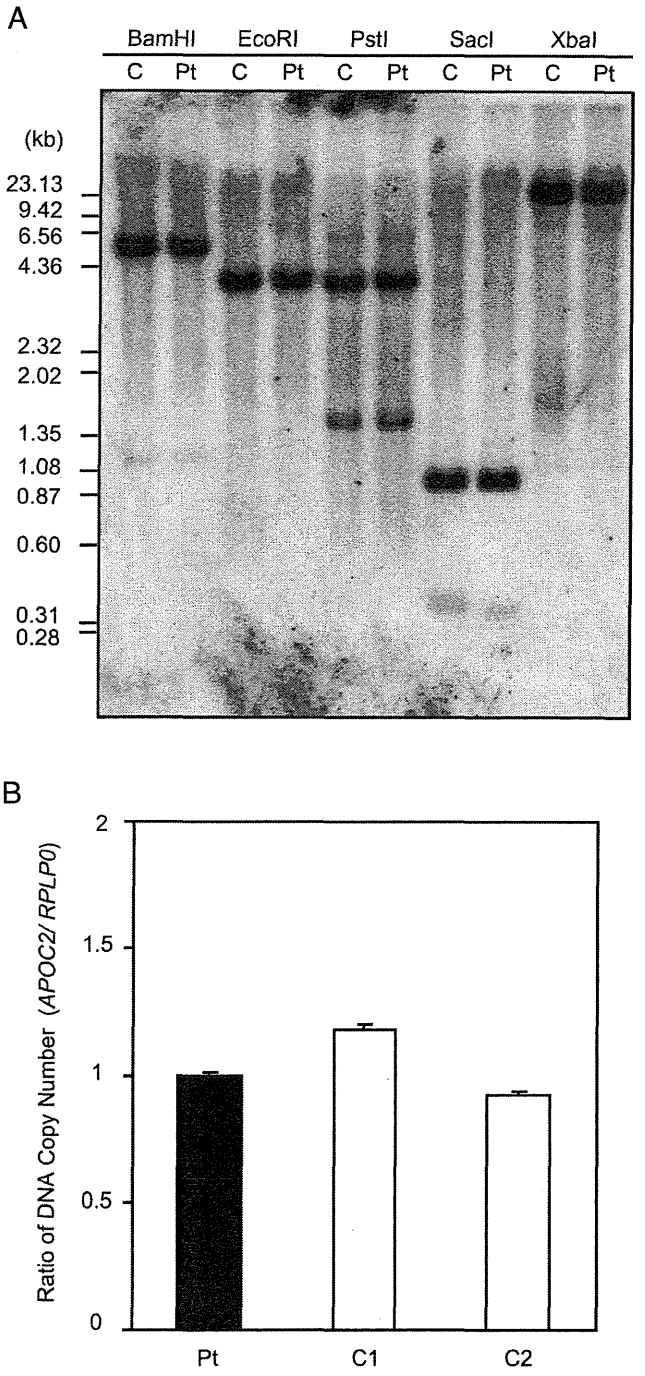

Fig. 3. Southern blot and genomic DNA copy number analyses.

(A) Ten μg of genomic DNA from the patient (Pt) and normal control subject (C) were digested by restriction enzymes overnight and then subjected to Southern blot analysis using apoC-II cDNA probe. (B) DNA copy number analysis. DNA from the patient (Pt) and two normal control subjects (C1 and C2) were used to amplify the genomic regions of *APOC2*, or *RPLP0*, flanking intron-exon boundaries, by real-time PCR. The DNA copy number of the *APOC2* gene was estimated by the relative amount of *APOC2* to *RPLP0*, and normalized to the value of the patient.

Table 6. Overview of the 50 SNPs identified by sequencing the patient's *APOC2* gene and comparison to the reference sequence (**NC_000019.9**)

Nucleotide number	GenBank base no.	Position in gene	RefSNP ID	Minor allele Frequency[a]	Alleles	
					Ref.	Pt.
c.-147C>G	45449199	Promoter	rs2288912	0.35 (C)	C	G
c.-62T>G	45449284	Exon 1	rs2288911	0.35 (T)	T	G
c.-14+157T>A	45449489	Intron 1	rs111268759	NA[b]	T	A
c.-14+159T>A	45449491	Intron 1	rs7249138	0.49 (T)	T	A
c.-14+162_-14+163delGA	45449494	Intron 1	rs10605424	NA[b]	GA	-
c.-14+701C>T	45450033	Intron 1	rs9304644	0.44 (C)	C	T
c.-14+1076T>C	45450408	Intron 1	rs9304646	0.43 (T)	T	C
c.-14+1090_-14+1091insAA	45450422	Intron 1	rs34457201	NA[c]	-	AA
c.-13-851A>G	45450872	Intron 1	rs4803774	0.43 (A)	A	G
c.-13-795T>C	45450928	Intron 1	rs4803775	0.35 (T)	T	C
c.-13-281A>G	45451442	Intron 1	rs7256684	0.43 (A)	A	G
c.-13-103T>A	45451620	Intron 1	rs5120	0.35 (T)	T	A
c.216-145G>C	45452271	Intron 3	rs3745152	0.45 (C)	G	C
c.216-143_216-142insTTC	45452273	Intron 3	rs3833214	0.48 (TTC)[d]	-	TTC
c.216-81T>C	45452335	Intron 3	rs4803776	0.36 (T)	T	C
c.*188_*189insT	45452694	3'-UTR	rs150448996	0.42 (T)[d]	-	T
c.*306C>T	45452812	3'-UTR	rs1130742	0.36 (T)	C	T
c.*638C>T	45453144	i.r.	rs7257468	0.44 (C)	C	T
c.*645T>G	45453151	i.r.	rs7258345	0.44 (T)	T	G
c.*659C>T	45453165	i.r.	rs7257476	0.44 (C)	C	T
c.*733G>A	45453239	i.r.	rs12709889	0.36 (A)	G	A
c.*1150G>A	45453656	i.r.	rs10423208	0.44 (G)	G	A
c.*1372G>A	45453878	i.r.	rs10402642	0.44 (G)	G	A
c.*1730G>A	45454236	i.r.	rs7246900	0.44 (G)	G	A
c.*1857G>A	45454363	i.r.	rs11083752	0.44 (G)	G	A
c.*2180T>C	45454686	i.r.	rs7248162	0.44 (T)	T	C
c.*2253A>G	45454759	i.r.	rs7247227	0.44 (A)	A	G
c.*2260G>A	45454766	i.r.	rs7247551	0.35 (G)	G	A
c.*2952G>A	45455458	i.r.	rs892101	0.44 (G)	G	A
c.*3200C>T	45455706	i.r.	rs7251501	0.44 (C)	C	T
c.*3209C>G	45455715	i.r.	rs7251503	0.44 (C)	C	G
c.*3395C>G	45455901	i.r.	rs12460346	0.46 (C)	C	G
c.*3399C>T	45455905	i.r.	rs12460347	0.50 (T)	C	T
c.*3415A>G	45455921	i.r.	rs7254723	0.47 (A)	A	G
c.*3460C>T	45455966	i.r.	rs12460352	0.44 (C)	C	T
c.*3479A>C	45455985	i.r.	rs35670684	NA[e]	A	C
c.*3481delT	45455987	i.r.	rs34356599	0.47 (T)	T	-
c.*3595G>A	45456101	i.r.	rs4803777	0.45 (G)	G	A
c.*3991T>C	45456497	i.r.	rs4803779	0.44 (T)	T	C
c.*4122C>T	45456628	i.r.	rs4803780	0.44 (C)	C	T
c.*4599A>G	45457105	i.r.	rs3760626	0.44 (A)	A	G
c.*4674T>C	45457180	i.r.	rs3760627	0.44 (T)	T	C
c.*4719G>A	45457225	i.r.	rs3760628	0.44 (G)	G	A
c.*4787C>T	45457293	i.r.	rs66867801	0.44 (C)	C	T
c.*4800C>T	45457306	i.r.	rs7259679	0.44 (C)	C	T

(Cont Table 6)

Nucleotide number	GenBank base no.	Position in gene	RefSNP ID	Minor allele Frequency[a]	Alleles	
					Ref.	Pt.
c.*4813G>A	45457319	i.r.	rs66771331	0.44 (G)	G	A
c.*4871G>A	45457377	i.r.	rs73047641	0.44 (G)	G	A
c.*4887T>C	45457393	i.r.	rs73047643	0.36 (C)	T	C
c.*5061T>C	45457567	i.r.	rs7245611	0.44 (T)	T	C
c.*5178G>C	45457684	i.r.	rs10426750	0.44 (G)	G	C

Standardized nomenclature according to the Human Genome Variation Society is given in column 1, with the correlating GenBank number in column 2 (ref no: **NC_000019.9**). Reference SNP IDs (RefSNP ID) and minor allele frequencies as reported in dbSNP database are given in column 4 and 5, respectively. The alleles of the reference (Ref.: **NC_000019.9**) and patient (Pt.) are given in column 6 and 7, respectively. UTR, untranslated region; i.r., intergenic region between *APOC2* and *CLPTM1*. [a]minor allele is given in parenthesis. [b]SNPs located in a $(TG)_n(AG)_m$ microsatellite; the patient has $(TG)_{20}(AG)_8$, which is reportedly a common variant (Fornage M et al. *Genomics* 1992; 12: 63-68). [c]The allele frequency not determined even by direct sequencing due to polyA repeat. [d]The allele frequency determined by direct sequencing of Japanese individuals (n=384). [e]The frequency of C allele (the patient type) was 15.2% in 112 individuals, for whom PCR was successful in the control cohort (n=384).

detectable (named hypoapoC-II). In these cases, homozygous mutations were found in the promoter region[19] or in the donor splice site[17, 18] of *APOC2*, resulting in decreased transcription or defective processing of apoC-II mRNA, respectively. Here we describe a new case of hypoapoC-II with no causative mutations found in the *APOC2* gene. Involvement of other genetic defects affecting plasma apoC-II levels is clearly implied.

Our case was not due to mutations in the protein-coding region of *APOC2* (**Fig. 3**, **Table 6**). Markedly reduced levels of plasma apoC-II protein (**Fig. 1**) could result from decreased apoC-II mRNA transcription, as reported in other cases of hypoapoC-II[17-19]. In fact, we found about 50% reduction in endogenous apoC-II mRNA levels in monocytes/macrophages of the patient (**Fig. 2A**) with no apparent change in the rate of mRNA degradation (**Fig. 2B**). We further detected about a 50% decrease in the transcriptional activity of the *cis*-regulatory region of the patient's *APOC2* gene (**Fig. 4B**); however, all the SNPs contained in the *cis*-regulatory regions are common variants. By scrutinizing every type of apoC-II deficiency reported so far, we conclude that this is a new case of apoC-II deficiency without any rare variants in the *APOC2* gene.

The major question here is what other genes cause extremely low levels of apoC-II proteins in the patient's plasma, and exactly what molecular mechanism is involved. Currently, we do not have the answer to these questions. We sequenced other genes involved in plasma TG metabolism (*LPL*, *APOA5*, *APOC3*, *LMF1* and *GPIHBP1*) and found no mutations or rare variants. The causative genetic defects in our patient would most likely involve other unknown genes that regulate plasma apoC-II concentration. The search for a defect (in genes, miRNAs, etc.) by whole-exome or whole-genome sequencing is of particular interest, which could reveal the yet-to-be-identified regulatory mechanisms of the apoC-II protein.

While our data clearly showed that the transcriptional activity of the patient's *APOC2* minigene was reduced by 50% compared with the normal subject (**Fig. 4B**) with an accompanying 50% reduction in apoC-II mRNA levels (**Fig. 2A**), apoC-II protein levels are more profoundly reduced in the patient's plasma (0.6 $mg \cdot dL^{-1}$; **Fig. 1A**): Compared with the apoC-II protein levels in normolipidemic Japanese population (2.9 $mg \cdot dL^{-1}$)[27], the patient has only 21% of the normal amount of plasma apoC-II protein. This discrepancy between apoC-II protein and apoC-II mRNA levels in the patient implies the yet-to-be-identified causative genes that regulate apoC-II protein levels at translational or post-translational levels. Alternatively, the causative genes could be some transcriptional factors that affect apoC-II mRNA transcription, as the discrepancy may result from 1) the difference between the *in vivo* and *in vitro* milieu of transcriptional machinery, or 2) from the decreased transcription of apoC-II mRNA in the liver or intestine *in vivo*.

Another important question is if this level of reduction in plasma apoC-II (0.6 $mg \cdot dL^{-1}$) could explain the observed massive hypertriglyceridemia in this patient. In the seminal paper by Breckenridge *et al.*, it was implied that only 5-10% of the normal levels of apoC-II would be sufficient to keep plasma triglycerides levels within the normal range[7]. Considering that plasma apoC-II concentration of the patient is only 21% of that of normolipidemic Japanese popu-

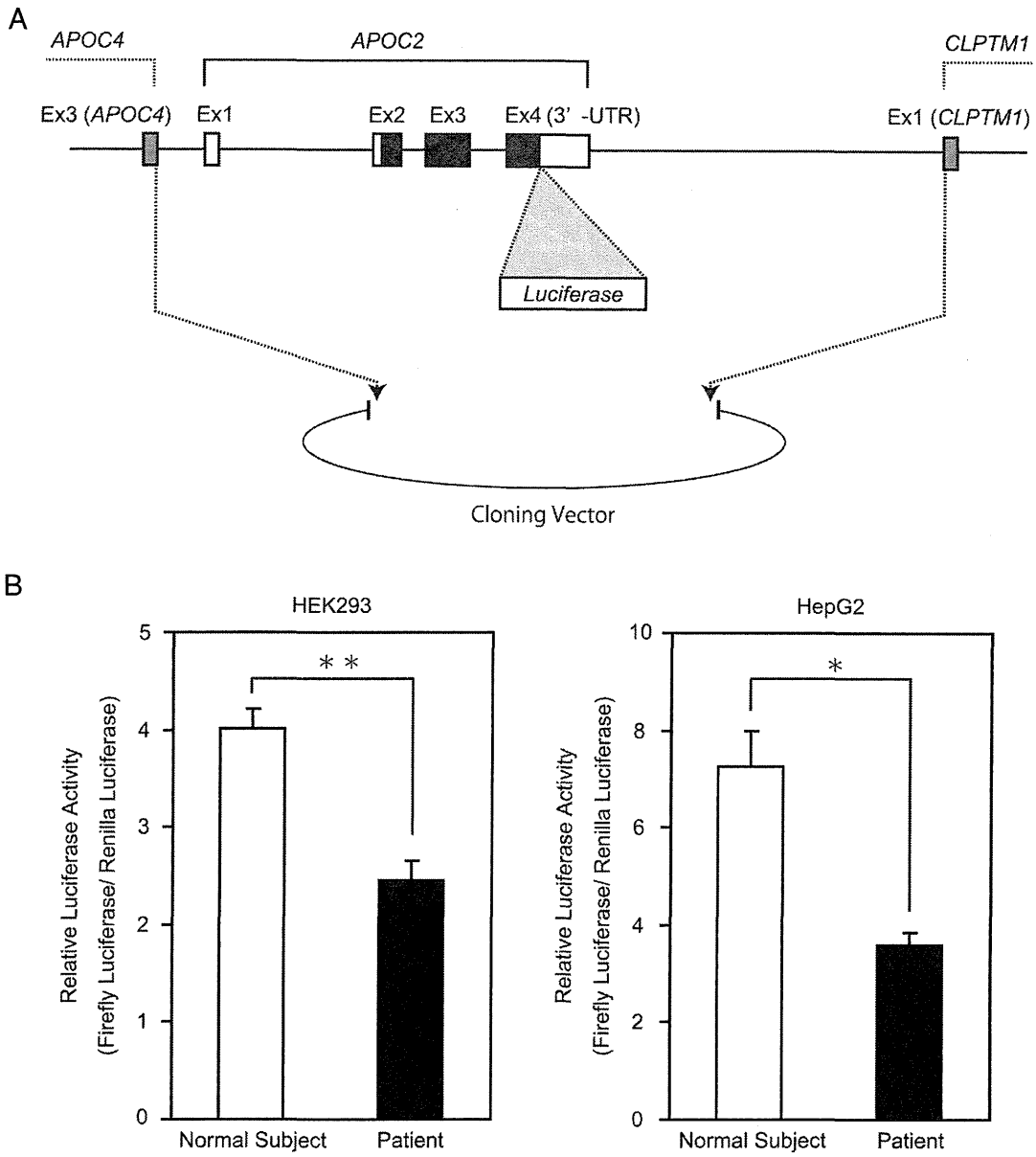

Fig. 4. Minigene reporter assay.

(A) The *APOC2* minigene, spanning from the 3' end of the last exon of *APOC4* to the 5' end of the first exon of *CLPTM1*, were cloned to pGEM-T easy vector, followed by insertion of the firefly luciferase gene just before the stop codon of *APOC2*. (B) HEK293 and HepG2 cells were transiently transfected with these minigene reporter constructs along with Renilla luciferase plasmid as a transfection control. Relative luciferase activity (RLU) was calculated by dividing firefly luciferase activity by Renilla luciferase activity. Data are expressed as the average ± SE of triplicate wells ($^{*}p<0.01$; $^{**}p<0.005$).

lation as calculated above[27], this level of hypoapoC-II would not be sufficient to explain hypertriglyceridemia in this patient *per se*. Indeed, another case of hypoapoC-II with similar plasma apoC-II concentration (0.5 mg·dL^{-1}) was reported by Kawano *et al.*, where the patient only had moderate postprandial hypertriglyceridemia (1.46 mmol·L^{-1} (129 mg·dL^{-1}) and 6.14 mmol·L^{-1} (543 mg·dL^{-1}), in the fasted and fed states, respectively)[31]. This phenotypic heterogeneity may result from additional nutritional, environmental, or other genetic factors such as apoE genotype, as mentioned previously[18, 32]. In other words, hypoapoC-II might be a common genetic predisposition to hypertriglyceridemia requiring additional

genetic/environmental/nutritional factors to develop severe hypertriglyceridemia. Further research on additional genetic defects in hypoapoC-II patients may address this issue.

A key feature of hypoapoC-II reported so far is the decreased rate of transcription of apoC-II mRNA[17-19], which should accompany a decrease in apoC-II mRNA levels in endogenous tissues; however, only one study directly demonstrated the reduction in apoC-II mRNA, where they utilized a liver biopsy specimen[17]. Since the measurement of endogenous levels of apoC-II is essential for the diagnosis of hypoapoC-II, our method to detect endogenous levels of apoC-II mRNA using monocytes/macrophages derived from peripheral blood provides a useful diagnostic tool for this subtype of apoC-II deficiency. The culture system is also useful to explore the mechanism of decreased apoC-II mRNA levels (**Fig. 2**).

Conclusion

We report a new case of apoC-II deficiency that is phenotypically identical to hypoapoC-II, but with no causative mutations detected in the *APOC2* gene. Our results clearly suggest the involvement of other genes that regulate plasma apoC-II protein levels. Further studies on hypoapoC-II may uncover the yet-to-be-identified molecular mechanisms that regulate apoC-II and plasma TG metabolism.

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Conflict of Interest

None.

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Cardiovascular Pharmacology

Locked nucleic acid antisense inhibitor targeting apolipoprotein C-III efficiently and preferentially removes triglyceride from large very low-density lipoprotein particles in murine plasma

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ABSTRACT

A 20-mer phosphorothioate antisense oligodeoxyribonucleotide having locked nucleic acids (LNA–AON) was used to reduce elevated serum triglyceride levels in mice. We repeatedly administered LNA–AON, which targets murine apolipoprotein C-III mRNA, to high-fat-fed C57Bl/6J male mice for 2 weeks. The LNA–AON showed efficient dose-dependent reductions in hepatic apolipoprotein C-III mRNA and decreased serum apolipoprotein C-III protein concentrations, along with efficient dose-dependent reductions in serum triglyceride concentrations and attenuation of fat accumulation in the liver. Through precise lipoprotein profiling analysis of sera, we found that serum reductions in triglyceride and cholesterol levels were largely a result of decreased serum very low-density lipoprotein (VLDL)-triglycerides and -cholesterol. It is noteworthy that larger VLDL particles were more susceptible to removal from blood than smaller particles, resulting in a shift in particle size distribution to smaller diameters. Histopathologically, fatty changes were markedly reduced in antisense-treated mice, while moderate granular degeneration was frequently seen the highest dose of LNA–AON. The observed granular degeneration of hepatocytes may be associated with moderate elevation in the levels of serum transaminases. In conclusion, we developed an LNA-based selective inhibitor of apolipoprotein C-III. Although it remains necessary to eliminate its potential hepatotoxicity, the present LNA–AON will be helpful for further elucidating the molecular biology of apolipoprotein C-III.

1. Introduction

Apolipoprotein C-III (apoC-III) is synthesized mainly in the liver and circulates in plasma (Bruns et al., 1984). The mechanism of apoC-III action is primarily thought to be the attenuation of hydrolysis of triglycerides in lipoproteins, principally by inhibiting capillary endothelial lipoprotein lipase activity. Thus, serum accumulation of apoC-III would cause reduced clearance of triglyceride-rich lipoprotein particles from blood, resulting in the blood accumulation of triglyceride-rich lipoproteins (Havel et al., 1973; Wang et al., 1985). ApoC-III is also known to reduce the clearance of triglyceride-rich lipoproteins and their remnants by blocking apolipoprotein B- or apolipoprotein E-mediated uptake of these lipoproteins to low-density lipoprotein (LDL) receptor (Clavey et al., 1995; Sehayek and Eisenberg, 1991). As growing evidence has shown that elevated plasma triglyceride levels are major risk factors for metabolic syndrome, type 2 diabetes and cardiovascular diseases, apoC-III is a potential therapeutic target for these diseases (Goldberg, 2001; Grundy et al., 2004; Hokanson and Austin, 1996; Sarwar et al., 2007). This notion is also supported by the observation that humans with a null mutation in *APOC3* gene show lower fasting and postprandial serum triglycerides and LDL cholesterol and higher high-density lipoprotein (HDL) cholesterol levels, as well as reduced coronary artery calcification, as compared to humans with normal apoC-III activity (Pollin et al., 2008), while some specific single-nucleotide polymorphism carriers in *APOC3* show increased plasma triglyceride levels and evidence of non-alcoholic fatty liver, in addition to

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elevated cardiovascular disease risk (Petersen et al., 2010). A number of studies using genetically engineered mouse models have also revealed the dyslipidemic or atherogenic effects of apoC-III (Gerritsen et al., 2005; Ito et al., 1990; Jong et al., 2001; Takahashi et al., 2003). In addition, attenuation of apoC-III has been shown to be beneficial for type I diabetes (Holmberg et al., 2011; Juntti-Berggren et al., 1993, 2004). Thus, the privation of apoC-III would lead to significant benefits, both indirectly and directly, in the reduction of cardiovascular disease risk (Ooi et al., 2008; Pollin et al., 2008).

There are currently several state-of-the-art gene silencing approaches available for target-specific disruption, such as antisense oligonucleotides (AONs), monoclonal antibodies and small interfering RNAs (siRNAs), which are showing promising results, particularly in dyslipidemia therapy (Norata et al., 2013). Graham et al. (2013) recently reported successful attenuation of apoC-III mRNA and plasma triglyceride levels in preclinical models and humans by using antisense oligonucleotides chemically modified with 2′-*O*-methoxyethyl RNAs, which are known to preferentially distribute to the liver, where apoC-III is synthesized (Graham et al., 2013). Our group has developed a series of conformationally constrained nucleic acids including 2′,4′-bridged nucleic acids (2′,4′-BNAs), which are also known as locked nucleic acids (LNAs) (Mitsuoka et al., 2009; Miyashita et al., 2007; Obika et al., 1997; Yahara et al., 2012). This class of modified nucleotides has been found to have superior potential for antisense therapeutics on account of their extraordinarily high mRNA binding, as well as systemic effects over 2′-*O*-methoxyethyl RNAs (Gupta et al., 2010; Lanford et al., 2010; Lindholm et al., 2012; Prakash et al., 2010; Seth et al., 2009; Yamamoto et al., 2012). Specifically, the in vivo potencies of LNA-based AONs are generally 5 to 10-fold greater than their 2′-*O*-methoxyethyl RNA-containing counterparts (Prakash et al., 2010; Seth et al., 2009). Thus, LNA-based anti-apoC-III AONs are expected to be better alternatives to 2′-*O*-methoxyethyl RNA-containing congeners. We here demonstrated the effective reduction in elevated serum triglyceride levels in mice using LNA-based AONs targeting hepatic apoC-III mRNA.

2. Materials and methods

2.1. Antisense oligonucleotides

LNA was partially incorporated into a 20-mer phosphorothioated oligodeoxyribonucleotide. We prepared two potential AONs, **A301S** (5′-tcttatccagctttattagg-3′) and **A301SL** (5′-TCtTaTCcagcttTaTTaGg-3′), in which lowercase and uppercase letters represent DNA and LNA, respectively. These AONs have an identical sequence targeting murine apoC-III mRNA, a sequence patented by ISIS pharmaceuticals as being highly potent (Crooke et al., 2009). These modified AONs were synthesized and provided by Gene Design (Osaka, Japan). Syntheses were conducted using standard phosphoramidite procedures, and products were carefully processed under aseptic conditions and purified. All products were endotoxin-free and contained low levels of residual salts for in vivo usage.

2.2. In vivo pharmacological experiments

All animal procedures were performed in accordance with the guidelines of the Animal Care Ethics Committee of the National Cerebral and Cardiovascular Center Research Institute (Osaka, Japan). All animal studies were approved by the Institutional Review Board. C57BL/6J mice were obtained from CLEA Japan (Tokyo, Japan). All mice were male, and studies were initiated when animals were aged 6–8 weeks. Mice were maintained on a 12-h light/12-h dark cycle and fed ad libitum. Mice were fed normal chow (CE-2; CLEA Japan) or Western diet (F2WTD; Oriental Yeast, Tokyo, Japan) for 2 weeks before the first treatment and throughout the experimental period. Mice received multiple treatments with AONs administered intraperitoneally at doses of 10 and 20 mg/kg/injection. Peripheral blood was collected from the tail vein in BD Microtainers (BD, Franklin Lakes, NJ) for separation of serum. At the time of sacrifice, livers were harvested and snap frozen until subsequent analysis. Collected blood was subjected to serum separation for subsequent analysis.

2.3. High performance liquid chromatography analysis of serum

The cholesterol and triglyceride profiles of serum lipoproteins were analyzed using a dual detection high performance liquid chromatography (HPLC) system with two tandem connected TSKgel LipopropakXL columns (300 mm × 7.8 mm; Tosoh, Tokyo, Japan), in accordance with the methods provided by Skylight Biotech (Akita, Japan). Individual subfractions were quantified by best curve fitting analysis, assuming that the particle sizes of all subfractions followed a Gaussian distribution. Particle sizes for individual subfractions were previously determined as 44.5–64 nm (large VLDL), 36.8 nm (medium VLDL), 31.3 nm (small VLDL), 28.6 nm (large LDL), 25.5 nm (medium LDL), 23 nm (small LDL), 16.7–20.7 nm (very small LDL), 13.5–15 nm (very large HDL), 12.1 nm (large HDL), 10.9 nm (medium HDL), 9.8 nm (small HDL) and 7.6–8.8 nm (very small HDL) (Okazaki et al., 2005; Usui et al., 2002).

2.4. mRNA quantification

Total RNA was isolated from cultured cells or mouse liver tissues using TRIzol Reagent (Life Technologies Japan, Tokyo, Japan) according to the manufacturer's protocols. Gene expression was evaluated by 2-step quantitative reverse transcription PCR (RT-PCR). Reverse-transcription of RNA samples was performed using a High Capacity cDNA Reverse-Transcription Kit (Life Technologies Japan, Tokyo, Japan), and quantitative PCR was performed by TaqMan Gene Expression Assay (Life Technologies Japan, Tokyo, Japan). mRNA levels of target genes were normalized against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA levels. The following primer sets were used for quantitative PCR: for assay ID, Mm00445670_m1 (apoc3) and Mm99999915_m1 (gapdh).

2.5. Western blotting analysis

Serum was diluted with buffer (150 mM NaCl, 1.0% IGEPAL® CA-630, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris, pH 8.0, 20 × Complete Mini protease inhibitor cocktail 1:20 (Roche, Indianapolis, IN)) and total protein concentrations were measured with a detergent compatible assay kit (Bio-Rad, Hercules, CA). Solutions were subjected to electrophoresis on 16% Tris-glycine gels (Life Technologies Japan, Tokyo, Japan) at 180 V for 30 min, and were transferred to a PVDF membrane (Bio-Rad). Apo-CIII Western blotting was performed at room temperature for 1 h with an anti-apo-CIII antibody (Santa Cruz Biotechnology, Santa Cruz, CA) at 200 mV for 120 min. Membranes were washed three times with PBS containing 0.3% Tween20. Blots were labeled with horseradish peroxidase-conjugated secondary goat anti-rabbit antibody (Santa Cruz Biotechnology, Santa Cruz, CA). Chemiluminescent detection was performed using an ECL prime Western blot detection kit (Amersham Biosciences, Buckinghamshire, UK), and bands were visualized using an LAS-4000 mini image analyzer (Fuji Film, Tokyo, Japan).

2.6. Serum chemistry and histopathology

Blood collected from the inferior vena cava upon sacrifice was subjected to serum chemistry. Assay kits (Wako, Osaka, Japan) were used to measure serum levels of aspartate aminotransferase, ALT, blood urea nitrogen and creatinine, which are biomarkers for hepatic and kidney toxicity. Formalin-fixed liver and kidney samples (#064–00406; Wako) were embedded in Histsec (Merck, Darmstadt, Germany), sliced at 5 μm using a microtome (Leica Microsystems, Wetzlar, Germany) and stained with Carrazzi's hematoxylin and Tissue-Tek eosin solutions (Sakura Finetek USA, Torrance, CA) for histopathological examination. Frozen liver tissues were placed in Tissue-Tek Intermediate cryomolds (#4566; Sakura Finetek USA) filled with precooled Tissue-Tek O. T.C embedding compound (#4583; Sakura Finetek USA) and flash-frozen by immersion in liquid nitrogen. Samples were sliced at 5 μm using a Leica CM1850 (Model 1850-11-1; Leica Biosystems, Wetzlar, Germany) and were air-dried for an hour. The resulting sections were rinsed with distilled water for 30 s and 60% 2-propanol (#03065-35; Nakarai Tesque, Kyoto, Japan) for 60 s. Oil Red O staining stock solution was prepared by dissolving 0.3 g of Oil Red O dye (#154–02072; Wako) in 100 mL of 2-propanol with gentle overnight incubation at 60 °C. Then, 30 mL of stock solution was diluted with 20 mL of distilled water to give a working solution. Samples were stained with this working solution at 37 °C for 15 min, rinsed with 60% 2-propanol and distilled water, and stained with hematoxylin (Gill's Formula) (#H-3401; Vector, Burlingame, CA) solution (25% in PBS) for 2 min at room temperature for histological analysis.

2.7. Statistical analysis

Pharmacological studies were performed with 4–9 mice per treatment group. All data are expressed as means ± SD. $P < 0.05$ was considered to be statistically significant in all cases. Statistical comparisons of results were performed by Dunnett's multiple comparison tests.

3. Results

3.1. Design and physicochemical properties of anti-apoC-III LNA–AON

We first designed AONs targeting apoC-III carrying LNAs (**A301SL**). We placed nine LNAs in the strand, keeping a six natural-nucleotide gap, which is thought to be sufficient for the introduction of RNase H-mediated scission of the mRNA strand (Yamamoto et al., 2012). At the same time, we prepared a corresponding conventional phosphorothioate AON designated **A301S** (Table 1). **A301SL**, **A301S** and 2′-*O*-methoxyethyl RNA-based apoC-III AON, reported previously by Graham et al. (2013), possess the phosphorothioate backbone, but they have different target sequences. As introduction of 2′-*O*-methoxyethyl RNAs into conventional phosphorothioate AONs moderately improves mRNA binding and in vivo antisense potency, **A301S** is speculated to have weaker potential than 2′-*O*-methoxyethyl RNA-based AON. Ideally, the potency and toxicity characteristics of **A301SL** should be compared with those of a corresponding 2′-*O*-methoxyethyl RNA-containing counterpart; however, as we were unable to obtain their phosphoroamidites, we herein utilized **A301S** as a non-LNA control. Note that the sequence, length and composition of AONs have not been fully optimized. A thermal melting study was carried out and T_m values of **A301SL** and **A301S** with their complementary RNA strands were determined. As expected, **A301SL** showed excellent target affinity when compared with conventional phosphorothioate AON (Table 1).

Table 1
Antisense oligonucleotides used in this study.

	Sequence ID	Sequence[a]	T_m (°C)
1	**A301S**	5′-tcttatccagctttattagg-3′	48
2	**A301SL**	5′-TCtTaTCcagcttTaTTaGg-3′	79

[a] Oligonucleotides with LNA (upper case letters) and DNA (lower case letters). All inter nucleotide linkages are phosphorothioated. Melting temperatures (T_m) of 1:1 mixtures of **A301S** and complementary RNA or **A301SL** and complementary RNA.

3.2. Hepatic reduction of apoC-III mRNA expression after systemic administration of LNA–AON

In order to assess the mRNA silencing potency of AONs, we repeatedly administered **A301SL** and **A301S** to C57Bl/6J male mice. After feeding 6-week-old male C57Bl/6J mice a high-fat diet for 2 weeks, mice were subjected to intraperitoneal (i.p.) injection of naked AON at a dosage of 10 and 20 mg/kg/injection five times over 2 weeks. Peripheral blood sampling was performed on day 0 just before the first injection, and on days 8 and 16 post-dose under feed-deprived condition for lipid component analysis and toxicity evaluation. Mice were dissected and their livers were harvested for measurement of gene expression on day 16 post-injection. As shown in Fig. 1, a significant dose-dependent decrease in hepatic apoC-III mRNA levels was only observed in **A301SL**-treated arms. **A301SL** suppressed hepatic apoC-III mRNA expression by ~29% and ~72% on average at a dosage of 10 and 20 mg/kg respectively, while **A301S** failed to achieve any reduction in apoC-III mRNA in the liver, even at the higher dose.

3.3. Serum reduction of apoC-III protein after systemic administration of LNA–AON

Changes in serum apoC-III protein concentration were confirmed by Western blot analysis. Although the quantitative capacity of Western blot analysis is very limited, we found that **A301SL** removed about half of apoC-III protein from sera at a dosage of 20 mg/kg on day 16, while **A301S** showed no significant reductions in apoC-III protein levels, which is consistent with the changes in hepatic apoC-III mRNA expression levels (Fig. 2). Collectively, we

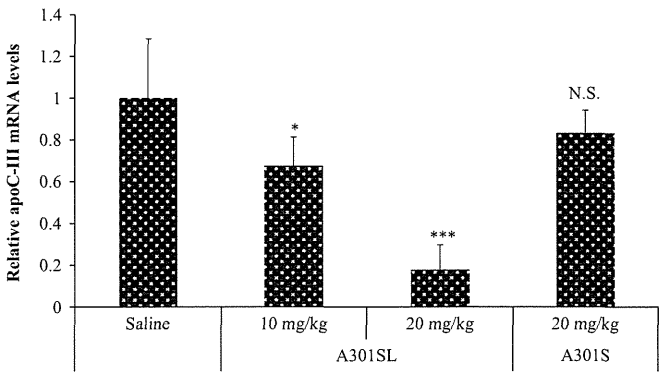

Fig. 1. Hepatic apoC-III mRNA silencing effects of **A301SL** and **A301S**. Western diet-fed mice received intraperitoneal administration of these two AONs at 10 or 20 mg/kg five times over 16 days. Relative hepatic apoC-III mRNA expression levels were determined by means of two-step real-time RT-PCR, and there was a significant reduction in A301SL-treated arms (Dunnett's multiple comparison test, $^{***}P < 0.001$, $^{**}P < 0.01$, $^{*}P < 0.05$, N.S.; not significant). Error bars represent group means + SD.

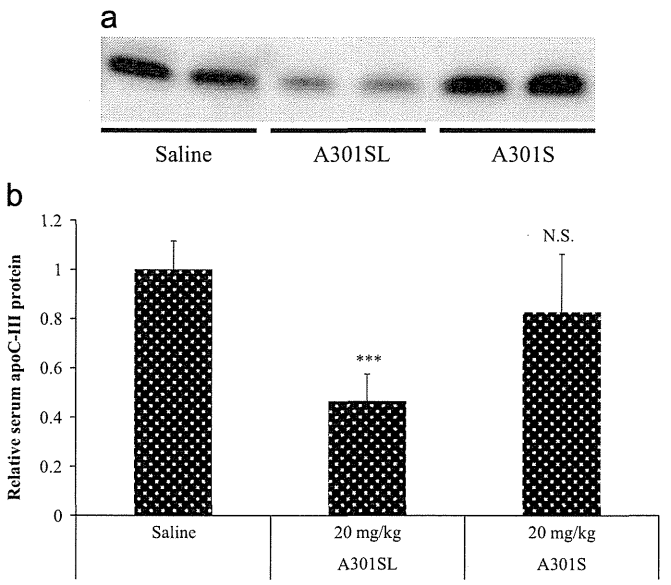

Fig. 2. Effects of **A301SL** and **A301S** on serum apoC-III protein levels. Western diet-fed mice received i.p. administration of these two AONs at 20 mg/kg for five times over 16 days. After completion of dosing, reductions in apoC-III protein level in serum were investigated by Western blotting. (a) Representative images of the membrane, and (b) there was a significant reduction in **A301SL**-treated arms (Dunnett's multiple comparison test, $^{***}P<0.001$, $^{**}P<0.01$, $^{*}P<0.05$, N.S.; not significant). Error bars represent group means ± S.D.

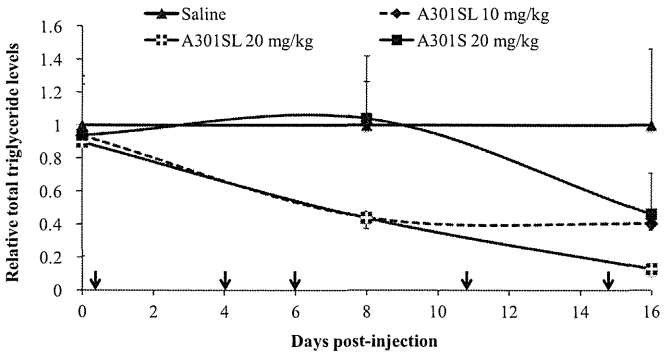

Fig. 3. Effects on serum triglyceride levels over time. Western diet-fed mice received intraperitoneal administration of two AONs, **A301SL** at 10 and 20 mg/kg/injection and **A301S** at 20 mg/kg/injection five times over 16 days. On days 0, 8 and 16, blood samples were collected from tail vein and total triglyceride levels were measured. Dose-dependent reductions were observed in **A301SL** groups, and delayed reductions were seen in the **A301S**-treated arm. Error bars represent group means ± S.D. Arrows indicate the date of administration.

successfully showed that the LNA–AON designed here is a potential inhibitor of apoC-III expression in vivo.

3.4. Serum changes in triglyceride-rich lipoprotein particles concentrations after systemic administration of LNA–AON

To confirm the ability of LNA–AON to modify serum lipids, we assessed the changes in triglyceride contents in fasting peripheral blood collected on days 0, 8 and 16 post-injection. As shown in Fig. 3, **A301SL** was confirmed to reduce serum total triglyceride concentration dose-dependently and more efficiently when compared to **A301S**. Total serum triglyceride levels with a 20 mg/kg/injection of **A301SL** were reduced by ~56% and ~87% over time, as compared to saline-treated controls, whereas **A301S** reduced total serum triglyceride levels by ~54% on day 16. We further conducted HPLC analysis of sera collected on day 8 to determine

Table 2
Serum lipoprotein profiles of hypertriglyceridemic mice on day 8.

	20 mg/kg		
	Saline	A301SL	A301S
Triglyceride [mg/dL]			
Total TG	54.9 ± 13.0	22.1 ± 5.7[b]	51.5 ± 13.1
Chylomicron	0.9 ± 0.5	0.2 ± 0.1[b]	0.5 ± 0.2
Large VLDL	30.3 ± 9.9	5.2 ± 2.3[a]	24.1 ± 7.2
Medium VLDL	10.8 ± 1.7	4.9 ± 1.5[a]	10.4 ± 2.5
Small VLDL	2.9 ± 0.3	2.0 ± 0.5[c]	3.1 ± 0.6
Large LDL	3.4 ± 0.3	2.7 ± 0.7	4.0 ± 0.8
Medium LDL	2.5 ± 0.2	2.3 ± 0.6	3.4 ± 0.8
Small LDL	1.4 ± 0.2	1.3 ± 0.4	1.9 ± 0.4
Very small LDL	1.1 ± 0.2	1.0 ± 0.3	1.3 ± 0.3
Very large HDL	0.28 ± 0.06	0.19 ± 0.05	0.29 ± 0.08
Large HDL	0.26 ± 0.04	0.27 ± 0.11	0.34 ± 0.09
Medium HDL	0.24 ± 0.04	0.49 ± 0.28	0.48 ± 0.18
Small HDL	0.11 ± 0.01	0.59 ± 0.40[c]	0.48 ± 0.23
Very small HDL	0.74 ± 0.09	1.17 ± 0.43	1.13 ± 0.21
Cholesterol [mg/dL]			
TC	133.4 ± 15.6	109.5 ± 11.7*	125.1 ± 14.9
Chylomicron	0.13 ± 0.05	0.04 ± 0.02[a]	0.07 ± 0.02[b]
Large VLDL	6.3 ± 1.8	1.3 ± 0.5[a]	3.9 ± 0.7[a]
Medium VLDL	4.7 ± 0.9	2.6 ± 0.6[a]	2.9 ± 0.4[a]
Small VLDL	3.0 ± 0.5	2.7 ± 0.8	2.4 ± 0.4
Large LDL	5.0 ± 0.6	5.0 ± 1.4	4.8 ± 0.7
Medium LDL	4.9 ± 0.6	5.2 ± 1.6	5.9 ± 0.8
Small LDL	3.4 ± 0.4	3.6 ± 1.1	4.4 ± 0.7
Very small LDL	6.1 ± 2.3	5.6 ± 2.0	11.7 ± 3.8[c]
Very large HDL	7.5 ± 2.3	7.2 ± 2.3	9.1 ± 1.7
Large HDL	32.1 ± 5.2	27.9 ± 3.3	29.4 ± 3.3
Medium HDL	35.3 ± 3.4	28.6 ± 1.1[b]	29.9 ± 3.2[c]
Small HDL	15.7 ± 1.0	12.1 ± 0.2[a]	12.5 ± 1.7[b]
Very small HDL	9.1 ± 0.8	7.6 ± 0.5[c]	8.0 ± 1.0

TG; triglyceride, TC; total cholesterol. Data are means ± S.D.
[a] $P<0.001$ vs. saline group.
[b] $P<0.01$ vs. saline group.
[c] $P<0.05$ vs. saline group.

the precise serum lipid profile. HPLC analysis revealed that **A301SL** markedly reduced VLDL-triglycerides, and larger VLDL-triglycerides were preferentially removed (Table 2). Moreover, substantial reductions in VLDL- and HDL-cholesterol were also observed in the **A301SL**-treated arm, and a much milder but similar trend was seen in the **A301S**-treated arm. These trends were particularly evident on day 16 (Fig. 4), and are consistent with the slight but not significant reductions in hepatic apoC-III mRNA and serum apoC-III protein levels, as shown in Figs. 1 and 2 on day 16.

3.5. Histopathological analysis of murine liver and kidneys

Pharmacological and toxicological characteristics of **A301SL** upon dosing were estimated by histopathological analysis. While all individuals in the saline group showed fat accumulation in the liver, induced by the Western diet, no such findings were observed in the **A301S**- and **A301SL**-treated arms (Fig. 5 and Table 3). We further visualized and compared fat drops in the livers by direct lipid staining with Oil Red O. As shown in Fig. 5, LNA–AON markedly reduced hepatic fat accumulation. Histopathologically, no severe cellular damage was noted, even at the highest doses in the centrilobular and perilobular hepatocytes, which were frequently seen after toxicological insult. On the other hand, moderate granulomas and granular degeneration were observed in the liver. Serum chemistry profiles showed slight increases in serum

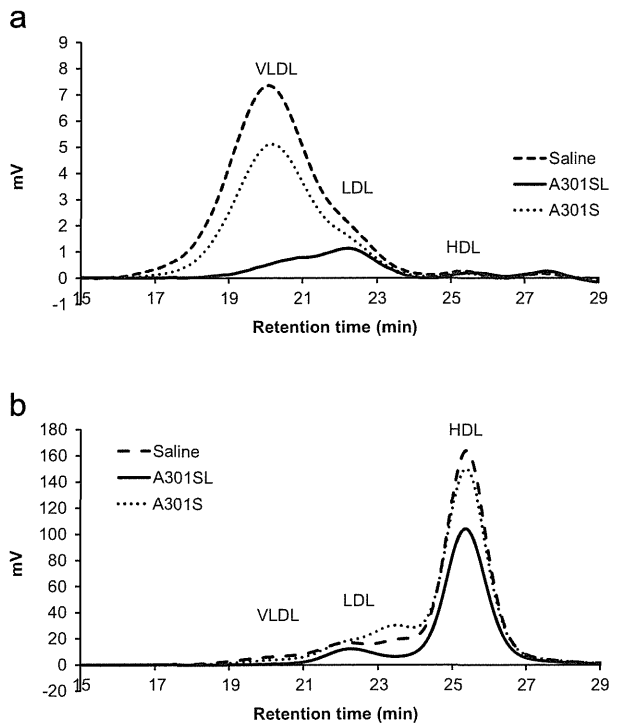

Fig. 4. Representative HPLC lipoprotein profiles of western diet-fed C57BL/6J mice received intraperitoneal administration of saline (dashed line), **A301SL** (solid line) at 20 mg/kg/injection or **A301S** (dotted line) at 20 mg/kg/injection five times over 16 days. Five saline-, **A301SL**- and **A301S**-treated mice were analyzed and the data from one representative individual mouse were presented. Corresponding (a) triglyceride and (b) cholesterol profiles were obtained from one identical mouse in each arm.

transaminases and slight decreases in blood urea nitrogen (Table 4). Elevations in transaminases may be due to the granular degeneration of hepatocytes. There were no significant changes in serum creatinine levels in each group.

4. Discussion

We have scarcely obtained selective inhibitors of apoC-III, which is thought to be a potential drug for the treatment of dyslipidemia, diabetes and cardiovascular diseases, as well as a useful tool for elucidation of the physiological roles of apoC-III. To develop a selective inhibitor of apoC-III, we designed an LNA-based 20-mer phosphorothioated AON (**A301SL**), in which LNAs are expected to greatly help with the target binding for the usage in vivo. As expected, **A301SL** achieved efficient dose-dependent reductions in hepatic apoC-III mRNA and decreased serum apoC-III protein concentration, which could be associated with the observation of efficient dose-dependent reductions in serum triglyceride concentration and attenuation of fat in the liver. *One limitation is that serum change of apoC-III protein was here confirmed by semiquantitative Western blot analysis.* For further study, we moved onto a precise lipoprotein profiling analysis of sera using HPLC methodology. Through this analysis, we found that serum reductions in triglycerides and cholesterol levels were largely a result of decreases in VLDL-triglycerides and VLDL-cholesterol from sera. It is also noteworthy that larger-sized VLDL was more susceptible to removal from blood, resulting in a shift of particle size distribution to smaller diameters (Table 2 and Fig. 4). Generally, large triglyceride-rich VLDL-1 are preferentially converted into atherogenic small, dense LDL, through a process mediated principally by cholesteryl ester transfer protein, lipoprotein lipase and hepatic lipase (Millar and Packard, 1998). Lipoprotein lipase activity is known to be modified by apoC-III protein and lipoprotein lipase preferentially hydrolyzes larger triglycerides-rich VLDL subfractions than smaller particles (Fisher et al., 1995). Thus, preferential removal of triglycerides from larger VLDL particles observed here can be explained as a result of derepression of lipoprotein lipase activity *via* successful silencing of apoC-III with LNA–AON. Combined with previous observations that, among triglycerides-rich lipoprotein subfractions in combined hyperlipidemia patients such as type IIb, VLDL-1 has the highest potential to induce accumulation of triglycerides and cholesterol in macrophages and foam cell formation (Milosavljevic et al., 2001), selective apoC-III inhibitors would possibly show anti-atherogenic phenotype.

Both apoC-III-null subjects and apoC-III-deficient mice generally possess reduced plasma total cholesterol levels, as well as total triglycerides, when compared to those of normal controls (Gerritsen et al., 2005; Jong et al., 2001; Pollin et al., 2008; Takahashi et al., 2003). We also observed a 33% reduction in total cholesterol levels along with apoC-III attenuation by the LNA–AON. This decrease in plasma cholesterol levels was reflected in both apolipoprotein B-containing and HDL fractions (Table 2 and Fig. 4). However, the mechanistic background for the reduction of plasma cholesterol upon apoC-III attenuation is controversial. A previous study showed that apoC-III deficiency in apolipoprotein E-knockout mice accelerated the kinetics of uptake of cholesterol ester, which is related to the function of hepatic lipase (Jong et al., 2001). In addition, hepatic lipase transgenic rabbits and hepatic lipase transgenic and adenovirus-transduced mice were reported to reduce plasma triglycerides and apolipoprotein B-containing lipoprotein cholesterols as well as HDL cholesterol (Applebaum-Bowden et al., 1996; Busch et al., 1994; Dichek et al., 1998; Fan et al., 1994). As our findings are in line with these previous observations, we speculate that activation of hepatic lipase resulting from apoC-III attenuation by the LNA–AON caused a reduction in plasma cholesterol levels. In contrast, Old Order Amish individuals with an *APOC3*-null mutation have higher plasma HDL cholesterol concentrations, as well as lower levels of triglycerides and non-HDL cholesterol than those of normal subjects (Pollin et al., 2008). In addition, knockout effects of apoC-III on plasma cholesterol levels also vary between genetic backgrounds of mice and experimental conditions (Jong et al., 2001; Takahashi et al., 2003). There are only a small number of reports focusing on the relationship between cholesterol metabolism and apoC-III (Kinnunen and Ehnholm, 1976). To determine the true effects of apoC-III modulation on cholesterol metabolism, further experimental data is necessary.

The toxicological characteristics of **A301SL** and **A301S** were estimated based on serum biochemistry characteristics and histopathological analysis. As phosphorothioated AONs accumulate mainly in the kidney and liver, hepatotoxicity and/or nephrotoxicity are primary concerns. Our experiments found only moderate hepatotoxicity for **A301SL** and **A301S,** as shown in the moderate increases in liver transaminases and decreases in blood urea nitrogen, while no significant changes in serum creatinine levels were noted. Histopathological observations supported these data (Fig. 5, Tables 3 and 4). Similar hepatotoxicity attributable to LNA-modified phosphorothioated AONs, which was avoidable by substituting 2′,4′-BNA^{NC} chemistry for LNA, has been reported (Prakash et al., 2010; Yamamoto et al., 2012). Dose-related hepatotoxicity could be tolerable based on the systemic AON recently approved by the US Food and Drug Administration (FDA) named "Kynamro", which also shows serum elevation of transaminases, specifically alanine aminotransferase (ALT) (http://www.kynamro.com/). However, it is necessary to determine how AONs trigger toxicity in order to resolve this issue (Levin, 1999). Therefore, we further conducted Oil Red O staining of liver samples. The results

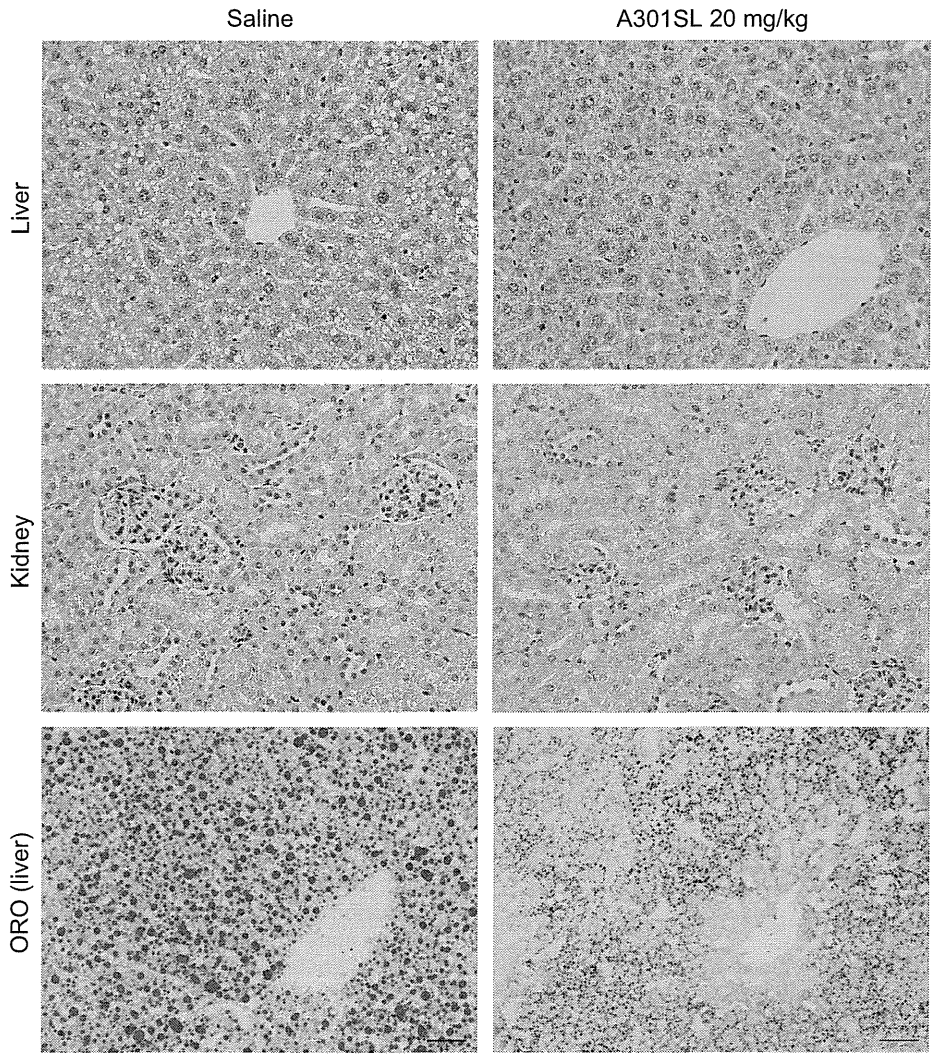

Fig. 5. Representative histopathological changes in livers and kidneys subjected to 16 days of saline (left) and **A301SL** (right) dosing were assessed by H&E or Oil Red O staining (× 200 magnification). Peripheral fatty changes were observed in the liver of the saline-treated mice (top and bottom), while periportal granular degeneration were seen in the highest dose of **A301SL**-treated mice with complete loss of fatty changes (top). No significant changes were observed in kidneys (middle).

Table 3
Histopathological findings.

		Saline	A301SL		A301S
	Dose	–	10 mg/kg	20 mg/kg	20 mg/kg
	Number of mice examined–	9	4	5	5
Organ Liver	Findings				
	Normal	0	3	0	2
	Fatty change, periportal	9	0	0	0
	Granuloma	0	1	1	2
	Granular degeneration, periportal	0	0	5	0
Kidney(s)					
	Normal	9	5	5	4
	Hemorrhage	0	0	0	1

All lesions showed a moderate grade.

Table 4
Effects on serum chemistry.

		AST (IU/L)	ALT (IU/L)	BUN (mg/dL)	Cre (mg/dL)
Saline		17.6 ± 2.8	9.1 ± 3.2	29.7 ± 6.4	0.2 ± 0.05
A301SL	10 mg/kg	22.5 ± 4.6	20 ± 9.2[b]	30.1 ± 4	0.1 ± 0.05
	20 mg/kg	44.2 ± 13.8[b]	14.5 ± 3.3	20.4 ± 3.3[b]	0.1 ± 0.00
A301S	20 mg/kg	22 ± 2	7.5 ± 0.9	18.4 ± 2.6[b]	0.1 ± 0.00

AST; aspartate aminotransferase, ALT; alanine aminotransferase, BUN; blood urea nitrogen, Cre; serum creatinine. Data are presented as means ± S.D.
[a] $P < 0.001$, [c] $P < 0.05$ vs. saline group.
[b] $P < 0.01$ vs. saline group.

showed that our AON does not induce steatosis, which is a typical feature of drug-induced hepatotoxicity (Begriche et al., 2011). Instead, we observed drastic regression of steatohepatitis in **A301SL**-treated arms (Fig. 5), which is presumably an on-target-based pharmacological effect.

In conclusion, we successfully developed an anti-apoC-III LNA-AON. Although this selective apoC-III inhibitor of **A301SL** shows improved potency and safety, it will nevertheless be of help to further elucidate the molecular biology and molecular physiology of apoC-III that other non-selective inhibitors of apoC-III and have failed to reveal.

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Committee Report 9

Familial Hypercholesterolemia

Executive Summary of the Japan Atherosclerosis Society (JAS) Guidelines for the Diagnosis and Prevention of Atherosclerotic Cardiovascular Diseases in Japan—2012 Version

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This is a collaborative work to describe the guidelines for familial hypercholesterolemia issued by the Committee for Epidemiology and the Clinical Management of Atherosclerosis, the Committee for the Diagnosis and Treatment of Familial Hypercholesterolemia and the Research Committee for Primary Hyperlipidemia, Research on Measures against Intractable Diseases.

Heterozygous Familial Hypercholesterolemia

1. Condition and Clinical Picture of FH

Familial hypercholesterolemia (FH) is an autosomal dominant disease caused by abnormal LDL receptors or LDL receptor-related genes, characterized by the triad of (1) hyper-LDL cholesterolemia, (2) premature coronary artery disease (CAD) and (3) tendon/cutaneous xanthoma. Arcus corneae is also characteristic of FH; however, the rate is approximately 30%.

FH by itself is a very high-risk condition for CAD. Untreated men 30 to 50 years of age and women 50 to 70 years of age are likely to develop CAD, such as myocardial infarction and angina pectoris[1]. Early diagnosis and appropriate treatment result in the prevention of premature death. Heterozygous FH exists in approximately one in 500 people, and it is estimated that there are approximately 300,000 patients in Japan. Therefore, heterozygous FH is one of the genetic diseases most frequently encountered by general practitioners.

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2. Diagnosis of Heterozygous FH

1) LDL-C Cutoff Value

Table 1 shows the diagnostic criteria. Using data obtained from a total of 1,397 untreated dyslipidemic patients, including 439 patients with FH and 958 patients without FH, an analysis was performed of

Table 1. Diagnostic Criteria for Heterozygous FH in Adults (15 Years of Age or Older)

1. Hyper-LDL cholesterolemia (an untreated LDL-C level of ≥ 180 mg/dL)
2. Tendon xanthoma (tendon xanthoma on the backs of the hands, elbows, knees, etc. or Achilles tendon hypertrophy) or xanthoma tuberosum
3. Family history of FH or premature CAD (within the patient's second-degree relatives)

- The diagnosis should be made after excluding secondary hyperlipidemia
- If a patient meets two or more of the above-mentioned criteria, the condition should be diagnosed as FH. In cases of suspected FH, obtaining a diagnosis using genetic testing is desirable.
- Xanthoma palpebrarum is not included in xanthoma tuberosum.
- Achilles tendon hypertrophy is diagnosed if the Achilles tendon thickness is ≥ 9 mm on soft X-ray imaging.
- An LDL-C level of ≥ 250 mg/dL strongly suggests FH.
- If a patient is already receiving drug therapy, the lipid level that led to treatment should be used as the reference for diagnosis.
- Premature CAD is defined as the occurrence of CAD in men < 55 years of age or women < 65 years of age.
- If FH is diagnosed, it is preferable to also examine the patient's family members.

major items, including an LDL-C level of ≥ 180 mg/dL, the presence of Achilles tendon hypertrophy or cutaneous xanthoma and a history of FH or premature CAD in relatives within the second degree. The results showed a sensitivity of 94.3% and a specificity of 99.1%. In cases involving an LDL-C level of ≥ 190 mg/dL, the sensitivity was 92.1% and the specificity was 99.1%. Therefore, 180 mg/dL, the level at which the specificity was the same and the sensitivity was higher than that observed at 190 mg/dL, was adopted as the LDL-C cutoff value[2]. Because this analysis showed that 5% of patients with an LDL-C level of ≥ 250 mg/dL do not have FH, a diagnosis of FH is thus strongly suspected in the presence of an LDL-C level of ≥ 250 mg/dL alone[3].

2) Soft X-Ray Radiography of the Achilles Tendon

Achilles tendon hypertrophy should be evaluated using soft X-ray radiography. Positioning is performed so that the lower leg bones and sole of the foot form a 90-degree angle, and radiation is administered so that the X-ray enters the center of the lateral malleolus from the side of the foot. The imaging distance should be 120 cm, and the imaging conditions should be 50 kV and 5.0 mA. When the greatest dimension is ≥ 9 mm, hypertrophy is diagnosed. Conducting the evaluations using ultrasonography is possible, although it has not yet been standardized.

3) Differential Diagnosis

Diseases that must be distinguished from FH include conditions that cause secondary hyperlipidemia (e.g., diabetes mellitus, hypothyroidism and nephrotic syndrome) and a similar disease, familial combined hyperlipidemia (FCHL). FCHL is distinguished by the absence of tendon xanthoma, the presence of small, dense LDL, the presence of other types of dyslipidemia (types IIa, IIb and IV) in the patient's family and, in children, a lower degree of increase in the LDL-C level compared with that observed in FH.

3. Management Targets for LDL-C in Heterozygous FH

Because FH is a disease associated with a very high risk of CAD, FH should be considered to correspond to secondary prevention, and it is desirable to set a management target for the LDL-C level at < 100 mg/dL. However, in many cases, it is difficult to achieve a management target for an LDL-C level of < 100 mg/dL in FH patients in clinical practice. Therefore, it is acceptable to aim for < 50% of the pretreatment level if the management target for LDL-C is not achieved. The achievement of the management target does not always assure the absence of future cardiovascular events. In the treatment of FH, risk assessment cannot be applied using the risk charts provided in these guidelines. This management target should be applied to patients with FH ≥ 30 years of age, and it is desirable to administer the treatment under the direction of a specialist, in principle. Treatment for FH in patients 15-29 years of age must be administered under the direction of a specialist.

4. Treatment of Heterozygous FH

1) Lifestyle Modification

Lifestyle modification should be performed in FH patients after diagnosis and continued as described in committee report 7A[4]. However, due to the high risk of cardiovascular disease (CVD), screening for CVD before administering exercise therapy is essential. CVD should be evaluated using patient interviews to determine the presence or absence of effort angina, and exercise electrocardiography and echocardiography should be performed. If the existence of ischemic heart disease is suspected, administering treatment for ischemic heart disease before initiating exercise therapy is thus preferred. Smoking cessation and obesity management are also important.

2) Drug Therapy

Statins are the first-line drugs for FH treatment. A retrospective analysis of 329 patients with heterozy-

gous FH conducted in Japan revealed that the use of statins delayed the onset of CAD[5]. If the patient does not respond to monotherapy with statins, other lipid-lowering drugs should be concomitantly used. Such concomitant drugs include ezetimibe, bile acid-binding resins (cholestyramine and colestimide), probucol, fibrates and nicotinic acid derivatives. Although there is no evidence that these combination therapies inhibit cardiovascular events in patients with FH more effectively than statin monotherapy, strict management of the LDL-C level is recommended in patients with FH. A retrospective investigation suggested that probucol delays the recurrence of CAD in patients with heterozygous FH[6].

3) Indications for LDL Apheresis

In heterozygous FH patients, LDL apheresis should be considered if the total cholesterol (TC) level does not decrease to ≤250 mg/dL following intensive drug treatment in the presence of CAD. If LDL apheresis is indicated, it is desirable to consult a specialist.

5. FH in Children

1) Diagnosis of Heterozygous FH in Children

The initial finding of heterozygous FH is hyper-LDL cholesterolemia. In childhood, many patients do not develop physical signs associated with hyper-LDL cholesterolemia, such as Achilles tendon xanthoma and arcus corneae. Therefore, FH in children is primarily diagnosed based on the presence of hyper-LDL cholesterolemia and family history. In the diagnosis of FH in children, if the parent(s) has/have hyper-LDL cholesterolemia, a diagnosis of FH in the parent(s) should be established. The diagnostic criteria for heterozygous FH in children are shown in **Table 2**. Because 95% of healthy children have an LDL-C level of ≤140 mg/dL[7], the cutoff value for screening is defined as 140 mg/dL.

2) Treatment for Heterozygous FH in Children

• Nutritional Guidance and Lifestyle Modification

If heterozygous FH is diagnosed, the affected child and their guardians should be directed to modify their lifestyle as soon as possible. Affected children with a smoking habit should be directed to stop smoking. In addition, they should be directed to avoid smoking throughout their life and receive an explanation of the risk of passive smoking; their family members should also be directed to stop smoking.

• Drug Therapy

Evidence pertaining to the age from which treatment should be administered in patients with heterozygous FH has not yet been established in Japan. Because atherosclerotic changes in the coronary arteries are observed from an earlier age in heterozygous FH patients, appropriate LDL-C management is recommended at an earlier age. According to the proposal of the American Academy of Pediatrics, if a patient has an "LDL-C level of ≥190 mg/dL" or an "LDL-C level of ≥160 mg/dL and a family history of premature CAD or at least two risk factors," lipid-lowering treatment should be initiated, even in children, and if lifestyle modification is inadequate, drug therapy should also be considered in boys aged 8 to 10 years or older and in girls after menarche[8]. Among patients who are at a very high risk, such as patients with tendon xanthoma or aortic stenosis or those with a family history of remarkable atherosclerosis, a differential diagnosis of heterozygous FH should be performed. With respect to drug therapy, in terms of safety for growth and development, bile acid-binding resins, which are not absorbed from the gastrointestinal tract, are typically used and are the first-line drugs. Drug therapy for children should be administered under the direction of a specialist.

Table 2. Diagnostic Criteria for Heterozygous FH in Children

1. Hypercholesterolemia: an untreated LDL-C level of ≥140 mg/dL (measure the LDL-C level if the TC level is ≥220 mg/dL)
2. Family history of FH or premature CAD within the patient's second-degree relatives

• Pediatric patients exhibit few symptoms, such as tendon xanthoma. Therefore, diagnosing FH in the patient's family members is important.
• The LDL-C level may vary during development. Providing careful follow-up is necessary.
• Premature CAD is defined as the occurrence of CAD in men <55 years of age or women <65 years of age.

6. Heterozygous FH in Women

Drug therapy, other than bile acid-binding resins, during pregnancy should be carefully considered due to concerns regarding the risk of fetal malformations. According to the National Institute for Health and Clinical Excellence[9], if pregnancy is diagnosed during drug therapy, lipid-lowering drugs other than bile acid-binding resins should be immediately discontinued, and, if there is a possibility of pregnancy, pregnancy after the discontinuation of drug treatment for three months should be recommended.

Homozygous Familial Hypercholesterolemia

1. Diagnosis of Homozygous FH

Homozygous FH is characterized by the presence of a TC level of ≥600 mg/dL, xanthoma and CVD from childhood, with both parents being heterozygous for FH. Therefore, making a clinical diagnosis is possible. If homozygous FH is suspected even when the TC level is <600 mg/dL, obtaining the diagnosis and therapeutic decisions from a specialist is essential.

2. Drug Therapy for Homozygous FH

Similar to that recommended for patients with heterozygous FH, lifestyle modification, including diet therapy, exercise therapy, smoking cessation and obesity management, provides the basis for treatment in patients with homozygous FH, although intensive LDL-C-lowering treatment is required at an earlier age because patients with homozygous FH face a considerable risk with respect to the development and progression of CAD. However, homozygous FH is much less responsive to drug treatment than heterozygous FH. Therefore, the administration of LDL apheresis once every one to two weeks is necessary. Probucol exerts LDL-C-lowering effects on homozygous FH and may cause the regression or disappearance of xanthoma in the skin or Achilles tendon. For patients with homozygous FH who wish to have children, screening for CAD and the presence of aortic stenosis and supravalvular stenosis should be performed, and appropriate measures should be taken as required to ensure the safe continuation of pregnancy and delivery[10].

3. LDL Apheresis for Homozygous FH

In patients with homozygous FH, it is difficult to decrease the LDL-C level sufficiently using existing drug therapies, and many patients require continued LDL apheresis with extracorporeal circulation starting in childhood. Considering the inhibition of the progression of CVD, the earlier LDL apheresis is initiated, the better; however, it is difficult to perform LDL apheresis until the affected child can be kept in bed during apheresis. Realistically, the timing of treatment initiation is 4 to 6 years of age, when children can lie in bed and extracorporeal circulation can be performed; however, it is recommended that the treatment be initiated as early as possible.

4. Pregnancy and Delivery of Patients with Homozygous FH

It is important to permit patients with homozygous FH to become pregnant as planned. Before pregnancy, screening for atherosclerosis should be performed using carotid ultrasonography, echocardiography and exercise tolerance tests to assess the status of atherosclerosis. By three months before the planned pregnancy, treatment with lipid-lowering drugs other than bile acid-binding resins should be discontinued. Because the cardiovascular system is greatly stressed during late pregnancy, particularly at delivery, performing LDL apheresis during pregnancy is desirable. LDL apheresis can also be safely administered during pregnancy.

5. Homozygous FH Designated as a Specified Disease

In October 2009, homozygous FH was designated as a specified disease in the Specified Disease Treatment Research Program. The criteria for designation are as follows: patients with homozygous FH definitively diagnosed using a genetic analysis of genes involved in the LDL metabolic pathway or measurement of the LDL receptor activity are definitively designated, and patients with remarkable hypercholesterolemia and those with cutaneous xanthoma starting in childhood who are refractory to drug treatment should be designated.

Footnotes

This is an English version of the guidelines of the Japan Atherosclerosis Society (chapter 9) published in Japanese in June, 2012. The details of this Committee Report 9 on Familial Hypercholesterolemia have been previously published as an original manuscript[11]; this is a brief summary.

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Increase in Secretory Sphingomyelinase Activity and Specific Ceramides in the Aorta of Apolipoprotein E Knockout Mice during Aging

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Atherosclerosis is caused by many factors, one of which is oxidative stress. We recently demonstrated that systemic oxidative stress increased secretory sphingomyelinase (sSMase) activity and generated ceramides in the plasma of diabetic rats. In addition, we also showed that the total ceramide level in human plasma correlated with the level of oxidized low-density lipoprotein. To investigate the relationship between ceramide species and atherogenesis during aging, we compared age-related changes in ceramide metabolism in apolipoprotein E knock out mice (apoE$^{-/-}$) and wild type mice (WT). Although the total plasma ceramide level was higher in apoE$^{-/-}$ than that in WT at all ages, it decreased with increasing age. sSMase activity increased at 65 weeks (w) of age in both strains of mice. When apoE$^{-/-}$ developed atherosclerosis at 15 w of age, C18:0, C22:0, and C24:0 ceramide levels in the apoE$^{-/-}$ aorta significantly increased. Furthermore, at 65 w of age C16:0 and C24:1 ceramide levels were significantly higher than those in WT. These results suggested that elevation in levels of specific ceramide species due to sSMase activity contributed to atherogenesis during aging.

Key words aging; apolipoprotein E; atherosclerosis; ceramide; sphingomyelinase

Cardiovascular disease is associated with aging. Hyperlipidemia and long-term exposure to various stimuli such as oxidative stress lead to atherosclerosis. Despite several indications that aging causes atherosclerosis, a specific factor associated with atherosclerosis that increases or decreases with aging is yet to be entirely identified. One possible factor is elevation of bioactive lipid such as ceramide. Ceramide regulates cell cycle arrest, apoptosis, and cellular senescence,[1] and serves as an intracellular second messenger in these processes.[2] Oxidative stress inducers such as UV light, antineoplastic drugs, and radiation stimulate ceramide accumulation in the cell.[3–6] Ceramide belongs to the sphingolipid family and comprises a saturated or unsaturated fatty acid of C16–C26 chain length bound to the amino group of sphingosine.

Ceramide is generated by *de novo* synthetic pathway as well as from the hydrolysis of sphingomyelin (SM) by sphingomyelinase (SMase). Sphingomyelinases are classified into five types on the basis of their optimum pH, subcellular localization, and cation dependence.[5,7] Among these enzymes acid SMase (aSMase) (optimal pH=4.8) operates in the endosomal/lysosomal compartment or plasma membrane.[8] Of note, the aSMase gene (*smpd1*) gives rise to two different enzymes lysosomal SMase *i.e.*, aSMase, and secretory SMase (sSMase) *via* alternative trafficking of the same protein precusor.[9,10] The vascular endothelium and macrophages secrete sSMase, which is the only enzyme responsible for sphingolytic activity in plasma.[11]

Elevated ceramide levels were recently shown to correlate with atherogenetic processes such as low-density lipoprotein (LDL) aggregation,[12] and form cell migration.[13] In relation to atherogenesis, we previously reported that the ceramide level in human plasma was positively correlated with both total cholesterol and oxidized apolipoprotein B-100 (oxLDL) levels.[14] Deevska *et al.*[15] reported that LDL-SM content and sSMase activity in LDL receptor knockout mice, which were fed an atherogenic diet, were increased. Excessive cholesterol intake increased plasma ceramides in apolipoprotein E knockout mice (apoE$^{-/-}$), a typical animal model for atherosclerosis.[16] In addition, we previously reported that the increase in plasma ceramide level was caused by increased activity of sSMase in the streptozotocin-induced diabetic rats,[17] thus suggesting that the elevation of plasma ceramide was an important factor in atherogenesis. sSMase activity also increased in response to stimulation of macrophages obtained from patients with chronic heart failure[18] and type 2 diabetes.[19] These studies indicated that oxidative stress resulted in ceramide accumulation by increasing sSMase activity.

To investigate the effect of aging on ceramide metabolism, we compared changes in tissue ceramides and related enzymes in apoE$^{-/-}$ with wild-type mice (WT), particularly focusing on SMase activity, which increased because of oxidative stress.[17,20,21]

MATERIALS AND METHODS

Materials All the solvents were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). All other reagents were obtained from Nacalai Tesque Inc. (Kyoto, Japan). Authentic ceramides were purchased from Avanti Polar Lipids Inc. (Alabaster, AL, U.S.A.). Nitrobenzofurazan (NBD) C_6-SM was purchased from Molecular Probes Inc. (Eugene, OR, U.S.A.).

Animals This study was approved by the Animal Care Committee of Nara Women's University. Female WT (SLC: C57BL/6J; 4 or 13 weeks of age) were obtained from Japan SLC Co. (Hamamatsu, Shizuoka, Japan). Female apoE$^{-/-}$ were obtained from the Jackson Laboratory. The animals were housed in a room maintained at 24±2°C, with a 12h/12h

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light/dark cycle. Animals were fed commercial laboratory chow (CE-2, Oriental Yeast Co., Osaka, Japan) and water *ad libitum*. Mice were sacrificed at the age of 7, 15, and 65 weeks (w) of age.

Analytical Methods Mice were anesthetized with Nembutal, and blood samples were collected by left ventricular puncture using a syringe containing sodium heparin as an anticoagulant. After perfusion with saline, the liver and aorta were dissected out. Plasma was separated from whole blood sample by centrifugation.

SMase Activity Assay SMase activities were measured using NBD C_6-SM as a substrate, and the assay was principally performed based on previous studies.[17,22] For sSMase analysis, a 800 μL assay mixture consisted of 15 μL of plasma and 785 μL of an assay buffer (0.1 mM $ZnSO_4$, 1 nmol NBD C_6-SM, 0.1% NP-40/62, 0.1 M sodium acetate buffer at pH 5.0) was incubated for 2 h at 37°C. The reaction was stopped by adding 1 mL of methanol. For aortic aSMase, which depended on zinc ion, the aorta was dissected and cleaned of visible fat debris. The tissue was homogenized on ice in 400 μL of phosphate buffered saline (10 mM, pH 7.4). Protein concentrations were determined according to the method of Lowry *et al.*[23] Aortic aSMase activity was determined by suspending the homogenate (1–2 mg protein/mL) in 800 μL assay mixture comprising of 0.1 mM $ZnSO_4$, 1 nmol NBD C_6-SM, 0.1% NP-40/62, and 0.1 M sodium acetate (pH 5.0). The reaction was continued for 2 h at 37°C and stopped by the addition of 2 mL methanol. NBD C_6-SM and generated NBD C_6-Ceramide were subsequently extracted according to the method of Bligh and Dyer.[24] The extract was dissolved in 0.5 mL methanol and analyzed using HPLC as described below. The samples (20 μL) were directly analyzed by HPLC with a Nova Pak 4 μm C18 column (3.9×150 mm, Waters Corporation, Milford, MA, U.S.A.). Elution was performed at a flow rate of 1 mL/min with a mixture of water, acetonitrile, and phosphoric acid at a volume ratio of 35:65:0.2. NBD fluorescence was determined using a fluorescence detector (Shimadzu, RF-10AXL, excitation at 466 nm and emission at 536 nm).

Data were expressed as mean±S.E.M., and analyzed by multiple comparison tests using Statcel software (OMS Publishing Inc., Tokyo, Japan). Using the Tukey–Kramer Procedure, the differences between group means were significant at $p<0.05$.

Ceramide Measurement Lipids were extracted according to the method of Folch *et al.*[25] Lipids were dissolved in chloroform and subjected to chromatography on Silica gel 60 thin-layer chromatography (TLC) plates (Merck, Darmstradt, Germany). Separation was performed using silica gel 60 TLC plates (Merck, Germany) as previous studies.[6,26] In brief, the first elution was made with a mixture of *n*-butanol–acetic acid–water (30:10:10, v/v/v) to the one third of the plate and the second elution was made to the top of the plate with a mixture of diethyl ether–*n*-hexane–acetic acid (90:10:1, v/v/v). The ceramide spot was visualized under the UV by staining with primulin spray. The ceramide spot was scratched from the TLC plates and collected into a glass tube. Extraction was made with 2 mL of a mixture of H_2O–CH_3OH–$CHCl_3$ (20:30:50, v/v/v) under shaking for 30 min. After centrifugation, the lower layer was collected. To the upper phase was added 1.5 mL of $CHCl_3$, and extraction was made an additional two times. The collected $CHCl_3$ solution was evaporated and resuspended in a mixture of 10 mL (liver) or 2 mL (aorta and plasma) of $CHCl_3$–CH_3OH (1:9, v/v). Standards and tissue ceramide extracts were stored at −20°C. The quantities of major ceramide species were measured by LC-MS/MS using a triple-quadrupole mass spectrometer [the ACQUITY TQD mass spectrometer (Waters Corporation, Milford, MA, U.S.A.)] equipped with the ACQUITY ultra performance liquid chromatography (UPLC) system (Waters Corporation, Milford, MA, U.S.A.). The ceramide species were separated using a column, [ACQUITY UPLC BEH, 1.7 μm, 2.1×50 mm, C18 (Waters Corporation, Milford, MA, U.S.A.)] at 50°C. UPLC gradient elution was applied beginning with 15% of mobile phase A (water containing 0.2% formic acid and 5 mM ammonium acetate) and 85% of mobile phase B (methanol containing 0.2% formic acid and 5 mM ammonium acetate) at a flow rate of 0.4 mL/min. The initial solvent condition was maintained for 3 min, and the percentage of the B solution was gradually increased with a linear gradient to 99% for 6 min. The column was equilibrated for 4 min with 85% mobile phase B prior to next injection. The total run time per injection was 13 min. The mass spectrometry settings were as follows: electrospray ionization (ESI) positive ion mode, capillary voltage, 3.0 kV; cone voltage, 20 V; source temperature, 120°C; and desolvation temperature, 350°C. The flow rates of nitrogen gas in the cone and desolvation gas were 50 and 600 L/h, respectively. Argon gas was used for collision-induced dissociation and maintenance of collision cell pressure at 10^{-4} mbar. Results were analyzed using multiple reaction monitoring (MRM). Mass spectra (*m/z*) for an internal standard (IS) and six major ceramides were set up at 426.4→264.2 for C8:0 ceramide (IS), and 538.4→264.2, 566.5→264.2, 622.5→264.3, 650.6→264.3, 648.6→264.3, 646.6→264.3 for C16:0, C18:0, C22:0, C24:0, C24:1, and C24:2 ceramides, respectively. Data acquisition was carried out by MassLynx software (version 4.1, Waters Corporation). Ceramide species were quantified using standard curves and ratios of the integrated peak areas of each ceramide species and C8:0 ceramide, which was used as an internal standard for quantification of the other ceramide species. Total ceramide content was calculated by addition of the amount of C16:0, C18:0, C22:0, C24:0, C24:1, and C24:2 ceramides. Data were expressed as an mean±S.E.M.

RESULTS

Change in SMase Activity Plasma sSMase activity was significantly elevated at 65 w of age in both apoE$^{-/-}$ and WT compared with level observed at 7 w of age (Table 1). At 7 w

Table 1. Changes in sSMase Activity in Plasma (pmol/mL/min) and Aortic aSMase Activity (pmol/mg protein/min) of Wild-Type and ApoE$^{-/-}$ Mice

	7 w WT	15 w WT	65 w WT	7 w apoE$^{-/-}$	15 w apoE$^{-/-}$	65 w apoE$^{-/-}$
sSMase	1599.6±171.6^{ab}	1666.7±83.6^{ab}	2688.7±175.1^{c}	657.3±86.9^{d}	1187.2± 121.7^{ad}	1990.2±207.1^{b}
Aortic aSMase	79.2±9.4^{a}	130.8±12.5^{bc}	271.5±14.3^{d}	113.8±13.5^{ab}	167.1±8.3^{c}	66.4±5.0^{a}

Values are presented as mean±S.E.M. for 4 or 5 animals in each group. Different superscript letters indicate significant differences at $p<0.05$ (Tukey–Kramer *post hoc* test).