GAO, W.Y., HAN, F.S., STORM, C., EGAN, W., and CHENG, Y.C. (1992). Phosphorothioate oligonucleotides are inhibitors of human DNA polymerases and RNase H: implications for antisense technology. Mol. Pharmacol. **41,** 223–229.

HARI, Y., OBIKA, S., OHNISHI, R., EGUCHI, K., OSAKI, T., OHISHI, H., and IMANISHI, T. (2006). Synthesis and properties of 2′-O,4-C-methyleneoxymethylene bridged nucleic acid. Bioorgan. Med. Chem. **14,** 1029–1038.

HUTVAGNER, G., and ZAMORE, P.D. (2002). A microRNA in a multiple-turnover RNAi enzyme complex. Science **297,** 2056–2060.

KOLLER, E., VINCENT, T. M., CHAPPELL, A., DE, S., MANOHARAN, M., and BENNETT, C. F. (2011). Mechanisms of single-stranded phosphorothioate modified antisense oligonucleotide accumulation in hepatocytes. Nucleic Acids Res. **39,** 4795–4807.

KURRECK, J., WYSZKO, E., GILLEN, C., and ERDMANN, V.A. (2002). Design of antisense oligonucleotides stabilized by locked nucleic acids. Nucleic Acids Res. **30,** 1911–1918.

LIMA, W.F., and CROOKE, S.T. (1997). Binding affinity and specificity of Escherichia coli RNase H1: impact on the kinetics of catalysis of antisense oligonucleotide-RNA hybrids. Biochemistry **36,** 390–398.

LIMA, W.F., ROSE, J.B., NICHOLS, J.G., WU, H.J., MIGAWA, M.T., WYRZYKIEWICZ, T.K., SIWKOWSKI, A.M., and CROOKE, S.T. (2007a). Human RNase H1 discriminates between subtle variations in the structure of the heteroduplex substrate. Mol. Pharmacol. **71,** 83–91.

LIMA, W.F., ROSE, J.B., NICHOLS, J.G., WU, H.J., MIGAWA, M.T., WYRZYKIEWICZ, T.K., VASQUEZ, G., SWAYZE, E.E., and CROOKE, S.T. (2007b). The positional influence of the helical geometry of the heteroduplex substrate on human RNase H1 catalysis. Mol. Pharmacol. **71,** 73–82.

MIYASHITA, K., RAHMAN, S.M.A., SEKI, S., OBIKA, S., and IMANISHI, T. (2007). *N*-Methyl substituted 2′,4′-BNA^{NC}: a highly nuclease-resistant nucleic acid analogue with high-affinity RNA selective hybridization. Chem. Commun. **2007,** 3765–3767.

MONIA, B.P., LESNIK, E.A., GONZALEZ, C., LIMA, W.F., MCGEE, D., GUINOSSO, C.J., KAWASAKI, A.M., COOK, P.D., and FREIER, S.M. (1993). Evaluation of 2′-modified oligonucleotides containing 2′-deoxy gaps as antisense inhibitors of gene expression. J. Biol. Chem. **268,** 14514–14522.

NOWOTNY, M., GAIDAMAKOV, S.A., GHIRLANDO, R., CERRITELLI, S.M., CROUCH, R.J., and YANG, W. (2007). Structure of human RNase h1 complexed with an RNA/DNA hybrid: insight into HIV reverse transcription. Mol. Cell **28,** 264–276.

OBIKA, S., NANBU, D., HARI, Y., ANDOH, J., MORIO, K., DOI, T., and IMANISHI, T. (1998). Stability and structural features of the duplexes containing nucleoside analogues with a fixed N-type conformation, 2′-*O*,4′-*C*-methyleneribonucleosides. Tetrahedron Lett. **39,** 5401–5404.

OBIKA, S., NANBU, D., HARI, Y., MORIO, K., IN, Y., ISHIDA, T., and IMANISHI, T. (1997). Synthesis of 2′-O,4′-C-methyleneuridine and -cytidine. Novel bicyclic nucleosides having a fixed C-3,-endo sugar puckering. Tetrahedron Lett. **38,** 8735–8738.

PRAKASH, T.P., SIWKOWSKI, A., ALLERSON, C.R., MIGAWA, M.T., LEE, S., GAUS, H.J., BLACK, C., SETH, P.P., SWAYZE, E.E., and BHAT, B. (2010). Antisense oligonucleotides containing conformationally constrained 2′,4′-(N-methoxy)aminomethylene and 2′,4′-aminooxymethylene and 2′-O,4′-C-aminomethylene bridged nucleoside analogues show improved potency in animal models. J. Med. Chem. **53,** 1636–1650.

SETH, P.P., SIWKOWSKI, A., ALLERSON, C.R., VASQUEZ, G., LEE, S., PRAKASH, T.P., WANCEWICZ, E.V., WITCHELL, D., and SWAYZE, E.E. (2009). Short antisense oligonucleotides with novel 2′-4′ conformationaly restricted nucleoside analogues show improved potency without increased toxicity in animals. J. Med. Chem. **52,** 10–13.

SINGH, S.K., NIELSEN, P., KOSHKIN, A.A., and WENGEL, J. (1998). LNA (locked nucleic acids): synthesis and high-affinity nucleic acid recognition. Chem. Commun. **1998,** 455–456.

SOHLENIUS-STERNBECK, A.K. (2006) Determination of the hepatocellularity number for human, dog, rabbit, rat and mouse livers from protein concentration measurements. Toxicol. In Vitro **20,** 1582–1586.

STANTON, R., SCIABOLA, S., SALATTO, C., WENG, Y., MOSHINSKY, D., LITTLE, J., WALTERS, E., KREEGER, J., et al. (2012). Chemical modification study of antisense gapmers. Nucleic Acid Ther. **22,** 344–359.

STEIN, C.A., HANSEN, J.B., LAI, J., WU, S., VOSKRESENSKIY, A., HOG, A., WORM, J., HEDTJARN, M., SOULEIMANIAN, N., MILLER, P., et al. (2010). Efficient gene silencing by delivery of locked nucleic acid antisense oligonucleotides, unassisted by transfection reagents. Nucleic Acids Res. **38,** e3.

STRAARUP, E.M., FISKER, N., HEDTJARN, M., LINDHOLM, M.W., ROSENBOHM, C., AARUP, V., HANSEN, H.F., ORUM, H., HANSEN, J.B., and KOCH, T. (2010). Short locked nucleic acid antisense oligonucleotides potently reduce apolipoprotein B mRNA and serum cholesterol in mice and non-human primates. Nucleic Acids Res. **38,** 7100–7111.

VESTER, B., BOEL, A.M., LOBEDANZ, S., BABU, B.R., RAUNKJAER, M., LINDEGAARD, D., RAUNAK, HRDLICKA, P.J., HOJLAND, T., et al. (2008). Chemically modified oligonucleotides with efficient RNase H response. Bioorg. Med. Chem. Lett. **18,** 2296–2300.

WATANABE, T.A., GEARY, R.S., and LEVIN, A.A. (2006). Plasma protein binding of an antisense oligonucleotide targeting human ICAM-1 (ISIS 2302). Oligonucleotides **16,** 169–180.

YAHARA, A., SHRESTHA, A.R., YAMAMOTO, T., HARI, Y., OSAWA, T., YAMAGUCHI, M., NISHIDA, M., KODAMA, T., and OBIKA, S. (2012). Amido-bridged nucleic acids (AmNAs): synthesis, duplex stability, nuclease resistance, and in vitro antisense potency. Chembiochem **13,** 2513–2516.

YAMAMOTO, T., HARADA-SHIBA, M., NAKATANI, M., WADA, S., YASUHARA, H., NARUKAWA, K., SASAKI, K., SHIBATA, M.A., TORIGOE, H., et al. (2012a). Cholesterol-lowering action of BNA-based antisense oligonucleotides targeting PCSK9 in atherogenic diet-induced hypercholesterolemic mice. Mol. Ther. Nucleic Acids **1,** e22.

YAMAMOTO, T., NAKATANI, M., NARUKAWA, K., and OBIKA, S. (2011). Antisense drug discovery and development. Future Med. Chem. **3,** 339–365.

YAMAMOTO, T., YASUHARA, H., WADA, F., HARADA-SHIBA, M., IMANISHI, T., and OBIKA, S. (2012b). Superior silencing by 22,42-BNA^{NC}-Based short antisense oligonucleotides compared to 22,42-BNA/LNA-based apolipoprotein B antisense inhibitors. J. Nucleic Acids, Article ID 707323.

ZHANG, Y., QU, Z., KIM, S., SHI, V., LIAO, B., KRAFT, P., BANDARU, R., WU, Y., GREENBERGER, L.M., and HORAK, I.D. (2011). Down-modulation of cancer targets using locked nucleic acid (LNA)-based antisense oligonucleotides without transfection. Gene Ther, **18,** 326–333.

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Received for publication October 23, 2013; accepted after revision March 13, 2014.

Volume 723 15 January 2014 ISSN 0014-2999

european journal of pharmacology

Drugs in Action

European Journal of Pharmacology 723 (2014) 353–359

Contents lists available at ScienceDirect

European Journal of Pharmacology

journal homepage: www.elsevier.com/locate/ejphar

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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ARTICLE INFO

Article history:
Received 23 May 2013
Received in revised form
25 October 2013
Accepted 2 November 2013
Available online 20 November 2013

Keywords:
Hyperlipidemia
Antisense oligonucleotide
Synthetic nucleic acid
Bridged nucleic acids
Locked nucleic acids
Apolipoprotein C-III

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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http://dx.doi.org/10.1016/j.ejphar.2013.11.004

European Journal of Pharmacology 723 (2014) 353–359

Contents lists available at ScienceDirect

European Journal of Pharmacology

journal homepage: www.elsevier.com/locate/ejphar

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ARTICLE INFO

Article history:
Received 23 May 2013
Received in revised form
25 October 2013
Accepted 2 November 2013
Available online 20 November 2013

Keywords:
Hyperlipidemia
Antisense oligonucleotide
Synthetic nucleic acid
Bridged nucleic acids
Locked nucleic acids
Apolipoprotein C-III

ABSTRACT

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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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http://dx.doi.org/10.1016/j.ejphar.2013.11.004

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

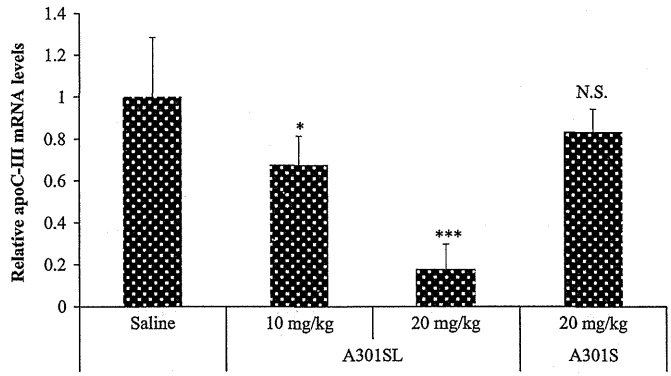

Fig. 1. Hepatic apoC-III mRNA silencing effects of **A301SL** and **A301S**. Western diet-fed mice received intraperitoneal administration of these two AONs at 10 or 20 mg/kg five times over 16 days. Relative hepatic apoC-III mRNA expression levels were determined by means of two-step real-time RT-PCR, 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.

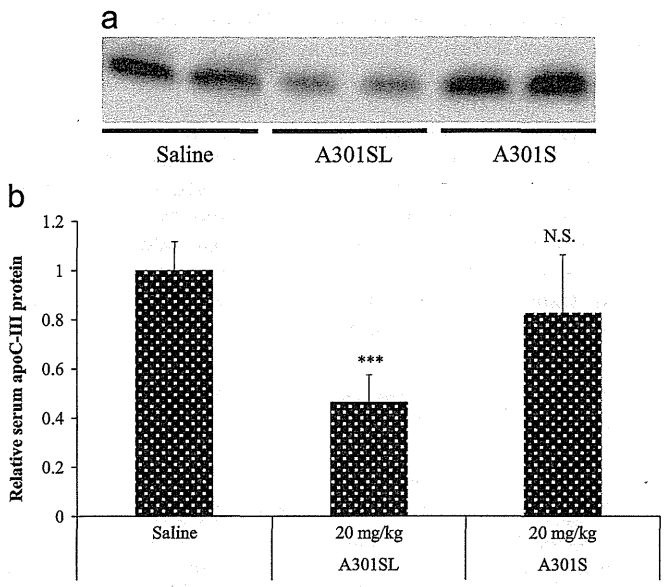

Fig. 2. Effects of **A301SL** and **A301S** on serum apoC-III protein levels. Western diet-fed mice received i.p. administration of these two AONs at 20 mg/kg for five times over 16 days. After completion of dosing, reductions in apoC-III protein level in serum were investigated by Western blotting. (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.

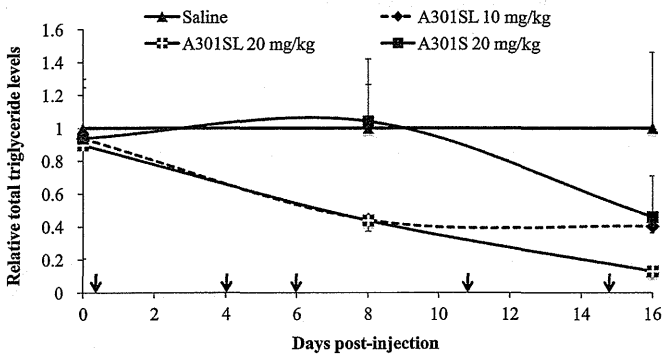

Fig. 3. Effects on serum triglyceride levels over time. Western diet-fed mice received intraperitoneal administration of two AONs, **A301SL** at 10 and 20 mg/kg/injection and **A301S** at 20 mg/kg/injection five times over 16 days. On days 0, 8 and 16, blood samples were collected from tail vein and 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

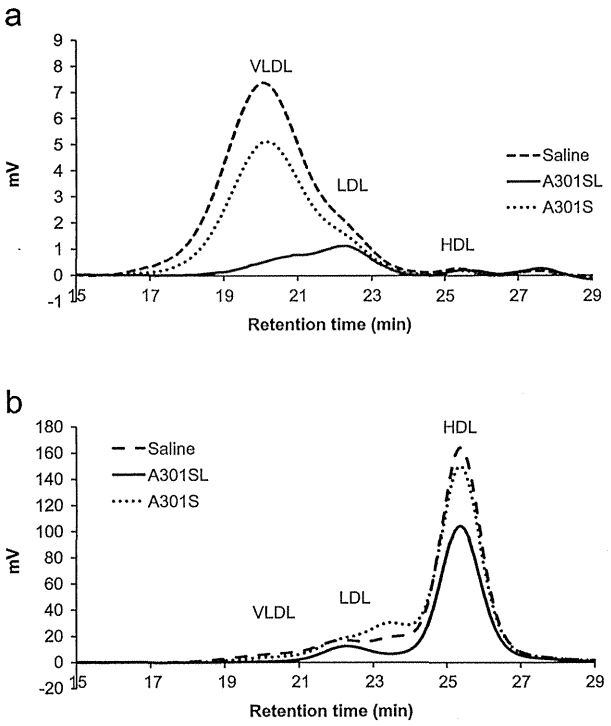

Fig. 4. Representative HPLC lipoprotein profiles of western diet-fed C57BL/6J mice received intraperitoneal administration of saline (dashed line), **A301SL** (solid line) at 20 mg/kg/injection or **A301S** (dotted line) at 20 mg/kg/injection five times over 16 days. Five saline-, **A301SL**- and **A301S**-treated 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

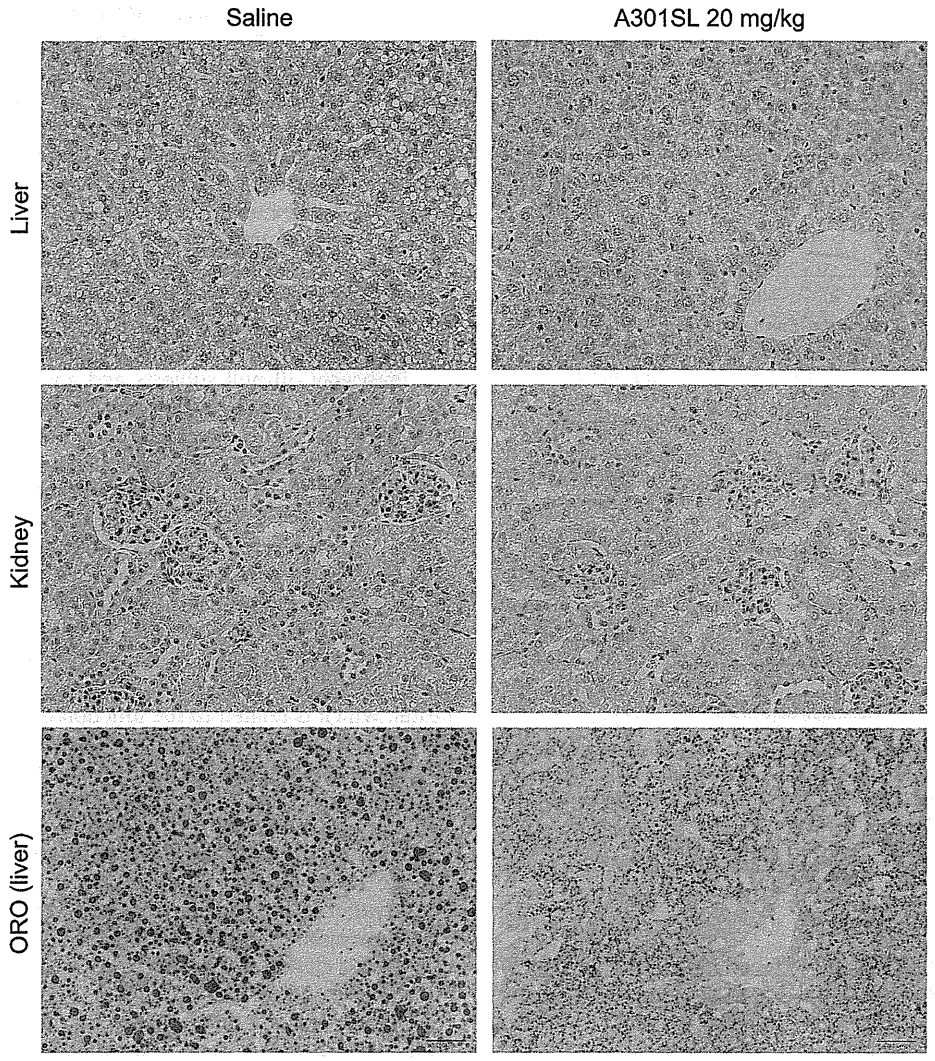

Fig. 5. Representative histopathological changes in livers and kidneys subjected to 16 days of saline (left) and **A301SL** (right) dosing were assessed by H&E or Oil Red O staining (× 200 magnification). Peripheral fatty changes were observed in the liver of the saline-treated mice (top and bottom), 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.

Acknowledgments and notice of grant support

This work was supported by a Grant-in Aid for Scientific Research from the Japanese Ministry of Health, Labour and Welfare (H23-seisaku tansaku-ippan-004).

References

Applebaum-Bowden, D., Kobayashi, J., Kashyap, V.S., Brown, D.R., Berard, A., Meyn, S., Parrott, C., Maeda, N., Shamburek, R., Brewer, H.B., Santamarina-Fojo, S., 1996. Hepatic lipase gene therapy in hepatic lipase-deficient mice—Adenovirus-mediated replacement of a lipolytic enzyme to the vascular endothelium. J. Clin. Invest. 97, 799–805.

Begriche, K., Massart, J., Robin, M.A., Borgne-Sanchez, A., Fromenty, B., 2011. Drug-induced toxicity on mitochondria and lipid metabolism: mechanistic diversity and deleterious consequences for the liver. J. Hepatol. 54, 773–794.

Bruns, G.A.P., Karathanasis, S.K., Breslow, J.L., 1984. Human apolipoprotein A-I-C-III gene complex is located on chromosome. Arteriosclerosis 4, 97–102.

Busch, S.J., Barnhart, R.L., Martin, G.A., Fitzgerald, M.C., Yates, M.T., Mao, S.J.T., Thomas, C.E., Jackson, R.L., 1994. Human hepatic triglyceride lipase expression reduces high-density-lipoprotein and aortic cholesterol in cholesterol-fed transgenic mice. J. Biol. Chem. 269, 16376–16382.

Clavey, V., Lestaveldelattre, S., Copin, C., Bard, J.M., Fruchart, J.C., 1995. Modulation of lipoprotein B binding to the Ldl receptor by exogenous lipids and apolipoprotein-Ci, apolipoprotein-Cii, apolipoprotein-Ciii, and apolipoprotein-E. Arterioscl. Throm. Vas. 15, 963–971.

Crooke, R.M., Graham, M.J., Lemonidis, K.M., Dobie, K.W., 2009. Modulation of Apolipoprotein C-III Expression. Isis Pharmaceuticals, Inc.. (Isis Pharmaceuticals, I. (Ed.).

Dichek, H.L., Brecht, W., Fan, J.L., Ji, Z.S., McCormick, S.P.A., Akeefe, H., Conzo, L., Sanan, D.A., Weisgraber, K.H., Young, S.G., Taylor, J.M., Mahley, R.W., 1998. Overexpression of hepatic lipase in transgenic mice decreases apolipoprotein B-containing and high density lipoproteins. Evidence that hepatic lipase acts as a ligand for lipoprotein uptake. J. Biol. Chem. 273, 1896–1903.

Fan, J.L., Wang, J.J., Bensadoun, A., Lauer, S.J., Dang, Q., Mahley, R.W., Taylor, J.M., 1994. Overexpression of hepatic lipase in transgenic rabbits leads to a marked reduction of plasma high-density-lipoproteins and intermediate density lipoproteins. Proc. Nat. Acad. Sci. U.S.A. 91, 8724–8728.

Fisher, R.M., Coppack, S.W., Humphreys, S.M., Gibbons, G.F., Frayn, K.N., 1995. Human triacylglycerol-rich lipoprotein subfractions as substrates for lipoprotein lipase. Clin. Chim. Acta 236, 7–17.

Gerritsen, G., Rensen, P.C., Kypreos, K.E., Zannis, V.I., Havekes, L.M., Willems van Dijk, K., 2005. ApoC-III deficiency prevents hyperlipidemia induced by apoE overexpression. J. Lipid Res. 46, 1466–1473.

Goldberg, I.J., 2001. Clinical review 124—diabetic dyslipidemia: causes and consequences. J. Clin. Endocr. Metab. 86, 965–971.

Graham, M.J., Lee, R.G., Bell, T.A., Fu, W.X., Mullick, A.E., Alexander, V.J., Singleton, W., Viney, N., Geary, R., Su, J., Baker, B.F., Burkey, J., Crooke, S.T., Crooke, R.M., 2013. Antisense oligonucleotide inhibition of apolipoprotein C-III reduces plasma triglycerides in rodents, nonhuman primates, and humans. Circ. Res. 112. (1479-U1221).

Grundy, S.M., Brewer, H.B., Cleeman, J.I., Smith, S.C., Lenfant, C., Participants, C., 2004. Definition of metabolic syndrome—Report of the National Heart, Lung, and Blood Institute/American Heart Association Conference on Scientific Issues Related to Definition. Circulation 109, 433–438.

Gupta, N., Fisker, N., Asselin, M.C., Lindholm, M., Rosenbohm, C., Orum, H., Elmen, J., Seidah, N.G., Straarup, E.M., 2010. A locked nucleic acid antisense oligonucleotide (LNA) silences PCSK9 and enhances LDLR expression in vitro and in vivo. PLoS One 5, e10682.

Havel, R.J., Fielding, C.J., Olivecro., T, Shore, V.G., Fielding, P.E., Egelrud, T., 1973. Cofactor activity of protein components of human very low-density lipoproteins in hydrolysis of triglycerides by lipoprotein-lipase from different sources. Biochemistry 12, 1828–1833.

Hokanson, J.E., Austin, M.A., 1996. Plasma triglyceride level is a risk factor for cardiovascular disease independent of high-density lipoprotein cholesterol level: a meta-analysis of population-based prospective studies. J. Cardiovasc. Risk 3, 213–219.

Holmberg, R., Refai, E., Hoog, A., Crooke, R.M., Graham, M., Olivecrona, G., Berggren, P.O., Juntti-Berggren, L., 2011. Lowering apolipoprotein CIII delays onset of type 1 diabetes. Proc. Nat. Acad. Sci. U.S.A. 108, 10685–10689.

Ito, Y., Azrolan, N., O'Connell, A., Walsh, A., Breslow, J.L., 1990. Hypertriglyceridemia as a result of human apo CIII gene expression in transgenic mice. Science 249, 790–793.

Jong, M.C., Rensen, P.C., Dahlmans, V.E., van der Boom, H., van Berkel, T.J., Havekes, L.M., 2001. Apolipoprotein C-III deficiency accelerates triglyceride hydrolysis by lipoprotein lipase in wild-type and apoE knockout mice. J. Lipid Res. 42, 1578–1585.

Juntti-Berggren, L., Larsson, O., Rorsman, P., Ammala, C., Bokvist, K., Wahlander, K., Nicotera, P., Dypbukt, J., Orrenius, S., Hallberg, A., Berggren, P.O., 1993. Increased activity of L-Type Ca^{2+} channels exposed to serum from patients with type-I diabetes. Science 261, 86–90.

Juntti-Berggren, L., Refai, E., Appelskog, I., Andersson, M., Imreh, G., Dekki, N., Uhles, S., Yu, L., Griffiths, W.J., Zaitsev, S., Leibiger, I., Yang, S.N., Olivecrona, G., Jornvall, H., Berggren, P.O., 2004. Apolipoprotein CIII promotes Ca^{2+}-dependent beta cell death in type 1 diabetes. Proc. Nat. Acad. Sci. U.S.A. 101, 10090–10094.

Kinnunen, P.K.J., Ehnholm, C., 1976. Effect of serum and C-apoproteins from very low-density lipoproteins on human postheparin plasma hepatic lipase. FEBS Lett. 65, 354–357.

Lanford, R.E., Hildebrandt-Eriksen, E.S., Petri, A., Persson, R., Lindow, M., Munk, M.E., Kauppinen, S., Orum, H., 2010. Therapeutic silencing of microRNA-122 in primates with chronic hepatitis C virus infection. Science 327, 198–201.

Levin, A.A., 1999. A review of the issues in the pharmacokinetics and toxicology of phosphorothioate antisense oligonucleotides. Biochim. Biophys. Acta 1489, 69–84.

Lindholm, M.W., Elmen, J., Fisker, N., Hansen, H.F., Persson, R., Moller, M.R., Rosenbohm, C., Orum, H., Straarup, E.M., Koch, T., 2012. PCSK9 LNA antisense oligonucleotides induce sustained reduction of LDL cholesterol in nonhuman primates. Mol. Ther. 20, 376–381.

Millar, J.S., Packard, C.J., 1998. Heterogeneity of apolipoprotein B-100-containing lipoproteins: what we have learnt from kinetic studies. Curr. Opin. Lipidol. 9, 197–202.

Milosavljevic, D., Griglio, S., Le Naour, G., Chapman, M.J., 2001. Preferential reduction of very low density lipoprotein-1 particle number by fenofibrate in type IIB hyperlipidemia: consequences for lipid accumulation in human monocyte-derived macrophages. Atherosclerosis 155, 251–260.

Mitsuoka, Y., Kodama, T., Ohnishi, R., Hari, Y., Imanishi, T., Obika, S., 2009. A bridged nucleic acid, 2,4-BNA(COC): synthesis of fully modified oligonucleotides bearing thymine, 5-methylcytosine, adenine and guanine 2,4-BNA(COC) monomers and RNA-selective nucleic-acid recognition. Nucleic Acids Res. 37, 1225–1238.

Miyashita, K., Rahman, S.M.A., Seki, S., Obika, S., Imanishi, T., 2007. *N*-Methyl substituted 2 ′,4 ′-BNA(NC): a highly nuclease-resistant nucleic acid analogue with high-affinity RNA selective hybridization. Chem. Commun., 3765–3767.

Norata, G.D., Ballantyne, C.M., Catapano, A.L., 2013. New therapeutic principles in dyslipidaemia: focus on LDL and Lp(a) lowering drugs. Eur. Heart J. 34, 1783–1789.

Obika, S., Nanbu, D., Hari, Y., Morio, K., In, Y., Ishida, T., Imanishi, T., 1997. Synthesis of 2′-*O*,4′-C-methyleneuridine and -cytidine. Novel bicyclic nucleosides having a fixed C-3,-*endo* sugar puckering. Tetrahedron Lett. 38, 8735–8738.

Okazaki, M., Usui, S., Ishigami, M., Sakai, N., Nakamura, T., Matsuzawa, Y., Yamashita, S., 2005. Identification of unique lipoprotein subclasses for visceral obesity by component analysis of cholesterol profile in high-performance liquid chromatography. Arterioscl. Throm. Vas. 25, 578–584.

Ooi, E.M., Barrett, P.H., Chan, D.C., Watts, G.F., 2008. Apolipoprotein C-III: understanding an emerging cardiovascular risk factor. Clin. Sci. (London) 114, 611–624.

Petersen, K.F., Dufour, S., Hariri, A., Nelson-Williams, C., Foo, J.N., Zhang, X.M., Dziura, J., Lifton, R.P., Shulman, G.I., 2010. Apolipoprotein C3 gene variants in nonalcoholic fatty liver disease. N. Engl. J. Med. 362, 1082–1089.

Pollin, T.I., Damcott, C.M., Shen, H., Ott, S.H., Shelton, J., Horenstein, R.B., Post, W., McLenithan, J.C., Bielak, L.F., Peyser, P.A., Mitchell, B.D., Miller, M., O'Connell, J.R., Shuldiner, A.R., 2008. A null mutation in human APOC3 confers a favorable plasma lipid profile and apparent cardioprotection. Science 322, 1702–1705.

Prakash, T.P., Siwkowski, A., Allerson, C.R., Migawa, M.T., Lee, S., Gaus, H.J., Black, C., Seth, P.P., Swayze, E.E., Bhat, B., 2010. Antisense oligonucleotides containing conformationally constrained 2′,4′-(*N*-methoxy)aminomethylene and 2′,4′-aminooxymethylene and 2′-*O*,4′-C-aminomethylene bridged nucleoside analogues show improved potency in animal models. J. Med. Chem. 53, 1636–1650.

Sarwar, N., Danesh, J., Eiriksdottir, G., Sigurdsson, G., Wareham, N., Bingham, S., Boekholdt, S.M., Khaw, K.T., Gudnason, V., 2007. Triglycerides and the risk of coronary heart disease: 10,158 incident cases among 262,525 participants in 29 Western prospective studies. Circulation 115, 450–458.

Sehayek, E., Eisenberg, S., 1991. Mechanisms of inhibition by apolipoprotein C of apolipoprotein-E-dependent cellular-metabolism of human triglyceride-rich lipoproteins through the low-density-lipoprotein receptor pathway. J. Biol. Chem. 266, 18259–18267.

Seth, P.P., Siwkowski, A., Allerson, C.R., Vasquez, G., Lee, S., Prakash, T.P., Wancewicz, E.V., Witchell, D., Swayze, E.E., 2009. Short antisense oligonucleotides with novel 2′-4′ conformationaly restricted nucleoside analogues show improved potency without increased toxicity in animals. J. Med. Chem. 52, 10–13.

Takahashi, T., Hirano, T., Okada, K., Adachi, M., 2003. Apolipoprotein CIII deficiency prevents the development of hypertriglyceridemia in streptozotocin-induced diabetic mice. Metabolism 52, 1354–1359.

Usui, S., Hara, Y., Hosaki, S., Okazaki, M., 2002. A new on-line dual enzymatic method for simultaneous quantification of cholesterol and triglycerides in lipoproteins by HPLC. J. Lipid Res. 43, 805–814.

Wang, C.S., McConathy, W.J., Kloer, H.U., Alaupovic, P., 1985. Modulation of lipoprotein lipase activity by apolipoproteins. Effect of apolipoprotein C-III. J. Clin. Invest. 75, 384–390.

Yahara, A., Shrestha, A.R., Yamamoto, T., Hari, Y., Osawa, T., Yamaguchi, M., Nishida, M., Kodama, T., Obika, S., 2012. Amido-bridged nucleic acids (AmNAs): synthesis, duplex stability, nuclease resistance, and in vitro antisense potency. Chembiochem 13, 2513–2516.

Yamamoto, T., Harada-Shiba, M., Nakatani, M., Wada, S., Yasuhara, H., Narukawa, K., Sasaki, K., Shibata, M.A., Torigoe, H., Yamaoka, T., Imanishi, T., Obika, S., 2012. Cholesterol-lowering action of BNA-based antisense oligonucleotides targeting PCSK9 in atherogenic diet-induced hypercholesterolemic mice. Mol. Ther. Nucleic Acids 1, e22.

Therapeutic Apheresis and Dialysis

Therapeutic Apheresis and Dialysis 2014; 18(1):93–102
doi: 10.1111/1744-9987.12056

Proteomic Analysis of Proteins Eliminated by Low-Density Lipoprotein Apheresis

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Abstract: Low-density lipoprotein apheresis (LDL-A) treatment has been shown to decrease serum LDL cholesterol levels and prevent cardiovascular events in homozygous patients with familial hypercholesterolemia. Recently, LDL-A treatment has been suggested to have beneficial effects beyond the removal of LDL particles. In this study, to clarify the preventive effects of LDL-A treatment on atherosclerosis, the waste fluid from the adsorption columns was analyzed. The waste fluid of LDL adsorption columns was analyzed by two-dimensional electrophoresis followed by mass spectrometry. Serum concentrations of the newly identified proteins before and after LDL-A treatment were measured by enzyme-linked immunosorbent assay. We identified 48 kinds of proteins in the waste fluid of LDL adsorption columns, including coagulation factors, thrombogenic factors, complement factors, inflammatory factors and adhesion molecules. In addition to the proteins that were reported to be removed by LDL-A treatment, we newly identified several proteins that have some significant roles in the development of atherosclerosis, including vitronectin and apolipoprotein C-III (Apo C-III). The serum levels of vitronectin and Apo C-III decreased by 82.4% and 54.8%, respectively, after a single LDL-A treatment. While Apo C-III was removed with very low-density lipoprotein (VLDL) and LDL, vitronectin was removed without association with lipoproteins. The removal of proteins observed in the waste fluid has a certain impact on their serum levels, and this may be related to the efficacy of LDL-A treatment. Proteomic analysis of the waste fluid of LDL adsorption columns may provide a rational means of assessing the effects of LDL-A treatment. **Key Words:** Apolipoprotein C-III, Low-density lipoprotein apheresis, Proteomic analysis, Vitronectin.

Familial hypercholesterolemia (FH) is an autosomal-dominant inherited disorder resulting from genetic mutation in the molecules related to low-density lipoprotein receptor (LDLR) pathways (1). Homozygous FH patients show severe symptoms of atherosclerosis such as coronary artery disease (CAD) and valvular heart disease at a young age due to their extremely high levels of serum LDL-cholesterol (LDL-C) from birth (2). Because drugs that act via the upregulation of LDLR activity are not effective in homozygous patients with FH, several attempts have been made to reduce their LDL-C levels and to prevent atherosclerosis. DeGenne et al. conducted plasma exchange (PE) in homozygous FH patients in 1967 (3), and in 1975 Thompson et al. reported the LDL-C-reducing effects of PE on the alleviation of angina pain with the improvement of coronary artery stenosis (4). Subsequently, attempts to remove LDL in more selective ways have been made by using a special double-filtration plasmapheresis (DFPP) method termed thermofiltration (5–7) and an LDL adsorption column (8,9). Collectively, these two methods have been called LDL-apheresis (LDL-A). The use of LDL adsorption columns in particular is selective for LDL removal (8); these columns have been shown to be effective not only for

Received November 2012; revised March 2013.
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homozygous FH patients but also for severe heterozygous FH patients, as well as for atherosclerotic diseases including arteriosclerosis obliterans (ASO) (10). Although there have been no randomized controlled studies, Mabuchi et al. and Nishimura et al. reported that LDL-A treatment had more beneficial effects, including the prevention of cardiac events and the inhibition of coronary stenosis, compared to treatment with a maximal dose of statins in heterozygous FH patients (11,12).

After the launch of strong statins such as atorvastatin, pitavastatin, and rosuvastatin, some patients on LDL-A treatment withdrew from the therapy because of the high medical costs. However, several patients who withdrew from LDL-A treatment later died of cardiac events, though their serum lipid levels were controlled by a maximal dose of a strong statin at the same level as under LDL-A treatment, as reported by studies from the National Cerebral and Cardiovascular Center and Kanazawa University (13,14). These results suggest that LDL-A treatment can prevent cardiovascular events by removing a series of proteins and substances from the blood in addition to apolipoprotein B (Apo B)-containing lipoproteins. LDL-A treatment using an adsorption column has been reported to reduce Lp(a), fibrinogen; coagulation factors II, V, VII, VIII, X, XI, and XII, serotonin; C-reactive protein (CRP), and amyloid proteins (15,16); while LDL-A treatment using DFPP has been reported to reduce fibrinogen, Lp(a), C3, C4, α_2-macroglobulin, and immunoglobulins (17). These studies were conducted by measuring the target molecules before and after LDL-A treatment. On the basis of the hypothesis that the adsorption column removed atherosclerosis-related proteins other than lipoprotein-binding proteins or positively charged proteins, proteomic analysis of the waste fluid was performed. Edwards et al. reported that proteomics has the potential to reveal proteins that are associated with pathogenesis, by providing a greater understanding of information flow in pathogenic situations (18). Proteomics has been used for analysis of the waste fluid of LDL-A treatment by DFPP, direct adsorption of lipoproteins (DALI), and heparin-mediated extracorporeal LDL precipitation (HELP) (19). However, it has not been applied to analysis of the waste fluid of the dextran sulfate column, which is a major treatment in Japan. In this study, we analyzed stepwise-eluted fluid from LDL adsorption columns by a proteomics approach to identify the proteins removed by the LDL-A treatment and, in turn, to present a possible mechanism underlying the preventive effects of LDL-A treatment on atherosclerosis.

PATIENTS AND METHODS

Patients and LDL-A treatment

The subjects were four FH patients, including one homozygous and three heterozygous patients, who were on regular LDL-A treatment. For LDL-A treatment, MA-03 (Kaneka, Osaka, Japan) and LDL adsorption columns (Liposorber LA-15; Kaneka) were used. The patients' backgrounds are shown in Table 1. All the patients used heparin for anticoagulation for LDL-A treatment, and the treated plasma volumes are shown in Table 1. Each patient gave written informed consent to participate in the study, and the study protocol was endorsed by the ethics committee of the National Cerebral and Cardiovascular Center (approval No. M20-26).

Serological investigation

Blood was collected from the blood removal line immediately before and after LDL-A treatment to determine changes in the levels of lipids, lipoprotein fractions, hematological values, electrolytes, serum proteins, and so on. Vitronectin was determined by the sandwich ELISA method (Human Total Vitronectin ELISA kit; Innovative Research, Novi, MI, USA). The measurements of fibrinogen, D-dimer and apolipoprotein C-III (Apo C-III) were consigned to SRL (Tokyo, Japan) and other items were consigned to the clinical laboratory at our hospital. Lipoprotein fractions were separated by ultracentrifugation. The data are presented as the means ± SEM of three

TABLE 1. *Background of study patients*

Patient No.	Sex (M/F)	Type of FH	HT	DM	Mode of LDL-A treatment			Drug for hypercholesterolemia		
					Duration of LDL-A (year)	Treated plasma volume (mL)	Anticoagulation	Statin	Ezetimibe	Other
1	M	Het.	+	–	2	4000	Heparin	+	+	–
2	M	Hom.	+	–	27	6000	Heparin	+	+	–
3	F	Het.	–	–	16	4000	Heparin	+	–	+
4	M	Het.	+	+	16	4000	Heparin	+	–	+

HT, hypertension; DM, diabetes mellitus; Het., Heterozygote; Hom., Homozygote. (+) Is an affected patient and (–) is a non-affected patient.

measurements in each patient. Comparisons of parameters before and after LDL-A treatment were made by a paired *t*-test using Excel analysis software (Microsoft, Redmond, WA, USA).

Proteomic analysis

Sampling

Three separate samples for proteomic analysis of the waste fluid were obtained twice from the LA-15 system of each patient. Sample 1 was the waste fluid obtained from 0.86 M NaCl solution during LDL-A treatment; Sample 2 was obtained after the removal of lipoproteins by the ultracentrifugation of Sample 1; and Sample 3 was the solution eluted from the column with 2 M NaCl solution.

Sample preparation

Samples 1 to 3 were dialyzed in dialysis buffer, 0.3 mM ethylenediaminetetraacetic acid (EDTA) (pH 7.4), and 0.15 M NaCl using cellulose tubing overnight. After dialysis, Sample 3 was concentrated using a 5 kDa molecular weight cutoff spin concentrator (5 kDa MWCO 4 mL; Agilent Technologies, Santa Clara, CA, USA). Albumin and immunoglobulin in Samples 1 and 2 were removed using an Albumin and IgG Removal kit (GE Healthcare, Chalfont St. Giles, UK).

Isoelectric focusing electrophoresis

Isoelectric focusing electrophoresis was performed using an Immobiline Dry Strip (IPG, pH 3–10, 24 cm, GE Healthcare). Samples 1, 2, and 3 from study patients were each adjusted to a protein concentration of 1 mg/450 μL with the rehydration buffer according to the manual supplied by the manufacturer. The passive hydration of the gels was carried out for 12 h at 20°C, and the isoelectric focusing was performed at 500 V for 1 h, 1000 V for 1 h, 8000 V for 8.2 h, and 500 V for 1 h.

SDS-PAGE

After the isoelectric focusing electrophoresis, the IPG strips were equilibrated in 6 M urea 30% glycerol v/v, 2% sodium dodecyl sulfate (SDS), 50 mM Tris–HCl (pH 8.8), and 0.1% (w/v) dithiothreitol (DTT) for 15 min. Subsequently, the IPG strips were immersed in the above buffer containing 0.25% (w/v) of iodoacetamide instead of DTT at room temperature for 15 min. SDS-polyacrylamide gel electrophoresis (PAGE) was performed using the 12.5% polyacrylamide gel with an Ettan DALT buffer kit (GE Healthcare). Electrophoresis was performed at 4–6 W for 18 h.

Staining and de-staining

After the electrophoresis, the gels were fixed with a fixative solution, 40% methanol (v/v) and 10% acetic acid (v/v), for 30 min and stained in the same mixture containing 0.2% Coomassie Brilliant Blue (CBB) (w/v) for 30 min. The gels were then de-stained with 20% methanol and 5% acetic acid (v/v) solution at room temperature until spots were clearly visible.

In-gel digestion with trypsin

The spots were cut out from the gels, and were further immersed in a solution of 50% acetonitrile (ACN) and 25 mM ammonium hydrogen carbonate. The gel pieces were then dehydrated and dried. Each gel piece was rehydrated with 15 μL of 100 mM ammonium bicarbonate containing 100 μg/mL of trypsin, 7% ACN, and 1% octyl-beta-glucoside and left to stand for 45 min on ice, after which the gel was incubated overnight at 37°C with shaking. An extraction solution containing 50% ACN and 1% trifluoroacetic acid (TFA) was added to the gel and left for 30 min. After centrifugation, the recovered extract was concentrated to 5 to 10 μL using a SpeedVac concentrator (Thermo Scientific, Waltham, MA, USA).

Desalting and condensation by C-tip

Desalting and condensation were performed using a solid phase extraction tip, C-tip (Nikkyo Technos, Tokyo, Japan). After pre-treatment, 5–10 μL of the tryptic digest solution was applied to the tip, washed with 0.1% trifluoroacetic acid (TFA) and 10% ACN, and eluted with a solution of 0.1% TFA and 60% ACN by centrifugation to recover the desalted tryptic digests.

Mass spectrometry (MS) analysis

Tryptic peptides eluted from the C-tip were spotted onto a sample plate (Opti-TOF 384 Well MALDI Plate Inserts; Applied Biosystems, Carlsbad, CA, USA), mixed with a matrix (0.175% alpha-cyano-4-hydroxycinnamic acid, 50% ACN and 0.1% TFA), and air-dried. Each spotted sample was then subjected to mass spectrometric (MS) and tandem mass spectrometric (MS/MS) analysis with a 4800 matrix-assisted laser desorption/ionization time-of-flight/time-of-flight (MALDI TOF/TOF) Analyzer (Applied Biosystems) to identify tryptic peptides. Each spot was analyzed by MS in reflector mode in a mass-to-charge ratio (*m/z*) range of 800 to 4000 (Fig. 1A). MS/MS was performed for digested peptide peaks with a signal-to-noise (S/N) ratio greater than 100 (Fig. 1B). Peak lists were generated by the "Launch Peaks to Mascot" function of the

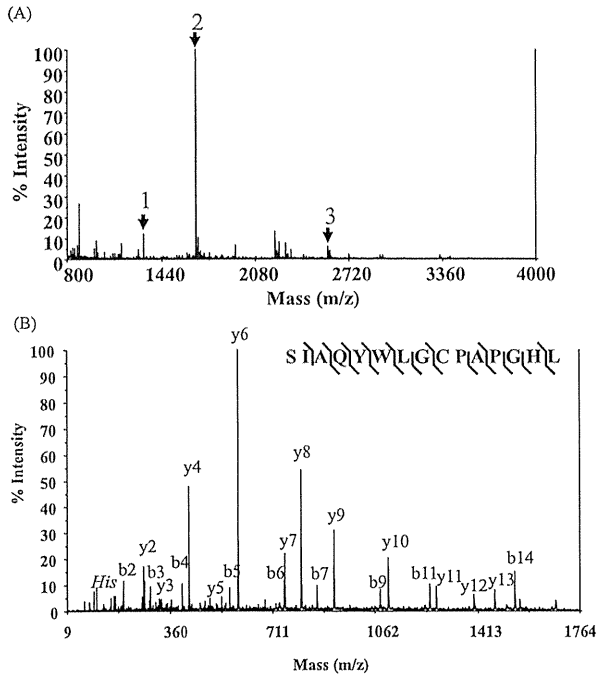

FIG. 1. Peptide mass fingerprint (A) and mass spectrometry/mass spectrometry (MS/MS) spectrum (B) of vitronectin obtained by a 4800 matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) analyzer. (A) 1, Vitronectin [453–463], RVDTVDPPYPR; 2, Vitronectin [464–478], SIAQYWLGCPA-PGHL (carbony-tarminal); 3, Vitronectin [422–443], MDWLVPAT-CEPIQSVFFFSGDK. (B) Tryptic peptide number 2 in (A) was identified by nine b-ions and 12 y-ions by MS/MS analysis. *His* shows the ammonium ion of His. The "y" shows the y-ion and the "b" shows the b-ion. The amino acid sequence of the peptide is shown.

4000 Series Explorer software (ver. 3.5; Applied Biosystems). Peak lists were searched against the human NCBInr database (80 128 entries on 18 June 2009) using the Mascot search algorithm (ver. 2.2), with trypsin specification. Carbamide-methylated cysteine was set as a fixed modification. Peptide tolerance was set to 125 ppm, and MS/MS tolerance was 0.4 Da. Peptide sequences with an expectation value lower than 0.05 were identified.

Separation of the lipoprotein and bottom fractions

To determine the removal rate of Apo C-III and vitronectin in the lipoprotein and bottom fractions by LDL-A treatment, the serum was separated by ultracentrifugation ($d < 1.006$: very low-density lipoprotein (VLDL), $1.006 \leq d < 1.019$: intermediate-density lipoprotein (IDL), $1.019d<1.063$: LDL, $1.063 \leq d < 1.210$: high-density lipoprotein (HDL), $1.210 < d$: bottom).

RESULTS

Low-density lipoprotein apheresis treatment was performed in one homozygous and three heterozygous patients by using the LDL adsorption method.

Hematologic test and blood chemical analysis data

Hematologic test and blood chemical analysis data before and after LDL-A treatment are shown in Table 2. After a single LDL-A treatment, serum LDL-C levels decreased by 80.9%, total cholesterol

TABLE 2. *Laboratory data before and after a single low-density lipoprotein apheresis treatment*

	Before treatment (Mean ± SD)	After treatment (Mean ± SEM)	Decrease (%)	*P*-value
Total cholesterol (mg/dL)	254 ± 102	73 ± 22	−71.4	**
LDL cholesterol (mg/dL)	194 ± 81	37 ± 19	−80.9	**
HDL cholesterol (mg/dL)	46 ± 22	39 ± 18	−15.4	n.s.
Triglycerides (mg/dL)	132 ± 76	24 ± 16	−81.8	**
Total protein (mg/dL)	7.4 ± 0.6	6.4 ± 0.7	−13.6	**
Albumin (g/dL)	4.6 ± 0.4	4.1 ± 0.5	−12	*
RBC (10×10^5 mm^3)	4.27 ± 0.81	4.52 ± 0.77	5.7	n.s.
WBC (10×10^5 mm^3)	5.8 ± 2.0	7.5 ± 2.5	28.2	n.s.
Hemoglobin (g/dL)	13.4 ± 2.5	13.8 ± 2.6	3	n.s.
Hematocrit (%)	38.5 ± 6.2	40.1 ± 6.4	4.2	n.s.
Plt (10^3/μL)	166 ± 65	152 ± 56	−8.4	n.s.
Na (mEq/L)	138 ± 1	142 ± 1	2.5	**
K (mEq/L)	4.0 ± 0.4	4.1 ± 1	2.5	n.s.
Cl (mEq/L)	104 ± 3	109 ± 4	4.3	n.s.
Ca (mEq/L)	9.6 ± 0.4	9.0 ± 0.3	−5.7	*
Fibrinogen (μg/dL)	203 ± 51	123 ± 31	−39.4	**
D-dimer (μg/mL)	1.04 ± 0.91	1.13 ± 1.013	8.7	n.s.

*$0.01 < P < 0.05$, **$P < 0.01$. HDL, high-density lipoprotein; n.s., not significant; RBC, red blood cell count; SEM, standard error of the mean; WBC, white blood cell count.

(TC) levels by 71.4% and triglyceride (TG) levels by 81.8%, while high-density lipoprotein-cholesterol (HDL-C) showed no significant change. Serum fibrinogen decreased significantly to 39.4%. These results are in good agreement with those previously reported.

Proteomic analysis

The proteomic analysis was performed using three kinds of samples (Sample 1, Sample 2 and Sample 3) from each study patient. Typical two-dimensional gel electrophoresis profiles obtained from a homozygous FH patient (Fig. 2A–C show the data from patient number 2) and from one of three heterozygous FH patients (Fig. 2D–F are the results for patient number 1) are shown. Sample 1, which was prepared from the waste fluid fraction, gave 110 spots (Fig. 2A) and 127 spots (Fig. 2D), while Sample 2, which was prepared by removing lipoproteins from Sample 1, gave 120 (Fig. 2B) and 145 spots (Fig. 2E), in the homozygous and heterozygous patients, respectively. The density and distribution patterns of the protein spots were similar in homozygous and heterozygous patients. Sample 3, the eluate from the column by 2 M NaCl, appeared to yield fewer spots compared to Samples 1 and 2: 30 spots in the homozygous patient (Fig. 2C) and 25 spots in the heterozygous patient (Fig. 2F). Sample 3 also showed similar density and distribution patterns between the homozygous and heterozygous patients.

By mass spectrum analysis of the spots obtained from Samples 1, 2, and 3 of four patients, 48 proteins were identified from 279 spots in Sample 1, 34 proteins from 140 spots in Sample 2, and 10 proteins from 36 spots in Sample 3. As numerous spots gave the same identification results, we identified 48 proteins in total from Samples 1–3 without redundancy. The identified proteins are listed in Table 3. The spots were distributed between 10 kDa and 260 kDa on the gel; the largest was fibronectin and the smallest was Apo C-III. The protein with the lowest pI was vitronectin, and that with the highest was fibrinogen-α-chain (Fig. 3A–C). The identified proteins included coagulation factors such as fibrinogens; antithrombin III; haptoglobin; heparin cofactor; thrombogenic factors such as β_2-glycoprotein I; fibronectin; kininogen I; complement factors; inflammation factors such as Apo C-III and α_1-acid glycoprotein I; and adhesion molecules such as vitronectin. The proteins identified from Sample 3 were mostly fibrinogen α, β, and γ chains; the protein α_1-microglobulin bikunin precursor (AMBP); antithrombin III; and the Ig mu chain C region. Of the proteins identified, β_2-glycoprotein I, clusterin, gelsolin, α_1-antitrypsin, apolipoprotein F (Apo F), CD5 antigen-like, amidase, pigment epithelium-derived factor, tetranectin, transthyretin, vitamin D-binding protein, histidine-rich glycoprotein, alpha-2 HS glycoprotein, and complement component C6 were first found to have been removed by the LDL-adsorption column. Fibrinogen (α, β, and γ chains), antithrombin III apolipoprotein A-I (Apo A-I), and apolipoprotein E (Apo E), which were previously reported to have been removed by LDL-A treatment, were also identified in the waste fluid in this study (Table 3).

Changes in identified protein levels in serum before and after LDL-A treatment

Among the identified proteins, vitronectin, and Apo A-I, A-II, B, C-II, and C-III levels in the serum were measured before and after LDL-A treatment. Vitronectin showed a substantial decrease of 82.4% after a single treatment. In addition, we measured vitronectin levels in the lipoprotein and the bottom fractions before and after LDL-A treatment. Before treatment, the amount of vitronectin in the bottom fraction accounted for 68.8% of the total amount, suggesting that most of the vitronectin was not associated with lipoproteins. After treatment, vitronectin levels were decreased by 83.6% in the bottom fraction and by 43.9% in the lipoprotein fraction. Among apolipoproteins, Apo A-I, A-II, B, C-III, and E decreased significantly after a single treatment. Most of the Apo C-III was found in the lipoprotein fraction, and a negligible amount was seen in the bottom fraction. In addition, Apo C-III levels in the VLDL and LDL fractions decreased by 22.8% and by 42.6% after LDL-A treatment. On the other hand, the amount of Apo C-III in the bottom fraction showed no change, suggesting that Apo C-III was removed in association with VLDL and LDL lipoproteins.

DISCUSSION

The adsorption column used in LDL-A treatment has been reported to remove many proteins, such as fibrinogen, antithrombin III, coagulation factors II, V, VII, VIII, IX, X, XI and XII, CRP, α_1-antitrypsin, serum amyloid A protein, and α_1-acid glycoproteins of inflammation factors and lipoproteins, such as Lp(a), MDA-LDL, sd-LDL, and ox-LDL (15,20,21). On the other hand, DFPP has been reported to remove fibrinogen, Lp(a), C3, C4, β_2-macroglobulin, and immunoglobulins (17). The mechanism of the removal of Apo B-containing lipoproteins is based on the molecular size in DFPP, whereas in the LDL adsorption column, the mechanism is the electrostatic binding to the negatively charged ligand.

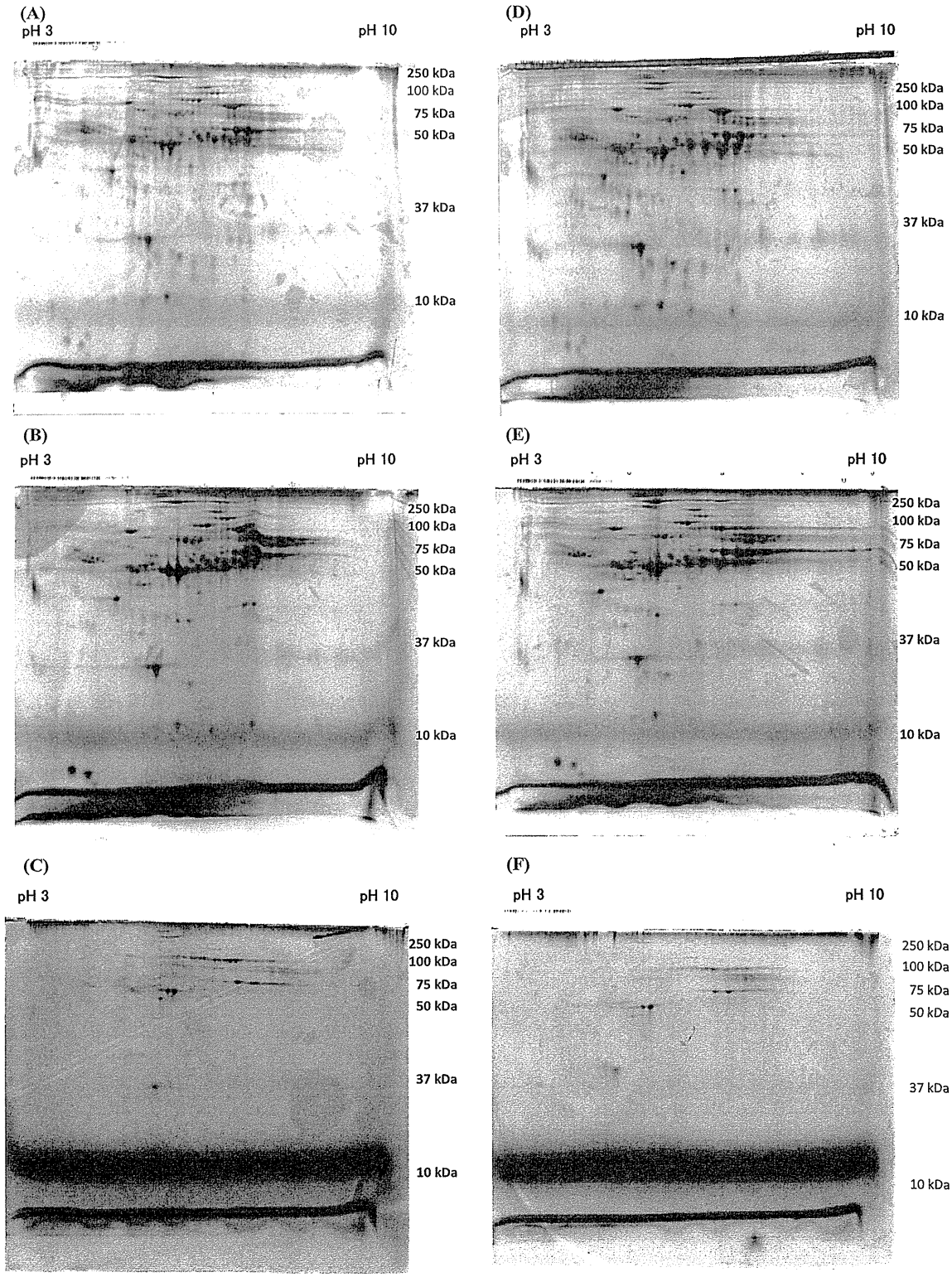

FIG. 2. Two-dimensional gel electrophoresis profiles of the samples prepared from one homozygous familial hypercholesterolemia (FH) patient (A–C) and one heterozygous FH patient (D–F). For each of the two patients, sample 1 (A,D), sample 2 (B,E), and sample 3 (C,F) were subjected to two-dimensional electrophoretic analysis and stained. The horizontal axis/bar shows isoelectric focusing with pH values of 3 to 10, and the vertical axis/bar shows the molecular weight.

TABLE 3. *Identified proteins in the eluate from the low-density lipoprotein apheresis column*

Group	Protein Name	MW	Sample 1	Sample 2	Sample 3
Coagulation factor	α-fibrinogen precursor	69 809	+	+	+
	Antithrombin III	52 618	+	+	+
	β-fibrinogen precursor	54 895	+		
	EGF-containing fibulin-like extracellular Matrix protein 2	49 405	+		
	Fibrinogen γ chain	49 481	+	+	+
	Heparin cofactor II precursor	57 098	+		
	Kininogen I	47 901	+	+	
	β2-glycoprotein I	36 254	+	+	
Thrombogenic factor	Histidine-rich glycoprotein	53 378	+		
	α-1-acid glycoprotein 1 precursor	23 511	+	+	
Inflammation factor	Apolipoprotein C-III	10 822	+	+	
	Inter-alpha-trypsin inhibitor family heavy chain-related protein	103 385	+	+	
	α-1 antitrypsin	46 706	+	+	+
	α-2-glycoprotein 1	34 258	+	+	
	α-2-HS-glycoprotein	39 324	+	+	
Adhesion molecule	Vitronectin	54 335	+	+	
	Fibronectin precursor	256 689	+	+	
Complement component	Complement component C3	187 163	+	+	
	Complement C1r subcomponent	80 173	+		
	Complement component C4A	192 861	+	+	
	Complement factor B	85 562	+	+	+
	Complement factor C6	104 843	+		
	C1-inhibitor	32 708	+		
	Complement C1s	37 208	+		
	Complement component C6	104 786	+		
Glycoproteins	α-1B-glycoprotein	54 254	+	+	
	Angiotensinogen	53 154	+		
	Clusterin	52 495	+	+	
	Hemopexin	51 676	+	+	
	Zinc-alpha-2-glycoprotein	34 259	+	+	
Apolipoproteins	Apolipoprotein A-I	30 778	+	+	+
	Apolipoprotein A-IV	45 399	+	+	
	Apolipoprotein C-II	11 284	+	+	
	Apolipoprotein E	36 154	+	+	
	Apolipoprotein F	35 399	+		
Immunogloblin	Ig kappa chain C region	11 609	+	+	
	Ig mu chain C region	49 307	+		
Others	CD5 antigen-like	38 088	+	+	
	Gelsolin	85 698	+	+	+
	N-acetylmuramoyl-L-alanine amidase	62 217	+		
	Pigment epithelium-derived factor	46 312	+	+	+
	Protein AMBP	38 999	+	+	
	Retinol-binding protein 4	23 010	+	+	
	Serotransferrin	77 064	+	+	+
	Serum albumin	69 367	+		
	Tetranectin	22 566	+	+	+
	Transthyretin	20 193	+	+	
	Vitamin D-binding protein	52 964	+	+	

(+) Protein was identified in the waste fluid and eluted solution from the adsorption column.

Therefore, the proteins removed by DFPP may be associated with VLDL and/or LDL. On the other hand, the removal of proteins by the LDL adsorption column has three mechanisms, association with VLDL and/or LDL, electrostatic binding of their positive charge to the ligands, or nonspecific binding to the column.

Dihazi et al. reported the clearance of proteins by three kinds of LDL-A methods: DFPP, DALI, and HELP (19). They reported that 74 proteins were identified and, among these, 15 proteins, that is, α-1 antitrypsin, α-2 antiplasmin, fibrinogen A alpha polypeptide, fibrinogen beta chain, fibrinogen gamma polypeptide, kininogen I, transthyretin, alpha-2-macroglobulin, complement C4 precursor, complement C3, complement component C4B, complement factor H –precursor, haptoglobin, Ig kappa chain C region and immunoglobulin J chain, were reported to be removed by all three methods. In our study, 48 proteins were found to be removed by the LDL adsorption columns, and among them, eight proteins, β-fibrinogen precursor, fibrinogen γ chain, kininogen

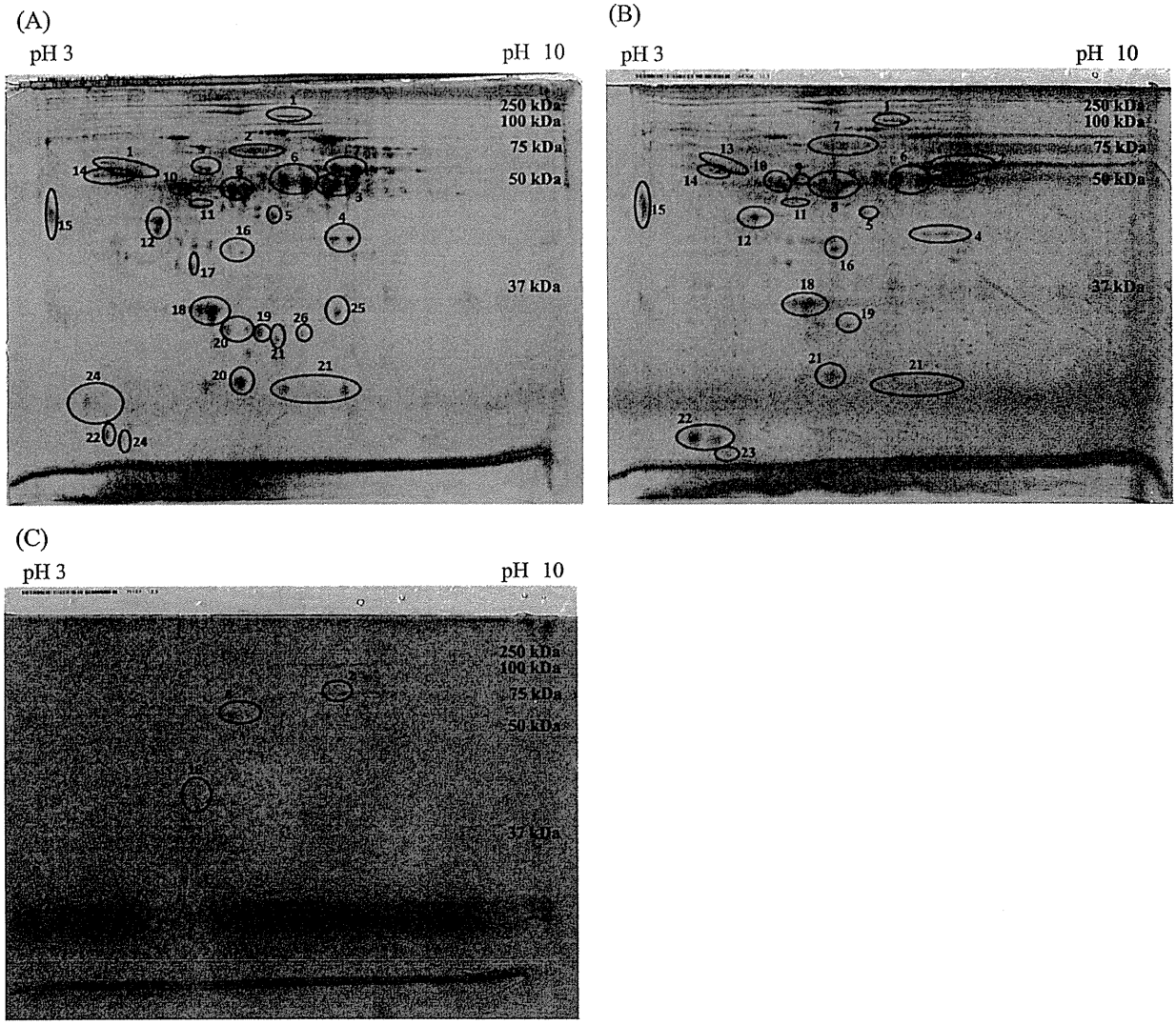

FIG. 3. Protein spot map on two-dimensional gel electrophoresis. Each spot was excised from the gel and subjected to in-gel trypsin digestion, followed by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry/mass spectrometry (MS/MS) analysis. (1) Fibronectin, (2) β_2 glycoprotein I, (3) fibrinogen β-chain, (4) complement factor CA4, (5) complement factor H, (6) apolipoprotein H, (7) hemopexin, (8) fibrinogen γ-chain, (9) vitamin D-binding protein, (10) α_1 antitrypsin, (11) apolipoprotein A-IV, (12) complement factor 3, (13) kininogen I, (14) α-HS glycoprotein, (15) vitronectin, (16) apolipoprotein E, (17) microglobulin binding protein, (18) apolipoprotein A-I, (19) haptoglobin, (20) transthyretin, (21) fibrinogen α-chain precursor, (22) apolipoprotein C-III, (23) apolipoprotein C-II, (24) vitronectin.

I, α-1 antitrypsin, fibronectin precursor, complement component C3, Ig kappa chain C region and transthyretin, were the proteins removed by all four methods—that is, HELP, DFPP, DALI and LDL adsorption—although the analytical methods were not the same. There have been no data on long-term clinical outcomes indicating that one of the existing lipid apheresis methods is superior to any of the others. Indeed, the report of Dihazi that analyzed the proteins removed by each LDL-A method does not support the superiority of any specific method. The fact that fibronectin and fibrinogen were removed by all four methods is of interest, because removal of these proteins has been reported to lower plasma viscosity which has been related to a significant improvement of peripheral flow.

Proteins that have heparin-binding domains, such as fibrinogen, antithrombin III, fibronectin, and α_1-β-glycoprotein, carry positive charges, which may be removed by ionic interaction. Apolipoproteins known as lipoprotein-associated proteins may be removed together with the lipoproteins. We identified not only proteins reported to be removed by LDL-A treatment (10,21,22), but also new molecules such

as vitronectin and Apo C-III, which are known to have important roles in atherogenesis. It is of interest that while vitronection was not removed with Apo B-containing lipoproteins but by ionic interaction, Apo C-III was removed with Apo B-containing lipoproteins. We also found that the serum levels of Apo C-III and vitronectin were significantly decreased after a single LDL-A treatment.

Vitronectin is a heparin-binding protein with a molecular weight of 54 kDa, 5.55 pI, and is contained at 200 to 400 μg/mL in human serum. Vitronectin carries positive charges in the heparin binding domain, suggesting that its binding mechanism to the negative charges of dextran sulfate is plausible. Serum vitronectin levels are reportedly increased in patients with significant stenosis in two or more segments of the coronary arteries compared to those with stenosis in no or only one segment (23). Peng et al. reported that vitronectin-knockout mice show reduced neointima formation after carotid injury ligation or chemical injury compared with wild-type mice (24). Although vitronectin does not directly change vascular smooth muscle cell (VSMC) proliferation, this protein was reported to promote neointima development by enhancing VSMC migration. Vitronectin reduction may be one of the beneficial effects of LDL-A treatment in the prevention of atherosclerosis.

Apo C-III is 10 kDa in molecular weight and 5.23 in pI, and is contained at 5.4 mg/dL to 10.0 mg/dL in human serum. Apo C-III is known to be distributed mainly in VLDL and HDL, and secreted as a component of VLDL from the liver (25). The physiological role of Apo C-III is the regulation of lipolysis through noncompetitive inhibition of endothelial cell-bound LPL that hydrolyzes TG in VLDL, transforming large TG-rich particles into smaller TG-depleted remnant lipoproteins (25). In several clinical studies, higher Apo C-III levels were associated with an increased severity of CVD in patients with angiographically defined coronary artery diseases (25). Pollin et al. reported that Lancaster Amish patients are heterozygous carriers of a null mutation in the gene encoding Apo C-III, and thus express half the amount of Apo C-III present in noncarriers. The carriers had higher HDL-C levels, lower TC and LDL-C levels (26), and less detectable coronary artery calcification than noncarriers (26). Null mutation in Apo C-III confers favorable lipid profiles and apparent cardioprotection without any obvious detrimental effect; this raised the possibility that therapies targeting Apo C-III would be clinically effective in reducing cardiovascular events (26). In our study, Apo C-III was decreased by 54.8% with a single LDL-A treatment. Moreover, the Apo C-III level in VLDL was decreased by 77.2%. This suggests that the decrease in Apo C-III by LDL-A treatment is one of the protective effects against cardiovascular disease.

There are some limitations to this study. First, some proteins that LDL-A treatment removes have beneficial effects on the inhibition of atherosclerosis development. Second, some proteins having physiological importance, such as immunoglobulin, albumin, transthyretin and so on, were removed by the adsorption column. In order to evaluate the effect of removal of each protein by LDL-apheresis, a comprehensive understanding of each factor involved in the pathogenesis and pathophysiology based on the analysis of both patients and animal models will be needed.

CONCLUSION

In this study, several proteins that might be involved in the cause and pathophysiology of atherosclerosis were identified in the waste fluid of lipoprotein apheresis treatment by proteomic analysis. Proteomic analysis may provide information on the mechanisms underlying the effects of LDL-A treatment on atherosclerosis, and also could provide data to broaden the application of the treatment, thus demonstrating this treatment's usefulness from these additional perspectives.

Acknowledgments: This work was supported by Grants-in-Aid for Scientific Research from the Japanese Ministry of Health, Labor, and Welfare (H23-seisaku tansaku-ippan-004 and H23-nanji-ippan-011) and the Cardiovascular Research Foundation (Suita, Japan). The authors thank Ms Mika Nishimura, Ms Eiko Shibata, Ms Mai Inoue, and Ms Megumu Morimoto of the Department of Molecular Innovation in Lipidology, National Cerebral and Cardiovascular Center Research Institute for their support and advice. We also thank Mr Teruyuki Hayashi, Mr Takayuki Nishigaki, Mr Kohji Ogawa, and the clinical engineers of the Department of Clinical Engineering, National Cerebral and Cardiovascular Center for their technical support.

REFERENCES

1. Goldstein JL, Hobbs HH, Brown MS. Familial hypercholesterolemia. In: Scriver CR, Beaudet A, Sly WS, Valle D, eds. *The Metabolic and Molecular Bases of Inherited Disease* 8th edn. New York: McGraw-Hil, 2001; 2863–2913.
2. Makino H, Harada-Shiba M. Long-term effect of low-density lipoprotein apheresis in patients with homozygous familial hypercholesterolemia. *Ther Apher Dial* 2003;7:397–401.
3. de Gennes JL, Touraine R, Maunand B, Truffert J, Laudat P. [Homozygous cutaneo-tendinous forms of hypercholesteremic xanthomatosis in an exemplary familial case. Trial of plasmapheresis ans heroic treatment]. *Bull Mem Soc Med Hop Paris* 1967;118:1377–402.

4. Thompson GR, Lowenthal R, Myant NB. Plasma exchange in the management of homozygous familial hypercholesterolaemia. *Lancet* 1975;1:1208–11.
5. Mineshima M, Agishi T, Hasuo Y, Kaneko I, Era K, Ota K. Effect of membrane trapping in plasma fractionator on separative characteristics. *Int J Artif Organs* 1988;11:191–4.
6. Usami M, Horiuchi T, Emura M et al. Thermal effect of LDL/HDL separation in membrane filtration; thermofiltration. *Trans Am Soc Artif Intern Organs* 1985;31:716–21.
7. Nosé Y, Usami M, Malchesky PS et al. Clinical thermofiltration: initial application. *Artif Organs* 1985;9:425–7.
8. Tani N. Development of selective low-density lipoprotein (LDL) apheresis system: immobilized polyanion as LDL-specific adsorption for LDL apheresis system. 1996. *Ther Apher* 2000;4:135–41.
9. Yokoyama S, Hayashi R, Satani M, Yamamoto A. Selective removal of low density lipoprotein by plasmapheresis in familial hypercholesterolemia. *Arteriosclerosis* 1985;5:613–22.
10. Kobayashi S, Oka M, Moriya H, Maesato K, Okamoto K, Ohtake T. LDL-apheresis reduces P-Selectin, CRP and fibrinogen—possible important implications for improving atherosclerosis. *Ther Apher Dial* 2006;10:219–23.
11. Mabuchi H, Koizumi J, Shimizu M et al. Long-term efficacy of low-density lipoprotein apheresis on coronary heart disease in familial hypercholesterolemia. Hokuriku-FH-LDL-Apheresis Study Group. *Am J Cardiol* 1998;82:1489–95.
12. Nishimura S, Sekiguchi M, Kano T et al. Effects of intensive lipid lowering by low-density lipoprotein apheresis on regression of coronary atherosclerosis in patients with familial hypercholesterolemia: Japan Low-density Lipoprotein Apheresis Coronary Atherosclerosis Prospective Study (L-CAPS). *Atherosclerosis* 1999;144:409–17.
13. Takata M, Kawashiri M, Yamagishi M, Mabuchi H. Study on prognosis of patients with familal hypercholesterolemia (FH) who have withdrawn from LDL apheresis. *Lipid* 2007;18:86–90.
14. Harada-Shiba M, Nakahama H, Nagumo S et al. Long-term prognosis of LDL-apheresis in homozygous and heterozygous familial hypercholesterolemia. *Jpn J Apher* 2006;25:65.
15. Kojima S, Harada-Shiba M, Toyota Y et al. Changes in coagulation factors by passage through a dextran sulfate cellulose column during low-density lipoprotein apheresis. *Int J Artif Organs* 1992;15:185–90.
16. Cucuianu M, Dican L. Coagulation factor XIII and atherothrombosis. A mini-review. *Rom J Intern Med* 2003;41:339–55.
17. Klingel R, Fassbender T, Fassbender C, Gohlen B. From membrane differential filtration to lipidfiltration: technological progress in low-density lipoprotein apheresis. *Ther Apher Dial* 2003;7:350–8.
18. Edwards AV, White MY, Cordwell SJ. The role of proteomics in clinical cardiovascular biomarker discovery. *Mol Cell Proteomics* 2008;7:1824–37.
19. Dihazi H, Koziolek MJ, Sollner T et al. Protein adsorption during LDL-apheresis: proteomic analysis. *Nephrol Dial Transplant* 2008;23:2925–35.
20. Kojima S, Harada-Shiba M, Yamamoto A. Plasma constituents other than low-density lipoprotein adsorbed by dextran-sulfate column. *Ther Apher* 1997;1:309–13.
21. Knisel W, Di Nicuolo A, Pfohl M et al. Different effects of two methods of low-density lipoprotein apheresis on the coagulation and fibrinolytic systems. *J Intern Med* 1993;234:479–87.
22. Mabuchi H, Michishita I, Takeda M et al. A new low density lipoprotein apheresis system using two dextran sulfate cellulose columns in an automated column regenerating unit (LDL continuous apheresis). *Atherosclerosis* 1987;68:19–25.
23. Ekmekci OB, Ekmekci H. Vitronectin in atherosclerotic disease. *Clin Chim Acta* 2006;368:77–83.
24. Peng L, Bhatia N, Parker AC, Zhu Y, Fay WP. Endogenous vitronectin and plasminogen activator inhibitor-1 promote neointima formation in murine carotid arteries. *Arterioscler Thromb Vasc Biol* 2002;22:934–9.
25. Ooi EM, Barrett PH, Chan DC, Watts GF. Apolipoprotein C-III: understanding an emerging cardiovascular risk factor. *Clin Sci (Lond)* 2008;114:611–24.
26. Pollin TI, Damcott CM, Shen H et al. A null mutation in human APOC3 confers a favorable plasma lipid profile and apparent cardioprotection. *Science* 2008;322:1702–5.