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Angiopoietin-Like Protein3 Regulates Plasma HDL Cholesterol Through Suppression of Endothelial Lipase

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Objectives—A low level of high-density lipoprotein (HDL) in plasma has been recognized as an aspect of metabolic syndrome and as a crucial risk factor of cardiovascular events. However, the physiological regulation of plasma HDL levels has not been completely defined. Current studies aim to reveal the contribution of angiopoietin-like protein3 (angptl3), previously known as a plasma suppressor of lipoprotein lipase, to HDL metabolism.

Methods and Results—Angptl3-deficient mice showed low plasma HDL cholesterol and HDL phospholipid (PL), and which were increased by ANGPTL3 supplementation via adenovirus. In vitro, ANGPTL3 inhibited the phospholipase activity of endothelial lipase (EL), which hydrolyzes HDL-PL and hence decreases plasma HDL levels, through a putative heparin-binding site in the N-terminal domain of ANGPTL3. Post-heparin plasma in Angptl3-knockout mice had higher phospholipase activity than did that in wild-type mice, suggesting that the activity of endogenous EL is elevated in Angptl3-deficient mice. Furthermore, we established an ELISA system for human ANGPTL3 and found that plasma ANGPTL3 levels significantly correlated with plasma HDL cholesterol and HDL-PL levels in human subjects.

Conclusions—Angptl3 acts as an inhibitor of EL and may be involved in the regulation of plasma HDL cholesterol and HDL-PL levels in humans and rodents. (***Arterioscler Thromb Vasc Biol.* 2007;27:366-372.**)

Key Words: angptl3 ■ high density lipoprotein ■ endothelial lipase ■ phospholipase ■ triglyceride

Plasma concentrations of high-density lipoprotein (HDL) cholesterol are inversely correlated with the risk of atherosclerotic cardiovascular disease.[1] HDL cholesterol levels are low in patients with metabolic disorders, such as obesity, insulin resistance, and diabetes.[2,3] However, the genetic and metabolic factors that regulate HDL metabolism remain to be elucidated. Recently, endothelial lipase (EL) has been recognized as one factor that influences HDL metabolism. EL was originally discovered as a member of the family of triglyceride (TG)-lipases together with lipoprotein lipase (LPL) and hepatic lipase (HL). In contrast to LPL or HL, however, EL has relatively lower triglyceride lipase activity and substantially higher phospholipid lipase activity and can hydrolyze HDL phospholipids (PL).[4] Overexpression of EL in mice resulted in reduced plasma HDL levels and EL knockout mice showed significant increase in HDL levels,[5–7] indicating that EL regulates HDL metabolism.

In the colony of KK mice, characterized by obesity, diabetes mellitus, and hypertriglyceridemia, we recently identified a mutant subgroup of KK/Snk mice with low plasma TG levels despite maintaining the phenotype of obesity and diabetes. Genetic mapping and positional cloning identified the gene of angiopoietin-like protein 3 (Angptl3), which was mutated in the KK/Snk mice. The Angptl3 gene in KK/Snk mice contained a 4-bp nucleotide insertion in exon 6, which caused a premature stop codon attributable to a frameshift, leading to a lack of production of the protein.[8] Angptl3 mRNA is expressed exclusively in the livers of humans and mice. ANGPTL3 protein contains a signal sequence of 18 amino acids at the N terminus, followed by a coiled-coil domain and a fibrinogen-like domain at the C-terminal side.[8,9] Treatment with recombinant ANGPTL3 or adenovirus-mediated overproduction of ANGPTL3 significantly elevated plasma levels of TG, nonesterified fatty acids (NEFA), and total cholesterol in mice.[8] In subsequent studies, we revealed that ANGPTL3 increased very low density lipoprotein (VLDL)-TG levels by inhibiting LPL activity via the putative heparin-binding motif in the N-terminal re-

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gion.[10,11] In another study, we also found that ANGPTL3 was able to bind to adipocytes and increase the release of NEFA through activating lipolysis.[12] Thus, the molecular mechanisms of ANGPTL3-mediated increase in plasma TG and NEFA have been investigated. However, the effects of ANGPTL3 on plasma total cholesterol, especially on plasma HDL which is the major lipoprotein carrying cholesterol in mice, have not yet been investigated.

Moreover, the amino acid sequence of EL is 44% identical to that of LPL, and in particular, the clusters of positively charged residues involved in heparin binding are conserved between EL and LPL,[13] suggesting that ANGPTL3 might affect EL activity, because it inhibits LPL activity. In the current study, we investigated the potential involvement of ANGPTL3 in HDL metabolism and its effects on EL activity.

Methods

Animals

Studies were conducted in 15- to 19-week-old male wild-type KK and Angptl3-deficient KK/Snk mice. To obtain a congenic strain, KK/Snk mice were backcrossed to C57BL/6J mice for 10 generations, and designated as C57BL/6J Angptl3^{hypl} mice.[14] Angptl3-knock out mice were made as described previously.[15] Experiments were conducted when the mice (males) were between 8 and 9 weeks of age. The mice were housed in a room under controlled temperature (23±1°C) with free access to water and mouse chow (Oriental Yeast). Blood samples were taken from the inferior vena cava after anesthetization with pentobarbital (50 mg/kg, injected intraperitoneally). Plasma Angptl3 was measured using an Enzyme-linked immunosorbent assay (ELISA) for mouse protein as described previously.[14,16] Plasma HDL cholesterol, total cholesterol, and TG concentrations were measured using assay kits from Wako Pure Chemical Industries. Briefly, to determine HDL-cholesterol and HDL-PL, plasma samples were mixed with reagent to precipitate a non-HDL fraction including magnesium chloride and phosphotungstic acid.[17] The supernatant containing the HDL fraction was harvested and the cholesterol and PL contents were measured with the assay kits (Wako).

Adenovirus Construction

Adenovirus expression vectors containing β-galactosidase (LacZ; designated Ad/lacZ) and human ANGPTL3 (designated Ad/ANGPTL3) cDNAs were constructed as described previously.[8,10] We injected 1 or 2×10^9 pfu of each recombinant adenovirus intravenously into C57BL/6J *Angptl3^{hypl}* mice.[14]

Lipoprotein Analysis

Plasma lipoproteins were analyzed by an upgraded high performance liquid chromatography (HPLC) analysis according to the procedure described by Usui et al[18,19] (Skylight Biotech Inc).

Recombinant ANGPTL3 Protein

Human recombinant ANGPTL3 protein was prepared as described previously, and it was confirmed to inhibit LPL in vitro and to increase plasma TG concentrations in mice.[8,10] Recombinant proteins of truncated and/or mutated human ANGPTL3 were prepared as described previously.[11]

Phospholipase Activity

To obtain EL protein, we constructed human EL cDNA adding an in-frame DNA sequence, as described previously.[13] Human expression constructs were transfected into HEK293 cells with Lipofectamine2000 (Life Technologies), and then a stable transfectant was obtained by G418 selection. The stable transfectant cells were incubated with Opti-MEM I (Invitrogen). After 48 hours, the conditioned (heparin-washed) media were harvested as the enzyme solution, and phospholipase activities were measured with recombinant ANGPTL3 proteins as described in supplemental Methods (available online at http://atvb.ahajournals.org). For the quantification of phospholipase activity in mouse plasma, studies were conducted in 11- to 13-week-old male C57/BL6 and C57/BL6 Angptl3-knockout mice. Plasma was collected into tubes, using a heparin-coated glass capillary, before and 10 minutes after the heparin (20U/kg) injection into the jugular vein. 20 μL of mouse plasma was used as an enzyme solution, and phospholipase activities were measured as described in supplemental Methods.

ELISA for Plasma ANGPTL3 in Humans

Two ANGPTL3 mouse antibodies were produced using the recombinant human ANGPTL3 as the antigen, and were introduced in a double-antibody sandwich enzyme immunoassay system (ELISA) to detect human ANGPTL3.[8,10,14,16] 45B1 mouse monoclonal antibody was fixed on the 96-well plates. 16-fold diluted plasma samples were immobilized on the 96-well plates at 4°C for 16 hours. Then, we washed the plates with PBS containing 0.1% tween20 (PBST) and added horseradish peroxidase (HRP)-conjugated No.1 rabbit polyclonal antibody to these plates. After 1 hour incubation at 37°C, we washed the plates with PBST and added the detection reagent for HRP. Thirty minutes later, we stopped the reaction by the addition of an equal volume of 1N H_2SO_4 and measured at 450 nm absorbance.

Western Blotting

Western blotting of recombinant human ANGPTL3 protein was conducted as described previously.[11] The plasma protein bound to the ELISA plate fixed with 45B1 mouse monoclonal antibody was subjected to western blotting with HRP-conjugated No.1 rabbit polyclonal antibody.

Human Studies

87 volunteers working at Sankyo Co. were enrolled in the study. All subjects gave informed consent. Several subjects with obesity, hypertriglyceridemia, hypertension, fatty liver, diabetes, kidney failure, low body weight, and detection of blood in the urea, were excluded from the correlation analyses. Subjects taking drugs for hyperlipidemia also were excluded. Plasma samples were collected under overnight fasting conditions. Total cholesterol and TG concentrations were measured using an automatic analyzer from Wako Pure Chemical Industries. HDL cholesterol and HDL-PL concentrations were measured as described above.

Statistical Analysis and Ethical Considerations

The correlation coefficient (r) and probability (p) were calculated in human studies using Microsoft Excel 2003. All data were expressed as the means±SEM or SD. Differences between the groups were examined for statistical significance using a Student *t* test. A probability value less than 0.05 denoted the presence of a statistically significant difference. All study protocols described in this report were approved by the Human and Animal Experimentations Ethics Review Committees of Sankyo.

Results

Low HDL lipids Were Observed in the Plasma of Angptl3-Deficient Mice

Figure 1A shows the plasma lipid concentrations in wild-type KK mice (n=5) and KK/Snk mice (n=5). Plasma Angptl3 was not detected in KK/Snk mice (29±4.9 ng/mL versus not detected, $P<0.001$, Figure 1A). The levels of plasma HDL cholesterol and HDL-PL were significantly lower in KK/Snk mice than in KK mice (41±4.1 versus 79±3.9 mg/dL, $P<0.001$; 105±13.7 versus 233±13.3 mg/dL, $P<0.001$,

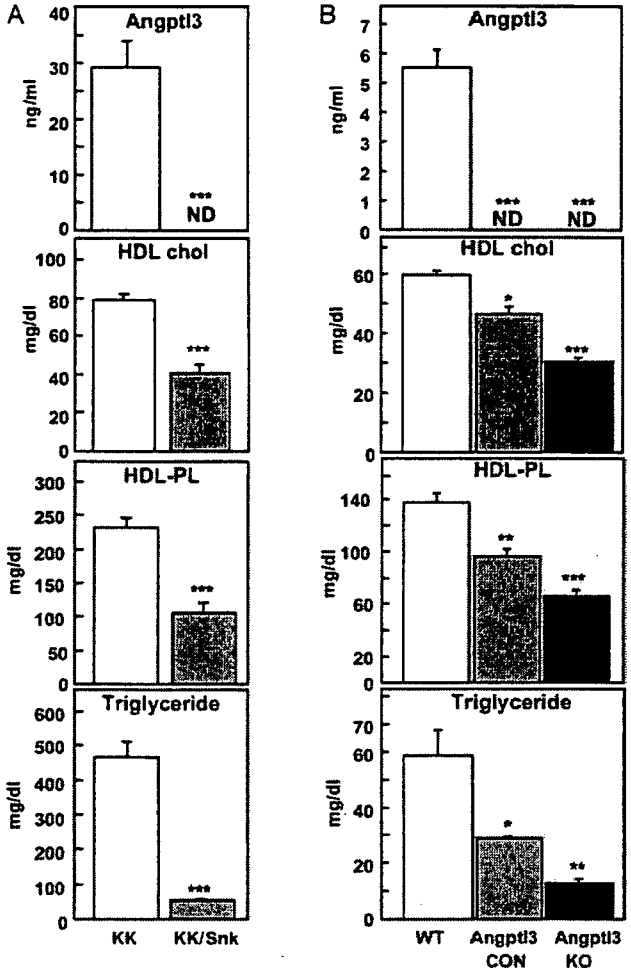

Figure 1. HDL cholesterol, HDL–PL, and triglyceride concentrations in Angptl3-deficient mice. A, Plasma levels of Angptl3, HDL cholesterol (HDL chol), HDL-PL, and triglyceride were measured in male wild-type KK (white bars, n=5) and Angptl3-deficient KK/Snk mice (gray bars, n=5). B, The same for those measured in wild-type C57BL/6J (WT, white bars, n=6), Angptl3-deficient congenic C57BL/6J *Angptl3*^{hypl} (CON, gray bars, n=3) and Angptl3-knockout mice (KO, black bars, n=4). Blood samples were taken under ad libitum conditions. Data are the means±SEM. ***P<0.001 vs wild-type KK mice; *P<0.05, **P<0.01 vs C57/BL6J mice. ND; not detected.

respectively; Figure 1A). Plasma TG levels were also lower in KK/Snk mice than in KK mice, as we reported previously.[7] To avoid the strain effect, we established angptl3-deficient congenic C57BL/6J *Angptl3*^{hypl} mice.[14] However, *Angptl3*^{hypl} mice showed a faint expression of angptl3 in liver.[8] To completely eliminate the expression of angptl3, we also generated Angptl3-knockout mice, whose backstrain was C57BL/6J.[15] Plasma Angptl3 in both congenic C57BL/6J *Angptl3*^{hypl} mice (CON, n=3) and Angptl3-knockout mice (KO, n=4) could not be detected with ELISA for mouse Angptl3, whereas its concentration was measurable in wild-type C57BL/6J mice (WT, n=6) (WT; 5.5±0.6 ng/mL versus CON and KO; not detected, P<0.05). In congenic C57BL/6J *Angptl3*^{hypl} mice and Angptl3-knockout mice, plasma levels of HDL cholesterol and HDL-PL, as well as TG, were significantly lower compared with C57BL/6J mice; HDL cholesterol, WT;60±1.3 versus CON;47±2.5 mg/dL, P<0.05 or versus KO;30±1.8 mg/dL, P<0.001: HDL-PL, WT;137±7.6 versus CON;96±6.1 mg/dL, P<0.001 or versus KO;66±5.2 mg/dL, P<0.001: TG, WT;13±1 mg/dL versus CON; 59±9 mg/dL, P<0.05 or versus KO; 29±0.8 mg/dL, P<0.01 (Figure 1B). These results suggested that lack of Angptl3 is associated with low plasma HDL cholesterol and HDL–PL concentrations.

ANGPTL3 Increased Plasma HDL Lipids in Angptl3-Deficient Mice

Next, we treated congenic C57BL/6J *Angptl3*^{hypl} mice with adenovirus expressing lacZ or human ANGPTL3. Plasma HDL cholesterol concentrations increased from day 4 (48±1.8 versus 32±1.3 mg/dL, P<0.001) and doubled on day 10 (69±3.0 versus 33±2.6 mg/dL, P<0.001) after treatment with adenovirus producing ANGPTL3, compared with the control (Figure 2A). Plasma HDL-PL levels were also increased from day 4 and doubled on day 7 (399±5.6 versus 216±23 mg/dL, P<0.001) by the ANGPTL3 adenovirus, compared with the control (Figure 2A). We also analyzed lipoprotein profiles of the pooled plasma collected from adenovirus-treated congenic C57BL/6J *Angptl3*^{hypl} mice on day 14 after adenoviral injection, with high-resolution HPLC. Cholesterol and PL concentrations increased mainly in the HDL fraction of the mice treated with ANGPTL3 adenovirus, compared with the control (Figure 2B). On the other hand, ANGPTL3 adenovirus increased only the VLDL fraction of TG (Figure 2B), a finding consistent with our previous reports.[8,10] These results suggest that ANGPTL3 does not only influence VLDL hydrolysis but also homeostasis of the HDL metabolism.

ANGPTL3 Inhibited EL Activity In Vitro

Next, we investigated whether EL might be a novel target of ANGPTL3, accounting for the association between ANGPTL3 and HDL levels in plasma. Both in vitro assays, using phophatidylcholine (Figure 3A) and human HDL particles (Figure 3B) as substrates, revealed that recombinant ANGPTL3 protein markedly inhibited the activity of EL in a dose-dependent manner. HDL particles did not inhibit phospholipase activities of EL by themselves (data not shown). To determine the domain of ANGPTL3 responsible for inactivation of EL, we produced truncated and/or mutated ANGPTL3 proteins, as shown in Figure 3C.[8] The N-terminal coiled-coiled region of ANGPTL3 (ANGPTL3-CC) protein suppressed EL activity in a manner similar to that by full-length ANGPTL3 protein (Figure 3C). This inhibitory effect was completely abolished when the region of the heparin-binding site was mutated (Figure 3C), suggesting that the putative heparin-binding site in the N-terminal region is important for ANGPTL3-induced suppression of EL activity.

Heparin-Releasable Phospholipase Activity Was Elevated in Angptl3-Deficient Mice

EL is responsible for the bulk of heparin-releasable phospholipase activity in mice.[7] To investigate whether Angptl3-deficiency leads to the elevation of EL activity in blood vessels, we measured the enzymatic activities of phospho-

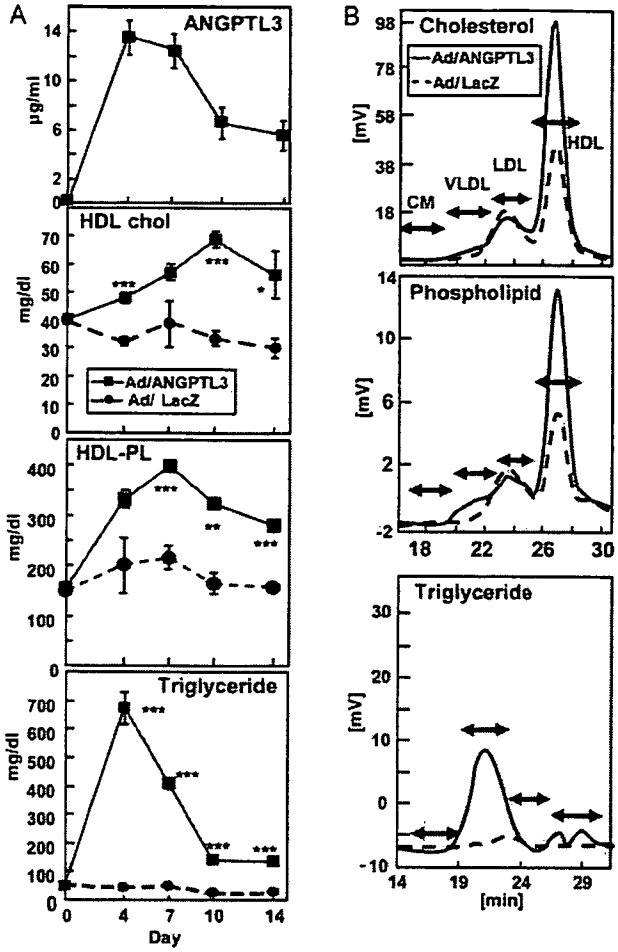

Figure 2. Alterations of plasma lipid profiles by supplementation of ANGPTL3 via adenovirus in Angptl3-deficient mice. A, Angptl3-deficient congenic C57BL/6J *Angptl3*^{hypl} mice were treated with recombinant adenoviruses carrying β-galactosidase (Ad/lacZ, circles) or human ANGPTL3 (Ad/ANGPTL3, squares). On the indicated days after the viral injection, HDL cholesterol, HDL-PL, and triglyceride concentrations in plasma were measured as described in Methods. Data are the mean±SE values of 4 mice per group. *P<0.05, **P<0.01 and ***P<0.001 vs Ad/lacZ group. B, Plasma samples were collected on day 14 from mice injected with Ad/LacZ (dotted line) or Ad/ANGPTL3 (bold line). Pooled plasma samples from each group were subjected to highly-sensitive HPLC. Cholesterol, phospholipid, and triglyceride profiles in lipoprotein fractions were determined as described in Methods. The indicated fractions are CM, chylomicron; VLDL, very low density lipoprotein; LDL, low density lipoprotein; HDL, high density lipoprotein.

lipase in the plasma of C57BL/6J and Angptl3-deficient mice before and after a heparin injection. Plasma phospholipase activities were slightly elevated by heparin-injection in C57BL/6 mice (100±2 versus 108±3%, Figure 4). On the other hand, in Angptl3-knockout mice, the elevation of plasma phospholipase activities by heparin-injection was marked compared with C57BL/6J mice (103±4 versus 163±19%, Figure 4). These results indicate that circulating Angptl3 should contribute to the inhibition of the phospholipase activity of EL via the heparin-binding site in vivo.

Plasma HDL Cholesterol, HDL-PL, and ANGPTL3 Levels Correlated in Humans

To date, the physiological role of Angptl3 has only been assessed in rodents. To investigate the physiological and pathological roles of ANGPTL3 in humans, we constructed an ELISA system to measure ANGPTL3 concentration in human plasma. To construct the sandwich ELISA system, mouse monoclonal antibody (45B1) and rabbit polyclonal antibody (No.1) were raised against human ANGPTL3. These antibodies specifically detected recombinant human ANGPTL3 protein (please see supplemental materials). In the sandwich ELISA system, we used the 45B1 monoclonal antibody as the first antibody and detected ANGPTL3 with HRP-conjugated No.1 polyclonal antibody. We confirmed that this sandwich ELISA system specifically detect ANGPTL3 protein in human plasma by western blotting (please see supplemental materials). Using this sandwich ELISA system, we were able to generate a linear calibration curve using serial dilutions of the recombinant human ANGPTL3 protein (please see supplemental materials).

We found that the presence of other plasma proteins in the sample hindered quantitative analysis, especially when the plasma samples were directly subjected to ELISA. This was avoided by dilution of the plasma samples by more than 1/16. Neither ethylenediaminetetraacetic acid (EDTA) nor heparin, which are anticoagulants used for collecting plasma samples, had any effect on the above measurement (data not shown). The quantifiable range of the ANGPTL3 concentration in human plasma was 50 to 800 ng/mL using our system. Furthermore, ANGPTL3 concentrations of plasma samples were stable throughout five freeze-thaw cycles (data not shown).

To investigate the significance of ANGPTL3 in lipid homeostasis in humans, we analyzed plasma lipids and ANGPTL3 concentration of Japanese healthy volunteers [n=87, mean age, 33.6±8.4 years (±SD, range, 21 to 57), male/female: 45/42] (Figure 5). This study revealed that plasma ANGPTL3 concentrations (470±122 ng/mL) correlated strongly with plasma HDL cholesterol (62±14 mg/dL; r=0.500, P<0.001) and HDL-PL levels (92±25 mg/dL; r=0.286, P=0.007), but not with plasma total cholesterol (182±33 mg/dL; r=0.169, P=0.117) or TG level (77±54 mg/dL; r=−0.125, P=0.249).

Discussion

A low level of plasma HDL has been recognized as an aspect of metabolic syndrome and is a crucial risk of cardiovascular events. Various factors have been demonstrated to influence plasma HDL-cholesterol level, including apoA1, ATP-binding cassette transporter (ABC) A1, lecithin:cholesterol acyltransferase (LCAT), PLTP, and cholesteryl ester transfer protein (CETP), etc.[20] However, to date, pathophysiological regulation of the HDL level in plasma is not completely defined. Recently, lines of study have revealed that EL is a crucial factor in determining the plasma HDL level. Overexpression of EL in mice resulted in reduced plasma HDL levels, and EL knockout mice showed significant increase of HDL levels.[5–7] In another study, injection of a neutralizing

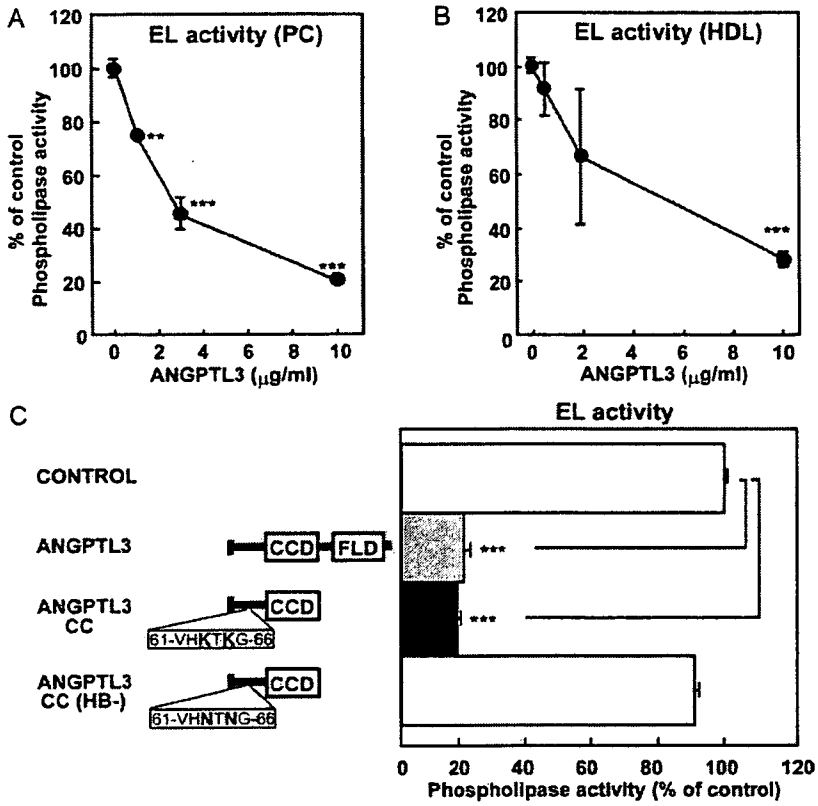

Figure 3. Inhibition of phospholipase activity of EL by ANGPTL3. A, Phospholipase activities of EL were determined using phosphatidylcholine emulsion (PC) as substrate as described in Methods, in the presence of recombinant human ANGPTL3 at the indicated doses (0, 1, 3, and 10 μg/mL). B, Similarly, they were determined using HDL particles as substrate, in the presence of the indicated dose of ANGPTL3 (0, 0.4, 2, and 10 μg/mL). Relative phospholipase activities of EL are expressed as a percentage of the value in the absence of ANGPTL3 treatment. C, The schemas indicate recombinant full-length ANGPTL3, N-terminal domain containing coiled-coil region (CCD) (ANGPTL3 CC), and the N-terminal domain containing CCD with mutation in the putative heparin-binding site [ANGPTL3 CC (HB-)]. Phospholipase activities of EL were determined in the absence of ANGPTL3 (control, open bar), in the presence of 10 μg/mL full-length ANGPTL3 (gray bar), 5 μg/mL ANGPTL3-CC (solid bar), or 5 μg/mL ANGPTL3-CC (HB-) (open bar). Relative phospholipase activities of EL are expressed as a percentage of the control. Data are the mean±SD of 3 experiments. $^{**}P<0.01$ and $^{***}P<0.001$ vs control.

antibody against EL increased plasma HDL in mice.[21] Human genetic analysis showed that a single nucleotide polymorphism (584C/T) in EL cDNA, causing one amino acid replacement (T111I), was significantly associated with plasma HDL concentrations, but not with plasma total cholesterol or TG.[6] However, the mechanism which regulates EL activity in vivo has not been clarified yet. In the present study, we showed that ANGPTL3, a hepatic secretory factor, significantly inhibited the activity of recombinant EL protein. We also found that the N-terminal domain, especially the putative heparin-binding region, is crucial for ANGPTL3-mediated suppression of EL activity. Furthermore, in Angptl3-deficiency, the phospholipase activity of post-heparin plasma was significantly elevated in vivo. Besides EL, LPL and HL also have phospholipase activity. However, McCoy et al previously demonstrated that the phospholipase activity of LPL and HL was extremely low compared with EL, whereas they had relatively high levels of triglyceride-lipase activity.[4] Moreover, the loss of EL in the homozygous knockout mice resulted in a significant decrease in the post-heparin augmentation of phospholipase activity.[7] These data clearly point to EL as a major contributor to heparin-releasable phospholipase activity in mice. Based on this previous evidence and our in vitro data, we assume that the elevation of heparin-releasable phospholipase activity in Angptl3-null mice should be explained by the lack of inhibitory effect of Angptl3 on EL. However, further analyses, eg, with double knockout mice of Angptl3 and EL, are still required to provide definitive evidence.

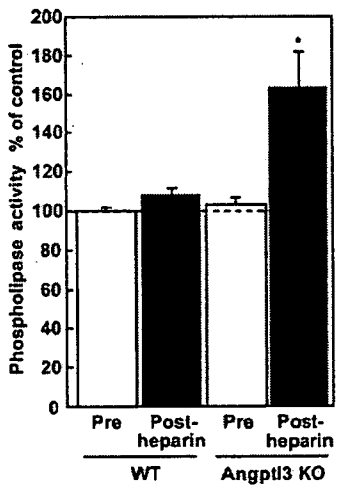

Figure 4. Phospholipase activities of pre- and post-heparin plasma in wild-type and Angptl3-knockout mice. Phospholipase activities of pre- (white bars) and post-heparin plasma (black bars) from wild-type (WT, n=5) and Angptl3-knockout mice (KO, n=5) were determined using 1,2 di[1-^{14}C] oleyl-L-3-phophatidylcholine and triolein as substrates. Relative phospholipase activities are expressed as a percentage of the values of the pre-heparin plasma in wild-type mice. Data are the mean±SEM of the values of 5 mice. $^{*}P<0.05$ vs the post-heparin plasma of wild-type mice.

Our previous and current studies demonstrated that ANGPTL3 suppressed the activities of two lipases, LPL and EL, in vitro, and Angptl3-deficiency led to a significant reduction of plasma TG and HDL levels, and supplementation of ANGPTL3 restored them in vivo. Furthermore, in the current study, we constructed an ELISA system for measuring ANGPTL3 concentrations in human plasma,

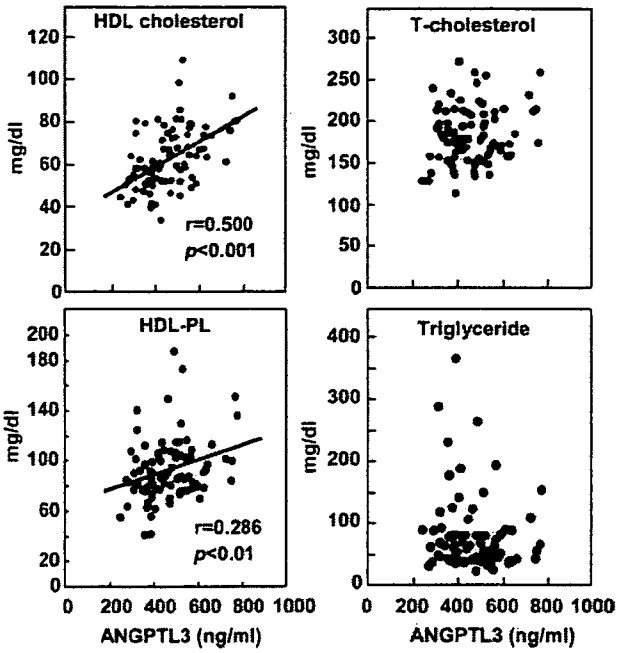

Figure 5. Plasma lipids and ANGPTL3 levels in humans. Plasma concentrations of ANGPTL3, HDL cholesterol, HDL-PL, total (T-) cholesterol and triglyceride were determined under overnight fasting conditions in healthy Japanese subjects (n=87). The values of correlation and probabilities are shown in the figures of ANGPTL3 and HDL cholesterol, and HDL-PL.

and revealed that the plasma ANGPTL3 level significantly correlated to the plasma HDL cholesterol, suggesting that ANGPTL3 should play an essential role as a regulatory factor of plasma HDL-cholesterol levels in humans, but not of plasma TG. Our previous studies showed that in mice, either the administration of ANGPTL3 protein or an injection of ANGPTL3-adenovirus promptly elevated the plasma TG level, but the elevated TG level started to decrease shortly afterward, in spite of the high level of ANGPTL3 in the plasma,[8,10] suggesting that the inhibition of LPL by ANGPTL3 does not appear to persist in vivo. In addition, plasma TG levels are easily affected by various nutritional and hormonal factors in humans. It is conceivable that these elements might be related to the finding that there was not a simple correlation between plasma ANGPTL3 and TG levels in human subjects.

Previously, we and other groups reported that insulin and leptin inhibited the production of Angptl3,[16,22] and liver X receptor (LXR) agonist upregulated the mRNA and protein expression of Angptl3 via the activation of its promoter by LXR/ retinoic X receptor (RXR).[14,23] In a recent study, downregulation of human ANGPTL3 gene by thyroid hormone was reported.[24] These previous data suggest that the expression of ANGPTL3 can be altered metabolically or nutritionally, and altered plasma levels of ANGPTL3 might be involved in the pathophysiological alterations of plasma HDL levels.

In conclusion, ANGPTL3 may be involved in the regulation of plasma HDL cholesterol levels through the inhibition of EL activity. Our findings provide new insight into understanding the regulation of EL activity and HDL metabolism via angptl3. Further epidemiological studies will provide more information for understanding the complicated HDL metabolism in humans.

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Disclosures

None.

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Physiological and pathological roles of a multi-ligand receptor CD36 in atherogenesis; insights from CD36-deficient patients

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Abstract

Oxidized low density lipoprotein (LDL) (Ox-LDL) plays an important role in the pathogenesis of atherosclerosis. Oxidized LDL is taken up by macrophages via scavenger receptors. CD36 is an 88 kDa glycoprotein expressed on platelets, monocyte-macrophages, microvascular endothelial cells, adipose tissue, skeletal muscles and heart. We found patients with CD36 deficiency and identified several mutations in the CD36 gene. We also reported that CD36-deficient macrophages showed a 50% reduction in the binding of Ox-LDL, suggesting that CD36 is one of the major receptors for Ox-LDL. CD36 was expressed on macrophages in the atherosclerotic lesions of human aorta and coronary arteries especially on foamed macrophages. The distribution of CD36 expression was slightly different from that of scavenger receptor class A types I and II. The expression of CD36 on macrophages was up-regulated by Ox-LDL and down-regulated by interferon γ. Since CD36 is a transporter of long-chain fatty acids (LCFA), CD36-deficient patients showed a defect in the uptake of an LCFA analog, BMIPP, by the heart. Furthermore, the secretion of IL-1β and TNF-α from monocyte-derived macrophages induced by Ox-LDL was markedly reduced and the activation of NF-κB was attenuated in CD36-deficient subjects compared with controls, suggesting that CD36-mediated signaling is also impaired in CD36 deficiency.

To elucidate the roles of CD36 *in vivo*, we characterized the clinical profile of CD36-deficient patients. Most of them were accompanied by hyperlipidemia (mainly hypertriglyceridemia), increased remnant lipoproteins and mild elevation of fasting plasma glucose level and blood pressure. Glucose clamp technique revealed mean whole body glucose uptake was reduced in CD36-deficient patients, indicating the presence of insulin resistance. The frequency of CD36 deficiency was higher in patients with coronary heart disease (CHD) than in control subjects. Taken together, CD36 deficiency is accompanied by (1) hyperlipidemia and increased remnant lipoproteins, (2) impaired glucose metabolism based upon insulin resistance, and (3) mild hypertension, and comprises one of the genetic backgrounds of the metabolic syndrome, leading to the development of CHD.

Key words: scavenger receptor, CD36 deficiency, atherosclerosis, fatty acid transporter

Introduction

Ox-LDL has been shown to play a crucial role in the pathogenesis of atherosclerosis. Modified lipoproteins such as Ox-LDL are taken up by scavenger receptors expressed on macrophages. A number of molecules including scavenger receptor class A have been identified so far. CD36 is an 88 kDa glycoprotein belonging to class B scavenger

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receptors [1]. Cells overexpressing CD36 bound and took up Ox-LDL [2], whereas CD36-deficient macrophages were resistant to foam cell formation *in vitro* [3]. Thus, CD36 could be one of the major receptors for Ox-LDL in humans. Furthermore, the expression of CD36 gene was upregulated by its own ligand, Ox-LDL, while it was downregulated by interferon γ [4, 5]. CD36 was abundantly expressed on the foam cells of human atherosclerotic plaques [6]. The distribution and localization of CD36 in the lesions were different from those of scavenger receptor class A [7]. CD36 is also expressed in multiple organs such as heart, skeletal muscles, and adipose tissues other than macrophages and platelets. Many *in vitro* experimental studies showed that CD36 could bind multiple ligands such as LCFA, thrombospondin-1, collagen, malaria-infected erythrocytes, and native lipoproteins [8]. These results indicated that CD36 may play some roles other than scavenger receptor for Ox-LDL. We found patients with genetic deficiency of CD36 in 1990 from those with refractoriness to platelet transfusion [9]. We analyzed the molecular bases and identified mutations in the CD36 gene [10, 11]. The aim of the present study was to investigate the phenotypic expression and pathophysiology of human CD36 deficiency.

Results

Characterization of macrophages from CD36-deficient patients

As mentioned earlier, CD36-deficient macrophages obtained from the patients showed an approximately 50% reduced binding and uptake of Ox-LDL, leading to the resistance to the foam cell formation *in vitro* [3]. CD36 could bind with Ox-LDL in transfected HEK293 cells [2]. As far as we know, CD36 deficiency is the only genetic deficiency state among scavenger receptors. Furthermore, CD36 was shown to be a receptor to collagen. We demonstrated that the CD36-deficient macrophages presented with a reduced binding to the collagen-coated culture dishes [12].

Ox-LDL and bacterial lipopolysaccharides (LPS) stimulate macrophages to secrete several proinflammatory cytokines, such as tumor necrosis factor α (TNF-α) and interleukin 1β (IL-1β). These reactions are regulated by a nuclear receptor, nuclear factor kappa B (NF-κB). We reported that the upregulation of inflammatory cytokine expression by Ox-LDL and activation of NF-κB were markedly reduced in CD36-deficient macrophages, whereas LPS-induced upregulation of NF-κB and cytokine expression were conserved [13].

Clinical features of patients with CD36 deficiency

Abnormal metabolic dynamics of LCFA and its possible relationship to cardiomyopathy

The *in vitro* experiments have suggested that CD36 plays a role in taking up LCFA [14]. With the use of a radioactive analogue for LCFA, [^{123}I-BMIPP: iodine-123 15-(p-iodophenyl)-(R, S), methylpentadecanoic acid], the heart of CD36-deficient patients was found to be defective in taking up BMIPP [15–17]. Cardiac muscle utilizes LCFA as a major energy source for maximum contractility, therefore CD36 deficiency may be related to the pathogenesis of cardiomyopathy [16].

Phenotypic expression of the metabolic syndrome and insulin resistance in CD36-deficient patients

We next investigated the phenotypic expression of 30 patients with type I CD36 deficiency (mean age: 62 years old). They had significantly higher serum triglycerides (TG) and lower HDL-cholesterol levels at fasting. Furthermore, oral fat loading test indicated CD36-deficient patients had a higher and delayed response of serum TG and apolipoprotein B48, a marker for intestine-derived lipids. They had a higher plasma glucose levels and blood pressure. The number of coronary risk factors in CD36 deficiency tended to be more than two. Thus, patients with CD36 deficiency showed some features of the metabolic syndrome. We tested whether these patients had insulin resistance. Although fasting plasma levels of glucose and insulin are used as parameters for insulin resistance in daily clinical settings, we used hyperinsulinemic euglycemic clamp, which is thought to be a more accurate way of evaluating insulin resistance. In all patients tested, whole body glucose uptake was reduced, indicating the presence of insulin resistance [18]. Furthermore, whole body glucose uptake was also reduced in younger subjects. Younger subjects with CD36 deficiency have been found to have a defective response of free fatty acids after glucose loading tests and some abnormalities in plasma lipoprotein levels [19, 20]. CD36-deficient patients showed postprandial hyperlipidmia with an increase in small intestine-derived lipids [21].

Relationship of CD36 deficiency to coronary heart disease (CHD)

A substantial number of CD36-deficient patients suffered from apparent CHD and cardiomyopathy. The frequency of CD36 deficiency was approximately 3-fold higher in patients with CHD than that in the control population (unpublished observation).

Discussion

In the current study, we have characterized the phenotypic expression of CD36-deficient patients. We found that CD36 deficiency was accompanied by (1) hyperlipidemia and increased remnant lipoproteins, (2) impaired glucose metabolism based upon insulin resistance, and (3) mild hypertension. CD36 deficiency thus comprises one of the genetic backgrounds of the metabolic syndrome, leading to the development of CHD. What is the mechanism for these phenotypic expressions of the metabolic syndrome in CD36 deficiency? There are a couple of data that may possibly explain the mechanism. One is the altered dynamics of LCFA *in vivo*, which was demonstrated by the cardiac scintigram using an analogue of LCFA, ^{123}I-BMIPP. We and others have reported that the uptake of BMIPP in the heart was totally absent in the CD36-deficient patients, indicating that CD36 may be a major receptor for LCFA in the human heart [15–17]. On the other hand, the uptake of LCFA was paradoxically increased in the patients' liver [22], where fatty acid transporters other than CD36 may be dominant. The increased flux of LCFA into the liver may lead to the enhanced production and secretion of very low density lipoproteins (VLDL) and hyperlipidemia as well as insulin resistance. The other is a possible impairment of the peroxisome proliferator activated receptor-γ (PPAR-γ) pathway. As mentioned earlier, we and others have reported that CD36 expression was upregulated by its ligand, Ox-LDL, as well as a synthetic ligand for PPAR-γ [23]. In studies with human macrophages, CD36 appears to mediate intracellular signaling through PPAR-γ. The synthetic ligands for PPAR-γ such as thiazolidinediones are known to increase insulin sensitivity. CD36 deficiency could interfere with this pathway, causing the insulin resistance *in vivo*.

In many cases with multiple risk factors, the combination of genetic and acquired factors as well as the impact of each is important. In CD36 deficiency, the phenotype with the metabolic syndrome does not always appear to be manifest, but they already had subclinical abnormalities at their younger age [20]. In rodent models for CD36 deficiency, diets affected the phenotypic expression [24, 25]. CD36 deficiency has been found in some spontaneous hypertensive (SHR) rats expressing insulin resistance [24, 26]. Pravenec *et al.* [27] showed that glucose intolerance was observed under a high fructose diet in SHR rats and the transgene of wild-type CD36 corrected this phenotype. The SHR rats with CD36 mutation appeared to have heavier hearts than did controls, suggesting that abnormalities in CD36 are related to myocardial hypertrophy [28]. In CD36 null mice, hyperlipidemia [29] and insulin resistance [25] have been reported, which is similar to the above data from SHR rats with CD36 mutation. On the other hand, the original

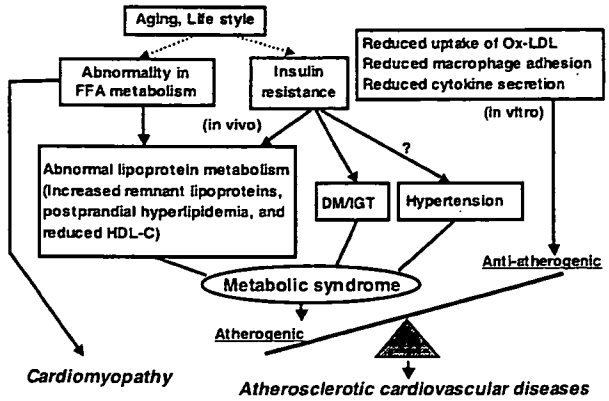

Fig. 1. Pathophysiology of human CD36 deficiency. Macrophages from patients with CD36 deficiency are resistant to Ox-LDL-induced foam cell formation at least *in vitro*, which might be antiatherogenic. However, CD36 deficiency may be related to insulin resistance, the metabolic syndrome, atherosclerosis and cardiomyopathy. Altered dynamics of LCFA may be involved in the phenotypic expression of these disorders. Some acquired factors, including lifestyle, may modify the phenotypic expression. FFA: free fatty acids.

SHR rats did not have mutations in the CD36 gene [30]. Thus, CD36 deficiency may not be the only cause of insulin resistance in all strains of SHR. CD36 null mice were resistant to atherosclerosis in the apolipoprotein E-negative background [31]. Further investigation of the pathophysiological aspects of CD36 deficiency would provide important insights into the molecular mechanisms for the development of the metabolic syndrome and atherosclerotic cardiovascular diseases.

Regarding the atherogenicity, it appears that CD36 deficiency has both atherogenic and antiatherogenic aspects (Fig. 1): the former is the expression of the metabolic syndrome, which is most commonly found in patients with CHD; and the latter is the fact that in *in vitro* experiments, macrophages obtained from CD36-deficient patients were resistant to Ox-LDL-induced foam cell formation. In general, CD36 deficiency appears to be associated with atherogenic aspects. However, further clarification is needed with regard to how these contrasting aspects affect the atherogenicity and the phenotypes of CD36 deficiency.

Conclusion

Human CD36 deficiency is accompanied by multiple risk factors such as hyperlipidemia, impaired glucose metabolism based upon insulin resistance, and a mild hypertension. Thus, it comprises one of the genetic backgrounds of the metabolic syndrome, leading to the development of CHD.

Acknowledgments

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Adiponectin deficiency suppresses ABCA1 expression and ApoA-I synthesis in the liver

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Abstract **Plasma high density lipoprotein (HDL)-cholesterol levels are inversely correlated with the incidence of cardiovascular diseases. HDL is mainly assembled in the liver through the ATP-binding cassette transporter (ABCA1) pathway. In humans, plasma HDL-cholesterol levels are positively correlated with plasma adiponectin (APN) concentrations. Recently, we reported that APN enhanced apolipoprotein A-I (apoA-I) secretion and ABCA1 expression in HepG2 cells. In the present study, we investigated HDL assembly in APN-knockout (KO) mice. The apoA-I protein levels in plasma and liver were reduced in APN-KO mice compared with wild-type-mice. The ABCA1 expression in liver was also decreased in APN-KO mice. APN deficiency might cause the impaired HDL assembly by decreasing ABCA1 expression and apoA-I synthesis in the liver.**

Keywords: ABCA1; Adiponectin; ApoA-I; ApoB-100; HDL; Reverse cholesterol transport

1. Introduction

Plasma high density lipoprotein (HDL)-cholesterol levels are negatively correlated with the incidence of coronary artery disease (CAD). It is thought that HDL prevents the development of atherosclerosis by removing excess cholesterol from atheroma and transporting it back to the liver in the protective system, so-called "reverse cholesterol transport" (RCT) [1].

The ATP-binding cassette transporters (ABCA1 and ABCG1), which are expressed in the liver, small intestine and peripheral tissues, are thought to be rate-limiting factors for HDL assembly in RCT system [2,3]. ABCA1, the responsible gene for familial HDL deficiency including Tangier disease [4–6], promotes apoA-I-mediated cholesterol efflux, which is the initial step in RCT system, decreasing cholesterol accumulation in macrophages and initiating HDL formation in the liver [2]. ABCG1 also stimulates cholesterol efflux to mature HDL in macrophages [3].

Adiponectin (APN), a bioactive peptide secreted from adipocytes is one of the important molecules to inhibit the development of atherosclerosis. Several clinical studies have demonstrated that plasma levels of APN are extremely low in patients with the metabolic syndrome which clusters risk factors for CAD such as visceral obesity, dyslipidemia, impaired glucose tolerance and hypertension. Plasma APN concentrations are positively correlated with plasma HDL-cholesterol levels [7–11]. Although these findings suggest that APN might have an ability to prevent the development of atherosclerosis by the acceleration of RCT system, the underlying mechanisms for it has not been clarified yet. Recently, we reported that human recombinant APN enhanced the expression of ABCA1 and accelerated the synthesis of apoA-I in a human liver cell line, HepG2 cells, suggesting that APN might increase HDL assembly in the liver [12]. Therefore, in the present study, we investigated the HDL assembly in APN knockout (APN-KO) mice.

2. Materials and methods

2.1. Animals

Adiponectin-knockout (APN-KO) mice were generated as described previously and backcrossed to wild-type (WT) C57BL/6J mice [13]. Both APN-KO and WT mice (male) were housed in temperature and humidity controlled facility with a 12-h light/dark cycle and fed a normal chow diet (MF, OrientalBio Laboratories, Chiba, Japan) and sacrificed for analysis at the age of 8–10 weeks old. The experimental protocol was approved by the Ethics Review Committee for Animal Experimentation of Osaka University School of Medicine.

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Abbreviations: ABCA1, ATP-binding cassette transporter A1; ABCG1, ATP-binding cassette transporter G1; APN, adiponectin; apoA-I, apolipoprotein A-I; apoB-100, apolipoprotein B-100; CAD, coronary artery disease; CM, chylomicron; FC, free cholesterol; HDL, high density lipoprotein; HPLC, high performance liquid chromatography; KO, knockout; LDL, low density lipoprotein; MTP, microsomal triglyceride transfer protein; PL, phospholipids; RCT, reverse cholesterol transport; TC, total cholesterol; TG, triglyceride; VLDL, very low density lipoprotein; WT, wild-type

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2.2. Lipid profile by high performance liquid chromatography (HPLC) analysis

One hundred microliter of blood from anesthetized mice (at the age of 8–10 weeks) were drawn from retro-orbital plexus *ad libitum* and plasma was immediately isolated from the collected blood by centrifugation at 4 °C. The lipid profile of plasma was analyzed by an online dual enzymatic method using high performance liquid chromatography (HPLC) at Skylight Biotech Inc. (Akita, Japan), according to the procedure as described by Usui et al. [14]. The plasma concentrations of total cholesterol (TC), triglyceride (TG), free cholesterol (FC) and phospholipids (PL) of four fractioned groups [chylomicron (CM): lipoprotein particle size >80 nm, very low density lipoprotein (VLDL): 30 < particle size <80 nm, low density lipoprotein (LDL): 16 < particle size <30 nm and HDL: 8 < particle size < 16 nm] were determined by using enzymatic reagents (Kyowa Medex, Tokyo, Japan).

2.3. Western blot analysis

Mice plasma or proteins isolated from the liver were separated by SDS–polyacrylamide gel electrophoresis and transferred to PVDF membranes (Bio-Rad, Germany). Incubations of antibodies with the membranes were performed in TBS including 0.1% Tween 20 and 2% skim milk at 4 °C overnight. Detection of the immune complexes was carried out by ECL Advance Western Blot Detection System (Amersham Biosciences, UK). Anti-mouse apoA-I antibody (Biodesign, USA), anti-mouse apolipoprotein B48/100 (apoB) antibody (Biodesign, USA), anti-mouse ABCA1 antibody (Novus, USA) and anti-mouse ABCG1 antibody (Santa Cruz, USA) were used for the assay.

2.4. cDNA synthesis and quantitative PCR

One microgram of total RNA isolated from tissues was primed with 50 pmol of oligo (dT) 20 and reverse-transcribed with SuperScript III (Invitrogen, USA) for first strand cDNA synthesis, according to the protocol of the manufacturer. Real-time quantitative PCR was performed according to the protocol of DyNamo HS SYBR Green quantitative PCR kit. Relative gene expression was quantified using GAPDH as an internal control.

2.5. Primers used in this study

The primes for mouse ABCA1 were ABCA1-forward: 5′-TGGGAACTCCTGCTAAAAT-3′ and ABCA1-reverse: 5′-CCATGTGGTGTGTAGACA-3′, for mouse apoA-I, apoA-I-forward: 5′-GTGGCTCTGGTCTTCCTGAC-3′ and apoA-I-reverse: 5′-ACGGTTGAACCCAGAGTGTC-3′, for mouse apoB, apoB-forward: 5′-TGGGATTCCTCTGCCATCTCGAG-3′ and apoB-reverse: 5′-GTAGAGATCCATCACAGGACAATG-3′, for mouse GAPDH, GAPDH-forward: 5′-ACTCCACTCACGGCAAATTC-3′ and GAPDH-reverse: 5′-TCTCCATGGTGGTGAAGACA-3′.

2.6. Statistical analysis

Values were expressed as means ± S.D. Statistical significance was assessed by Student's *t*-test for paired values and set at $P < 0.05$.

3. Results

3.1. Plasma VLDL-TG levels were increased in APN-KO mice, while there was no significant difference in plasma levels of HDL-cholesterol between WT and APN-KO mice

Blood samples from anesthetized APN-KO ($n = 6$) and WT ($n = 6$) mice at the age of 8–10 weeks were drawn from retro-orbital plexus ad libitum and plasma was immediately isolated by centrifugation at 4 °C. The lipid profile of plasma was analyzed by automatic HPLC and enzymatic methods. The plasma levels of TG, in particular VLDL-TG, were significantly increased in APN-KO mice compared with WT mice (Fig. 1). However, there was no significant difference in plasma TC, HDL-cholesterol and PL levels between APN-KO and WT mice.

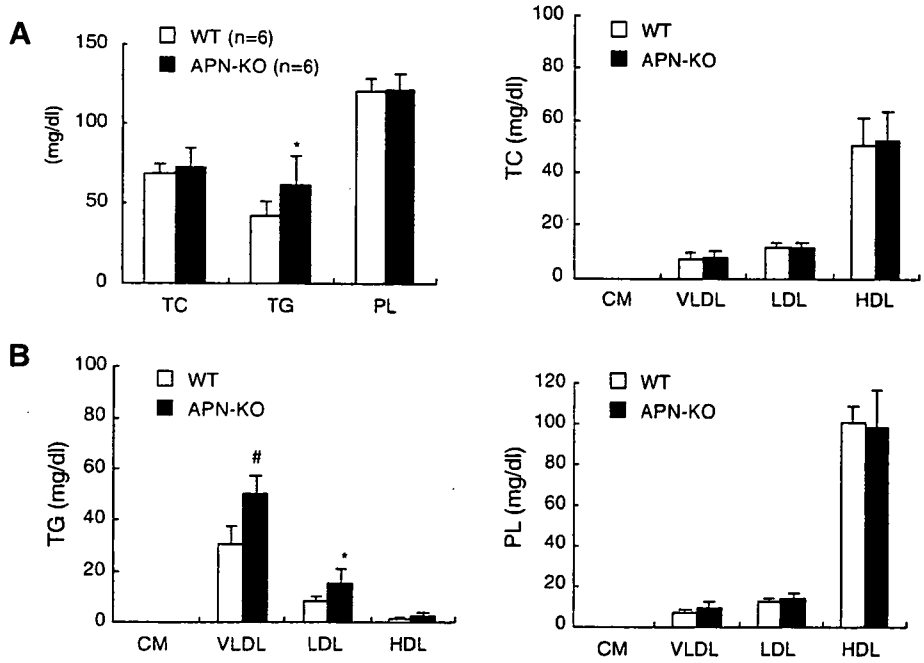

Fig. 1. Plasma lipid profile of APN-KO mice. Blood samples from anesthetized APN-KO ($n = 6$) and WT mice ($n = 6$) at the age of 8–10 weeks were drawn from retro-orbital plexus *ad libitum*. (A) Plasma TC, TG and PL levels. Plasma TG levels of APN-KO mice were significantly higher than those of WT mice. (B) Lipid composition of lipoproteins (CM, VLDL, LDL, and HDL). VLDL-TG concentrations were significantly increased in APN-KO mice compared with WT mice. Values are expressed as means ± S.D. $^{*}P < 0.05$, $^{\#}P < 0.01$, vs. WT mice.

3.2. ApoA-I levels in plasma and the liver were decreased in APN-KO mice

Recently, we reported that APN increased the secretion of apoA-I and decreased the release of apolipoprotein B-100 (apoB-100) from HepG2 cells. Therefore, first, plasma levels of apolipoproteins (apoA-I and apoB-100) in APN-KO mice were investigated by Western blot. As shown in Fig. 2A, plasma levels of apoA-I were slightly decreased in APN-KO mice compared with WT mice, while plasma concentrations of apoB-100 were increased in APN-KO mice. Furthermore, both the mRNA and protein levels of apoA-I in the liver were definitely reduced in APN-KO mice compared to WT mice (Fig. 2B and C). However, there was no significant difference in the mRNA levels of apoB-100 in the liver between APN-KO and WT mice.

3.3. ABCA1 expressions were reduced in APN-KO mice

Finally, we investigated in APN-KO mice the expression levels of ABC transporters (ABCA1 and ABCG1) to generate HDL in the liver. Both the protein (Fig. 3A) and mRNA (Fig. 3B) levels of ABCA1 were significantly decreased in the liver of APN-KO mice compared with WT mice. However, there was no significant difference in the levels of ABCG1 protein between APN-KO and WT mice (Fig. 3A).

4. Discussion

In the present study, we demonstrated for the first time that the expression levels of apoA-I in plasma and the liver were decreased in APN-KO mice as expected from our previous report [12]. Furthermore, we found that the ABCA1 expressions were also reduced in APN-KO mice. These data suggest that low plasma APN concentrations might suppress HDL assembly in the liver, suggesting that the subjects with low serum APN show low plasma HDL-cholesterol levels. However, there was no significant difference in plasma HDL-cholesterol levels between APN-KO and WT mice fed a normal chow diet despite the decrease of apoA-I levels in plasma and the liver of APN-KO mice. There might be some difference in the lipid composition of HDL particles, for example, in the apoA-I mass of HDL particles between APN-KO and WT mice. Abnormal HDL particles with low apoA-I concentrations might have a decreased ability in promoting cholesterol efflux to prevent against atherosclerosis.

Recently, Otabe et al. reported that overexpression of human APN in transgenic mice results in suppression of visceral fat accumulation and reduction of plasma fasting glucose, insulin and leptin levels compared with WT mice [15]. However, these differences were observed only when mice were

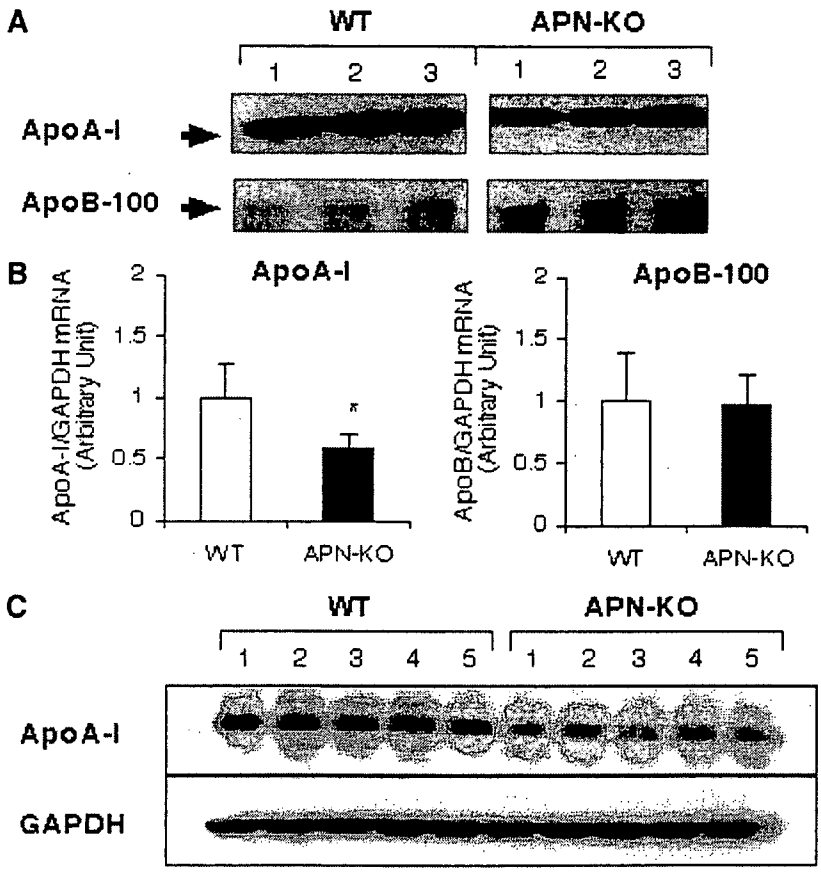

Fig. 2. ApoA-I levels in plasma and the liver were decreased in APN-KO mice. (A) Analysis of plasma apolipoprotein (apoA-I and apoB-100) levels by western blot. Plasma apoA-I levels were slightly decreased in APN-KO mice ($n = 3$) compared with WT mice ($n = 3$), while plasma apoB100 levels were increased in APN-KO mice. (B) The mRNA and (C) the protein expression levels of apoA-I or apoB-100 in the liver. Both the mRNA and protein of apoA-I levels were significantly reduced in the liver of APN-KO mice ($n = 5$) compared with WT mice ($n = 5$). Relative gene expression determined by quantitative PCR was quantified using GAPDH as an internal control. Values are expressed as means ± S.D. $^*P < 0.05$, vs. WT mice.

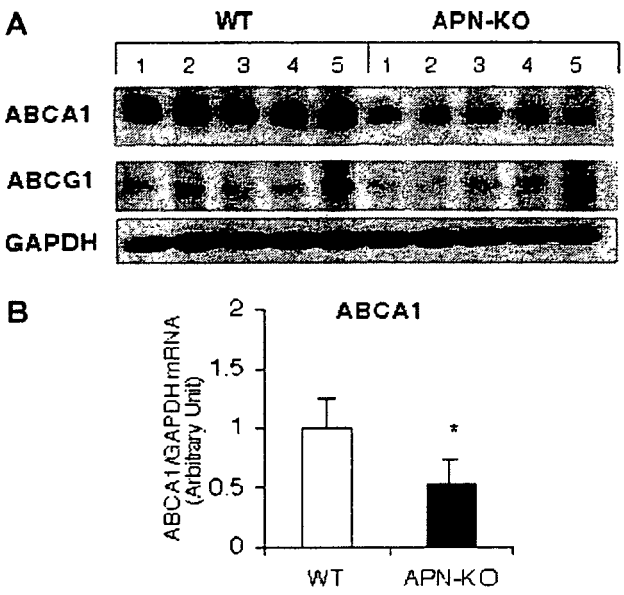

Fig. 3. ABCA1 expressions were reduced in the liver of APN-KO mice. Both the protein (in A) and mRNA (in B) levels of ABCA1 were significantly reduced in the liver of APN-KO mice ($n = 5$) compared with WT mice ($n = 5$). Relative gene expression determined by quantitative PCR was quantified using GAPDH as an internal control. Values are expressed as means ± S.D. $^*P < 0.05$, vs. WT mice.

fed a high fat/high sucrose diet, but not a normal chow diet. In our study, the lipid profiles of APN-KO mice were examined with feeding only a normal chow diet. The effect of APN on some parameters including HDL-cholesterol levels might be dependent on the nutritional condition. Therefore, possibly, low plasma HDL-cholesterol might be observed in APN-KO mice fed with over nutrition like a high cholesterol/high fat diet. These issues will be studied in the near future.

We found that apoB-100-containing lipoproteins (VLDL and LDL)-TG, in particular, VLDL-TG levels were increased in APN-KO mice. Recently, it is clinically focused that the accumulation of TG-rich lipoprotein like VLDL in plasma is also strongly linked to CAD as well as that of an atherogenic lipoprotein, LDL [16]. Therefore, plasma VLDL accumulation might be in part associated with the development of atherosclerosis in APN deficiency.

Although plasma VLDL-TG levels were significantly increased in APN-KO mice, there was no significant difference in the apoB-100 expression in the liver between APN-KO and WT mice. As shown in our previous and Neumeier's reports [12,17], in vitro, the apoB secretion from HepG2 cells or primary human hepatocytes was enhanced by recombinant APN, while the mRNA expression levels of apoB were not influenced by APN. Therefore, APN might be involved in the assembly or secretion of VLDL in the liver, but not in apoB-100 synthesis. Microsomal triglyceride transfer protein (MTP) is well known to be an intracellular lipid transfer protein, closely associated with VLDL output from the liver [18,19]. We need to examine the effect of APN on MTP expression (or activity) in the liver.

In summary, we clarified that apoA-I synthesis and ABCA1 expression in the liver were suppressed in APN-KO mice. APN might play an important role in preventing the development of atherosclerosis by the acceleration of HDL assembly in RCT system.

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Role of LCAT in HDL remodeling: investigation of LCAT deficiency states

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Abstract **To better understand the role of LCAT in HDL metabolism, we compared HDL subpopulations in subjects with homozygous (n = 11) and heterozygous (n = 11) LCAT deficiency with controls (n = 22). Distribution and concentrations of apolipoprotein A-I (apoA-I)-, apoA-II-, apoA-IV-, apoC-I-, apoC-III-, and apoE-containing HDL subpopulations were assessed. Compared with controls, homozygotes and heterozygotes had lower LCAT masses (−77% and −13%), and LCAT activities (−99% and −39%), respectively. In homozygotes, the majority of apoA-I was found in small, disc-shaped, poorly lipidated preβ-1 and α-4 HDL particles, and some apoA-I was found in larger, lipid-poor, discoidal HDL particles with α-mobility. No apoC-I-containing HDL was noted, and all apoA-II and apoC-III was detected in lipid-poor, preβ-mobility particles. ApoE-containing particles were more disperse than normal. ApoA-IV-containing particles were normal. Heterozygotes had profiles similar to controls, except that apoC-III was found only in small HDL with preβ-mobility. Our data are consistent with the concepts that LCAT activity: *1*) is essential for developing large, spherical, apoA-I-containing HDL and for the formation of normal-sized apoC-I and apoC-III HDL; and *2*) has little affect on the conversion of preβ-1 into α-4 HDL, only slight effects on apoE HDL, and no effect on apoA-IV HDL particles.**—Asztalos, B. F., E. J. Schaefer, K. V. Horvath, S. Yamashita, M. Miller, G. Franceschini, and L. Calabresi. **Role of LCAT in HDL remodeling: investigation of LCAT deficiency states.** *J. Lipid Res.* **2007. 48: 592–599.**

Supplementary key words HDL subpopulations • apolipoproteins • reverse cholesterol transport

LCAT is a 416 amino acid protein that binds to lipoproteins or is present in lipid-free form in plasma and is secreted by the liver in humans (1). LCAT synthesizes the majority of cholesteryl esters in plasma by transferring a fatty acid from lecithin (phosphatidyl choline) to the 3-hydroxyl group of cholesterol. It is generally believed that LCAT maintains the unesterified cholesterol gradient between peripheral cells and HDL. Efflux of free cholesterol (FC) from cells occurs by a passive diffusion of FC between cellular membranes and acceptors and by mechanisms facilitated by scavenger receptor type B-I (SR-BI) and ABCs. In the presence of LCAT, the bi-directional movement of cholesterol between cells and HDL results in net cholesterol efflux (2, 3). Therefore, LCAT plays a central role in the initial steps of reverse cholesterol transport. LCAT is activated primarily by apolipoprotein A-I (apoA-I), but can also be activated by apoA-IV, apoC-I, and apoE (4, 5). Both the binding and activation of LCAT on the surface of HDL are essential for esterification of FC and accumulation of cholesteryl esters in the core of HDL.

Familial LCAT deficiency (FLD) is characterized by the absence of LCAT activity and reduced HDL cholesterol (HDL-C) level in plasma. In affected individuals, LCAT is either absent or present but inactive in plasma (6). LCAT has two distinct substrates: HDL and LDL. LCAT activity on HDL is called α-activity, and LCAT activity on LDL is called β-activity (7, 8). Lack of α-LCAT activity causes fish eye disease (FED). Homozygous subjects with FLD have corneal opacification, anemia, proteinuria, hematuria, and ultimately, renal failure, often requiring kidney transplantation (9). FED subjects have no clinical manifestation other than an age-dependent corneal opacification. Although it is not clear whether LCAT deficiency is directly linked to premature coronary artery disease (CAD), increased risk for CAD has been reported in some patients (9). Data obtained from cholesterol-fed human-LCAT transgenic rabbits indicated that HDL-C increased

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Abbreviations: apoA-I, apolipoprotein A-I; CAD, coronary artery disease; CETP, cholesteryl ester transfer protein; EL, endothelial lipase; FC, free cholesterol; FED, fish eye disease; FLD, familial LCAT deficiency; HDL-C, HDL cholesterol; sPLA2, secretory phospholipase A2; SR-BI, scavenger receptor type B-I; TG, triglyceride.

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