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Original Article

The Lipoprotein Fraction between VLDL and LDL Detected by Biphasic Agarose Gel Electrophoresis Reflects Serum Remnant Lipoprotein and Lp(a) Concentrations

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We analyzed lipoprotein profiles in 616 Japanese by biphasic agarose gel electrophoresis using Chol/Trig Combo™ to yield HDL, VLDL, LDL and CM fractions which were stained with cholesterol and triglyceride reagents, respectively. To further evalute the pattern of electrophoresis, we analyzed the fraction between VLDL and LDL to confirm the possibility of a MidBand by using an automatic-five-fraction function. The cholesterol concentrations in MidBand (MidBand-C) showed a good correlation to remnant-like particle-cholesterol (RLP-C) (r = 0.95) in 23 consecutive samples (TC < 220 mg/dl, Lp(a) < 30 mg/dl). However, MidBand-C concentrations of subjects with high Lp(a) levels (Lp(a) > 30 mg/dl) were also high compared to RLP-C concentrations. The average MidBand-C levels in elderly normolipidemic control subjects (TC < 220, TG < 150) were 5.2 ± 2.4 mg/dl in 30 males (mean age, 70 ± 10 years) and 5.4 ± 2.0 mg/dl in 40 females (64 ± 11 years). The average MidBand-C levels of normolipidemic patients with coronary artery diseases (CAD; TC < 220, TG < 150) were 9.4 ± 4.1 mg/dl in 126 males (mean age, 66 ± 10 years) and 9.1 ± 4.0 mg/dl in 44 females (67 ± 10 years). These levels were significantly higher than control values (p < 0.0001). Areas under ROC curves were greater for MidBand-C than for TC, LDL-C and TG when used to discriminate between the patients with CAD and normolipidemic control subjects for each sex. There results suggest that the MidBand-C level may be useful as an indicator of risk for CAD. *J Atheroscler Thromb, 2006; 13: 55–61.*

Key words: Agarose gel electrophoresis, Automatic five-fraction, Remnant like particles-cholesterol, Coronary artery disease.

Introduction

The major remnant lipoproteins are a mixture of the exogenous chylomicron remnant derived from the intestine and the endogenous very low density lipoprotein (VLDL) remnant (IDL) derived from the liver. Methods have been developed to measure remnant lipoproteins. Remnant lipoproteins have been identified as atherogenic (1).

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We reported that serum levels of remnant-like particle cholesterol (RLP-C) were increased in patients with diabetes and impaired glucose tolerance (2). We also reported that remnant lipoproteins had unique biological effects such as the induction of apotosis of cultured endothelial cells (3). VLDL remnants can be separated by the ultracentrifugal method at a density of 1.006–1.019 g/ml (4). However, this method is not suitable for routine analysis, because it takes hours, and the processing of multiple specimens is difficult (5).

Agarose gel electrophoresis is an authentic method of detecting remnant lipoprotein which appears as a broad band. The mid-band in polyacrylamide gel electrophoresis is also well known as the band of remnant lipoprotein (4). However, these methods are qualitative not quantitative. Recently a quantitative assay for RLP-C has been developed (6), and an elevation of plasma RLP-C levels was reported in patients with coronary artery diseases (CAD) (7). But there are considerable numbers of patients with type I and V hyperlipidemia whose levels of RLP-C appear abnormal in immunoadsorption assays with antibody. Therefore, it is important to develop simple and comprehensive methods to measure remnant lipoprotein levels.

We analyzed the lipoprotein fractions by using the agarose gel electrophoresis with Chol/Trig Combo™, which can quantify each lipoprotein fraction on the basis of the separate staining of cholesterol and TG and by automatic analysis. Based on this method, it is possible not only to measure the levels of HDL- cholesterol (HDL-C) and LDL-cholesterol (LDL-C) quantitatively, but also to recognize the entire pattern of lipoprotein fractions (8–11). We identified the fraction between the VLDL and LDL fractions using the Automatic five-fraction function of Chol/Trig Combo™ and elucidated that this fraction was closely related to the levels of RLP-C and increased in CAD patients compared with normolipidemic subjects. We named this fraction MidBand and clarified it's clinical significance.

Methods

Study subjects

We measured RLP-C and Lp(a) concentrations in 64 patients [31 males (mean age 60 ± 11 years, age range 31–81 years) and 33 females (63 ± 16, 26–80 years)], who requested a RLP-C test between 2000.03 and 2003.03.

The elderly control subjects were the 80 normolipidemic (TC 114–219 mg/dl, TG < 150 mg/dl) staff members of the Kobe University hospital enrolled in an annual medical checkup and patients 60 years or older who were free from CAD, liver disease, kidney disease and diabetes mellitus who were selected from among patients at Kobe University Hospital. These were 35 males (mean age 66 ± 13years, age range 41–90 years) and 45 females (64 ± 12, 50–88 years)

To evaluate the clinical usefullness of MidBand-C in CAD, 168 patients with CAD (TC 80–219 mg/dl, TG < 150 mg/dl) were admitted to Kobe University hospital and their diagnosis was confirmed by coronary angiography. This group consisted of 125 males (mean age 67 ± 10 years, age range 35–88 years) and 43 females (68 ± 9, 47–83 years).

Hospital staff were ordered with informed consent to come to blood sampling in the fasting state. Sera from the patients with CAD were drawn in a 12-hour fasted state with venopuncture. These samples were made anonymous without linking.

Biochemical analysis

Serum total cholesterol (TC) and TG concentrations were measured using an auto analyzer TBA-80M (TOSHIBA, Tokyo, Japan) by the enzymatic method. The RLP-C concentration was measured by the immune adsorption method (JIMROII, Japan). The serum Lp(a) concentration was measured using an auto analyzer TBA-80FR (TOSHIBA) by the latex agglutination immunoassay.

Agarose gel electrophoreisis analysis

The serum samples were subjected to the lipoprotein analysis using agarose gel electrophoresis (Rapid Electrophoresis, Helena Laboratories, Beaumont, Texas) and the gels were stained with cholesterol and TG reagent. The conditions for electrophoresis were as follows; serum application volume was 1μ, and electrophoresis time was 18 min at 400 volts, and 20°C. Elution profiles were analyzed by an automatic densitometer, Chol/Trig Combo™ (Helena Kennkyusyo, Saitama, Japan).

Apolipoprotein B48 (apoB48) and Apolipoproteon B100 (B100) were identified with immunoblotting on the nitrocellulose film where the agarose gels were transferred. For detecting apo B48 and apo B100, we used anti-apoB48-151 monoclonal antibody (Fujirebio Co, Tokyo, Japan) and anti-apo B100 monoclonal antibody (Biochemical Division, Ohio), respectively. Ofuto® cream (Milk fat 35%, 547 kcal. Jyoumou Shokuhin company, Gunma, Japan) was used in the fat load. The samples which could not be measured on the day were stored at –40°C with the addition 1/20 (vol/vol) of dimethyl sulfoxide (8). This preservation method was effective up to four weeks (data not shown).

Statistical analysis

Data are expressed as the mean ± SD. Pearson's Correlation coefficient was used for the relationship between MidBand-C and RLP-C. Differences were examined with Student's *t* test. The computer analysis was done using Stat View Ver. 5.0. A *p*-value less than 0.01 was considered statistically significant. Receiver-operative charac-

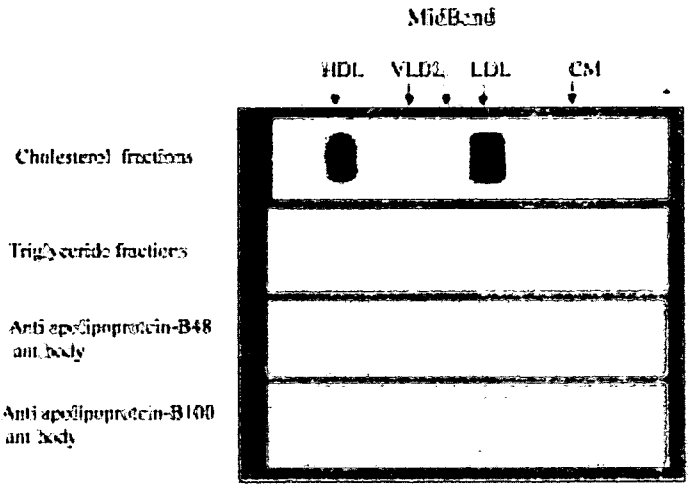

Fig. 1. Electrophoregram of cholesterol fractions, triglyceride fractions, apo B48 or apo B100. Apo B48 and apo B100 were detected by immunoblotting with anti -apo B48 or anti-apo B100 antibody. Sera were electrophoresed 4 hours after fat load (Ofuto™)

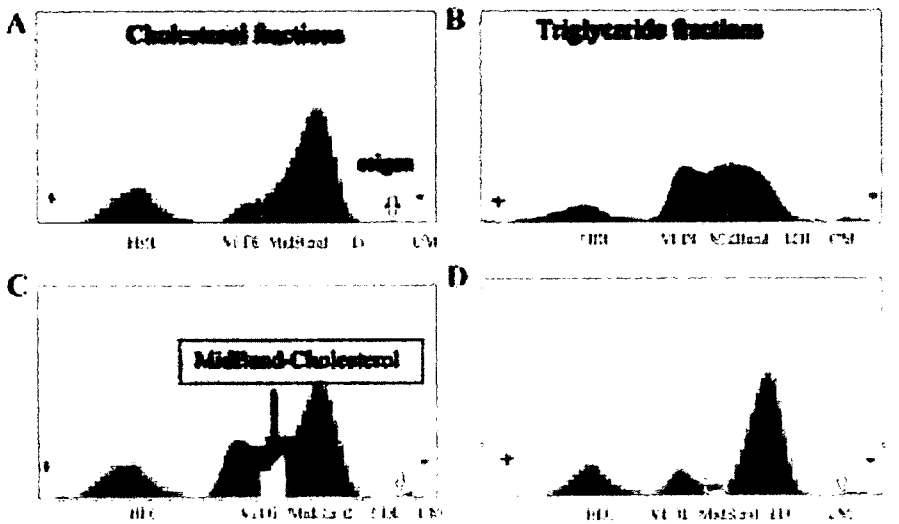

Fig. 2. The representative densitometric scanning patterns for Chol/Trig Combo™.
(A): cholesterol stain (B): triglyceride stain (C): layer in A and B (D): an example of a normolipidemic control subject, TC 202, TG 86, HDL-C 65, Mid Band-C 2.0, RLP-C < 2.5 (mg/dl)

teristics curve analysis was used to show performance figures. For the computation and analysis of ROC curves, we used the software program Med Calc, Ver. 6.01 (Med Calc Software).

Results

Determination of MidBand

We established automatic-five-fraction on the strength of the electric charge with apolipoprotein. The fraction eluted between the VLDL and LDL fractions in agarose gel electrophoresis of Chol/Trig Combo™ was defined as MidBand.

To confirm whether MidBand was rich in apoB48, serum was selected 4 hours after the loading of fat (Ofuto™) and electrophoresed and transfered to a nitrocellulose membrane. Nitrocellulose membrane was immunoblotted with anti-human apoB48 antibody and compared with the electrophoregram of cholesterol fractions, TG fractions and fractions immunoblotted with anti apoB100 antibody. MidBand was rich in apoB48, and the LDL fraction was rich in apoB100 (Fig. 1).

For example, agarose gels were stained for cholesterol (Fig. 2A) or TG (Fig. 2B) after electrophoresis and analyzed by the automatic densitometer Chol/Trig Combo™. After both patterns were imposed as shown in Fig. 2C,

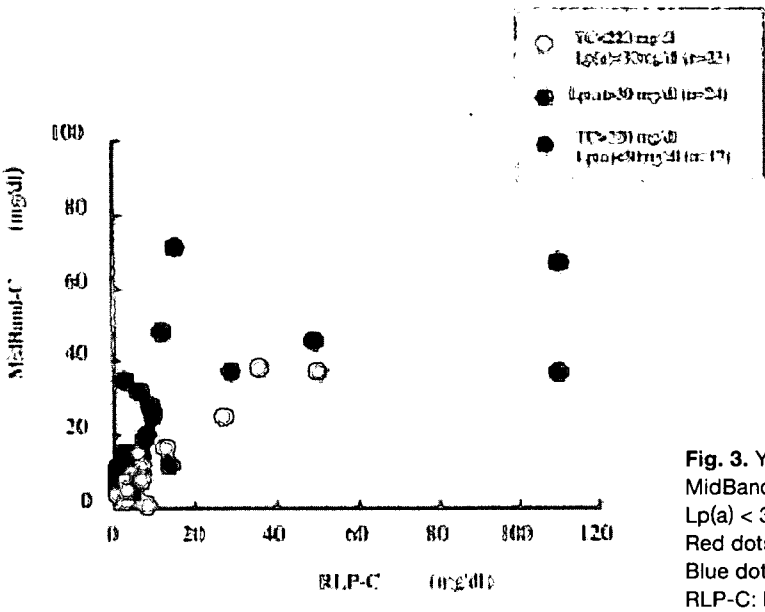

Fig. 3. Yellow dots: A significant positive correlation between MidBand-C levels and RLP-C levels when TC < 220 mg/dl and Lp(a) < 30 mg/dl.
Red dots: Lp > 30 mg/dl
Blue dots: TC > 220 mg/dl and Lp(a) < 30 mg/dl
RLP-C: Remnant-like particle cholesterol

MidBand was determined as the fraction between LDL and VLDL fractions. A representative profile of a normolipidemic subject is shown in Fig. 2D. The MidBand -cholesterol (MidBand-C) concentration was calculated from the serum cholesterol level and percentage of MidBand in the cholesterol fraction of the agarose gel electrophoretogram.

Relationship among MidBand-C, RLP-C and Lp(a)

To evaluate the relationship between MidBand-C and RLP-C, we measured RLP-C concentrations of 64 subjects and compared them to MidBand-C concentrations. We selected 23 subjects with both a TC of less than 220 mg/dl and Lp(a) concentration of less than 30 mg/dl out of the 64 subjects. As shown in Fig. 3 (yellow dot), there was a significant positive correlation between concentrations of MidBand-C and RLP-C in subjects with a correlation coefficient(r) of 0.95 ($p < 0.001$). However, MidBand-C concentrations of hyperlipidemic patients deviated from the standard line (Fig. 3, red dot and blue dot). MidBand-C concentrations of subjects with high Lp(a) levels were also high compared with RLP-C concentrations. As we have already reported, Lp(a) at levels of more than 30 mg/dl migrates as a peak of cholesterol between VLDL and LDL using this method (13). This position is the same as that of MidBand. Representative elution profiles are shown in Fig.4. In sample A, the Lp(a) concentration was 142 mg/dl showing a sharp MidBand-C peak. Sample B was from a type IIb hyperlipidemic patient and MidBand-C is the mid-band. Sample C was obtained from a type III hyperlipidemic patient and had a broad beta-band in the lipoprotein fraction. Sample D showed the presence of chylomicron and a high concentration of VLDL-TG, indicating type V hyperlipidemia.

Comparison of MidBand-C levels of normolipidemic subjects with patients with CAD

Table 1 shows a summary of the clinical characteristics of the elderly control subjects and the patients with CAD. Significant differences in HDL-C and MidBand-C concentrations were observed between the two groups in each sex.

To elucidate the clinical significance of MidBand-C in CAD, we measured the MidBand-C concentrations of the patients with CAD. Mean MidBand-C concentrations were 9.4 ± 4.1 and 9.2 ± 4.0 mg/dl in the male and female patients, and significantly higher than in control subjects ($p > 0.0001$) (Fig. 5).

A ROC analysis was performed for MidBand-C, HDL-C, LDL-C, TC and TG in the CAD patients and control subjects in males (Fig.6) and females (Fig.7). The area under the ROC curve (AUC) for MidBand-C in males and females was 0.80 and 0.79, respectively, showing a significant difference from that for LDL-C, TC and TG. ROC curves of HDL-C and MidBand-C almost overlapped and there was no significant difference.

Discussion

In the present study, we demonstrated that MidBand-C concentrations analyzed by agarose gel electrophoresis and automatic densitometer showed a significant correlation with RLP-C concentrations. MidBand is consid-

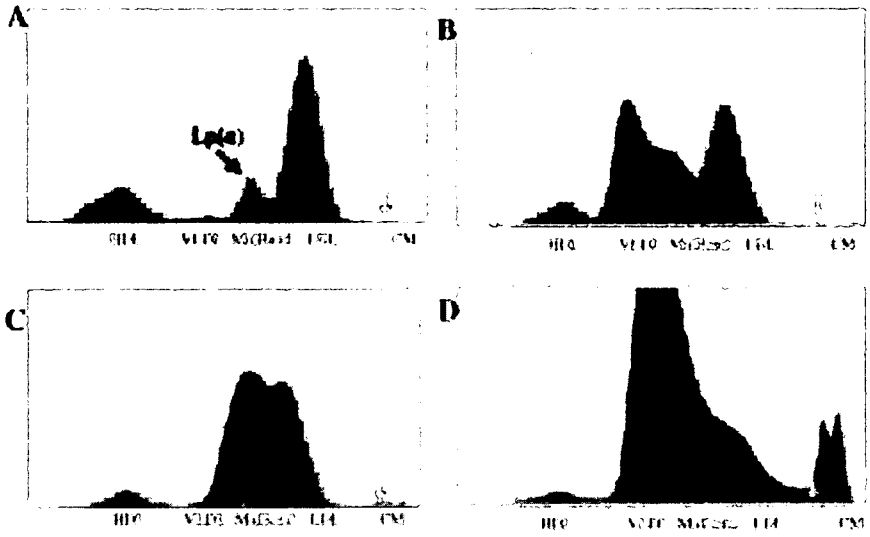

Fig. 4. Densitometric scanning patterns of hyperlipidemia.
Patient A had type IIa hyperlipidemia, and the mid-band; TC 311, TG 41, RLP-C 2.9, MidBand-C 39.5, Lp(a) 142 mg/dl. Patient B had type IIb hyperlipidemia, and the mid-band; TC 252, TG 264, RLP-C 9.0, MidBand-C 46.4 mg/dl. Patient C had type III hyperlipidemia, and a broad beta-band in the lipoprotein fraction; TC 232, TG 342,RLP-C 12.0, MidBand-C 58.0 mg/dl. Patients D had the type V hyperlipidemia, and an increase of chiromicron; TC 167, TG967, RLP-C 71.0, MidBand-C 14.0 mg/dl.

Table 1. Clinical characteristics of CAD patients and elderly control subjects

	Men		Female	
	control (n = 35)	CAD (n = 125)	control (n = 45)	CAD (n = 43)
Age (year)	66.1 ± 12.6	66.7 ± 9.6	63.7 ± 11.5	68.1 ± 9.3
TC (mg/dl)	164.7 ± 30.7	169.3 ± 29.7	178.7 ± 22.3	173.3 ± 24.5
TG (mg/dl)	86.0 ± 28.8	92.2 ± 30.7	92.0 ± 23.4	98.5 ± 28.3
LDL-C (mg/dl)	104.7 ± 24.2	103.9 ± 26.4	92.1 ± 20.8	104.5 ± 22.5
HDL-C (mg/dl)	61.3 ± 14.4	42.4 ± 13.3*	69.4 ± 17.5	47.4 ± 13.9**
MidBand-C (mg/dl)	5.2 ± 2.3	9.4 ± 4.1*	5.5 ± 2.0	9.2 ± 3.9**

* Significantly different from the value of control men, $p < 0.0001$
** Significantly different from the value of control female, $p < 0.0001$

ered similar to the mid-band observed in polyacrylamide gel electrophoresis (PAGE). However, it is not possible to conduct a quantitative analysis of remnant lipoproteins by PAGE. Additionally, preparative and analytical ultracentrifugation methods have been used to separate VLDL and IDL. However these methods are labor-intensive and complex. With our method using Chol/Trig Combo™, the operation is simple and semi-automated. Moreover, one is able not only to recognize the entire lipoprotein profile but also to quantitate the cholesterol and TG of MidBand that is equivalent to the IDL or remnant lipoprotein. We demonstrated that the MidBand richly contained apo B48.

To investigate the relation between MidBand-C and RLP-C, we measured RLP-C concentrations of 23 subjects with both a TC concentration of less than 220 mg/dl and a Lp(a) concentation of less than 30 mg/dl. In these subjects, there was a significant correlation between RLP-C and MidBand-C. Moreover, Lp(a) at levels of more than 30 mg/dl migrates as the same peak as MidBand-C. These results suggested that MidBand-C reflected VLDL remnants and Lp(a). Both are known to be atherogenic.

We analyzed MidBand-C in 80 elderly control subjects. To investigate the significance of the MidBand-C con-

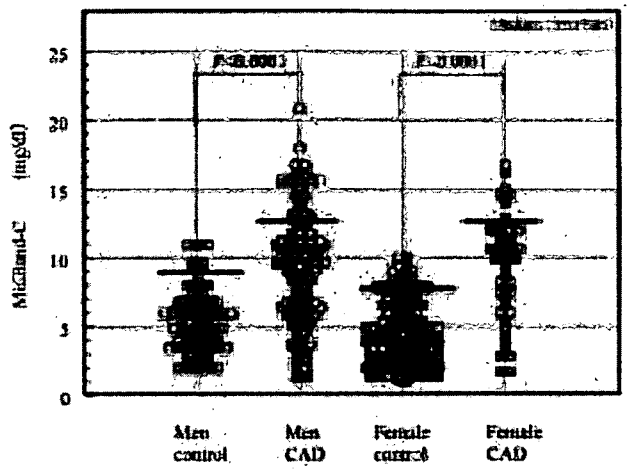

Fig. 5. Levels of MidBand-C in the elderly normolipidemic control subjects and the normolipidemic patients with CAD. There was a significant difference in MidBand-C levels between the control and CAD in each sex.

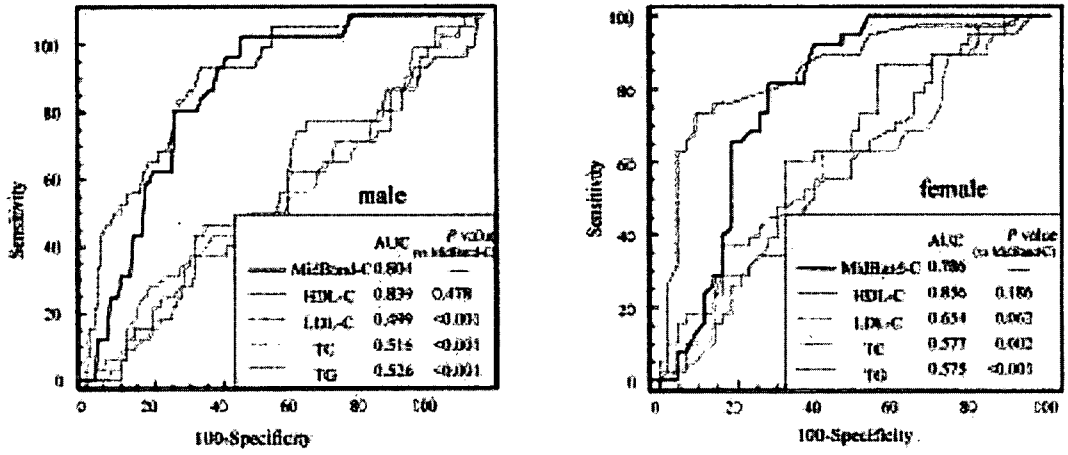

Fig. 6. and Fig. 7. Receiver operating characteristic curves of MidBand-C, HDL-C, LDL-C, TC and TG when used to discriminate between the patients with CAD and control subjects in males and females. AUC: Area under the curve.

centration in CAD, we measured levels in 168 patients with CAD. Mean MidBand-C levels of CAD patients (9.4 ± 4.1 mg/dl and 9.2 ± 3.9 mg/dl in males and females, respectively) were significantly higher than those of elderly control subjects (5.2 ± 2.3 mg/dl and 5.5 ± 2.0 mg/dl, $p < 0.0001$). In the ROC analysis performed on CAD patients and control subjects, the AUC for MidBand-C was 0.80 for males and 0.79 for females. In addition, the AUC of MidBand-C was significantly higher than that for LDL-C, TC or TG, suggesting that the elevation in the concentration of MidBand-C could be a useful marker of the risk of developing CAD.

Considering these results, measurements of MidBand-C made using Chol/Trig Combo™ may be important to evaluate the risk of CAD.

Conclusion

We defined MidBand as the fraction between LDL and the VLDL fractions in Chol/Trig Combo™. MidBand-C reflected the serum levels of RLP-C and Lp(a), and may be an important index with which to estimate the risk of CAD.

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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

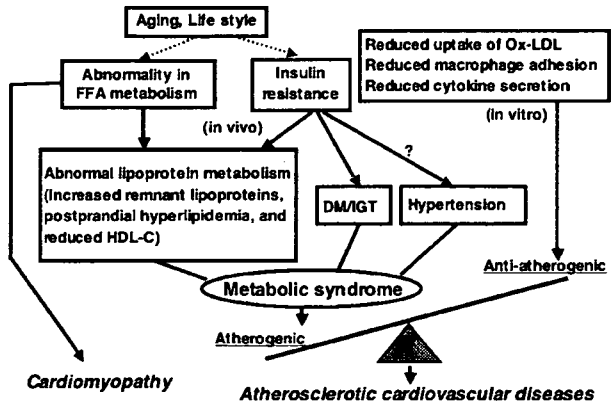

Fig. 1. Pathophysiology of human CD36 deficiency. Macrophages from patients with CD36 deficiency are resistant to Ox-LDL-induced foam cell formation at least *in vitro*, which might be antiatherogenic. However, CD36 deficiency may be related to insulin resistance, the metabolic syndrome, atherosclerosis 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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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, $P<0.001$,

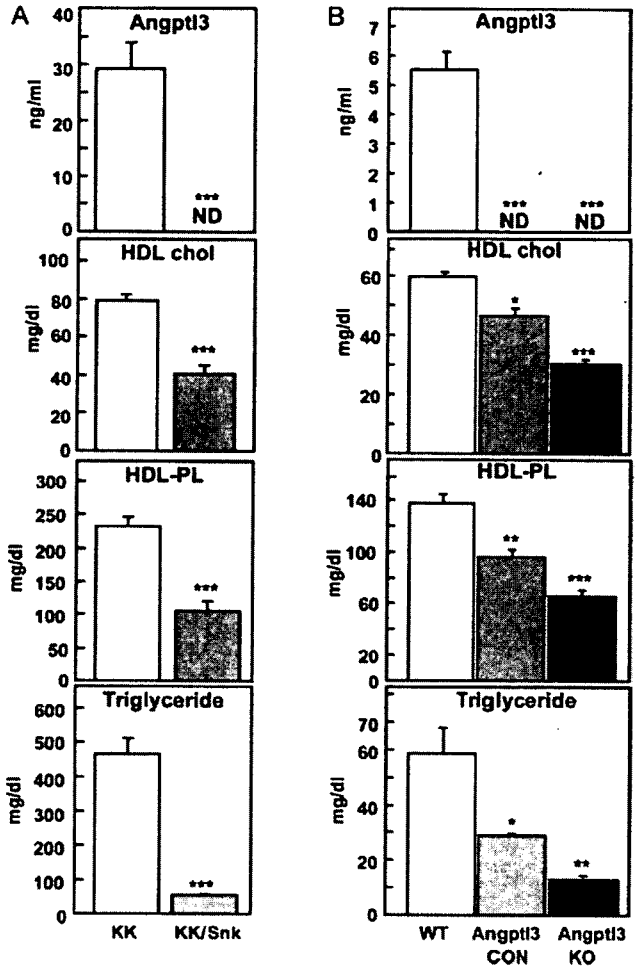

Figure 1. HDL cholesterol, HDL–PL, and triglyceride concentrations in Angptl3-deficient mice. A, Plasma levels of Angptl3, HDL cholesterol (HDL chol), HDL-PL, and triglyceride were measured in male wild-type KK (white bars, n=5) and Angptl3-deficient KK/Snk mice (gray bars, n=5). B, The same for those 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 phosphatidylcholine (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-

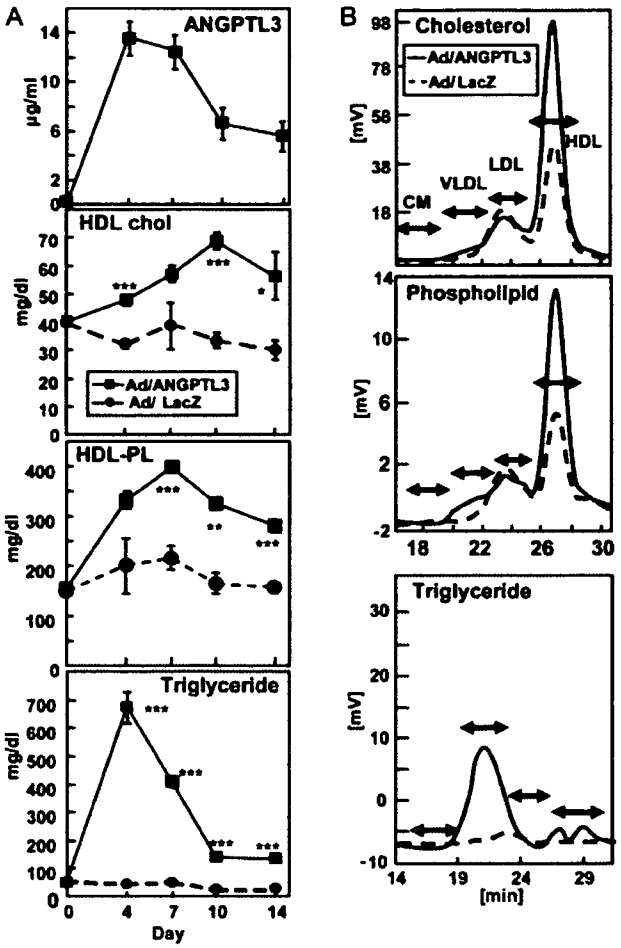

Figure 2. Alterations of plasma lipid profiles by supplementation of ANGPTL3 via adenovirus in Angptl3-deficient mice. A, Angptl3-deficient congenic C57BL/6J *Angptl3*^{hypl} mice were treated with recombinant adenoviruses carrying β-galactosidase (Ad/lacZ, circles) or human ANGPTL3 (Ad/ANGPTL3, squares). 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

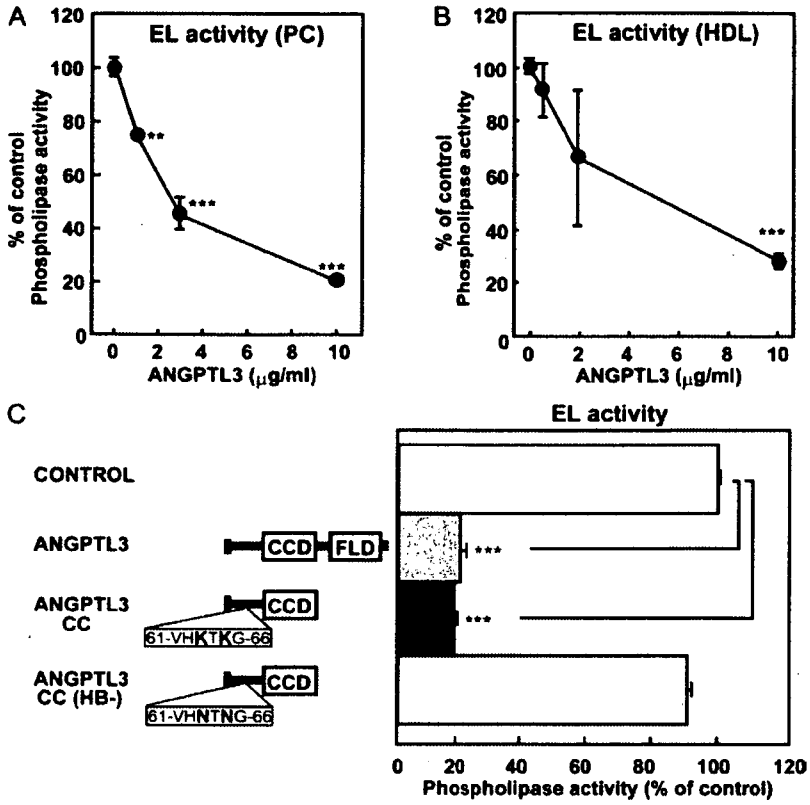

Figure 3. Inhibition of phospholipase activity of EL by ANGPTL3. A, Phospholipase activities of EL were determined using phosphatidylcholine emulsion (PC) as substrate as described in Methods, in the presence of recombinant human ANGPTL3 at the indicated doses (0, 1, 3, and 10 μg/mL). B, Similarly, they 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.

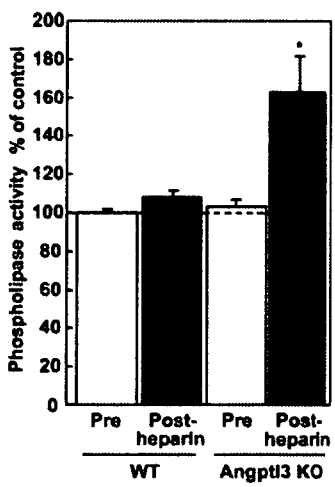

Figure 4. Phospholipase activities of pre- and post-heparin plasma in wild-type and Angptl3-knockout mice. Phospholipase activities of pre- (white bars) and post-heparin plasma (black bars) from wild-type (WT, n=5) and Angptl3-knockout mice (KO, n=5) were determined using 1,2 di[1-^{14}C] oleyl-L-3-phophatidylcholine 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,

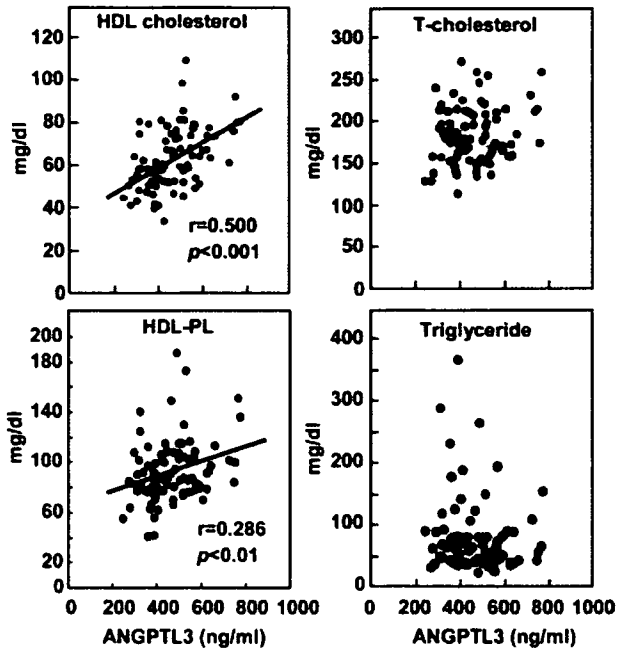

Figure 5. Plasma lipids and ANGPTL3 levels in humans. Plasma concentrations of ANGPTL3, HDL cholesterol, HDL-PL, total (T-) cholesterol and triglyceride were determined under overnight fasting conditions in healthy Japanese subjects (n=87). The values of correlation and probabilities are shown in the 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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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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due to decreased catabolism of larger HDL particles, suggesting that the size of HDL may modulate the selective HDL-C uptake by the liver (10). In human-LCAT transgenic mice, the liver uptake of HDL was reduced by 41%, resulting in a substantial increase of large HDL particles that might be atherogenic (11) due to the fact that mice lack cholesteryl ester transfer protein (CETP) and that continued increase of cholesteryl ester in HDL by high levels of LCAT changes both the size and lipid composition of HDL. When CETP was coexpressed in LCAT transgenic mice, HDL size and composition changed and the animals were protected from atherosclerosis (12). These data suggest that under normal conditions in which CETP is present as in humans, increased LCAT activity is likely to increase HDL cholesterol and size and might reduce the risk for atherosclerosis. Our previous data suggest that the two largest, spherical, cholesteryl ester-rich HDL particles, α-1 and α-2, are good substrates for SR-BI in a human hepatoma cell line (13).

Our aim was to gain insight into the role that LCAT plays in HDL metabolism as well as to better understand LCAT deficiency states. We have examined apoA-I-, -A-II-, -A-IV-, -C-I-, -C-III-, and -E-containing HDL subpopulation profiles in LCAT-deficient homozygotes and heterozygotes and in control subjects. The data we present indicate that LCAT plays a very significant role in HDL particle metabolism, composition, and remodeling.

MATERIALS AND METHODS

Subjects

We examined plasma obtained from 11 homozygous LCAT-deficient subjects of Italian (n = 7), Japanese (n = 3), and US (n = 1) origin, as well as from 11 heterozygous LCAT-deficient subjects from Italy. Plasma obtained from gender-matched control subjects from the US (n = 15), Italy (n = 4), and Japan (n = 3) was used in this comparison. Homozygous and heterozygous subjects from Italy have been described previously (14). All homozygous subjects had primary hypoalphalipoproteinemia as defined by a plasma HDL-C level below the 5th percentile for the age- and gender-matched general populations of the specific countries. One homozygous subject from Japan had FED; however, none of the measured parameters of this subject were different by more than 1 SD from those of the other 10 homozygotes.

Sample handling and measurements

Blood was collected from all subjects after an overnight fast and immediately placed on ice. Plasma was separated by low-speed centrifugation at 4°C and was stored at − °80 C until use. Samples were sent to the Lipid Metabolism Laboratory at Tufts University on dry ice and were thawed in a 37°C water bath for 1–2 min and then placed on ice just before use. Plasma total cholesterol, HDL-C, and triglyceride (TG) levels were determined using standard enzymatic techniques. Plasma concentrations of apoA-I, -A-II, and -B were determined by immunoturbidimetry. Plasma concentrations of apoA-IV, -C-I, -C-III, and -E were estimated by dot-blot analyses and expressed as arbitrary units. LCAT gene analyses, activity, and mass measurements were performed as described previously (14). HDL subpopulations were determined by nondenaturing two-dimensional PAGE, immunoblotting, and image analysis as described previously (15). Four microliters of plasma was applied and electrophoresed on a vertical-slab agarose gel (0.7%) in the first dimension at 250 V until the α-mobility front moved 3.5 cm from the origin. The agarose gel was sliced, and the strips were applied onto 3–35% nondenaturing concave gradient polyacrylamide gels. In the second dimension, gels were electrophoresed to completion at 250 V for 24 h at 10°C, followed by electrotransfer to nitrocellulose membranes at 30 V for 24 h at 10°C. The specific apolipoproteins were immuno-localized on the membrane with mono-specific goat anti-human primary and ^{125}I-labeled secondary antibodies [immunopurified rabbit F(ab')$_2$ fraction against goat IgG]. The bound ^{125}I-labeled secondary antibody was quantified in a FluoroImager (Molecular Dynamics). Each membrane was first probed for the apolipoprotein of primary interest and than reprobed for apoA-I for reference.

Data analysis

Means and standard deviations were calculated for all study groups. Data obtained from homozygotes and heterozygotes were compared with data from controls using ANOVA analyses. A two-tailed $P < 0.05$ was considered as significant.

RESULTS

Table 1 shows data on LCAT mass and activity as well as on lipids and apolipoproteins in controls (n = 22), heterozygotes (n = 11), and homozygotes (n = 11) for LCAT deficiency. Heterozygotes had 39% of the LCAT activity and 87% of the LCAT mass of controls. They had lower apoA-I (−22%), apoA-II (−19%), HDL-C (−15%), and

TABLE 1. Characteristics of study participants

	Controls (n = 22)	Heterozygotes (n = 11)	Homozygotes (n = 11)
Male/female	17/5	7/4	10/1
LCAT mass (μg/ml)	4.60 ± 1.01	4.02 ± 1.07	1.04 ± 0.96*
LCAT activity (nmol/ml/h)	33.0 ± 18.1	20.21 ± 1.6*	0.44 ± 0.66*
Total cholesterol (mg/dl)	200 ± 38	171 ± 37*	112 ± 63*
LDL-C (mg/dl)	126 ± 33	99 ± 33*	65 ± 54*
HDL-C (mg/dl)	54 ± 13	46 ± 12*	9 ± 5*
TG (mg/dl)	137 ± 88	127 ± 45	203 ± 146*
apoA-I (mg/dl)	140 ± 25	109 ± 17*	34 ± 11*
apoA-II (mg/dl)	38 ± 4	31 ± 5	11 ± 6*
apoB (mg/dl)	96 ± 16	97 ± 25	31 ± 17*

ApoA-I, apolipoprotein A-I; HDL-C, HDL cholesterol; TG, triglyceride. Data are mean ± SD.
*Significantly different ($P < 0.05$) from controls.