Table 1. Lipid profiles of the patient's family

	Father	Mother	Patient	Sister
Age (ys)	39	39	11	9
Total cholesterol	486	206	524	154
Triglyceride	194	44	273	53
LDL-C	384	136	446	82
HDL-C	48	50	23	55

Values are expressed as mg/dL

not have his serum lipids tested before visiting the regional hospital. His father had been diagnosed with hetero FH at 20 years of age; a coronary arteriogram at 33 years of age revealed 50% stenosis of the coronary arteries. He was initially treated with statin and colestimide, but neglected to continue treatment with these drugs. At 39 years of age, his coronary artery stenosis increased to a maximum of 90% on three branches, and he underwent percutaneous transluminal coronary angioplasty (PTCA). No history of coronary artery disease or hypercholesterolemia was detected in the patient's paternal uncle and aunts. The patient's paternal grandfather had suffered acute myocardial infarction at 45 years of age (lipid data was not available) and died of coronary artery disease at 71 years. The patient's maternal grandfather had coronary artery stenosis detected at 72 years of age and underwent PTCA. In addition, the patient's maternal great-grandfather and great-grandmother had died of heart disease, but the details were unclear. **Table 1** shows the lipid profiles of patient's family. No lipid abnormalities were found in his mother or younger sister.

The results of physical examination were as follows: height, 135.4 cm; weight, 31.9 kg; abdominal circumference, 56.8 cm; body mass index (BMI), 17.4 kg/ ; blood pressure, 116/78 mmHg. Xanthomas were present on both elbows (**Fig. 1**). Small xanthomas (about 1-mm diameter) were also found just beneath the eyes. Radiographs of the Achilles tendons revealed thickening of both tendons (right: 16 mm, left: 18 mm; **Fig. 2A**), which decreased following treatment. As shown in **Table 2**, serum concentrations of total cholesterol (TC), triglyceride (TG), LDL-C and apoB were very high, and that of HDL-C was very low (95th percentiles of TC, TG and LDL-C in Japanese schoolchildren were 220, 140, and 140 mg/dL, respectively). The 5th percentile of HDL-C in Japanese schoolchildren is 40 mg/dL[5]. The 90th percentile of apoB in schoolchildren at our hospital is 101 mg/dL (unpublished data). Serum concentration of Lp(a) was within the normal range. Serum concentrations of plant-derived sterols, such as sitosterol and cholestanol, were

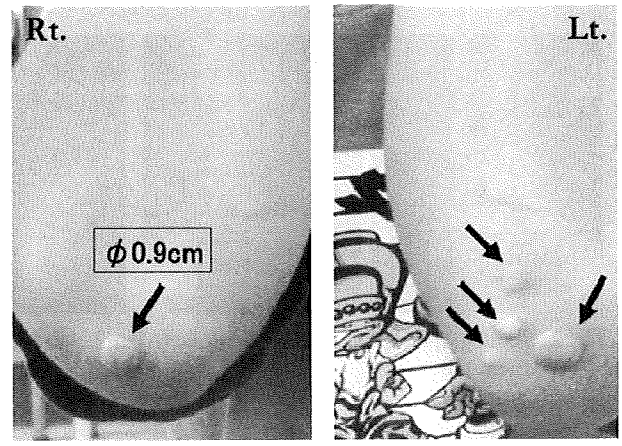

Fig. 1. Xanthomas on both elbows (arrows).

all within normal ranges. ApoE phenotype was E3/E3. The activity of LDL receptor was reduced to 32% by lymphocyte assay[6]. Arterial intima-media thickness (IMT) was assessed by carotid echogram, which revealed plaque formation and hyperplasia of the intima-media complex (**Fig. 2B**: maximum thickness, 1.3 mm; IMT in control children in our department <0.5 mm). Atherosclerotic change of the coronary arteries was not detected on 3D-computed tomography. As the patient's father had been diagnosed with hetero FH, patient had followed a low-fat diet.

Effect of Lipid-Lowering Therapy

After the serum concentrations of plant-derived sterols and LDL-receptor activity were determined, the patient was tentatively diagnosed with severe hetero FH and began treatment with HMG-CoA reductase inhibitor (rosuvastatin). At first, he received cholestimide (1–2 g/day), but there was no marked reduction of LDL-C. The rosuvastatin dose was increased every 4 weeks, based on the LDL-C levels, to a maximum of 15 mg/day. After 6 months of statin therapy, his LDL-C level had decreased by 22% but remained above 300 mg/dL; therefore, he received cholestimide in addition to statin. His LDL-C level decreased to 220 mg/dL after 12 months of treatment (rosuvastatin 15 mg/day, cholestimide 1.5 g/day), while HDL-C levels increased from 23 to 42 mg/dL. After 12 months of treatment, the thickness of the Achilles tendons had decreased from 16–18 mm to 13 mm, IMT from 1.3 mm to 0.7 mm, and the small xanthomas (about 1 mm in diameter) located beneath the eyes disappeared. LDL-C levels, however, did not fall below 220 mg/dL with rosuvastatin 15 mg/day and cholestimide 1.5 g/day; cholestimide was therefore changed to ezetimibe (10 mg/day). After 2 months of therapy with ezeti-

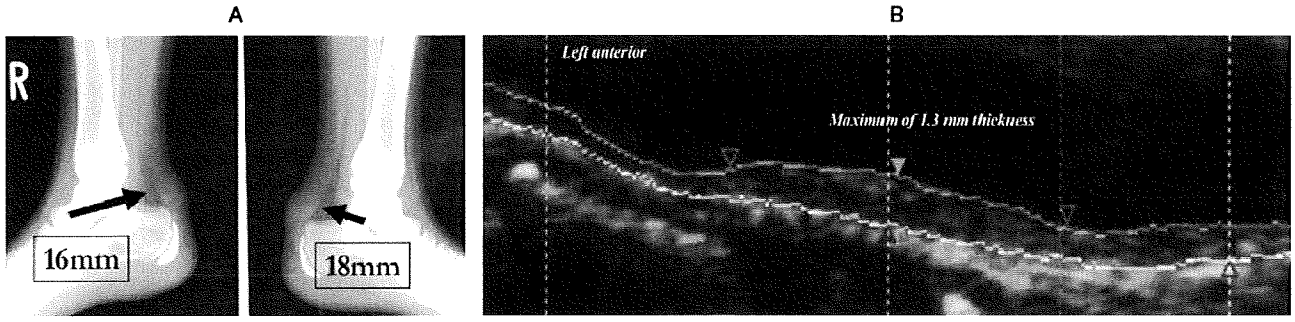

Fig. 2. Radiograph of the Achilles tendons (A) and carotid echogram images of arterial intima-media thickness before statin treatment (B).

Table 2. Results of laboratory tests on admission

Total Cholesterol	524 mg/dL (<220 mg/dL)
LDL-Cholesterol	446 mg/dL (<140 mg/dL)
Triglyceride	273 mg/dL (<140 mg/dL)
HDL-Cholesterol	23 mg/dL (>40 mg/dL)
ApoA-I	59 mg/dL (112-162 mg/dL)
ApoB	249 mg/dL (59-99 mg/dL)
ApoE	11.0 mg/dL (3.0-5.5 mg/dL)
Lp (a)	27 mg/dL (<40 mg/dL)
AST	20 u/dL (14-33 IU/L)
ALT	8 u/dL (4-24 IU/L)
Glucose	75 mg/dL (68-105 mg/dL)
Insulin	9.4 μu/mL (2.6-30.7 μu/mL)
Cholestanol	3.4 μg/mL (1-7.9 μg/mL)
Sitosterol	5.1 μg/mL (5.1-9.9 μg/mL)
ApoE Phenotype	E3/E3
LDL-receptor activity	32% (101 ± 10.8%, mean ± SD)

Rerefence intervals are shown in parenthesis.

mibe, the LDL-C level was relatively unchanged (220 -240 mg/dL); we are currently adjusting the dosage of rosuvastatin by measuring the IMT and the thickness of the Achilles tendons.

Discussion

In children, xanthomas with hypercholesterolemia are usually found in patients with homo FH or sitosterolemia or cerebrotendinous xanthomatosis (CTX)[7,8]. Similar to sitosterolemia, serum concentrations of cholestanol increase in patients with CTX[8]. Thus, after determining serum concentrations of cholestanol and sitosterol (**Table 2**, all within normal ranges), sitosterolemia and CTX were excluded from the differential diagnosis in our patient. The leading cause of homo FH is a loss of function of the LDL receptor that is inherited in an autosomal-dominant manner. In addition, homo FH is recessively inherited when mutations disrupt the function of an adaptor protein for endocytosis of the LDL receptor, known as autosomal recessive hypercholesterolemia (ARH)[9]. The pedigree of our patient shows vertical transmission of hypercholesterolemia from father to son, thereby suggesting an autosomal-dominant manner. In addition, a low level of LDL-receptor activity and good response to lipid-lowering therapy using statin suggested that our patient was very likely to hve hetero FH.

Pathologically, atherosclerotic changes in the coronary arteries originate during childhood, with the extent of atherosclerotic lesions correlating positively with plasma LDL-C levels and negatively with plasma HDL-C levels, even in children and young adults[4]. In our preliminary studies (unpublished data), serum levels of LDL-C, Triglyceride (TG) and HDL-C in schoolboys with untreated FH were 220 ± 42 mg/dL, 86 ± 56 mg/dL and 62 ± 12 mg/dL, respectively (n=24, mean ± SD). Serum levels of LDL-C, TG and HDL-C in our patient were more than twice (LDL-C and TG) and less than half (HDL-C) when compared with average levels, respectively. The phenotype of dyslipidemia in our patient and his father was type IIb. As reported previously, children with IIb usually showed lower HDL-C levels than those with normolipidemia and IIa[10,11]. In Japanese adults patients with hetero FH, IIb showed increased coronary artery disease compared to cases of IIa[12]. Furthermore, cases of low HDL-C had increased coronary artery disease in herero FH[12]. Thus, in our patient, early development of xanthomas and advanced atherosclerosis (increased carotid IMT and thickened Achilles tendons) may have been caused by the interactions of high LDL-C, high TG and low HDL-C levels. In this patient, however, coronary artery disease was common in his maternal family, although their detailed medical histories are not available at present. Because his mother's serum levels of LDL-C and HDL-C were within normal ranges, fac-

tors other than dyslipidemia may have contributed to the early development of atherosclerosis in our patient. Although the possibility may be very low, interactions of FH and ARH should also be considered. Recently, as a cause of severe phenotype in hetero FH, dominant gain-of-function mutations in the gene encoding a member of the proprotein convertase family, PCSK9, have been reported[9]. To clarify the mechanism behind the early development of atherosclerosis in our patient, further studies, including genetic analysis, are needed.

In Japan, there are currently no guidelines for the pharmacological treatment of children with hetero FH; however, recent studies of children and adolescents with hetero FH have established the effectiveness and safety of statin therapy[13, 14]. Based on these data, the American Academy of Pediatrics (AAP) released revised recommendations for the management of hypercholesterolemia[15]. According to this report, statins are recommended as first-line pharmacologic agents, and drug therapy may be instituted from 8 years of age. Although AAP recommendations for drug therapy have elicited controversy[16], the urgency for statin treatment in our patient may override any possible adverse effects of statins. Thus far, we have not observed any adverse effects of statin therapy.

In our patient, drug therapy enabled a 50% reduction in LDL-C levels and 100% increase in HDL-C levels, but these values remained greater than 220 mg/dL and less than 50 mg/dL, respectively. This 50% reduction in LDL-C and 100% increase in HDL-C delivered a greater than expected improvement in the thickness of the Achilles tendons and carotid IMT. As reported[17], initiation of statin treatment in children may be more effective than in adults to achieve regression and/or delay the progression of atherosclerosis. In the present patient, we will control the statin dosage by measuring carotid IMT and the thickness of the Achilles tendons in addition to serum levels of LDL-C and HDL-C.

In conclusion, careful evaluation of atherosclerosis should be considered, even in children with hypercholesterolemia. If advanced atherosclerosis is detected, pharmacological therapy should be considered in addition to dietary treatment.

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Enhanced circulating soluble LR11 in patients with coronary organic stenosis

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ABSTRACT

LR11, an LDL receptor family member, is expressed in intimal smooth muscle cells. It was found that the soluble form of LR11 (sLR11) is detected in serum, and the circulating sLR11 levels are positively correlated with intima-media thickness of carotid arteries in dyslipidemic subjects. To clarify the significance of serum sLR11, the circulating sLR11 levels in patients with organic coronary stenosis and the contributing risk factors for them were studied. The subjects, 150 patients with symptoms of coronary artery disease, underwent coronary angiographic examination, and were divided into sex- and age-matched two groups; one is organic coronary stenosis group (OCS) and the other is normal coronary group (NC). Serum sLR11 levels were significantly higher in OCS than in NC (4.9 ± 2.7 U vs 3.6 ± 1.8 U, $p < 0.05$). Multivariate regression analysis showed that circulating sLR11 is independent contributing factor for the OCS, as well as diabetes mellitus and dyslipidemia. Among various coronary risk factors for sLR11 level, HbA1c showed the highest correlation coefficient ($p < 0.01$).

These results suggest that the circulating sLR11 might reflect coronary organic stenosis, and that hyperglycemic condition might be promoting factor for expression of LR11 in intimal smooth muscle cells.

1. Introduction

In the formation of atherosclerosis, migration of vascular smooth muscle cells (SMCs) from the media to the intima is the key step [1,2]. Following migration, SMCs change the phenotype and proliferate in the intima, resulting in intimal thickness. Furthermore, proliferating SMCs secrete matrices and proteases to form atheromatous lesions under the influence of stimulatory cytokines [3–6].

Recently, we [7] identified LR11, which is an LDL receptor family member with poorly defined function, and observed the expressing of LR11 specifically in intimal SMCs, but not in medial SMCs, macrophages or lymphocytes in the arterial wall [7–10]. LR11 as both the membrane-spanning and the shed soluble (sLR11) forms bind to urokinase-type plasminogen activator receptor (uPAR) on the cell surface [8,11]. Over-expression of LR11 in SMCs enhances their migration via elevated levels of uPAR, and appears to thereby increase the activation of the uPA system [7,12].

Interestingly, the sLR11 was detected in the serum, and that the circulating sLR11 levels are positively correlated with intima-media thickness of carotid arteries in dyslipidemic subjects [13]. The relationship of the sLR11 levels in serum with other risk factors for atherosclerosis, such as age, sex, smoking, blood pressures, serum lipids, and plasma glucose was not observed. But, the precise was not clear yet.

It is reported that arterial intimal thickening after balloon catheter injury was enhanced in diabetic animals than control [6,14]. Clinically, increased intimal-medial thickness of carotid artery in type 2 diabetes was reported [15,16]. Thus, a relationship between coronary stenosis and sLR11 level, and also a relationship between sLR11 and diabetic condition were suspected.

In this report, we investigated the significance of circulating sLR11 in organic coronary stenosis (OCS) of the patients with a suspicion of coronary artery diseases (CAD). Contributing factors for the elevation of serum sLR11 were also analyzed.

2. Subject and methods

The subjects were 150 persons who were suspected to have coronary artery disease and who underwent coronary angiography at Toho University Sakura Hospital Cardiovascular Center were

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recruited for the study. Patients suffered from chronic heart disease with ejection fraction < 50% or chronic renal failure with serum creatinine > 1.3 mg/dl were excluded from the study analysis. The study protocol was approved by the Human Investigation Review Committee of Toho University Sakura Hospital, and informed consent was given by each patient.

The angiographical severity of coronary stenosis was assessed in the worst view position, and the percentage of luminal narrowing was recorded according to the American Heart Association reporting system [17]: organic coronary stenosis was defined as a stenosis with ≥75% diameter, and normal coronary artery (NC) was defined as without significant stenosis. Blood sample were collected in the morning after an overnight fast. Lipid variables and fasting blood glucose were measured by standard laboratory techniques. Serum insulin was measured by an enzymatic-immunological assay. Homeostasis model assessment insulin resistance index (HOMA-IR) was defined as: (plasma glucose × serum insulin)/405 [18]. Potential risk factors for atherosclerosis were analyzed, including age, sex, body mass index (BMI), smoking, and histories of hypertension, diabetes mellitus or dyslipidemia. Hypertension was defined as a history of hypertension (systolic pressure > 140 mmHg or diastolic pressure >90 mmHg). Diabetes mellitus was defined as a history of diabetes mellitus having fasting blood glucose > 126 mg/dl and HbA1c > 5.8%. Dyslipidemia was defined as a history of serum total cholesterol >220 mg/dl and/or triglyceride >150 mg/dl in the fasting and/or HDL-cholesterol <40 mg/dl.

3. Measurement of serum sLR11 concentration

Fasting blood samples were collected and were centrifuged immediately after collection to measure serum sLR11 levels. Fifty microliters of serum was purified using a 39 kDa receptor-associated protein (RAP)-GST affinity beads (Cosmo Bio). For immunoblotting, equal amount of protein extracted from pelleted beads was subjected to 10% SDS-PAGE after heating to 95 °C for 5 min as described [13] under reducing conditions, and transferred to a nylon membrane. Incubations were with antibody against LR11 (5-4-30-19-2 at 1:500 dilution) [13], followed by peroxidase-conjugated anti-mouse IgG. Development was performed with the ECL detection reagents (Amersham Pharmacia). The signals were quantified by densitometric scanning using NIH image™ software. The sLR11 levels in each human serum (50 μl) was determined as an averaged value of three quantified signal intensities resulting from independent assays using samples with blind indication, and expressed as a ratio to that of a standard serum. The immunological estimation indicated that the signal of 1 U (in 50 μl serum) corresponded to approximately 50 ng/ml of recombinant sLR11.

4. Statistics

The results are shown as mean ± SD or proportion (%) for each index. Statistical analysis was performed with SPSS version 13.0 (SPSS Japan Inc.). The unpaired *t*-test and the *chi*-square test were used to compare the continuous and the categorized variables, respectively. Pearson's correlation coefficient analysis was used to assess association between measured parameters. Subsequently, multiple linear regression analyses were used to calculate the ORs for the OCS (i) by controlling for all risk factors (age, sex, BMI, smoking, diabetes, hypertension, dyslipidemia and sLR11) (Model 1); (ii) by additionally controlling for BMI, diabetes, dyslipidemia, and sLR11, which are significantly associated with OCS by above analyses (Model 2). These risk factors were scored as explanatory factors, and subordinate variable was OCS = 1 and NC = 0. A value of $p < 0.05$ was considered significant. Multivariate analysis was performed by multiple regression analysis.

Table 1
Characteristics of the normal coronary artery and organic coronary stenosis subjects.

	NC	OCS	*p* value
n	55	95	
Male (%)	65.5	74.7	0.23
Age (y)	66.1 ± 8.4	66.5 ± 9.7	0.87
BMI (kg/m^2)	23.9 ± 3.0	25.1 ± 3.4	<0.05
Diabetes (%)	5.5	33.7	<0.01
Hypertension (%)	56.4	64.2	0.34
Dyslipidemia (%)	52.7	85.3	<0.01
sLR11(U)	3.6 ± 1.8	4.9 ± 2.7	<0.01
Fasting blood sugar (mg/dl)	106.7 ± 13.4	111.5 ± 27.1	0.22
Insulin (μU/dl)	6.2 ± 3.9	7.9 ± 4.9	<0.05
HOMA-IR	1.7 ± 1.1	2.2 ± 1.9	<0.05
Medications			
Administration of statin (%)	18.2	66.3	<0.01
Administration of ACE-I or ARB (%)	29.1	42.1	0.13

Plus–minus values are means ± SD. The unpaired *t*-test was used for continuous variables, and the *chi*-square test was used for categorized variables. BMI: Body mass index. Circulating sLR11 levels in NC group and OCS group were 3.6 ± 1.8 U and 4.9 ± 2.7 U, respectively, indicating that the sLR11 levels in OCS were significantly higher than those in NC ($p < 0.01$).

5. Results

5.1. Circulating sLR11 levels in NCA and OCS groups

The subjects were classified into age- and sex-matched two groups, according to the angiographical evaluation. Normal coronary artery group is composed of 55 subjects and organic coronary stenosis group is composed of 95 subjects (Table 1). BMI, histories of diabetes and dyslipidemia were significantly increased in the OCS group comparing with the NC group. Smoking and the history of hypertension were not different between the two groups. Insulin levels and HOMA-IR levels were significantly increased in the OCS group. Circulating sLR11 levels in NC group and OCS group were 3.6 ± 1.8 U and 4.9 ± 2.7 U, respectively, indicating that the sLR11 levels in OCS were significantly higher than those in NC ($p < 0.01$). Note that we have reported that the mean circulating sLR11 levels in four-hundreds dyslipidemic subjects are 3.0 ± 1.0 U [13]. Thus, circulating sLR11 levels increased in the patients with organic coronary stenosis among the patients taking angiographical examination with a suspicion of coronary arterial diseases.

5.2. Multivariate analysis of sLR11 and other risk factors for OCS

We next analyzed the significance of sLR11 in comparison to other risk factors for OCS in all subjects (Table 2). The multivariate analysis of all variables (Model 1) for OCS showed that circulating sLR11 and the histories of diabetes or dyslipidemia were explanatory factor for OCS independent from other variables. The Model 2 analysis using the limited variables which have been shown to be significantly increased in OCS (see Table 1) showed that circulating sLR11 is still an independent factor for OCS. These results showed that the circulating sLR11 level was enhanced in OCS group among patients with a suspicion of CAD and taking coronary angiography.

5.3. Correlation of serum sLR11 with other various parameters in all subjects

As shown in Table 3, a negative correlation between sLR11 concentration and HDL-cholesterol ($r = -0.161$, $p < 0.05$) and a positive correlation between sLR11 and triglyceride ($r = -0.161$, $p < 0.05$) were found. Furthermore, BMI ($r = 0.182$, $p < 0.05$), insulin (0.186, $p < 0.05$) and HOMA-IR (0.242, $p < 0.01$) and HbA1c ($r = 0.272$, $p < 0.01$) showed significant positive correlations with sLR11, respectively. But there was no significant correlation between cir-

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Table 2
Multivariate assessment of the effect of sLR11 and other risk factors on OCS BMI, sLR11 and age were analyzed per 0.1 kg/m², 0.1 U, and 10 y increase, respectively. Models 1 and 2 are described in Methods. BMI: Body mass index.

	OR (95% CI)	*p* values
Model 1		
BMI, per 0.1 kg/m² increase	1.00 (0.99–1.02)	0.85
Diabetes	7.98 (2.29–27.77)	<0.01
Dyslipidemia	4.03 (1.61–10.08)	<0.01
Male	1.48 (0.54–4.07)	0.44
Hypertension	1.53 (0.67–3.48)	0.31
Smoking	2.14 (0.88–5.21)	0.09
sLR11, per 0.1 U increase	1.03 (1.00–1.05)	<0.01
Age, per 10 y increase	1.15 (0.72–1.82)	0.93
Model 2		
BMI, per 0.1 kg/m² increase	1.04 (0.91–1.15)	0.57
Diabetes	6.36 (1.97–20.51)	<0.01
Dyslipidemia	4.55 (1.86–1.61)	<0.01
sLR11, per 0.1 U increase	1.02 (1.00–1.04)	<0.05

The multivariate analysis of all variables (Model 1) for OCS showed that circulating sLR11 and the histories of diabetes or dyslipidemia were explanatory factor for OCS independent from other variables. The Model 2 analysis using the limited variables which have been shown to be significantly increased in OCS (see Table 1) showed that circulating sLR11 is still an independent factor for OCS.

culating sLR11 levels and age, sex, white blood cell, red blood cell, platelet, total cholesterol, LDL-cholesterol, non-HDL-cholesterol or fasting blood sugar.

5.4. Multiple regression analysis for sLR11 in all subjects

Table 4 shows multiple regression analysis for sLR11concentratin. Variables in simple liner regression analysis with $p < 0.05$ were included into the multiple regression analysis model. The levels of sLR11 significantly correlated with HDL-cholesterol, triglyceride, HbA1c, BMI and HOMA-IR. Among these variables, only HbA1c concentration showed independent correlation with sLR11 levels (t-value = 3.02 $p < 0.01$).

Table 3
Correlation of Serum Soluble Form of LR-11 with various parameters in all subjects.

	Pearsons correlation coefficient	*p* values
Age	0.121	0.14
Male	−0.150	0.07
Total cholesterol (mg/dl)	0.067	0.42
HDL-cholesterol (mg/dl)	−0.160	<0.05
Triglyceride(mg/dl)	0.161	<0.05
LDL-cholesterol(mg/dl)	0.101	0.11
Non-HDL-cholesterol (mg/dl)	0.144	0.08
Fasting blood sugar (mg/dl)	0.077	0.35
Insulin(μU/dl)	0.186	<0.05
HOMA-IR	0.242	<0.01
HbA1c (%)	0.272	<0.01
Body Mass Index (kg/m²)	0.182	<0.05

A negative correlation between sLR11 concentration and HDL-cholesterol ($r = -0.161$, $p < 0.05$) and a positive correlation between sLR11 and triglyceride ($r = -0.161$, $p < 0.05$) were found. Furthermore, BMI ($r = 0.182$, $p < 0.05$) and HbA1c ($r = 0.272$, $p < 0.01$) showed significant positive correlations with sLR11, respectively.

Table 4
Results of multiple regression analysis for soluble form of LR11 in all subjects.

	Partial regression coefficient (b)	t-value	*p* value
X			
HbA1c (%)	0.21	2.50	<0.01
HOMA-IR	0.11	1.23	0.22
Body Mass Index (kg/m²)	0.10	1.16	0.25
HDL-cholesterol (mg/dl)	−0.09	−1.05	0.29
Triglyceride (mg/dl)	0.05	0.59	0.56
Y Soluble form of LR 11			

X, explanatory factor; Y, subordinate variables; Correlation coefficient (R) = 0.35 F value = 4.1, $p = 0.002$, ($n = 150$); The levels of sLR11 significantly correlated with HDL-cholesterol, triglyceride, HbA1c, and BMI. Among these variables, only HbA1c concentration showed independent correlation with sLR11 levels (t-value = 3.02 $p < 0.01$).

6. Discussion

Coronary organic stenosis is formed mainly with intimal thickness which is composed of proliferative intimal smooth muscle cells and matrix components accompanying with lipid pool [19–21]. In our cases, sLR11 was higher in OCS group than that of in NCA group. We have recently reported that LR11 is produced by the intimal SMCs, and considerable amounts of the shed sLR11 enhance SMC migration in vitro [13]. Therefore, high sLR11 concentration may reflect the pathophysiological condition of intimal SMCs. And we also reported that sLR11 is a circulating marker for IMT independent from the other classic risk factor for atherosclerosis in dyslipidemic subjects without CAD or diabetes [13]. Considering the facts that LR11 is highly expressed in intimal SMCs, macrophages, or lymphocytes [7–10], the above results strongly suggest that circulating sLR11 level reflect the amount of intimal SMC in coronary arteries. Next, the contributing risk factors for elevation of circulating sLR11 were studied. Although multiple regression analysis showed HbA1c was only significant factor correlated with sLR11, insulin resistance relating factors such as BMI, HOMA index, low HDL-cholesterol, high triglyceride were related with sLR11 in single regression analysis, indicating that sLR11 might be induced with the state of insulin resistance in addition to diabetes mellitus.

The reason why diabetic condition is tightly correlated with sLR11 levels in serum is not available yet, but it has been reported that arterial intimal thickening after balloon catheter injury was enhanced in diabetic animals than controls [6,14]. From these observations, diabetic condition may induce the expression of sLR11 directly, and also modify the phenotype of smooth muscle cell into so-called synthetic type in the arterial wall. Those possibilities are currently under investigation.

In summary, the results obtained from the patients with a suspicion of coronary artery diseases suggested that circulating sLR11 may relate to coronary organic stenosis, and that hyperglycemic condition is a promoting factor for expression of LR11 in vascular SMCs.

6.1. The limitation

The limitation of the present investigation is at first a lack of information about the serum sLR11 data at acute phases of coronary artery diseases. Second, the data may be influenced by the continuous medication. More of subjects have received the treatments against dyslipidemia with statins in OCS group than in NC group, and most of subjects have received against hypertension with angiotensin II receptor type 1 blockers (ARBs) in the present study (Table 1). Considering the facts that statins and ARBs inhibit the sLR11 expression in the cultured SMCs [10,13], the circulating sLR11 levels may be influenced by these treatments in addition to coronary artery diseases. Further studies to investigate the effects of these drugs on the circulating LR11 levels are in progress. Third, HOMA-IR was assessed as a marker of insulin resistance. If this is the case, glucose clamp method might be better than HOMA-IR; but they are problematic in daily practice, and HOMA-IR considered a reliable insulin resistance marker, in vivo, especially in subjects whose fasting blood glucose concentration were not so high. Therefore we used HOMA-IR as a marker of insulin resistance, considering the burden on patient and medical stuff.

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Development of an Immunoassay for the Quantification of Soluble LR11, a Circulating Marker of Atherosclerosis

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BACKGROUND: Vascular smooth muscle cells (SMCs) migrate from the arterial media to the intima in the progression of atherosclerosis, and dysfunction of SMCs leads to enhanced atherogenesis. A soluble form of the LDL receptor relative with 11 ligand-binding repeats (sLR11) is produced by the intimal SMCs, and the circulating concentrations of sLR11 likely reflect the pathophysiological condition of intimal SMCs. Furthermore, polymorphism of the LR11 gene has been found to be related to the onset of Alzheimer disease. This study describes the development of a sandwich immunoassay for quantifying sLR11 in human serum and cerebrospinal fluid.

METHODS: We used synthetic peptides or DNA immunization to produce monoclonal antibodies (MAbs) A2-2–3, M3, and R14 against different epitopes of LR11.

RESULTS: sLR11 was immunologically identified as a 250-kDa protein in human serum and cerebrospinal fluid by SDS-PAGE separation, and was purified from serum by use of a receptor-associated protein and MAb M3. An immunoassay for quantification of sLR11 with a working range of 0.25–4.0 μg/L was developed using the combination of MAbs M3 and R14. Treatment of serum with 5.25% n-nonanoyl-N-methyl-d-glucamine reduced the matrix effects of serum on the absorbance detection in the ELISA system. The linear dynamic range of the ELISA spanned the variation of circulating sLR11 concentrations in individuals with atherosclerosis.

CONCLUSIONS: A sandwich ELISA was established for quantifying sLR11 in serum and cerebrospinal fluid. This technique provides a novel means for assessing the pathophysiology of atherosclerosis, and possibly neurodegenerative diseases.

The LDL receptor relative with 11 ligand-binding repeats (LR11)[4] (also known as SorLA) *(1, 2)* is a member of the LDL receptor family and is highly expressed in atheromatous plaques, particularly in the intimal smooth muscle cells (SMCs) at the border between the arterial intima and the media *(3)*. Overproduction of LR11 protein promotes the enhanced migration of SMCs via the upregulation of urokinase-type plasminogen activator receptor *(4, 5)*. LR11 plays an essential role in the angiotensin II–induced mobility of SMCs, and angiotensin II type 1 receptor blockers have been found to reduce intimal thickness through the inhibition of the LR11/urokinase-type plasminogen activator receptor–mediated pathway of intimal SMCs in cuff-injured mice *(6)*. The extracellular domain of the membrane-spanning LR11 is released to yield an active soluble form of LR11 (sLR11) *(5, 7, 8)*. Recombinant sLR11 stabilizes urokinase-type plasminogen activator receptor and enhances the activation of the integrin/FAK/Rac1 pathway in SMCs and macrophages *(6, 8)*. The concentrations of sLR11 in arteries increased 2 weeks after endothelial injury in rats *(8)*, and the neutralization of sLR11 activity by specific antibodies reduced the intimal thickness after cuff injury in mice *(5)*. Statins, as well as angiotensin II type 1 receptor blockers, have been reported to inhibit the migration of intimal SMCs via the downregulation of LR11 expression and to attenuate LR11 expression in the intimal SMCs of aortic arteriosclerotic plaques in hyperlipidemic rabbits *(9)*.

Circulating sLR11 can be immunologically detected in serum by use of specific antibodies against LR11 *(6)*. Circulating concentrations of LR11 were positively correlated with intimal-media thickness in dyslipidemic individuals, and the correlation was independent of other classical risk factors for atherosclerosis *(6)*. In addition, neuronal LR11 expression is characteristically reduced in mild cognitive impairment and in the brains of individuals with Alzheimer disease (AD) *(10–13)*. Single nucleotide polymorphism anal-

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[4] Nonstandard abbreviations: LR11, LDL receptor relative with 11 ligand-binding repeats; SMC, smooth muscle cells; sLR11, soluble form of LR11; AD, Alzheimer disease; MAb, monoclonal antibody; CSF, cerebrospinal fluid; RAP, receptor-associated protein; PBST, PBS with Tween.

ysis of the gene for LR11 [sortilin-related receptor, L(DLR class) A repeats-containing (*SORL1*)] has been used to predict AD onset *(14, 15)*.

In this study, we produced specific monoclonal antibodies (MAbs) that bind to intact sLR11 without prior purification. Using these antibodies, we developed a novel sandwich ELISA method to quantify circulating sLR11 concentrations in both serum and cerebrospinal fluid (CSF). This technique provides a means for quantifying sLR11 to be used potentially as a marker for atherosclerosis and a predictor of AD and other neurodegenerative diseases.

Materials and Methods

BIOLOGICAL SAMPLES

Human and animal sera were purchased from Tennessee Blood Services and Cosmo Bio, respectively. Commercial human CSF samples (n = 13) were obtained from Scipac. To evaluate the normal concentration range of circulating sLR11, human serum was obtained from 87 healthy normolipidemic individuals (41 males and 46 females), who gave informed consent for participation in this study, which was approved by the Human Investigation Review Committee of the Chiba University Graduate School of Medicine.

EXTRACTION OF sLR11 WITH A RECEPTOR-ASSOCIATED PROTEIN AFFINITY RESIN

Recombinant human receptor-associated protein (RAP) was prepared as a glutathione *S*-transferase fusion protein *(16)* and applied to a glutathione-Sepharose resin (GE Healthcare), which was used to extract sLR11 from serum. Briefly, samples were incubated overnight at 4 °C at a RAP affinity resin–to–sample volume ratio of 1:20, and the resin was packed into a separation column. The column was washed with 20 mmol/L Na,K-phosphate buffer (pH 7.2) containing 150 mmol/L NaCL, and sLR11 was eluted with 50 mmol/L sodium citrate buffer (pH 5.0) containing 150 mmol/L NaCl. The sLR11 from cultured human IMR32 cells was extracted with a RAP affinity resin as described previously *(5)*.

PREPARATION OF MAb BY SYNTHETIC PEPTIDE IMMUNIZATION

Anti-LR11 MAb for use in the immunoblot analyses of human and animal sera was prepared by immunizing mice with a synthetic peptide (SMNEENMRSVITFDKG) corresponding to amino acid residues 432–447 of LR11 *(2, 17)* coupled to keyhole-limpet hemocyanin. The peptide–keyhole-limpet hemocyanin complex was emulsified with complete Freund's adjuvant (Gibco) and subcutaneously injected into BALB/c mice, 4 times at 2-week intervals. The spleen cells extracted from the immunized mice were fused with mouse myeloma cells (Sp2/0) in the presence of 50% polyethylene glycol. A single clone was selected to yield MAb A2-2-3 (IgG1,k), which reacted with both human and rabbit sLR11 in immunoblot analyses.

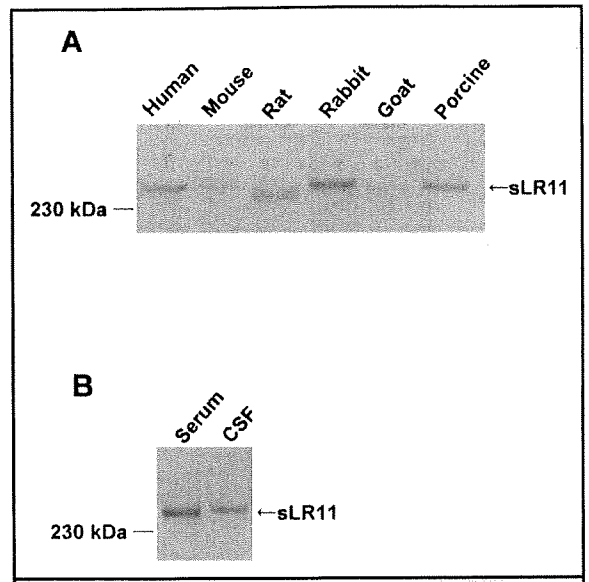

Fig. 1. Identification of sLR11 in serum and CSF.
(A), Samples (50 μL) extracted from human and animal sera by RAP affinity resin were separated by SDS-PAGE (2%–15% gradient) under reducing conditions. The sLR11 was detected by using an immunoblot assay with MAb A2-2–3. (B), Human CSF (10 μL) was separated by SDS-PAGE (2%–15% gradient) under reducing conditions, with RAP affinity-treated human serum as a control. The sLR11 protein was detected as above. A representative photo is shown. A 230-kDa marker is shown at the left in both panels.

IMMUNOBLOT ANALYSIS

Before immunoblot analysis, serum proteins were boiled in SDS-Tris buffer, with or without β-mercaptoethanol (reducing or nonreducing condition, respectively), and then separated by SDS-PAGE and transferred to a polyvinylidene difluoride membrane (Millipore). The membrane was blocked with 1% BSA in PBS containing 0.05% Tween 20 (PBST), incubated with MAb A2-2–3, reacted with horseradish peroxidase–conjugated rabbit antimouse IgG using a VECTASTAIN ABC kit (Vector Laboratories) according to the manufacturer's instructions, and subsequently stained with diaminobenzidine.

PREPARATION OF MAbs BY DNA IMMUNIZATION

Anti-LR11 MAbs for the sandwich ELISA were prepared via DNA immunization at Nosan Corporation *(18–20)*. Briefly, cDNA encoding amino acid resi-

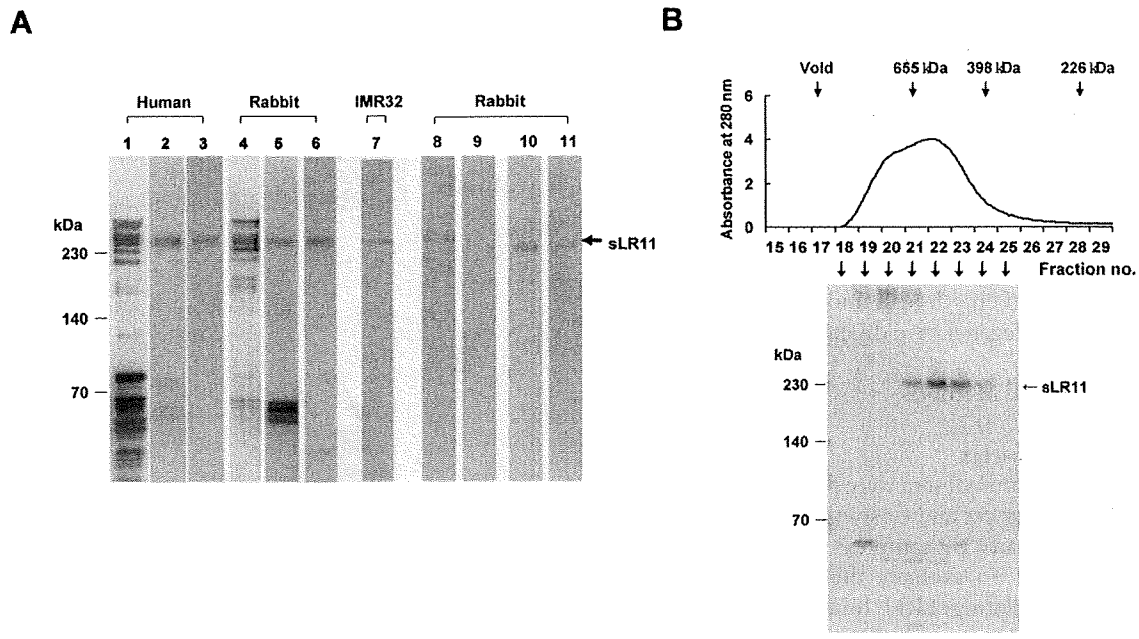

Fig. 2. Purification of sLR11 from human and rabbit serum.

(A), Samples extracted from a RAP affinity column with (lanes 2, 3, 5, 6, and 8–11) or without (lanes 1, 4, and 7) subsequent purification using an MAb-M3 affinity column were separated by SDS-PAGE (2%–15% gradient) under reducing (lanes 1–9) or nonreducing (lanes 10 and 11) conditions. Following electrophoresis, the samples were silver stained (lanes 1, 2, 4, and 5), or subjected to immunoblot analysis using MAb A2-2–3 (lanes 3, 6, and 7), R14 (lanes 8 and 10), or M3 (lanes 9 and 11). Lanes 1–3, human serum; lanes 4–6 and 8–11, rabbit serum; lane 7, human IMR32 cells. (B), Purified rabbit sLR11 protein was fractionated by gel filtration chromatography (HiLoad Superdex 200), and absorbance was monitored at 280 nm. The eluted proteins in each fraction were separated by SDS-PAGE under nonreducing conditions, and then silver stained. Markers at 230, 140, and 70 kDa are shown at the left.

dues 1000–1550 of LR11 *(2, 17)* was cloned into an expression plasmid (in-house vector, Nosan), and we immunized BALB/c mice or Wistar rats by intradermal application of DNA-coated gold particles, using a hand-held device for particle bombardment (Gene Gun, Bio-Rad). Antibody-producing cells were isolated and fused with Sp2/0 myeloma cells by use of polyethylene glycol, according to standard procedures. Five mouse and 5 rat MAbs were selected based on their reactivity with extracted rabbit sLR11, and preliminary sandwich ELISAs were performed using various combinations of these MAbs and A2-2–3. Mouse MAb M3 (IgG2a,k) and rat MAb R14 (IgG2b,k) were identified as the most sensitive for rabbit and human sLR11, respectively, and the combination of these antibodies gave the strongest reactivity against serum sLR11 in our ELISA system. Rat MAb R14 was then conjugated with sulfo-NHS-LC-biotin (Pierce), according to the manufacturer's instructions.

PURIFICATION OF sLR11 FROM HUMAN AND RABBIT SERA

The RAP affinity resin described above was used to extract sLR11 from 2.5 L of human serum or 1.0 L of rabbit serum. The eluted proteins were concentrated and applied to a HiLoad Superdex 200 gel filtration column (GE Healthcare) equilibrated with PBS. The fractions containing immunologically detected sLR11 were pooled, concentrated, and incubated overnight at room temperature with anti-LR11 MAb M3-Sepharose resin. After the resin was rinsed with PBS, immunologically bound sLR11 was eluted with 100 mmol/L sodium citrate buffer (pH 3.0). The sLR11 content was quantified by comparison with BSA standards on silver-stained gels.

SANDWICH ELISA

The wells of a polystyrene microtiter plate (Nunc) were coated with 100 μL of MAb M3 (10 mg/L in PBS) and incubated for 2 h. After extensive washing with PBST, the wells were blocked by incubation with 200 μL of

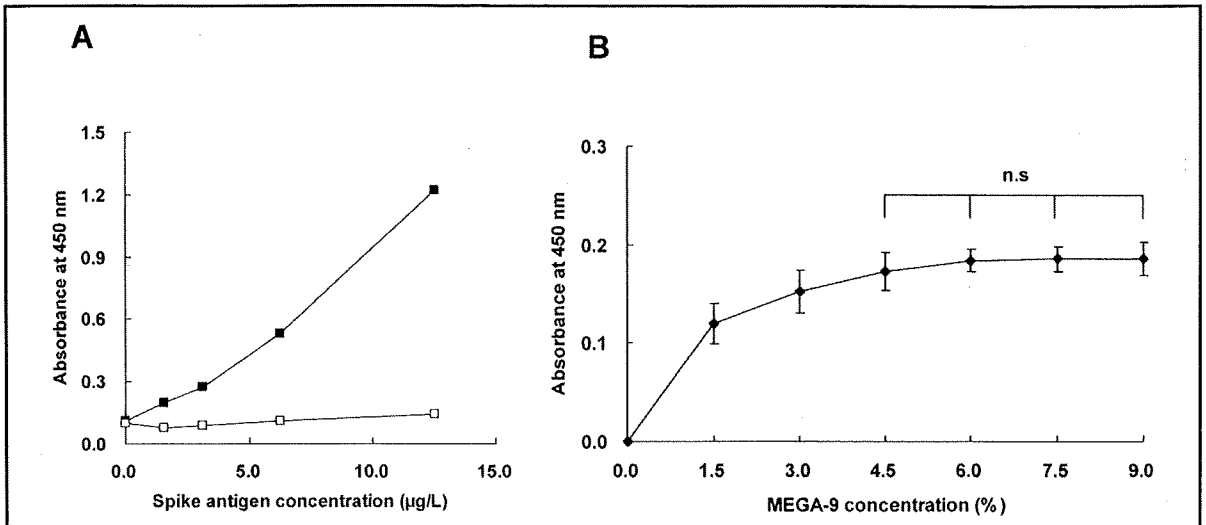

Fig. 3. The effect of MEGA-9 on ELISA measurements of sLR11 in the presence or absence of human serum.
(A), Extracted human sLR11 was measured by ELISA; sLR11 samples were diluted with PBS in the presence (□) or absence (■) of 10% human serum. (B), The sLR11 concentrations were quantified by ELISA (n = 8) in the sample buffer containing 10% human serum in the presence of various concentrations of MEGA-9. n.s., not significant.

1% BSA-PBST for 1 h. The samples (10 μL) were diluted with 100 μL of sample buffer, which consisted of 5.25% n-nonanoyl-N-methyl-d-glucamine (MEGA-9; Dojindo) and 25% heterophilic blocking reagent (Scantibodies Laboratory) in PBS. The calibration samples (0–4.0 μg/L rabbit sLR11) serially diluted in sample buffer together with the above diluted samples (100 μL) were placed into wells and then incubated for 6–16 h. After extensive washing with PBST, 100 μL of biotinylated MAb R14 was added to each well, and the plate was subsequently incubated for 4 h. After extensive washing with PBST, the LR11-MAb complex was reacted with horseradish peroxidase–conjugated streptavidin (Pierce) for 1 h. The trapped complexes were washed and incubated with 100 μL of substrate solution (tetramethyl-benzidine in citrate buffer, pH 3.65, containing hydrogen peroxide) for 30 min. The chromogenic reaction was stopped with 100 μL H_2SO_4, and the absorbance of each sample was determined at 450 nm. All steps were performed at room temperature. ELISA data (μg/L) were significantly and positively correlated with the immunoblotting data (U) previously observed following purification with RAP affinity chromatography ($r = 0.781$, $P < 0.001$, $y = 1.31x + 8.34$) *(6)*.

STATISTICAL ANALYSIS

Statistical analyses were performed with commercial software (Stat Flex, Ver. 5.0). The effect of sample dilution with various concentrations of MEGA-9 on the ELISA results was examined using a paired *t*-test, with $P < 0.05$ considered significant. The correlation between variables was evaluated using Pearson correlation analysis. Furthermore, sLR11 concentrations in individuals with atherosclerosis vs those in normal individuals were compared by use of box plot analysis.

Results

IDENTIFICATION OF sLR11 IN VARIOUS SERA AND HUMAN CSF

sLR11 was isolated as a 250-kDa protein from rabbit SMCs and human IMR32 cells by use of immunoblot techniques under reducing conditions and an antibody against a recombinant protein corresponding to a partial amino acid sequence of rabbit LR11 *(5)*. For comparison, sLR11 was extracted from both human and animal sera, using RAP–glutathione *S*-transferase resin. A single 250-kDa protein was detected in human serum by use of MAb A2-2–3 (Fig. 1A). The migration distance of the protein during electrophoresis was consistent with that of sLR11 from rabbit SMCs and human IMR32 cells *(5)*. A single protein band, similar in size to that obtained from human serum, was detected immunologically by MAb A2-2–3 in mouse, rat, rabbit, goat, and porcine sera. The relative intensities of the immunological signals suggested that sLR11 was most abundant in rabbit serum.

We also assessed the presence of sLR11 in human CSF (Fig. 1B), where it was identified by MAb A2-2–3 without the need for RAP extraction. Although sLR11

obtained from CSF was slightly larger than that in serum, the protein appeared as a single band at 250-kDa in both cases.

PURIFICATION OF sLR11 FROM HUMAN AND RABBIT SERA

Using DNA immunization, we established 2 MAbs, M3 and R14, against different epitopes of human sLR11; these MAbs were then used to purify intact sLR11 from serum and construct a sandwich ELISA assay. Intact sLR11 protein was first purified from human and rabbit sera using RAP affinity resin and was released from the resin with an eluting buffer, without decoupling from RAP–glutathione *S*-transferase (Fig. 2A, lanes 1 and 4). The eluted samples were treated with anti-LR11 MAb M3-Sepharose resin. Silver staining after electrophoresis indicated that the M3-reactive samples contained sLR11 as a single protein at 250 kDa as well as low molecular weight proteins, in both human and rabbit sera (lanes 2 and 5). The purified sLR11, but no other low molecular weight protein, was specifically bound to MAb A2-2–3 (lanes 3 and 6). The migration distance of sLR11 from human and rabbit sera was not different from that of sLR11 in the culture medium of IMR32 cells (lane 7). Therefore, 2-step affinity chromatography with RAP and MAb M3 can be used to specifically purify serum sLR11 as a soluble protein identical to that released from cultured cells. R14, as well as A2-2–3 and M3, showed reactivity against the purified sLR11, but did not bind any other low molecular weight protein (lanes 8–11). Notably, R14 reacted with sLR11 under both reducing and nonreducing conditions, whereas M3 reacted with sLR11 under nonreducing condition only.

Silver staining of the purified protein after gel filtration chromatography showed that the position to which purified sLR11 eluted corresponded to an estimated molecular weight >398 kDa (Fig. 2B). Notably, no other distinct protein proportional to the level of the stained sLR11 protein was detected in these fractions. The apparent molecular weight of sLR11 estimated from gel filtration was greater than that determined by use of gel electrophoresis (see Figs. 1 and 2A).

PREPARATION OF SAMPLES WITH MEGA-9 FOR SANDWICH ELISA

Sample conditions for the sandwich ELISA were determined using the above MAbs and purified samples. The absorbance level of immunologically detected sLR11 was proportional to the volume of extracted human sLR11 when diluted with PBS. However, the expected change in absorbance was not observed when samples were diluted with human serum instead of PBS (Fig. 3A). To measure sLR11 in human serum accurately, the matrix effects were mitigated by the addition of MEGA-9 detergent. The effects of MEGA-9 on absorbance recovery increased with increasing amounts

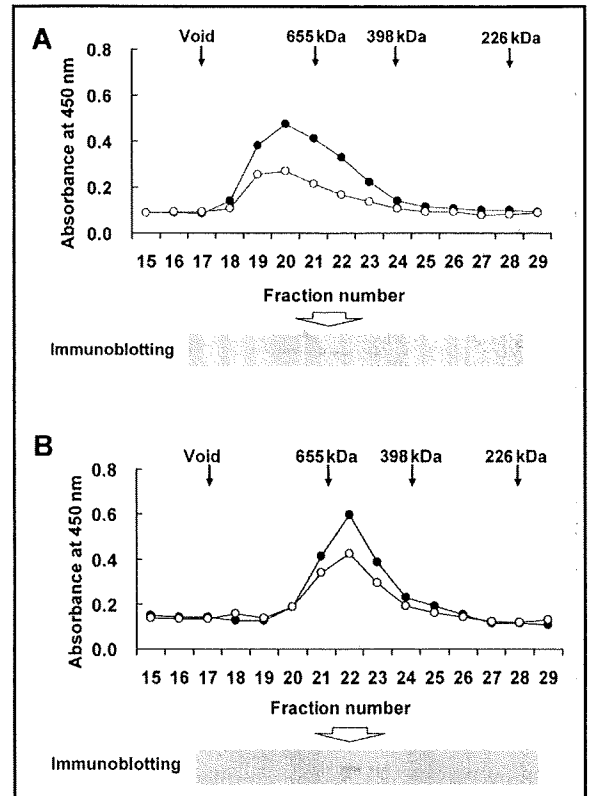

Fig. 4. Gel filtration elution profiles of sLR11 in human serum and CSF.

Human serum (A) and pooled CSF (B) samples (2 mL each) were fractionated by gel filtration chromatography (HiLoad Superdex 200), and the sLR11 concentration in each fraction was measured by ELISA in the presence (●) or absence (○) of MEGA-9. The amount of sLR11 in each fraction was also visualized by immunoblotting after extraction with the RAP affinity resin.

of MEGA-9, up to 4.5%. No significant differences were observed at higher concentrations (Fig. 3B). These results suggest that human serum contains unknown factors that interfere with sLR11 quantification, and that this interference could be diminished by the presence of MEGA-9. Therefore, samples were diluted with 5.25%, which was chosen as the middle concentration of 4.5% and 6.0%.

CHARACTERIZATION OF sLR11 IN SERUM AND CSF BY GEL FILTRATION

To assess whether ELISA can specifically detect naturally occurring sLR11, each fraction of human serum and CSF was analyzed for sLR11, following to separation by gel filtration chromatography; the results were then compared with the immunologically purified

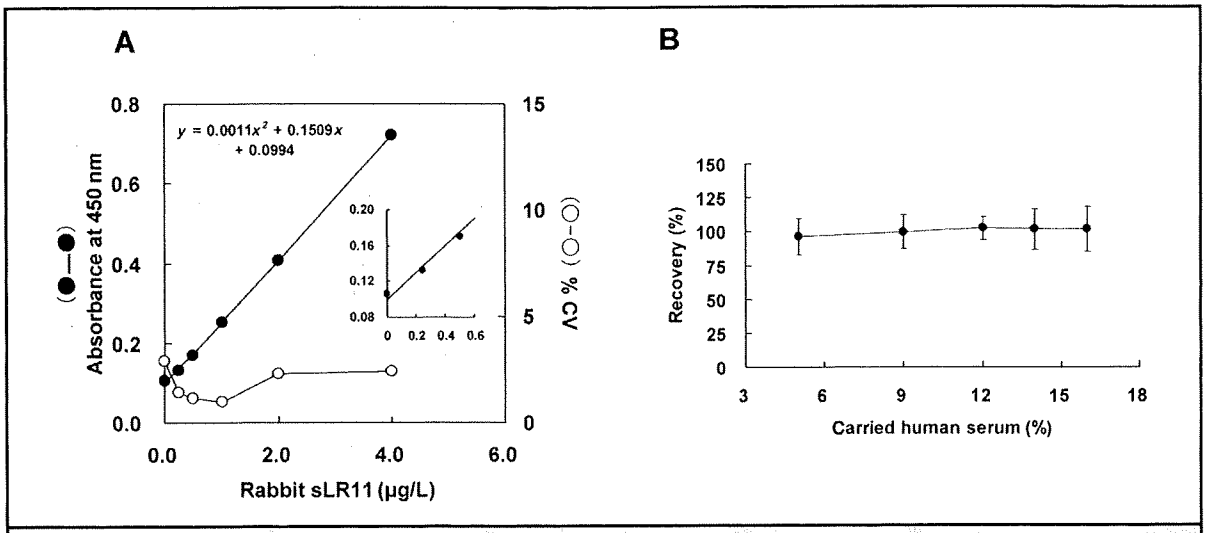

Fig. 5. Calibration curve and sample dilution test.

(A), Using the ELISA system developed in this study, we constructed a typical calibration curve (●) for sLR11 extracted from rabbit serum and the % CV of each point (○). Imprecision was assessed based on 5 replicates of the calibration curve. The insert graph is a close-up of the low-concentration area of the calibration curve. (B), Percentage recovery of purified sLR11 (2 μg/L) measured in the presence of various concentrations of human serum (5%–16%) in sample buffer. The concentration of each sample was measured using ELISA, and the percentage recoveries were calculated as a ratio of the actual-to-theoretical sLR11 concentrations.

sLR11 protein as a quantitative calibrator. The ELISA of serum and CSF samples that had been diluted with or without MEGA-9 showed abundant sLR11 in fractions with molecular weights >398 kDa (Fig. 4), similar to the results of the purified protein (see Fig. 2B). Immunoblot analyses showed that the concentration of sLR11 was proportional to the signal intensity of the gel-filtered 250-kDa proteins in both the serum and CSF samples. These results strongly suggest that this ELISA based on the immunologically purified 250-kDa sLR11 protein is also appropriate for quantifying the naturally occurring sLR11 in serum and CSF, although the gel filtration analyses suggested that the naturally occurring protein may be involved in a high molecular weight complex.

ELISA PERFORMANCE: ASSAY CHARACTERISTICS

A representative calibration curve is shown in Fig. 5A. The working range of this ELISA was 0.25–4.0 μg/L. A quadratic equation was applied to the calibration curve in the working range. The sensitivity, defined as the mean back-fit value for the lowest standard giving acceptable precision (CV = 10%), was 0.25 μg/L. With this ELISA method the lower limit of detection for sLR11 was 0.1 μg/L, which corresponds to the mean blank signal plus 3 SDs. The intraassay CVs (n = 10) were 3.0% and 3.7% at sLR11 concentrations of 7.6 μg/L in serum and 4.4 μg/L in CSF, respectively. The interassay CVs (n = 4) were 3.9% and 10.5% at sLR11 concentrations of 7.6 μg/L in serum and 4.1 μg/L in CSF, respectively. When we used samples containing 5%–16% human serum, percentage recovery ranged from 96.5% to 102.6% (Fig. 5B).

VARIATIONS OF sLR11 IN SERUM AND CSF

Measurements of sLR11 in 87 serum samples and 13 CSF samples obtained from normal individuals gave mean (SD) sLR11 concentrations of 8.7 (2.1) μg/L (range, 4.5–14.2 μg/L) and 8.5 (3.5) μg/L (range, 3.7–13.0 μg/L) in serum and CSF, respectively. We observed no significant difference in serum sLR11 concentrations between males [8.4 (1.9) μg/L, n = 41] and females [9.8 (5.8) μg/L, n = 46].

sLR11 CONCENTRATIONS IN INDIVIDUALS WITH ATHEROSCLEROSIS

To evaluate whether this ELISA method is useful for detecting variation in circulating sLR11 under pathophysiological conditions, we measured sLR11 concentrations in individuals with atherosclerosis. The sLR11 concentrations determined by immunoblotting after RAP affinity chromatography were positively correlated with the degrees of atherosclerosis in the carotid arteries of individuals with dyslipidemia *(6)*. The sLR11 concentrations in individuals with atherosclerosis were compared to those of healthy individuals (see

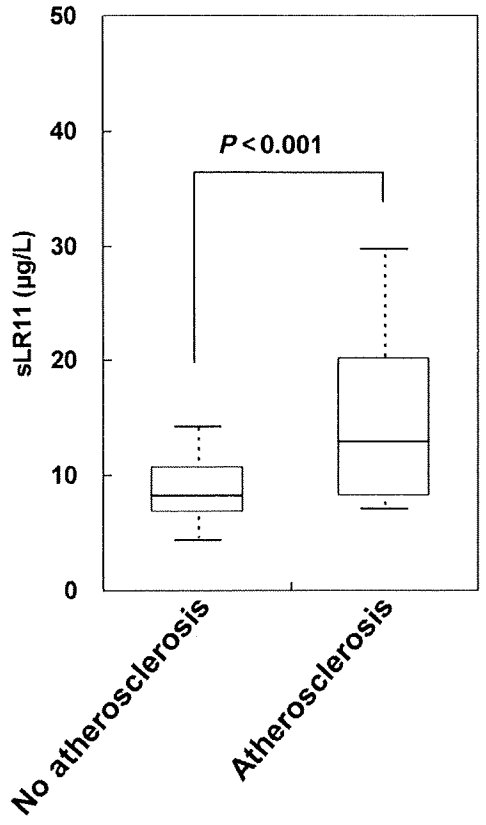

Fig. 6. Circulating sLR11 concentrations in individuals with atherosclerosis vs those in individuals without atherosclerosis.

Center horizontal lines indicate median values. Upper and lower edges of boxes, mean ± 1 SD; upper and lower bars, maximum and minimum.

previous section on variation of sLR11 in serum and CSF). The sLR11 concentrations [14.2 (6.0) µg/L] in individuals with atherosclerosis were significantly higher than those in healthy individuals (Fig. 6). The variation in circulating sLR11 concentrations in individuals with atherosclerosis was within the dynamic range of the ELISA.

Discussion

Three MAbs were established against different epitopes of human sLR11, and an ELISA method was developed for the quantitative measurement of sLR11 in serum and CSF. One of the MAbs (M3), in combination with RAP affinity extraction, enabled the purification of sLR11 from human and rabbit sera. The purified human and rabbit sLR11 was immunologically identical to sLR11 released from cultured cells, strongly suggesting that circulating sLR11 corresponds to the soluble form of membrane-bound LR11. This soluble form has been identified in the media of IMR32 and SMC cultures *(5, 6, 9)*. The combination of MAb M3 and MAb R14 yielded an ELISA that is highly specific for sLR11 in serum and CSF, without the need for prior RAP affinity extraction.

Strong matrix effects interfered with the accurate determination of sLR11 in serum by ELISA. However, these effects were diminished by pretreatment with MEGA-9 detergent (see Fig. 3). This pretreatment may dissociate complexes of sLR11 and serum components or may induce a conformational change in sLR11 such that it more efficiently interacts with the MAbs. Previous studies have shown that several serum components, including apolipoprotein E–containing lipoproteins, urokinase plasminogen activator–plasminogen activator inhibitor type 1 complex, and amyloid-β, can interact with membrane-bound LR11 *(1, 16, 21)*. The observation that MEGA-9 increased the absorbance of the isolated protein at 450 nm in gel filtration fractions obtained from both serum and CSF, and did so in proportion to the signal intensity of the sLR11 protein detected immunologically in the absence of MEGA-9 (see Fig. 4), suggests that epitope recognition by MAbs was strengthened by MEGA-9. The mechanism of MEGA-9–mediated absorbance enhancement requires further elucidation, specifically with regard to the interaction between naturally occurring sLR11 and various matrices in serum and with homomeric or heteromeric complexes under various column conditions (see Fig. 2B).

Using the established ELISA conditions, we investigated the mean sLR11 concentrations in serum and CSF. In 74% of healthy individuals, serum sLR11 concentrations were <10 µg/L. The sLR11 concentrations in the sera of individuals with atherosclerosis ranged from 6 to 30 µg/L. Therefore, the ELISA technique described here provides sufficient sensitivity for detecting circulating sLR11 concentrations in individuals with atherosclerosis and in normal populations.

Given that sLR11 is abundantly expressed in intimal SMCs *(3)* and that circulating sLR11 concentrations are positively correlated with the carotid intima-media thickness in dyslipidemic individuals *(6)*, variation in the circulating sLR11 concentration may be indicative of the condition of intimal SMCs. Metabolic disorders such as dyslipidemia and diabetes can cause pathological changes in intimal SMC function, possibly leading to accelerated progression of atherosclerosis *(22–24)*. The expression level of LR11 is drastically higher in intimal SMCs relative to that in medial SMCs *(3)*, and a large proportion of the LR11 in the cell membrane is released into the culture medium of

SMCs *(5)*. Therefore, the concentration of circulating sLR11, rather than the LR11 expression level in intimal SMCs, may be more effective as a novel marker for pathogenic changes in SMCs.

Recent studies have highlighted the pathological function of sLR11 in neurodegenerative diseases. Immunological analyses indicate that sLR11 exists in CSF at concentrations similar to those in serum. Neuronal LR11 expression is significantly reduced in individuals with mild cognitive impairment and AD *(10–13)*, and polymorphism of the gene for LR11 is highly associated with the onset of AD *(14)*. Therefore, methods for determining sLR11 concentrations in CSF may be vital for future research into neuronal diseases, particularly AD.

In conclusion, we established a sensitive ELISA method for determining sLR11 concentrations in serum and CSF. This ELISA method constitutes a useful tool for monitoring the pathological condition of intimal SMCs and the progression of atherosclerosis *(25)*. Use of this ELISA method to measure sLR11 as an indicator of intimal SMC function may enable novel strategies for treating atherosclerosis and help to determine risk factors for vascular disease. Furthermore, the ELISA described here has adequate sensitivity and dynamic range for determining sLR11 concentrations in CSF and may allow significant progress in AD-related research.

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Plasma preβ1-HDL level is elevated in unstable angina pectoris

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ABSTRACT

Preβ1-HDL, a minor HDL subfraction consisting of apolipoprotein A-I (apoA-I), phospholipids and unesterified cholesterol, plays an important role in reverse cholesterol transport. Plasma preβ1-HDL levels have been reported to be increased in patients with coronary artery disease (CAD) and dyslipidemia. To clarify the clinical significance of measuring plasma preβ1-HDL levels, we examined those levels in 112 patients with CAD, consisting of 76 patients with stable CAD (sCAD) and 36 patients with unstable angina pectoris (uAP), and in 30 patients without CAD as controls. The preβ1-HDL levels were determined by immunoassay using a specific monoclonal antibody (Mab55201) that we established earlier. The mean preβ1-HDL level in the CAD patients was significantly higher than the level in the controls (34.8 ± 12.9 mg/L vs. 26.6 ± 6.9 mg/L, $p < 0.001$). In addition, the mean preβ1-HDL level was markedly higher in the uAP subgroup than in the sCAD subgroup (43.1 ± 11.5 mg/L vs. 30.9 ± 11.7 mg/L, $p < 0.0001$). These tendencies remained even after excluding dyslipidemic subjects.

These results suggest that elevation of the plasma preβ1-HDL level is associated with the atherosclerotic phase of CAD and may be useful for identifying patients with uAP.

1. Introduction

Preβ1-HDL, an HDL subfraction consisting of one or two molecules of apolipoprotein A-I (apoA-I), small amounts of phospholipids and unesterified cholesterol, plays an important role in reverse cholesterol transport, although it comprises only 1–5% of total apoA-I in blood plasma [1–4]. The initial step of reverse cholesterol transport, called cholesterol efflux, is a reaction by which excessively accumulated cholesterol in peripheral tissues is removed by HDL. Preβ1-HDL is known as the initial plasma acceptor of cell-derived cholesterol [1–5].

Three pathways have been suggested as routes by which preβ1-HDL is generated [6–11]. The first is a pathway in which preβ1-HDL is formed when lipid-free apoA-I or lipid-poor apoA-I removes cell-derived, unesterified cholesterol, mediated by ATP-binding cassette transporter A1 (ABCA1) located on cell membranes [6–8]. The second is a pathway in which preβ1-HDL is directly secreted from the liver [9,10], and the third is a pathway in which preβ1-HDL is released from α-migrating HDL during its remodeling [11]. As the catabolic pathway of preβ1-HDL, a lecithin–cholesterol acyltransferase (LCAT)-dependent conversion pathway has been suggested. The unesterified cholesterol on the preβ1-HDL is esterified by LCAT, and the preβ1-HDL removes cellular cholesterols, increases in size and is converted to an α-migrating HDL [12–14].

Thus, preβ1-HDL is proposed to be a key component of reverse cholesterol transport. We previously reported development of a monoclonal antibody (Mab55201) specifically recognizing an epitope of apoA-I that is exposed only in preβ1-HDL, and we established an ELISA system for direct measurement of preβ1-HDL using Mab55201 [15]. The method provides a way to investigate the clinical significance of measuring plasma preβ1-HDL levels.

Plasma preβ1-HDL levels have been reported to be elevated in patients with coronary artery disease (CAD) and dyslipidemia [15–17]. However, the mechanism responsible for elevation of the preβ1-HDL level has not been clarified. In this study we examined whether the preβ1-HDL level is elevated in normolipidemic CAD patients and whether the levels differ between patients with unstable angina pectoris (uAP) and those with stable CAD (sCAD), including stable effort angina pectoris and old myocardial infarction. In addition, we studied the relationship between

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Table 1
Baseline characteristics of the study subjects.

	CAD (n = 112)	p^*	Normolipidemic CAD (n = 37)	p^*	Controls (n = 30)
Age (years)	65.8 ± 9.1	n.s.	65.5 ± 9.0	n.s.	66.3 ± 8.3
BMI (kg/m^2)	24.0 ± 2.7	n.s.	24.1 ± 3.4	n.s.	23.3 ± 2.9
SBP (mmHg)	127 ± 18.8	n.s.	123 ± 19.7	<0.05	134 ± 14.2
DBP (mmHg)	74.1 ± 11.7	n.s.	71.7 ± 12.5	<0.05	77.9 ± 9.7
HbA1c (%)	7.3 ± 1.4	<0.05	7.1 ± 1.4	n.s.	6.4 ± 1.2
T-Cho (mg/dL)	203 ± 41.4	n.s.	181 ± 22.9	n.s.	193 ± 20.3
TG (mg/dL)	142 ± 77.9	<0.001	92.2 ± 25.4	n.s.	85.3 ± 29.3
LDL-C (mg/dL)	131 ± 34.7	0.051	110 ± 21.7	n.s.	115 ± 16.1
HDL-C (mg/dL)	43.9 ± 9.4	<0.0001	49.8 ± 8.6	<0.05	55.2 ± 11.2
ApoA-I (mg/dL)	115 ± 18.5	<0.0001	123 ± 16.6	<0.01	137 ± 21.0
ApoB (mg/dL)	108 ± 27.3	<0.01	90.0 ± 16.1	n.s.	93.4 ± 12.3
Creatinine (mg/dL)	0.93 ± 0.32	<0.01	0.93 ± 0.34	<0.01	0.73 ± 0.18
BUN (mg/dL)	17.7 ± 7.3	n.s.	16.8 ± 4.0	n.s.	15.4 ± 4.0
AST (IU/L)	37 ± 19.5	n.s.	38.1 ± 16.3	n.s.	32.0 ± 11.0
ALT (IU/L)	27.4 ± 20.2	<0.05	29.1 ± 22.8	<0.05	19.0 ± 9.0
ChE (U/L)	325 ± 77.6	n.s.	308 ± 77.9	n.s.	316 ± 67.2
Medications, n (%)					
Aspirin	90 (80.4%)	<0.0001	28 (75.7%)	<0.0001	2 (6.7%)
Nitrates	87 (77.7%)	<0.0001	24 (64.9%)	<0.0001	1 (3.3%)
Calcium channel blockers	51 (45.5%)	n.s.	17 (45.9%)	n.s.	13 (43.3)
Beta blockers	37 (33.0%)	n.s.	10 (27.0%)	n.s.	5 (16.7%)
ACE inhibitors	26 (23.2%)	n.s.	8 (21.6%)	n.s.	3 (10.0%)
Diuretics	10 (8.9%)	n.s.	3 (8.1%)	n.s.	1 (3.3%)
Digoxin	6 (5.4%)	n.s.	0 (0.0%)	n.s.	1 (3.3%)
ARBs	2 (1.8%)	n.s.	1 (2.7%)	n.s.	1 (3.3%)
Antiplatelet	27 (24.1%)	<0.01	9 (24.3%)	<0.01	0 (0.0%)
Antidiabetics	45 (40.2%)	n.s.	13 (35.1%)	n.s.	9 (30.0%)
Lipid-lowering agents	3 (2.7%)	n.s.	1 (2.7%)	n.s.	0 (0.0%)

Data are shown as mean ± S.D. or number. ARBs: angiotensin II receptor blockers.
* Significance vs. controls.

the preβ1-HDL level and lipid metabolism by examining for correlations between the preβ1-HDL level and the concentrations or activities of various lipid metabolic markers.

2. Methods

2.1. Study subjects

One hundred and twelve coronary artery disease patients were recruited from inpatients and outpatients of Chiba Cardiovascular Center (Chiba, Japan). The diagnosis of CAD was based on a history of myocardial infarction, clinical symptoms including prolonged chest pain, and the presence of angiographically demonstrated stenosis (≥75% obstructive lesions). The CAD group was divided into 36 patients with uAP and 76 patients with sCAD based on the clinical symptoms. uAP was diagnosed in accordance with the American Heart Association (AHA) classification (1975): the presence of chest pain which began during the previous 3 weeks and most recently occurred within the previous 1 week; and the absence of both ST segment elevation on the electrocardiogram and serum biochemical markers of cardiac necrosis. All patients with uAP belonged to class I or II in severity and class B or C in the clinical circumstances according to Braunwald's classification (1989). The sCAD subgroup was composed of 32 patients with stable effort angina pectoris and 44 patients with old myocardial infarction. We also enrolled 30 age- and BMI-matched subjects as the control group. The control subjects were recruited from outpatients of Chiba Cardiovascular Center and included type 2 diabetics and/or hypertension patients without dyslipidemia and no history of CAD. The control subjects were all confirmed to have no cardiac disorders on the exercise-loaded electrocardiogram. Normolipidemic subjects in the CAD group and the control group were determined on the basis of the concentrations of four serum lipid markers, i.e., total cholesterol (T-Cho) <220 mg/dL, LDL-cholesterol (LDL-C) <140 mg/dL, triglyceride (TG) <150 mg/dL, and HDL-cholesterol (HDL-C) ≥40 mg/dL. Patients with renal and/or liver dysfunction were excluded from this study.

We obtained informed consent from all participants at entry. This study was conducted in accordance with the Declaration of Helsinki of the World Medical Association.

2.2. Blood collection

Venous blood samples for plasma and serum were drawn from the subjects after fasting for one night. The blood samples for plasma were drawn into plastic tubes containing EDTA-2Na, immediately chilled in ice water and centrifuged at 2 °C. The plasma was diluted with 20 volumes of 50% sucrose solution for stabilization and then stored at −80 °C until preβ1-HDL was assayed. The blood samples for serum were separated and stored at −80 °C until assay for serum lipids, apolipoproteins, LCAT activity and other markers of liver or renal function.

2.3. Measurement of preβ1-HDL and biochemical parameters

Preβ1-HDL levels were measured by a sandwich enzyme immunoassay using Mab55201 [15,18]. The preβ1-HDL level was expressed as both an absolute value and a relative value. The absolute value indicates the preβ1-HDL concentration (mg/L) in the plasma, and the relative value indicates the percentage of preβ1-HDL in the total apolipoprotein A-I in the plasma.

The T-Cho, TG, LDL-C, HDL-C, creatinine, blood urea nitrogen (BUN), aspartate transaminase (AST), alanine transaminase (ALT) and cholinesterase (ChE) concentrations were determined enzymatically using an automated analyzer. Apolipoprotein concentrations were determined by immunoturbitometry with commercial reagents from Daiichi Pure Chemicals (Tokyo, Japan), using an automated analyzer. Hemoglobin A1c (HbA1c) was determined by an automated liquid-chromatographic system. LCAT

Table 2
Comparisons of preβ1-HDL levels between CAD and control groups and between uAP and sCAD subgroups.

	n	Preβ1-HDL (mg/L)	p	Preβ1-HDL/apoA-I (%)	p
CAD	112	34.8 ± 12.9	<0.001*	3.05 ± 1.04	<0.0001*
Normolipidemic CAD	37	36.2 ± 12.8	<0.001*	2.94 ± 0.95	<0.0001*
CAD (HDL-C ≥ 40 mg/dL)	74	36.9 ± 13.1	<0.0001*	2.98 ± 0.97	<0.0001*
CAD (high LCAT**)	28	36.5 ± 12.3	<0.001*	3.15 ± 0.93	<0.0001*
Controls	30	26.6 ± 6.9	–	1.97 ± 0.51	–
uAP	36	43.1 ± 11.5	<0.0001	3.66 ± 0.95	<0.0001
sCAD	76	30.9 ± 11.7		2.76 ± 0.95	
Normolipidemic					
uAP	16	43.1 ± 9.1	<0.01	3.42 ± 0.76	<0.01
sCAD	21	30.9 ± 12.8		2.58 ± 0.94	
HDL-C ≥ 40 mg/dL					
uAP	26	44.9 ± 11.6	<0.0001	3.54 ± 0.87	<0.001
sCAD	48	32.6 ± 11.9		2.68 ± 0.89	
HDL-C < 40 mg/dL					
uAP	10	38.3 ± 10.3	<0.05	4.00 ± 1.10	<0.01
sCAD	28	28.1 ± 11.0		2.90 ± 1.05	

Data are shown as mean ± S.D.
* Significance vs. controls.
** LCAT activity ≥80 nmol/mL/h/37 °C.

activities were determined only in 58 randomly selected CAD (9 uAP and 49 sCAD) patients, by the method of Nagasaki and Akanuma using an endogenous substrate [19].

2.4. Statistics

Statistical analyses were performed using Stat Flex for Windows ver. 5.0 (Artech Inc., Osaka, Japan). The difference between two groups was assessed using Student's paired *t*-test. Categorical variables were compared using the χ^2-test. The relationship between two parameters was examined using Pearson's Correlation Coefficient. Receiver-operating characteristic (ROC) curves were plotted, and the area under the curve (AUC) was analyzed to compare the predictive powers of preβ1-HDL, HDL-C and LDL-C for uAP using the sCAD and the control group as reference groups. The AUC indicates the diagnostic accuracy of tests [20]. For all analyses, $p < 0.05$ was considered statistically significant.

Table 3
Comparisons of baseline characteristics between sCAD and uAP subgroups.

	uAP (n = 36)	sCAD (n = 76)	p
Age (years)	67.0 ± 8.8	65.3 ± 9.2	n.s.
BMI (kg/m²)	24.6 ± 3.4	23.8 ± 2.4	n.s.
SBP (mmHg)	129 ± 22.3	126 ± 16.9	n.s.
DBP (mmHg)	76.8 ± 13.5	72.9 ± 10.7	n.s.
HbA1c (%)	7.5 ± 1.3	7.3 ± 1.4	n.s.
T-Cho (mg/dL)	191 ± 41.8	209 ± 40.2	<0.05
TG (mg/dL)	129 ± 67.4	147 ± 82.1	n.s.
LDL-C (mg/dL)	118 ± 35.6	132 ± 33.5	<0.05
HDL-C (mg/dL)	44.4 ± 9.8	43.6 ± 9.2	n.s.
ApoA-I (mg/dL)	119 ± 20.3	112 ± 17.3	n.s.
ApoB (mg/dL)	100 ± 26.3	112 ± 27.0	<0.05
Creatinine (mg/dL)	0.96 ± 0.29	0.91 ± 0.34	n.s.
BUN (mg/dL)	17.7 ± 4.8	17.8 ± 8.2	n.s.
AST (IU/L)	39.8 ± 21.2	35.7 ± 18.7	n.s.
ALT (IU/L)	28.6 ± 20.6	26.9 ± 20.1	n.s.
ChE (U/L)	30.5 ± 89.8	335 ± 69.6	n.s.
LCAT activity (nmol/mL/h/37 °C)*	72.9 ± 21.0	72.9 ± 12.4	n.s.
Medications, n (%)			
Aspirin	28 (77.8%)	62 (81.6%)	n.s.
Nitrates	27 (75.0%)	60 (78.9%)	n.s.
Calcium channel blockers	16 (44.4%)	35 (46.1%)	n.s.
Beta blockers	12 (33.3%)	25 (32.9%)	n.s.
ACE inhibitors	10 (27.8%)	16 (21.1%)	n.s.
Diuretics	4 (11.1%)	6 (7.9%)	n.s.
Digoxin	1 (2.8%)	5 (6.6%)	n.s.
ARBs	0 (0.0%)	2 (2.6%)	n.s.
Antiplatelet	11 (30.6%)	16 (21.1%)	n.s.
Antidiabetics	9 (25.0%)	36 (47.4%)	<0.05
Lipid-lowering agents	1 (2.8%)	2 (2.6%)	n.s.

Data are shown as mean ± S.D. or number (%). ARBs: angiotensin II receptor blockers.
* Determined in 9 uAP and 49 sCAD patients.

3. Results

3.1. Comparison between CAD patients and controls

Table 1 shows the baseline characteristics of the study subjects. Age, BMI and blood pressure were comparable between the CAD and control groups. The concentrations of HbA1c, TG and apoB were significantly higher, and the concentrations of HDL-C and apoA-I were significantly lower, in the CAD group than in the control group. In the normolipidemic subjects, the only differences were that the concentrations of HDL-C and apoA-I were slightly higher in the control group. Medications were comparable between the CAD and control groups except for aspirin, nitrates and antiplatelet drugs. The absolute and relative values for the preβ1-HDL level were markedly higher in the CAD group than in the control group. These differences were also seen even in the normolipidemic subjects only (Table 2). We then compared the preβ1-HDL levels between the CAD subgroups with high HDL-C (≥40 mg/dL) or high LCAT activity (≥80 nmol/mL/h/37 °C) and the control group. The preβ1-HDL levels were markedly higher in both the CAD subgroups than in the control group (Table 2).

3.2. Comparison between uAP and sCAD subgroups

We divided the CAD group into a uAP subgroup and an sCAD subgroup and compared the preβ1-HDL levels between them. The absolute and relative values for the preβ1-HDL level were markedly higher in the uAP subgroup than in the sCAD subgroup. Moreover, even in the comparisons using only the normolipidemic subjects, only the high HDL-C (≥40 mg/dL) subjects and only the low HDL-C (<40 mg/dL) subjects, the differences remained significant between the two subgroups (Table 2). On the other hand, the concentrations of lipid markers, age, BMI, blood pressure, renal and hepatic function markers and medications did not differ between the uAP and

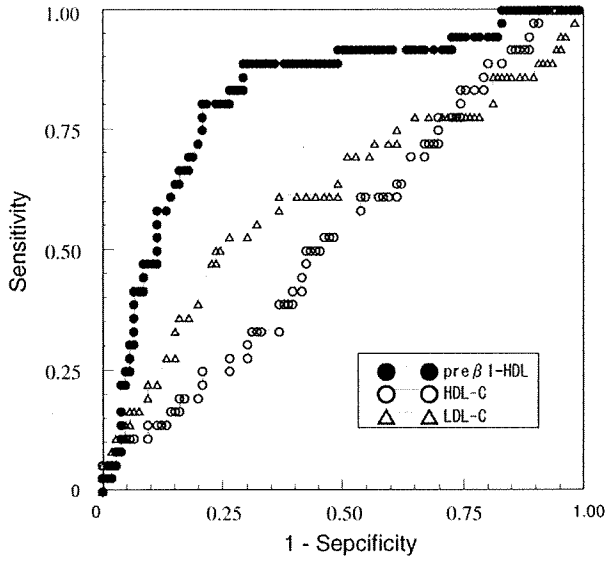

Fig. 1. ROC curves of preβ1-HDL, HDL-C, LDL-C for diagnosis of uAP. The true-positive rate (sensitivity as *y* axis) was plotted vs. the false-positive rate (1-specificity as *x* axis) by changing the cutoff values for the test. The areas under the curves were 0.821 (95% CI, 0.780–0.863) for preβ1-HDL, 0.536 (95% CI, 0.482–0.591) for HDL-C and 0.616 (95% CI, 0.557–0.675).

sCAD subgroups, except that the concentrations of T-Cho, LDL-C and apoB were slightly lower and that the patients taking antidiabetics were slightly fewer in number in the uAP subgroup (Table 3).

3.3. *Preβ1-HDL as a diagnostic marker of uAP*

ROC analyses were performed to evaluate preβ1-HDL as a diagnostic marker of uAP. The AUC of preβ1-HDL was significantly greater than that of either HDL-C or LDL-C (vs. HDL-C, $p < 0.0001$; vs. LDL-C, $p < 0.01$) (Fig. 1).

3.4. *Correlations between preβ1-HDL level and clinical factors*

We examined for correlations between the absolute preβ1-HDL concentration and various clinical factors in the CAD patients and in the control subjects. In the CAD patients, the preβ1-HDL concentration showed a strong, significant positive correlation with apoA-I and a significant positive correlation with HDL-C. On the other hand, no correlation was found with the LDL-C, T-Cho or LCAT activity. TG showed a slightly positive correlation with preβ1-HDL. The only other marker correlating significantly with preβ1-HDL was ALT, which correlated slightly. In the control subjects, none of the factors showed a significant correlation with preβ1-HDL (Table 4).

Table 4
Correlations between preβ1-HDL and clinical factors.

	CAD group ($n = 112$)		Control group ($n = 30$)	
	r	*p*	*r*	*p*
Age	0.163	n.s.	−0.122	n.s.
BMI	0.134	n.s.	−0.026	n.s.
SBP	−0.037	n.s.	0.117	n.s.
DBP	0.003	n.s.	0.115	n.s.
HbA1c	0.127	n.s.	−0.039	n.s.
T-Cho	0.146	n.s.	−0.013	n.s.
TG	0.200	<0.05	−0.157	n.s.
LDL-C	0.025	n.s.	−0.054	n.s.
HDL-C	0.247	<0.01	0.194	n.s.
ApoA-I	0.400	<0.0001	0.189	n.s.
ApoB	0.070	n.s.	−0.157	n.s.
Creatinine	0.172	n.s.	−0.012	n.s.
BUN	0.183	n.s.	0.181	n.s.
AST	0.182	n.s.	0.035	n.s.
ALT	0.207	<0.05	−0.195	n.s.
ChE	0.068	n.s.	−0.033	n.s.
LCAT activity*	0.204	n.s.	–	–

* Determined in 58 CAD patients.

We then examined for correlations between the preβ1-HDL and the HDL-C, apoA-I or LCAT activity in the uAP and sCAD subgroups separately. HDL-C showed a significant positive correlation with preβ1-HDL in the sCAD subgroup, but not in the uAP subgroup (Fig. 2A). ApoA-I showed a significant positive correlation with preβ1-HDL in both the uAP and sCAD subgroups (Fig. 2B), whereas LCAT activity did not (Fig. 2C).

4. Discussion

The present study clearly showed that the plasma preβ1-HDL level was high in the CAD group even when excluding dyslipidemic patients (Table 2). Earlier studies reported the plasma preβ1-HDL concentration to be elevated in patients with CAD, dyslipidemia and obesity, and also in hemodialysis patients [15–17,21,22]. The present study excluded patients with renal disorders, including hemodialysis patients, and BMI-matched control subjects were

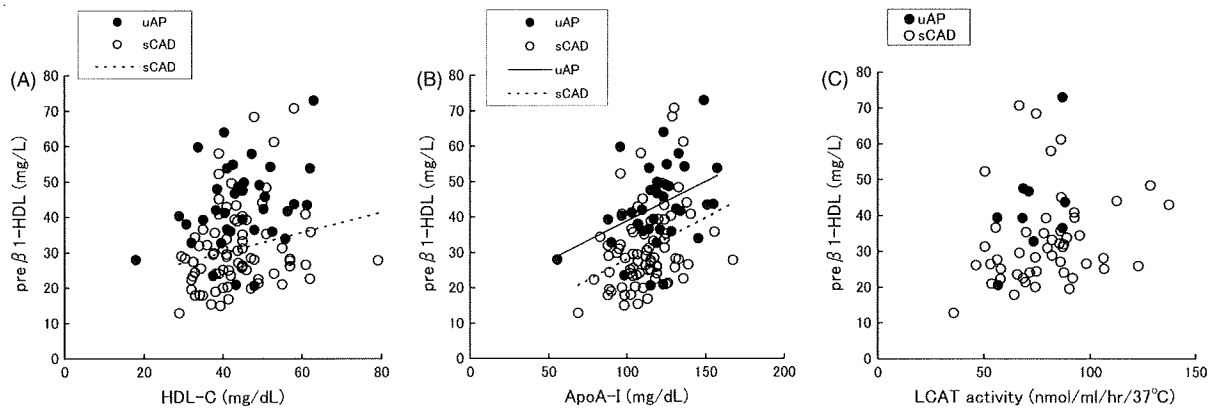

Fig. 2. Correlations (Pearson's Coefficients) between preβ1-HDL levels and HDL-C levels (A), apoA-I levels (B) and LCAT activities (C) in uAP and sCAD subgroups. A: uAP ($n = 36$), $r = 0.313$, $p = 0.063$; sCAD ($n = 76$), $r = 0.227$, $p < 0.05$. B: uAP ($n = 36$), $r = 0.394$, $p < 0.05$; sCAD ($n = 76$), $r = 0.350$, $p < 0.01$. C: uAP ($n = 9$), $r = 0.538$, $p = 0.135$; sCAD ($n = 49$), $r = 0.215$, $p = 0.139$.

used to exclude any effect of obesity. When the CAD group was divided into uAP and sCAD subgroups, the preβ1-HDL level was markedly higher in the uAP subgroup than in the sCAD subgroup. Moreover, the difference remained significant even when dyslipidemic patients were excluded from the two subgroups (Table 2). The age, BMI, blood pressure and concentrations of HbA1c, hepatic function markers, renal function markers, HDL-C and apoA-I did not differ significantly between the two subgroups, although T-Cho, LDL-C and apoB were somewhat lower in the uAP subgroup than in the sCAD subgroup (Table 3). ROC analyses were performed to investigate the potential of preβ1-HDL as a predictive marker for uAP. Preβ1-HDL showed better diagnostic accuracy than other lipid markers, suggesting that preβ1-HDL may be useful for identifying patients with uAP (Fig. 1).

Two earlier studies reported elevation of the preβ1-HDL levels in CAD patients [16,17]. However, the mechanism responsible for that elevation has not been elucidated. Miida et al. reported that delayed catabolism of preβ1-HDL, specifically, delayed LCAT-dependent conversion of preβ1-HDL into α-migrating HDL, causes elevation of the preβ1-HDL level in CAD patients. However, they also described that some CAD patients had a high preβ1-HDL level despite the high LCAT activity, suggesting that some other mechanism may be responsible for preβ1-HDL elevation [16]. Asztalos et al. reported that CAD patients with low HDL-C levels (≤35 mg/dL) have high preβ1-HDL levels and suggested that delayed catabolism of preβ1-HDL is responsible for the elevated preβ1-HDL [17]. In our study, the normolipidemic CAD patients, excluding those with low HDL-C levels (<40 mg/dL), also showed elevated preβ1-HDL levels (Table 2). We speculate that the many uAP patients included in the present study may have been the cause of the elevated preβ1-HDL level in CAD patients without dyslipidemia. If, as has been suggested [17], delayed catabolism of preβ1-HDL is responsible for preβ1-HDL elevation, the HDL-C concentration and LCAT activity should be lower in the uAP subgroup than in the sCAD subgroup and should correlate negatively with the preβ1-HDL concentration. However, we could not find any difference in either the HDL-C concentration or the LCAT activity between the uAP and sCAD subgroups (Table 3), and there was no negative correlation between the preβ1-HDL concentration and either the HDL-C concentration or the LCAT activity in the CAD patients. In fact, the preβ1-HDL concentration conversely showed a significant and positive correlation with the HDL-C concentration in the CAD patients (Table 4 and Fig. 2A). In addition, the CAD patients with either a high HDL-C level or high LCAT activity also showed an elevated preβ1-HDL level (Table 2). These results suggest that some other mechanism must be responsible for preβ1-HDL elevation.

Perhaps that mechanism is enhancement of preβ1-HDL formation. The following three formation pathways are known: synthesis in the liver [9,10], new formation through interaction of apoA-I and peripheral cells [6–8] and dissociation through remodeling of α-HDL [11]. In the case of CAD, the last two of these preβ1-HDL formation pathways seem most likely and are discussed below.

Preβ1-HDL formation is increased in atherosclerotic CAD due to accelerated interaction of apoA-I and peripheral cells. It was reported that foam cell formation enhanced expression of ATP-binding cassette transporter A1 and apoA-I-mediated cholesterol efflux from cells in *in vitro* experiments [23,24]. Since preβ1-HDL is formed by the cellular cholesterol efflux of lipid-free apoA-I or lipid-poor apoA-I mediated by ABCA1 [3,4,6–8], the formation of preβ1-HDL in atherosclerotic CAD caused by accumulation of excess cholesterol might be accelerated by enhancement of that efflux in the peripheral cells.

The other most likely pathway of preβ1-HDL formation in uAP is that preβ1-HDL generation is enhanced by α-HDL remodeling caused by an increase in acute-phase proteins during inflammation. Serum amyloid A (SAA), group IIa secretory phospholipase A2 (sPLA2-IIa) and phospholipid transfer protein (PLTP), whose blood concentrations or activities are elevated in the acute-phase, are known to be factors that facilitate α-HDL remodeling [25–28]. For example, it was reported that the amount of SAA in HDL particles increases markedly during the acute inflammatory phase [28] and that it dissociates preβ1-HDL from α-HDL when it binds to α-HDL [29]. The blood concentration of SAA increases in uAP [30], and SAA is highly expressed in atherosclerotic lesions [31]. van der Westhuyzen et al. suggested a model for the acute-phase response in CAD in which SAA and sPLA2-IIa, present at sites of inflammation and tissue damage, play protective roles by enhancing cellular cholesterol efflux, thereby promoting the removal of excess cholesterol from macrophages [25]. Thus, acute-phase proteins, including SAA, seem to be factors promoting preβ1-HDL elevation in uAP, although the results of the present study are not sufficient to prove this hypothesis.

In summary, we demonstrated that the preβ1-HDL level is elevated in CAD patients, especially in uAP patients, even when excluding dyslipidemic subjects. These results suggest that elevation of the plasma preβ1-HDL level is associated with the atherosclerotic phase of CAD. Elevation of plasma preβ1-HDL may be useful for the identification of patients with uAP. Moreover, that elevation may be caused by a different mechanism from the previously proposed delayed catabolism of preβ1-HDL due to low LCAT activity.

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