of monocyte chemoattractant protein 1 (MCP-1) and adhesion molecules *(11–13)*. In addition to oxidized LDL, LOX-1 binds various ligands, e.g., apoptotic cells, activated platelets, leukocytes, and C-reactive protein *(14–17)*. Accumulating evidence suggests that LOX-1 is involved in endothelial dysfunction, inflammation, atherogenesis, myocardial infarction, and intimal thickening after balloon catheter injury *(16, 18–23)*.

Recently, we developed a system to measure the biological activity of apolipoprotein B (ApoB)-containing lipoprotein based on binding to LOX-1 *(24)*. The activity of LOX-1 ligand containing ApoB (LAB) might reflect atherogenicity of LDL better than measurements of oxidized lipids, oxidized LDL, and LDL. In addition, recent reports have shown that the serum concentrations of soluble LOX-1 (sLOX-1), which is released from the cell surface by proteolysis of LOX-1, might be a useful biomarker for the diagnosis of acute coronary syndrome *(25, 26)*. Accordingly, we hypothesized that the product of LAB and sLOX-1, here designated "LOX index," might be an even better marker reflecting the interaction of atherogenic lipoproteins and their receptors.

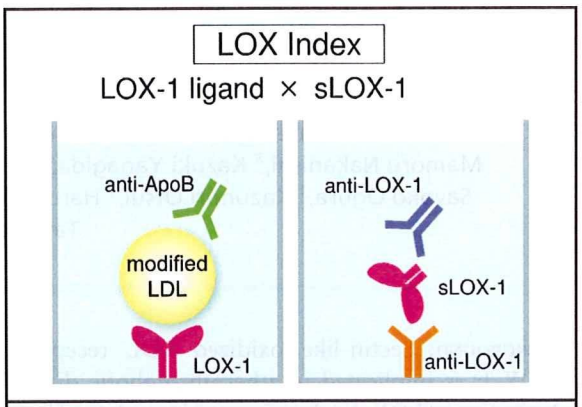

Fig. 1. Scheme of the detection systems for LAB and sLOX-1 to determine LOX index.

Materials and Methods

STUDY POPULATION

The Suita Study is a population-based cohort study in an urban area performed by the National Cardiovascular Center, the details of which have been reported *(2, 27, 28)*. Briefly, in 1989, 6485 men and women, aged 30–79 years, were enrolled as study participants randomly selected from the community of Suita City. They underwent medical examinations every 2 years. In these participants, we set the baseline of the present study as the medical examination held between April 1994 and February 1995, since at that time serum samples were collected and stored at −80 °C. During this 10-month time period, 2437 participants were followed until December 31, 2007. Of these, 142 participants were excluded or the following reasons: history of CHD or stroke (n = 94), lost to follow-up (n = 17), and other reasons such as missing data (n = 31). Data from the remaining 2295 participants (1094 men and 1201 women) were included in the analysis. Informed consent was obtained from all participants. This study was approved by the institutional review board at the National Cardiovascular Center.

BASELINE MEDICAL EXAMINATION

A baseline survey included questionnaires, anthropometric measurements, and blood sample testing after overnight fasting (at least 10 h). Height and weight were measured in light clothing, and body mass index (BMI) was calculated as weight (kg) divided by height (m) squared. Blood pressure of participants in a sitting position after at least 5 min of rest was measured 3 times by well-trained physicians, using a standard mercury sphygmomanometer *(27)*. The average of the second and third measurement was used in the analysis. Hypertension was defined as systolic blood pressure ≥140 mmHg, diastolic blood pressure ≥90 mmHg, and/or the use of antihypertensive agents. Serum total cholesterol (TC), HDL cholesterol, and fasting serum glucose were analyzed with an automated analyzer at the laboratory of the National Cardiovascular Center. Non-HDL cholesterol was calculated by subtracting HDL cholesterol from TC. Diabetes was defined as serum glucose concentrations ≥7.0 mmol/L (126 mg/dL) in fasting or ≥11.1 mmol/L (200 mg/dL) in nonfasting samples and/or current use of medications for diabetes. Well-trained health nurses obtained information on the smoking, alcohol drinking, and medical histories of the participants.

MEASUREMENT OF LAB

A schematic presentation of the detection system for sLOX-1 and LAB is shown in Fig. 1.

We immobilized recombinant human LOX-1(61–273) (0.25 μg/well) on 384-well plates (Greiner 384 Plate High Bind 781061) by incubating overnight at 4 °C in 50 μL PBS *(9, 24)*. After 3 washes with PBS, we blocked the plates with 80 μL of 3% BSA in HEPES buffer (10 mol/L HEPES, 150 mmol/L NaCl, pH 7.0). After 3 washes with PBS, the plates were incubated for 2 h at room temperature with 40 μL standard oxidized LDL or samples. We prepared samples by 20-fold dilution of serum with EDTA-BSA-HEPES buffer [2 mmol/L EDTA, 5% BSA/HEPES buffer (10 mmol/L HEPES, 150 mmol/L NaCl, pH 7.0)] and standards by dilution of oxidized LDL with EDTA-BSA-HEPES buffer. After 3 washes with PBS, the plates were incu-

bated for 1 h at room temperature with 83 μg/L chicken monoclonal anti-ApoB antibody (HUC20) in EDTA-BSA-HEPES buffer *(24)*. After 3 washes with PBS, the plates were incubated for 1 h at room temperature with peroxidase-conjugated donkey antichicken IgY (AP194P; Chemicon) diluted 6000 times with EDTA-BSA-HEPES buffer. After 5 washes with PBS, we added the substrate solution containing 3,3′5,5′-tetramethylbenzidine (TMB solution; Bio-Rad) to the plates and incubated them for 30 min at room temperature. The reaction was terminated with 2 mol/L sulfuric acid. We determined peroxidase activity by measuring absorbance at 450 nm; the functional sensitivity of the measurement was 7.8 μg/L, and the range of the measurement was 7.8–500 μg/L oxidized LDL. Imprecision (CV) was 7.5% intraassay and 12.5% interassay at 50 μg/L (n = 10).

MEASUREMENT OF sLOX-1

We immobilized antihuman LOX-1 antibody (TS92, 0.25 μg/well) on 384-well plates (Corning 384 Plate High Bind 3700) by incubating overnight at 4 °C in 50 μL PBS *(9)*. After 3 washes with PBS, the plates were blocked with 20% ImmunoBlock (DS Pharma). After 3 washes with PBS, the plates were incubated with 40 μL standard oxidized LDL or samples for 2 h at room temperature. We prepared samples by 4-fold dilution of the serum with 1% BSA/PBS containing 0.04% Tween20 and 2 mmol/L EDTA and also by dilution of recombinant extracellular LOX-1(61–273) with the same buffer. After 3 washes with PBS, the plates were incubated with 0.16 μg/mL chicken monoclonal antihuman LOX-1 antibody (HUC5–40) in PBS containing 0.04% Tween20 and 2 mmol/L EDTA for 1 h at room temperature *(29)*. After 3 washes with PBS, the plates were incubated with the peroxidase-conjugated donkey antichicken IgY diluted 5000 times. After 5 washes with PBS, the substrate solution containing TMB solution was added to the plates and incubated for 30 min at room temperature. The reaction was terminated with 2 mol/L sulfuric acid. We determined peroxidase activity by measuring absorbance at 450 nm; the functional sensitivity of the measurement was 15.6 ng/L and the range of the measurement of sLOX-1 was 15.6–2500 ng/L. Imprecision (CV) was 8.5% intraassay and 14.7% interassay at 150 ng/L (n = 10).

ENDPOINT DETERMINATION

The method of endpoint determination for the Suita Study has been reported *(2, 27, 28)*. The endpoints of the current follow-up study were (1) date of first CHD or stroke event; (2) date of death; (3) date of leaving Suita city; and (4) December 31, 2007.

The first step in the survey for CHD and stroke involved checking the health status of all participants by repeated clinical visits every 2 years and yearly questionnaires sent by mail or conducted by telephone. In the second step, in-hospital medical records of participants who were suspected of having CHD or stroke were reviewed by registered hospital physicians or research physicians who were blinded to the baseline information. To complete the surveillance for fatal CHD and stroke, we conducted a systematic search for death certificates. The criteria for stroke were defined according to US National Survey of Stroke criteria *(30)*. Classification of patients into stroke subtypes (ischemic stroke, intracerebral hemorrhage, and subarachnoid hemorrhage) was based on examination of computed tomography, magnetic resonance imaging, or autopsy. Definite and probable myocardial infarction (MI) were defined according to the criteria of the MONICA (Monitoring Trends and Determinants of Cardiovascular Disease) project *(31)*. The criteria for a diagnosis of CHD included first-ever MI, coronary artery bypass surgery, or angioplasty. Sudden deaths of unknown origin that occurred within 24 h from onset were classified as CHD in the present study. We also defined cardiovascular disease (CVD) as a composite outcome of CHD or stroke.

STATISTICAL ANALYSIS

In addition to sLOX and LAB, we calculated the LOX index by multiplying serum concentrations of sLOX-1 by those of LAB. We set the cutoff points of sLOX, LAB, and LOX index according to the quartile ranges. Statistical methods of data analysis included ANOVA for assessing mean differences between groups and χ^2 tests for proportions. Multivariable analysis combining patients of both sexes was performed because there was no interaction between sex and LOX index (or LAB, sLOX) on the incidence of CHD or stroke. We calculated the multivariable-adjusted hazard ratios (HRs) of sLOX, LAB, and LOX index for CHD or stroke using a proportional hazards regression model after adjusting for sex, age, hypertension, diabetes, use of lipid-lowering agent, BMI, and current smoking and alcohol drinking (model 1). Further adjustment for non-HDLC was also performed (model 2). All CIs were estimated at the 95% level, and significance was set at $P < 0.05$. We used the SAS Statistical Package (release version 8.2, SAS Institute) for all the analyses.

Results

LAB and sLOX-1 concentrations at baseline [mean (SD)] were 516.1 (17.1) μg/L and 1060.1 (8.6) ng/L in men and 782.3 (23.7) μg/L and 797.8 (0.2) ng/L in women. The mean baseline serum TC was 181.5 (1.3) mg/dL [4.70 (0.034) mmol/L] in men and 224.5 (2.0) mg/dL [5.81 (0.052) mmol/L] in women in this population. Table 1

Table 1. Sex-specific means and prevalence of risk factors according to LAB or sLOX-1 quartiles at the baseline survey.[a]

	Mean LAB, μg/L (range)					Mean sLOX1, ng/L (range)				
	Q1, 217 (21–349)	Q2, 496 (350–644)	Q3, 870 (649–1128)	Q4, 1931 (1133–6379)	*P* (trend)	Q1, 558 (85–754)	Q2, 925 (755–1085)	Q3, 1289 (1084–1534)	Q4, 2367 (1540–9874)	*P* (trend)
Men										
n	261	257	288	288		229	251	284	330	
Age, years	61 (12)	60 (12)	60 (12)	59 (12)	0.41	62 (12)	61 (13)	60 (13)	59 (12)	0.01
TC										
mg/dL	186 (30)	195 (30)	192 (33)	203 (30)	<0.001	195 (33)	194 (29)	193 (34)	196 (32)	0.65
mmol/L	4.82 (0.78)	5.05 (0.78)	4.97 (0.85)	5.26 (0.78)		5.05 (0.85)	5.02 (0.75)	5.00 (0.88)	5.08 (0.83)	
HDL cholesterol										
mg/dL	54 (14)	56 (14)	54 (15)	53 (12)	0.06	54 (14)	55 (15)	55 (14)	53 (13)	0.33
mmol/L	1.40 (0.36)	1.45 (0.36)	1.40 (0.39)	1.37 (0.31)		1.40 (0.36)	1.42 (0.39)	1.42 (0.36)	1.37 (0.34)	
Non-HDL cholesterol										
mg/dL	132 (31)	139 (32)	139 (34)	151 (32)	<0.001	141 (33)	139 (30)	138 (34)	142 (34)	0.35
mmol/L	3.42 (0.80)	3.60 (0.83)	3.60 (0.88)	3.91 (0.83)		3.65 (0.85)	3.60 (0.78)	3.57 (0.88)	3.68 (0.88)	
Hypertension, %[b]	35	37	37	39	0.96	34	37	37	39	0.99
Lipid-lowering agent use, %	4	4	3	5	0.53	5	4	4	4	0.88
Diabetes, %[c]	7	6	5	6	0.79	6	6	7	5	0.62
Current smoking, %	41	44	43	39	0.71	32	37	45	48	<0.001
Current alcohol drinking, %[d]	69	68	71	67	0.75	70	73	65	67	0.21
Women										
n	312	317	286	286		344	323	290	244	
Age, years	57 (12)	59 (12)	57 (12)	57 (12)	0.05	58 (12)	57 (12)	57 (13)	58 (13)	0.71
TC										
mg/dL	200 (32)	204 (33)	207 (32)	216 (37)	<0.001	208 (33)	207 (35)	203 (34)	207 (35)	0.19
mmol/L	5.18 (0.83)	5.28 (0.85)	5.36 (0.83)	5.59 (0.96)		5.39 (0.85)	5.36 (0.91)	5.26 (0.88)	5.36 (0.91)	
HDL cholesterol										
mg/dL	62 (14)	62 (14)	61 (13)	59 (13)	0.06	62 (14)	62 (13)	60 (13)	60 (13)	0.04
mmol/L	1.61 (0.36)	1.61 (0.36)	1.58 (0.34)	1.53 (0.34)		1.61 (0.36)	1.61 (0.34)	1.55 (0.34)	1.55 (0.34)	
Non-HDL cholesterol										
mg/dL	138 (32)	142 (33)	146 (34)	156 (39)	<0.001	146 (34)	145 (36)	143 (35)	148 (36)	0.48
mmol/L	3.57 (0.83)	3.68 (0.85)	3.78 (0.88)	4.04 (1.01)		3.78 (0.88)	3.76 (0.93)	3.70 (0.91)	3.83 (0.93)	
Hypertension, %	33	27	30	32	0.84	29	30	30	34	0.42
Lipid-lowering agent use, %	8	5	7	6	0.27	8	5	7	7	0.62
Diabetes, %	3	3	3	5	0.26	3	3	4	4	0.72
Current smoking, %	7	12	10	10	0.31	6	8	11	16	<0.001
Current alcohol drinking, %	25	28	26	24	0.84	25	27	23	28	0.52

[a] Data are mean (SD) unless noted otherwise.
[b] Hypertension was defined as systolic blood pressure ≥140 mmHg, diastolic blood pressure ≥90 mmHg, or use of antihypertensive agents.
[c] Diabetes was defined as fasting serum glucose ≥7.0 mmol/L (126 mg/dL), use of antidiabetic agents, or both.
[d] Alcohol drinking was defined as consuming at least 1 drink per week.

shows the baseline characteristics of the participants in each LAB or sLOX-1 quartile. In both sexes, there were significant differences in the mean concentrations for TC and non-HDL cholesterol according to LAB quartile, with higher concentrations in the higher LAB quartiles. Conversely, serum TC and non-HDLC were not found to be associated with sLOX-1 quartiles. In women, HDL cholesterol was lower in the higher LOX-1 quartiles. The prevalence of smoking was higher in the upper sLOX-1 quartiles but was not associated with LAB. There were no significant differences across quartiles in the prevalence of hypertension and diabetes.

During the mean follow-up period of 11 years, there were 68 incident cases of CHD and 91 cases of stroke, including 60 cases of ischemic stroke. The number of incident cases and multivariable-adjusted HRs for CVD, stroke, ischemic stroke, and CHD stratified by LAB and sLOX-1 are shown in Table 2. The HRs for stroke, ischemic stroke, and CHD were highest in the highest LAB quartile, and except for CHD, the trends in HRs across quartiles were statistically significant. The HR for CVD was highest in the top quartile of LAB, and the trend across quartiles was statistically significant. For sLOX-1, however, the across-quartile trends in HRs did not reach statistical significance.

The number of incident cases and multivariable-adjusted HRs for CVD, stroke, ischemic stroke, and CHD stratified by LOX index (LAB × sLOX) are shown in Table 3. The HR for ischemic stroke was constantly high from the second to the highest quartile: 3.39 (95% CI 1.34–8.53), 3.15 (1.22–8.13), and 3.23 (1.24–8.37), respectively. Furthermore, the HR of the highest quartile of LOX index was 2.09 (1.00–4.35) for CHD. In the highest LOX index quartile, the incidence of CVD was approximately 2-fold that in the lowest LOX index quartile, and the associated HR was 1.83 (1.03–2.96).

After additional adjustment for HDL cholesterol or the exclusion of sudden cardiac death from CHD, the results of all the analyses listed above remained the same (data not shown).

Discussion

In the present study, we followed 1094 men and 1201 women for a mean period of 11 years to investigate the impact of LAB, sLOX-1, and LOX index (LAB × sLOX) on the incidence of CVD. We found LAB and LOX index to be significantly associated with the incidence of CVD and CHD, especially ischemic stroke. This investigation is the first cohort study on the relationship between CVD and LOX index–related or oxidized LDL–related parameters in a general population.

Instead of oxidized LDL, here we measured the serum concentrations of LAB. Researchers have applied several methods to measure circulating concentrations of oxidized LDL, including measurement of oxidation-dependent epitopes in the ApoB moiety of LDL. These methods, however, which evaluate the amount of oxidized moiety on LDL, do not necessarily reflect biological activity. In contrast, the present assay system using recombinant LOX-1 and anti-ApoB antibody has the ability to evaluate the biological activity of the atherogenic lipoproteins (Fig. 1). On the other hand, circulating sLOX-1 concentrations might reflect the expression levels of LOX-1, the target site of the atherogenic lipoproteins in vascular wall. Therefore, LOX index (LAB × sLOX-1) could represent ligand (LAB)–receptor (LOX-1) interaction leading to vascular dysfunction. The present results confirmed LOX index as a predictor of the incidence of CVD. This suggests that LOX-1 may be important in the pathogenesis of CVD, and indicates that the evaluation of LOX-1–mediated signaling may serve as a potential tool for risk stratification.

It is well known that increased blood pressure, smoking, diabetes, and atrial fibrillation are major risk factors for stroke, especially for ischemic stroke *(32)*. In contrast with these risk factors, we found either no relationship or a weakly positive one between TC or LDL cholesterol and ischemic stroke in several cohort studies performed in the Japanese population *(32–34)*. A large metaanalysis of individual data from 61 prospective studies performed mainly in Western populations also showed no association between TC or non-HDL cholesterol and stroke mortality *(4)*. We recently reported that we found no association between LDL cholesterol or non-HDL cholesterol concentrations and the incidence of ischemic stroke in this cohort using another baseline survey, shortly before that of the present study *(2)*. In contrast, the present investigation demonstrated that the LOX index is a predictor of not only CHD but also ischemic stroke. A strong association of LOX-1 with ischemic stroke in experimental models has been reported. For example, expression of LOX-1 and MCP-1 is increased in the early stage of atherosclerotic changes of common carotid arteries in spontaneously hypertensive rats *(35)*. Schwarz et al. *(36)* reported that LOX-1 expression was induced >10-fold at ischemic core sites during experimental stroke. Furthermore, we found that LOX-1 contributed to the formation of arterial thrombus (unpublished data). Thus, activation of LOX-1 might facilitate the pathophysiological conditions leading to stroke. In the present study, the risk for ischemic stroke was significantly increased from the second to the fourth quartile of LOX index, which suggests a protective role

Table 2. Age- and multivariable-adjusted odds ratios (95% CIs) for the incidence of cardiovascular disease and its subtypes according to LAB and sLOX-1 quartiles.

	Mean LAB, μg/L (range)					Mean sLOX1, ng/L (range)				
	Q1, 217 (21–349)	Q2, 496 (350–644)	Q3, 870 (649–1128)	Q4, 1931 (1133–6379)	*P* (trend)	Q1, 558 (85–754)	Q2, 925 (755–1085)	Q3, 1289 (1084–1534)	Q4, 2367 (1540–9874)	*P* (trend)
Person-years	6447	6343	6323	6156		6376	6446	6173	6276	
Stroke										
Cases, n	16	27	22	26		21	20	25	25	
Age-adjusted	1	1.60 (0.86–2.99)	1.44 (0.75–2.75)	1.86 (0.99–3.50)	0.09	1	0.93 (0.50–1.72)	1.18 (0.66–2.12)	1.17 (0.65–2.11)	0.45
Multivariable-adjusted, model 1[a]	1	1.68 (0.90–3.15)	1.49 (0.77–2.89)	2.07 (1.09–3.91)	0.05	1	0.89 (0.48–1.65)	1.16 (0.64–2.09	1.12 (0.61–2.03)	0.52
Multivariable-adjusted, model 2	1	1.69 (0.90–3.17)	1.50 (0.77–2.90)	2.09 (1.10–3.98)	0.05	1	0.89 (0.48–1.66)	1.17 (0.64–2.10)	1.12 (0.61–2.04)	0.52
Ischemic stroke										
Cases, n	9	18	13	20		12	17	13	18	
Age-adjusted	1	1.99 (0.89–4.44)	1.51 (0.64–3.56)	2.62 (1.18–5.79)	0.04	1	1.37 (0.65–2.87)	1.02 (0.46–2.25)	1.34 (0.64–2.81)	0.63
Multivariable-adjusted, model 1	1	2.12 (0.94–4.77)	1.63 (0.68–3.90)	3.18 (1.41–7.12)	0.01	1	1.38 (0.65–2.94)	1.02 (0.46–2.25)	1.29 (0.60–2.74)	0.72
Multivariable-adjusted, model 2	1	2.10 (0.93–4.74)	1.62 (0.67–3.88)	3.11 (1.37–7.04)	0.15	1	1.38 (0.65–2.94)	1.03 (0.46–2.29)	1.29 (0.60–2.74)	0.71
CHD										
Cases, n	12	18	15	23		12	17	23	16	
Age-adjusted	1	1.46 (0.69–3.07)	1.35 (0.63–2.90)	2.02 (1.00–4.08)	0.06	1	1.47 (0.69–3.16)	2.12 (1.03–4.37)	1.44 (0.66–3.12)	0.24
Multivariable-adjusted, model 1	1	1.54 (0.73–3.26)	1.44 (0.66–3.16)	2.18 (1.06–4.44)	0.04	1	1.53 (0.71–3.30)	1.97 (0.95–4.08)	1.42 (0.64–3.07)	0.31
Multivariable-adjusted, model 2	1	1.42 (0.67–3.02)	1.35 (0.62–2.96)	1.82 (0.88–3.76)	0.13	1	1.66 (0.77–3.58)	2.13 (1.02–4.42)	1.47 (0.67–3.21)	0.27
Cardiovascular disease										
Cases, n	28	45	37	49		33	37	48	41	
Age-adjusted	1	1.52 (0.94–2.45)	1.38 (0.84–2.26)	1.89 (1.18–3.02)	0.02	1	1.13 (0.70–1.81)	1.50 (0.96–2.35)	1.26 (0.79–2.02)	0.18
Multivariable-adjusted, model 1	1	1.60 (0.99–2.59)	1.44 (0.87–2.38)	2.05 (1.28–3.29)	0.01	1	1.11 (0.69–1.80)	1.43 (0.91–2.24)	1.21 (0.75–1.94)	0.27
Multivariable-adjusted, model 2	1	1.56 (0.96–2.52)	1.41 (0.85–2.32)	1.91 (1.18–3.08)	0.02	1	1.13 (0.70–1.83)	1.48 (0.94–2.33)	1.21 (0.76–1.95)	0.26

[a] Model 1 adjusted for age, sex, BMI, smoking, drinking, hypertension, diabetes, and use of lipid-lowering agents; model 2 as model 1 with the addition of non-HDL cholesterol.

Table 3. Age- and multivariable-adjusted odds ratios (95% CIs) for the incidence of cardiovascular disease and its subtypes according to LOX index ($\times 10^6$) quartiles.

	Mean LOX index (range)				
	Q1, 0.21 (0.017–0.363)	Q2, 0.52 (0.364–0.7040)	Q3, 0.97 (0.7043–1.314)	Q4, 2.64 (1.315–44.22)	*P* (trend)
Person-years	6416	6396	6314	6144	
Stroke					
Cases, n	17	24	25	25	
Age-adjusted	1	1.49 (0.80–2.80)	1.50 (0.80–2.79)	1.68 (0.90–3.14)	0.12
Multivariable-adjusted, model 1[a]	1	1.44 (0.76–2.71)	1.59 (0.85–2.99)	1.74 (0.92–3.28)	0.08
Multivariable-adjusted, model 2	1	1.44 (0.76–2.71)	1.60 (0.85–3.00)	1.74 (0.92–3.30)	0.09
Ischemic stroke					
Cases, n	6	20	17	17	
Age-adjusted	1	3.54 (1.41–8.87)	2.80 (1.10–7.14)	3.03 (1.18–7.74)	0.07
Multivariable-adjusted, model 1	1	3.40 (1.35–8.56)	3.22 (1.25–8.29)	3.31 (1.28–8.56)	0.03
Multivariable-adjusted, model 2	1	3.39 (1.34–8.53)	3.15 (1.22–8.13)	3.23 (1.24–8.37)	0.04
CHD					
Cases, n	12	19	12	25	
Age-adjusted	1	1.81 (0.85–3.81)	1.07 (0.47–2.44)	2.40 (1.17–4.93)	0.05
Multivariable-adjusted, model 1	1	1.70 (0.80–3.64)	1.04 (0.45–2.40)	2.37 (1.15–4.90)	0.05
Multivariable-adjusted, model 2	1	1.67 (0.78–3.59)	1.02 (0.44–2.35)	2.09 (1.00–4.35)	0.11
Cardiovascular disease					
Cases, n	29	43	37	50	
Age-adjusted	1	1.58 (0.98–2.55)	1.31 (0.80–2.15)	1.92 (1.20–3.07)	0.02
Multivariable-adjusted, model 1	1	1.49 (0.92–2.42)	1.35 (0.82–2.23)	1.95 (1.21–3.13)	0.01
Multivariable-adjusted, model 2	1	1.48 (0.91–2.41)	1.31 (0.80–2.17)	1.83 (1.13–2.96)	0.03

[a] Model 1 adjusted for age, sex, BMI, smoking, drinking, hypertension, diabetes, and use of lipid-lowering agents; model 2 as model 1 with the addition of non-HDL cholesterol.

against ischemic stroke. Additional epidemiologic studies to establish clinical cut points of LOX index are warranted.

The positive relationship between LOX index and ischemic stroke bridged, for the first time, the missing link between stroke and cholesterol-related parameters. In those with high LOX index, breaking the interaction between LAB and LOX-1 might be effective in preventing stroke. The most straightforward approach would be to apply a LOX-1 antagonist, which is yet to be developed. Although most of the randomized controlled trials failed to find a beneficial effect of antioxidants for the prevention of CVD *(21)*, we may reduce LAB concentration per se by statin therapy, thereby increasing LDL receptor expression, leading to an increase in the turnover of LDL and a decrease in the chance of LDL modification *(37)*. Actually, in the present study, serum concentrations of LAB showed a significant association with TC and non-HDLC. In addition, a positive relationship between smoking and LOX-1 suggests the possibility of smoking cessation to reduce sLOX-1 concentration by decreasing the expression level of LOX-1.

The present study has some limitations. First, a recent report from the Hisayama study showed a positive relationship between LDL cholesterol and atherothrombotic infarction, which accounts for one fourth of all ischemic stroke *(38)*. Therefore, the relation between LOX index and each subtype of ischemic stroke would be worth analyzing; however, the relatively small sample size of the current study precludes such analysis. Second, the participants in the present investigation were all Japanese; therefore, the study should be repeated in other ethnic populations. Because the incidence of stroke in Japan is much greater than in other countries *(39)*, some Japanese-specific factors might be affecting the present findings. Finally, since the number of cardiovascular events was not sufficiently large to enable a sex-specific analysis, especially in women, we did not perform such analysis.

In conclusion, LOX index is associated with an increased risk of CVD, especially ischemic stroke, in a Japanese urban population. From a public health viewpoint, the novel biochemical marker may provide new insights into not only risk stratification but also therapeutic strategy for CVD.

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Generation of monoclonal antibodies against a soluble form of lectin-like oxidized low-density lipoprotein receptor-1 and development of a sensitive chemiluminescent enzyme immunoassay

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ABSTRACT

Lectin-like oxidized low-density lipoprotein receptor-1 (LOX-1), expressed prominently in atherosclerotic lesions, is cleaved and released as a soluble LOX-1 (sLOX-1), which is a specific biomarker to diagnose acute coronary syndrome (ACS) at an early stage. Although sLOX-1 levels in patient's blood were successfully measured with our previously established enzyme-linked immunosorbent assay (ELISA), the assay was not sensitive enough to detect normal serum levels of sLOX-1 in healthy human subjects. We therefore developed sensitive and specific monoclonal antibodies (mAbs) against sLOX-1 in order to establish a more sensitive immunoassay. Mice were immunized with recombinant human LOX-1 extracellular domain. mAbs were subsequently generated by standard myeloma cell fusion techniques with a novel screening method using time-resolved fluorescence immunoassay. Using two anti-human sLOX-1 mAbs and alkaline phosphatase as a label, a sandwich chemiluminescent enzyme immunoassay (CLEIA) was developed. In total, nine mAbs were obtained. The dissociation constant (K_d) values of these mAbs for sLOX-1 were 0.12–1.32 nM. Characteristics of these mAbs were estimated and the best combination for CLEIA was selected. The newly established CLEIA could determine sLOX-1 levels as low as 8 pg/mL, and thus, was sensitive enough to measure serum sLOX-1 levels in normal human subjects and to evaluate subtle differences. Values for sLOX-1 measured by monoclonal CLEIA and polyclonal ELISA were highly correlated ($r^2 = 0.7594$, $p < 0.0001$). Area under the curve values of the receiver-operating characteristic curves in detecting ACS were 0.948 and 0.978 for monoclonal CLEIA and polyclonal ELISA, respectively. Thus, a more sensitive sLOX-1 CLEIA was established using newly developed mAbs against sLOX-1. In addition to its advantage in early diagnosis of ACS, this assay may also be useful in predicting cardiovascular disease risk in disease-free subjects.

1. Introduction

It is widely recognized that acute coronary syndrome (ACS) is caused by rupturing of lipid-rich atheromatous plaques followed by thrombus formation [1,2]. Biomarkers for plaque instability or rupture would allow prediction or accurate diagnosis of ACS at an early stage. Currently, the MB fraction of creatine kinase (CK-MB), troponin T, and heart-type fatty acid-binding protein (H-FABP) are used as serum biomarkers of ACS [3–5]. However, these markers are not markedly elevated during the early stage of ACS, before ischemic myocardial damage becomes apparent. In addition, if ischemic myocardial damage remains minimal, these biomarkers may not be significantly elevated even several hours after the onset of ACS. Several other serum biomarkers, including high-sensitivity C-reactive protein (hs-CRP), were reported to be associated with ACS [6]. However, none of these markers have been established as clinical tests for ACS because they do not directly or specifically indicate plaque instability or plaque rupture before myocardial damage becomes apparent.

For this purpose, we focused on lectin-like oxidized low-density lipoprotein receptor-1 (LOX-1) [7]. In human atherosclerotic lesions, LOX-1 is prominently expressed by intimal smooth muscle cells and macrophages in advanced atherosclerotic plaques and by vascular endothelial cells covering early atherosclerotic lesions

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[8,9]. In contrast, LOX-1 expression is undetectable in normal aortas without visible atherosclerosis [8,9]. LOX-1 expressed on the cell surface can undergo proteolysis at its membrane-proximal extracellular domain (ECD) and be released as soluble LOX-1 (sLOX-1) [10]. Therefore, we previously developed a sandwich enzyme-linked immunosorbent assay (ELISA) for determination of sLOX-1 concentration in human sera, using rabbit polyclonal antibodies and colorimetric detection [11]. Using this technique, we measured serum samples from 427 patients undergoing coronary angiography and from 94 patients with other acute and chronic noncardiovascular diseases, and found that serum sLOX-1 levels are markedly higher in ACS, but not in noncardiovascular acute inflammatory diseases. In addition, receiver-operating characteristic (ROC) curves of sLOX-1 showed higher sensitivity and specificity for the diagnosis of ACS than hs-CRP [12].

Although sLOX-1 in ACS patient sera was successfully measured by our sandwich ELISA, this assay was not sensitive enough to determine the normal serum sLOX-1 levels in healthy human subjects as they are below its lower detection limit. In this study, therefore, sensitive and specific monoclonal antibodies (mAbs) against human sLOX-1 were generated and a more sensitive sandwich chemiluminescent enzyme immunoassay (CLEIA) was developed using a combination of two different mAbs.

2. Materials and methods

2.1. Reagents

All chemicals purchased were of analytical grade unless otherwise specified.

2.2. Preparation of LOX-1 ECD

Recombinant human LOX-1 ECD was produced in *Escherichia coli* by transforming a cDNA fragment corresponding to the ECD of human LOX-1 (GenBank accession no. AB010710), which had been subcloned into a pQE vector (Qiagen, Valencia, CA, USA) [13]. LOX-1 ECD was extracted from *E. coli*, solubilized with 8 M urea, and purified with an Ni-NTA agarose column (Invitrogen, Carlsbad, CA, USA). The eluate with 250 mM imidazole was subjected to stepwise dialysis against 4, 2, 1, and 0 M urea in phosphate buffer (pH 9.0; 0.05 M). The supernatant of the dialysate was used as an assay standard [11] after determination of its concentration by amino acid analysis with a Hitachi L-8800 analyzer (Hitachi High-Technologies, Tokyo, Japan). The amino acid sequence of LOX-1 ECD was obtained as Met Arg Gly Ser His His His His His His Gly – Ser85–Gln273 (of LOX-1) from its cDNA sequence analysis.

2.3. Preparation of human sLOX-1 from CHO cell culture

CHO-K1 cells stably expressing human LOX-1 (LOX-1–CHO) were prepared and cultured as described previously [13]. A small amount of sLOX-1 in the conditioned medium of LOX-1–CHO was concentrated and purified as described previously in bovine sLOX-1 [10].

2.4. Immunization of mice

LOX-1 ECD was also used as an antigen to immunize mice. A solution containing the precipitate of LOX-1 ECD was emulsified with Freund's complete adjuvant and injected intraperitoneally (0.1 mg/0.1 mL) into female mice (A/J Jms Slc; Japan SLC, Hamamatsu, Japan). Mice were immunized a total of four times at 3 week intervals. Small samples of blood were collected from the mice 10 days after each immunization and antibody titers were monitored with a time-resolved fluorescence immunoassay (TR-FIA) as described below.

2.5. Generation of mAb-secreting hybridoma clones

Stimulated mouse spleen cells were harvested after the fourth immunization and mixed with the cells of P3-X63-Ag8-U1 mouse myeloma cell line at a ratio of 5:1. Cell fusion was performed by the polyethylene glycol method. After hypoxanthine, aminopterin, and thymidine (HAT) selection, hybridomas were screened using competitive TR-FIA as described below. Hybridomas that secreted abundant anti-sLOX-1 antibodies were cloned by limiting dilution.

2.6. Production and purification of mAbs

Anti-sLOX-1 antibody-producing clones were cultured in 50 mL flasks or injected intraperitoneally into peristan-pretreated mice. The hybridoma cell culture supernatant or ascites of the hybridoma-injected mice was collected and purified using a protein A affinity column (MAPS-II Kit; Bio-Rad, Hercules, CA, USA).

2.7. Characterization of mAb

2.7.1. Titers of antibodies

Titers of mAbs were determined using TR-FIA. Anti-mouse IgG antibodies (goat IgG, 1 μg/100 μL) in an immobilization buffer [Tris–HCl buffer (pH 7.8; 0.05 M) containing 0.5 g/L sodium azide] were added to microplates (MaxiSorp FluoroNunc; Nalge Nunc, Rochester, NY, USA) and incubated overnight at 4 °C to immobilize on the surface of microplates. After two washes with a blocking buffer [Tris–HCl buffer (pH 7.8; 0.025 M) containing 0.25 g/L sodium azide, 20 g/L Block Ace (Dainippon Sumitomo Pharma, Osaka, Japan), and 100 g/L sucrose], the plates were incubated with the blocking buffer for 2 h at room temperature, dried under vacuum at room temperature, and then stored at 4 °C until use.

LOX-1 ECD was subjected to biotinylation using Sulfo-NHS-LC-Biotin (Thermo, Rockford, IL, USA). The biotin-labeled antigen was mixed with europium-labeled streptavidin (DELFIA Eu-labeled Streptavidin; PerkinElmer, Fremont, CA, USA) to prepare a europium-labeled antigen.

The test antiserum or culture supernatant of hybridoma was diluted serially with an assay buffer [Tris–HCl buffer (pH 7.4; 0.05 M) containing 0.5 g/L sodium azide, 5 g/L bovine serum albumin (BSA), 9.8 mg/L diethylenetriaminepentaacetic acid, 0.1 g/L Tween 80, and 9 g/L sodium chloride] to determine the titer of the antibody. The europium-labeled antigen (100 μL) and the test antibody (50 μL) were added to anti-mouse IgG antibody-immobilized microplates after two washes with a TR-FIA washing solution (saline containing 0.1 g/L Tween 20 and 0.5 g/L sodium azide). The microplates were incubated for 16 h at 4 °C, then washed three times with the TR-FIA washing solution, and then 150 μL of an enhancement solution (1.39 g potassium hydrogen phthalate, 6.0 g acetic acid, 19.3 mg tri-n-octylphosphine oxide, 4.59 mg 2-naphthoyltrifluoroacetone, and 1.0 g Triton X-100 in 1 L distilled water) was added to measure time-resolved fluorescence using a multilabel counter for microplate (Wallac 1420 Arvo$_{sx}$, PerkinElmer). Titers of the antibody were estimated as the dilution of sample solution giving a time-resolved fluorescence intensity of 100,000 counts/s (cps).

2.7.2. Affinity of mAb

Affinities of mAbs were determined by competitive TR-FIA. The europium-labeled antigen solution (50 μL), the culture supernatant of the hybridoma (50 μL) and LOX-1 ECD standard solution (50 μL) or sLOX-1 solution (50 μL) from the culture supernatant of LOX-1–CHO were added to the anti-mouse IgG antibody-immobilized

microplates. Then the microplates were incubated for 16 h and the time-resolved fluorescence intensity was measured as described above. An inhibition curve was obtained using different concentrations of LOX-1 ECD or sLOX-1 derived from the culture supernatant of LOX-1–CHO. Dissociation constants were calculated by Scatchard analysis of the inhibition curves for the mAbs [14].

Immunoglobulin class and subclass were determined using mouse immunoglobulin isotyping ELISA kit (BD Biosciences, San Jose, CA, USA).

2.7.3. Epitope mapping

Epitopes recognized by the mAbs were determined by scanning 63 peptides (15-meric peptides in which sequences of 12 amino acids overlapped) derived from LOX-1 ECD (residues 85–273) using an epitope mapping method [15]. The assay was performed by JPT Peptide Technologies (Berlin, Germany) using their protocols [16].

2.8. Development of CLEIA

2.8.1. Preparation of enzyme-labeled antibody

The mAbs were digested by pepsin (Sigma, St. Louis, MO, USA) and purified to $F(ab')_2$, which was further reduced to Fab′ by 10 mM 2-mercaptoethylamine in buffer A [phosphate buffer (pH 6.0; 0.1 M) containing 5 mM EDTA] by the standard method [17]. Alkaline phosphatase (ALP; 2.0 mg or 14.2 nmol from calf intestine; Kikkoman, Chiba, Japan) in 0.475 mL of a buffer [Tris–HCl buffer (pH 7.0; 0.1 M) containing 1 mM $MgCl_2$ and 0.1 mM $ZnCl_2$] was mixed with 31 μg (71 nmol) of *N*-(8-maleimidocapryloxy) sulfosuccinimide (Sulfo-HMCS; Dojindo, Kumamoto, Japan) in 0.05 mL water for 1.5 h on ice. HMCS-activated ALP was purified using a gel filtration column (PD-10; GE Healthcare, Chalfont St. Giles, UK) and concentrated using an ultracentrifugal concentrator (Centricon YM-30; Millipore, Billerica, MA, USA). The HMCS-activated ALP solution (0.96 mg in 0.192 mL) was added to 0.441 mg of the Fab′ in 0.15 mL of buffer A and mixed for 16 h at 4 °C. The reaction mixture was purified by gel-permeation HPLC [column, TSK gel G2000SW_{XL}, id 7.8 mm × 300 mm (TOSOH, Tokyo, Japan); mobile phase, Tris–HCl buffer (pH 6.8; 0.1 M) containing 1 mM $MgCl_2$ and 0.1 mM $ZnCl_2$; flow rate, 0.5 mL/min; detection, UV 280 nm] to obtain ALP-labeled mAb.

2.8.2. Preparation of antibody-immobilized plates

The anti-sLOX-1 mAb (IgG; 1.5 μg in 150 μL immobilization buffer) was added to the microplate wells (MaxiSorp FluoroNunc) and incubated overnight at 4 °C for them to immobilize. After two washes with CLEIA washing solution (saline containing 0.1 g/L Tween 20 and 0.2 g/L sodium azide), the microplates were incubated with the blocking buffer for 2 h at room temperature, dried under vacuum at room temperature, and stored at 4 °C until use. The microplates were washed with the CLEIA washing solution immediately before use.

2.8.3. CLEIA procedure

Standard sLOX-1 solutions (8–25,000 pg/mL LOX-1 ECD) in CLEIA buffer [Tris-buffered saline (pH 7.4; 0.05 M) containing 10 g/L BSA, 0.1 g/L Tween 80, 1 mM $MgCl_2$, 0.1 mM $ZnCl_2$, 10 mg/L mouse γ-globulin, 1000 KIU/mL aprotinin, and 0.5 g/L sodium azide] or serum samples from human subjects (10 μL) were added to the anti-sLOX-1 mAb-immobilized microplate wells in duplicates, containing 100 μL of the CLEIA buffer (serum sample without pretreatment was diluted 1:11). The microplate was sealed after gentle mixing and incubated for 4 h at room temperature (first incubation), which was followed by washing three times with 350 μL CLEIA washing solution. Then, the ALP-labeled mAb solution (100 μL; approximately 94.5 ng/mL in the CLEIA buffer) was added to the microplate wells, and the wells were incubated for 1 h at room temperature (second incubation). This was followed by washing four times and addition of substrate solution (100 μL) containing APS-5 (Lumigen, Southfield, MI, USA). The chemiluminescence (CL) intensity (in cps) of each well was measured using a plate reader (Wallac 1420 $Arvo_{SX}$).

Table 1
Characteristics of anti-sLOX-1 monoclonal antibody clones.

Clone	Affinity to LOX-1 ECD	Affinity to sLOX-1 (CHO)[b]	Growth	Sub class
6B11	0.34 nM[a]	++	++	IgG_1
1G2	+++	+++	++	IgG_1
2E4	+++	+++	++	IgG_1
2E5	++	++	++	IgG_1
2G11	0.16 nM[a]	++	++	IgG_1
3E12	1.32 nM[a]	++	++	IgG_1
7G1	0.12 nM[a]	++	++	IgG_1
1A7	0.51 nM[a]	++	++	IgG_1
1B8	++	++	++	IgG_{2b}

[a] Dissociation constant (K_d).
[b] sLOX-1 was derived from the cell culture supernatant of LOX-1–CHO.

3. Results and discussion

3.1. Assay standard and immunogen

A small amount of sLOX-1 from LOX-1–CHO was obtained and used for sequence analysis and characterization of mAb. The sequence of sLOX-1 was defined as a mixture of residues 88–273 and 92–273. However, appropriate amounts of natural sLOX-1 for use as an assay standard and immunogen could not be obtained after various trials. We decided to use LOX-1 ECD (residues 85–273) as the assay standard and immunogen. We overcame some of the differences between natural sLOX-1 and LOX-1 ECD by taking advantage of the characteristics of immunological methods described below.

3.2. Production of mAbs

In our initial attempt, we produced mAbs against LOX-1 ECD by a standard method; however, the antibodies we obtained reacted only with precipitated (denatured) LOX-1 ECD and not with sLOX-1. We then sought to obtain mAbs that could react with sLOX-1 by immunizing A/J mice in order to obtain higher antibody titers using a novel screening method called competitive TR-FIA.

Consequently, using LOX-1 ECD as an antigen, we obtained nine mAbs (6B11, 1G2, 2E4, 2E5, 2G11, 3E12, 7G1, 1A7, and 1B8) which bound not only to LOX-1 ECD but also to sLOX-1 derived from the cell culture supernatant of LOX-1–CHO. The characteristics of these anti-sLOX-1 mAbs are summarized in Table 1. Affinities of the mAbs to LOX-1 ECD were estimated by Scatchard analysis, although some of them could not be calculated because of absence of linear relationships in their Scatchard plots. Most of the dissociation constants (K_d) were less than 1 nM, indicating that the antigen-binding affinities of these antibodies were high enough for use in sensitive immunoassays.

3.3. Selection of mAbs for sensitive sandwich immunoassay

Seventy-two combinations using eight biotin-labeled mAbs (1B8 could not be labeled) and nine immobilized antibodies were investigated to select suitable mAb pairs for the ability to detect sLOX-1 in a two-site sandwich TR-FIA system. As shown in Table 2, the combination of 6B11 and 1A7 showed the best response and we selected them for the two-site sandwich immunoassay.

Table 2
Combination of monoclonal antibodies for sLOX-1 sandwich immunoassay.

No.	Immobilized antibodies								
	6B11	1A7	3E12	1G2	2G11	2E4	7G1	2E5	1B8
Biotinylated antibodies									
6B11	–	++	–	–	–	–	–	–	–
1A7	++	–	–	–	–	–	–	–	–
3E12	+	–	–	–	–	–	–	–	–
1G2	+	–	–	–	–	–	–	+	–
2G11	+	–	–	–	–	–	–	+	–
2E4	–	–	–	–	–	–	–	–	–
7G1	–	–	–	–	–	–	–	–	–
2E5	–	–	–	–	–	–	–	–	–
1B8	None	None	None	None	None	None	None	None	None

LOX-1 ECD was used as the analyte (antigen).

3.4. Antibody-binding site

The C-type lectin-like domain (CTLD, residues 143–273) of LOX-1 forms a disulfide-linked homodimer [18,19] with Cys140, and therefore, natural sLOX-1 and LOX-1 ECD should exist as a homodimer. Generally, a labeled antibody and an immobilized antibody derived from the same mAb clone would bind to the two structurally identical sites of one homodimer molecule if the mAb can bind to the monomer. However, LOX-1 ECD was not sandwiched by any of the identical mAb pairs, as shown in Table 2. Moreover, neither 6B11 nor 1A7 was able to bind to the denatured LOX-1 ECD in western blotting (data not shown). These results suggest that 6B11 and 1A7 may bind to sites that appear on the natural homodimeric form of sLOX-1.

From the results of epitope mapping, the mAbs 6B11 and 1A7 had almost the same binding profiles at residues 147–158, 195–203, 201–215, and 222–236. Among these residues, 195–203 and 201–205, which are positioned at the engaging surface of the sLOX-1 homodimer, showed stronger binding to both the mAbs. Thus, we presumed that 6B11 and 1A7 may bind to the two steric structures around the engaging surface. Although the precise binding positions have not yet been fully clarified, CLEIA using 6B11 and 1A7 appears to be sufficiently specific because it measures only the homodimeric structure of sLOX-1.

3.5. Development of CLEIA

3.5.1. Assay conditions

We selected 1A7 as the immobilized antibody on microplate wells and 6B11 as the enzyme-labeled antibody for detection. Binding to the immobilized 1A7 IgG (first incubation) was equilibrated for approximately 2 h at room temperature without shaking (data not shown). Therefore, the first incubation time was fixed at 4 h. Binding of ALP-labeled 6B11 Fab′ (second incubation) was equilibrated within 1 h at room temperature without shaking (data not shown). Consequently, 1 h was selected as the standard second incubation time. The incubation times for CLEIA using these mAbs were shorter than those of ELISA (18 h) using polyclonal antibodies [11], and the assay results could be obtained within a day.

3.5.2. CLEIA standard curve, recovery, and precision

Our previous ELISA using polyclonal antibodies was not sensitive enough to be able to determine the circulating sLOX-1 levels in normal healthy human subjects [11,12]. In this study, we developed a more sensitive CLEIA using newly developed anti-sLOX-1 mAbs, ALP as an enzyme label, and APS-5 as a chemiluminescent substrate. CLEIA standard curve for LOX-1 ECD was linear on log (sLOX-1 concentration)-log (chemiliminescence intensity after subtraction of the blank value, 1163 cps) scale, ranging from 8 to 25,000 pg/mL. Precisions were 14.4, 6.3, 5.2, 4.2, 4.6, and 3.3% of coefficient of variation (CV, $n = 10$) at 8, 40, 200, 1000, 5000, and 25,000 pg/mL of standard concentrations, respectively.

Table 3
Intra- and inter-assay precision of monoclonal chemiluminsecent enzyme immunoassay.

STD[a] Added	Measured (pg/mL) Mean ± S.D.[c]	CV[b] (%)	Bias (%)
Intra-assay ($n = 5$)			
0	200 ± 13	6.5	–
100	297 ± 15	5.1	−3.0
1000	1130 ± 50	4.4	−7.0
5000	4880 ± 310	6.4	−6.4
Inter-assay[d] ($n = 15$)			
0	198 ± 12	6.1	–
100	298 ± 12	4.0	0.0
1000	1160 ± 50	4.3	−3.8
5000	4880 ± 230	4.7	−6.4

[a] LOX-1 ECD was used as the assay standard (STD) and added to normal human serum.
[b] Coefficient of variation (CV).
[c] Standard deviation (S.D.).
[d] Overall mean, S.D., and CV for three batches were calculated.

Next, we estimated intra- and inter-assay precision using sera spiked with LOX-1 ECD. As shown in Table 3, CV values (intra-assay CV 4.4–6.5% and inter-assay CV 4.0–6.1%) were good enough. Effects of serum on sLOX-1 measurement were negligible, although standard LOX-1 ECD solutions were prepared without a serum component. We were able to measure 8–25,000 pg/mL of sLOX-1 from the standard curve. Thus, this new CLEIA was 125-fold more sensitive than our previous polyclonal antibody ELISA with colorimetric detection (quantification range 1–100 ng/mL) [11]. In addition, samples with concentration higher than 25,000 pg/mL (25, 50, 100 ng/mL of spiked samples) could be measured accurately by CLEIA after several fold dilution with the CLEIA buffer (1/1, 1/4, 1/16, 1/64, 1/256, and 1/1024) (recovery 91–104%).

3.5.3. Specificity

We were unable to obtain sufficient amounts of natural human sLOX-1 to be used as a standard; therefore, we examined the reactivity of the mAbs with a natural form of sLOX-1 that was produced in cell-conditioned medium from human aortic endothelial cells (HAEC; Cascade Biologics, Portland, OR, USA) and activated by TNF-α (R&D Systems, Minneapolis, MN, USA). We successfully detected natural human sLOX-1 by CLEIA, as shown in Fig. 1.

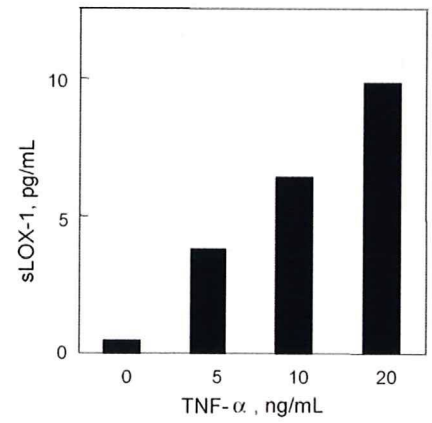

Fig. 1. TNF-α induced sLOX-1 release from human aortic endothelial cells (HAEC). After HAEC were incubated with the indicated concentrations of TNF-α for 24 h, they were further incubated for 24 h in 1% FCS/199 medium. Cell-conditioned media were subjected to the sLOX-1 CLEIA. Values below the assay range (<8 pg/mL) were for reference, calculated by extrapolation.

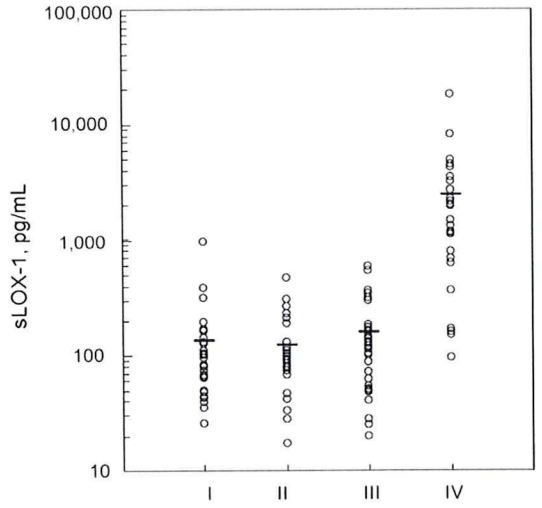

Fig. 2. Diagnosis of ACS by CLEIA. Serum sLOX-1 concentrations in four groups of subjects were measured by CLEIA. Groups I–III consisted of subjects without ACS (total, 90 subjects) and Group IV comprised patients with ACS (30 patients). See Section 3.6 for details of the four groups. Group IV differed significantly from the other groups ANOVA ($p < 0.0001$).

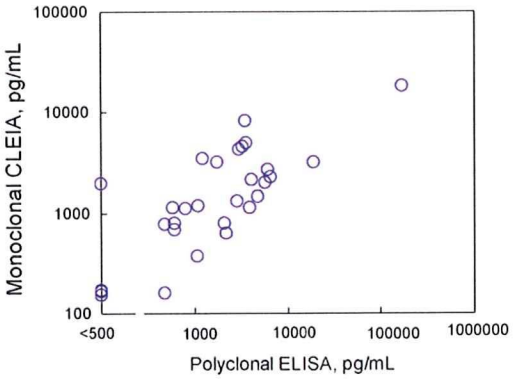

Fig. 3. Correlation of values measured by monoclonal CLEIA and polyclonal ELISA. In ACS (Group IV) samples, the equation relating polyclonal ELISA (x) to monoclonal CLEIA (y) values was $y = 0.09633x + 1751$ ($r^2 = 0.7594$, $p = 0.0000907$, $n = 26$). Values for six points in the polyclonal ELISA that were between the detection limit (0.5 ng/mL) and the limit of quantification (1 ng/mL) were for reference, calculated by extrapolation. Four points below 0.5 pg/mL in the polyclonal ELISA were omitted from the calculations.

The effect of lipoprotein ligands on CLEIA was investigated. Various concentrations of oxidized LDL (up to 500 μg/mL, Biomedical Technologies, Stoughton, MA, USA) or native LDL (as a negative control, up to 1250 μg/mL; Biomedical Technologies) were added to sLOX-1 solutions derived from LOX-1-CHO cell supernatant (600 pg/mL), and then sLOX-1 concentrations were measured by CLEIA after 24 h incubation. All measured values were approximately 600 pg/mL and assay results of CLEIA were not significantly changed by the presence of LOX-1 ligand, oxidized LDL or native LDL (data not shown). These results suggest that the mAb binding site is far from the top (opposite the *N*- and *C*-terminal part) of the CTLD, where oxidized LDL is bound, which agrees with the antibody-binding position described in Section 3.4.

Similarly, various concentrations of bilirubin F, bilirubin C, hemolytic hemoglobin, and chyle (2.3–18.7 mg/dL, 2.6–20.9 mg/dL, 61–484 mg/dL, and 183–1460 FTU, respectively, in final concentration; Interference Check A Plus; Sysmex, Kobe, Japan) were added to normal human serum. Samples were then measured by CLEIA; the assay values were not significantly altered by addition of increasing amounts of these interfering components (data not shown).

It is important to assess cross-reactivities with sLOX-1-like proteins, but we did not find appropriate proteins which had CTLD or identical amino acid sequence. Further studies would be necessary to find any cross-reactive compounds that are present in human serum, especially from patients with diseases including ACS.

3.5.4. *Stability of spiked samples*

LOX-1 ECD (1000 pg/mL) was added to normal human serum and the samples were measured after five freeze–thaw cycles between −80 °C and room temperature. Remaining immunoreactivity after the five cycles was 97–105%. The assay reagents and the spiked samples were stable under the proposed assay conditions.

3.6. *Measurement of serum samples*

We measured 26 serum samples collected from normal subjects (11 males and 15 females, age 28–57) after informed consent and successfully determined the sLOX-1 concentrations in all samples by CLEIA. Their mean (±standard deviation) value was 191 (±89) pg/mL. The proposed CLEIA was sensitive enough to measure levels of sLOX-1 in normal human sera.

We then measured 120 serum samples in four patient groups (30 patients each; Group I, intact coronary; Group II, medically controlled stable coronary heart disease [CHD] patients with significant coronary stenosis; Group III, stable CHD patients who required elective percutaneous coronary intervention [PCI] or coronary artery bypass graft surgery [CABG]; and Group IV, ACS) randomly selected from corresponding groups among 427 patients who underwent diagnostic coronary angiography as described previously [12]. As shown in Fig. 2, the current monoclonal CLEIA was able to discrim-

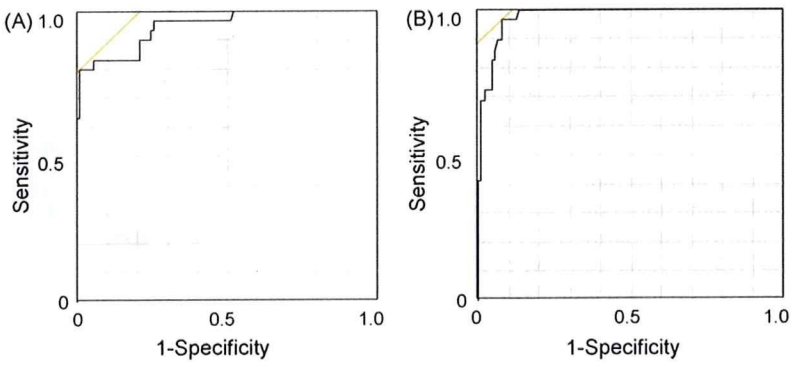

Fig. 4. Comparison of ROC curves of sLOX-1 from monoclonal CLEIA and polyclonal ELISA for the diagnosis of ACS. ROC curves of sLOX-1 measured by monoclonal CLEIA (A) and polyclonal ELISA (B) for the diagnosis of ACS. The true-positive fraction (sensitivity, y axis) was plotted against the false-positive fraction (1–specificity, x axis) by changing cutoff values for the test (sLOX-1). AUC values for monoclonal CLEIA and polyclonal ELISA were 0.948 and 0.978, respectively.

inate ACS patients from others in a similar manner to the previous polyclonal ELISA [12].

There were fairly good correlations between values measured by monoclonal CLEIA and polyclonal ELISA (Fig. 3). The correlation would be improved by better understanding of the stability of sLOX-1 and the differences in assay specificity between polyclonal ELISA and monoclonal CLEIA.

Fig. 4 compares the ROC curves of sLOX-1 values measured by monoclonal CLEIA and the previously reported polyclonal ELISA for the detection of ACS [12]. The area under the curve (AUC) values for monoclonal CLEIA and polyclonal ELISA were 0.948 and 0.978, respectively, indicating sufficient specificity and sensitivity of both the assays in detecting ACS.

We developed a time saving and a very sensitive immunoassay using newly produced mAbs. We believe that this CLEIA could become a useful diagnostic tool for risk prediction or early detection of ACS and other diseases in which sLOX-1 levels are elevated. Furthermore, this assay may prove useful in developing novel drugs to prevent ACS, using sLOX-1 as a surrogate biomarker.

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