

Detection of a High Ratio of Soluble to Membrane-Bound LOX-1 in Aspirated Coronary Thrombi From Patients With ST-Segment–Elevation Myocardial Infarction

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Background—The circulating level of soluble lectin-like oxidized low-density lipoprotein receptor-1 (sLOX-1) is a valuable biomarker of acute myocardial infarction (AMI). The most electronegative low-density lipoprotein, L5, signals through LOX-1 to trigger atherogenesis. We examined the characteristics of LOX-1 and the role of L5 in aspirated coronary thrombi of AMI patients.

Methods and Results—Intracoronary thrombi were aspirated by performing interventional thrombosuction in patients with ST-segment–elevation myocardial infarction (STEMI; n=32) or non–ST-segment–elevation myocardial infarction (n=12). LOX-1 level and the ratio of sLOX-1 to membrane-bound LOX-1 were higher in thrombi of STEMI patients than in those of non–ST-segment–elevation myocardial infarction patients. In all aspirated thrombi, LOX-1 colocalized with apoB 100. When we explored the role of L5 in AMI, deconvolution microscopy showed that particles of L5 but not L1 (the least electronegative low-density lipoprotein) quickly formed aggregates prone to retention in thrombi. Treating human monocytic THP-1 cells with L5 or L1 showed that L5 induced cellular adhesion and promoted the differentiation of monocytes into macrophages in a dose-dependent manner. In a second cohort of AMI patients, the L5 percentage and plasma concentration of sLOX-1 were higher in STEMI patients (n=33) than in non–ST-segment–elevation myocardial infarction patients (n=25), and sLOX-1 level positively correlated with L5 level in AMI patients.

Conclusions—The level of LOX-1 and the ratio of sLOX-1 to membrane-bound LOX-1 in aspirated thrombi, as well as the circulating level of sLOX-1 were higher in STEMI patients than in non–ST-segment–elevation myocardial infarction patients. L5 may play a role in releasing a high level of sLOX-1 into the circulation of STEMI patients. (*J Am Heart Assoc.* 2020;9:e014008. DOI: 10.1161/JAHA.119.014008.)

Key Words: acute myocardial infarction • coronary thrombus • electronegative LDL • LOX-1

Acute myocardial infarction (AMI), including ST-segment–elevation myocardial infarction (STEMI) and non–ST-segment–elevation myocardial infarction (NSTEMI), compromises quality of life and is a leading cause of death worldwide.¹ The principal underlying mechanism of AMI is coronary plaque rupture with acute thrombosis formation.² In

general, the amount of intracoronary thrombosis and the extent of coronary blockage are greater in patients with STEMI than in those with NSTEMI. In patients with AMI, unstable coronary plaque is manifested by key features that include neovascularization, gelatinase hyperactivity, and apoptosis of endothelial cells and macrophages.^{3,4} After rupture, the eroded plaque

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Clinical Perspective

What Is New?

- Aspirated thrombi from patients with ST-segment–elevation myocardial infarction showed a higher ratio of soluble lectin-like oxidized low-density lipoprotein receptor-1 (LOX-1) to membrane-bound LOX-1 and a higher level of total LOX-1, which correlated with apoB100 content, than did aspirated thrombi from patients with non–ST-segment–elevation myocardial infarction.
- Deconvolution microscopy showed that L5 has a high aggregation capability, which is important for retention in coronary thrombi, and in vitro studies in cultured human monocytic cells showed that L5 induces macrophage transformation and LOX-1 expression.
- In a separate cohort of patients with acute myocardial infarction, we observed higher levels of circulating soluble LOX-1 and electronegative L5 in ST-segment–elevation myocardial infarction patients than in non–ST-segment–elevation myocardial infarction patients, and a positive correlation was identified between plasma soluble LOX-1 and L5 levels.

What Are the Clinical Implications?

- Our findings suggest that L5 may play a role in the release of a high level of soluble LOX-1 into the circulation of ST-segment–elevation myocardial infarction patients.
- L5 and soluble LOX-1 may serve as novel biomarkers and provide additional diagnostic value for the early detection of acute cardiovascular events.

surface serves as a site for platelet attachment, activation, and aggregation, which leads to acute thrombogenesis.⁵

Recently, the role of the lectin-like oxidized low-density lipoprotein receptor-1 (LOX-1) in plaque vulnerability and rupture has received increasing attention.⁶ LOX-1 is the main scavenger receptor on endothelial cells for oxidized low-density lipoprotein (ox-LDL) and is responsible for binding, internalizing, and degrading of ox-LDL.⁷ LOX-1 has been shown to be present in human coronary endothelial cells, smooth muscle cells, platelets, macrophages, fibroblasts, and cardiomyocytes.⁸ The membrane-bound form of LOX-1 (ie, mbLOX-1) can be cleaved to generate a soluble form of LOX-1 (ie, sLOX-1) before being released into the circulation. sLOX-1 has been shown to be a useful diagnostic and prognostic marker for the development of atherosclerosis, particularly in acute settings.^{9–12} However, the key entities that contribute to the increased level of plasma sLOX-1 and to the composition of mbLOX-1/sLOX-1 in the context of occluded coronary arteries remain to be clarified.

In search of candidate entities, we have previously isolated a highly negatively charged subfraction of LDL (ie, L5) from

patients with cardiometabolic risk factors.¹³ In a series of studies, we have defined the unique chemical, physical, and functional properties of this lipoprotein particle, which has enabled us to differentiate it from other more electropositive LDL subfractions.^{14,15} In cultured vascular cells, L5 is the only LDL subfraction capable of inducing a spectrum of atherogenic responses through LOX-1 signaling.¹⁶ Furthermore, L5 exposure induces the synthesis of LOX-1.¹⁷ We have previously shown that the plasma L5 level is increased in patients with AMI, specifically those with STEMI.¹⁸ Therefore, we sought to determine whether L5 plays a role in the release of sLOX-1 and in coronary plaque rupture. We hypothesized that in vulnerable plaque, L5 induces LOX-1 expression and the cleavage of mbLOX-1 to sLOX-1, eventually leading to plaque rupture. To explore this possibility in vivo, we obtained intraluminal thrombi aspirated from the culprit arteries of AMI patients during primary percutaneous coronary intervention and examined plaque tissues for sLOX-1 level, as well as L5-associated lipid and protein components. Here, we present novel evidence that connects L5 with coronary artery plaque formation and rupture.

Methods

All data and supporting materials have been provided with the article. The authors declare that all supporting data are available within the article and its online supplementary files.

Patient Population

This study was approved by the institutional review board of China Medical University Hospital in Taiwan (DMR 100-IRB-134), and all participants gave written informed consent in accordance with the Declaration of Helsinki. We prospectively recruited 44 consecutive patients with AMI who underwent a thrombectomy during primary percutaneous coronary intervention at China Medical University Hospital (Cohort 1). Cohort 1 was recruited for comparing thrombus constituents between STEMI and NSTEMI patients. All patients enrolled in this study were 20 years of age or older and presented to the emergency department, where they fulfilled the diagnostic criteria of AMI according to clinical symptoms, electrocardiography, or cardiac enzyme levels. All patients underwent cardiac catheterization and thrombectomy during interventional therapy. The classification of STEMI or NSTEMI was dependent on ECG findings. ECG criteria for STEMI were defined as ST-segment elevation >1 mm in 2 contiguous limb leads or >2 mm in the precordial leads, or as the presence of new-onset left bundle branch block.¹⁹ Patients without ECG findings as described above were categorized as NSTEMI patients. Exclusion criteria included the following: (1) the

onset of chest pain >12 hours before the patient presented to the emergency department; (2) the previous use of fibrinolytic agents before intervention; (3) no thrombus formation but only tight stenotic lesions noted in coronary angiography; or (4) failure to obtain informed consent. Clinical data were obtained from the database of study patients' electronic medical records. To determine the circulating levels of sLOX-1 and L5 in AMI patients, we enrolled a second cohort of 58 patients with AMI (Cohort 2). Cohort 2 was recruited for generally comparing plasma levels of sLOX-1 and L5 between STEMI and NSTEMI patients. Of those patients, 25 had NSTEMI, and 33 had STEMI; all patients fulfilled the above-described inclusion criteria. Patient blood samples were collected via an antecubital vein within 3 days after percutaneous coronary intervention.

Thrombosuction Procedure

All patients included in this study were treated with 300 mg of aspirin, 300 mg of clopidogrel, and a 5000-IU bolus of intravenous heparin on admission. After initial medical therapy, STEMI patients were immediately transferred from the emergency room to the catheterization laboratory to receive primary percutaneous coronary intervention. Within 24 hours of admission, NSTEMI patients with stable hemodynamics received elective early intervention. In the presence of unstable hemodynamics or refractory chest pain under initial medical control,

urgent percutaneous coronary intervention was arranged. The infarct-related artery was engaged by using a 7F guiding catheter to allow the introduction of a 0.014-inch guide wire to pass through the lesion via the femoral artery. Then, thrombosuction was performed with the 7-Fr Kaneka thrombuster II catheter (Kaneka Corp., Japan). Continuously negative pressure suction was applied to the thrombuster while the catheter was advanced forward and backward across the occlusive lesion several times. Each suction usually lasted for 5 to 10 s, and several suctions were performed at the occlusive site during the interventional procedure. The aspirated intracoronary thrombi were collected in the collection filter (Figure 1A). After thrombosuction, balloon angioplasty and/or coronary stent placement was performed according to the operator's judgment.

Immunohistochemical Analysis of Coronary Thrombi

After thrombosuction, all aspirated coronary thrombi were fixed in formalin for 24 hours. The samples were then embedded in paraffin and sliced into 5- μ m-thick sections. Hematoxylin and eosin staining was performed, and thrombi were classified as follows according to age on the basis of their morphologies^{20,21}: (1) fresh or recent thrombus, which was estimated to form in <5 days, was composed of a layered pattern of platelets, nondegenerated fibrin, erythrocytes, macrophages, and intact granulocytes; (2) organized thrombus

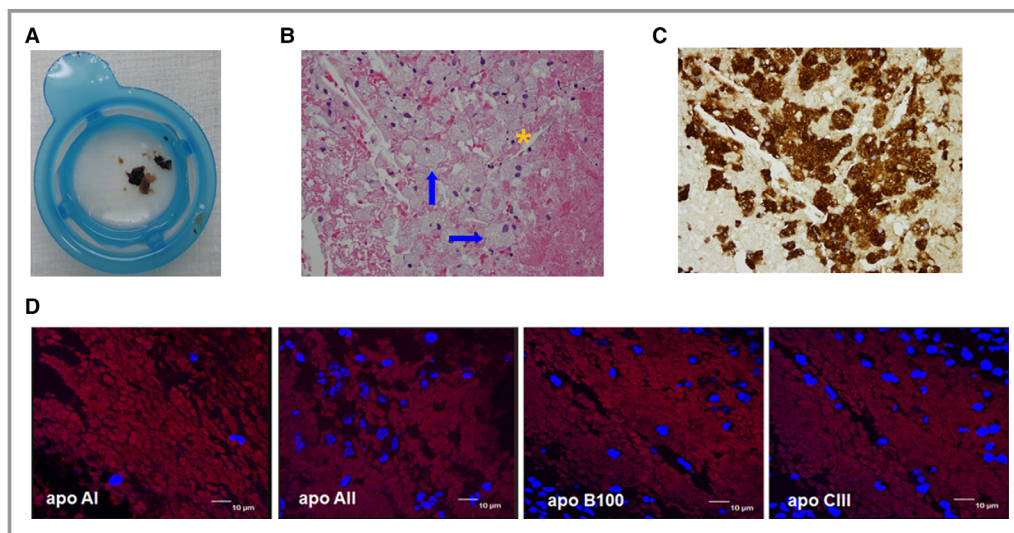


Figure 1. Component analysis of aspirated coronary thrombi. **A**, Representative image showing the gross appearance of coronary thrombotic tissue from a patient with acute ST-segment-elevation myocardial infarction. **B**, Hematoxylin and eosin staining showing soft plaque constituents consisting of foam cells (arrows) and cholesterol crystals with a scratch-like appearance (asterisk). **C**, CD68 staining confirming the presence of foam cells. The stain-free, scratch-like area indicates cholesterol crystals. **D**, Immunofluorescence staining showing the presence of apolipoproteins apoAI, apoAII, apoCIII, and apoB100 in aspirated coronary thrombi (red). The nuclei of macrophages were stained blue with 4',6-diamidino-2-phenylindole.

consisting of degenerated fibrin and erythrocytes, with the in-growth of smooth muscle cells typically within ≥ 5 days. Thrombi with mixed compositions were classified according to the oldest portion. Immunohistochemical analysis was performed with antibodies against CD68 to detect macrophage foam cells (clonePG-M1, 1/100 dilution; Dako, CA). For immunofluorescence studies, thrombi tissues were incubated with anti-LOX-1 antibody (Ab 28-1, T. Sawamura) for 24 hours at 4°C, followed by treatment with AlexaFluor488 donkey anti-mouse IgG. Nuclei were counterstained with 4',6-diamidino-2-phenylindole, and immunohistochemical staining was examined by using fluorescence microscopy. Of the 44 thrombi samples, 20 with a sufficient amount of tissue were selected for the costaining of LOX-1 and apolipoproteins. Samples were incubated with anti-LOX-1 antibody (Ab 28-1) and anti-apoB100, anti-apoAI, anti-apoAII, or anti-apoCIII antibody (Academy Bio-Medical Co., TX) for 24 hours at 4°C. Thrombi were then cotreated with AlexaFluor488 donkey anti-mouse IgG (for Ab 28-1, Abcam, UK) and AlexaFluor647 goat anti-rabbit IgG (for apoproteins, Abcam) for 1 hour. After nuclear staining with 4',6-diamidino-2-phenylindole, images were examined by using fluorescence microscopy. Immunohistochemical analyses were performed by experienced pathologists who were blinded to the patients' clinical history.

LDL Isolation and Ion-Exchange Purification of Electronegative LDL

LDL particles with a final density of 1.019 to 1.063 g/mL were isolated from patient plasma by using sequential potassium bromide density centrifugation to remove chylomicrons, very low-density lipoprotein, and intermediate-density lipoprotein.²² Whole LDL was equilibrated by performing dialysis in a column loaded with buffer A (20 mmol/L TrisHCl, 0.5 mmol/L EDTA, and 0.01% NaN₃ at pH 8.0). Approximately 100 mg of LDL was injected onto a UnoQ12 anion-exchange column (Bio-Rad, CA) by using a fast-protein liquid chromatography system (GE Healthcare, Chicago, IL) and was eluted by using a multistep sodium chloride gradient, as previously described.¹³

Western Blotting of Coronary Thrombi Protein Extracts

Protein extracts from coronary thrombi were used for immunoblotting analyses. Thrombi were lysed in RIPA buffer (Pierce Biotechnology, Inc, MA) with protease inhibitor cocktail. Protein concentration was measured by using a bicinchoninic acid assay (Thermo Fisher Scientific, MA). To detect sLOX-1, we used 2 antibodies against LOX-1: one that recognizes only mbLOX-1 (Ab 1-1, T. Sawamura), and another that binds both membrane-bound and sLOX-1 (Ab 5-2, T. Sawamura).

Enzyme-Linked Immunosorbent Assay for Detecting sLOX-1

Enzyme-linked immunosorbent assays of human plasma were performed by using antibodies against sLOX-1 (Aviscera Bioscience, Inc, CA) according to the manufacturer's instructions. Absorbance was measured with the Infinite M1000 multifunctional monochromator-based microplate reader (TECAN, Switzerland).

Effects of LDL Subfractions on the Differentiation of THP-1 Monocytes into Macrophages

The THP-1 human monocytic cell line (American Type Culture Collection, Manassas, VA) was cultured according to methods described previously.²³ THP-1 cells were incubated with 25 mg/mL L1, 25 mg/mL L5, or 50 mg/mL L5 for 8 hours to examine the amount of differentiated macrophages. Phosphate-buffered saline was used as negative control. After incubation, nonadherent cells were removed by aspiration and washing with C-RPMI. Adherent macrophages were counted under the view of a microscope. LOX-1 expression in macrophages was examined by using immunofluorescence microscopy as described above.

Particle Aggregation in LDL Subfractions

We prepared fresh L1 and L5 in Tris-EDTA at a final concentration of 0.2 mg/mL. DiO-L1 and DiI-L5 labels were prepared as previously described.²⁴ Samples were directly loaded onto glass slides as 100% DiO-L1, 100% DiI-L5, or 50% DiO-L1 +50% DiI-L5 and were examined by using a deconvolution fluorescence light microscope.

Statistical Analysis

Continuous data are expressed as the mean \pm SE for normally distributed variables. Differences between the 2 groups were compared by using Student *t* test. To determine the minimal sample sizes required to detect differences in thrombus LOX-1 levels (Cohort 1) and plasma sLOX-1 levels (Cohort 2) between patients with STEMI and patients with NSTEMI, we performed power analyses. Assuming an effect size of 0.8, 55 patients with STEMI and 17 patients with NSTEMI were required in Cohort 1 to reach a statistical power of 80% when using a 2-sided hypothesis test with a significance level of 0.05. Similarly, in Cohort 2, 31 patients with STEMI and 23 patients with NSTEMI were needed to reach a statistical power of 80%.²⁵ The correlation between LOX-1 expression level in aspirated coronary thrombi and cardiometabolic or immunohistochemical factors was assessed by using the Pearson correlation coefficient. The correlation between peripheral sLOX-1 level and L5 percentage was measured by using the Spearman rank

correlation coefficient. A 2-tailed $P < 0.05$ was considered statistically significant. All analyses were performed by using SPSS 12.0 software (SPSS Inc, Chicago, IL).

Results

Composition of Aspirated Coronary Thrombi From Study Patients

Table 1 shows the demographic and clinical characteristics of 44 consecutive patients with AMI (STEMI, $n=32$; NSTEMI, $n=12$) who underwent successful thrombosuction during percutaneous coronary intervention. No statistically significant difference was observed between STEMI and NSTEMI patients with respect to age, sex, prevalence of hypertension or diabetes mellitus, body mass index, peak cardiac enzyme levels, hsCRP (high-sensitivity C-reactive protein) level, or lipid profile component levels. Of the 12 NSTEMI patients, 2 (16.7%) had in-hospital major adverse cardiac events, whereas none of the STEMI patients had major adverse cardiac events ($P=0.02$). For 35 of the 44 patients (80%), the aspirated materials were consistent with fresh or recent thrombi, whereas those for the remaining 9 (20%) appeared organized on aspiration (Figure 1A). Hematoxylin and eosin staining showed the universal presence of cholesterol crystals in all 44 thrombectomy specimens (Figure 1B). In addition, foam cells were observed in 12 of the 44 samples (27%), which was further confirmed with positive CD68 staining (Figure 1C). Immunofluorescence staining showed the presence of apolipoproteins including apoAI, apoAII, apoCIII, and apoB100 in aspirated coronary thrombi (Figure 1D).

Total LOX-1 Levels and Ratio of sLOX-1 to mbLOX-1 in Aspirated Coronary Thrombi

Immunofluorescence staining showed that LOX-1 protein was more robustly expressed in the aspirated thrombi of STEMI patients than in those of NSTEMI patients (17.8 ± 22.6 versus 6.4 ± 7.8 au, $P=0.016$, Figure 2A and 2B). To further discriminate the type and quantity of LOX-1 expressed in aspirated thrombi, we performed Western blot analysis with the following 2 LOX-1 antibodies (Figure 2C): antibody that recognized both mbLOX-1 and sLOX-1, and antibody that recognized only mbLOX-1. The ratio of sLOX-1 level to mbLOX-1 level was significantly higher in STEMI patients than in NSTEMI patients (3.2 ± 0.9 versus 1.1 ± 0.2 , $P=0.008$, Figure 2D).

Correlation Between Plasma sLOX-1 and L5 Level in AMI Patients

To study the levels of plasma sLOX-1 and L5 in peripheral blood, we enrolled a second cohort of patients with AMI (Cohort 2; Table 1; STEMI, $n=33$; NSTEMI, $n=25$). We found

that both the plasma level of sLOX-1 and the percentage of L5 (ie, L5%) were significantly higher in STEMI patients than in NSTEMI patients (sLOX-1, 131.34 ± 19.87 ng/mL versus 61.62 ± 5.78 ng/mL, respectively, $P < 0.001$; L5%, $6.04 \pm 2.27\%$ versus $2.19 \pm 0.48\%$, respectively, $P=0.02$; Figure 3A and 3B). Notably, we also found that the sLOX-1 level was positively correlated with L5% in all AMI patients ($r=0.41$, $P=0.002$, Figure 3C), suggesting that L5 is associated with the release of a high level of sLOX-1 into the circulation of STEMI patients. We further analyzed the correlation between plasma sLOX-1 level and cardiometabolic risk factors by using the Pearson correlation coefficient. Plasma sLOX-1 level was marginally inversely correlated with plasma high-density lipoprotein level ($r=-0.029$, $P=0.04$), but no significant correlation was observed between sLOX-1 level and other traditional risk factors (Table 2 and Figure S1).

Correlation Between LOX-1 and ApoB100 in Aspirated Thrombi in AMI Patients

In Cohort 1 ($n=44$), we performed a correlation analysis between LOX-1 protein levels in the aspirated coronary thrombi and key cardiometabolic risk factors or lipoprotein constituents in the thrombi, as shown in Table 3. LOX-1 level was not correlated with clinical cardiometabolic risk factors such as diabetes mellitus, lipid profile component levels, or hsCRP level. However, immunofluorescence studies showed that LOX-1 level was strongly correlated with apoB100 content ($r=0.69$, $P=0.001$, $n=20$) but not apoAI, apoAII, or apoCIII content in coronary thrombi. This was consistent with our immunofluorescence findings showing that LOX-1 and apoB100 colocalized in aspirated thrombi (Figure 4). Because apoB100 is the most abundant lipoprotein in L5 or L1 (the least electronegative subfraction of LDL), we next examined whether L5 or L1 plays a role via the conjugation of LOX-1 in coronary thrombi formed after plaque rupture. Using deconvolution microscopy, we observed the quick aggregation of DiI-labeled L5 particles prone to retention in the aspirated coronary thrombi, whereas DiO-labeled L1 particles remained heterogeneously dispersed (Figure S2).

L5-Induced Differentiation of Monocytes into Macrophages

Because lipid-laden macrophages (ie, foam cells) were present in $\approx 30\%$ of aspirated thrombi, we conducted an in vitro study to compare the effects of L5 and L1 on the transformation of monocytes into macrophages. Human monocytic THP-1 cells were treated with L1 (25 $\mu\text{g}/\text{mL}$) or subapoptotic concentrations of L5 (25 and 50 $\mu\text{g}/\text{mL}$) for 8 hours. Subapoptotic doses of L5 induced cellular adhesion to the plastic surface of the culture plate and promoted the differentiation of monocytes

Table 1. Baseline Characteristics of the 2 Cohorts of Patients With STEMI or NSTEMI

	Cohort 1			Cohort 2		
	STEMI (n=32)	NSTEMI (n=12)	P Value	STEMI (n=33)	NSTEMI (n=25)	P Value
Age, y	51.0±2.2	53.4±3.8	0.58	56.9±1.9	59.5±2.4	0.17
Men*	31 (96.9)	9 (75)	0.28	33 (100)	21 (84)	0.02 [†]
Smoker*	21 (65.6)	7 (58.3)	0.92	24 (72.7)	5 (20)	<0.001 [†]
Hypertension*	13 (40.6)	2 (16.7)	0.23	18 (54.5)	18 (72)	0.18
DM*	7 (21.9)	2 (16.7)	0.81	11 (33.3)	13 (52)	0.16
Hyperlipidemia*	8 (25)	3 (25)	1.000	9 (27.3)	8 (32)	0.71
CKD*	1 (3.1)	1 (8.3)	0.49	2 (6.1)	3 (12)	0.44
ESRD*	1 (3.1)	0 (0)	0.58	1 (3.0)	2 (8)	0.41
CVA history*	0 (0)	0 (0)	1.000	0 (0)	0 (0)	1.00
BMI	26.0±1.0	28.1±1.3	0.13	26.5±0.6	26.9±0.8	0.97
Culprit vessel						
LCX*	25 (78.1)	10 (83.3)	0.06	7 (21.2)	9 (36)	0.22
RCA*	14 (43.8)	7 (58.3)	0.87	16 (48.5)	4 (16)	0.01 [†]
LAD*	24 (75)	10 (83.3)	0.67	11 (33.3)	15 (60)	0.046 [†]
Killip status	1.5±0.2	1.8±0.4	0.23	1.7±0.2	1.9±0.2	0.16
Peak CPK, IU/L	2581.3±367.7	2468.8±1056.2	0.14	1494.4±308.3	974.9±218.3	0.17
Peak CKMB, ng/mL	172.5±32.2	192.5±89.5	0.25	134.8±25.1	66.9±15.4	0.06
Peak Tnl, ng/mL [§]	104.2±15.7	80.5±39.4	0.08
eGFR, mL/min per 1.73 m ² ‡	82.8±4.5	72.8±3.9	0.19	74.7±3.4	78.0±6.2	0.75
Cr, mg/dL‡	1.0±0.1	1.0±0.0	0.63	1.1±0.1	1.2±0.2	0.16
hsCRP, mg/dL	3.1±1.4	2.9±2.4	0.96	0.4±0.1	0.9±0.3	0.72
T-CHOL, mg/dL	183.0±8.0	170.7±12.7	0.43	183.0±8.9	179.6±7.2	0.96
TG, mg/dL	144.0±17.3	120.1±21.3	0.81	202.1±35.9	117.6±12.2	0.02 [†]
HDL, mg/dL	36.4±2.3	33.0±3.0	0.52	39.5±2.0	42.6±2.1	0.20
LDL, mg/dL	114.7±6.0	111.4±10.7	0.78	173.3±62.4	116.9±6.7	0.23
LVEF	52.7±2.6	46.4±5.3	0.33	54.8±1.8	49.8±2.6	0.11
In-hospital MACE	0 (0)	2 (16.7)	0.02 [†]	1 (3.0)	0 (0)	0.38

Data are expressed as the mean±SE, unless otherwise indicated. BMI indicates body mass index; CKMB, creatine kinase MB; CKD indicates chronic kidney disease; CPK, creatine phosphokinase; Cr, creatinine; hsCRP, high-sensitivity C-reactive protein; CVA, cerebral vascular accident; DM, diabetes mellitus; ESRD, end-stage renal disease; GFR, glomerular filtration rate; HDL, high-density lipoprotein; LAD, left anterior descending; LCX, left circumflex; LVEF, left ventricular ejection fraction; LDL, low-density lipoprotein; MACE, major adverse cardiac events; NSTEMI, non-ST-segment-elevation myocardial infarction; RCA, right coronary artery; STEMI, ST-segment-elevation myocardial infarction; T-CHOL, total cholesterol; TG, triglyceride; Tnl, troponin I.

*Number (%).

[†]P<0.05.

[‡]Excluding ESRD cases.

[§]A total of 10 values of Tnl in Cohort 2 (STEMI=8, NSTEMI=2) exceeds the upper limit; thus the levels cannot be precisely quantified and compared.

into macrophages in a dose-dependent manner (Figure 5), which was confirmed with immunofluorescence staining for CD68 (data not shown). Furthermore, in transformed macrophages, treatment with L5 enhanced LOX-1 expression in a dose-dependent manner. In contrast, L1 did not trigger the transformation of monocytes to macrophages, nor was LOX-1 expression enhanced by treatment with L1 (Figure 5).

Discussion

For the first time to our knowledge, we report that the ratio of sLOX-1 to mbLOX-1 is higher in aspirated thrombi from STEMI patients than in thrombi from NSTEMI patients. Furthermore, in a separate cohort of AMI patients, we found that peripheral sLOX-1 and L5% are higher in STEMI patients than in NSTEMI

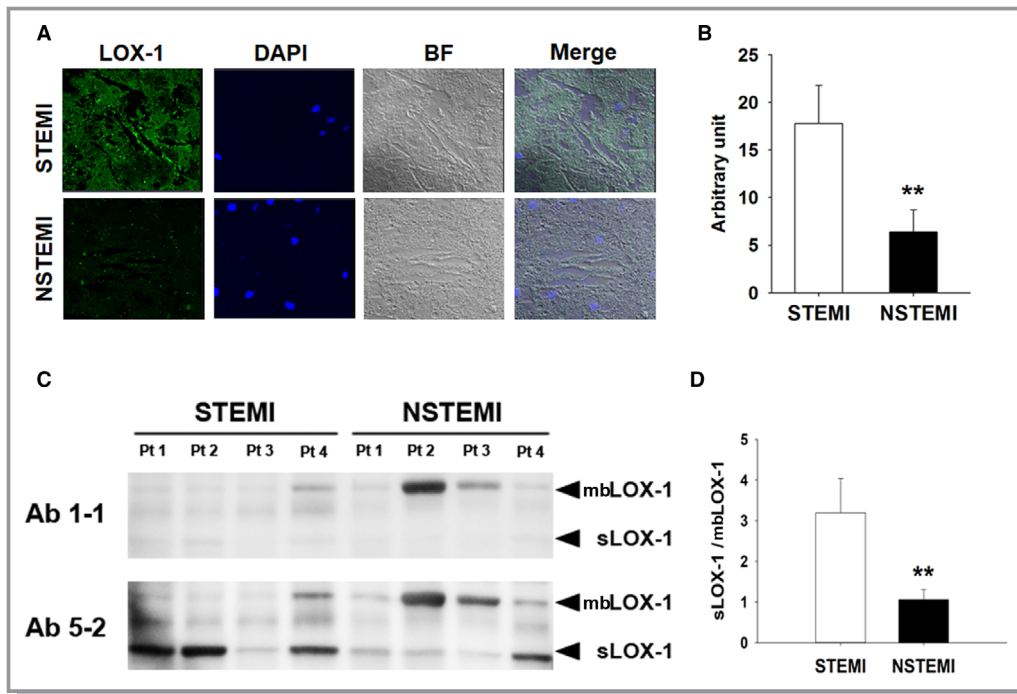


Figure 2. LOX-1 expression in aspirated coronary thrombi of patients with acute myocardial infarction (AMI). **A**, Immunofluorescence staining showing that LOX-1 (green) is expressed in the coronary thrombi of patients with ST-segment-elevation myocardial infarction (STEMI) and of patients with non-segment-elevation myocardial infarction (NSTEMI). **B**, LOX-1 expression levels were significantly higher in the coronary thrombi of STEMI patients (n=32) than in those of NSTEMI patients (n=12). **C**, Representative Western blot showing the protein expression of both membrane-bound LOX-1 (mbLOX-1) and soluble LOX-1 (sLOX-1). Antibody #5-2 recognizes both mbLOX-1 and sLOX-1, and antibody #1-1 recognizes only mbLOX-1. **D**, Comparison showing the ratio of sLOX-1 to mbLOX-1 in thrombi from STEMI patients and from NSTEMI patients. ***P*<0.02. BF indicates bright-field microscopic images; DAPI, 4',6-diamidino-2-phenylindole; LOX-1, lectin-like oxidized low-density lipoprotein receptor-1; pt, patient.

patients and that sLOX-1 and L5 levels are positively correlated in all AMI patients. We also discovered that there is a significant correlation between LOX-1 expression and apoB100 content in the aspirated coronary thrombi of AMI

patients and that L5 has a higher aggregation capability than L1. Finally, we found that treating human monocytic THP-1 cells with L5 induced macrophage transformation and LOX-1 expression in vitro. Together, these findings indicate that L5

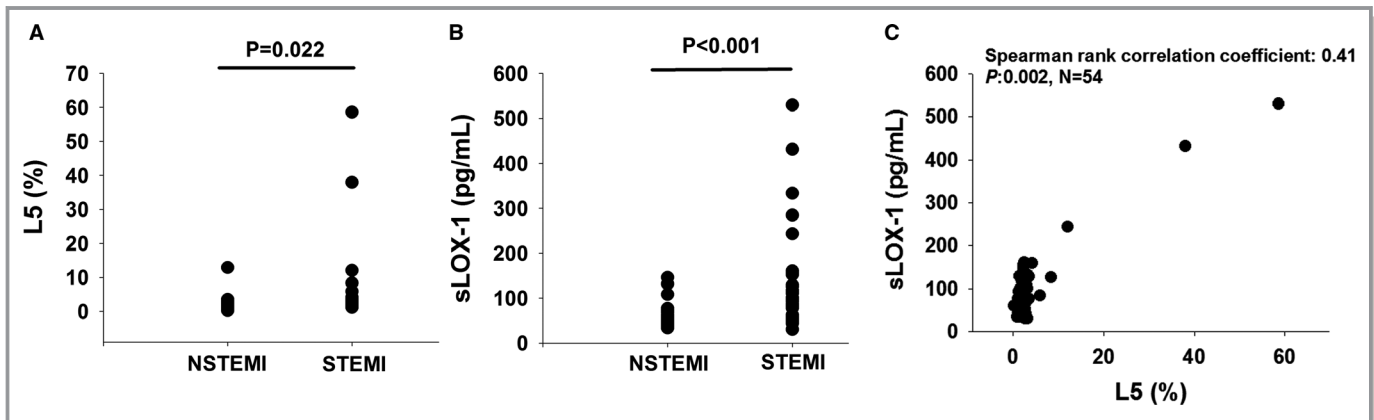


Figure 3. Correlation between plasma L5 percentage (L5%) and soluble LOX-1 (sLOX-1) level in patients with acute myocardial infarction. **A**, Comparison of plasma L5% between patients with ST-segment-elevation myocardial infarction (STEMI) and patients with non-ST-segment-elevation myocardial infarction (NSTEMI). **B**, Comparison of sLOX-1 levels between patients with STEMI and patients with NSTEMI. **C**, Correlation between plasma L5% and sLOX-1 level in all patients (n=54). LOX-1 indicates lectin-like oxidized low-density lipoprotein receptor-1.

Table 2. Correlation Between sLOX-1 Level in Peripheral Plasma and Cardiometabolic Risk Factors

	DM	T-CHOL	TG	HDL	LDL	hsCRP	Peak CPK	Peak CKMB	GFR	Cr	LVEF
<i>r</i>	0.01	-0.07	0.18	-0.29	0.09	-0.09	0.06	0.11	-0.12	0.09	0.04
<i>P</i> Value	0.91	0.64	0.19	0.04*	0.52	0.71	0.68	0.411	0.40	0.52	0.77

CKMB indicates creatine kinase MB; CPK, creatine phosphokinase; Cr, creatinine; hsCRP, high-sensitivity C-reactive protein; DM, diabetes mellitus; GFR, glomerular filtration rate; HDL, high-density lipoprotein; LDL, low-density lipoprotein; LVEF, left ventricular ejection fraction; sLOX1, soluble lectin-like oxidized low-density lipoprotein receptor-1; T-CHOL, total cholesterol; TG, triglyceride.

**P*<0.05.

may play a role in the release of high-level sLOX-1 into the circulation in patients with STEMI.

LOX-1 Expression in Aspirated Coronary Thrombi

LOX-1, a primary endothelial ox-LDL receptor, was first cloned by Sawamura et al⁷ in 1997 from bovine aortic endothelial cells. LOX-1 is primarily expressed on endothelial cells, macrophages, and smooth muscle cells²⁶ and is believed to be responsible for the formation of atherosclerosis in humans and various animals. LOX-1 has been found in human atherosclerotic plaque tissues and contributes to the initiation, progression, and destabilization of atherosclerotic plaque, leading to eventual rupture and intravascular thrombosis formation.^{10,27-31} Unlike ox-LDL, which is oxidized *ex vivo* by copper, naturally occurring electronegative LDL (ie, L5) can be isolated from human plasma and signals through LOX-1 to activate vascular endothelial cell apoptosis.¹⁶ Furthermore, L5 was found to upregulate LOX-1 expression in endothelial cells, which leads to a positive feedback loop of cellular apoptosis.¹⁷ To our knowledge, we are the first to characterize the expression of LOX-1 in aspirated coronary thrombi from AMI patients, and our findings substantiate the connection between LOX-1 signaling and plaque rupture with coronary thrombosis. Notably, we found that the expression level of LOX-1 in aspirated thrombi was significantly higher in STEMI patients than in NSTEMI patients. In STEMI patients, the thrombus burden is typically large, causing total occlusion of the culprit coronary artery, whereas the thrombus burden is often relatively smaller in NSTEMI patients, leading to a subtotal coronary flow obstruction. Thus, the greater abundance of thrombus LOX-1 in STEMI patients than in NSTEMI

patients correlates with the higher thrombus burden in STEMI patients.

We have previously shown that L5 isolated from the plasma of STEMI patients can signal through LOX-1 and platelet-activating factor receptor to enhance adenosine diphosphate-induced platelet activation via the protein kinase C- α -mediated pathway.³² L5 also induces tissue factor and P-selectin expression in human aortic endothelial cells, in turn triggering platelet activation and aggregation. In an experimental model of stroke, L5 potentiated amyloid β -mediated platelet activation, platelet aggregation, and hemostasis via IKK2/NF- κ B signaling.³³ All of these findings indicate that the increased level of LOX-1 promotes a greater extent of intravascular thrombosis in STEMI patients that may be mediated by the L5-LOX-1 signaling pathway.

Increased sLOX-1 Level in Aspirated Coronary Thrombi and Peripheral Blood

Studies in humans have shown that mbLOX-1 can be cleaved to form sLOX-1 before being released into the circulation. In addition, circulating levels of sLOX-1 have been proposed as a valuable biomarker of AMI.^{9,34} The circulating sLOX-1 particles may arise from activated platelets, endothelial cells, or other constituents in atheromatous plaque^{6,9,10,34}—all of which have been shown to be targeted by the L5-LOX-1 signaling cascade.^{16,17,32,33} In this study, we showed that both the total LOX-1 level and the ratio of sLOX-1 to mbLOX-1 were higher in aspirated thrombi from STEMI patients than in those from NSTEMI patients. This finding supports the notion that the proteolytic cleavage of mbLOX-1 may occur in ruptured plaque tissues. Furthermore, we showed in a

Table 3. Correlation Between LOX-1 Level in Aspirated Coronary Thrombi and Cardiometabolic Risk Factors or Lipoprotein Constituents in Thrombi

	DM	T-CHOL	TG	HDL	LDL	hsCRP	ApoB100	ApoAI	ApoAII	ApoCIII	Peak CPK	Peak CKMB	Peak Tnl	GFR	Cr
<i>r</i>	-0.06	0.08	-0.13	0.16	0.03	0.04	0.69	0.36	0.29	0.31	0.10	0.06	0.18	-0.07	0.10
<i>P</i> Value	0.72	0.64	0.44	0.34	0.87	0.85	0.001*	0.12	0.22	0.37	0.53	0.69	0.24	0.66	0.53

Apo indicates apolipoprotein; CKMB, creatine kinase MB; CPK, creatine phosphokinase; Cr, creatinine; DM, diabetes mellitus; hsCRP, high-sensitivity C-reactive protein; GFR, glomerular filtration rate; HDL, high-density lipoprotein; LDL, low-density lipoprotein; LOX-1, lectin-like oxidized low-density lipoprotein receptor-1; T-CHOL, total cholesterol; TG, triglyceride; Tnl, troponin I.

**P*<0.05.

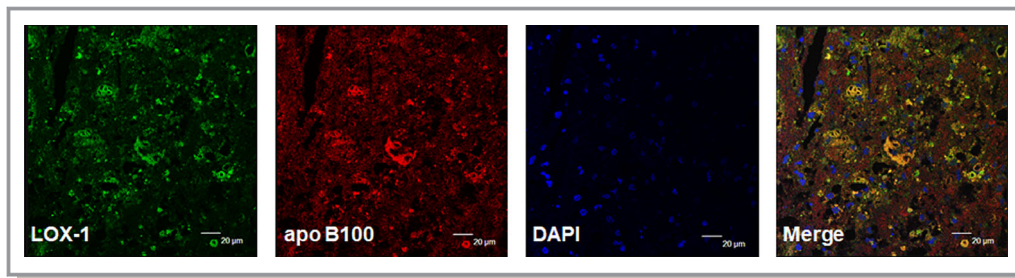


Figure 4. Immunofluorescence costaining of LOX-1 and apoB100 in aspirated coronary thrombi. Representative immunostaining of LOX-1 and apoB100 showing that LOX-1 expression is strongly correlated with apoB100 content ($r=0.69$, $P=0.001$, $n=20$) in patients with acute myocardial infarction. apoB100 indicates apolipoprotein B100; DAPI, 4',6-diamidino-2-phenylindole; LOX-1, lectin-like oxidized low-density lipoprotein receptor-1.

separate cohort that sLOX-1 level and L5% were positively correlated in the peripheral blood of AMI patients. This evidence collectively supports the notion that L5 may be the upstream stimulator that leads to the release of a high level of sLOX-1 into the circulation in STEMI patients by directly binding to mbLOX-1 in platelets, endothelial cells, macrophages, and smooth muscle cells of atheroma.

Interestingly, our data showed that plasma sLOX-1 level was marginally inversely correlated with plasma high-density lipoprotein level, but no correlation was observed between sLOX-1 level and other traditional risk factors. This finding was in line with the report by Inoue et al,³⁵ who also observed an inverse correlation between high-density lipoprotein level and sLOX-1 level but not between sLOX-1 level and other conventional risk factors, except for smoking, which was identified in a larger number of individuals in the general

population. In addition, the vascular expression of LOX-1 has been shown to be increased in hypertensive³⁶ and diabetic³⁷ rats. However, because these studies were performed in animal models, there are limitations in the comparisons that can be made with our data. In our study, although plasma sLOX-1 level was not correlated with traditional risk factors, it was positively correlated with L5% in all AMI patients. Further studies are needed to elucidate whether plasma sLOX-1 level or coronary thrombi LOX-1 expression is positively correlated with other novel risk factors.

LOX-1 and L5: Co-Players in Acute Atherothrombosis

In this study, we discovered that the amount of LOX-1 expressed in aspirated coronary thrombi is independent of traditional

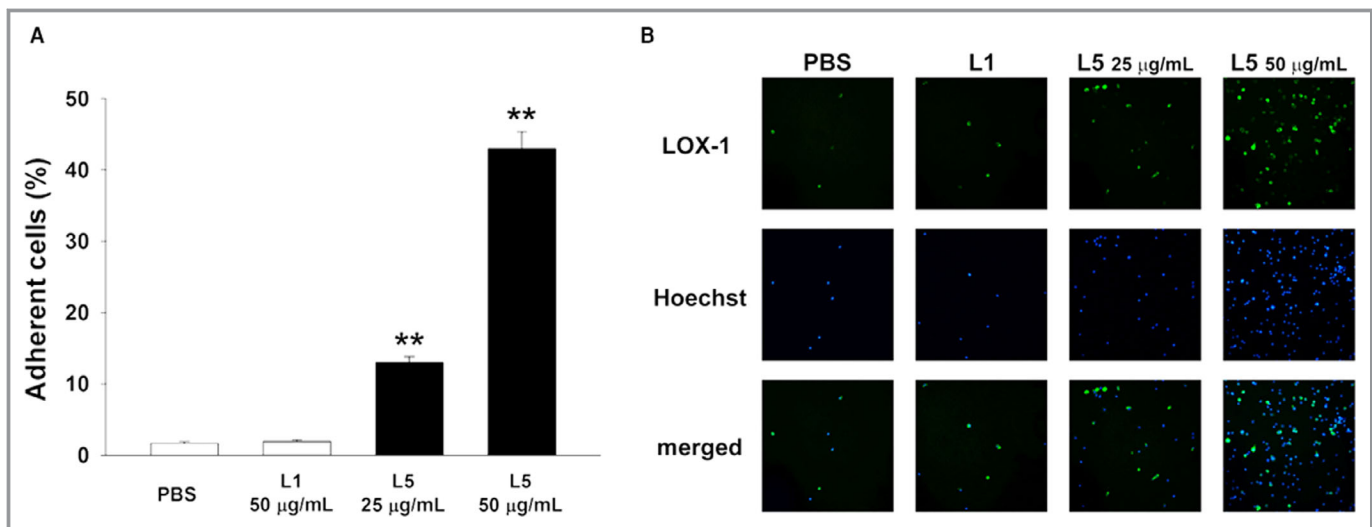


Figure 5. L5-induced differentiation of monocytes into macrophages. **A**, Treatment of THP-1 human monocytic cells with a subapoptotic concentration (25 or 50 µg/mL) of L5 but not treatment with L1 promoted the differentiation of monocytes to macrophages in a dose-dependent manner after 8 hours of incubation. ****** $P<0.01$. **B**, Immunofluorescence staining showing the expression of the scavenger receptor LOX-1 (green) in adherent cells. The transformed macrophages induced by L5 adhered to the bottom of plastic tissue culture plates and were stained with Hoechst 33342 (blue). LOX-1 indicates lectin-like oxidized low-density lipoprotein receptor-1; PBS, phosphate-buffered saline.

cardiovascular risk factors such as hypertension, diabetes mellitus, lipid profile component levels, or plasma hsCRP level. However, LOX-1 expression was significantly correlated with apoB100 content in coronary thrombi, which is the major surface apolipoprotein of LDL, including L5. Using deconvolution microscopy, we found that L5 but not L1 (the least electronegative subfraction of LDL) particles quickly formed aggregates prone to retention in thrombi. The enhanced ability of L5 to aggregate, which may arise from its highly electronegative surface, further enables it to penetrate into the vascular wall to activate subsequent atherosclerotic changes, similar to previous observations of oxidatively modified LDL.^{38–40} Our *in vitro* findings further indicated that the enhanced ability of L5 to aggregate may also induce the transformation of monocytes into macrophages and stimulate macrophages to express LOX-1 in a dose-dependent manner. All of these findings are consistent with the currently accepted model of atherogenesis in which LDL modification promotes the phagocytosis of modified LDL by monocytes or macrophages, in turn activating the migration of monocytes into the arterial wall and promoting the formation of foam cells.⁴¹ In contrast to ox-LDL, which is artificially oxidized by copper, L5 is the only known naturally occurring pathogenic LDL derived from the human plasma. From this perspective, we believe that L5 is most likely to be the principal upstream ligand-specific stimulator of LOX-1 that leads to the initiation, progression, and instability of atherosclerotic plaque and intravascular thrombosis formation in patients with acute coronary syndrome. Nevertheless, in addition to L5, various stimuli such as advanced glycation end-products, cytokines, or shear stress may also upregulate the expression of LOX-1 in patients with AMI.⁴² Further studies are needed to delineate the individual contribution of LOX-1 to the pathologic evolution of acute coronary syndrome (ACS) in the context of multifactorial milieu.

Clinical Implications and Potential Mechanisms for sLOX-1 Elevation in ACS

We have previously demonstrated that the plasma level of naturally occurring, atherogenic L5 LDL is significantly higher in patients with acute STEMI³² and acute ischemic stroke³³ than in normal individuals. However, in those studies, we found no significant difference in the plasma LDL level between diseased and normal individuals. Similarly, serum sLOX-1 level was previously shown to be significantly higher in patients with ACS than in control individuals with no apparent coronary artery disease; thus serum sLOX-1 levels may begin to rise before the onset of ACS.³³ In addition, sLOX-1 level has been shown to independently predict long-term all-cause mortality and major adverse cardiac events after STEMI.⁴³ Therefore, both L5 and sLOX-1 may serve as novel biomarkers and provide additional diagnostic value for

the early detection of acute cardiovascular and cerebrovascular events.

In addition to troponin T, creatine kinase MB, and hsCRP,^{34,43} sLOX-1 is a useful biomarker for ACS; however, little is known about how sLOX-1 level is increased upon AMI. One possibility is that sLOX-1 level increases in parallel with the expression of LOX-1. This is conceivable because of the property of LOX-1 as an acute-phase reactant that can be induced by electronegative L5.¹⁷ Another possibility is that the conversion rate from full-length LOX-1 to sLOX-1 is increased. Our finding that the ratio of sLOX-1 to membrane-bound LOX-1 in aspirated coronary thrombi of patients with STEMI is higher than that in patients with NSTEMI has shed light on this process for the first time, to our knowledge.

Study Limitations

Our study had some limitations. First, the analysis of LOX-1 level in thrombi and plasma was not performed on the same group of patients. At our institute, coronary thrombi can be obtained by aspiration thrombectomy during percutaneous coronary intervention in only 10% to 20% of NSTEMI patients, which is much lower than that in STEMI patients (90%). Our experience with lower coronary thrombus burden in NSTEMI patients is in line with a previous report showing that the percentage of coronary thrombus formation in non-Q wave MI patients was only 24%.⁴⁴ Therefore, Cohort 1 was intended for comparing thrombus constituents between patients with STEMI and patients with NSTEMI. However, because Cohort 1 represented only a minority of NSTEMI patients, it may not have been the most appropriate cohort for generally comparing plasma levels of sLOX-1 and L5 between STEMI and NSTEMI patients. Thus, to avoid the underrepresentation of general NSTEMI patients, we recruited a second cohort of patients to compare the plasma levels of sLOX-1 and L5 between STEMI and NSTEMI patients.

Another limitation of our study was that the sample size in Cohort 1 was small, especially in the NSTEMI subgroup, resulting in an underpowered comparison of thrombus LOX-1 between STEMI and NSTEMI patients. This was because, unlike in STEMI patients, coronary thrombus formation in NSTEMI patients is seldom observed. Indeed, further large-scale studies are needed to confirm our findings. In addition, our study did not show direct tissue evidence of LOX-1 expression in intact coronary plaque, warranting future studies. Last, whether electronegative L5 can be taken up by transformed macrophages through the LOX-1 receptor remains to be shown. On the basis of our previous studies of L5^{16,17} and the observations of others,⁴⁵ we believe that in the subendothelial space, L5 not only transforms monocytes into LOX-1-expressing macrophages but also promotes its

uptake by macrophages via the LOX-1 receptor, which leads to a positive feedback loop of foam cell formation.

Conclusions

LOX-1 expression and the ratio of sLOX-1 to mbLOX-1 were higher in aspirated coronary thrombi of STEMI patients than in those of NSTEMI patients. Furthermore, the circulating level of sLOX-1 was greater in STEMI patients than in NSTEMI patients. Our findings support that electronegative L5 may play a role as the upstream stimulator of LOX-1 and in the release of a high-level sLOX-1 into the circulation of STEMI patients.

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Disclosures

None.

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SUPPLEMENTAL MATERIAL

Figure S1. Correlation between plasma sLOX-1 level and cardiometabolic risk factors.

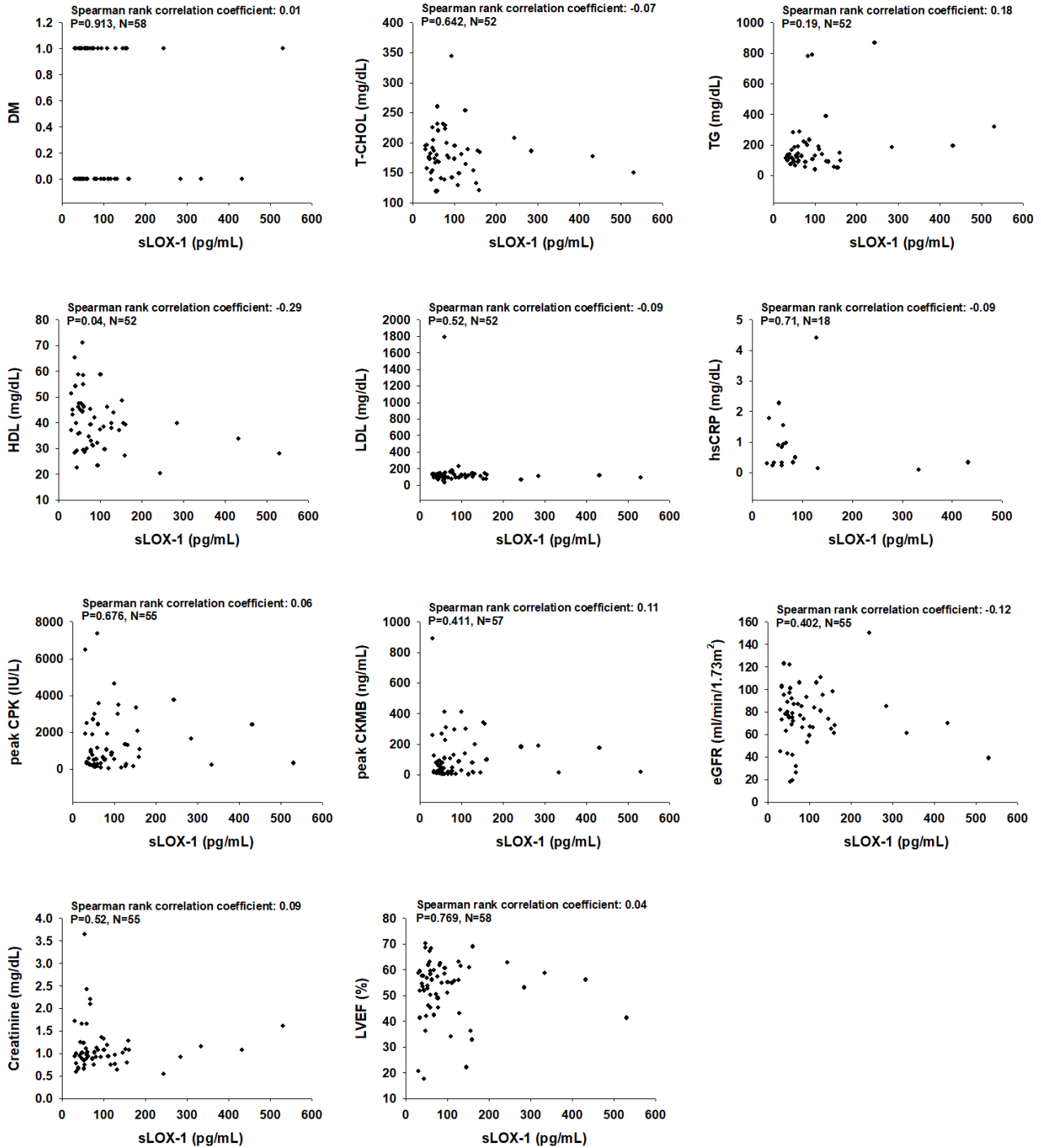
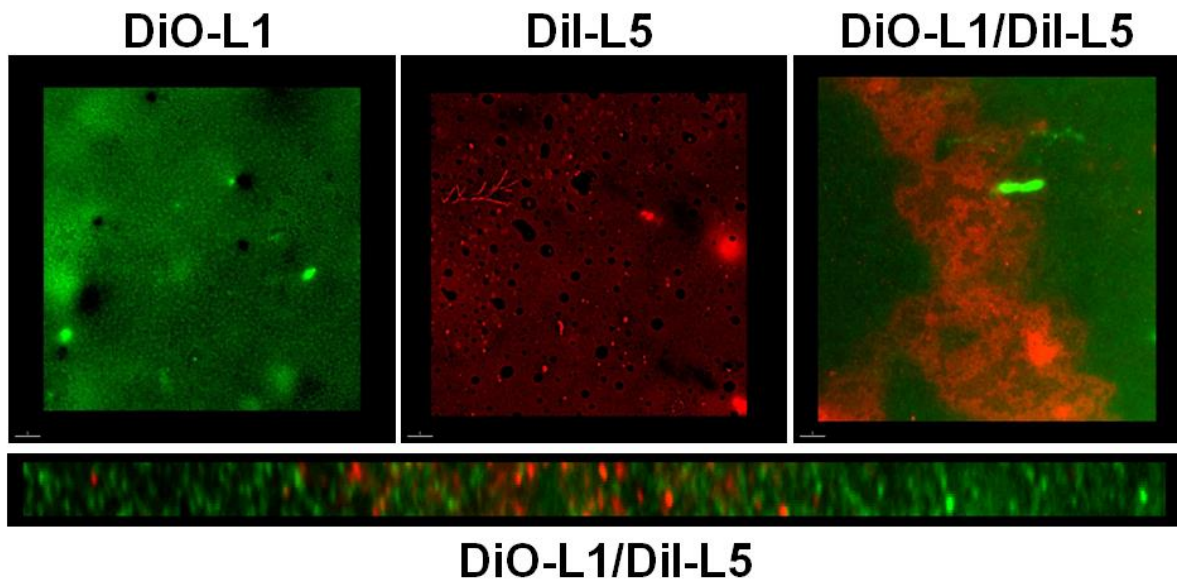


Figure S2. Particle aggregability of L1 and L5 evaluated using deconvolution microscopy imaging.



L5 particles labeled with the fluorescent probe DiI (DiI-L5, red) formed aggregates on glass, whereas L1 particles labeled with the fluorescent probe DiO (DiO-L1, green) remained homogeneously dispersed.