A Novel Cell-Free, Non-Fluorescent Method to Measure LOX-1-Binding Activity Corresponding to The Functional Activity of HDL

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Aims: A functional abnormality in high-density lipoprotein (HDL) particles rather than a quantitative abnormality in HDL cholesterol levels has been suggested to promote atherosclerosis. The modification of HDL may underlie functional changes to HDL such as gaining the ability to bind and activate the lectin-like oxidized low-density lipoprotein (LDL) receptor-1 (LOX-1). We aimed to develop a novel method for measuring modified HDL on the basis of its binding to LOX-1.

Methods: We designed a LOX-1 binding–based enzyme-linked immunosorbent assay (ELISA) with recombinant LOX-1 and anti-apoAI antibody. A lipid-free standard was devised by making a chimeric fusion protein containing anti-LOX-1 antibody and human apoAI fragment. We used this system to detect modified HDL, designated as LOX-1 ligand containing apoAI (LAA).

Results: With our ELISA system, we detected HDL modified by copper oxidation, hypochlorous acid, 4-hydroxynonenal, and potassium cyanate, but not native HDL. Upon oxidation, HDL showed increased LOX-1 binding activity and decreased cholesterol efflux and paraoxonase-1 activities. In the ELISA, the chimeric fusion protein standard showed minimal variation in reference binding curves in contrast to copper-oxidized HDL preparations, suggesting better quality control of the chimeric fusion protein as the standard for measuring modified HDL activity. LAA was detectable in the plasma of healthy individuals and of mice fed a high-fat diet.

Conclusion: We have developed a novel ELISA by using recombinant LOX-1 and anti-apoAI antibody to measure the activity of modified HDL in plasma.

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Key words: Modified HDL, HDL quality, LOX-1, ELISA

Introduction

Previously, an inverse correlation between high-density lipoprotein (HDL) cholesterol concentration and coronary artery disease (CAD) had generally been accepted in the field of epidemiology. However, results of recent epidemiologic studies have suggested that very high concentrations of HDL cholesterol may actually increase the risk of CAD[1-5]. Furthermore, clinical trials performed with cholesterol ester transfer

protein inhibitors, which significantly increase HDL cholesterol levels, failed to show a decreased risk of CAD[6-8]. Another report showed that genetically high plasma HDL concentrations did not lower the risk of myocardial infarction[9].

Although cholesterol is one of the major constituents of HDL, attention has recently been drawn to the function of HDL particles rather than the cholesterol component of HDL. It has been suggested that the quality of HDL may be more important in the pathogenesis of CAD and atherosclerosis than the quantity of HDL cholesterol. In a cross-sectional study, the cholesterol efflux capacity of HDL from macrophages was shown to have a strong inverse association with carotid intima-media thickness and possibly CAD[10]. Furthermore, in a cohort study, the cholesterol efflux capacity of HDL was inversely associated with the incidence of cardiovascular events[11]. In addition, Huang *et al.*[12] reported that dysfunctional apolipoprotein (apo)AI, which has lost its ATP-binding cassette transporter A1(ABCA1)-dependent cholesterol efflux activity, accumulates in atheroma. Biochemical analyses of HDL-binding proteins with a shotgun proteomics approach have shown that protein constituents of HDL from CAD patients are different from those of healthy individuals, namely HDL from CAD patients is enriched in apoC-IV, paraoxonase 1 (PON1), and complement C3[13]. Other reports have shown that apoC-III content was high in HDL from CAD patients[14, 15]. We have previously shown that the most electronegative subfraction of HDL, named H5, has reduced cholesterol efflux capacity compared with the least electronegative, major subfraction of HDL[16].

Because assessing the quality of HDL particles has become an important area of interest[17], researchers have focused on identifying ways to quantitatively study HDL function to determine which modifications may result in its loss of function. The primary function of HDL in cholesterol efflux has been quantitatively studied by using a cell-based assay with macrophages[11]. Recently, a cell-free assay was developed by using fluorescence-labeled cholesterol liposomes[18]. Some researchers have also attempted to measure oxidatively modified HDL. Huang *et al.*[12] focused on quantifying the oxidation of apoAI on Trp 72 by myeloperoxidase, which leads to a loss of cholesterol efflux capacity. They measured the oxidation on Trp 72 of apoAI by using a monoclonal antibody against the neoantigen that forms upon its oxidation (ie, 2-OH-Trp72 [2-oxindolyl alanine]). With this method, the authors found that 2-OH-Trp72 apoAI was present at a detectable concentration in human plasma, and higher concentrations of 2-OH-Trp72 apoAI were associated with an increased risk of cardio-

vascular disease[12].

Similar to the study by Huang *et al.*[12], Besler *et al.*[19] reported that the concentration of malondialdehyde (MDA)-modified HDL in serum was higher in patients with CAD than in healthy individuals and that HDL from CAD patients induced vascular cell adhesion protein-1 (VCAM-1) expression and nuclear factor (NF)-κB activation in endothelial cells. This finding indicated that a gain of function occurred in HDL particles, promoting atherogenic changes in endothelial cells. These authors have further shown that this proatherogenic property of dysfunctional HDL is mediated by the lectin-like oxidized low-density lipoprotein (LDL) receptor 1 (LOX-1).

Aim

We previously developed a system as a tool for measuring the modification of LDL by quantifying its LOX-1 binding activity, named LOX-1 ligand containing apoB (LAB)[20, 21]. LAB values correlate well with the risk of cardiovascular disease and the state of arteriosclerosis[22, 23]. In the present study, we applied a technology similar to the LAB system to measure the functional activity of HDL that reflects the change in HDL quality resulting from its modification, with the goal of quantifying HDL dysfunction.

Materials and Methods

Materials

Recombinant human LOX-1 purified to homogeneity (**Fig. 1A**) and anti-apoB antibody (HUC20) were prepared as previously described[21]. Sheep anti-apoAI polyclonal antibody was obtained from Binding Site (Birmingham, United Kingdom). Chicken anti-apoAI polyclonal antibody was generated by immunizing chickens with subcutaneous injections of purified human apoAI protein by contract manufacturing with SCRUM Inc. (Tokyo, Japan).

Sandwich Enzyme-Linked Immunosorbent Assay (ELISA) for LOX-1 Ligand Containing ApoAI (LAA) and LAB

Recombinant human LOX-1 (0.15 µg/well) dissolved in phosphate-buffered saline (PBS) (30 µL/well) was immobilized on 384-well plates by incubating overnight at 4℃. After the wells were washed twice with PBS, 3% (w/v) bovine serum albumin (BSA) blocking solution was added, and the plates were incubated for 2 h at 25℃. After the wells were washed three times with PBS, the plates were incubated with 20 µL of the modified HDL or plasma diluted four times with HEPES–NaCl buffer (10 mM
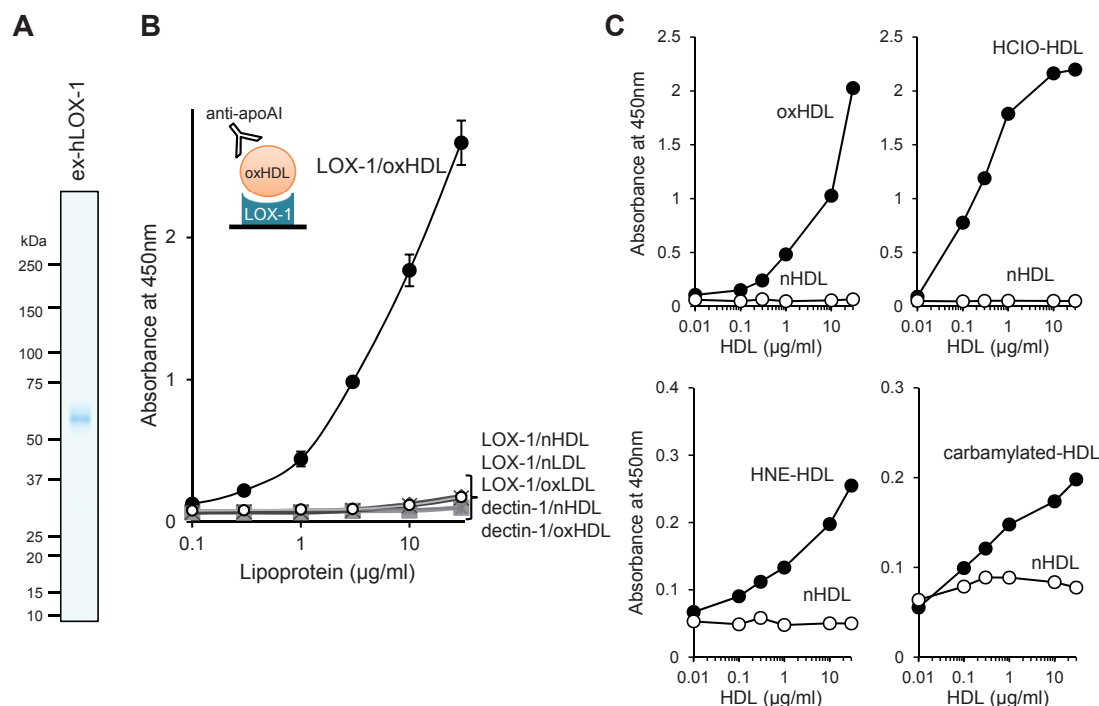
Fig. 1. Specificity of a novel ELISA system for detecting the binding of modified HDL to LOX-1

(A) Coomassie Brilliant Blue staining of purified recombinant human LOX-1 extracellular domain (ex-hLOX-1) separated by using SDS-PAGE under nonreducing conditions. (B) Dose-dependent binding of oxHDL to LOX-1. (C) Binding of various modified types of HDL to LOX-1. nHDL, native HDL; oxHDL, copper-oxidized HDL; HClO-HDL, hypochlorous acid (HClO)-modified HDL; HNE-HDL, 4-hydroxynonenal (HNE)-modified HDL.

HEPES, 150 mM NaCl, pH 7.4) containing 0.3% (w/v) BSA. Then, the wells were washed three times with PBS, and the plates were incubated for 1 h at 25℃ with 1.0 µg/mL anti-apoAI antibody in HEPES–NaCl buffer containing 1% (w/v) BSA. After the wells were washed three times with PBS, the plates were incubated for 1 h at 25℃ with peroxidase-conjugated goat anti-chicken IgG (Kirkegaard & Perry Lab Inc., Gaithersburg, Maryland) that was diluted 4000 times with HEPES–NaCl buffer containing 1% (w/v) BSA. After the wells were washed five times with PBS, the substrate solution (tetramethylbenzidine [TMB] solution, Bio-Rad Laboratories, Hercules, California) was added to the plates and incubated at room temperature. The reaction was terminated with 2 M sulfuric acid. Peroxidase activity was determined by measuring absorbance at 450 nm.

ELISA for LAB was performed with recombinant human LOX-1 and anti-human apoB antibody as described previously[24].

Generation of Chimeric Fusion Protein Containing LOX-1 Single-Chain Antibody and ApoAI

A cDNA fragment encoding human apoAI (amino acids 31-267) (GenBank accession number NM000039.2) was cloned from human liver cDNA. A chimeric fusion protein was generated by connecting the apoAI fragment to the C-terminus of anti-human LOX-1 single-chain antibody with linker sequences. The chimeric fusion protein was expressed by using the Expi293 Expression System (Invitrogen Corp., Carlsbad, California). The chimeric fusion protein was secreted into culture media and purified by using Ni-sepharose excel (GE Healthcare, Little Chalfont, United Kingdom).

ELISA for Human and Mouse ApoAI

The binding of chicken anti-apoAI antibody to human and mouse apoAI was analyzed with ELISA. A 384-well ELISA plate was coated with purified human apoAI (Sigma-Aldrich Corp., St. Louis, Missouri), recombinant mouse apoAI (Thermo Fisher Scientific, Waltham, Massachusetts), or chimeric fusion protein. The wells were washed with PBS, and PBS containing 20% Immunoblock (Dainippon Sumitomo Pharma Co., Ltd., Osaka, Japan) was added for blocking. The plate was incubated for 1 h at 25℃. After the wells were washed with PBS, chicken anti-apoAI antibody

was added, and the plate was incubated for 1 h at 25℃. The wells were washed, and horseradish peroxidase (HRP)-labeled anti-chicken IgY secondary antibody (1:4000; MilliporeSigma, Burlington, Massachusetts) was added. The plate was incubated for 1 h at 25℃. Peroxidase activity was detected with a TMB peroxidase enzyme immunoassay substrate kit (Bio-Rad) and quantified by measuring the absorbance at 450 nm on a microplate reader.

Immunoblot Analysis

Purified apoAI protein and plasma were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions and transferred to polyvinylidene (PVDF) membrane (iBlot2; Invitrogen). After the membrane was blocked with Immunoblock (Dainippon Sumitomo), it was incubated with chicken anti-apoAI antibody (1.0 µg/mL) and then treated with HRP-labeled anti-chicken IgY (1:4000; Millipore). Blots were developed with chemiluminescent HRP substrate (Immobilon; Millipore), and images were obtained with an ImageQuant LAS-4000 mini (GE Healthcare).

Blood Chemistry

Serum was obtained from 12 healthy volunteers. Total cholesterol, LDL-C, HDL-C, triglyceride, apoAI, apoB, glucose, C-reactive protein, phospholipid, alanine aminotransferase, and γ-GTP levels were measured at Shinshu University Hospital Clinical Laboratory. For the use of human blood samples, including those used for the LAA and LAB assays, written informed consent was obtained from each study participant. The study protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the ethics committee on research in humans at Shinshu University Faculty of Medicine.

Preparation of Lipoproteins from Human Plasma

HDL, LDL, and very low-density lipoprotein (VLDL) were isolated by performing sequential ultracentrifugation of freshly obtained plasma from healthy human volunteers, as previously described[25]. For the Cu^{2+}-oxidation of HDL and LDL, 3 mg protein/mL native HDL or LDL were incubated with 7.5 µM $CuSO_4$ in PBS for 16 h at 37℃. For the HClO modification of HDL, 1 mg protein/mL of native HDL was incubated with 25 µM NaClO in PBS with 0.1 mM EDTA for 1 h at 37℃. For the hydroxynonenal modification of HDL, 2 mg/mL native HDL was incubated with 2 mM 4-hydroxy-2-nonenal (HNE) at 37℃ for 24 h. For the carbamylated HDL, HDL (1 mg/mL) in 50 mM sodium phosphate, pH 7.0, was incubated with sterile KOCN (Sigma-Aldrich) at 37

℃ for 8 h. Excess reagents were removed by performing dialysis at 4℃ against PBS with 0.1 mM EDTA or by using a PD MiniTrap G-25 column (GE Healthcare).

Thiobarbituric Acid Reactive Substance (TBARS) Assay

Lipid peroxidation levels were determined by measuring levels of malondialdehyde (MDA) with a TBARS Assay Kit (Cayman Chemical, Ann Arbor, MI, USA) according to the manufacturer's protocol. The concentration of MDA was measured by reading fluorescence at an excitation wavelength of 530 nm and an emission wavelength of 550 nm. Data were recorded and analyzed by using a SpectraMax Gemini EM fluorometer and SoftMax Pro software (Molecular Devices).

Paraoxonase-1 (PON1) Activity

PON1 activity was measured by using a Paraoxonase Assay Kit (Nikken Seil Co., Ltd., Shizuoka, Japan) according to the manufacturer's protocol. Data were recorded and analyzed by using a PrectraMax 340PC Microplate Reader and SoftMax Pro software (Molecular Devices).

Cholesterol Efflux

Human monocytic THP-1 cells were grown in RPMI 1640 medium with 10% fetal bovine serum at 37℃ under 5% CO_2. Three days before the experiment, THP-1 cells were differentiated into macrophages by adding 100 nM phorbol 12-myristate 13-acetate (Sigma-Aldrich Corp., St. Louis, Missouri, USA). Cholesterol efflux was measured by using a Cholesterol Efflux Assay Kit (ab196985; Abcam, Cambridge, MA, USA) according to the manufacturer's protocol.

Animal Studies

Animal studies were conducted in accordance with the institutional guidelines for animal experiments at Shinshu University. Male C57BL/6J mice were purchased from the CLEA Japan, Inc. (Tokyo, Japan). At 7 weeks of age, the mice were fed a high-fat diet without vitamin E (1.25% cholesterol, 0.5% cholic acid, 20% milk casein, and 15% cocoa butter chow) for 2 weeks. After high-fat loading, blood pressure and heart rates of mice were measured by using tail cuff plethysmography (BP-98A; Softron, Tokyo, Japan). Then, the mice were euthanized and subjected to analyses. Total cholesterol and HDL in EDTA-treated plasma were measured by using Fuji Dri-Chem7000Z (Fujifilm, Tokyo, Japan). Triglycerides, phospholipids, and non-esterified fatty acids were
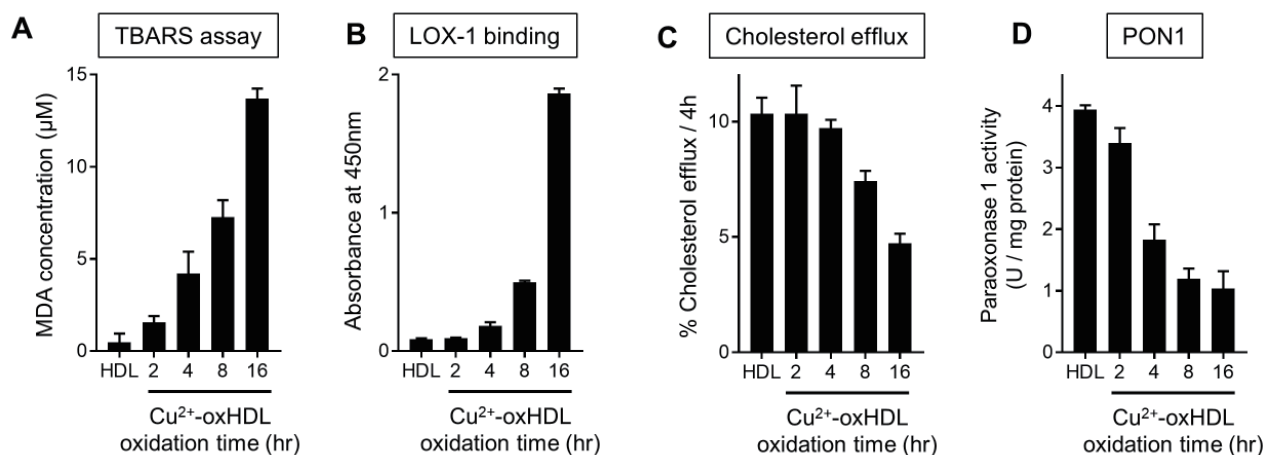
Fig. 2. Biochemical and functional changes in HDL as a function of oxidation time

The oxidative modification of HDL was performed by incubating HDL with 7.5 μM CuSO$_4$ for 2 to 16 h at 37℃. The graphs in A–D show the MDA concentration measured by using the TBARS assay (A), LOX-1 binding activity (B), cholesterol efflux activity (C), and PON1 activity (D) in HDL ($n=3$) as a function of oxidation time.

measured by using commercially available kits (TG-E, PL-C, and NEFA-C, respectively; Wako Pure Chemical Industries, Ltd., Tokyo, Japan).

Statistical Analysis

Data are expressed as the mean ± standard error. A Student's t-test was used for comparing the two data sets. One-way analysis of variance in conjunction with Tukey's test was used for comparisons among multiple data sets. Statistical calculations were performed in GraphPad Prism (version 7.0) for Windows (GraphPad Software Inc., La Jolla, California).

Results

Binding of Modified HDL to LOX-1

First, we examined the binding of HDL with or without modification to LOX-1 by using anti-apoAI antibody. HDL oxidized under the presence of Cu^{2+} ion was selectively bound to LOX-1 in dose-dependent manner but not to dectin-1, which is the protein with the highest homology to LOX-1 within the same protein family (**Fig. 1B**). However, native HDL (ie, without modification) was not bound to either LOX-1 or dectin-1. In addition, the binding of oxidized LDL was not detected with this method, which is expected with the use of anti-apoAI antibody.

Because the modification of HDL mediated by Cu^{2+} is known to cause various chemical reactions on HDL, we also examined HDL modified by using other chemicals, namely, HClO⁻, HNE, and KOCN, to generate HClO⁻-modified HDL (HClO-HDL), HNE-modified HDL (HNE-HDL), and carba-

mylated HDL, respectively. Each modified HDL was dose-dependently bound to LOX-1, whereas native HDL was not (**Fig. 1C**), indicating that this novel system can be used to detect various kinds of modified HDL. Hereafter, we designate LOX-1 bound to HDL as LOX-1 ligand containing apoAI (LAA).

HDL Function and LOX-1 Binding

To examine the relationship between HDL function and LOX-1 binding activity, namely, LAA activity, we analyzed changes in representative HDL functions, including cholesterol efflux from macrophages and PON1 activity, during its oxidation with Cu^{2+}. As oxidation time increased, the concentration of the oxidation product MDA increased in HDL (**Fig. 2A**) and was accompanied by increased LOX-1 binding activity of HDL (**Fig. 2B**). Concurrently, cholesterol efflux activity mediated by HDL and PON1 activity in HDL were decreased (**Fig. 2C, D**), showing a clear inverse correlation of these HDL functions with LOX-1 binding activity.

Analysis of LAA in Human Lipoprotein Fractions

Although the presence of apoAI is the essential property of HDL, the possibility remains that apoAI may also be present in a certain subfraction of LDL[26] and that the LAA activity results from fractions other than HDL. Therefore, we examined which fraction of human lipoprotein contained LAA by using our LAA detection assay, which involves the use of anti-apoAI antibody. We separated human lipoprotein into LDL, HDL, and VLDL fractions by using ultracentrifugation (**Fig. 3A**). As expected, we found that LAA was
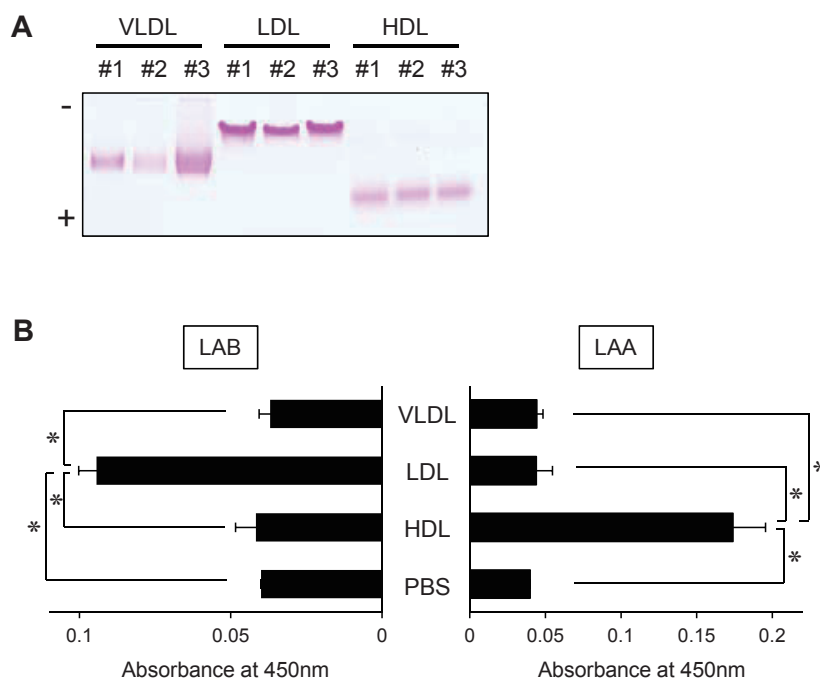
Fig. 3. Determination of LAA and LAB in human plasma

(A) Relative electrophoretic mobility on agarose gel for various lipoprotein fractions. (B) Reactivity of lipoprotein fractions to LOX-1. Lipoproteins (100 μg protein/mL) were isolated from human plasma by performing sequential ultracentrifugation and were subjected to the sandwich ELISA assay for LAA or LAB. $n=3$, each fraction. $^*p<0.005$.

predominantly concentrated in the HDL fraction, whereas LAB was predominantly concentrated in the LDL fraction (**Fig. 3B**). These results suggest that most of the LAA activity in lipoprotein is attributed primarily to the HDL fraction of lipoprotein, most likely to modified HDL.

A Chimeric Protein Standard for the LAA Detection System

To establish the reproducibility of our LAA detection system, we designed a chimeric fusion protein that can be used as a standard in place of *in vitro*–oxidized or modified HDL. To mimic modified HDL, the standard protein should bind to both LOX-1 and anti-apoAI antibody. Therefore, we constructed a chimeric fusion protein consisting of anti-LOX-1 antibody and apoAI fragments (**Fig. 4A**). The chimeric fusion protein was synthesized and purified as recombinant protein that retained reactivity to anti-apoAI (**Fig. 4B**).

When we used the sandwich ELISA (ie, the LAA detection system) utilizing immobilized LOX-1 and anti-apoAI antibody with chimeric protein standard, we observed a clear sigmoid binding curve between chimera concentrations of 0.01 and 10 μg/mL

(**Fig. 4C**). Although the standard curves generated by using different preparations of Cu^{2+}-modified HDL varied significantly (**Fig. 4D**), the standard curves generated by using different preparations of the chimeric fusion protein were almost identical (**Fig. 4E**), suggesting better quality control with the use of the fusion protein as the standard to measure modified HDL activity.

Next, as a pilot study, we determined LAA in plasma from 12 healthy volunteers (mean age, 34.0 ± 2.5 years) by using the LAA detection system with the chimeric protein standard. As shown in **Table 1**, this group contained no current smokers or individuals with hypertension, diabetes mellitus, or dyslipidemia. The mean LAA activity was 143.1 ± 30.1 ng/mL ($n=$ 12), and the intra- and interassay coefficients of variance were within 11.6% ($n=16$) and 17.9% ($n=9$), respectively.

Elevation of LAA in the Plasma of Wild-Type Mice Fed a High-Fat Diet

Given that mice are commonly used for the study of atherogenesis, we sought to determine whether our LAA detection system could be applied to the plasma of wild-type mice, whose lipoprotein is
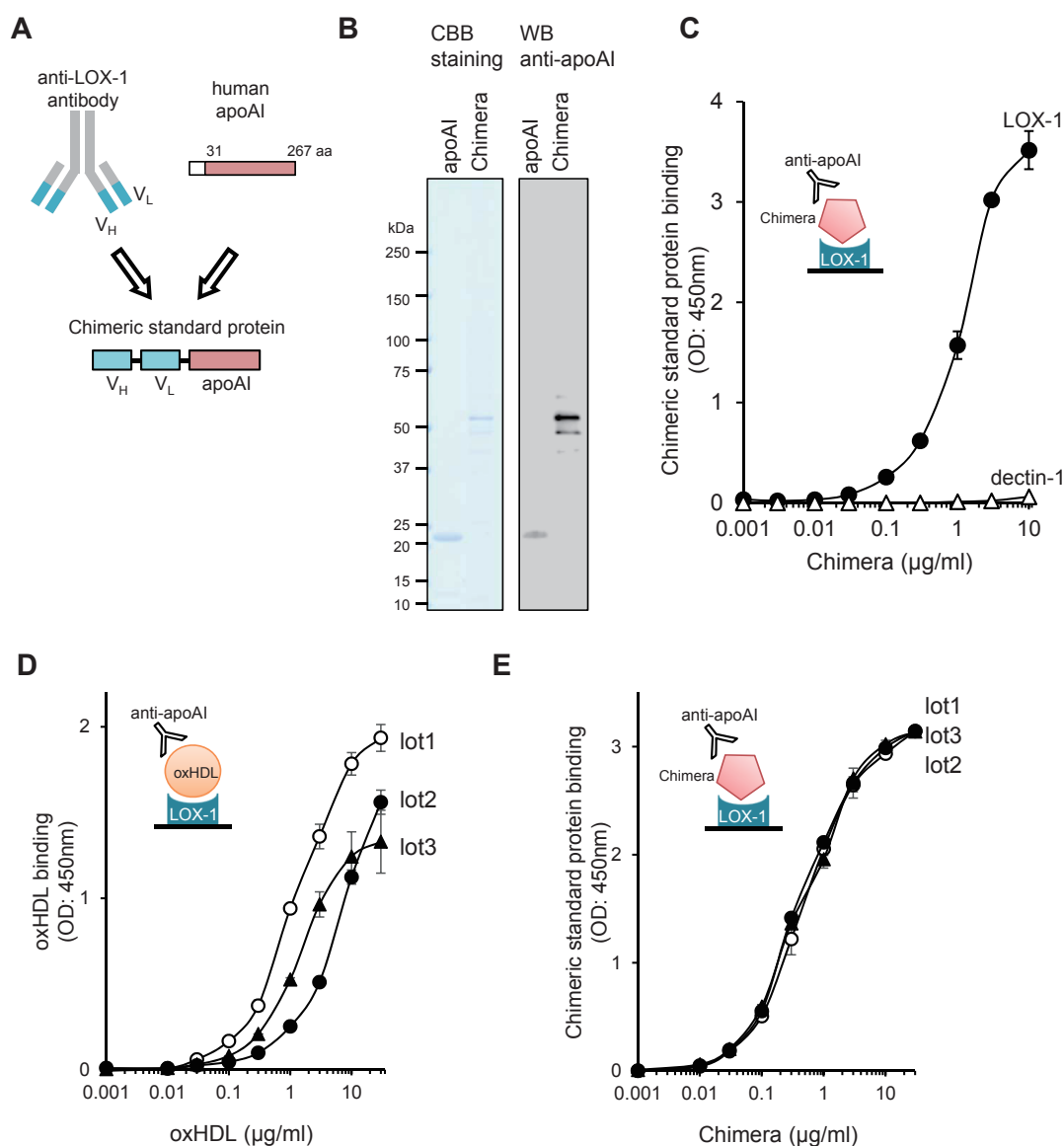
Fig. 4. Generation of the chimeric fusion protein standard

(A) Strategy for constructing the chimeric protein standard (chimera). cDNA for single-chain antibody against human LOX-1 and a fragment of human apoAI were linked to produce the fusion protein of anti-LOX-1 antibody-apoAI. (B) Purified fusion protein or human apoAI were subjected to SDS-PAGE and detected with anti-apoAI antibody. (C) Binding of the fusion protein to immobilized LOX-1 was determined by using anti-apoAI antibody. (D, E) The standard curves of different preparations of copper-oxidized HDL (D) and the fusion protein designated as the chimeric protein standard (E).

mostly composed of HDL. Importantly, we found that the polyclonal antibody that we generated against human apoAI cross-reacted with mouse apoAI, although with a sensitivity 10 times lower than that for human apoAI (**Fig. 5A**).

We next measured LAA in mouse plasma after mice were fed a normal chow diet or a high-fat diet for 2 weeks. LAA activity in mice fed a normal chow diet was almost undetectable, whereas LAA activity was significantly elevated in mice fed a high-fat diet

(**Fig. 5B**), despite comparable HDL cholesterol concentrations between the two groups (**Table 2**). In addition, LAB activity was undetectable in the plasma of mice fed a high-fat diet or a normal chow diet.

Discussion

### Quantifying the Quality of HDL

Although HDL has been recognized as a lipoprotein fraction with beneficial properties, it has been

Table 1. Characteristics and biochemical profiles of healthy volunteers ($n=12$)

Variable	Value
Men, *n* (%)	7 (58)
Age (years)	34.0 ± 2.5
Current smoking habit, *n* (%)	0 (0)
Habitual alcohol consumption, *n* (%)	1 (8.3)
Hypertension, *n* (%)	0 (0)
Diabetes mellitus, *n* (%)	0 (0)
Body mass index (kg/m^2)	21.4 ± 0.5
CRP (mg/dL)	0.02 ± 0.005
LDL cholesterol (mg/dL)	105.3 ± 10.2
HDL cholesterol (mg/dL)	61.6 ± 4.3
Total cholesterol (mg/dL)	188.2 ± 9.7
Triglyceride (mg/dL)	88.2 ± 19.0
ApoAI (mg/dL)	142.3 ± 6.6
ApoB (mg/dL)	81.1 ± 6.8
Phospholipid (mg/dL)	204.7 ± 8.1
ALT (U/L)	17.8 ± 2.4
γ-GPT (U/L)	19.7 ± 2.6
LAA (ng/mL)	143.1 ± 30.1

Values are expressed as the mean ± standard error of the mean, unless otherwise indicated.
Hypertension was defined as systolic blood pressure ≥ 140 mmHg or diastolic blood pressure ≥ 90 mmHg. Diabetes was defined as fasting serum glucose ≥ 126 mg/dL (7.0 mmol/L). Habitual alcohol consumption was defined as consuming at least 1 drink per week. CRP, C-reactive protein; LDL, low-density lipoprotein; HDL, high-density lipoprotein; ALT, alanine aminotransferase; γ-GPT, γ-glutamyl transpeptidase.
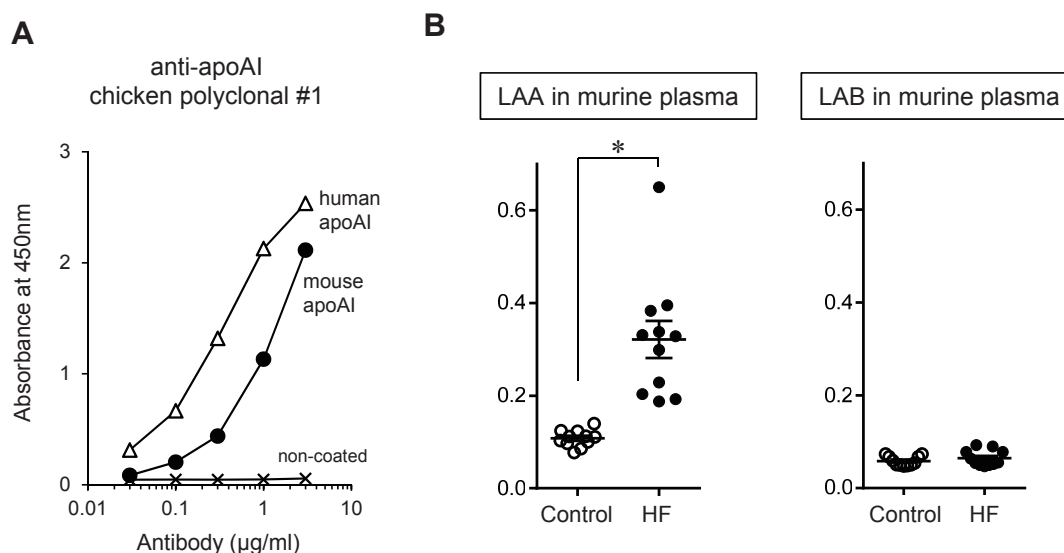
Fig. 5. Determination of LAA and LAB in murine plasma

(A) ELISA reactivity of anti-apoAI antibody against mouse apoAI (closed circles) and human apoAI (open triangles). (B) Individual plasma LAA or LAB measurements in wild-type mice fed a normal chow diet (control) or a high-fat (HF) diet. Scatter plots show individual measurements and the mean ± standard error of the mean. $n=11$, each group. *$p<0.0001$ vs. control diet.

Table 2. Hemodynamic and plasma lipid indices of the wild-type C57BL/6J mice used for the determination of LAA

	Normal chow diet ($n = 11$)	High-fat diet ($n = 11$)	p-value
Body weight (g)	21.8 ± 0.2	21.1 ± 0.3	n.s.
SBP (mmHg)	106.9 ± 2.4	100.6 ± 2.7	n.s.
MBP (mmHg)	76.3 ± 3.1	67.7 ± 4.7	n.s.
DBP (mmHg)	86.5 ± 2.5	78.7 ± 3.8	n.s.
Heart rate (BPM)	540.7 ± 27.9	551.3 ± 25.7	n.s.
Total cholesterol (mg/dl)	77.5 ± 1.9	129.1 ± 3.4	$p < 0.001$
HDL cholesterol (mg/dl)	74.5 ± 1.8	69.9 ± 1.6	n.s.
Triglyceride (mg/dl)	83.6 ± 5.5	79.4 ± 5.2	n.s.
Phospholipid (mg/dl)	132.4 ± 2.9	207.3 ± 3.0	$p < 0.001$
NEFA (mEq/L)	0.63 ± 0.08	0.82 ± 0.08	n.s.

Data were collected after mice were fed a normal chow diet or a high-fat diet for 2 weeks.
SBP, systolic blood pressure; MBP, mean blood pressure; DBP, diastolic blood pressure; BPM, beats per minute; HDL, high-density lipoprotein; NEFA, non-esterified free fatty acid; n.s., not significant. Values are expressed as the mean ± standard error mean.

suggested that the quality of HDL in patients with CAD may be altered from its native state possibly because of HDL modifications. In this context, a compelling need exists for a reliable and reproducible methodology for measuring the quality of modified HDL. In this study, we have established a novel method, designated as the LAA method, for quantifying the biologic activity of modified HDL.

Advantages of the LAA Methodology

LOX-1, originally discovered as the receptor for oxidized LDL more than two decades ago[27-29], has also been reported to bind to modified HDL[19, 30]. Utilizing this property of modified HDL, we have successfully established a methodology for evaluating the biologic activity of modified HDL. As described here, this method is used to measure the binding activity of apoAI-containing lipoprotein to LOX-1 protein on the solid phase, designated as LAA activity. We showed that LOX-1 binds any Cu^{2+}-oxidized HDL, HClO-HDL, HNE-HDL, and carbamylated HDL, depending on the affinity of the ligand to LOX-1.

Despite the common use of terms such as "oxidized HDL" or "modified HDL," there are actually various types of HDL modifications, and their effects on the biologic activity of HDL further vary depending on the type of modification. The possibility remains that several modifications, potentially multiple kinds, may be present on a single particle of HDL. Therefore, for evaluating the whole dysfunctional activity of HDL, it is not sufficient to measure the binding of a single epitope on HDL to a single antibody, such as anti-MDA-HDL, anti-HNE-HDL, or anti-carbamylated-HDL antibodies, nor is it sufficient to simply measure the absolute amount of modified

HDL. What is important is to measure the altered biologic activity of HDL after it is modified. The LAA method described here does not simply involve the recognition of various epitopes of chemically modified HDL by LOX-1 based on the principle of recognition alone, but it allows for the measurement of the biologic activity of modified HDLs exerted via LOX-1. Notably, this novel method does not require the purification of HDL, the use of cultured cells, or fluorescent labeling, while all or either of them have been previously required to assess the cholesterol efflux–inducing activity of HDL.

Ensuring Reproducibility

Measuring modified lipoproteins has presented various challenges, partly because of lipid instability. The problem of specimen instability may be overcome by optimizing the preservation method and by shortening the measurement time. On the other hand, preparing virtually identical and stable standards for measuring modified HDL is difficult when using HDL as a source of reference material. This is because the modification of HDL is not consistent among preparations, and HDL may be further altered during long-term storage by additional modifications such as oxidation through exposure to air. Here, we have identified an alternative solution to this problem by developing a lipid-free protein standard with an affinity for both the LOX-1 protein and the anti-apoAI antibody. This protein standard can be synthesized as a recombinant protein, and it showed better reproducibility than oxidized HDL.

HDL Function and LAA

A recent report showed that decreased cholesterol efflux activity in HDL was associated with an

increased risk of CAD[18]. The *in vivo* relationship between decreased cholesterol efflux activity and the modification of HDL remains unclear. Nevertheless, it has been shown that an oxidative reaction by myeloperoxidase reduces the cholesterol efflux activity of HDL[12]. In addition, the most electronegative subfraction of HDL, H5, was shown to exhibit impaired cholesterol efflux capacity[16]. Therefore, it is conceivable that the various modifications of HDL in some way affect the cholesterol efflux activity of HDL. We found that the oxidative modification of HDL decreased the cholesterol efflux activity and PON-1 activity of HDL. Furthermore, the oxidative modification of HDL increased LOX-1 binding activity. Thus, the LAA determined by the method described here may be a good surrogate indicator of the impaired physiologic function of HDL.

Effective Tool for Human and Mouse Research

Although the concentration of HDL cholesterol in human plasma is usually much lower than that of LDL cholesterol, we detected LAA activity in the plasma of healthy volunteers, even relatively young healthy individuals who were non-smokers (mean age, 34.0 ± 2.5 years). According to a previous report[19], the activity of oxidized HDL was much higher in CAD patients than in healthy individuals. Therefore, LAA activity may be detectable at higher levels in CAD patients, which will be a topic of interest in future clinical studies.

It is also noteworthy that the LAA method could be useful for evaluating the quality of HDL in mice, although the anti-apoAI antibody used in our study was less sensitive to mouse apoAI than to human apoAI. Previously in mice, it was reported that oxidized HDL was not detectable with an anti-oxidized HDL antibody until 10 weeks after mice were fed a high-fat diet[31]. In contrast, we showed that just 2 weeks of feeding wild-type C57BL/6J mice with a high-fat diet was sufficient to significantly increase LAA activity in the plasma, illustrating an important advantage of our methodology for detecting modified HDL activity in mouse plasma. This also implies that, at least in wild-type mice that have a high concentration of HDL, 2 weeks of feeding mice with a high-fat diet was enough to change HDL *quality*, whereas HDL cholesterol *quantity* was unchanged. Previously, we have shown that, in mice fed with the same high-fat diet for a longer duration[32], atheroma developed at the aortic root, suggesting that the abnormality of HDL can be detected with the LAA detection method long before the development of atherosclerosis, at least in mice.

Currently in laboratory research, mice are widely used for studies of dyslipidemia and atherosclerosis. In most cases, apoE knockout mice and LDL receptor knockout mice are used for the analysis of atherosclerosis because their high concentration of VLDL/LDL accelerates atherogenesis, making the study period shorter than that with wild-type mice. However, when analyzing the pathologic metabolism of HDL, it may be better to use wild-type mice in which the HDL concentration is naturally high.

Conclusion

In conclusion, we have developed a novel assay to evaluate the quality of HDL, which presumably reflects the proatherogenic biologic activity of HDL. To evaluate the advantages and disadvantages of this methodology, the application of this method to population and clinical studies is warranted. We have used similar logic to develop the LAB assay for the analysis of proatherogenic LDL[20, 21]. Previously, we concluded that LAB was an independent risk factor for carotid atherosclerosis and cardiovascular events, suggesting its possible use as a biomarker for stratifying residual risk[22-24]. Therefore, we believe that the LAA detection system for HDL presented here may also be a good surrogate biomarker for evaluating residual risk. However, whether the LAA provides additional information beyond what the LAB provides is a crucial point to consider for determining the significance of this method. According to the results of our ongoing research, LAA and LAB concentrations do not correlate, and each independently affects the risk of cardiovascular disease.

Conflict of Interest

The authors declare no conflict of interest to the present study.

Author Contributions

AK performed all the ELISA and animal experiments, analyzed and interpreted the data, and wrote the manuscript. YU performed lipoprotein fractionation experiments. SH and YF set up protein reagents. KK, CHC, and TO commented on and edited the manuscript. TS designed, conducted, and supervised the study and wrote the manuscript. All authors reviewed the content and approved the submission of the manuscript.

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