ELISA System for Human Endothelial Lipase

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BACKGROUND: Endothelial lipase (EL) regulates the metabolism of HDL cholesterol (HDL-C). However, the role of EL in regulating plasma HDL-C concentrations and EL’s potential involvement in atherosclerosis in humans has not been fully investigated due to the lack of reliable assays for EL mass. We developed an ELISA system for serum EL mass.

METHODS: Human recombinant EL proteins, purified from cultured media of human EL–transfected Chinese hamster ovary cells, were used as antigen and calibrator. Two specific monoclonal antibodies were generated in mice against recombinant EL protein for a sandwich ELISA. We measured EL mass in human serum using EL recombinant protein as a calibration standard.

RESULTS: The EL antibodies did not cross-react with lipoprotein lipase and hepatic triglyceride lipase. The detection limit of the ELISA was 20 pg/mL, which is approximately 10 times lower than that of previous ELISA systems. Recovery of spiked EL in serum was 90%–105%. Assay linearity was intact with a >4-fold dilution of serum. Intra- and interassay CVs were <5%. The serum EL mass in 645 human subjects was [mean (SE)] 344.4 (7.7) pg/mL (range 55.2–1387.7 pg/mL). Interestingly, serum EL mass was increased in patients with diagnosed cardiovascular disease and inversely correlated with serum HDL-C concentrations. There was no difference in EL mass between pre- and postheparin plasma samples.

CONCLUSIONS: This ELISA should be useful for clarifying the impact of EL on HDL metabolism and EL’s potential role in atherosclerosis.

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A large number of studies have established an inverse relationship between HDL cholesterol (HDL-C)6 and risk for cardiovascular disease (CVD) in humans (1–5), and low HDL-C is considered one of the most important negative risk factors for atherosclerotic CVD (6). Even after LDL cholesterol (LDL-C) is intensively controlled to low concentrations with statin therapy, low HDL-C remains a clinically significant cardiovascular risk factor (7, 8). Furthermore, low HDL-C is frequently accompanied by hypertriglyceridemia, and these lipid disorders synergistically contribute to an increased risk for CVD (9). Increased plasma triglyceride (TG) concentrations and low plasma concentrations of HDL-C have emerged as diagnostic criteria for the metabolic syndrome. Despite the therapeutic potential of HDL in combating CVD, there is a limited therapeutic strategy available for selectively raising HDL-C concentrations. Moreover, because of the multiplicity of HDL metabolism in humans, it is difficult to make an etiological diagnosis for the cause of high or low HDL-C concentrations in clinical settings.

Endothelial lipase (EL), a member of the triacylglyceride lipase family, is synthesized by vascular endothelial cells (10–13). Experiments in engineered mice with a disrupted native EL locus, as well as in mice overexpressing human EL (hEL), have revealed an inverse relationship between plasma HDL-C concentration and EL expression (11, 14). Previous studies have shown that plasma EL mass measured by ELISA is inversely correlated with HDL-C concentrations in humans (15, 16). Association-based human genetic studies have provided evidence that variations in the EL genomic LIPG locus such as T111I, G26S, and N396S are linked to differences in circulating HDL-C concentrations or CVD (17–21), although recent studies with a large number of subjects have established associations between LIPG single-nucleotide polymorphism

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6 Nonstandard abbreviations: HDL-C, HDL cholesterol; CVD, cardiovascular disease; LDL-C, LDL cholesterol; TG, triglyceride; EL, endothelial lipase; hEL, human EL; CHO, Chinese hamster ovary; FBS, fetal bovine serum; LPL, lipoprotein lipase; HTGL, hepatic triglyceride lipase; HRP, horseradish peroxidase.
and HDL-C concentrations, but none with CVD (21–23). Thus, genetic variation in EL can modulate plasma HDL-C concentrations, although the relationship with CVD remains controversial. Although EL is still expected to be an attractive pharmacological target for raising HDL-C concentrations, the clinical significance of plasma EL concentration on plasma HDL-C concentrations and atherosclerosis in humans has not been fully elucidated due to the lack of a standard and reliable assay system. To obtain a better understanding of the association of EL and plasma concentrations of HDL-C in humans, our goal was to establish a new assay system for EL mass measurement using newly generated antibodies.

**Materials and Methods**

**MONOCLONAL ANTIBODY PREPARATION**

We transfected Chinese hamster ovary (CHO) cells (American Type Culture Collection) with the human EL-c-myc/pHBAP-3-neo plasmid (10) and selected stable transfectants with 500 μg/mL G418 (Invitrogen). We screened EL expression in the stable transfectants using an ELISA system with monoclonal antibodies against EL (clones 11–9B and 2–12E) (16) and chose 1 of the high expression clones, referred to as hEL-myc/CHO 53A5, for subsequent experiments. The cells were cultured for 24 h in TIL medium (Immuno-Biological Laboratories) containing 10% fetal bovine serum (FBS) (PAA Laboratories), after which the medium was changed for serum-free TIL medium. After 3 days, the supernatants were collected and measured. We purified EL protein from the concentrated conditioned medium of hEL-myc/CHO 53A5 using an immunofinity column containing anti-EL carboxy-terminus antibody (clone 2–12E) (14, 16), emulsified with Freund complete adjuvant, and then immunized into BALB/c mice (Charles River). After a boost with immunogen, we carried out fusion of spleen cells with X63-Ag8.653 myeloma cells (Immuno-Biological Laboratories) with PEG1500 (Roche Applied Science) followed by screening for desired hybridoma reactive only to EL by immunoblotting. We identified 2 monoclonal antibodies that reacted with amino terminus (26A1) and carboxy terminus (48A1) of EL among 15 clones.

**PREPARATION OF EL CALIBRATORS AND OTHER LIPASES**

To obtain standard full-length EL protein, the concentrated culture medium of hEL-myc/CHO 53A5 was incubated with 70% NH₄SO₄ and then put through immuno-affinity columns containing monoclonal antibodies against amino (clone 5–3B) and carboxy (clone 2–12E) terminus of EL (14, 16). We estimated the purity of the recombinant hEL protein by densitometry using a Multi Gauge (Fujifilm) and determined the concentration of the protein by comparison with BSA (PAA Laboratories) as an indicator after electrophoresis. In addition, we used the culture supernatant of hEL-myc/CHO 53A5 cells as a working standard for the ELISA system.

To test the cross-reactivity of the ELISA with other lipase members, including lipoprotein lipase (LPL) and hepatic triglyceride lipase (HTGL), we purchased recombinant human LPL protein from BioVendor and generated recombinant human HTGL as follows. Full-length human HTGL cDNA was identified in human liver cDNA (Clontech) by PCR using primers 5'-ATCGGAGAAATGGACAC AAGTCCC-3' and 5'-CGCTCGAGTCTGATCTTT CGCTTTGATGTTTT-3'. A FLAG-epitope tag was added to the 3' end of cDNA before subcloning. To generate the plasmid encoding hHTGL-Full, the cDNA was inserted into pcDNA3.1(+) expression vector (Invitrogen). Human HTGL exhibits cell surface binding through the 5 carboxyl-terminal residues (KKKIR) (24). Therefore, to promote secretion to a culture supernatant, we prepared a truncated human HTGL mutant (hHTGL-471) by deleting the 5 carboxy-terminal residues. We used antisense PCR primer (5'- CGCTCGAGTGATGTTTTAGACTTTATTTCACA-3') to generate the plasmid encoding hHTGL-471. The PCR product was inserted into pcDNA3.1(+) expression vector after a FLAG-epitope tag was added to the 3’ end.

CHO cells were transfected with the hHTGL-471 plasmid and selected with 500 μg/mL G418 to establish stable transfectants. We screened hHTGL-471 expression in the transfectants by using anti-c-FLAG rabbit IgG (Immuno-Biological Laboratories) and generated 1 of the high-expression clones, referred to as human HTGL-471/CHO 3B1. We purified HTGL protein from the concentrated conditioned medium of human HTGL-471/CHO 3B1 using an anti-FLAG M2 affinity gel (Sigma-Aldrich). We estimated the purity of the recombinant human HTGL protein by densitometry using a Multi Gauge and determined the concentration of the protein by comparison with BSA as an indicator after electrophoresis.

**IMMUNOBLOTTING AND IMMUNOPRECIPITATION-IMMUNOBLOTTING**

We analyzed the supernatant from hEL-myc/CHO 53A5 cells by SDS-PAGE, followed by staining with Coomassie Brilliant Blue. For immunoblotting, 5 μL conditioned medium of hEL-myc/CHO 53A5 was used for 12% SDS-PAGE and transferred to nitrocellulose membrane (Bio-Rad). The membrane was incubated with 2 μg of the 26A1 or 48A1 antibody followed by incubation with secondary antibody conjugated with...
horseradish peroxidase (HRP) (1:4000, Immuno-Biological Laboratories). We assessed the reactivity of antibodies by immunoprecipitation-immunoblotting. The supernatant from the hEL-myc/CHO 53A5 cells was incubated with 2 μg 26A1 or 48A1 or mouse IgG (as negative control) and then with added Protein-G Sepharose (GE Healthcare Japan). After further incubation, the supernatant was centrifuged, and the resulting pellet was washed 3 times with 500 μL TNE buffer (10 mmol/L Tris-HCl, pH 7.8, 150 mmol/L NaCl, 1 mmol/L EDTA, 1% Nonidet P-40). The pellet was subsequently lysed, and we performed immunoblot analysis using biotinylated anti-EL amino-terminus monoclonal antibody (clone 11–9B) (14, 16) followed by HRP-conjugated streptavidin system. We visualized the EL signal by use of an ECL reagent (Amersham Biosciences).

IMMUNOFLUORESCENCE

The hEL-myc/CHO 53A5 and control mock-CHO cells cultured on coverslips were washed with prewarmed PBS (137 mmol/L NaCl, 10 mmol/L phosphate, 2.7 mmol/L KCl, pH 7.4), fixed with 4% paraformaldehyde in PBS for 15 min, and permeabilized with 0.1% Triton X-100 for 60 min. After saturation of unspecific sites with 100 g/L BSA/PBS, the cells were first incubated with the EL 26A1 or 48A1 antibody (1:200) or negative control (mouse mAb IgG Isotype Control, Cell Signaling Technology), and then with fluorescence-labeled secondary antibody (Alexa Fluor594 goat anti-mouse IgG, Invitrogen, 1:200). DAPI was used for nuclear staining. Images were captured by use of the Biozero BZ-8000 microscope (Keyence).

EL SANDWICH ELISA PROTOCOL

Microtiter plates (96 wells) were coated by adding 100 μL of 100 mmol/L carbonate buffer (pH 9.5) to each well that also contained 1.0 μg purified 48A1 mouse monoclonal IgG, followed by incubation overnight at 4 °C. The plates were then washed with PBS-T and blocked with 200 μL of 1% (wt/vol) BSA in PBS containing 0.05% NaN₃/well overnight at 4 °C. After two washings with PBS-T, test samples and recombinant EL, as a standard, that had been serially diluted with 1% BSA in PBS-T per 100 μL were added to the wells of the coated microtiter plate in duplicate and incubated at 4 °C overnight. After 4 washes with PBS-T, 100 μL HRP-conjugated 26A1 mouse IgG Fab′ was added to each well and the samples were incubated for 30 min at 4 °C. The wells were washed 5 times with PBS-T, and 100 μL tetramethyl benzidine solution (Kem-En-Tec) was added to each well as a substrate, followed by incubation in the dark for 30 min at room temperature. The reaction was terminated by adding 100 μL of 0.5 mol/L H₂SO₄. We measured absorbance of the solution at 450 nm by means of an ELISA reader (E-Max; Molecular Devices).

To assess the intra- and interassay precision for the ELISA, we established 3 QC samples covering the high, middle, and low range of the calibration curves. We determined intraassay precision by 24 repeated measurements of each QC sample in a plate, and interassay precision by assessing each QC sample across 6 different plates with quintuple wells. Additionally, for assessing the recovery rate in blood samples, different concentrations of recombinant EL added to samples were measured, and the recovery rate was validated as the difference between the measured concentration and the theoretical concentration. The analytical limit of quantification for this kit was determined on the basis of the guidelines provided by CLSI evaluation protocols.

The ELISA assay system was finally designed as a kit (Immuno-Biological Laboratories, code 27182).

PREPARATION OF BLOOD SAMPLES

The investigation conformed to the principles outlined in the Declaration of Helsinki, and the clinical study was approved by the Institutional Review Board of Kobe University Graduate School of Medicine.

We conducted the first set of experiments to determine the effect of heparin administration on plasma EL mass. We collected pre- and postheparin plasma samples from overweight and obese participants in a nutritional research study conducted at the University of California, Davis Clinical and Translational Science Center’s Clinical Research Center as described previously (25). The activity of HTGL in the plasma samples was measured as described previously (26).

The second set of experiments was conducted to evaluate EL mass in patients with diagnosed existing CVD. Whole blood was obtained from 645 Japanese patients consecutively admitted to Kobe University Hospital, Kobe, Japan, from April 2008 to March 2011, with written informed consent. Because it has been reported that preheparin EL mass is significantly correlated with postheparin EL mass, blood was collected without administration of heparin in the fasting state, while both pre- and postheparin plasma was obtained from some patients. The sera and plasma were immediately separated and kept frozen at –80 °C until assay. In some preliminary experiments, we obtained plasma and sera of healthy subjects (Veritas). Plasma concentrations of HDL-C, LDL-C, and TG were measured enzymatically.
STATISTICAL ANALYSIS
We conducted statistical analysis with Stat View version 5.0 (SAS Institute). We used Spearman correlation coefficient analysis to assess associations between measured parameters. Results are expressed as mean (SE), and P < 0.05 was considered significant.

Results
IDENTIFICATION OF RECOMBINANT EL AND CHARACTERIZATION OF ANTIBODIES AGAINST EL
We generated a pair of antibodies recognizing the amino terminus (26A1) and carboxy terminus (48A1) that exhibited a highly specific reactivity with the EL protein derived from hEL-myc/CHO 53A5 cells. Immunoblotting revealed a strong signal for 68-kDa mature EL protein (Fig. 1A). To confirm whether the antibodies had the ability to react against native EL in aqueous conditions, we performed an immunoprecipitation-immunoblotting analysis. Both 26A1 and 48A1 antibodies were able to immunoprecipitate EL originated from the conditioned medium of hEL-myc/CHO 53A5 (Fig. 1B). Immunofluorescence revealed that EL expression was abundantly detected in the cytosol of hEL-myc/CHO 53A5 (Fig. 1C). No signal was detected in negative controls, i.e., mock-transfected cells with primary antibodies or hEL-myc/CHO 53A5 cells without primary antibodies. Thus, we were able to identify EL expression in culture medium of hEL-myc/CHO 53A5 cells and confirm the specific reactivity of the 26A1 and 48A1 antibodies with EL protein.

SPECIFICITY, RECOVERY, AND IMPRECISION OF THE ELISA
Because the 26A1 and 48A1 antibodies had a highly specific reactivity with the EL protein, we chose them for establishment of the new sandwich ELISA system. The standard dose–response curve for the EL ELISA system exhibited a linear shape when plotted on a log/log scale over a range from 31 to 2000 pg/mL, and the linearity was excellent ($R^2 = 0.99$) (Fig. 2A). The new ELISA system worked for both serum and EDTA plasma samples equally. Although EL has a 44% and 41% amino acid sequence homology with LPL and HTGL (10), the cross-reactivity of this ELISA against human LPL and HTGL was <0.1% (Fig. 2B). Imprecision was determined with 3 supplemented QC controls (high, middle, and low). The intraassay imprecision exhibited CVs of 1.9% in the high, 2.7% in the middle, and 3.0% in the low controls (Table 1). Additionally, the interassay results for the CVs were 3.7% in the high, 2.0% in the middle, and 2.8% in the low controls. Thus, we considered the ELISA system to be reliable from the standpoint of imprecision. The recoveries were >85.6% for human EDTA plasma samples at 4× dilution, >74.7% for human serum, and nearly 100% for TIL media supplemented with 10% FBS (Table 2). We calculated the assay limit of quantification as 5.7 pg/mL using CLSI protocols.

Because EL has several heparin-binding domains, we investigated the effect of heparin administration on plasma EL mass. Unexpectedly, there was no significant difference in EL mass between pre- and postheparin samples (Fig. 3, A and B), in contrast to the marked heparin-releasable HTGL activity in the same samples.
Although there are heparin-binding sites in the carboxy-terminal end of EL, we confirmed that heparin does not block the binding sites of the amino-terminus (26A1) or carboxy-terminus (48A1) antibody.

Figure 2. Characteristic of the hEL ELISA system. (A), Calibration curve for calculation of EL concentrations. (B), Specificity of the EL ELISA system. A, absorbance.

Table 1. Intra- and interassay imprecision.

<table>
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<th>Measured value, pg/mL</th>
<th>SD, pg/mL</th>
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<td>3.7</td>
<td>6</td>
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<tr>
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<td>91.4</td>
<td>2.54</td>
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Table 2. Recovery validation.

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<th>Measured value, pg/mL</th>
<th>% Recovery</th>
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<td>557.9</td>
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<td>397.9</td>
<td>357.4</td>
<td>89.8</td>
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<td>1098.6</td>
<td>820.6</td>
<td>74.7</td>
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<td></td>
<td>598.6</td>
<td>449.3</td>
<td>75.1</td>
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<td></td>
<td>348.6</td>
<td>274.3</td>
<td>78.7</td>
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<tr>
<td>TIL media supplemented with 10% FBS</td>
<td>1000.0</td>
<td>941.1</td>
<td>94.1</td>
</tr>
<tr>
<td></td>
<td>500.0</td>
<td>483.0</td>
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</tr>
<tr>
<td></td>
<td>250.0</td>
<td>245.7</td>
<td>98.3</td>
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</table>

Inverse Correlation of Serum EL Mass and HDL-C Levels in CVD

The serum EL mass in 645 consecutive human subjects was 344.4 (7.7) pg/mL, and ranged from 55.2 to 1387.7 pg/mL. No patients had an EL concentration below the limit of quantification. The distribution of EL mass was skewed to the left (Fig. 4A). The EL mass was not correlated with serum HDL-C (Fig. 4B and Supplemental Fig. 1B, which accompanies the online version of this article at http://www.clinchem.org/content/vol58/issue12) or LDL-C concentrations (data not shown) in this population. Because our previous study showed that EL mass was associated with plasma HDL-C concentrations in patients with CVD (16), we next investigated the serum EL mass in patients having atherosclerotic CVD. The EL mass concentration in these 228 patients with CVD was 395.8 (15.1) (range 57.7–1387.7) pg/mL, which was significantly higher than that in the 645 consecutive patients (P < 0.001), and the EL distribution was again skewed to the left (Fig. 5A). Concomitantly, the patients with CVD had significantly lower concentrations of serum HDL-C than those without CVD {46.2.0 (1.0) vs 52.0 (0.6) mg/dL [1.20 (0.03) vs 1.35 (0.02) mmol/L]}, P < 0.001). When serum EL concentration was compared to the lipid profile in the CVD patients, it was inversely correlated with plasma HDL-C concentrations (R = –0.250, P < 0.001) (Fig. 5B and online Supplemental Fig. 2B), but not with LDL-C (R = –0.055, NS), or triglyceride (R = 0.078, NS) concentrations.

Discussion

We generated specific EL monoclonal antibodies against recombinant EL that reacted with the amino (26A1) and carboxy (48A1) terminus. Both antibodies had strong reactivity with native EL protein and en-
abled the detection of full-length EL by the sandwich ELISA. The limit of quantification of 5.7 pg/mL for hEL is much lower than that of previous ELISA systems, probably because the new antibodies are more specific for full-length EL protein than the old ones, which were generated against peptide fractions of EL (16). The serum EL mass in preheparin plasma was approximately 70–1000 pg/mL in the present study. Previous studies by our group and another group reported that the concentrations of EL mass in preheparin plasma were approximately 10–1000 μg/L, concentrations approximately 1000-fold higher than measured with the new assay (15, 16). These differences have resulted in confusion as to whether the plasma concentration of EL is really higher than the concentrations of LPL and HTGL without heparin infusion. The concentrations of other lipases, including LPL and HTGL, in preheparin plasma have been reported to be <30–100 μg/L (27, 28). When compared with the low plasma concentrations of other lipase members, we speculate that the range of EL concentrations determined by the new ELISA is more reasonable than those by the previous one. We consider the specificity of the antibodies used for this assay to be suitable for determining specific EL mass concentrations in human plasma, which showed different reactivity from the antibodies generated against peptide fractions in EL (16). The new ELISA may also be useful for identifying cases of genetic deficiency of EL in humans.

In the present study, a modest but significant inverse correlation between serum EL and HDL-C concentrations in patients with CVD was noted, whereas the relationship was not observed in all patients. The EL concentration was not correlated with serum LDL-C or TG concentrations. These findings confirmed the previous notion that EL is a determinant of

Fig. 3. Effect of heparin administration on plasma EL mass.
(A), Administration of heparin did not affect plasma EL mass but markedly increased plasma HTGL activity in the same pre- and postheparin samples (n = 480). *P < 0.001 vs. without heparin. (B), Scatter plot showing association between pre- and postheparin EL mass.

Fig. 4. Serum EL mass and HDL-C concentrations in all patients.
(A), Histogram showing the skewed distribution of EL mass (n = 645). (B), Lack of correlation of serum EL mass with serum HDL-C concentrations. NS, not statistically significant.
plasma HDL-C concentrations, particularly in patients with CVD. It has been reported that EL expression is highly regulated by a variety of factors including inflammatory cytokines, biomechanical forces, lipopolysaccharide, angiotensin II, and oxidized LDL (29–31). In contrast, statins reduce EL expression and plasma EL mass (16, 34), which is accompanied by increased plasma HDL-C concentrations in humans (16). These findings suggest that a change in EL expression associated with inflammatory states may at least in part account for the variation of HDL-C concentrations in CVD patients.

Cell culture experiments revealed that cytokine-stimulated EL expression was concomitant with an increase in EL activities (35). However, EL activity is partly regulated through posttranscriptional mechanisms. It has been reported that EL forms a head-to-tail dimer in the human plasma, and the homodimer formation is essential for the maintenance of EL activity (36), as is the case with LPL and HTGL. In addition, EL is proteolytically processed into 40- and 28-kDa fragments and inactivated by proprotein convertases (37). In this regard, our sandwich ELISA system can recognize the dimer of full-length EL with the intact enzymatic activity. On the other hand, angiopoietin-like 3 is known to act as an endogenous EL inhibitor (38). In addition, human heat-inactivated serum inhibited EL phospholipase activity (39), indicating the existence of some endogenous EL inhibitor in human serum. Furthermore, a naturally occurring variant in the EL gene (LIPG), glycine-26 to serine, which is associated with increased HDL, exhibits impaired synthesis (20).

It has been reported that EL has several heparin-binding domains and binds to heparan sulfate proteoglycans on the vascular endothelium (12, 15, 40). Therefore, EL should be released into plasma by heparin treatment (15). In the present study, however, there was no difference in EL mass between pre- and postheparin plasma samples, whereas control HTGL activity was markedly increased by the heparin administration. When we evaluated EL mass by our previous ELISA system (16), we confirmed that the administration of heparin did not affect plasma EL protein. Thus, the interaction of EL with heparan sulfate proteoglycans needs to be determined by further studies.

In conclusion, we developed a sandwich ELISA using newly generated monoclonal antibodies specific to human plasma EL. The limit of quantification, range of linearity, and imprecision for EL quantification are suitable for both experimental and clinical use. From the preliminary study of a healthy reference range, we found that reference-range EL concentrations were between approximately 50 and 1400 pg/mL in human plasma. This range is much lower and, we suggest, more reasonable than the range measured with previous EL assays. It is possible that patients with low (EL deficiency) and high serum EL concentrations associated with high or low HDL-C concentrations will be identified by use of this new assay for EL.

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