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# Enzyme-linked immunosorbent assay of lipoprotein(a) in serum and cord blood

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#### Summary

We have developed a new sensitive method for quantifying lipoprotein(a) (Lp(a)) in human serum, using a 'sandwich' type noncompetitive enzyme-linked immunosorbent assay (ELISA). The solid-phase used was a polystyrene plate. The anti-Lp(a) antibody-enzyme conjugate was labelled by linking Fab' fragments to peroxidase (EC 1.11.1.7) by the maleimide method.

The minimum detectable concentration was 0.5 ng/well. Routinely, the assay was carried out with 1000-fold diluted serum, and Lp(a) was quantified between 4.0 and 500 mg/l. Within-run coefficients of variation (CVs) ranged from 3.5% to 10.4% and between-run CVs from 5.0% to 11.1%. Results by the ELISA were in good agreement with those by radial immunodiffusion (r = 0.955).

The distribution of Lp(a) in serum from 820 healthy donors was highly skewed: mean 141.1 mg/l, medium 97.9 mg/l. In cord blood, the mean and median were 15.6 and 9.8 mg/l, respectively. This ELISA for Lp(a) has the advantages of being highly sensitive and specific, simple to perform, and does not use radioisotopes.

#### Introduction

Lipoprotein(a) (Lp(a)), originally described as a genetic variant of low-density lipoprotein (LDL) and thus a genetic marker [1], has been considered to be an

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independent risk factor for the development of coronary artery disease [2,3]. Lp(a) lipoprotein closely resembles LDL in some respects. Its density (1.05-1.12 kg/l)overlaps those of LDL and high-density lipoprotein (HDL), but its electrophoretic mobility on agarose corresponds to the pre- $\beta$  band in contrast with the  $\beta$  mobility of LDL. The protein moiety of Lp(a), apolipoprotein (Apo)a, is covalently linked to Apo B-100 through disulfide bonds [4] in the Lp(a) lipoproteins. A number of different assays, such as radial immunodiffusion (RID) [5], radioimmunoassay (RIA) [2], electroimmunoassay [3], immunonephelometry [6], and recently latex immunoassay [7], have been developed for quantitating Lp(a) in human sera. Each of these is based upon an immunochemical reaction between the Apo(a) antigen and an antibody directed against one or more of its determinants. We have already reported an RID method for Lp(a) determination [8]. This method is less than ideal, however, because of the frequency with which the concentration of Lp(a) in whole human plasma, including normal subjects, lies below its lower limit of sensitivity of 20 mg/l (approximately 10% of serum samples tested). Therefore, we have developed a highly sensitive 'sandwich'-type noncompetitive enzyme-linked immunosorbent assay (ELISA) for Lp(a) levels in human sera and cord blood sera.

# Materials and methods

#### Blood samples

Fresh normolipidemic sera (n = 820) were obtained from the Red Cross Blood Center, Gifu, Japan. They were from healthy adult subjects, aged 16–64 yr (523 men, mean  $\pm$  sD age 32.8  $\pm$  10.3 yr, and 297 women, 24.3  $\pm$  10.5 yr). Cord blood serum samples were obtained from 85 healthy neonates born at Hirano Hospital, Gifu, Japan. All sera were collected in the presence of 1 mmol of disodium EDTA, 3 mmol of NaN<sub>3</sub>, and 220 Kallikrein units of proteases inhibitor (Aprotinin, Sigma Chemical Co., St. Louis, MO, USA) per liter, final concentration, and then either stored at 4°C until they were analyzed within 1 wk, or frozen at -40°C.

# Isolation of Lp(a) antigen

Lp(a) antigen was prepared as described previously [8] from human Lp(a)-rich sera, as checked by agarose electrophoresis. Briefly, the LDL fraction containing Lp(a) was selectively precipitated with 0.5  $\mu$ mol of dextran sulfate sodium salt ( $M_r = 500\,000$ ; Pharmacia, Uppsala, Sweden) and 45 mmol of CaCl<sub>2</sub>/1 [9] from pooled sera, dissolved in 100 mmol/l NaCl and 45 mmol/l CaCl<sub>2</sub> solution, and isolated in a narrow density range (1.05–1.12 kg/l) by preparative ultracentrifuge (Model L8–70; Beckman Instruments, Fullerton, CA, USA). The dissolved precipitate was adjusted to 1.05 kg/l with solid NaBr. Ultracentrifugation was carried out in a Beckman 60 Ti rotor at 120 000 × g at 4°C for 20 h. The infranatant was collected, readjusted to 1.12 kg/l and recentrifuged at 140 000 × g at 4°C for 40 h. The Lp(a)-containing fraction recovered in the top was dialyzed overnight at 4°C against 100 mmol/l NaCl containing 100 mmol/l EDTA, pH 7.4. The purity of this fraction was checked by polyacrylamide gel electrophoresis [10].



Fig. 1. Results of immunoelectrophoresis. Sample wells contained 5  $\mu$ l of Lp(a)-rich serum. Position 1 contained anti-human whole serum; 2, anti-apolipoprotein B serum; 3, anti-Lp(a) serum.

#### Preparation of Lp(a) antiserum

Isolated Lp(a)-containing fraction (approximately 1 mg) was emulsified with an equal volume of complete Freund's adjuvant (Nakarai Chemicals Ltd., Kyoto, Japan), and injected intramuscularily into Japanese white male rabbits (body weight, approximately 2 kg) for immunization, as described earlier [8]. The rabbits were given booster injections of approximately 1 mg Lp(a) antigen at 2-wk intervals and were bled when adequate titers were achieved. Cross-reactivity in these antisera against apoA, apoB, apoC and other apolipoproteins was removed by absorption on an affinity chromatographic column of Sepharose 4B (Pharmacia) coupled with pooled sera obtained from Lp(a)-negative subjects. In order to check the specificity of the antiserum, immunoelectrophoresis was carried out and showed only a single precipitin line against human serum (Fig. 1). This figure shows that anti-Lp(a) serum clearly differ from that against the anti-apolipoprotein B serum (Dakopatts a/s, Denmark). Immunoglobulin (Ig)G fraction was isolated from the antiserum by chromatography on Affi-gel Blue column  $(3.0 \times 60 \text{ cm}; \text{Bio-rad}, \text{Richmond}, \text{CA}, USA).$ 

# Preparation of anti-Lp(a) antibody-enzyme conjugate

The conjugated antibody was prepared by the method of Kato et al [11]. We dialyzed overnight 50 mg of anti-Lp(a) IgG in 5 ml solution against 0.1 mol/l sodium acetate buffer, pH 4.5, at 4°C and digested the dialysate to  $F(ab')_2$  fragments by incubation with 3.6 mg of pepsin (EC 3.4.23.1; Boehringer Mannheim Yamanouchi, Tokyo, Japan) at 37°C for 4 h. After adjusting the mixture to pH 8.0 with 0.1 mol/l NaOH, the  $F(ab')_2$  fragments were isolated by affinity chromatography on a column of Lp(a)-Sepharose 4B (1.0 × 10 cm). Monitoring the absorbance at 280 nm, the adsorbed  $F(ab')_2$  fragments were eluted with 1 mol/l glycine buffer, pH 3.0.

The  $F(ab')_2$  fragments were concentrated to a volume of 400  $\mu$ l by ultrafiltration using an Amicon B15 membrane (Amicon Co., Lexington, MA, USA), and then incubated with 10 mmol/l 2-mercaptoethylamine reagent in 0.1 mol/l phosphate buffer, pH 6.0 at 37 °C for 90 min. The  $F(ab')_2$  fragments were almost completely converted to Fab' fragments. After incubation, the mixture was chromatographed on a Sephadex G-25 column (1.5 × 25 cm; Pharmacia) with 0.1 mol/l phosphate buffer, pH 6.0, containing 5 mmol of EDTA/l, and the Fab' fragments were concentrated to a volume of 400  $\mu$ l using an Amicon B15 membrane (Amicon).

Meanwhile, we prepared maleimide-peroxidase conjugate by incubating 4.8  $\mu$ mol/1 *N*-hydroxysuccinimide ester of *N*-(4-carboxycyclohexylmethyl)maleimide (Gieben Chemicals, Tokyo, Japan), suspended in dimethylsulfonamide, with 2 mg of horseradish peroxidase (POD, EC 1.11.1.7; Toyobo, Osaka, Japan) in a volume of 300  $\mu$ l of 0.1 mol/l phosphate buffer, pH 7.0 at 37 °C for 1 h. After centrifuging the mixture, we applied 300  $\mu$ l of the supernate to a column of Sephadex G-25 and eluted the conjugate with 0.1 mol/l phosphate buffer, pH 6.0, which was concentrated to 400  $\mu$ l.

In order to conjugate the Fab' fragments with POD, we mixed the maleimidetreated POD with an equal volume (400  $\mu$ l) of Fab' fragments and incubated at 4°C for 20 h. The mixture was then purified by column chromatography with Sephacryl S-200 (1.5 × 60 cm; Pharmacia), being eluted with 0.1 mol/l phosphate buffer, pH 6.0. The anti-Lp(a) antibody-enzyme conjugate was thus obtained.

#### Coating of the solid-phase

Anti-Lp(a) IgG was diluted 1 000-fold with 50 mmol/l sodium carbonate buffer, pH 9.6, to make a final concentration of 3.4 mg/l. Polystyrene plates (Nunc Immunoplate I; Intermed, Denmark) were coated with 300  $\mu$ l of anti-Lp(a) IgG per well at 4°C by overnight incubation. After washing three times with phosphatebuffered saline (PBS) containing 0.5 g/l of Tween 20, the solid-phase was incubated again at room temperature for 1 h in 300  $\mu$ l of PBS containing 0.5 g/l bovine serum albumin (BSA) to minimize non-specific binding. PBS used in these procedures contained, per liter, 5.59 mmol of Na<sub>2</sub>HPO<sub>4</sub>, 1.47 mmol of KH<sub>2</sub>PO<sub>4</sub>, 137 mmol of NaCl, 2.68 mmol of KCl, and 3 mmol of NaN<sub>3</sub>. Each well of the solid-phase microplates was filled with PBS and was stored sealed. The coated plates could be stored at 4°C for at least one month without loss of activity.

#### Lp(a) standard

A commercial human Lp(a) (475 mg/l expressed in terms of total Lp(a) lipoprotein, lot no. 290001; Immuno Diagnostika, Vienna, Austria) was employed for the standard reference. A pooled serum, which was used as the secondary standard and control, was prepared by collecting sera with high concentrations of Lp(a), adding EDTA, NaN<sub>3</sub> and Aprotinin for stabilization, and filtering the mixture through a 0.22  $\mu$ m pore-size Millipore filter (Millipore, Bedford, MA). It was divided into portions of 200  $\mu$ l and stored at -40 °C.

Secondary Lp(a) standards were calibrated by determining Lp(a) cholesterol in lipoprotein precipitated with Lp(a) antiserum. They were converted to total Lp(a)

lipoprotein by multiplying by 3.3 (Lp(a) lipoprotein is assumed to contain 29.8% cholesterol [5]). We found no discrepancy between our in-house secondary standards and a commercially available standard in the concentration of Lp(a) lipoprotein.

# ELISA procedures

Each determination was carried out in duplicate, including standards and controls. All procedures of the sandwich ELISA for Lp(a) were performed at room temperature. Serum samples and standards were diluted 1000-fold and cord blood samples 100-fold with PBS containing 0.5 g/l BSA. We placed 200  $\mu$ l of diluted samples in each solid-phase well and incubated them for 2 h. After washing three times with PBS containing 0.5 g/l of Tween 20, 200  $\mu$ l of conjugated antibody, diluted 2000-fold with PBS, was added to the wells and incubated for 1 h. The plates were washed three times again and incubated in the dark for 1 h with 200  $\mu$ l of freshly prepared *o*-phenylene diamine hydrochloride (2.2 mmol/l) in 50 mmol/l citric acid buffer, pH 5.5, containing hydrogen peroxide (3.5 mmol/l) per well. On completion, the reaction was terminated by addition of 100  $\mu$ l of 250 mmol/l H<sub>2</sub>SO<sub>4</sub> per well, and the absorbance read at 486 nm with 620 nm reference, using a microplatereader (SLT-210; Labinstruments, Glodig, Austria). The results were calculated from the best-fitting least-square parabola with an on-line microcomputer (NEC 9801-VM2; NEC Co., Japan).

# RID method

RID was carried out in 1% agarose, pH 8.6 (Agarose Immunodiffusion Tablets, Bio-Rad), containing 2% anti-Lp(a) serum. Antiserum-agarose solution was poured on polyester film coated Agarose ( $100 \times 150$  mm, Gelbond; FMC Co., Maine, PA, USA). Five microliters of standards and serum samples were added to each well of 2.5 mm diameter. The plates were placed in a humid chamber at 37°C. After 48 h, the ring-shaped immunoprecipitates were measured using a digital micrometer scale (NSK Co., Tokyo, Japan)

# Results

#### Specificity

In order to investigate the specificity of the anti-Lp(a) serum used in the present study, Western blotting after SDS-PAGE of serum was performed using the antiserum for detection. SDS-PAGE and Western blotting were carried out according to Gaubatz et al [12]. As shown in Fig. 2, a single band of Lp(a) lipoprotein was detected with immunoblotting procedure.

# Standardization and assay range

A standard serum was diluted to appropriate concentrations with PBS containing 0.5 g/l BSA. The standard curve is shown in Fig. 3. The assays were carried out ranging from 0.5 to 50 ng/well, which corresponded to Lp(a) concentration in serum of 4.0-500 mg/l for 1000-fold dilution.



Fig. 2. Western immunoblot to Lp(a) after 3.25% SDS-PAGE. Lane 1 only was prestained for lipid with Sudan Black B. Lane 1-4 were applied 10, 5, 10, 20  $\mu$ l of serum, respectively, and subjected to Western immunoblotting using anti-Lp(a) serum. Electrophoresis is from top to bottom.



Fig. 3. Calibration curve for Lp(a) lipoprotein quantitation.

TABLE I

Precision	of	the	method	
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Samples	Lp(a) (mg/l)	Lp(a) (mg/l)		
	Mean	SD		
Within-assay $(n =$	20)			
А	4.9	0.51	10.4	
В	16.4	0.58	3.5	
С	53.2	2.41	4.5	
Between-assay (n	= 7)			
D	6.2	0.69	11.1	
E	20.2	1.01	5.0	
F	103.8	7.19	6.9	

# Sensitivity and precision

The present method could detect Lp(a) lipoprotein at a minimum concentration of 0.5 ng/well, defined as the concentration significantly different from zero at the 95% confidence level. Results of within-assay and between-assay precision are given in Table I. Within-assay variation, determined by assaying three samples 20 times and calculating the CV in the sample values, ranged from 3.5% to 10.4%. The between-assay reproducibility was evaluated by determining three samples once per day for consecutive days over a 1-wk in duplicate assay and the CVs ranged from 5.0% to 11.1%.

# Analytical recovery

Recovery, calculated from data (n = 10) on several concentrations of purified Lp(a) adding to pooled sera as the ratio of the observed concentrations to the expected concentrations multiplied by 100%, was from 102.3% to 105.7% (Table II).

#### Interference studies

Possible interfering substances were investigated by the usual addition technique, with use of pooled human serum. Bilirubin (250 mg/l, ditaurobilirubin; Dojin,

#### TABLE II

n	Lp(a) (mg/l)				
	Added	Observed	Expected	Recovery (%)	
10	15	35.3±1.1 <sup>b</sup>	33.4	$105.6 \pm 3.3$ <sup>b</sup>	
10	30	$51.2 \pm 1.8$	48.4	$105.7 \pm 3.7$	
10	60	$82.7 \pm 3.1$	78.4	$105.4 \pm 4.0$	
10	120	$141.6\pm6.3$	138.4	$102.3\pm4.6$	

Analytical recovery of Lp(a) added to pooled serum <sup>a</sup>

<sup>a</sup> Pooled serum contained 18.4 mg of Lp(a)/l.

<sup>b</sup> Results are given as mean  $\pm$  SD.

Kumamoto, Japan) and 100 g/l of hemoglobin had no effect on the present Lp(a) assay. We also determined Lp(a) in hyperlipidemic sera with chylomicrons after 100-fold predilution with PBS containing 0.5 g/l Tween 20, and demonstrated that this treatment had no effect on the apparent concentration of Lp(a) in serum.

# Comparison with RID method

Serum samples from 70 subjects, including a wide range of Lp(a) concentrations, were assayed for Lp(a) lipoprotein by both the present ELISA method (y) and the RID method (x). Lp(a) determined by the ELISA procedure was highly correlated with the level of Lp(a) determined by the RID method (r = 0.955,  $S_{y/x} = 25.97$  mg/l). The equation for the regression line was y = 0.98x + 1.61 mg/l.

#### Reference values

Since the frequency distribution of serum Lp(a) levels determined by the present ELISA method for the normolipidemic adult and cord blood were highly skewed, the logarithmic conversion was carried out (Fig. 4). Mean and median values for adult serum was 141.1 and 97.9 mg/l, respectively, and those for cord blood 15.6 and 9.8 mg/l, respectively. 95% ranges for the normal Lp(a) concentrations were between 5.1 and 421.9 mg/l for adults, and between 0.15 and 55.3 mg/l for cord blood. Of all adult sera assayed, approximately 10% of samples were shown to have concentrations below 20 mg/l. The values showed no sex-related difference in either



Fig. 4. Distribution of Lp(a) concentrations in normal adults (left) and cord bloods (right) determinated by ELISA. Log transformation was performed on Lp(a) value for adults and cord bloods because of skewness. Left n = 820, mean = 141.1 mg/l, median = 97.9 mg/l; right n = 85, mean = 15.6 mg/l, median = 9.8 mg/l.

mean or median Lp(a) levels; men having a mean of 132.7 mg/l and a median of 84.7 mg/l, while women a mean of 148.8 mg/l and a median 91.9 mg/l.

#### Discussion

The present paper describes a new quantification of human serum Lp(a) by an ELISA procedure. Lp(a) concentration in serum has been considered to be determined genetically. Initially, interest in Lp(a) determinations was limited to cardiovascular disease [3]. With the advent of more sensitive and precise techniques Lp(a) has been measured in other diseases. Recently, Parra et al [13] reported that Lp(a) was increased in patients with chronic renal failure who were treated with hemodialysis. Also we have found that Lp(a) in serum increased during the acute phase after surgical operations and attacks of myocardial infarction, and conversely decreased to a very great degree in patients with obstructive jaundice [14,15].

A sensitive latex immunoassay for Lp(a) was reported and the assay range by it was between 0.05 to 1.15 mg/l for 1000-fold diluted serum [7]. On the other hand, an RIA method could determine Lp(a) beyond the 5 mg/l concentration [2]. In the present study, we used POD-Fab' (anti-Lp(a) antibody) as the enzyme-conjugated antibody, because Kato et al [11] suggested that the immunoassay with Fab'-enzyme conjugate was more sensitive than with IgG-enzyme conjugate. The sensitivity of the present ELISA method was equal to or greater than that found in the RIA method. The precision of our assay was comparable to other immunoassay methods. Although RID has the disadvantage in the case of larger lipoprotein molecules such as cylomicrons that diffused poorly in agarose plates, the present ELISA is not affected by chylomicrons and other interfering substances such as bilirubin and hemoglobin.

Using an RID method, Albers et al [5] reported the normal value of Lp(a) in serum, which were collected from 340 healthy and fasting company employees ranging from 20-65 years. Their report showed that the frequency distribution of Lp(a) was highly skewed with a mean of 140 mg/l and a median of 80 mg/l, and that no sex- and age-related differences were observed. As mentioned above, our results in Japanese compare well with that in the previous report [5].

Lp(a) concentration in the cord blood serum was very low, approximately one-tenth of that in adult serum. After birth, Lp(a) is assumed to be produced rapidly and the level in serum increases to the adult level [16].

In conclusion, the present ELISA technique for Lp(a) offers the advantages of a simple procedure, high sensitivity and no use of radioisotopes. The sensitivity of the ELISA is almost equivalent to that of an RIA method. Thus we believe that the present ELISA technique for Lp(a) can be used both for experimental research and in routine clinical analysis.

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