

## Polymorphism and Distribution of Apo(a)

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Several apo(a) isoforms, controlled by a series of alleles Lp(a)<sup>F</sup>, Lp(a)<sup>B</sup>, Lp(a)<sup>S1</sup>, Lp(a)<sup>S2</sup>, Lp(a)<sup>S3</sup>, Lp(a)<sup>S4</sup> and null, were found in 470 healthy Japanese by 4% SDS-PAGE and immunoblotting techniques. There was a strong inverse relationship between the apparent molecular weight of apo(a) isoforms and plasma concentrations of Lp(a). Lp(a) in d < 1.006 fraction increased 2–4 h after oral fat load. Lp(a) exhibited a marked avidity for triglyceride-rich lipoprotein (TRL), and we suggest that the TRL-bound Lp(a) is the intact Lp(a) derived from serum. We demonstrated that the lipid-free apo(a) does not contain apo B-100 in serum, and has a molecular mass of ca 200 kDa. The free apo(a) level in normal subjects was 1.75 mg/dl (as Lp(a)) and was no different from the level in CAD patients.

**Key words :** Lipoprotein (a) [Lp(a)], Isoform, Phenotype, Free apo(a), TG-rich lipoprotein

Lipoprotein (a) [Lp(a)] resembles low-density lipoprotein in particle size and lipid composition, and its density distribution is between 1.05 and 1.12 kg/l. The amino acid sequence of apo(a) was determined by McLean *et al.* (1), and shows a remarkable homology to human plasminogen. After that, part of the factor for atherosclerotic diseases was considered its function in the fibrinolytic system. Apo(a) has several isoforms that vary in molecular mass between 400 and 700 kDa, exist even in a lipid-free form, and show an affinity to triglyceride-rich lipoproteins (TRL).

The present study investigated size heterogeneity and multi-distributions of apo(a).

### Materials and Methods

#### Blood collection

Control subjects were hospital staff or blood donors. Subjects with coronary artery diseases (CAD) were hospital patients undergoing cine angiography. Blood samples were routinely collected from subjects, and centrifuged to separate serum at a final concentration of 1 mmol Na<sub>2</sub>EDTA, 3 mmol sodium azide and kallikrein inhibitor (200 units/5 ml blood). Sera were examined within a week and stored at –40°C until use.

#### Oral fat load

After a 14 h fast, each subject was given butter (50 g/person) as an oral fat load. Blood was drawn at 2, 4 and 6 h after loading.

#### Antibody against Lp(a)

Lp(a) antigen and antiserum were prepared as described previously (2). Briefly, Lp(a) antigen purification was performed using ultracentrifugation (1.05–1.12 kg/l) of LDL fraction precipitated with dextran sulfate and calcium chloride from human Lp(a)-rich sera. Antibody against Lp(a) was produced in rabbits by immunization with the isolated Lp(a)-containing fraction. The anti-apo(a) antibodies were purified by affinity chromatography by cyanogen-bromide-activated Sepharose 4B (Pharmacia, Uppsala, Sweden) coupled with pooled sera obtained from Lp(a)-negative subjects. Specificity of the antibody was characterized by Western blotting with human serum, and tested cross-reactivity against apo B-100 and plasminogen.

#### Electrophoretic procedure

A ten  $\mu$ l sample was prepared by diluting 1:4 with sample buffer, which was mixed with 1.0 ml of 0.5 mol/L Tris-HCl buffer, pH 6.8, 1.6 ml of 10% SDS, 0.8 ml of glycerol, 0.4 ml of 2-mercaptoethanol, 0.2 ml of 0.05% bromphenol blue, and 4.0 ml of distilled water. The solution was heated for 4 min at 95 °C. 10  $\mu$ l aliquots of

the mixture, corresponding  $\sim 2 \mu\text{l}$  serum, were used for electrophoresis.

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was routinely performed on T=4.0% slab gels using a discontinuous buffer. We used SDS mini gel (catalog #01-055; Tefco Company, Japan), which is  $8 \times 8 \text{ cm}$ , 10 wells per gel with 5 mm width and 1.0 mm thick. The buffer of the electrophoresis chamber was used as the discontinuous buffer. Upper cathode buffer was 0.04 mol Tris buffer, pH 8.64, with 0.04 mol boric acid and 0.1% SDS, and the lower anode was 0.43 mol Tris-buffer, pH 9.18, with 0.03 mol hydrochloric acid, per liter. Gels were run at 20 mA per plate as  $25^\circ\text{C}$  for 90 min.

#### Western blotting

The gels were transferred onto a sheet of nitrocellulose membrane (Trance-Blot,  $0.45 \mu\text{m}$ , catalog #162-0115; Bio-Rad, Richmond CA, USA) and the proteins were electro-blotted with a constant voltage of 100 V in 25 mmol/l Tris, 192 mmol/l glycine and 20% methanol of a transcription buffer, pH 8.3 for one hour.

The Lp(a) or apo B bands were visualized on the membrane by a double-antibody procedure, involving rabbit anti-apo(a) or goat anti-apo B (catalog #AB742; Chemicon Inc., CA, USA) antisera as the first antibody, and anti-rabbit IgG (Gar-Gold, catalog #170-6625, Bio-Rad) or anti-goat IgG (catalog #GAF-013; EY labs, Inc., CA, USA) as the second antibody which were labeled with colloidal gold ( $E_{520} = 0.2$ , 20 nm diameter). The gold-labeled bands were enhanced with a physical developer consisting of silver lactic acid and hydroquinone.

#### Analytical methods

Lp(a) concentrations were determined by enzyme-linked immunosorbent assay (ELISA) as described previously (2) using rabbit anti-apo(a) and Lp(a) reference standard (Immuno, Vienna, Austria).

#### Ultracentrifugation

Serum were brought to  $d > 1.210 \text{ kg/l}$  with two volume of  $d > 1.308 \text{ kg/l}$  NaBr solution. The sample was obtained from the top fraction for HPLC assay of apo(a) in TRL and the bottom fraction for assay of free apo(a).

Samples were centrifuged for 20 h in a Beckman L7-80 ultracentrifuge at 40,000 rpm ( $15^\circ\text{C}$ ) using a 50.3 Ti rotor.

#### HPLC procedure

Samples were injected  $100 \mu\text{l}$  of lipoprotein fraction into a Model CCPE/UV-8010 HPLC unit (Tosoh Co., Tokyo) equipped with G4000SW column ( $7.5 \text{ mm} \times 60 \text{ cm}$ ) and separated with 150 mmol/l NaCl at a flow rate of 0.5 ml/min at room temperature. The separated fractions were collected 40 parts and assayed Lp(a) by ELISA.

#### Statistical method

Pairwise comparison of Lp(a) concentrations between phenotypes was performed by two-tailed Mann-Whitney test. Comparison of Lp(a) phenotype frequencies between two groups was done by  $\chi^2$  analysis. Lp(a) allele frequencies were calculated according to Wiener *et al.* (3).

### Results

#### Identification of Lp(a) phenotypes by immunoblotting

Serum samples from blood donors were subjected to SDS-PAGE under reducing conditions, and the Lp(a) protein was demonstrated by immunoblotting (Fig. 1).

Of the 470 tested subjects, 91% exhibited apo(a) band(s) upon immunoblotting. Two types of patterns characterized by the presence of single band Lp(a) phenotype or by two bands Lp(a) phenotype were observed. According to their relative mobilities compared with apo B-100, Lp(a) patterns were categorized into phenotypes F, B, S1, S2, S3, and S4, and into the respective double-band phenotypes. These phenotypes ranged in apparent

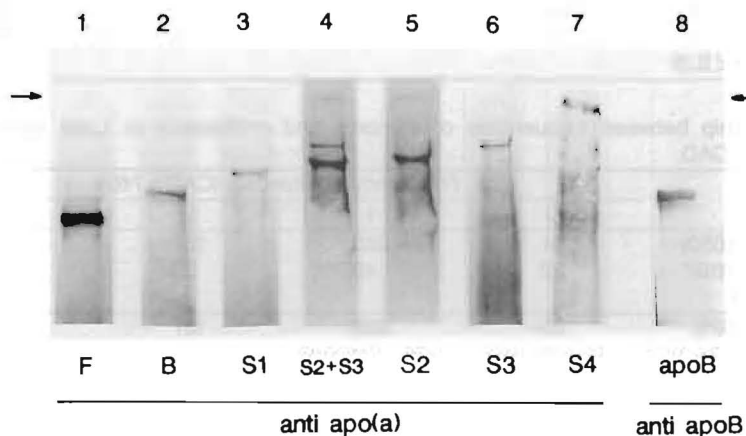


Fig. 1. A typical Western blot of apo(a).

molecular mass from approximately 400 to 700 kDa.

### Frequency of phenotype in Japanese

Lp(a) phenotypes were determined in 470 unrelated blood donors. The frequencies of phenotypes in the blood donors are given in Table 1: the incidence concur- red with that calculated theoretically as five alleles of Lp(a)<sup>B</sup>, Lp(a)<sup>S1</sup>, Lp(a)<sup>S2</sup>, Lp(a)<sup>S3</sup> and Lp(a)<sup>S4</sup> and null Lp(a)<sup>O</sup>. The distribution of the incidence of estimated Lp(a) type

was not significantly different among males and females ( $\chi^2=9.1$ ). There was only one individual with the F phenotype in this group. 9.1% of subjects had no detect- able Lp(a) on immunoblotting due to low serum Lp(a) concentrations. When we compared the observed phenotype frequencies with those expected from the genetic hypothesis and assuming Hardy-Weinberg equi- librium, there was no significant deviation between ob- served and expected frequencies ( $\chi^2=14.4$ ). In our

**Table 1.** The frequencies of Lp(a) phenotype and Lp(a) concentration.

	Number		Lp(a), mg/dl	
	Observed	Expected	Mean	SD
B	4	3.7	42.3	6.7
S1	14	12.8	22.0	18.0
S2	62	64.6	17.0	14.6
S3	133	114.3	12.5	8.4
S4	99	78.8	8.1	6.1
O	43	61.9	4.4	5.9
B+S1	0	0.4		
B+S2	2	1.6	24.9	15.4
B+S3	2	2.5	34.7	16.7
B+S4	2	1.9	8.9	8.9
S1+S2	10	5.2	43.9	28.2
S1+S3	7	8.4	25.1	16.1
S1+S4	2	6.2	27.8	24.0
S2+S3	35	36.6	23.3	12.7
S2+S4	26	27.0	19.7	12.0
S3+S4	28	43.1	16.9	11.8
F+S3	1		20.7	
Total	470	469	15.5	13.6

**Table 2.** Apo(a) isoform and Lp(a) concentration in CAD.

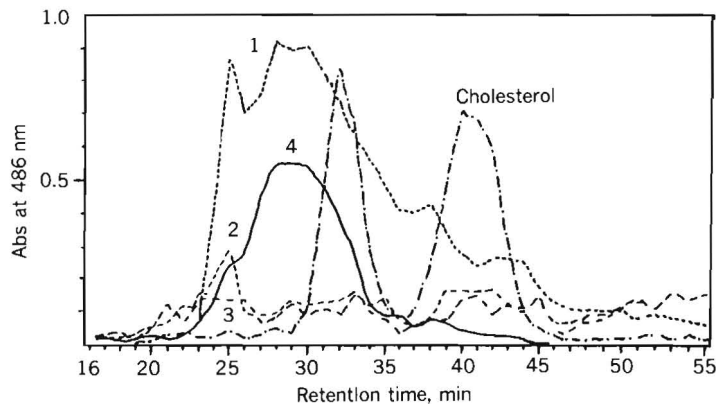
**a.** Relationship between frequencies of apo(a) isoform and cholesterol or Lp(a) level in 316 patients with CAD.

		CAD=316							
		Single band	O	S4	S3	S2	S1	B	Double band
Chol<220	247	148	5	70	61	9	2	1	99
Chol≥220	69	39	4	14	19	1	1		30
$\chi^2=6.3$ NS									
Lp(a)<30	236	164	9	80	68	6	1		72
Lp(a)>30	80	23	0	4	12	4	2	1	57
$\chi^2=52.4$ P<0.01									

**b.** Relationship between frequencies of stenosis and cholesterol or Lp(a) level in 769 patients with CAD.

	Frequency of Stenosis (CAD=769)			
	0	1	2	3
Chol < 220 (650)	91	283	157	119
Chol ≥ 220 (119) <sup>a</sup>	22	48	23	26
$\chi^2=2.3$ NS				
Lp(a) < 30 (525)	85	239	121	80
Lp(a) ≥ 30 (244) <sup>b</sup>	28	92	59	65
$\chi^2=16.0$ P<0.01				
Chol ≥ 220 (34) <sup>c</sup>	8	8	7	11
Lp(a) ≥ 30				

a vs. c:  $\chi^2=3.6$  NS; b vs. c:  $\chi^2=3.0$  NS



**Fig. 2.** Lp(a) affinity for TRL. Lp(a) profile in lipoprotein by HPLC and ELISA technique. Sample is used a high level of VLDL. 1: Lp(a) in  $d > 1.21$  fraction (A), 2: Lp(a) in  $d < 1.006$  of A, 3: Lp(a) in  $d < \sim 1.000$ , 4: Lp(a) in  $d > \sim 1.000$ .

study, frequency of "O" was 9.1% and concentration (mean  $\pm$  SD) of Lp(a) in this phenotype was  $4.4 \pm 5.9$  mg/dl. We found Lp(a) allelic frequencies of  $Lp(a)^B = 0.011$ ,  $Lp(a)^{S1} = 0.036$ ,  $Lp(a)^{S2} = 0.156$ ,  $Lp(a)^{S3} = 0.250$ ,  $Lp(a)^{S4} = 0.184$ , and  $Lp(a)^O = 0.363$ .

#### Lp(a) concentration in the respective phenotypes

We examined the distribution of Lp(a) levels of every single isoform. Pairwise comparison by a two-tailed Mann-Whitney test showed that the four frequent phenotypes may be split into two pairs, S1 and S2, and S3 and S4, with non-significant differences between the Lp(a) concentration within a pair, but significant differences between the Lp(a) concentrations of phenotypes in different pairs, e.g., S1 vs. S3 ( $P < 0.01$ ), S1 vs. S4 ( $P < 0.01$ ), S2 vs. S3 ( $P < 0.01$ ), and S2 vs. S4 ( $P < 0.01$ ).

#### Apo(a) isoform frequencies of CAD

We determined Lp(a) levels and apo(a) isoforms of 769 CAD patients. When we defined hyperLp(a)emia as above 30 mg/dl, the frequency was higher among CAD patients and in higher numbers of diseased arteries. However, serum cholesterol level did not relate to the number of stenosed arteries (Table 2). The phenotype of B, S1 and S2 type in single isoform was suggested high risk factor for CAD. However, the frequencies of alleles did not differ between CAD and normal subjects.

#### Affinity of apo(a) to TRL

Lp(a) antigen in  $d < 1.006$  fraction increased 2–6 hrs after the oral fat load, and was  $\sim 10$ -fold higher than during fasting. Lp(a) had high affinity to TRL and dissociated easily at a density lower than  $d \sim 1.000$  (Fig. 2). We detected lipid moiety in Lp(a) dissociated from TRL.

#### Free apo(a)

Free apo(a) was found in  $d > 1.21$  fraction obtained by

ultracentrifugation. Lp(a) antigen in this fraction was demonstrated as lipid-free apo(a) not containing apo B-100, and as a molecular mass of ca 200 KDa. We found that our apo(a) antibody has no cross reactivity to plasminogen in the  $d > 1.21$  fraction. Free apo(a) was observed in the sera of both normal and CAD subjects. Although Lp(a) of CAD patients was higher ( $P < 0.001$ ) than that of normal subjects. Mean free apo(a) level in normal subjects was 1.75 mg/dl (SD=0.18) and that in CAD patients was not significantly different from normal subjects (Table 3).

#### Discussion

The Lp(a) contains two major protein components, apo(a) and apo B-100 linked by disulfide bridge formation in the intact particle. In the presence of a reducing agent, such as 2-mercaptoethanol, apo(a) can be removed from Lp(a). To distinguish Lp(a) phenotypes in the Japanese population, we demonstrated that the apparent molecular weight ranged from 400 kDa to 700 kDa using SDS-PAGE followed by immunoblotting. These bands were designated as B, S1, S2, S3 and S4. Individual sera contained only one or two of these isoforms. These phenotypes were controlled by a series of autosomal alleles at a single locus designated as  $Lp(a)^B$ ,  $Lp(a)^{S1}$ ,  $Lp(a)^{S2}$ ,  $Lp(a)^{S3}$ ,  $Lp(a)^{S4}$  or  $Lp(a)^O$ , operationally defined as a null allele (4). Apo(a) isoforms were suggested to have a significant effect on serum Lp(a) levels. The number of kringle 4 repeats related directly to the size of the apo(a) isoform (5).

Frequencies of Lp(a) phenotypes in Europeans were reported by Utermann *et al.* (4–6). Frequencies of Lp(a) alleles in the present study were not significantly different from those in Europeans. Huang *et al.* (7) recently reported the frequencies in the American population, and described the S3 type as the most frequent indicating that

**Table 3.** Lp(a) and free apo(a) concentration in controls and CAD patients.

	Controls (83)	CAD (97)
Lp(a), mg/dl	14.5 ±12.8	27.4 ±28.2
Free apo(a), mg/dl	1.75± 2.02	1.65± 2.12
% to Lp(a)	15.4 ±14.7	9.3 ±12.1

mean ± SD

the white north American population was different from Europeans. Sandholzer *et al.* reported Lp(a) concentrations and Lp(a) phenotypes in 7 ethnic groups. Average Lp(a) concentrations were significantly different among these populations and frequencies of Lp(a) phenotype were also different. Levels of Lp(a) in Sudanese Blacks were highest in the groups and apo(a) isoform effects on Lp(a) levels were not significantly different among populations (8).

Since we used highly sensitive stains with lactic acid silver to increase the sensitivity of the immunoblot procedure, the frequency of Lp(a)<sup>0</sup> allele was considerably lower. Recently, Gaubatz *et al.* (9) detected 11 different isoforms and Lackner *et al.* (10) identified 19 different alleles by the DNA hybridization technique. Their data did not follow the Hardy-Weinberg equilibrium. Although differences in isoforms are the results of variation in the number of kringle repeats, they have also differences of primary structure in carbohydrate moiety, especially in neuraminic acid content. Utermann's and our classification is within the Hardy-Weinberg equilibrium and is practically suited to clinical concept.

Although we could find 0.2% of F phenotype among the blood donors, it was observed among 2.5% of the CAD group. High levels of Lp(a) are strongly associated with atherosclerosis (4). The present results suggest that serum Lp(a) above 30 mg/dl increases the risk of CAD.

Lp(a) increased in the TRL fraction of  $d < 1.006$  after fat feeding. Zioncheck *et al.* demonstrated the binding of Lp(a) and pure recombinant apo(a) protein to macrophages. The binding of modified lipoproteins to macrophages may lead to atherogenesis (11). We also found that Lp(a) in TRL isolated with HPLC was significantly higher in patients with cerebral infarction.

Pfaffinger *et al.* (12) reported that apo(a)-apo B 100, not apo(a), was associated with TRL and this complex might derive from *de novo* synthesis rather than from a pre-existing Lp(a) plasma pool. However, our result suggests that Lp(a) has a high affinity for TRL and is transferred to TRL from serum. In any event, high plasma triglycerides and Lp(a) are associated with an increased prevalence of athero-thrombotic factors.

A very small amount of apo(a) can exist in serum in a lipid-free form. In abetalipoproteinemia, Lp(a) was detect-

ed and the Lp(a) was apo(a)-apo B as a lipid-poor complex (13). Apo(a) is a glycoprotein and increases in response to inflammation (14). Lp(a) is suggested to relate to acute phase proteins. Free apo(a) was considered to have a definite level in serum, although the function and metabolism of free apo(a) is still unclear.

## References

- (1) McLean JW, Tomlinson JE, Kuang W-J, *et al.*: cDNA sequence of human apolipoprotein (a) is homologous to plasminogen. *Nature*, 330: 132-137, 1987
- (2) Abe A, Maeda S, Makino K, *et al.*: Enzyme-linked immunosorbent assay of lipoprotein (a) in serum and cord blood. *Clin Chim Acta*, 177: 31-40, 1988
- (3) Wiener AS, Lederer M, and Polayes SH: Studies in isohemagglutination. I. Theoretical considerations. *J Immunol*, 16: 469-482, 1929
- (4) Utermann G, Menzel HJ, Kraft HG, *et al.*: Lp(a) glycoprotein phenotypes. *J Clin Invest*, 80: 458-465, 1987
- (5) Utermann G: The mysteries of lipoprotein (a). *Science*, 246: 904-910, 1989
- (6) Utermann G, Kraft HG, Menzel HJ, *et al.*: Genetics of the quantitative Lp(a) lipoprotein trait. I. Relation of Lp(a) glycoprotein phenotypes to Lp(a) lipoprotein concentrations in plasma. *Hum Genet*, 78: 41-46, 1988
- (7) Huang CM, Kraft HG, and Gregg RE: Lp(a) phenotype frequency in a normal north American population (Abstract). *Clin Chem*, 35: 1070, 1989
- (8) Sandholzer C, Hallman DM, Saha N, *et al.*: Effects of the apolipoprotein (a) size polymorphism on the lipoprotein (a) concentration in 7 ethnic groups. *Hum Genet*, 86: 607-614, 1991
- (9) Gaubatz JW, Ghanem KI, Guevara J, *et al.*: Polymorphic forms of human apolipoprotein (a): inheritance and relationship of their molecular weights to plasma levels of lipoprotein (a). *J Lipid Res*, 31: 603-613, 1990
- (10) Lackner C, Boerwinkle E, Leffert CC, *et al.*: Molecular basis of apolipoprotein (a) isoform size heterogeneity as revealed by pulsed-field gel electrophoresis. *J Clin Invest*, 87: 2153-2161, 1991
- (11) Zioncheck TF, Powell LM, Rice GC, *et al.*: Interaction of recombinant apolipoprotein (a) and lipoprotein (a) with macrophages. *J Clin Invest*, 87: 767-771, 1991
- (12) Pfaffinger D, Schuelke J, Kim C, *et al.*: Relationship between apo(a) isoforms and Lp(a) density in subjects with different apo(a) phenotype: a study before and after a fatty meal. *J Lipid Res*, 32: 678-683, 1991
- (13) Menzel HJ, Dieplinger H, Lackner C, *et al.*: Abetalipoproteinemia with an apo B-100-lipoprotein (a) glycoprotein complex in plasma. *J Biol Chem*, 265: 981-986, 1990
- (14) Kawade M, Maeda S, Abe A, and Yamashiro M: Alternation in plasma Lp(a) lipoprotein Lp(a) and acute phase proteins after surgical operation (Abstract). *Clin Chem*, 30: 941, 1984