

ed that the Lp(a)<sup>S1</sup> and Lp(a)<sup>S2</sup>, which are associated with high Lp(a) levels, were more frequent among the patients with CAD, whereas the allele Lp(a)<sup>S4</sup>, which is associated with low Lp(a) levels, was more frequent among those without CAD. However, the number of patients used in their study appeared to be too small to determine the phenotype frequency in patients with or without CAD.

It remains to be established whether or not the difference in plasma Lp(a) levels between healthy subjects and patients with CAD was determined by the apo(a) gene locus. Although we indicated the importance of the apo(a) allele in the relationship between Lp(a) phenotypes and plasma concentrations in the preceding paper [5], we maintained that it is not possible to predict plasma Lp(a) concentrations from Lp(a) phenotypes, or vice versa.

In the present paper, we have studied the frequencies of Lp(a) phenotypes and alleles, the association between the Lp(a) phenotypes and plasma levels and the relationship between the severity of CAD and cholesterol and Lp(a) concentrations in plasma with respect to Lp(a) phenotypes in 470 patients with angiographically defined CAD.

## Materials and Methods

### Subjects

The coronary artery disease (CAD) group was composed of 470 patients undergoing cardiac catheterization and coronary arteriography for suspected ischemic heart disease at the Division of Cardiology, Juntendo University Hospital. The group consisted of 402 males and 68 females with a mean ( $\pm$  S.D.) age of  $50 \pm 9$  years.

Selective coronary cineangiography was performed using Sone's technique. Coronary artery segments were identified and categorized according to the reporting system proposed by the American Heart Association.

A significant coronary stenosis was defined as a narrowing of a major coronary artery branch by at least 75%. For our study, all cineangiography was reviewed by one of the authors (Y.J.L.), who is an experienced angiographer and was blinded to the

patients' symptomatic status and lipid findings. Cineangiography results indicated that 85 patients had no significant coronary stenosis, 183 patients had single-, 111 had double- and 91 had triple-vessel coronary artery disease. Informed consent was obtained from all patients.

### Blood sampling

Blood samples were collected in the morning by venipuncture on the day of the coronary catheterization and after an overnight fast and were allowed to clot at room temperature for 1–2 h. All sera were obtained under conditions minimizing proteolysis with EDTA, NaN<sub>3</sub> and a protease inhibitor and were kept at 4°C for immediate assay or at –40°C for further analysis.

### Preparation of Lp(a) antibody

Lp(a) antigen and antibody were prepared as described in the preceding paper [5].

### Lp(a) determination

Lp(a) concentrations were determined as reported previously [6], using rabbit anti-apo(a) antibody and Lp(a) reference standard (Immuno AG, Vienna, Austria).

### Electrophoretic procedures

Electrophoretic procedures for separation of apo(a) isoforms were performed as described in the preceding paper [5], using sodium dodecyl sulfate (SDS)-4.0% polyacrylamide gel electrophoresis (PAGE), immunoblotting and gold-silver staining.

### Lipid and lipoprotein analyses

Cholesterol, triglyceride and high density lipoprotein (HDL)-cholesterol in serum were measured enzymatically using commercial kits (International Reagent Corp., Kobe, Eiken Chemical Co., Ltd., Tokyo and Daiichi Pure Chemicals, Co., Ltd., Tokyo, respectively).

### Statistical methods

Overall comparison of Lp(a) concentrations among phenotypes was done by the non-parametric Kruskal-Wallis test. Comparison of Lp(a) phenotype frequencies between subgroups with high and low Lp(a) levels was done using  $\chi^2$

analysis. Lp(a) allele frequencies were calculated according to Wiener et al. [7].

## Results

### *Lp(a) concentrations and their distribution*

The frequency distribution for serum Lp(a) concentrations in the healthy and CAD groups was highly skewed, with a mean  $\pm$  S.D. of  $14.6 \pm 13.6$  mg/dl and  $23.5 \pm 23.3$  mg/dl and medians of 11.0 and 16.4 mg/dl, respectively (Table 1). The Lp(a) distribution in the CAD patients was markedly shifted to higher values.

Furthermore, as shown in Table 1, serum Lp(a) concentrations were significantly increased in proportion to the number of diseased vessels in the CAD patients. Frequencies of subjects with Lp(a) concentrations above 15 mg/dl also increased proportionally to the number of diseased coronary arteries. On the other hand, serum cholesterol and HDL-cholesterol levels were also significantly different in the CAD patients compared with those in the healthy group, whereas no change was observed in the subgroups divided according to the number of diseased vessels.

In order to confirm these results, the relationships between severity of CAD and either Lp(a) or cholesterol levels are shown in Table 2, using 769 CAD patients with known Lp(a) and cholesterol levels and known numbers of diseased vessels. We defined hypercholesterolemia as a plasma cholesterol level above 220 mg/dl and hyper-

TABLE 2

RELATIONSHIPS BETWEEN THE NUMBER OF DISEASED VESSELS AND SERUM CHOLESTEROL AND Lp(a) LEVELS IN CAD PATIENTS ( $n = 769$ )

	No.	Number of diseased vessels			
		0	1	2	3
Chol < 220	650	91	283	157	119
Chol $\geq$ 220	119	22	48	23	26
$\chi^2 = 2.3$ , N.S.					
Lp(a) < 30	525	85	239	121	80
Lp(a) $\geq$ 30	244	28	92	59	65
$\chi^2 = 16.0$ , $P < 0.01$					

Lp(a)emia as a plasma Lp(a) level above 30 mg/dl. As shown in Table 2, there was no significant difference in the frequencies of the number of diseased vessels in hyper- and normo-cholesterolemia, whereas there was a significant difference between hyper- and normo-Lp(a)emia ( $\chi^2 = 16.0$ ,  $P < 0.01$ ). Furthermore, the frequencies of hyper-Lp(a)emia increased with the number of diseased vessels.

### *Frequencies of Lp(a) phenotypes and putative alleles*

The frequencies of Lp(a) phenotypes in the CAD patients are given in Table 3, and were considerably different when compared with those in

TABLE 1

SERUM LEVELS OF CHOLESTEROL, HDL-CHOLESTEROL AND Lp(a) ( $M \pm S.D.$ ), AND PERCENTAGES OF CASES WITH Lp(a) LEVEL ABOVE 15 mg/dl AND DOUBLE-BAND PHENOTYPES IN RELATION TO THE NUMBER OF DISEASED VESSELS

Group	No.	Cholesterol (mg/dl)	HDL-cholesterol (mg/dl)	Lp(a) (mg/dl)	% of Lp(a) > 15 mg/dl	% of double-band phenotype
Healthy	465	$177 \pm 33$	$46.1 \pm 11.8$	$14.6 \pm 13.6$	40.0	24.7
CAD	470	$192 \pm 43^*$	$35.7 \pm 10.8^*$	$23.5 \pm 23.3^*$	56.6	43.2
0 <sup>a</sup>	85	$199 \pm 42$	$40.5 \pm 11.9$	$19.2 \pm 21.9$	41.2	36.2
1 <sup>a</sup>	183	$189 \pm 40$	$34.8 \pm 11.0$	$20.2 \pm 19.7$	45.9	38.3
2 <sup>a</sup>	111	$193 \pm 48$	$33.9 \pm 9.4$	$26.8 \pm 23.9$	55.9	45.0
3 <sup>a</sup>	91	$190 \pm 44$	$35.1 \pm 11.0$	$30.1 \pm 27.9$	62.6	51.4

<sup>a</sup>The number of diseased vessels.

\* $P < 0.001$  vs. healthy group.

TABLE 3

FREQUENCIES OF Lp(a) PHENOTYPES AND SERUM Lp(a) CONCENTRATIONS IN DIFFERENT PHENOTYPES IN CAD PATIENTS

Phenotype	Frequency		Lp(a) concentrations (mg/dl)		
	No.	%	Mean	S.D.	Median
F	1	0.2	47.7		
B	1	0.2	85.5		
S1	4	0.9	38.0	31.4	31.5
S2	13	2.8	25.2	18.5	16.6
S3	106	22.6	18.5	13.8	14.7
S4	132	28.1	13.6	11.8	10.0
Null	10	2.1	3.9	4.4	1.9
B + S1	2	0.4	30.4		
B + S2	3	0.6	37.5	10.5	35.5
B + S3	16	3.4	34.6	29.8	21.4
B + S4	16	3.4	34.2	29.8	15.2
S1 + S2	4	0.9	38.7	25.4	31.1
S1 + S3	25	5.3	37.3	28.7	27.3
S1 + S4	9	1.9	34.4	40.9	20.3
S2 + S3	23	4.9	37.7	28.2	30.1
S2 + S4	33	7.0	28.9	34.1	13.1
S3 + S4	66	14.0	31.9	22.9	30.4
F + S3	3	0.6	35.0	21.5	30.4
F + S4	3	0.6	25.5	4.3	23.3
Total	470	99.9	23.5	23.3	16.4

the healthy population described in the preceding paper [5]. In particular, the frequency of double-band phenotypes was significantly higher in the CAD patients than that in the healthy group. The

TABLE 5

RELATIONSHIPS BETWEEN Lp(a) PHENOTYPES AND SERUM CHOLESTEROL AND Lp(a) LEVELS IN CAD PATIENTS

	No.	Single-band							Double-band	
		No.	Null	S4	S3	S2	S1	B	F	
Chol < 220	367	209	6	108	82	9	3	1	0	158
Chol ≥ 220	103	58	4	24	24	4	1	0	1	45
$\chi^2 = 0.01$ , N.S.										
Lp(a) < 30	340	227	10	119	88	8	2	0	0	113
Lp(a) ≥ 30	130	40	0	13	18	5	2	1	1	90
$\chi^2 = 49.7$ , $P < 0.01$ .										

TABLE 4

FREQUENCIES OF Lp(a) ALLELES IN CAD PATIENTS

Lp(a) <sup>B</sup>	0.042
Lp(a) <sup>S1</sup>	0.045
Lp(a) <sup>S2</sup>	0.086
Lp(a) <sup>S3</sup>	0.302
Lp(a) <sup>S4</sup>	0.334
Lp(a) <sup>0</sup>	0.190

frequencies of double-band phenotypes were also consistently increased in proportion to the number of diseased vessels (Table 1). In order to rule out a possibility that the detection of double-band phenotypes in sera from the CAD patients is due to their higher Lp(a) concentrations, sera with Lp(a) levels below 20 mg/dl were applied to the gels in normal volume and double the usual volume. No difference was observed for Lp(a) phenotypes between them, except for greater staining of the bands.

The frequencies of these alleles were calculated and shown to be significantly different ( $\chi^2 = 13.3$ ,  $P < 0.05$ ) from those in the healthy population (Table 4), although those in phenotypes were not statistically significant ( $\chi^2 = 8.8$ ) when the null type was excluded. Therefore, the differences in serum Lp(a) concentrations between the healthy and CAD groups cannot be explained by allele differences (see the data in the preceding paper [5]). Furthermore, there were no consistent changes in the frequency distributions of Lp(a) phenotypes with respect to the number of diseased vessels.

TABLE 6

EFFECT OF Lp(a) PHENOTYPES ON SERUM Lp(a) AND CHOLESTEROL CONCENTRATIONS IN HEALTHY SUBJECTS AND CAD PATIENTS

Phenotype	Healthy subjects			CAD patients		
	No.	Lp(a) (mg/dl)	Cholesterol (mg/dl)	No.	Lp(a) (mg/dl)	Cholesterol (mg/dl)
Null	38	2.6 ± 2.2	172.8 ± 33.3	10	3.9 ± 4.4	205.0 ± 56.8
Single	312	12.8 ± 11.3	167.2 ± 35.1	257	17.0 ± 14.9	190.6 ± 46.2
Double	115	23.5 ± 16.7	162.7 ± 30.4	203	33.9 ± 29.3	189.5 ± 44.0

\* $P < 0.001$ .*Relationship between Lp(a) phenotypes and Lp(a) levels*

Serum Lp(a) concentrations in patients with various phenotypes are shown in Table 3. There is a strong inverse relationship between the apparent molecular weights of apo(a) isoforms and plasma Lp(a) concentrations, especially in the single-band phenotypes.

In order to confirm the relationships between the Lp(a) phenotypes and either serum cholesterol or Lp(a) levels, frequencies of hypercholesterolemia and hyper-Lp(a)emia in various Lp(a)

phenotypes were investigated (Table 5). With regard to cholesterol levels, there was no significant difference in the frequencies of hyper- and normo-cholesterolemia in various Lp(a) phenotypes and also in double-band phenotypes. On the contrary, the frequencies of hyper- and normo-Lp(a)emia were significantly different in various phenotypes ( $\chi^2 = 49.7$ ,  $P < 0.01$ ) as well as within single-band phenotypes ( $\chi^2 = 25.7$ ,  $P < 0.01$ ). Frequencies of hyper-Lp(a)emia tended to increase in phenotypes with lower molecular weight isoforms, compared with those of normo-

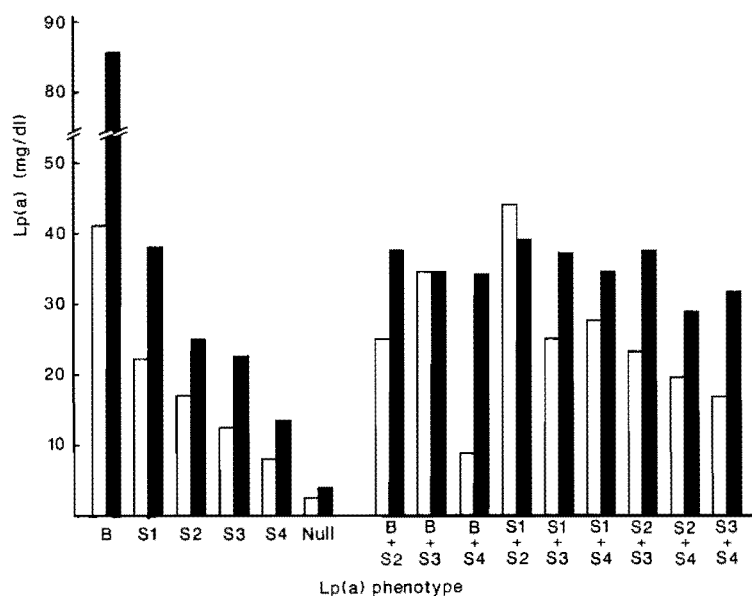


Fig. 1. Comparison of serum Lp(a) concentrations in different Lp(a) phenotypes between the healthy and CAD groups. □, healthy group; ■, CAD group.

Lp(a)emia in types with higher molecular weight isoforms. Furthermore, only one-third of patients with normo-Lp(a)emia showed the double-band phenotypes, whereas nearly 70% of hyper-Lp(a)emic patients had them. Nevertheless, the frequency of double-band phenotypes in normo-Lp(a)emic patients was considerably higher than that in normo-Lp(a)emic healthy subjects (113/340 = 33.2 vs. 86/422 = 20.4%).

The effects of Lp(a) phenotypes on serum Lp(a) and cholesterol concentrations in the healthy subjects and the CAD patients are shown in Table 6. There are significant differences in serum Lp(a) levels among null, single-band and double-band phenotypes in both groups. In contrast, no differences in cholesterol levels were observed among the different phenotypes.

Lp(a) mean levels in each phenotype were compared between the healthy subjects and the CAD patients and are shown in Fig. 1. Lp(a) levels were significantly higher in the CAD patients than those in the healthy subjects, except for the double-band phenotypes of B + S3 and S1 + S2.

All patients with single-band phenotypes were arbitrarily subdivided into those with Lp(a) levels below and above 15 mg/dl. As shown in Table 7, the frequency distributions of Lp(a) phenotypes were also significantly different between the subgroups with high and low Lp(a) concentrations in these patients. Distributions in the subgroups

with high Lp(a), except for the null type, were higher in the CAD patients than those in the healthy population.

## Discussion

A number of studies have demonstrated the association between the risk of coronary heart disease and elevated Lp(a) concentrations in plasma [1-3]. The present study provides data on elevated Lp(a) concentrations in serum as well as frequencies of Lp(a) phenotypes and alleles in Japanese patients with coronary heart disease.

Our results show that serum Lp(a) concentrations are inversely related to the apparent molecular weight of apo(a) in CAD patients and also in healthy subjects [5].

The frequency of double-band phenotypes was significantly higher in CAD patients than in healthy subjects and was proportional to the number of diseased vessels in the CAD patients. Although the possibility cannot be ruled out that the detection of double-band phenotypes in the sera of CAD patients is due to their higher Lp(a) levels, no difference in Lp(a) phenotypes was observed when SDS-PAGE was carried out with two-fold increased sample volumes. Furthermore, the frequency of double-band phenotypes was also higher in patients than in healthy subjects, even when only those with Lp(a) levels lower than 30 mg/dl were selected. There are more single-band phenotypes than would be expected from the number of different isoforms in the population. Kamboh et al. [8] recently reported, however, that their high-resolution SDS-agarose electrophoresis method identified a total of 23 different apo(a) isoforms and that only about 20% of the subjects tested revealed single-band phenotypes. These results suggest that there is a need for improving the technology for Lp(a) phenotyping, establishing the actual number of apo(a) isoforms and deciding the frequencies of single- and double-band phenotypes.

Subjects with the double-band phenotypes had significantly higher Lp(a) levels than those with the single-band phenotypes in both the healthy and CAD groups. These results are in agreement with those reported by Gaubatz et al. [9]. They also mentioned that there was a great over-

TABLE 7

### SINGLE-BAND Lp(a) PHENOTYPES IN CAD PATIENTS WITH LOW AND HIGH Lp(a) CONCENTRATIONS

Frequencies (% of phenotype) are in parenthesis.  $\chi^2 = 23.95$ ,  $P < 0.01$ .

Pheno- type	No.	Lp(a) $\leq$ 15 mg/dl	Lp(a) $>$ 15 mg/dl
F	1	0 (0)	1 (100.0)
B	1	0 (0)	1 (100.0)
S1	4	1 (25.0)	3 (75.0)
S2	13	5 (38.5)	8 (61.5)
S3	106	54 (50.9)	52 (49.1)
S4	132	93 (70.5)	39 (29.5)
Null	10	10 (100.0)	0 (0)
Total	267	163 (61.0)	104 (39.0)

representation in the population of the single-band compared with the double-band phenotypes, if the polymorphs were inherited by a simple Mendelian mechanism. The present results suggest that the double-band phenotypes have greater risks for CAD than the single-band ones.

Although Lp(a) levels were elevated 1.5- to 2-fold in the sera from patients with CAD, this elevation was not due to higher frequency of those Lp(a) phenotypes that are associated with high Lp(a) concentrations, but was due to an elevation of Lp(a) in each common Lp(a) phenotype. These results agree with those from patients with familial hypercholesterolemia [10], but not with those from CAD patients with familial hypercholesterolemia [4]. Unfortunately in the latter study, however, the number of patients used appeared to be too small to determine the phenotype frequency in patients with or without CAD.

The present results suggest that it is important to consider some post-translational or environmental modifications and other factors, e.g. other serum lipid and lipoprotein fractions, besides the genetic factor, as possible contributors to plasma Lp(a) levels. Boerwinkle et al. [11] estimated that 41.9% of the inter-individual variability of Lp(a) levels was attributable to difference in Lp(a) phenotypes. These results suggest that the cut-off value or therapeutic value of Lp(a) level should be decided separately for each Lp(a) phenotype in order to determine whether therapy is needed for hyper-Lp(a)emia and stress the importance of phenotypes in our understanding of the pathophysiology of this particle.

#### Acknowledgements

The authors wish to acknowledge Dr. Gunther M. Fless, Department of Medicine, University of Chicago, for his kind help in preparing the manuscript. The authors also wish to thank Mrs. Kayoko Fukui and Kiyomi Takahata for their skillful technical assistance. This work was supported by a Grant-in-Aid for Scientific Research

from the Ministry of Education, Science and Culture, Japan (No. 02454498).

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