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ATHERO 04884

Studies on apolipoprotein(a) phenotypes. Part 1. Phenotype frequencies in a healthy Japanese population

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(Received 29 January, 1992) (Revised, received 22 April, 1992) (Accepted 30 April, 1992)

Summary

The frequency distribution for serum lipoprotein(a) (Lp(a)) concentrations in healthy Japanese was highly skewed, with a mean \pm S.D. of 14.6 \pm 13.6 mg/dl and a median of 11.0 mg/dl. The present study provides the first evidence on the frequencies of Lp(a) phenotypes and alleles in healthy Japanese subjects. There was a strong inverse relationship between the apparent molecular weights of apo(a) isoforms and plasma Lp(a) concentrations, as reported previously. However, because of the considerable overlap between the Lp(a) concentrations of the different phenotypes, it was impossible to predict Lp(a) concentration from Lp(a) phenotypes, or vice versa. The present results suggest that the distribution of Lp(a) concentrations, mean and median values and Lp(a) phenotype and allele frequencies in healthy Japanese are not significantly different from the results for Europeans, whereas they are significantly different from other Asian populations, i.e. Chinese, Indians and Malaysians.

Key words: Japanese; Lp(a); Phenotype; Allele

Introduction

Lipoprotein(a) (Lp(a)) was first described by Berg [1] as a genetic variant of low density lipoprotein and is believed to be inherited as an autosomal dominant trait. A great deal of clinical interest was generated by quantitative studies that demonstrated a strong association between high Lp(a) concentrations and both cardiovascular and cerebrovascular diseases [2–4]. The lipid composition of Lp(a) is similar to that of low density lipoprotein, but its protein moiety contains, in addition to apo B-100, the Lp(a)-specific glycoprotein, apolipoprotein(a) (apo(a)), which is linked to apo B-100 by disulfide bridge(s) [5,6]. The Lp(a) lipoprotein exhibits a density heterogeneity within and between individuals, as well as a size heterogeneity of apo(a) [7,8]. Several recent reports by

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Utermann and his colleagues [8–11] have described the resolution of six different types of apo(a) by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) followed by immunoblotting. In addition, more recently the existence of 11, 19 or 23 different isoforms has been reported by Gaubatz et al. [12], Lackner et al. [13] and Kamboh et al. [14], respectively, but the relationships of these to the originally described genetic isoforms [8] are presently unclear.

The nucleotide sequence of cloned cDNA of apo(a) revealed a high homology to plasminogen [15]. Apo(a) contains a serine protease domain, one kringle-5-like domain and multiple copies resembling kringle-4 domains of plasminogen. The differences in size of apo(a) isoforms are due to allelic differences in the number of kringle-4-like domains [16,17]. The apo(a) size polymorphism is reportedly controlled genetically by at least seven alleles [8,11] at a locus closely linked to the plasminogen gene on chromosome 6 [18,19].

Although the relationships between Lp(a) phenotypes and plasma concentrations have been studied in Caucasian and other populations [8–12,20], no similar work has been carried out in a population of Japanese. The present study was designated to fill this gap with our investigation of Lp(a) concentrations and phenotype frequencies in healthy Japanese by using a sensitive immunoblotting technique on serum specimens.

Materials and Methods

Subjects and blood sampling

The subjects were 465 unrelated blood donors with no history of angina, myocardial infarction, or other atherosclerotic diseases and were from 16 to 64 years old with a mean age of 31 years (252 males, 33 ± 12 years, 213 females, 29 ± 13 years). Blood samples were collected in the morning by venipuncture 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, NaN₃ and a protease inhibitor (Aprotinin, Sigma Chemical Co., St. Louis, MO) and were either kept at 4°C for immediate assay or stored at -40°C for further analyses. Storage under these conditions did not affect Lp(a) analysis.

Preparation of Lp(a) antibody

Lp(a) antigen and antiserum were prepared as described previously [21]. Briefly, Lp(a) antigen was prepared using ultracentrifugation (1.05-1.12g/ml) of the precipitated fraction with dextran sulfate and calcium chloride from human Lp(a)rich pooled sera. Antibody against Lp(a) was produced in rabbits by immunization with the crude Lp(a) fraction. The anti-Lp(a) antibodies were purified by affinity chromatography with Sepharose 4B (Pharmacia Fine Chemicals, Uppsala, Sweden) coupled with pooled sera obtained from apparently Lp(a)-negative subjects and pure plasminogen.

Lp(a) determination

Lp(a) concentrations were determined by enzyme-linked immunosorbent assay (ELISA) as described previously [21], using rabbit anti-human apo(a) antibody and Lp(a) reference standard (Immuno AG, Vienna, Austria).

Electrophoretic procedures

Serum (10 μ l) was mixed with 40 μ l of the sample buffer, which was composed of 1.0 ml of 0.5 M 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% bromophenol blue and 4.0 ml of distilled water. A $10-\mu$ l aliquot of the mixture, containing $2 \mu l$ serum, was used as a sample for electrophoresis. SDS-PAGE was routinely performed using a 4.0% acrylamide slab gel and a discontinuous buffer. SDS mini gels (Tefco Corp., Tokyo, Japan) were used, with dimensions of $8 \times 8 \times 0.1$ cm and 10 5-mm wide wells per gel. The upper cathode buffer consisted of 0.04 M Tris (pH 8.64). adjusted with 0.04 M boric acid and 0.1% SDS; lower anode buffer was 0.43 M Tris (pH 9.18), with 0.03 M HCl. Gels were run at 20 mA per plate at 25°C for 90 min. The proteins in gels were electrophoretically transferred to a nitrocellulose membrane (0.45 μ m, Bio-Rad, Richmond, CA) in a pH 8.3 solution containing 25 mmol Tris, 192 mmol of glycine and 200 ml of methanol, per liter, by holding 100 volts constant for 1 h.

The apo(a) and apo B bands were visualized on the membranes by a double-antibody procedure, using rabbit anti-apo(a) or goat anti-apo B (Chemicon International, Inc., CA) antiserum as a first antibody and anti-rabbit IgG (GAR-Gold, Bio-Rad) or anti-goat IgG (EY Laboratories, Inc., CA) as a second antibody, which was labeled with colloidal gold ($E_{520} = 0.2$, 10 nm diameter). The gold-labeled bands were enhanced with a physical developer consisting of silver lactate and hydroquinone.

Lipid and lipoprotein analyses

Serum cholesterol and triglyceride were measured enzymatically using commercial kits (International Reagents Corp., Kobe, and Eiken Chemical Co., Ltd., Tokyo, respectively). High density lipoprotein (HDL)-cholesterol in serum was also determined using a commercial kit (Daiichi Pure Chemicals, Co., Ltd., Tokyo) with dextran sulfate-magnesium precipitation.

Statistical methods

Overall comparison of Lp(a) concentrations among phenotypes was done by the nonparametric Kruskal-Wallis test. Pairwise comparison of Lp(a) levels among phenotypes was performed by two-tailed Mann-Whitney test. Comparison of Lp(a) phenotype frequencies between subgroups with high (>15 mg/dl) and low (≤ 15 mg/dl) Lp(a) levels were done by χ^2 analysis. Lp(a) allele frequencies were calculated according to Wiener et al. [22].

Results

Distribution of serum Lp(a) concentrations

The frequency distribution for serum Lp(a) concentrations in the healthy Japanese was highly skewed (Fig. 1), as has been observed for white populations, with a mean \pm S.D. of 14.6 \pm 13.6 mg/dl and a median of 11.0 mg/dl. There was no significant difference in serum Lp(a) concentrations between males (13.8 \pm 13.0 mg/dl, median 10.3 mg/dl) and females (15.5 \pm 14.3, median 12.2 mg/dl).

Identification of apo(a) by immunoblotting

Serum samples were subjected to SDS-PAGE under reducing conditions and the Lp(a) proteins were demonstrated by immunoblotting using polyclonal rabbit anti-apo(a) antibody (Fig. 2). For visualization of weak apo(a) bands, the gold-



10 20 30 40 50 60 70 80 Lp(a) (mg/dl)

Fig. 1. Frequency distribution (at intervals of 5 mg/dl) of Lp(a) concentrations in serum samples obtained from 465 healthy Japanese.

20

10

Frequency (%)

labeled blots were further developed by silver staining. The method was sensitive enough to detect Lp(a) concentrations above 0.7 mg/dl, with 91.8% of the subjects tested exhibiting single or double band(s). According to their relative mobilities as compared with that of apo B-100, the Lp(a) patterns were classified into phenotype F (faster than apo B-100), B (identical to apo B-100), S1, S2, S3 and S4 (slower to different degrees than apo B-100), and into the respective double-band phenotypes. Lp(a) phenotype should be controlled by at least five alleles that code for the Lp(a)^B, $Lp(a)^{S1}$, $Lp(a)^{S2}$, $Lp(a)^{S3}$ and $Lp(a)^{S4}$ forms of apo(a). For cases with no detectable apo(a) band, a null allele, Lp(a)⁰ was postulated, but the frequency of this allele depends on the sensitivity of the immunoblotting assay.

Frequencies of Lp(a) phenotypes and putative alleles

The frequencies of Lp(a) phenotypes, determined in a population of healthy Japanese, are given in Table 1. The distribution of the estimated Lp(a) phenotype frequencies in this group did not vary significantly between males and females $(\chi^2 = 9.1)$. When we compared the observed



Fig. 2. Demonstration of different apo(a) phenotypes and apo B in individual sera with varying Lp(a) concentrations by immunoblotting analysis using anti-apo(a) and anti-apo B antibodies. Lane 1, F type; lane 2, B type; lane 3, S1 type; lane 4, S2 + S3 type; lane 5, S2 type; lane 6, S3 type; lane 7, S4 type; lane 8, apo B.

TABLE I

FREQUENCIES OF Lp(a) PHENOTYPES IN HEALTHY JAPANESE

$\chi^2 = 14.4$, N.S.

Phenotype	Number		Frequency (%)	
	Observed	Expected	Observed	Expected
B	4	3.7	0.9	0.8
S1	14	12.8	3.0	2.8
S2	62	64.6	13.0	13.9
\$3	133	114.3	28.6	24.6
S4	99	78.8	21.3	16.9
Null	38	57.9	8.2	12.5
B + S1	0	0.4	0	0.1
B + S2	2	1.6	0.4	0.3
B + S3	2	2.5	0.4	0.5
B + S4	2	1.9	0.4	0.4
S1 + S2	10	5.2	2.2	1.1
S1 + S3	7	8.4	2.2	1.1
S1 + S4	2	6.2	0.4	1.3
S2 + S3	35	36.6	7.5	7.9
S2 + S4	26	27.0	5.6	5.8
S3 + S4	28	43.1	6.0	9.3
F + S3	1		0.2	
Total	465	465	99.6	100.0

TABLE 2

FREQUENCIES OF Lp(a) ALLELES

0.011	
0.036	
0.158	
0.253	
0.187	
0.356	
	0.011 0.036 0.158 0.253 0.187 0.356

phenotype frequencies with those expected from the genetic hypothesis and assuming Hardy-Weinberg equilibrium, there was no significant deviation of observed from expected frequencies.

The frequencies of these alleles are illustrated in Table 2.

Relationship between Lp(a) phenotypes and Lp(a) levels

Serum Lp(a) concentrations of subjects with various single- or double-band 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, as reported by other researchers [9,23]. The mean Lp(a) level in the B type is almost two times higher than in the S1 type and five times higher than in the S4 type.

Pairwise comparison by a two-tailed Mann-Whitney test showed that the four frequent phenotypes may be split up into two pairs, S1 and S2, and S3 and S4, with no significant difference between Lp(a) concentrations within a pair but significant (P < 0.01) differences between the Lp(a) levels of phenotypes in different pairs, i.e. S1 vs. S3, S1 vs. S4, S2 vs. S3 and S2 vs. S4. Hence phenotypes S1 and S2 are associated with high Lp(a) concentrations and phenotypes S3 and S4 with low Lp(a) concentrations in serum. Phenotypes F and B were not included in the pairwise comparison because of the relatively small number of individuals, but seem to be associated with the highest Lp(a) levels.

In order to confirm these results, all subjects with single-band phenotypes and with known Lp(a) concentrations were arbitrarily subdivided into two groups, those with Lp(a) levels below and above 15 mg/dl. As shown in Table 4, the frequency distributions of Lp(a) phenotypes were

TABLE 3

SERUM LD(a) CUNCENTRALIONS (MP/O) IN DIFFERENT LD(a) FHENULTPES IN HEALTHY JAPANE	SERUM Lp(a) CONCENTRATIONS	(mg/dl) IN DIFFERENT I	Lp(a) PHENOTYPES IN HEALTHY	JAPANESE
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Phenotype	No.	Mean	S.D.	Median
В —	4	42.3	6.7	45.1
SI	14	22.0	18.0	18.5
\$2	62	17.0	14.6	13.8
53	133	12.5	8.4	10.9
54	99	8.1	6.1	6.8
Null	38	2.6	2.2	1.7
3 + S1	0			
3 + S2	2	24.9	15.4	
3 + S3	2	34.7	16.7	
3 + S4	2	8.7	8.9	
S1 + S2	10	43.9	28.2	39.5
$s_1 + s_3$	7	25.1	16.1	22.4
S1 + S4	2	27.8	24.0	
S2 + S3	35	23.3	12.7	24.9
S2 + S4	26	19.7	12.0	16.4
83 + 84	28	16.9	11.8	14.4
F + S3	1	20.7		
Гotal	465	14.6	13.6	11.0

TABLE 4

SINGLE-BAND Lp(a) PHENOTYPES IN SUBJECTS WITH LOW AND HIGH Lp(a) CONCENTRATIONS

Phenotype	No.	$Lp(a) \le 15 \text{ mg/dl}$	Lp(a) > 15 mg/dl
B	4	0 (0)	4 (100.0)
S1	14	5 (35.7)	9 (64.3)
S2	62	34 (54.8)	28 (45.2)
\$3	133	92 (69.2)	41 (30.8)
S 4	99	87 (87.9)	12 (12.1)
Null	38	38 (100.0)	0 (0)
Total	350	256 (73.1)	94 (26.9)

Frequencies (% of phenotype) are in parenthesis. $\chi^2 = 57.4$, P < 0.01.

significantly different between the subgroups with high and low Lp(a) concentrations (P < 0.01). Phenotypes B, S1 and S2 are clearly associated with elevated Lp(a) concentrations, whereas phenotypes S3 and S4 are associated with lower Lp(a).

Discussion

The frequency distribution of serum Lp(a) concentrations in healthy Japanese was highly skewed to the low concentration, as observed in previous studies. The distributions, together with mean and median values, were in good agreement with previous studies from European white populations, whereas they were significantly different from those for Asian populations, i.e. Chinese, Indians and Malaysians [20].

The first systematic study on Lp(a) heterogeneity was carried out by Fless et al. [7], who found that Lp(a) exhibited both inter- and intraindividual density heterogeneity and that this heterogeneity was associated with differences in protein and lipid compositions. They also observed that apo(a) exhibited size heterogeneity owing to species that have different electrophoretical mobility.

Utermann and his colleagues later established the techniques and concepts on apo(a) polymorphisms in their several studies [8-11]. They described specifically the resolution of six apo(a) polymorphs with no individual possessing more than two molecular weight species. According to them, apo(a) is under the control of a single gene locus that specified for several alleles, each coding for apo(a) isoforms differing in size. Other investigators clearly demonstrated that the differences in size of apo(a) isoforms are due to allelic differences in the number of its tandemly repeated plasminogen kringle-4-like domains [16,17].

The present study provides the first evidence of the apo(a) isoforms and their allele frequencies in a Japanese population. In early reports [8,9], Utermann's group was unable to detect apo(a) polymorphs in 44-49% of the subjects tested. In their more recent reports [10,11], however, the frequency of the null type was decreased due to a higher sensitivity of the improved immunoblotting method. The present results of apo(a) phenotype and allele frequencies in healthy Japanese were not significantly different from Utermann's recent results for Tyrolean, Hungarian and Icelandic populations, but significantly different from those in Chinese, Indian, Malaysian and Sudanese populations [20]. In particular, serum Lp(a) concentration $(7.2 \pm 13.1 \text{ mg/dl})$ and the estimated frequencies of apo(a) alleles in S3 and S4 types (0.102 and 0.502, respectively) in a Chinese population [20] were significantly different from those in our study. During the preparation of our manuscript, Helmhold et al. [24] published their findings on the ethnic variations in the contribution of apo(a) phenotype to plasma Lp(a) concentrations. The mean Lp(a) level (22.9 mg/dl) of Chinese from Wuhan in central China in their study was notably higher than that of Singapore Chinese (7.2 mg/dl) reported by Sandholzer et al. [20] and the skewed distributions of serum Lp(a)concentrations also differed considerably between them. Our results, however, on serum Lp(a) concentrations and their distribution were significantly different from those of Chinese reported not only by Sandholzer et al. [20], but by Helmhold et al. [24]. As for the apo(a) phenotypes, the present results are unable to be compared directly to those of Helmhold et al., because they observed higher number of apo(a) polymorphs using a gel system with a lower acrylamide concentration. These results are ethnologically very interesting, because it has been accepted that the ancient Japanese came from China. These results also suggest the possibility that natural selection has occurred since that time due to changes in environmental conditions.

The present results also indicate that serum Lp(a) concentrations are inversely related to the apparent molecular weight of apo(a). Phenotypes B, S1 and S2 are associated with high and phenotypes S3 and S4 with low Lp(a) levels. There is, however, considerable overlap between the Lp(a) concentrations of the different phenotypes and it is impossible to predict Lp(a) concentrations from Lp(a) phenotypes, or vice versa.

Although we used the apo(a) isoforms designated by Utermann et al. in the present study, many investigators [12-14] recently reported developments of extremely sensitive methods with high resolving powers for determining the size of apo(a) polymorphs in plasma samples. Furthermore, they could detect apo(a) bands in more than 99% of samples tested by standardizing the amount of Lp(a) in the sample load for SDS-PAGE rather than using a constant amount of serum. We are now trying to carry out our resolution method by standardizing the amount of Lp(a) protein in the sample load. However, the relationships of higher numbers of apo(a) polymorphs to the originally described isoforms or to each other are unclear at present. Since the precise number of different alleles at the apo(a) locus is not yet known, there is a need for improving the technology for apo(a) phenotyping, for establishing the actual number of the apo(a) isoforms and for defining their physicochemical characteristics.

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 for her 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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