APO(a) ISOFORMS AND LP(a) CONCENTRATION IN PREDICTING RISK FOR CORONARY ARTERY DISEASE: A STUDY IN MEN < 55 YEARS OF AGE

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Abstract - Lipoprotein (a) [Lp(a)] is formed by assembly of LDL-particles and the carbohydrate rich protein, apolipoprotein(a) [apo(a)]. Elevated plasma Lp(a) levels are an independent predictor of the development of premature coronary artery disease (CAD), but it is not clear whether the apo(a) isoform plays an additional and independent role or not. To investigate the possible effect of apo(a) isoform on premature CAD (in patients <55 years of age), we have analyzed apo(a) isoforms, Lp(a) level and their relation with many recognized CAD risk factors, in 60 male patients with angiographically defined CAD and in 60 male control with no angiographic evidence of CAD. The results show elevated Lp(a) concentration (29.4±16.1, vs 16.5±9.9 p<0.01) and frequency of S2 isoform (31.7%, vs 6.7% p<0.01) and B isoform (10% vs 1.7% p<0.01) in patients with premature CAD. Patients with S2 isoform exhibited significantly higher plasma Lp(a) concentration than control subject with the same isoform (39.8±15.9 vs 20.5±6.9, p<0.05), but patient with B isoform exhibited no significant Lp(a) concentration as compared to the controls (49.5±9.46 vs 45). In addition, all patients had a low frequency of S4 and null isoforms. The distribution of apo(a) Isoforms was significantly shifted towards small isoform size (band S2) in the CAD as compared to the controls. This study provides evidence that CAD patient <55 years of age have a different pattern of apo(a) isoforms than controls, and therefore apo(a) isoform may play an important role in predicting premature CAD.
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Key Words: Lipoprotein(a) [Lp(a)], apolipoprotein(a) [apo(a)], coronary artery disease (CAD), isoform, angiography

INTRODUCTION

Lipoprotein(a) [Lp(a)] is a complex macromolecule in human plasma that consists of low density lipoprotein (LDL) and a large glycoprotein designated apolipoprotein(a) [apo(a)] (1). Apo(a) is linked by a disulfide bond to the apoB-100 component of LDL (2). Lp(a) concentration is increasingly recognized to be associated quantitatively with coronary artery disease (CAD). It is also predicative of cardiac events and is therefore a true risk factor for CAD (3,4). The mechanism by which Lp(a) concentration and cardiac events are related is not known (4). Fless et al and Utermann et at found that the apo(a) glycoprotein varied in size among individuals (5). The gene for apo(a) has been localized on chromosome 6q 2.6 - 2.7. It codes for a protein with a high degree of homology to plasminogen (6). Different apo(a) isoforms originally designated F, B, S1, S2, S3, S4, > 54 which range in size from about 280 KD to > 850 KD were described (1).

To investigate the possible role of apo(a) isoform on premature CAD, we examined plasma Lp(a) levels, apo(a) isoforms and their relation with many recognized CAD risk factors in a group of male pateints with angiographically defined CAD and in a male control subjects so that both groups were matched for age and body mass index (BMI).

MATERIALS AND METHODS

A control group of 60 men were obtained from Dr Shariati hospital; they were selected from patients referred to the cardiology division for suspected CAD, they had not any angiography for CAD. The patients group consisted of 60 men admitted to the cardiology division of the Dr Shariati hospital because of premature CAD, and all of these subjects had angiographically proven CAD. Diagnostic selective coronary angiography was performed in all patients to evaluate the extent of coronary disease. According to the Gensini scoring system and Peardon procedure and the basis of coronary atherosclerosis score all patients were choiced as positive CAD (CAD+) (7,8). All subjects were questioned for the family history of premature CAD, presence of matabolic disorders, diabetis mellitus, cigarette smoking, alcohol intake and drug use. In all subjects, weight and height were measured at the time of admission and BMI were

calculated (9). Systemic hypertension was defined either as systolic blood pressure $\geq$ 160 mmHg and/or diastolic blood pressure $\geq$ 95 mmHg in the presence of antihypertensive therapy. Positive family history for CAD was defined as having one first-degree relative or one sibling who had had an acute myocardial infarction before age 55 years. Cigarette smokea was defined as the one who smokes an average $\geq$ 5 cigarettes per day, and finally all individuals had Iranian origins.

Total cholesterol, triglycerides and high density lipoprotein (HDL-C) in serum were measured by enzymatic methods with commerical kits. The concentration of low density lipoprotein cholesterol (LDL-C) was calculated with the formula of Friedwalet al (10). Lp(a) levels were determined by nephelometric procedure (11).

The apo(a) size isoforms were determined by a high resolution SDS-agarose electrophoresis, followed by immunoblotting which was based on the description made by Utermann and Kamboh et al (11-14). Reduction of the specimens was performed by mixing 10 μl of serum with 85 μl Lp(a) specimen, buffer [containing 4 ml SDS (50 g/l in water), 800 μl of 750 ml/l glycerol solution and 200 μl of 10 g/l bromophenol blue reagent] and 5μl mercaptoethanol, and leaving at room temperature for 20-30 minutes to allow for reduction of the serums. The standard was reconstituted and then treated in the same way as serum samples.

In the second step, isoforms were separated according to their molecular weights by SDS-polyacrylamide/ agarose gel electrophoresis. For the preparation of separating gel we used 25 ml 0.4% SDS with tris/HCL (pH:8.8, C:1.5 mol/l) and 750mg agarose plus 62.5 ml aqua bidest. After heating this mixture in a boiling water bath for approx 15 minutes until the agarose completely melted, 12.5 ml monomeric stock solution [acrylamide 30%, Bis [N, N' methylenebis acrylamide 0.8%] was added and after cooling the solution to 60° C , 150 μl TEMED and 2.5 ml of a 10% ammonium persulfate solution was added and immediately injected between 2 prewarmed glass plates. After 30 minutes, the collection gel containing 0.5 ml monomeric stock solution and 1.25 ml collection buffer (SDS 0.4% Tris/HCL (pH=6.8, 0.5 mol/l) plus 3.25 ml aqua bidest and 12 1 μl TEMED and finally 150 μl ammonium persulfate (10%) was injected between two glass plates and overlaid the separating gel. After polymerization was completed, 50 μl of the pretreated specimen was placed into the gel by means of a Hamilton syringe. Electrophoresis was carried out at constant current of 25 mA for approximately 5 hours with electrophoresis buffer (25 mmol/l tris, 0.2 mol/l glycine, 0.1% SDS, pH 8.3-8.6).

In order to transfer the separated proteins from the gel to the nitrocellulose membrane, we used a flat vessel, transfer buffer (25 mol/1 tris, 0.2 mol/l glycine, 20% methanol) and prepared transfer sandwich. Transfer was carried out at constant voltage of 50 volts to approximately 15 hours. In next step, after blocking the remaining protein-binding sites for 45 minutes with 3% bovine albumin solution, the memberane was incubated using a 1:500 polyclonal sheep antiserum Lp(a) solution (Immuno-AG, Vienna, Austria) as the first antibody and a 1:500 alkaline phosphatase labeled rabbit antisheep IgG solution (Immuno AG, Vienna, Austria) as the second antibody. After extensive washing the membrane was visualized using a substrate of the enzyme (13-15).

RESULTS

The clinical characteristics of two subject groups studied are shown in Table 1.

Patient with CAD were approximately older and had BMI and systolic blood pressure higher than the control group.

Table 1. Clinical characteristics and fasting plasma lipid, Lp(a), lipoprotein levels in pateints <55 years of age with CAD and in control subjects

	control (n=60)	patient (n=60)
Genetic - geographic origin	Iranian	Iranian
Age(years)	44±9	47±17
Body mass index (kg/m^2)	22.8±2.8	26±2.9
Smoker	20(33.3)	31 (51.7)
Systemic hypertension	12(20)	28(46.7)
Positive family history for CAD <55 years of age	5 (8.3)	23(38.3)
Total cholesterol	197±31	197±19
LDL-cholesterol	121±22	129±21
HDL-cholesterol	45.5±7.5	38±6
Triglycerides	141±41.5	185±14
Lp(a)	16.5±9.9	29.4±16.1

The number in parentheses indicates the percent value. Lipids are expressed in mg/dl. Values are mean ± SD.

The frequency of systemic hypertension (p<0.001), smokers (p<0.01) and positive family history of premature CAD (p<0.001) were significantly higher in the patients than in the control group. Patients with CAD had higher concentration of Lp(a) (p <0.01) and the concentration of total cholesterol, LDL cholesterol and triglyceride was not significantly different between both groups. The frequency of distribution of plasma Lp(a) levels in the patient group was shifted to the right compared with the control group. It was interesting to note that 16.67% of patients, but none of the control subjects, had a concentration of Lp(a) >55 mg/dl.

Table 2. Frequency of apolipoprotein(a) isoforms and lipoprotein(a) concentration in patients <55 years of age with CAD and in control subjects

| | Control Subjects | | | Patients | | |
| | Frequency | | Lp(a) | Frequency | | Lp(a) |
isoform	No.	(%)	(mg/dl)±SD	No.	(%)	(mg/dl)±SD
B	1	1.7	45	6	10	49.5±9.46
S1	2	3.3	22.5±1.5	1	1.7	25
S2	4	6.7	20.5±6.9	19	31.7	39.8±15.9
S3	13	21.7	22±7	12	20	20.9±3.7
S4	23	38.3	17.2±4	9	15	15.78±6.57
Null	9	15	2.8±2.2	2	3.3	4.5±2.5
SIS2	2	3.3	22.5±4.5	2	3.3	25±4
SIS3	-	-	1	1.7	47	
S2S3	1	1.7	48	4	6.6	37±17.9
S2S4	-	-	-	1	1.7	29
S3S4	5	8.3	12.4±5.31	3	5	17±6.5

Table 3. Plasma lipid, lipoprotein, and apolipoprotein levels in patients <55 years of age with CAD and in control subjects according to apolipoprotein(a) isoforms

| | Control Subjects | | Patients | |
	Low Molecular Weight	High Molecular Weight	Low Molecular Weight	High Molecular Weight
Number of patients	9	50	28	26
Total cholesterol	189±28	205±35	195±26	199±28
LDL cholesterol	116±16	127±28	128±19	130±23
HDL cholesterol	41±8	46±7	39±4	37±8
Triglycerides	145±36	135±47	167±20	159±34
Lp(a)	24.1±9	14.72±8	40.32±15	17.42±7

Values are mean ±SD

Lipids are expressed in mg/dl

Apo(a) isoform in the patients and control subjects was determined. Results have been shown in table 2. The percentage of S2 isoforms was sigificantly higher in the patient group than in the control group (31.7% vs 6.7% p<0.01), therefore the S2 isoform occured 4.7 fold as often in patient group than in the control group and B isoform was more in patients compared to the control subjects (10% vs 1.7% p<0.01), therefore the B isoforms occured 6 fold as often in patient group than in the control group.

Table 2 also shows mean (±SD) levels of Lp(a) associated with the various isoforms in both patients and control subjects. Patients with S2 isoform had significantly higher plasma Lp(a) level than control subjects carrying the same isoform (p<0.05).

In table 3 the lipid, lipoprotein and apolipoprotein levels according to apo(a) molecular weight: high (S3, S4 and null isoforms) and low molecular weight (B, S1 and S2 isoforms) are summarized. The HDL-C level was significantly lower (p<0.005) in patients with high molecular weight isoforms than in the corresponding group of control subjects.

Finally patients with low molecular weight isoforms had significantly higher concentrations of Lp(a) than control subjects with the same isoforms (p<0.05).

DISCUSSION

Similar to Bowden et al studies, our study has also shown that circulating Lp(a) levels and apo(a) isoform play an additional role in the development of CAD. Lp(a) levels are significantly increased in patient group as compared to control subject and besides, patients had a pattern of apo(a) isoforms that was significantly different from control subject. In this study, a sensitive method was used to separate and classify apo(a) isoforms into 6 categories of isoform mobility as the original 6 Uterman isoform classes (13, 17, 18). This study showed that a high frequency of S2 and B isoforms of apo(a), and conversely, a low frequency of S4 isoform were observed in patients. A major finding in this study is the high frequency of S2 isoform that is strictly related to the reduction of S4 isoforms in patients. This finding could explain the higher Lp(a) levels of the patients. However patients with S2 isoform exhibited significantly higher plasma Lp(a)

concentration than control subjects carrying the same apo(a) isoform (19). Our study confirmed the Kark et al studies that low molecular weight isoform (particulary S2) have important role in the etiology of atherosclerotic disease (20). But our finding were slightly different to the results that low molecular weight isoforms were independent of plasma Lp(a) levels in Israelis populations (20). An association of low molecular weight isoform with symptomatic peripheral atherosclerosis has also been demonstrated, and also our results conform with study results in the Chinese and Indian populations showing that Lp(a) concentration was elevated in patients with S2 isoform compared to controls (22).

The explanation for the significant variation in Lp(a) levels between patients and control subjects with the same S2 isoform is not simple. Only a few studies on in vivo metabolism of Lp(a) in human have been reported, and all of them conclude that the variation in Lp(a) levels is caused primarily by difference in Lp(a) production rate, but is not due to differences in Lp(a) catabolism (23, 24). Our study results have shown that patient with B isoform exhibit no significant Lp(a) concentration as compared to the control but conversely a high frequence of B isoform are observed in patients compared to the control groups. Rader et al clearly showed that in healthy control subjects, the fractional catabolic rate of S4 Lp(a) was absolutely unrelated to plasma Lp(a) levels, whereas a certain inverse correlation between 2 variables was demonstrated for S2 isoform (22, 23). Therefore, the higher Lp(a) levels in patients with S2 isoform could also be explained by a decreased fractional catabolic rate of Lp(a) particles compared to the control subjects carrying the same isoform (24,25). Our results show that the frequency of S2S3 double-band isoforms was higher in patients than in control subjects, and the frequency of S3S4 double bond isoforms was lower in patients than in control. This result would confirm data from a previous study on the importance of S2S3 double - bond isoforms as a risk factor for CAD (26).

Finally the results of our study demonstrate the risk of high Lp(a) levels and association of this phenomenon with high frequency of low molecular weight isoform, and provides a confirmed evidence that predicates of premature CAD in patient with <55 years of age.

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