LP(a) PHENOTYPES AND LEVELS IN ANGIOGRAPHICALLY PROVEN CORONARY HEART DISEASE PATIENTS AND CONTROLS.

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ABSTRACT

Lipoprotein Lp(a) excess has been identified as a powerful predictor of premature atherosclerotic vascular diseases. To evaluate this in a North-Indian population, 130 CAD patients and 130 controls were analyzed. The size of the apo(a) phenotypic isoforms was inversely proportional to Lp(a) concentrations. The mean concentration of Lp(a) in the CAD patients was 42±34 mg/dl whereas in the normal subjects it was much lower, 27±27 mg/dl. 157 subjects out of the total 260 subjects showed plasma levels of > 20mg/dl. The frequency of high Lp(a) levels was much higher in patients(73%) than controls (43%). These data suggest (1) that there is heterogeneity of the Lp(a) polymorphism, (2) Higher Lp(a) levels were found in patients than in the controls, (3) Patients showed 1.5 fold increase in Lp(a) levels as compared to the controls. We conclude that low molecular weight apo(a) isoforms are significantly associated with increased risk of CAD in the North-Indian population.

KEYWORDS: Lipoprotein(a); Apolipoprotein(a); Cholesterol; Coronary artery disease.

INTRODUCTION

Lipoprotein(a) [Lp(a)] is a strong and independent risk factor for atherosclerotic disease (1). Plasma Lp(a) concentrations vary widely in humans from <1mg/dl to > 300 mg/dl, whereas risk-associated levels are in the range above 30 mg/dl. Lp(a) levels are genetically determined (2) and are not influenced by diet or drug intervention (3). High Lp(a) levels in plasma are associated with an increased risk of premature coronary heart disease. Lp(a) is a cholesteryl-ester rich lipoprotein of unknown function consisting of an LDL-like core to which apolipoprotein (a) [apo(a)] is disulfide linked. Apo(a), a glycoprotein, exhibits a characteristic structure consisting of numerous kringle-IV(K-IV) repeats, one kringle-V and a protease domain all of which possess high homology to corresponding structures in plasminogen (3). Apo(a) exists in multiple

genetically determined isoforms with molecular weights ranging from approximately 350 to 1000 kDa. Presently, more than 30 different apo(a) isoforms are known, which differ from each other by one additional 'repetitive K-IV'. One repetitive K-IV has a theoretical molecular weight including sugars of 16kDa. The number of K-IV repeats is inversely correlated with the plasma Lp(a) concentration(4,5).

There are several reports suggesting a possible causal link of Lp(a) with the atherosclerotic process. It has been observed that Lp(a) accumulates in atherosclerosis plaques (6), stimulates smooth muscle cell proliferation(7), avidly binds to arterial proteoglycans (8) and fibronectins(9) and promotes cholesterol accumulation in cells (6). Because of its high structural homology with plasminogen, apo(a) was suggested to play a role in inhibiting fibrinolysis at several steps(10).

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Several studies have shown that increased plasma Lp(a) levels are strongly associated with coronary heart disease (11,12). Both the mean

Lp(a) concentration and the impact of apo(a) size polymorphism on Lp(a) concentration are heterogeneous across populations(13). The relation of apo(a) isoforms to Lp(a) concentrations has been extensively studied in different ethnic groups. It has been reported that the size polymorphism affects Lp(a) levels in all populations but to a different extent (4,13).

So far no study has been conducted to analyze the Lp(a) isoforms and levels in the CAD patients & normal subjects of the North-Indian population. Increased plasma Lp(a) level was reported not to be linked to cardiovascular diseases in all ethnic groups; in the African-Americans Lp(a) was not an independent risk factor for coronary artery disease (CAD)(14).

Analysis of the apo(a)/Lp(a) system in the CAD patients & controls of the North-Indian population may provide important insights into the differences in Lp(a) concentrations and its role in CAD in this population. Knowledge of the factors controlling Lp(a) concentration and genetic-epidemiological data may be of medical importance. As a first step towards these goals, we have performed a study of Lp(a) isoforms, Lp(a) levels & lipid levels and their correlation in 130 North-Indian CAD patients and 130 age and sex matched control subjects. Our study demonstrates large differences in apo(a) isoforms and that the lower isoforms of apo(a) are significantly associated with increased risk of CAD. High Lp(a) levels are associated with CAD patients than the controls.

MATERIALS AND METHODS

Blood was collected into Na₂EDTA-containing tubes from a total of 130 angiographically proven CAD patients (average age 50.7 years) and 130 normal, healthy control subjects. The controls were age and sex matched with the patient group. Samples from CAD patients were collected from the Department of Cardiology of CN Centre, All India Institute of Medical Sciences.

Plasma was separated from blood by low speed centrifugation (800 rpm for 20 minutes) to determine Lp(a) plasma concentration and apo(a) protein phenotypes. One aliquot of the plasma was used freshly to determine lipid and lipoprotein

levels. Another was stored frozen at - 70°C. Complete lipid profile could be obtained of only 41 CAD patients and 48 control subjects due to inadequate sample volume.

Lp(a) plasma concentration

This was determined using an enzyme-linked immunosorbent Sandwich assay (ELISA) exactly as described in Kronenberg et al (15). A mouse monoclonal anti-human apo(a) antibody MAB 1A2 (Boehringer Mannheim) was used to detect Lp(a) concentration.

Apo (a) protein phenotyping

Immunoblotting was performed using a modified version of the SDS-agarose system described by Kamboh et al (16). Between 2 and 10µl plasma, according to the Lp(a) plasma concentration, were mixed with sample buffer to give a final 100µl volume and boiled at 100°C for 10 minutes. The sample buffer consisted of 3.85% SDS, 7.7% β mercapto ethanol, 0.077% bromophenol blue and 0.77% glycerol. 2µl of sample was loaded onto a 1.5% agarose gel (ultrapure agarose, Bio Rad, USA), of dimension 20x20 x0.4 cm. The electrode buffer used was 90mM Tris HCl, 90mM boric acid, 2mM EDTA and 0.1% SDS. Electrophoresis was performed at 25W (constant) for 7 hours at room temperature. The proteins were transferred from the gel to nitrocellulose filters (Whatmann) using a transblot apparatus (LKB, Pharmacia). The filters were blocked with 1% BSA, 10mM Tris HCl, 85 mM NaCl and 0.2% triton X-100 for 1 hour at room temperature. The anti-apo(a) MAB 1A2 antibody was used to detect the apo(a) isoforms. Horse radish peroxidase (HRP)-conjugated goat anti-mouse antibody was used as the secondary antibody followed by the ECL detection system (Amersham). Extensive washings were performed between the incubation steps.

Lipids and lipoproteins were estimated by using fully enzymatic kits (Boehringer, Randox)

Student's 't' test for continuous data and chi square test for categorical data were applied. Pearson's correlation coefficient was worked out

to study the associations between the lipid and lipoprotein levels.

To obtain a sufficient occupation of each cell, the large number of phenotypes were reduced by binning of five adjacent kringle IV allele isoforms into one group for graphic presentations.

RESULTS

One hundred and thirty angiographically proven CAD patients were included in the study. One hundred and thirty individuals with no heart disease were taken as controls. The patients and controls were comparable with respect to age, sex and socio-economic status. The lipid and lipoprotein levels in 41 angiographically assessed CAD patients and 48 controls is shown in Table-1

Table 1 Lipid and lipoprotein levels in angiographically assessed CAD patients and controls

Parameters	Patients (n=41)	Controls (n=48)	p-Value
TC	190 ± 38	201 ± 50	NS
LDL-C	99 ± 40	116 ± 35	NS
HDL-C	42 ± 5	45 ± 8	NS
LDL/HDL Ratio	2.3 ± 0.7	2.5 ± 0.8	NS
VLDL-C	39 ± 13	41 ± 12	NS
TG	156 ± 74	134 ± 63	NS

All values are Mean ± SD in mg/dl

Table 2. Quartile distribution of serum Lp(a) levels (mg/dl) in CAD patients and controls

Group	Mean±SD	Quartile 1-25	50	Distribution 75-100	Skew
Patients (n=130)	42 ± 24	0.79-16.0	31.1	62.9-160	1.21
Controls (n=130)	27±27	0.69-7.8	17.7	36.6-147	2.13

Plasma Lp(a) concentrations

In both CAD patients and controls, Lp(a) concentrations were distributed over a broad range from <1mg/dl to 160 mg/dl. 157 out of 260 subjects showed plasma levels of >20mg/dl. The distribution is highly skewed towards lower concentrations in both patient and control groups (Fig.1). The mean and median Lp(a) concentrations are significantly higher in patients (mean=42±34 mg/dl) than in the controls (mean=27±27.mg/dl. The frequency of high Lp(a) levels was relatively much higher in patients (73%) than controls (43%) (Fig.1). Patients showed more than 1.5 fold increase in Lp(a) levels as compared to the controls (Table I). Lp(a) concentrations were not related to age or sex in either of the groups.

Apo(a) phenotype

Apo(a) isoform phenotypes were determined from whole plasma of all 260 subjects by SDS-agarose gel electrophoresis and immunoblotting using the monoclonal apo(a) antibody 1A2 (Boehringer Mannheim, Germany). 160 phenotypes were observed in the total population of 260 indicating hypervariability of apo(a) protein. 86% of these phenotypes had higher Lp(a) levels. The frequency of the apo(a) isoform with lower number of KIV repeats was higher in the CAD patients than in the control subjects.

Associations of apo(a) isoforms with plasma Lp(a) levels and lipids

A size polymorphism (KIV VNTR) and largely unknown sequence variation in the apolipoprotein (a) [apo(a)] gene together determine most of the extreme variation in apo(a) glycoprotein expression and Lp(a) plasma concentration in some populations (17).

Apo(a) phenotyping showed hypervariable expression of apo(a) protein. The smallest and the largest isoform having 12&48 KIV repeats, respectively, were observed in the study groups. Among patients, the most frequent isoform had 23 KIV repeats whereas in controls it was 32 (Fig.2). An inverse correlation was observed between apo(a) isoform size (KIV repeats) and Lp(a) concentration in the above study (Fig.3)

Lipid profile

Lp(a) levels exhibited significant negative correlation with LDL, VLDL and LDL/HDL ratio among the patients. On the other hand, in controls, it was positively correlated. No statistical differences were observed in the levels of total cholesterol (TC), HDL-cholesterol (HDL-C), LDL-cholesterol(LDL-C), VLDL-cholesterol(VLDLC),

LDL/HDL ratio and triglycerides(TG) (Table-1)

DISCUSSION

We have analyzed the frequencies of Lp(a) concentrations, apo(a) isoform size and expression in the plasma of angiographically proven CAD patients and normal subjects of the North Indian

Frequency Distribution of Lp(a) in Controls

Fig. 1 Histograms of the distribution of Lipoprotein(a) concentrations in CAD patients and controls.

Frequency distribution of Phenotypic Isoform of Apo (a) KIV repeats in CAD patients and Controls

Fig. 2 Histogram showing the distribution of phenotypic isoform of KIV repeats in CAD patients and controls.

population. The lipid and lipoprotein levels were also analyzed in these two groups.

The results of our study indicate that, in a random sample of angiographically proven CAD patients and age & sex matched controls, low molecular weight apo(a) phenotypes predict CAD. This finding confirms retrospective studies (11,12).

The present study is the first to investigate the predictive role for the apo(a) polymorphism in CAD in the North Indian population. The association between high plasma concentrations of Lp(a) and CAD is in agreement with findings in some nested case-control studies (12,16) and two recent prospective studies (18-21), whereas other studies with similar design did not support such an

association (14).

In an extensive study carried out in five geographical areas of Europe (20), significantly different Lp(a) levels in cases and controls were seen in Northern Europe and Great Britain, but not in Middle Europe, Southern Europe or Finland. Thus it is possible that importance of Lp(a) as a

risk factor for cardiovascular disease not only differs between races but also in populations within the same race.

LP(a) levels may not be solely under genetic control in all groups (eg. patients with atherosclerotic vascular disease) as has been demonstrated for healthy population samples. The associations

Fig. 3 Relationship between the sum of KIV repeats from both apo(a) phenotypic isoforms and plasma Lp (a) concentrations in CAD patients and controls.

observed in case control and even in relatively short time prospective studies may therefore be biased by an effect of atherosclerotic vascular disease on Lp(a) levels. We have therefore analyzed the apo(a) isoform phenotypic polymorphism in patients with CAD. A study of the apo(a) protein polymorphism in CAD patients and controls in the North Indian population showed that low molecular apo(a) isoforms (which are associated with high Lp(a) levels) are significantly expressed in the CAD patients than controls. Utermann (1) has reported similar findings in a study from six ethnic groups around the world which included over 1000 CAD patients. This suggests that variation at the apo(a) gene locus that is unaffected by disease and stable throughout life is associated with CAD(13).

Salient results of our study were entirely as expected. In both patient and normal subjects, the skewness of the Lp(a) concentrations and the inverse association of apo(a) size from phenotyping and Lp(a) concentration results were as expected from studies in other white populations (21).

In conclusion, our study demonstrates that the apo(a) gene polymorphism predicts CAD, suggesting that the association of Lp(a) with CAD is indeed causal.

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