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Association of Apo(a) Isoform Size with Dyslipoproteinemia in Male Venous Thrombosis Patients

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Abstract

Background—Lp(a) is a proatherogenic lipoprotein that may also be prothrombotic. Apo(a) size isoforms have differential effects on fibrinolysis. Whereas Lp(a) concentrations have been linked to venous thromboembolic disease (VTE) risk, apo(a) polymorphisms in VTE have not been studied.

Methods—We used a standardized high resolution agarose gel electrophoresis technique to determine apo(a) isoform size, and a Lp(a) immunoassay insensitive to apo(a) size to measure Lp(a) concentration in 46 men with VTE and 46 age-matched healthy controls.

Results—Apo(a) isoform distribution in VTE cases and controls was bimodal and VTE patients tended to have more medium-sized isoforms K₄-(19–27) (54.3% vs. 34.8%, p=0.06). Cases and controls had the same median predominant apo(a) size isoform (23.5 K₄ repeats) and comparable Lp(a) concentrations. However, subgroup analysis based on apo(a) isoform size (K₄ 23 or K₄ 24) revealed that cases in the K₄ 24 subgroup had higher Lp(a) concentrations than the controls in this isoform subgroup (14.5mmol vs. 6.6mmol, p=0.029). Also, dyslipoproteinemia (smaller LDL and HDL particles, higher LDL and lower HDL parameters) was strongly associated with VTE only in this larger apo(a) isoform group.

Conclusions—These observations provide the first evidence that determination of apo(a) isoforms may provide useful novel insights into VTE risk.

INTRODUCTION

Venous thromboembolic disease (VTE) is associated with dyslipoproteinemia and hypercoagulability and is a polygenic disease with pathogenic contributions from both genetic and environmental risk factors [1,2]. Plasma lipids and lipoproteins can influence both procoagulant and anticoagulant reactions in plasma [3,4]. We discovered that plasma concentrations of large HDL particles are lower in VTE patients than matched controls and that HDL and apolipoprotein AI concentrations are low in VTE patients who have recurrent

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VTE [5,6]. Two reports confirm that low HDL concentrations are linked to VTE [7,8] while one did not confirm it [9].

Lp(a) is a lipoprotein formed by an LDL-like particle and a unique protein component, apo(a), covalently linked to apoB-100 [10]. Apo(a) is a highly hydrophilic glycoprotein with multiple repeated copies of a sequence that closely resembles plasminogen kringle 4 (K₄), followed by sequences homologous to kringle 5 and the serine protease domains of plasminogen. The K₄-like sequences in apo(a) can be classified into 10 types based on amino acid sequence designated K₄ types 1–10. Each apo(a) molecule contains a single copy of K₄ types 1, 3–10 but variable identical repeats (3–>40) of K₄ type 2 motif, thus accounting for apo(a) or Lp(a) isoform size heterogeneity.

Lp(a) has long been recognized as having proatherogenic potentials, but has recently gained attention as a prothrombotic molecule [11]. Potential prothrombotic and antifibrinolytic properties of Lp(a) have been reviewed [11–13]. Lp(a) can bind to and inactivate tissue factor pathway inhibitor, thus promoting coagulation. It can also inhibit plasminogen activation through various mechanisms and attenuate fibrinolysis. Due to these potential prothrombotic and antifibrinolytic properties, Lp(a) has been studied as a prothrombotic risk factor [14–20], and a recent meta-analysis of 6 case-control studies concludes that high Lp(a) (>300mg/l) is associated with increased occurrence of VTE [18]. However, this conclusion remains controversial as significant statistical variations were noted among the studies, and opposite conclusions have been reported [19, 20].

Apo(a) size isoforms bind fibrin with different avidity and inhibit plasmin formation to different extents [12]. They are also inversely correlated to plasma Lp(a) concentration [21] due, in part, to the longer post-translational processing and retention in the endoplasmic reticulum of the larger isoforms compared to the smaller isoforms [22]. However, apo(a) size heterogeneity in VTE has never been investigated. Since apo(a) size isoforms have differential effects on fibrinolysis, we hypothesized that the apo(a) size distribution in VTE cases may contribute to increase risk for VTE. To test this hypothesis, we determined apo(a) isoforms in 46 well-characterized men with VTE and their age-matched healthy controls, and analyzed their relationship with Lp(a) concentrations and with the dyslipoproteinemia that has been linked to this disease.

MATERIALS and METHODS

Study Subjects

The subjects include 46 Caucasian male VTE patients and 46 healthy, age-matched (± 2 y) Caucasian men (controls) ages <55 y recruited from the Scripps Venous Thrombosis Registry and the General Clinical Research Center, respectively. In this study, subjects of African-American ancestry (3 cases and 3 controls) were excluded from the original registry (49 cases and 49 controls) in view of their significantly different pattern of Lp(a)/apo(a) distribution as compared with Caucasians [21]. Detailed information of the subjects has been reported [5] and their characteristics are shown in Table 1. The VTE patients had objectively documented deep venous thrombosis with (n=16) or without (n=30) pulmonary embolism. They did not have cancer and were not on lipid-lowering medications. Thirty-seven of the 46 patients presented with idiopathic VTE, one with diabetes, and 3 with hypertension. The control subjects had none of the above conditions. History of cigarette use was comparable between patients and controls with 8 patients and 5 controls being prior smokers, and 5 patients and 4 controls being current smokers. Thirty-eight patients were taking warfarin when blood was drawn. This study was approved by the institutional review board of the Scripps Clinic. Blood from the patients was collected at least 3 months after VTE diagnosis, and after 12 h of fasting in all subjects. Serum and EDTA plasma were prepared, and were

either used immediately for routine clinical laboratory tests or stored at -70°C for Lp(a) and other studies.

Laboratory Measurements

Apo(a) isoform size was determined by a high-resolution SDS-agarose gel electrophoresis method coupled with immunoblotting [23]. This procedure has a sensitivity of 5 fmol of apo(a). To enhance the isoform detection, variable volume of plasma calculated based on Lp(a) concentration was applied onto the gel. In this system, a constant relationship exists between the relative migration of apo(a) in the agarose gel and the number of K_4 type 2 repeats determined by pulsed-field electrophoresis [24]. Apo(a) size isoform was therefore identified from its migration distance on the gel and was expressed as the number of K_4 repeats (Fig. 1). In heterozygous subjects when the immunoblotting signal of 1 of the 2 isoforms predominated, the predominant form was used to describe the apo(a) size of the subject. When both isoforms appeared to be equally expressed, the mean K_4 number was used.

Lp(a) concentration was measured by a direct-binding double monoclonal antibody ELISA [25]. The capture antibody (a-5) is directed to an epitope present in apo(a) K_4 type 2, and the detection antibody (a-40) is directed to an epitope present in apo(a) K_4 type 9. Since K_4 type 9 is present in only 1 copy per apo(a) molecule, this immunoassay is insensitive to apo(a) size heterogeneity. The assay used calibrator with value traceable to the WHO/IFCC Reference Preparation and expressed in terms of molar concentration, allowing for the measurement of Lp(a) concentrations in nmol/l. Frozen sera from 5 individuals, representing a broad range of Lp(a) concentrations were used as quality controls for the ELISA.

Serum lipid profile data were obtained from routine clinical laboratory using standard techniques. Immunoturbometric assay kits (DiaSorin) were used to measure apoA-I and apoB. Lipoprotein subclass particle concentrations and the average particle diameters of very low density lipoproteins (VLDL), low-density lipoproteins (LDL), and high-density lipoproteins (HDL) were measured by Nuclear Magnetic Resonance Spectroscopy (NMR) at LipoScience, Inc (Raleigh, NC). Data of 9 lipoprotein subclasses based on particle diameter were used in this study. They were large VLDL (and chylomicrons if present) (> 60 nm), medium VLDL (35–60 nm), small VLDL (27–35 nm), intermediate density lipoproteins (IDL) (23–27 nm), large LDL (21.2–23 nm), small LDL (18–21.2 nm), large HDL (8.8–13 nm), medium HDL (8.2–8.8 nm) and small HDL (7.3–8.2 nm) [26].

Statistical Analysis

Data were summarized by mean and standard deviation or median and quartile. To minimize any possible contribution of outliers, the Mann-Whitney U test was used for group comparisons, and Spearman rank order correlation analyses were used to determine the relationship between Lp(a) concentration and apo(a) isoform. Contingency table analysis (using χ^2) was used for categorical data. Analyses were carried out using the Statistica software (StatSoft, Tulsa, OK) or Prism 4.0 (GraphPad Prism, San Diego, CA).

RESULTS

Apo(a) isoform size distribution in VTE and controls

Apo(a) isoform analysis showed the presence of 2 bands in 36 VTE and 31 control subjects. In most of these heterozygous cases (35 in VTE, 29 in controls), 1 of the 2 isoforms was predominantly expressed and this was used for statistical analysis. In the 3 cases where both isoforms were similarly expressed, the mean of the K_4 number of the 2 isoforms was used. With this approach, the predominantly expressed apo(a) isoforms in VTE (mean $\pm$ SD: 23.3

± 5.7 K₄ repeats, median 23.5, range 13–34) and controls (24.3 ± 6.3 K₄ repeats, median 23.5, range 15–36) were comparable. As previously reported in Caucasians without VTE, the frequency distribution of the predominant apo(a) size isoform in VTE cases and controls was bimodal. However, the locations of the modes in the VTE cases [K₄-(19–21) (23.9%) and K₄-(25–27) (17.4%)] and controls [K₄-(16–18) (23.9%) and K₄-(28–30) (21.7%)] were different (Fig.2). Chi² test showed that VTE patients tended to have more medium-sized isoforms K₄-(19–27) (54.3% vs. 34.8%, $p=0.06$).

Association of Lp(a) with VTE in apo(a) isoform size subgroups

No statistical difference was observed between the plasma Lp(a) concentration of the VTE patients (median: 17.9 nmol/l, quartiles range 7.3–42.1 nmol/l) and the controls (median: 14.2 nmol/l, quartiles range 5.1–44.3 nmol/l), and their distribution was positively skewed as expected (Table 2). Apo(a) size of the predominant isoform (but not the weaker one) was significantly and inversely correlated with plasma Lp(a) concentration in VTE ($R_s = -0.467$, $p=0.001$) and in the controls ($R_s = -0.593$, $p<0.001$).

Given the bimodal distribution of apo(a) isoform size, further comparison between VTE and controls was carried out in subjects with apo(a) isoforms in the 2 modes. The median size of the apo(a) isoform of the study participants was used to divide the VTE and controls into 2 isoform subgroups for analysis (K₄ 23 and K₄ 24). The subject profile of these subgroups is shown in Table 1. Most of the characteristics between the VTE patients and controls in each subgroup is similar except for factor V Leiden which is significantly more prevalent in the VTE patients in the smaller isoform K₄ 23 subgroup ($p=0.0008$), but not in larger isoform K₄ 24 subgroup ($p=0.38$).

As expected from the inverse relationship between plasma Lp(a) concentration and isoform size, subjects in the smaller isoform K₄ 23 subgroup had significantly higher plasma Lp(a) concentration than those in the larger K₄ 24 subgroup in VTE patients (median 34.3 vs. 14.5 nmol/l, $p=0.015$) and in the controls (median 44.3 vs. 6.6 nmol/l, $p<0.001$) (Table 2). The spread of plasma Lp(a) concentration was much wider in the smaller isoform subgroup (1.5–211.5 nmol/l) than the larger isoform subgroup (0.6–46.1 nmol/l) (Figure 3). When Lp(a) concentrations were compared between VTE and control in these two isoform size subgroups, Lp(a) was higher in VTE cases in the K₄ 24 subgroup than the controls (median: 14.5 vs. 6.6 nmol/l, $p=0.029$), although there was no difference for the K₄ 23 subgroup or for the entire group (Table 2). Using the 90th percentile of the controls in this size subgroup (20 nmol/l) as a cut-off point, an odds ratio was calculated that indicated that the risk of VTE for the larger apo(a) size subgroup was 5.60 (95% CI: 1.04–30).

Association of dyslipoproteinemia with VTE in apo(a) isoform size subgroups

We reported that dyslipoproteinemia (higher LDL and lower HDL parameters) was strongly associated with VTE for this cohort under study (Table 2) [5]. Similar subgroup analysis was performed to determine the relationship of apo(a) isoform size to the association of dyslipoproteinemia with VTE. As seen in Table 2, body mass index (BMI) which is known to be associated with VTE [27] was higher in VTE cases than in controls for both apo(a) isoform subgroups. Remarkably, dyslipoproteinemia including low concentrations of apoAI, HDL-cholesterol, total HDL particles and large HDL particles and high concentrations of apoB, LDL-cholesterol, total LDL particles, IDL particles, and small LDL particles were associated with VTE only for the K₄ 24 subgroup, but not the K₄ 23 subgroup. Thus, the distinct VTE-associated dyslipoproteinemia previously reported for this study population [5] was present only in the K₄ 24 subgroup.

DISCUSSION

The bimodal distribution of apo(a) isoform size, their inverse relationship with plasma Lp(a) concentration, and the positively skewed Lp(a) distribution observed in the VTE cases and controls in this study were all consistent with that found in 985 Caucasian men without VTE from four US communities [21]. Although median apo(a) isoform size in VTE and controls was identical, VTE patients had more intermediate isoform K₄-(19–27) (54.3% vs 34.8%) (Fig. 1). This difference is of interest as increased presence of intermediate size polymorphs [21] was also seen in men of black ethnicity who are known to have a higher prevalence of VTE [28].

Although no significant differences in plasma Lp(a) concentration were detected between VTE cases and controls in our molar based Lp(a) assay, remarkably, when the subjects in each group were divided into 2 subgroups based on their isoform size, VTE cases in the K₄ 24 subgroup had significantly higher Lp(a) than the controls in this isoform size subgroup. Equally remarkable was the observation that the strong association of dyslipoproteinemia with VTE which we reported earlier for this cohort was also present only in this larger sized K₄ 24 subgroup, but not the smaller K₄ 23 isoform size subgroup. The association of the larger size apo(a) isoforms with dyslipoproteinemia in VTE was not related to the few cases with higher Lp(a) concentrations (>30 nmol/l) in this larger isoform size subgroup, as all the lipoprotein differences between VTE and controls in this subgroup (Table 2) remained the same even when these cases were removed (data not shown). Thus, the diagnostic approach to identify Lp(a) concentrations and characterize the dyslipoproteinemia associated with VTE risk may be improved by determining the apo(a) isoform size distribution.

A clear pathophysiologic mechanism for the finding that dyslipoproteinemia (higher LDL and lower HDL parameters) was strongly associated with VTE only in the larger apo(a) isoform subgroup is unclear, but the following considerations may be relevant. Apo(a) is a plasminogen-like lipoprotein and it can inhibit fibrinolysis by competitive mechanisms. Lp(a) particles containing smaller apo(a) isoforms have the most profound influence on fibrinolysis by acting as a prominent competitive antagonist for fibrin binding by plasminogen [12]. In contrast, larger apo(a) phenotypes are associated with the reduction of plasmin generation [29]. Here we found the association of dyslipoproteinemia with VTE only in the larger apo(a) size group, i.e., with apo(a) isoform sizes that are linked to better reduction of plasmin generation. This might suggest that a dyslipoproteinemia-related prothrombotic state plus an antifibrinolytic activity state are simultaneously present in VTE patients. Additionally or alternatively, our findings might suggest that Lp(a) or apo(a) plays a previously unknown role in the metabolism of other lipoproteins and that the extent of its interaction with other lipoproteins differ among the apo(a) polymorphs.

In summary, we determined apo(a) isoform sizes in 46 well-characterized Caucasian men with VTE and their age-matched healthy controls, and have also analyzed their relationship with Lp(a) concentrations and dyslipoproteinemia, parameters that have recently gained attention as VTE risk factors. In cases and in controls, apo(a) isoform size distribution was bimodal. However, VTE patients appeared to have more intermediate-sized apo(a) isoforms. Also, VTE cases in the K₄ 24 subgroup (but not the K₄ 23 subgroup) had significantly higher Lp(a) than the controls, and there were multiple significant metabolic, lipid, and lipoprotein differences between cases and controls.

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Figure 1. Immunoblot of the human plasma apo(a) isoforms. Lane 1, apo(a) kringle 4 standards; lanes 2–6, plasma samples.

% Frequency

Figure 2.
Distribution of the predominant apo(a) isoform in VTE cases (solid bars) and controls (open bars).

Figure 3.

Plasma Lp(a) concentration in VTE and controls with predominant apo(a) size isoform $K_4 \leq 23$ (left panel) and $K_4 \geq 24$ (right panel). The solid line indicates the median concentration for each group.

Table 1

Study Population

Variables	Entire Group			Apo(a) Isoform K ₄ 23			Apo(a) Isoform K ₄ 24		
	Control N=46	VTE N=46	p	Control N=23	VTE N=23	p	Control N=23	VTE N=23	p
Age, y (SD)	46.3 (8.4)	46.7 (8.9)	NS	46.5 (9.6)	46.6 (9.2)	NS	46.2 (7.3)	46.9 (8.8)	NS
Ethnicity, (%)									
Non-Hispanic A	45 (97.8)	45 (97.8)	NS	22 (95.6)	22 (95.6)	NS	23 (100)	23 (100)	NS
Risk factors number (%)									
Family History for VTE	4 (8.7)	16 (34.8)	0.002	1 (4.3)	6 (26.0)	0.040	3 (13.0)	10 (43.4)	NS
Factor V Leiden	2 (4.3)	13 (28.3)	0.002	0 (0)	9 (39.1)	<0.001	2 (8.7)	4 (17.4)	NS
Prothrombin 20210A	2 (4.3)	7 (15.2)	NS	1 (4.3)	3 (13.0)	NS	1 (4.3)	4 (17.4)	NS
Idiopathic		37 (80.4)			17 (73.9)			20 (87.0)	
Others number (%)									
Smoking (prior)	5 (10.1)	8 (17.4)	NS	2 (8.7)	5 (21.7)	NS	3 (13.0)	3 (13.0)	NS
Smoking (current)	4 (8.7)	5 (10.1)	NS	2 (8.7)	2 (8.7)	NS	2 (8.7)	3 (13.0)	NS
Diabetes	0 (0)	1 (2.2)	NS	0 (0)	1 (4.3)	NS	0 (0)	0 (0)	NS
CAD	0 (0)	3 (6.5)	NS	0 (0)	2 (8.7)	NS	0 (0)	1 (4.3)	NS
Warfarin (+)		38 (82.6)			18 (78.3)			20 (87.0)	

CAD denotes coronary artery diseases. P values for age are from student t-test comparing mean values between VTE and Controls. P values for the remaining parameters are from χ^2 test.

Lipid, lipoprotein, and metabolic variables showing significant differences between VTE cases and controls in the 2 apo(a) size isoform subgroups

Table 2

Variables	Entire Group		p	Apo(a) Isoform K ₄ 23			Apo(a) Isoform K ₄ 24		
	Controls (n=46)	VTE (n=46)		Controls (n=23)	VTE (n=23)	p	Controls (n=23)	VTE (n=23)	p
Lp(a) (nmol/L)	14.2 (5.1–44.3)	17.9 (7.3–42.1)	NS	44.3 (9.5–84.3)	34.3 (6.7–103)	NS	6.6 (4.5–16.7)	14.5 (7.4–25.6)	0.029
BMI (kg/m ²)	27.4 ± 4.3	30.3 ± 5.7	0.001	27.0 ± 3.9	30.1 ± 6.2	0.046	27.3 ± 4.8	30.6 ± 5.4	0.014
Apo A-I (mg/dL)	104 ± 21	93 ± 27	0.005	97 ± 15	92 ± 22	NS	111 ± 24	95 ± 32	0.024
HDL cholesterol (mg/dL)	51.5 ± 12.3	45.5 ± 16.3	0.003	50.6 ± 11.4	49.0 ± 20.5	NS	52.4 ± 13.4	41.9 ± 9.8	0.005
HDL particles (total) (μmol/L)	26.3 ± 4.4	23.5 ± 4.8	0.002	25.0 ± 3.2	23.7 ± 3.7	NS	27.6 ± 5.1	23.4 ± 5.7	0.012
HDL particles (large) (μmol/L)	4.46 ± 2.79	3.4 ± 3.1	0.020	4.38 ± 2.95	3.57 ± 2.68	NS	4.53 ± 2.68	3.28 ± 3.52	0.027
HDL particle size (mean) (nm)	8.84 ± 0.43	8.66 ± 0.40	0.028	8.83 ± 0.48	8.75 ± 0.38	NS	8.84 ± 0.40	8.56 ± 0.41	0.003
Apo B (mg/dL)	80 ± 21	91 ± 21	0.023	83 ± 25	87 ± 23	NS	77 ± 17	95 ± 17	0.002
LDL cholesterol (mg/dL)	122 ± 34	136 ± 40	NS	130 ± 39	126 ± 39	NS	114 ± 27	146 ± 40	0.006
LDL particles (total) (nmol/L)	1043 ± 359	1215 ± 329	0.011	1058 ± 02	1137 ± 317	NS	1028 ± 318	1293 ± 342	0.010
LDL particles (small) (nmol/L)	36.0 ± 38.1	50.9 ± 37.8	0.017	39.1 ± 39.8	43.4 ± 34.8	NS	32.8 ± 36.8	58.3 ± 40.0	0.007
LDL particle size (mean) (nm)	747 ± 409	933 ± 351	0.005	765 ± 465	857 ± 318	NS	730 ± 354	1009 ± 372	0.006
	20.6 ± 0.84	20.3 ± 0.7	0.038	20.6 ± 0.9	20.4 ± 0.7	NS	20.7 ± 0.7	20.3 ± 0.7	0.044

Lp(a) is expressed in median (25th and 75th quartile). All other numbers are expressed in mean ± SD. *P* values are from Mann-Whitney test comparing median values between VTE and Controls for all isoforms, or within each apo(a) size isoform subgroup