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Combined effects of fibrinogen genetic variability on atherosclerosis in patients with or without stable angina pectoris: Focus on the coagulation cascade and endothelial function

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ABSTRACT

Background: Fibrinogen is a coagulation/inflammatory biomarker strongly associated with atherogenesis. Data have reported that the genetic variability on fibrinogen chains may affect the atherosclerotic process and the risk of coronary artery disease (CAD). We examined the combined effects of the G455A and the G58A fibrinogen genetic polymorphisms on prothrombotic profile, endothelial function and the risk of CAD in a Caucasian population.
Methods: We recruited 422 patients with angiographically documented CAD and 277 controls matched for age and gender. The two polymorphisms were genotyped by polymerase chain reaction and restriction endonuclease digestion. Fibrinogen and D-Dimers levels, as well as factors' (f) V, X activity were measured by standard coagulometry techniques. Endothelial function was assessed by the flow mediated dilatation (FMD) of the brachial artery.
Results: The two polymorphisms had no significant effect on the risk for CAD. Although the 58AA subjects had not significantly different levels of fibrinogen compared with the 58GG + GA in both groups ($p = NS$), we importantly found that the 455AA homozygosity was associated with increased fibrinogen levels not only in the control group ($p = 0.035$), but also in the CAD group ($p < 0.001$) compared to the G allele carriers. Moreover, both the 58AA ($p = 0.016$) and 455AA homozygotes ($p = 0.022$) presented with higher levels of D-Dimers in the CAD group. Interestingly, the 455AA homozygotes had increased fV activity in the CAD group ($p = 0.048$). However, no significant effects were observed on fX activity and FMD.
Conclusions: Both fibrinogen polymorphisms are capable to modify the atherosclerotic process via their effects on the coagulation cascade.

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1. Introduction

It is well established that underlying processes such as coagulation and endothelial function are strongly related to the initiation and progression of atherosclerosis [1]. Coagulation factors, such as the tissue factor is a crucial cofactor in the coagulation pathway, promoting thrombosis via the promotion of thrombin generation. Thrombin in turn activates platelets and also cleaves fibrinogen resulting in a thrombotic plug [2]. However, the exact mechanisms for the coagulation-dependent mechanisms of atherogenesis are still obscure. Moreover, it is well known that impaired endothelial function represents a major factor for atherosclerosis, while data have demonstrated a strong association of endothelial function with future cardiovascular events [3].

During the last decades several studies have focused on fibrinogen and its' relation to increased risk for coronary artery disease (CAD) [4]. Plasma fibrinogen levels have been found higher in patients with acute coronary syndromes compared to that of patients with stable coronary heart disease or healthy controls and also higher plasma fibrinogen levels may be predictors of poor long-term prognosis [5]. Additionally, the effect of fibrinogen levels on cardiovascular risk has been found equal that of known risk factors such as hypertension, diabetes mellitus and smoking [6].

Evidence suggests that the genetic variations of fibrinogen are associated with the prevalence of CAD and the occurrence of myocardial infarction (MI), even though they have yielded to date inconsistent results [7–9]. In particular, two polymorphisms such as the G455A and the G58A of the b-chain and a-chain genes of fibrinogen respectively have been suspected (several conflicting reports) for altered fibrinogen levels and for a potential role in the risk for cardiovascular disease [10,11]. However, the impact of these genetic variations of fibrinogen on the mechanisms of atherosclerosis such as endothelial function and coagulation cascade remains unclear.

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Therefore, in the present study we investigated whether the combination of the two aforementioned fibrinogen single nucleotide polymorphisms (SNPs) could modify the levels of coagulation markers such as fibrinogen, D-Dimers, factors V, X (fV, fX) and FMD, as well as the risk for CAD in patients with or without stable angina pectoris (SAP).

2. Methods

2.1. Study population

In the present association study we enrolled 699 subjects of Caucasian origin. Consecutive patients with significant coronary stenoses, but without symptoms of acute MI constituted the group with SAP (n = 422), while subjects without SAP (CAD) or previous MI, matched for age and gender were considered as controls (n = 277). The diagnosis of CAD was established angiographically in the presence of >50% stenosis in at least one of the three major coronary arteries or major branches. Subjects were considered eligible as controls when their coronary arteries were angiographically normal. According to the guidelines of the European Society of Cardiology, subjects were considered to have essential hypertension if their systolic and/or diastolic blood pressure was ≥140 and/or ≥90 mmHg on two different occasions or if they were currently being treated with anti-hypertensive drugs [12]. Subjects were considered to have hyperlipidemia, if total cholesterol levels ≥220 mg/dl were revealed in biochemical tests or if they were under hypolipidemic medication [13]. Similarly, subjects were considered to have diabetes mellitus if they had fasting plasma glucose levels ≥126 mg/dl, non-fasting plasma glucose levels ≥200 mg/dl, or when they were currently being treated with hypoglycemic agents [14]. As current smokers were considered those participants smoking 1–10 cigarettes/day for at least the last year [15]. Exclusion criteria were the existence of any inflammatory or infective disease, liver or renal disease, malignancy, heart failure defined as ejection fraction <45%, or a history of deep venous thrombosis or pulmonary embolism and MI < 6 months before (for CAD patients). To avoid possible influences of interventional procedures blood samples were obtained before coronary angiography or percutaneous coronary intervention. The demographic characteristics of the participants are presented in Table 1. Informed consent was obtained from each patient and the study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki as reflected in a priori approval by the institution's human research committee.

2.2. DNA extraction and genotyping

Approximately 5 ml blood was collected in tubes containing ethylenediaminetetraacetic acid. Genomic DNA was extracted from 2–5 ml of whole blood using standard methods (QIAamp DNA blood kit; Qiagen, West Sussex, UK). For the detection of both the G455A polymorphism (beta chain) and the G58A polymorphism (alpha chain), we used polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis. In order to amplify a part of the gene (by PCR) we used the following flanking intronic primers, forward: 5′-GAACATTTTACCTTATGTGAATTAAGG-3′ and reverse: 5′-GAAGCTCCAAGAAACCATCC-3′ for the first polymorphism. In addition, the following primers were used for the second polymorphism, forward: 5′-TGCAACAGCTTATCGGAAGC-3′ and reverse: 5′-GTGGAATAAACACCAGAGAG-3'. The resulting products were digested by the HaeIII and AciI restriction enzymes respectively (resolution by electrophoresis at 2% agarose gel). Digested fragments were visualized after ethidium bromide staining under ultraviolet light. For PCR quality control, 5% of the samples were randomly selected and genotyped twice for quality assurance, which yielded 100% concordance.

Table 1
Demographic characteristics of the study population.

	Controls	CAD	
Subjects	277	422	
Age (years)	62.7 ± 9.97	64.0 ± 9.96	p = 0.120
Hypertension, n (%)	165 (59.5)	320 (75.8)	p < 0.001
Dyslipidemia, n (%)	151 (54.5)	287 (68.0)	p = 0.001
Diabetes mellitus, n (%)	56 (20.2)	161 (38.2)	p < 0.001
Smoking, n (%)	138 (49.8)	249 (59.0)	p = 0.020
Ejection fraction	61.8 ± 5.54	51.2 ± 9.82	p < 0.001
Fibrinogen (mg/dl)	376.2 ± 99.0	436.8 ± 131.0	p < 0.001
D-Dimers (mg/l)[$]	2.46 [2.28–2.60]	2.60 [2.40–2.70]	p < 0.001
Factor V (%)	113.2 ± 23.1	121.0 ± 24.0	p = 0.002
Factor X (%)	108.7 ± 22.4	103.8 ± 23.6	p = 0.05
FMD (%)	6.26 ± 3.23	3.86 ± 2.35	p < 0.001
Genotype distribution			
58GG, n (%)	99 (38.1)	128 (37.7)	
58GA, n (%)	114 (43.8)	151 (44.5)	
58AA, n (%)	47 (18.1)	60 (17.8)	
455GG, n (%)	148 (54.4)	209 (50.6)	
455GA, n (%)	103 (37.8)	171 (41.4)	
455AA, n (%)	21 (7.8)	33 (8.0)	
Recessive model			
58AA, n (%)	47 (18.1)	60 (17.8)	*p = NS
58GG + 58GA, n (%)	213 (81.9)	279 (82.2)	
455AA, n (%)	21 (7.8)	33 (8.0)	†p = NS
455GG + 455GA, n (%)	251 (92.2)	380 (92.0)	

All values are expressed as mean ± SD. CAD: coronary artery disease, FMD: flow-mediated dilation, [$]Values expressed as median (25–75th percentile), *58AA vs 58GG + 58GA, [†] 455AA vs 455GG + 455GA by chi-square analysis.

2.3. Biochemical measurements

All of the participants were asked to abstain from tobacco, alcohol and caffeine containing beverages during the evening before (12 h) blood sampling. Venous blood samples were centrifuged at 3,500 rpm at 4 °C for 15 min, and plasma or serum was collected and stored at −80 °C until assayed. Serum levels of fibrinogen were measured by the Clauss method (Multifibren U, Siemens), while D-Dimers by a immunonephelometric method (Innovance D-Dimer, Siemens). In addition, fV activity in plasma was measured by a one-stage assay based on prothrombin time (factor V deficient, Dade Behring) and factor X activity was measured by a standard one-stage method using single-donor factor X–deficient plasma on a Dade Behring BCS Coagulation Analyzer (Deerfield, IL).

2.4. Assessment of endothelial function

Flow-mediated dilation (FMD), an index of endothelial dysfunction, was assessed in the brachial artery by ultrasound as described previously [16]. Briefly, after 10 minute rest, the right brachial artery was scanned in longitudinal section, 5 cm above the antecubital fossa, using a using a 7–12 MHz linear array transducer (General Electric, USA). After baseline diameter of the brachial artery was recorded. Then, a pneumatic cuff placed on the forearm was inflated to suprasystolic (at 50 mmHg) pressure for 5 min to induce reactive hyperemia. The longitudinal image of the artery was recorded continuously from 30 second before to 2 min after cuff deflation. A midartery pulsed Doppler signal was obtained upon immediate cuff release and no later than 15 s after cuff deflation to assess hyperemic velocity. The diameter of the brachial artery was measured at rest and at 60 s (maximal diameter) and 90 s post-cuff release. Flow mediated dilation was measured as absolute and percentage change in vessel diameter after cuff release from rest to the maximal diameter (60s).

2.5. Statistical analysis

Qualitative variables are presented as relative frequencies, while continuous variables were tested for normal distribution by Kolmogorov–Smirnov test. Normally distributed variables are presented as mean values plus/minus standard deviation (mean ± SD), whereas not normally distributed data were log-transformed for analysis and are presented in the non-logarithmic format as median (25–75th percentile values). Genotype and allele frequencies were compared between groups by chi-square analysis, while Cochran's and Mantel–Haenszel statistics were used to estimate odds ratios (OR) [95% confidence interval (CI)], of the development of CAD as an effect of the 2 polymorphisms. Odds ratios were adjusted for age, hypertension, smoking, hyperlipidemia and diabetes mellitus. Comparisons of continuous variables between the genotypes were performed by unpaired Student's t-test or the non parametric Mann–Whitney test. We tested that the allele frequencies conformed to Hardy-Weinberg equilibrium proportions by using Chi-square test. Linkage disequilibrium (LD) between the two SNPs was tested by using the online software of Rodriguez et al. [17]. Further binary logistic regression analysis was performed in order to investigate the potential associations between humoral behavior and clinical manifestations/diseased vessels. All reported p values are based on two-sided tests and compared to a significance level of 5%. SPSS version 18.0 (SPSS, Chicago, IL) software was used for all the statistical calculations.

3. Results

Our results indicated that the two SNPs were not in LD and the genotypes' distribution did not deviate from Hardy–Weinberg equilibrium (for G455A: $X^2 = 0.02$, p = NS and for G58A: $X^2 = 3.66$, p = NS).

3.1. Effects of the G58A and G455A polymorphisms on the risk for CAD, fibrinogen and D-Dimers levels

In the present study we have found that there was no significant difference in the prevalence of the different genotypes as shown in Table 1. In addition, we observed that none of the 2 study polymorphisms was associated with increased risk for CAD even after adjustment for known risk factors (Table 2).

Our study has shown that the two polymorphisms exerted significant effects on fibrinogen and D-Dimers levels. Regarding to fibrinogen levels, a non significant effect was observed for the G58A polymorphism in both the study groups (p = NS, despite a trend in the control group

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