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Plasma levels of soluble fibroblast activation protein in arterial thrombosis; determinants and cleavage of its substrate alpha-2-antiplasmin ☆

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ABSTRACT

Background: Fibroblast activation protein (FAP) is a transmembrane glycoprotein with dipeptidyl-peptidase and endopeptidase activities and circulates in blood in a truncated, soluble form (sFAP). Fibrinolysis inhibitor α2-antiplasmin (α2AP) has been described as a potential in vivo substrate of sFAP. We aimed to investigate sFAP levels and α2AP cleavage in young arterial thrombosis patients and in control individuals, study the correlation between sFAP levels and α2AP cleavage and investigate determinants of these variables.
Methods: sFAP levels and α2AP cleavage were determined by ELISA in the plasma samples of 391 coronary heart disease (CHD) patients, 221 ischemic stroke patients, 51 peripheral arterial disease patients and 501 control individuals.
Results: Median sFAP levels were similar in arterial thrombotic patients and in control individuals, but in CHD patients sFAP levels significantly increased with time (number of months) between the event and study inclusion (Spearman's rho: 0.209, $p < 0.001$), indicating reduced sFAP levels at time of event. sFAP levels and percentage α2AP cleavage significantly correlated in controls and in patients. Furthermore, sex, use of oral contraceptives and hyperlipidemia were significant determinants of sFAP levels.
Conclusions: sFAP levels were reduced in the CHD patient population, but only in the first months after the event, indicating that over time sFAP levels may normalize. The significant correlation between sFAP level and α2AP cleavage indicates that in vivo sFAP (at least partly) regulates cleavage of α2AP, irrespective of disease status. Differences in sFAP level due to sex, use of oral contraceptives and hyperlipidemia might suggest hormonal control of sFAP levels.

1. Introduction

Fibroblast activation protein (FAP) is a serine protease with both dipeptidyl-peptidase and endopeptidase activities, cleaving substrates at a post-proline bond (extensively reviewed in [1]). FAP is a homodimeric transmembrane glycoprotein, which in a truncated, soluble form (sFAP) also circulates in blood [2,3]. Substrates of FAP include collagen type I [4], alpha-2-antiplasmin (α2AP) [5] and the neuropeptides B-type natriuretic peptide, neuropeptide Y, substance P and peptide YY [6]. As α2AP was found to be a substrate of sFAP in vitro, sFAP has also been termed antiplasmin-cleaving enzyme (APCE) [5]. sFAP cleaves 12 amino acids of the N-terminus of native α2AP turning Met-α2AP (with a methionine (Met) at the N-terminus) into Asn-α2AP (with an asparagine (Asn) at the N-terminus). This occurs in approximately 70% of all circulating α2AP.

Lately, FAP has attracted increasing research interest as a selective marker of carcinoma-associated fibroblasts and more generally, of activated fibroblasts in tissues undergoing remodeling of the extracellular matrix due to chronic inflammation, fibrosis or wound healing. It has been shown that intrahepatic FAP expression strongly and significantly correlated with the severity of liver fibrosis [7]. We recently showed that plasma sFAP levels were also increased in patients with liver cirrhosis and corresponded to the severity of disease [8]. Furthermore, Brokopp et al. showed that tissue expression of FAP correlated with the progression of atherosclerotic plaques and with fibrous cap thinning, as they detected increased levels of FAP in thin fibrous caps compared to thick fibrous caps [9]. However, rather unexpectedly, Tillmanns

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et al. found reduced sFAP levels in the plasma of patients with acute coronary syndrome compared to the sFAP plasma levels of healthy blood donors [10]. This prompted us to investigate plasma sFAP levels in a large cohort of patients with arterial thrombosis and in control subjects. Additionally, with α2AP being one of the potential in vivo substrates of (s)FAP, we investigated the association with N-terminal cleavage of α2AP in this study population and studied determinants of sFAP.

2. Material and methods

2.1. Study population

The case–control population used in this study, the 'Genetic risk factors for Arterial Thrombosis at young age: the role of TAFI and other Coagulation factors'(ATTAC) study, has been described previously [11]. In short, consecutive patients who presented with a first arterial thrombotic event in the cardiac, cerebral or peripheral vascular system at young age were eligible for inclusion if they were 45 years or younger (males) or 55 years or younger (females). The cohort consisted of 663 patients divided into three subgroups: I] coronary heart disease (CHD), containing 391 patients with acute myocardial infarction or unstable angina pectoris, II] ischemic stroke (IS), containing 221 patients with an IS or transient ischemic attack, and III] peripheral arterial disease (PAD) containing 51 patients with PAD. The 501 control subjects were partners, friends or neighbors of the patients of the same age, without a history of cardiovascular disease. All individuals enrolled in this study provided written informed consent. Blood samples were collected in citrate (0.105 mol/L) usually one to three months after the event to minimize acute phase response effects. Plasma was obtained by centrifugation at 2000 *g* for 10 min at 4 °C, followed by centrifugation at 20,000 *g* for 10 min at 4 °C, and stored at −80 °C. Genomic DNA was isolated from blood collected in EDTA using standard salting-out procedures and stored at 4 °C.

2.2. sFAP antigen measurements

sFAP antigen levels were measured in plasma with the FAP DuoSet ELISA development kit (DY3715) from R&D Systems as recommended by the manufacturer and as recently extensively described [8,10]. Inter- and intra-assay variation was 12.5% and 3.5%, respectively.

2.3. α2AP antigen measurements

In addition to N-terminal cleavage, approximately 35% of circulating α2AP is also cleaved at its C-terminus, which leads to the loss of the plasminogen binding (PB) domain of α2AP and thus of α2AP activity. We previously developed two new ELISA assays to measure the plasma antigen levels of 1) total PB-α2AP (Met-PB-α2AP plus Asn-PB-α2AP) and 2) Met-PB-α2AP. The ELISA methods and validation have extensively been described [8]. To measure the Met-PB-α2AP levels, we used a custom-made rabbit affinity-purified α2AP antibody raised against a peptide corresponding to the N-terminal 12 amino acids (MEPLGRQLTSGP) of the α2AP protein (Charles River GmbH, UK; Squarix GmbH, Marl, Germany). As this peptide contained an arginine (Arg, R) on position 6, the position of polymorphism Arg6Trp (rs2070863), the ELISA only responded to Met-PB-α2AP(R6). Therefore, the statistical tests of the Met-PB-α2AP levels and the percentage N-terminal cleavage were only performed in Arg6 homozygous individuals (404 patients and 318 control individuals). Inter- and intra-assay variation was 9.7% and 2.4%, respectively, for the total PB-a2AP ELISA and 9.4% and 3.4%, respectively, for the Met-PB-a2AP ELISA.

2.4. Genetic analyses

All subjects were genotyped for the Arg6Trp polymorphism in α2AP using the TaqMan assay [12]. The polymerase chain reaction with fluorescent allele-specific oligonucleotide probes was performed on a geneamp PCR system (Applied Biosystems, Forster City, CA, USA), using ABsolute QPCR ROX Mix CM-205/A (Westburg, Leusden, the Netherlands). Fluorescence endpoint reading for allelic discrimination was performed on an ABI 7900HT with SDS 2.1 software for genotype clustering (Applied Biosystems, Forster City, CA, USA). DNA samples were available from 651 patients and 493 control subjects.

2.5. Statistical analyses

Distributions of the sFAP antigen levels, α2AP antigen levels and the percentage N-terminal cleavage were tested for normality using the Kolmogorov–Smirnov test. Data are presented as mean ± standard deviation (sd) or in a bar graph for normally distributed data and as median with interquartile ranges for non-normally distributed data. The percentage of N-terminal cleavage was calculated by subtracting the percentage Met-PB-α2AP of total PB-α2AP, from 100%. The body mass index (BMI)-based Framingham Risk Score was calculated in the control individuals based on the variables age, BMI, systolic blood pressure, antihypertensive medication use, smoking and diabetes status [13].

The non-normally distributed variables sFAP and total PB-α2AP were natural log transformed to obtain normal distributions. Differences in levels between groups were tested for significance using Student's t-test or analyses of variance (ANOVA) for normally distributed variables and Kruskal–Wallis analyses for non-normally distributed variables. Adjustments for classical risk factors for cardiovascular disease, such as age, sex, BMI, smoking, diabetes, hypertension (systolic blood pressure ≥140 mm Hg and/or diastolic blood pressure ≥90 mm Hg or use of anti-hypertensive drugs on day of event), and hypercholesterolemia (total cholesterol level > 5.0 mmol L^{-1} or lipid lowering treatment on day of event) were made using linear regression. The significance of correlations between variables was studied using Pearson's r for normally distributed variables and Spearman's rho for non-normally distributed variables. P-values < 0.05 were considered to indicate statistical significance. All statistical analyses were carried out using SPSS software version 21.

3. Results

The baseline characteristics of the study subjects are shown in Table 1. The patients were on average slightly older (43 years, range: 19–55) than the control individuals (39 years, range: 18–57). Compared to the control individuals, there were less females among the CHD patients, whereas there were more females among the IS patients and the PAD patients. Classical risk factors for arterial disease such as obesity, diabetes, smoking, hypertension and hypercholesterolemia were more prevalent in the patient subgroups than in the control individuals. The patients of our study population have predominantly been included one to three months after the event. Therefore, most patients were already taking medication at time of inclusion (Table 1).

Table 1
Baseline characteristics of the control individuals and the patients of the ATTAC Study.

	Controls (n = 501)	CHD patients (n = 391)	IS patients (n = 221)	PAD patients (n = 51)
Age (years), mean (range)	39 (18–57)	44 (23–55)	42 (19–55)	43 (21–55)
Female sex, n (%)	318 (63.5)	172 (44.0)	167 (75.6)	35 (68.6)
Risk factors				
BMI (kg/m^2)	25.6	27.4	26.3	26.0
Diabetes, n (%)	9 (1.8)	38 (9.7)	24 (10.9)	11 (21.6)
Hypertension, n (%)	31 (6.2)	99 (25.3)	71 (32.1)	19 (37.3)
Hypercholesterolemia, n (%)	170 (33.9)	155 (39.6)	75 (33.9)	23 (45.1)
Current smoker, n (%)	126 (25.1)	152 (39.9)	71 (32.1)	25 (49.0)
Former smoker, n (%)	134 (26.7)	172 (44.0)	66 (29.9)	23 (45.1)
Medication[a]				
Oral anticoagulation, n (%)	4 (0.8)	27 (6.9)	16 (7.3)	14 (27.5)
Antiplatelet drugs[b], n (%)	6 (1.2)	384 (98.2)	193 (87.7)	34 (66.7)
Statins, n (%)	9 (1.8)	371 (94.9)	145 (65.9)	28 (54.9)
Blood pressure lowering drugs[c], n (%)	28 (5.6)	367 (93.9)	91 (41.4)	28 (54.9)

[a] At time of study inclusion and drawing of blood samples (after the event).
[b] Aspirin or clopidogrel.
[c] Diuretics, beta blockers, ACE inhibitors, calcium antagonists or α2-antagonists.