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Measurement of activated factor XII in health and disease

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We investigated a new ELISA for measuring activated factor XII (FXIIa) in plasma. The intra-assay coefficient of variation was 3.5% and 5.1% for plasma containing 2.5 and 8.2 ng/ml FXIIa. The inter-assay coefficient of variation was 6.2% and 6.6%. FXIIa correlated with age in women older than 55 years ($r = 0.55$, $P = 0.0003$). Mean levels in the whole population of 160 healthy individuals included in this study were not different between men and women, but women younger than 55 years had lower levels than older women and men of the corresponding age. In a group of 25 healthy centenarians FXIIa was significantly higher (3.2 ng/ml, 95% CI 2.3–3.6) than in controls (2.1 ng/ml, 95% CI 1.8–2.4). Increased levels were also found in pregnancy, with higher levels in the third trimester (4.7 ng/ml, 95% CI 3.9–5.5) than in the first trimester (2.9 ng/ml, 95% CI 2.2–3.9). FXIIa was unmeasurable in patients with FXII deficiency, but normal in patients with FXI deficiency and C1-inhibitor deficiency. FXIIa was significantly higher than in normal controls in patients with severe sepsis (3.9 ng/ml, 95% CI 2.8–5.4) and septic shock (5.4 ng/ml, 95% CI 3.7–7.7). After treatment with thrombolytic agents, a marked increase of FXIIa was found in patients with myocardial infarction. In conclusion, the immunoassay of FXIIa permits to study more directly the contact phase of blood coagulation in situations in which the involvement of this system may play a pathophysiological role.

Key words: Factor XII, contact activation, aging, pregnancy, sepsis, thrombolysis, C1-inhibitor deficiency.

Introduction

Activation and inhibition of the contact phase of blood coagulation are involved in the regulation of fibrinolysis, of the complement system, and in the generation of kinins.[1–6] Factor XII (FXII), the first component of the contact system, is a single-chain protein with a molecular weight of 80 kDa that circulates in human plasma as an inactive zymogen with a concentration of 30 μg/ml.[7] Activation of the zymogen results in an active enzyme with the features of serine proteases (FXIIa). FXIIa plays a central role in contact activation, through the limited proteolysis of prekallikrein[8] and the reciprocal activation of FXII by plasma kallikrein. Activation of FXII by kallikrein results in two enzymatic forms, an 80 kDa protein consisting of two disulfide-linked polypeptide chains (FXIIa or αFXIIa), and a 28–30 kDa fragment (βFXIIa).[8] αFXIIa binds to negatively charged surfaces and activates factor XI, whereas βFXIIa has no surface-binding properties but retains its ability to activate prekallikrein and C1. The major plasma inhibitor of FXIIa is C1 inhibitor (C1-INH) which forms covalent, bimolecular complexes with the protease.[10,11]

The recent availability of a facile immunoassay of FXIIa[12] allowed us to measure directly the central enzyme of the contact system in plasma of normal individuals, to establish the range of its concentrations, and to investigate age- and sex-related changes. Furthermore, we measured FXIIa in the plasma of individuals having clinical or physiological conditions thought to be associated with an activation of the contact system, such as sepsis,[14–16] congenital C1-INH deficiency (hereditary angio-

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edema),[17–19] pregnancy,[20] and acute myocardial infarction treated with thrombolytic therapy.[21]

Materials and methods

Blood sampling and processing

Venous blood was collected in siliconized Vacutainer tubes containing 0.13 M trisodium citrate, nine parts of blood to one part of trisodium citrate. Samples were centrifuged at 2000 × *g* for 15 min at room temperature to obtain platelet-poor plasma, frozen in aliquots in liquid nitrogen, and stored in polypropylene tubes at −80°C until analysis.

Enzyme immunoassay of activated factor XII

Plasma levels of FXIIa were measured with a sandwich ELISA (Shield Diagnostics Ltd, Dundee, UK). Undiluted plasma samples (100 μl) were incubated for 1 h at room temperature in antibody-coated microtiter plate wells. The monoclonal capture antibody is specific for FXIIa and binds only negligible amounts of zymogen FXII.[12,13] After washing, wells were incubated for another hour at room temperature with 100 μl of a sheep polyclonal antibody to FXII conjugated to alkaline phosphatase. Wells were developed for 15 min with 100 μl of the substrate phenolphthalein monophosphate. Absorbances were read at 550 nm on a Titertek Twinreader Plus (Flow Laboratories, Lugano, Switzerland), and results were calculated from a calibration curve of human FXIIa (0, 1, 5, 10, 20 ng/ml). More details on the method are published in Ref. 13.

Samples

Normals: plasma was obtained from 160 healthy individuals (mean age 52 ± 21 years, age range 21–99 years, 82 women and 78 men): blood donors, hospital staff members, and old people from a nursing home. None of the women was taking oral contraceptives. Individuals older than 70 years were all ambulant, self-sufficient and healthy on the basis of the criteria set by the Senieur Protocol,[22] which includes both physical examination and laboratory analysis. Every decade between 21 and 99 years of age was represented by approximately 20 subjects, equally split between men and women.

Centenarians: plasma was collected from 25 centenarians (age range 100–102 years, 16 women and 9 men), who were healthy according to the Senieur Protocol.[22]

Pregnancy: plasma from 14 pregnant women (mean age 29 ± 5 years) was collected both during the first and third trimester of normal gestation.

Congenital factor XI and factor XII deficiencies: plasma from six patients with factor XI deficiency (three severe and three moderate) and three patients with severe FXII deficiency was obtained.

C1-inhibitor congenital deficiency: plasma from seven patients (four women and three men, age range 24–68 years), with hereditary angioedema (HAE) was obtained. Their levels of functional C1-INH were 23 ± 17% of normal pooled plasma (range 2–59%).

Sepsis and septic shock: plasma samples from twelve leukemic patients with severe sepsis (mean age 48 ± 20 years) and 13 with septic shock (mean age 54 ± 15) were collected within the first 24 h after the onset of fever. According to established criteria,[23] severe sepsis was diagnosed if a febrile episode in the presence of suspected infection was associated to one or more signs of inadequate organ perfusion, such as elevated plasma lactate levels, hypoxemia or oliguria. Septic shock was diagnosed if, in addition, a sustained decrease in systolic blood pressure for at least 1 h was found.[24]

Acute myocardial infarction and unstable angina: samples from 28 patients with acute myocardial infarction and 28 with unstable angina (15 women and 41 men, mean age 65 ± 11 years, range 32–84 years) were included in the study. Thirty patients with myocardial infarction were also studied after a 90 min infusion of thrombolytic agents and/or heparin (in ten patients, streptokinase 1 500 000 IU plus heparin 5000 IU/bolus; in ten patients, recombinant tissue plasminogen activator 100 mg plus heparin 5000 IU/bolus; and heparin 5000 IU/bolus alone in ten additional patients). Criteria for diagnosis and the treatment regimens are described in detail elsewhere.[25,26]

Statistical analysis

The values were analyzed after log-transformation using the StatView (BrainPower Inc., Calabasas, CA, USA) software package. The normal range was defined as the 95% confidence intervals (95% CI). The relationship between variables was tested by linear regression analysis. Comparison between groups was performed by analysis of variance and between-groups differences were assessed with the Scheffe test; *P* values below 0.01 were considered statistically significant.

Results

Assay validation

The reproducibility of the assay was established by testing two plasma samples with different concentra-

tions of FXIIa (2.5 and 8.2 ng/ml) 13 times intra-assay, and ten times inter-assay. The coefficient of variation was 3.5–5.1% intra-assay and 6.2–6.6% inter-assay. Plasma from individuals with severe FXII deficiency (less than 1% of average normal plasma) gave no colour in the assay mixture, indicating little or no cross-reactivity of the antibody with other plasma proteins.

Age- and gender-related changes in FXIIa levels

The mean FXIIa level in 160 healthy individuals was 2.3 ng/ml (95% CI 2.2–2.5), with no significant difference between women and men. Age-related variability was studied by arbitrarily dividing women and men in two age groups: < 55 and > 55 years. The only significant difference was observed in women, FXIIa levels being lower in those younger than 55 years than in older women ($P = 0.0085$; Table 1). In younger women FXIIa levels tended to be lower than in the corresponding men age group but the difference did not reach statistical significance ($P = 0.053$). Linear regression analysis showed a significant correlation between FXIIa and age only in the group of women older than 55 years ($n = 40$, $r = 0.55$, $P = 0.0003$; Figure 1).

Definition of control groups

Because of these age- and gender-related changes in FXIIa, three different control groups were chosen for comparison with the various groups of patients. For analysis of centenarians and patients with sepsis or C1-INH deficiency, 50 controls (control group A; median age 47 years, 20 men and 30 women) were chosen with the goal of reproducing on a small scale the frequencies of distribution according to classes of age of the adult Italian population. Accordingly, 25% of them were 20–35 years old, 36% 36–50 years old and 39% 51–69 years old. The mean FXIIa level in this control group was 2.1 ng/ml (95% CI 1.8–2.4). Different age- and sex-matched controls were selected for pregnancy (control group B; 14 women; median age 29 years, range 22–38 years; FXIIa 2.0 ng/ml, 95% CI 1.3–2.8), and for acute myocardial infarction and unstable angina (control group C; 15 women and 41 men; median age 63 years, range 38–84 years; FXIIa 2.4 ng/ml, 95% CI 2.1–2.7).

Table 1. Geometric means (95% confidence intervals) of FXIIa levels (ng/ml) in relation to gender and age

All women ($n = 82$)	2.3 (2.0–2.6)
All men ($n = 78$)	2.4 (2.2–2.7)
P women *vs* men	n.s.
Women ≤ 54 years ($n = 42$)	1.9 (1.6–2.3)
Women ≥ 55 years ($n = 40$)	2.7 (2.3–3.1)
P older *vs* younger women	0.0085
Men ≤ 54 years ($n = 40$)	2.5 (2.1–3.0)
Men ≥ 55 years ($n = 38$)	2.4 (2.0–2.8)
P older *vs* younger men	n.s.
Centenarians ($n = 25$)	3.2 (2.2–3.9)
Control group A ($n = 50$)	2.1 (1.8–2.4)
P controls *vs* centenarians	0.0018

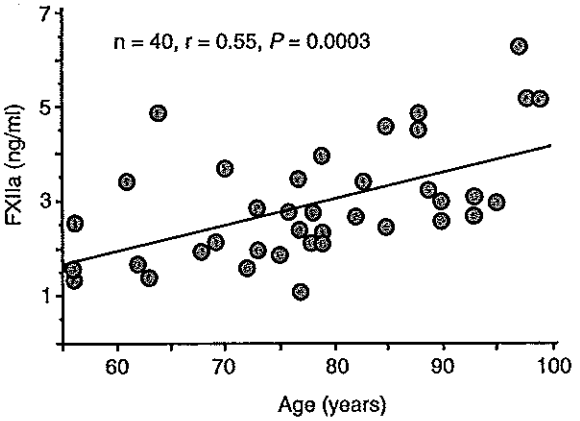

Figure 1. Correlation between plasma levels of activated factor XII and age in women older than 55 years ($n = 40$).

Centenarians

In 25 healthy centenarians, a significant increase ($P = 0.0018$) of FXIIa was observed (mean 3.2 ng/ml, 95% CI 2.2–3.90; Table 1).

Pregnancy

In 14 women during both the first and third trimester of gestation, levels of FXIIa were significantly increased compared with control group B (first trimester: mean 2.9 ng/ml, 95% CI 2.3–3.6, $P = 0.007$; third trimester: mean 4.7 ng/ml, 95% CI 3.9–5.5, $P = 0.0001$). The levels measured during the third trimester were significantly higher than those measured during the first trimester ($P = 0.0014$; Figure 2).

Contact system deficiencies

FXIIa levels were normal in six patients with congenital deficiency of FXI, those measured in three patients with severe FXII deficiency were undetectable (Table 2). Seven patients with C1-INH congenital deficiency, studied during a period of remission from angioedema, had normal plasma levels of FXIIa compared with age- and sex-matched controls (Table 2).

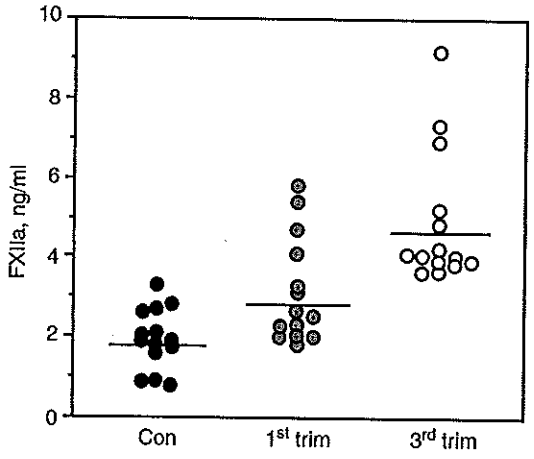

Figure 2. Plasma levels of activated factor XII in pregnancy. Con: controls; 1st trim: first trimester; 3rd trim: third trimester.

Table 2. Factor XIIa measured in patients with congenital deficiences of factor XI, factor XII and C1-inhibitor

Patient	Sex	Age	FXI activity (%)	FXII activity (%)	C1-INH activity (%)	FXIIa (ng/ml)
N.B.	F	28	< 1			2.9
G.R.	M	59	2			2.3
C.A.	F	56	< 1			4.9
F.A.	F	25	20			1.8
A.M.	M	15	20			2.3
P.P.	M	35	10			1.6
P.M.	M	46		< 1		unmeasurable
E.M.	F	39		< 1		unmeasurable
S.M.	F	49		< 1		unmeasurable
C.C.	F	68			17	1.5
L.G.A.	F	28			22	1.9
N.G.	M	42			2	2.5
R.S.	F	60			18	2.6
L.G.C.	M	24			18	2.0
M.A.	M	28			59	1.9
D.A.	F	57			22	4.1

Acute myocardial infarction and unstable angina

FXIIa levels in 28 patients with acute myocardial infarction (mean 2.2 ng/ml, 95% CI 1.8–2.7) and in 28 patients with unstable angina (mean 2.7 ng/ml, 95% CI 2.3–3.1) were not significantly different from control group C. FXIIa levels did not change after heparin infusion, while 90 min after thrombolysis with streptokinase or recombinant tissue plasminogen activator FXIIa showed a marked increase (Figure 3).

Sepsis and septic shock

Mean levels of FXIIa were not different between patients with sepsis and septic shock (mean 3.9 ng/ml, 95% CI 2.8–5.4; 5.4 ng/ml, 95% CI 3.7–7.7), but both were significantly higher than in the control group A ($P = 0.005$ and < 0.0001 respectively).

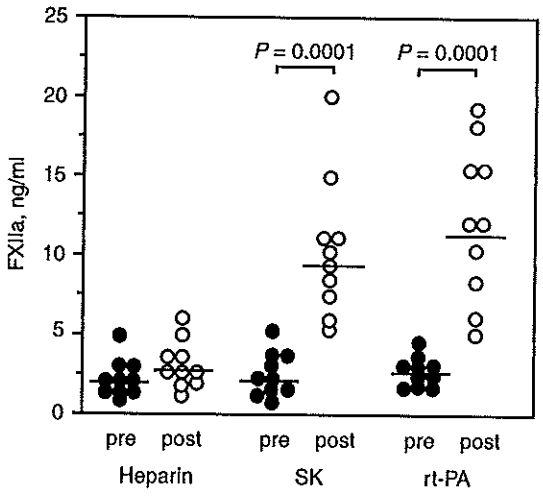

Figure 3. Plasma levels of activated factor XII in myocardial infarction before (pre) and after (post) heparin, streptokinase (SK), and recombinant plasminogen activator (rt-PA). Horizontal lines indicate geometric means, differences between post- and pre-treatment values were determined with the Scheffe test.

Discussion

The intra- and inter-assay coefficients of variation of this FXIIa assay indicate a good reproducibility. The undetectable levels of FXIIa in severe FXII deficiency provide an indirect demonstration of the specificity of the assay.

Plasma levels of FXIIa were significantly lower in women younger than 55 years than in older women. Based on the same study group of 160 normal individuals, we previously reported that FVIIa increases with age mainly in women,[27] and it has been suggested that hormonal changes induced by menopause may play a role in this phenomenon. Since *in vitro* studies have shown that FXIIa can activate FVII,[28] the increase with age of both FXIIa and FVIIa in post-menopausal women would indicate that the plasma levels of the two enzymes are related and may recognize common regulatory mechanisms. In support of this hypothesis, we found that FXIIa and FVIIa plasma levels in women older than 55 years were significantly correlated ($n = 40$, $r = 0.52$, $P = 0.0009$; data not shown). Like FVIIa and other coagulation enzyme markers, FXIIa levels are elevated in centenarians, confirming that there is a state of heightened activity of coagulation enzymes in these individuals.[29]

FXIIa levels in plasma of women in late pregnancy were higher than in women during early pregnancy. These results are in agreement with previous reports which showed an increased activation of FXII in late pregnancy.[20] Moreover, we found that FXIIa levels were significantly higher than in the age- and sex-matched control group already in the first trimester, indicating that contact system activation occurs early in pregnancy.

An activated contact system participates in inflammation through generation of kinins, interaction with complement system and stimulation of neutrophils.[7] Recently, the involvement of the complement and kinin systems was demonstrated after thrombolytic therapy in patients with acute myocardial infarction.[21] In addition, plasmin-mediated activation of purified FXII was demonstrated *in vitro*.[30] Our data are consistent with these results, showing that FXIIa levels, normal in untreated unstable angina or acute myocardial infarction, increased after thrombolytic therapy, when plasmin and kallikrein form in large amounts in plasma.[21] It is unclear why higher levels of FXIIa were found after recombinant tissue plasminogen activator than after streptokinase. Heparin therapy did not significantly affect plasma levels of FXIIa in patients with myocardial infarction, in agreement with *in vitro* studies showing that heparin does not induce activation of the contact system.[31]

Several studies have reported activation of the contact system in sepsis, using different indirect methods: evaluation of the ratio between activity and antigen of the various components,[32] measurement of cleavage products,[15] and quantification of complexes between proteases and inhibitors.[14,16] With our approach, which consists in measuring directly the central enzyme of the contact system, a significant increase in the plasma levels of FXIIa was observed both in severe sepsis and in septic shock. These findings are consistent with the involvement of FXII activation in the pathophysiology of sepsis, but since no significant differences were observed between patients with severe sepsis and with septic shock, the assay does not seem to have a prognostic value in this setting.

Hereditary angioedema is caused by functional deficiency of C1-INH,[33] inherited as an autosomal dominant trait. Symptoms are represented by localized increases in vascular permeability that only recur occasionally. Physical trauma, a potential contact system-activating stimulus, frequently acts as a triggering factor. The activation of the contact system during acute attacks of edema in C1-INH deficiency has been surmised on the basis of the presence of markers of activation such as α_2 macroglobulin–kallikrein complexes[14] and cleavage of high molecular weight kininogen,[17–19] whereas knowledge on the status of contact system activation during remission is relatively poor.[19,34] Our findings of normal levels of FXIIa during remission in HAE indicate that low levels of C1-INH *per se* do not affect FXIIa plasma levels. So far, we had no opportunity to measure FXIIa during acute attacks of HAE.

In conclusion, the immunoassay of FXIIa is a useful tool to study the role of the contact system in health and disease. The assay is simple and reproducible, and results are altered in situations that epitomize contact system activation, such as sepsis and thrombolytic therapy. The clinical value of the assay remains to be further defined by studies in larger populations and in other clinical conditions.

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