Increased factor XI but not factor XII is associated with enhanced inflammation and impaired fibrin clot properties in patients with eosinophilic granulomatosis with polyangiitis

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Abstract

Objective

In eosinophilic granulomatosis with polyangiitis (EGPA) a prothrombotic state, including formation of denser fibrin networks with reduced lysability has been observed. Little is known about the intrinsic pathway in EGPA. We investigated whether coagulation factors (F)XI and FXII are associated with eosinophil-driven prothrombotic state.

Methods

In 34 consecutive EGPA patients with remission we assessed FXI and FXII levels along with plasma fibrin clot permeability (Ks), fibrin clot morphology using scanning electron microscopy, and efficiency of fibrinolysis, expressed as lysis time (t50%) and maximum rate of increase in D-dimer levels (D-D_{rate}).

Results

Increased FXI level (>130%, the upper reference limit) was found in 8 (23.5%) patients. Compared to patients with FXI levels ≤130%, those with increased FXI had higher eosinophil count (+365%) and reduced percentage of neutrophils (-20.4%), along with reduced K_s (-20.5%). In patients with FXI>130% clots were composed of thinner fibrin fibres (-17.5%). FXI was not associated with C-reactive protein and fibrinogen levels or anti-neutrophil cytoplasmic antibodies titers. There were no correlations between FXI and FXII levels as well as between FXII and eosinophil count (all p>0.05).

Conclusion

To our knowledge, this study is the first to show association between FXI and a prothrombotic state in EGPA. Given clinical trials on FXI inhibition as an antithrombotic option, our findings suggest that this therapeutic approach could be useful in diseases with hypereosinophilia.

Key words

eosinophilic granulomatosis with polyangiitis, factor XI, factor XII, neutrophils

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Introduction

Eosinophilic granulomatosis with polyangiitis (EGPA) is a rare disease defined as an eosinophil-rich and necrotising granulomatous inflammation, involving the respiratory tract associated with asthma and eosinophilia and necrotising vasculitis in small and medium blood vessels (1-3). It has been reported that venous thromboembolism (VTE) occurs in EGPA patients, especially during the active phase of the disease, and its rate was about 8% (4-6). EGPA patients were characterised by persistent prothrombotic state, expressed by enhanced thrombin generation capacity and unfavourably modified fibrin clot properties, both during disease exacerbation and in remission (6, 7). Chronic inflammation, peripheral blood hypereosinophilia, and coexisting heart failure (HF) have been suggested as possible causes of thromboembolism in EGPA patients (8). Eosinophils store and can rapidly translocate TF to the cell membrane (9), expressing low amounts of TF, which can enhance thrombin generation amplification by FXI (10). Epidemiologic studies showed that high FXI levels are associated with VTE and ischaemic stroke incidence as well as according to the SMILE study with the risk of MI in men (11, 12). Moreover, circulating active FXI predicted cardiovascular mortality in patients with advanced coronary artery disease (13).

FXI has also been shown to decrease inflammatory response, including reduced eosinophils and neutrophils chemotaxis, trafficking, and decreased synthesis of proinflammatory cytokines in mouse models (14, 15). Moreover, recent data obtained using HF mouse model with preserved ejection fraction indicated that liver-derived FXI can protect against HF (16). In the intrinsic pathway the activation of FXII leads to FXI activation. FXII, in contrast to FXI, has been reported to exert proinflammatory actions via activating monocytes to secrete interleukin 1, neutrophils to degranulate, and complement activation (17).

Given rapidly growing evidence for the benefits from FXI inhibition in arterial and venous thrombosis prevention in various clinical settings (18), the aim of our work was to investigate associations between circulating FXI and FXII levels and a prothrombotic state, including fibrin clot properties in EGPA patients in remission.

Materials and methods

We enrolled 34 consecutive white patients with EGPA. The Bioethics Committee of the Jagiellonian University approved the study protocol and informed written consent was obtained from the participants in accordance with the Declaration of Helsinki. The patients were enrolled in the study if they met the following criteria: the EGPA diagnosed according to the American College of Rheumatology (ACR) classification criteria (1990), age over 18 years, and clinically stable disease according to the Birmingham Vasculitis Activity Score version 3 (BVAS v.3) (19, 20). The exclusion criteria were: any acute illness, cancer, hepatic injury, chronic kidney disease stadium 4 or more, and current anticoagulant therapy. We collected clinical data based on the EGPA, BVAS v.3, Vasculitis Damage Index (VDI), Five-Factor Score (FFS), and the Asthma Control Test (ACT), which correlates with asthma control according to the Global Initiative for Asthma (GINA) guidelines (21).

Laboratory investigations

After an overnight fast blood was collected to determine blood cell count, glucose, and creatinine using routine laboratory assays. Plasma fibrinogen was measured by the Clauss method and C-reactive protein (CRP) by nephelometry (Siemens, Munich, Germany). Eosinophil count was determined manually with a Bürker's chamber. FXI and FXII levels were determined using one-stage coagulometric assays using factor-deficient plasma on a Siemens BCS XP analyzer (Siemens Healthcare Diagnostics Products GmbH).

Antineutrophil cytoplasmic antibodies (ANCA) were measured using an immunofluorescence (Euroimmun, Lubeck, Germany). The further identification of anti-proteinase (PR) 3 and anti-myeloperoxidase (MPO) antibodies was performed by the immu-

noenzymatic assays (ELISA anti-PR3 and ELISA anti-MPO; Euroimmun, Lubeck, Germany).

Fibrin clot properties

Fibrin clot permeation was assessed as previously described (7). Briefly, plasma samples were recalcified with 20 mmol/L calcium chloride and then 1 U/mL human thrombin (Sigma-Aldrich, St. Louis, MO, USA) was added. After 2 h of incubation at room temperature, tubes containing the clots were connected via plastic tubing to a reservoir of a Tris buffer (0.05 M Tris-HCI, 0.15 M NaCL, pH 7.5) and its volume flowing through the gels was measured. A permeation coefficient (K_s), which indicates the pore size, was calculated from the equation: $K_s=QxLx\eta/txAx\Delta p$, where Q is flow rate in time t, L is the length of fibrin gel, ŋ is the viscosity of liquid, A is the cross-section area, Δp is differential pressure in dyne/cm^2.

Plasmin-mediated fibrinolysis was evaluated using two assays as previously described (7). In the first assay fibrin clots, formed as described above, were perfused with the same buffer containing 0.2 μmol/L recombinant tissue plasminogen activator (rtPA; Boehringer Ingelheim, Ingelheim, Germany). The lysis rate was determined by measuring D-dimer (American Diagnostica, Stanford, CA, USA), a marker of fibrin degradation, in the effluent every 20 min. Maximum rates of increase in D-dimer levels (D-D_{rate}) detected at 80 or 100 min were analysed.

In the second assay, 100 μL of citrated plasma were diluted with 100 μL of a Tris buffer, containing 20 mmol/L calcium chloride, 1 U/mL human thrombin (Sigma-Aldrich, St. Louis, MO, USA), and 1 μg/mL rtPA. Assembly kinetics was monitored by spectrophotometry at 405 nm. The time required for a 50% decrease in clot turbidity ($t_{50\%}$) was a marker of the clot susceptibility to fibrinolysis.

Scanning electron microscopy

Scanning electron microscopy (SEM) was performed as previously described (7). Briefly, fibrin clots were fixated in 2.5% glutaraldehyde, dehydrated in graded ethanol solutions, dried by the critical point procedure, and sputter coated with gold. Finally, clots were scanned in ten randomly selected areas taken from different clot parts (microscope HITACHI S-4700). We analysed fibrin clots from 8 patients with FXI>130% and 8 clots from 8 patients with FXI≤130%. Fibrin diameter based on 40–50 fibres per clot was evaluated using ImageJ software (US National Institutes of Health, Bethesda, MD, USA) at magnification of ×5,000.

Ethics

The Bioethics Committee of the Jagiellonian University approved the study protocol (protocol no. KBET/139/B/2010 of December 18, 2014). Written informed consent was obtained from the participants in accordance with the Declaration of Helsinki.

Statistics

Data were expressed as numbers (percentage) for categorical variables and mean±SD or median (Q1-Q3) for continuous variables, as appropriate. The Shapiro-Wilk test was used to assess normality. Categorical variables were compared using the Pearson's Chi-squared test or Fisher's exact test as appropriate. The two groups were compared using the Mann-Whitney U-test with the Holm adjustment for multiple comparisons. Differences among three groups were compared by the one-way ANOVA or Kruskal-Wallis test depending on the equality of variances and data distribution. Post-hoc comparisons were performed with the Tukey-Kramer HSD test. The Spearman's rank correlation coefficient was calculated to assess correlations between continuous variables. P-value <0.05 was considered statistically significant. The analysis of the power of statistical tests used was performed. The comparison of the studied groups using the Student's t-test for unrelated variables showed that at the significance level of p=0.05, it allows to detect differences equal to 0.7 of standard deviation between the means with a probability of 80%. Regarding the correlations analysis in the group of 34 patients, the 80% power of the test is achieved with correlation coefficients of 0.40. Statistical analysis was performed using STATISTICA 13 software (StatSoft STATISTICA, Poland 2022).

Results

All enrolled EGPA patients were in remission of the disease. Median duration of EGPA was 36 (5-144) months, while median duration of asthma was 72 (36-144) months. The median BVAS v.3 score was 2. As many as 7 patients (20.5%) had score 1, 10 patients (29.4%) had score 2, 8 patients (23.5%) had score 3, 7 patients (20.6%) had score 4, while 2 EGPA patients (5.9%) had score 5. Regarding the FFS scale the median score was 0. In details, 26 EGPA patients (76.5%) had score 0, 7 patients (23.8%) had score 1, and 1 patient (2.94%) had score 2. The median VDI score was 3. The VDI equal to 1 was calculated for 1 patient (2.94%), equal to 2 for 11 patients (32.4%), equal to 3 for 9 patients (26.5%), equal to 4 for 6 patients (17.6%), equal to 5 in 5 patients (14.7%) and equal to 6 for 2 (5.88%). As many as 12 (35.3%) patients were ANCA-positive, including 5 (41.7%) with pANCA and 7 (58.3%) with cANCA. A VTE history was present in 3 (8.8%) patients.

Factor XI

The median FXI level in the whole EGPA group was 111 [98-130]%. Neutrophil (p=0.0055 for ANOVA, Fig. 1A) and eosinophil (p=0.0012 for ANOVA, Fig. 1B) counts as well as K_s (p=0.0052 for ANOVA, Fig. 1C) differed with regard to FXI levels categorised below 100%, between 100-130% and above 130%, while $t_{1/2}$ tended to be prolonged in patients with FXI >130% (p=0.059 for ANOVA, Fig. 1D). FXI level exceeding 130% was found in 8 (23.5%) patients. FXI levels exceeding 130% were more often observed in ANCA-positive patients (p=0.044) but FXI levels were not associated with antibodies titer. Patients with FXI levels >130% had 365% higher eosinophil count, 32.7% higher platelet count, and 20.4% reduced percentage of neutrophils (Table I). Increased FXI levels were also associated with 20.5% reduced K_s, 15.2% prolonged $t_{1/2}$, and with 11.8% reduced D-D_{rate} with no difference in fibrinogen levels (Table I). After adjust-

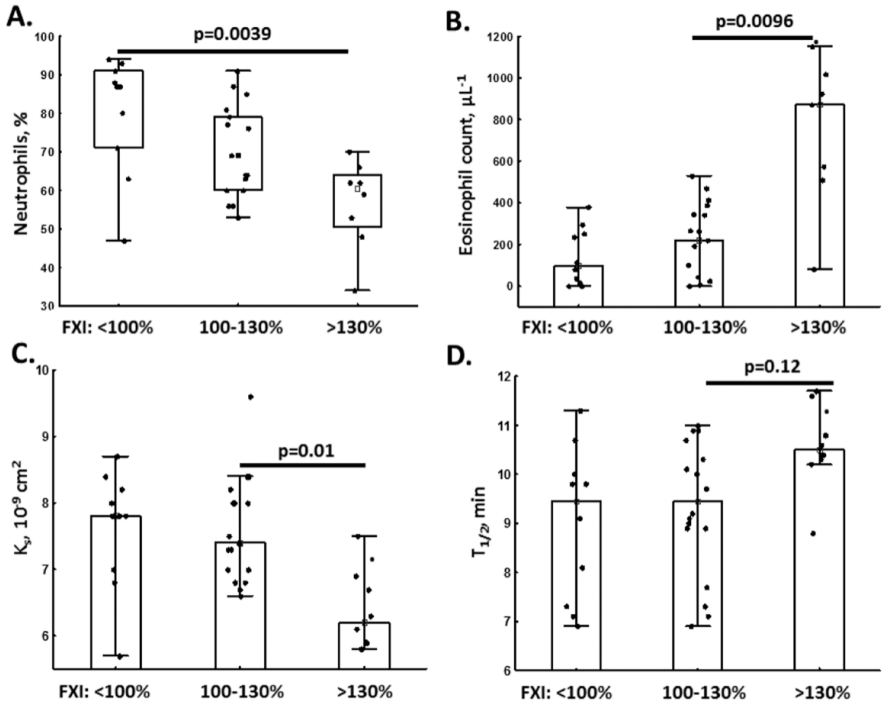

Fig. 1. Neutrophil (panel A) and eosinophil counts (panel B), fibrin clot permeability (K_s, panel B), and time to half lysis ($t_{1/2}$, panel C) in EGPA patients with factor (F)XI levels <100% *vs*. 100-130%, and >130%.

Table I. Characteristics of EGPA patients stratified according to factor (F)XI levels.

	EGPA patients with FXI >130% n=8	EGPA patients with FXI ≤130% n=26	*p*	*p* adjusted*
Age, years	46.5 (37.0-53.0)	52 (39-57)	0.69	-
Women, n (%)	3 (37.5)	6 (23.1)	0.39	-
Cigarette smoking (current/past), n (%)	1 (12.5)	6 (23.1)	0.55	-
Heart failure, n (%)	0	4 (15.4)	0.32	-
Coronary artery disease, n (%)	0	4 (15.4)	0.32	-
Arterial hypertension, n (%)	4 (50)	11 (42.3)	0.70	-
VTE history, n (%)	0	3 (11.5)	0.43	-
Laboratory tests				
WBC, 10^3/uL	9.5 (8.6-10.5)	7.2 (5.3-8.8)	0.067	0.34
Neutrophils, %	60.5 (50.5-64.0)	76 (64-87)	0.0061	0.046
Eosinophil count, μL^{-1}	898 (543-1084)	193 (37-343)	0.0001	0.001
Lymphocytes, %	27 (21-39)	18 (9-27)	0.034	0.24
IgE, IU/mL	45.0 (19.3-151.5)	54.5 (18.7-99.6)	0.89	0.99
Platelets, x10^3/µL	288 (234-366)	216 (187-255)	0.046	0.28
Fibrinogen, g/L	3.9 (3.3-4.5)	3.3 (2.7-4.0)	0.52	0.99
CRP, mg/L	1.61 (0.68-5.26)	1.65 (0.36-5.54)	0.77	0.99
D-dimer, ng/mL	419 (243-576)	304 (221-481)	0.69	0.99
K_s, x10^{-9}cm^2	6.2 (5.9-6.8)	7.8 (7.0-8.2)	0.0006	0.007
$T_{1/2}$, min	10.5 (10.3-11.2)	9.2 (7.7-10.3)	0.017	0.14
D-D_{rate}, mg/L/min	0.064 (0.061-0.069)	0.072 (0.070-0.075)	0.012	0.11

*Holm adjustment for multiple comparisons.
EGPA: eosinophilic granulomatosis with polyangiitis; VTE: venous thromboembolism, WBC: white blood cell count; CRP: C-reactive protein; K_s: fibrin clot permeability; $T_{1/2}$: time to half lysis; D-D_{rate}: maximum rate of increase in D-dimer levels in the clot lysis assay.

ment for multiple comparisons, solely differences in neutrophil and eosinophil counts as well as in K_s remained significant (Table I). SEM analysis showed that patients with FXI>130% had 17.5% thinner fibrin fibres compared with those who had FXI≤130% (85.8±11.4 *vs*. 104±13.2 nm, *p*<0.0001) (Fig. 2).

In the whole EGPA group FXI correlated positively with eosinophil count (rho=0.65, *p*<0.0001), percentage of lymphocytes (rho=0.48, *p*=0.0036), and negatively with neutrophil count (rho=-0.57, *p*=0.0003). FXI levels were also associated with K_s (rho=-0.43, *p*=0.01).

Factor XII

The median level of FXII in the whole group of EGPA patients was 108 [98-121]% (min-max. 74-145%). Patients with FXI>130% compared to those with FXI≤130% had similar FXII level (109 [97-119] *vs*. 107 [98-121]%, *p*=0.98). FXII showed no association with sex, body-mass index, eosinophil count or ANCA titers (all *p*>0.05). Moreover, we found no associations between FXII and fibrinogen (rho=0.17, *p*=0.31), K_s (rho=-0.07, *p*=0.70), $t_{1/2}$ (rho=-0.05, *p*=0.77), and D-D_{rate} (rho=0.95, *p*=0.59).

Discussion

Our study is the first to show that increased FXI level in EGPA patients is linked with prothrombotic fibrin clot phenotype and formation of thinner fibrin fibres. Moreover, increased FXI level was positively associated with eosinophil count but it limited neutrophil influx, while FXII showed no similar associations. FXII is involved in the intrinsic coagulation pathway, however its contribution to the prothrombotic state in EGPA patients seems to be of minor importance.

Previously, an antithrombotic effect of decreased FXI levels has been suggested in patients with coronary artery disease treated with a high-dose statin (22), precisely the reduction of FXI plasma levels was associated with both increased clot permeability and shortened clot lysis time in this group of patients. Moreover, severe FXI deficiency in humans was associated with delayed clot formation and surprisingly with prolonged clot lysis time in thromboelastometry, which is however in line with a lack of correlation between the severity of bleedings and FXI levels (23), probably related to thrombin-activatable fibrinolysis inhibitor (TAFI) pathway impairment (24).

Our previous data also showed associations between prothrombotic fibrin clot

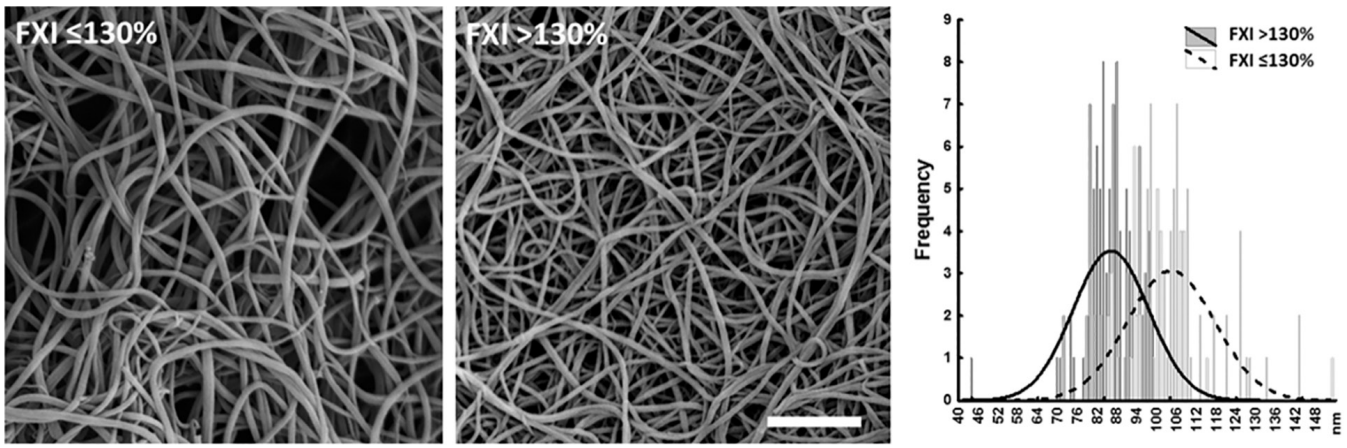

Fig. 2. Representative scanning electron microscopy images of plasma fibrin clots of EGPA patients with plasma FXI levels above or below 130% along with a histogram presenting fibrin fiber diameter frequency in both subgroups.
Magnification, 5000x. Scale bar, 200 nm.

phenotype, enhanced thrombin generation, and increased eosinophil count in EGPA patients (7). The current observation that increased FXI levels contribute to eosinophil-driven prothrombotic state may indicate that FXI is an important regulator of eosinophil-associated fibrin clot properties impairment. Moreover, in our opinion the frequency of increased FXI levels associated with elevated eosinophil count in EGPA patients reported here (about 25%) may, at least in part, explain up to 30% increased risk of thrombosis associated with hypereosinophilia in this disease observed previously (25). On the other hand, FXI-deficient mice showed increased influx of eosinophils into the lungs mediated by increased levels of eosinophil chemoattractant, along with enhanced allergic response (11), while in our study higher eosinophil count was associated with increased FXI levels. This discrepancy may be due to that in EGPA eosinophils play a pivotal role in all three stages of the disease pathology, constantly secreting several cytokines and chemokines, such as eotaxins or eicosanoids, which chronically activate immune system and coagulation (26). This in turn may lead to an increase in FXI level. Moreover, increased number of eosinophils, expressing TF, may contribute to thrombin generation amplification and the subsequent FXI activation. We found no associations of FXII with FXI and fibrin clot properties in EGPA patients. This observation may additionally support our hypothesis that hypereosinophila predisposes to higher FXI activation and more prothrombotic clot phenotype observed in EGPA patients, however, further studies are needed.

Another important issue is that in our study increased FXI levels were associated with the decreased number of neutrophils. It has been shown that neutrophils or mediators they release, can contribute to the procoagulant state and that this action is mediated to a greater extent by FXI than by FXII (27). Moreover, FXI-deficiency in mice exhibited reduced coagulation activation and leucocyte migration associated with increased survival during microbial-induced severe peritonitis (15). Binding of FXIa to human neutrophils also reduced their chemotaxis to the inflammation site (15). Thus, the previously reported protective effect of FXI (15, 16), in EGPA patients may be reflected by limited number or activation of neutrophils. Besides the contribution of neutrophils in the inflammatory response, they also modulate prothrombotic state through the release of a few active proteases, such as cathepsin G that activates platelets, FVII, FV, and FXI (15). Therefore, we hypothesise that reduced neutrophil count associated with increased FXI may represent a potential compensatory mechanism to attenuate the crosstalk between inflammation and coagulation activation in EGPA. This issue remains however further investigation.

This study has several limitations. First, the study group was limited, however EGPA is a rare condition (1, 8), therefore the associations presented here do not necessarily mean the cause-effect relationship and this is a hypothesis-generating study. Second, we did not assess concentration of proteins released by eosinophils as well as TF. However, our previous study showed increased thrombin generation potential in EGPA patients and a positive association between eosinophil count and eosinophil cationic protein levels (7).

In conclusion, our study is the first to show that in EGPA patients in remission of the disease increased FXI but not FXII is associated with the prothrombotic state and eosinophilic inflammation. Follow-up studies are needed to elucidate whether elevated FXI can contribute to higher incidence of thromboembolic events in EGPA patients, and if the reduced neutrophil count could be a potential compensatory mechanism. It would also be of interest to investigate if FXI inhibitors could improve clot phenotype in EGPA patients.

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