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Thrombin generation in rheumatoid arthritis: dependence on plasma factor composition

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Summary

Background—Growing evidence indicates that rheumatoid arthritis (RA) is associated with an increased risk for thromboembolic cardiovascular events.

Objectives—We investigated thrombin generation profiles in RA patients and their dependence on plasma factor/inhibitor composition.

Patients/Methods—Plasma factor (F) compositions (FII, V, VII, VIII, IX, X), antithrombin and free tissue factor pathway inhibitor (TFPI) from 46 consecutive RA patients with no cardiovascular events (39F, 7M, aged 57 [range, 23–75] years; DAS28 [Disease Activity Score] 5.2±1.1) were compared with those obtained in age- and sex-matched apparently healthy controls. Patients on anticoagulant therapy were excluded. Using each individual's plasma coagulation protein composition, tissue factor-initiated thrombin generation was assessed both computationally and empirically.

Results—RA patients had higher fibrinogen (4.18 [IQR 1.09] vs 2.56 [0.41] g/l, $p<0.0001$), FVIII (226±40 vs 113±15%, $p<0.001$), PC (107 [16] vs 100 [14]%, $p<0.001$), and free TFPI levels (22.3 [2.2] vs 14.7 [2.1] ng/ml, $p<0.001$). DAS28, but not age, RA duration, or C-reactive protein, was associated with FV, FVIII, FIX, FX, antithrombin, and free TFPI (r from 0.27 to 0.48, $p<0.05$). Intergroup comparison of computational thrombin generation profiles showed that in RA patients, maximum thrombin levels ($p=0.01$) and the rate of thrombin formation ($p<0.0001$) were higher, whereas the initiation phase of thrombin generation ($p<0.0001$) and the time to maximum thrombin levels ($p<0.0001$) were longer. Empirical reconstructions of the populations reproduced the thrombin generation profiles generated by the computational model.

Conclusions—Simulations of thrombin formation suggest that blood plasma composition, *i.e.* a marked increase in FVIII, somewhat counterbalanced by free TFPI, contributes to the prothrombotic phenotype in RA patients.

Keywords

inflammation; plasma composition; prothrombotic phenotype; rheumatoid arthritis; tissue factor pathway inhibitor; thrombin generation

Introduction

Growing evidence indicates that rheumatoid arthritis (RA) is associated with higher mortality and an increased risk for myocardial infarction and ischemic stroke that often occurs at a younger age than in the general population, although traditional demographic and cardiovascular risk factors significantly contribute to this heightened risk in RA.[1–3] Moreover, enhanced systemic inflammation in RA appears to represent an additional risk factor essential to accelerate atherogenesis and promote unstable plaque formation in coronary arteries.[4]

Aside from well established risk factors, it has been shown that RA is characterized by increased platelet count, together with elevated platelet activation markers and their subsequent expression on platelets.[5] Some investigators,[6]but not all, [5]suggested that RA is associated with increased thrombotic potential, reflected by increased levels of thrombin markers such as thrombin-antithrombin complexes and prothrombin fragments 1+2. Elevated fibrinogen, von Willebrand factor and D-dimer, that are mostly driven by systemic cytokine elevation, are observed in RA patients even during remission.[4,7] Impaired fibrinolysis combined with increased antithrombin levels have also been reported in RA.[8]Elevated levels of circulating tissue factor (TF) have been correlated with tissue factor pathway inhibitor (TFPI) activity in RA patients [6] along with elevated factor (F) VIII levels.

It has been postulated that hemostatic alterations, thrombotic in nature, might be involved in life-threatening manifestations of cardiovascular disease within the RA population. Recently, a 2-fold higher relative risk for pulmonary embolism and/or deep vein thrombosis has been found to be associated with RA.[9] To the best of our knowledge, however, no comprehensive analysis of coagulation factors and inhibitors, as well as thrombin generation in a RA population has been published. In this study, we sought to investigate thrombin generation profiles computationally and empirically in RA patients and their dependence on plasma factor/inhibitor composition.

Materials and Methods

Materials

HEPES and EDTA were purchased from Sigma Chemical Co (St. Louis, MO). Human coagulation FII, FVII, FIX and FX, were isolated from fresh frozen plasma and were purged of trace contaminants and traces of active enzymes using previously published methods[10, 11]. Human FV and antithrombin were isolated from freshly frozen plasma [12,13]. Recombinant FVIII and recombinant TF (residues 1–242) were provided as gifts from Drs. Liu and Lundblad (Hyland division, Baxter Healthcare Corp, Duarte, CA). Recombinant human FVIIa was provided as a gift from Dr. Hedner (Novo Nordisk, Denmark). Recombinant full-length TFPI was provided as a gift from Dr. Johnson (Chiron Corp, Emeryville, CA). The preparation of the TF/lipid reagent was performed as described elsewhere [14]. 1,2-Dioleoyl-*sn*-Glycero-3-Phospho-L-Serine (PS) and 1,2-Dioleoyl-*sn*-Glycero-3-Phosphocholine (PC) were purchased from Avanti Polar Lipids, Inc (Alabaster, AL). Phospholipid vesicles (PCPS) composed of 25% PS and 75% PC were prepared as described [15]. Spectrozyme TH was purchased from American Diagnostica, Inc (Greenwich, CT).

Study Population

Forty-six consecutive Caucasian patients who fulfilled the American College of Rheumatology criteria for RA and 46 apparently healthy control subjects matched for age and sex (recruited from the hospital staff) were included in the study. Exclusion criteria were

as follows: any acute illness, cancer, hepatic or renal dysfunction (creatinine >120 $\mu\text{mol/l}$), diabetes mellitus, previous myocardial infarction, stroke or another acute vascular event, venous thromboembolism and current anticoagulant therapy. At the time of the blood collection, patients with RA were treated with methotrexate 7.5–20 mg/week (n=32, 69.6%), prednisone 2.5–15 mg/d (n=35, 76.1%), nonsteroidal anti-inflammatory drugs (NSAIDs; n=36, 78.3%), disease modifying antirheumatic drugs (DMARDs; n=16, 34.8%), and statins (n=8, 17.4%).

Disease activity in RA patients was measured by the number of tender joints (out of 68 joints), number of swollen joints (out of 66 joints), assessing the duration of morning stiffness, patient's global assessment of disease activity, physician's global assessment of disease activity and patient's assessment of pain using 100 mm visual analogue scales (VAS). A Disease Activity Score (DAS 28) was calculated for all individuals. The University Ethical Committee approved the study, and patients provided written, informed consent.

Blood Collection

Blood samples were collected from fasting individuals into 0.1 volume of 3.2% trisodium citrate from an antecubital vein with minimal stasis on the same day that clinical data were recorded. Citrated blood samples were centrifuged within 15 minutes of collection at 3000 g for 20 minutes and stored in aliquots at -80°C until further use. Lipid profiles, blood cell counts, glucose, creatinine, and C reactive protein (CRP) were assayed by routine laboratory techniques.

Coagulation Protein Analyses

Fibrinogen was determined using the Clauss method. Free TFPI was determined by ELISA (Diagnostica Stago, Asnieres, France). FII, FV, FVII, FVIII, FIX, FX were measured by one-stage clotting assays using factor-deficient plasmas (Dade Behring Liederbach, Germany). Antithrombin activity was measured using Berichrom (Dade Behring). Protein C (PC) activity was measured using a chromogenic substrate assay (Dade Behring).

Computational Model

The current mathematical model is based upon previous publications[16,17]and yields concentration versus time profiles for selected species when electronic mixtures of procoagulant FII, FV, FVII/FVIIa, FVIII, FIX and FX, and anticoagulants TFPI and antithrombin are exposed to 5 pM TF. All procoagulant and anticoagulant protein levels were determined for each individual. Factor levels expressed as a percentage were translated into molar concentrations by using literature values for mean plasma concentrations. Each individual's plasma factor concentration was entered into the computer database, and simulated reactions were initiated with TF. Simulated reactions were solved for active thrombin over 1200 s. The outputs of these active thrombin curves are evaluated by parameters that describe the initiation, propagation and termination phases of thrombin generation: maximum level of thrombin generation, maximum rate of thrombin generated, time to 10 nM thrombin (clot time), time to maximum level of thrombin generated and total thrombin generated (area under the curve, AUC). [16,17]

The contribution of each individual factor to the overall thrombin generation profile of the RA population was evaluated by successively holding each protein (eight total) at their mean physiologic value while the other seven were used at their mean RA population value. We evaluated the outcomes by comparing the magnitude of the shift in the thrombin generation profile induced by normalizing each factor individually as previously described. [18]

Synthetic Coagulation Proteome Model

The procedure used was a modification of Lawson *et al.* [19] and van 't Veer *et al.* [11]. Briefly, two solutions were prepared: I. Procofactor solution. Relipidated TF (10 pM; molar ratio PCPS:TF=5000) is incubated with PCPS (100 μ M) in HBS (20 mM HEPES and 150 mM NaCl, pH 7.4) and CaCl_2 (2 mM) with 0.1% PEG for 10 min at 37 °C. Prior to the initiation of the reaction, FV and FVIII are added; II. Zymogen-inhibitor solution. FII, FVII, FVIIa, FX, FIX, TFPI and antithrombin are preheated for 3 min at 37 °C in HBS, 2 mM CaCl_2 with 0.1% PEG at twice their desired concentrations. For the comparison of RA to the control population we used the mean values presented in the Table. Factor levels were translated into Molar (M) concentrations using literature values for the mean plasma concentrations [20].

The reaction is started by mixing equal volumes of both Ca^{2+} preequilibrated solutions resulting in physiological concentrations of the zymogens, pro-cofactors and inhibitors, 5 pM TF, 2 mM CaCl_2 and 50 μ M PCPS. Following the start of the reaction, at selected time points, 10 μ l aliquots are withdrawn from the reaction mixture and quenched in 20 mM EDTA in HBS (pH 7.4) with 0.1% PEG containing 0.2 mM Spectrozyme TH and assayed immediately for thrombin activity. The hydrolysis of the substrate is monitored by the change in absorbance at 405 nm using a V_{max} spectrophotometer (Molecular Devices Corp., Menlo Park, CA). Thrombin generation is calculated from a standard curve prepared by serial dilutions of α -thrombin.

Statistical Analysis

Data are expressed as mean $\pm$ SD or median (interquartile range). The Kolmogorov-Smirnov test was used to assess conformity with a normal distribution. Categorical values were analyzed using the χ^2 test or Fisher's exact test as appropriate. Continuous variables were compared by the Student *t* test when normally distributed or by the Mann-Whitney U test for non-normally distributed variables. Correlations between the individual parameters were calculated using the Pearson or Spearman rank correlation as appropriate. A p-value <0.05 was considered statistically significant.

Results

A total of 4s6 RA patients (39F, 7M, aged 57 [range, 23–75] years) were analyzed. A median duration of RA was 5 (IQR 9.0) years. Most patients were categorized as functional class 2 (76.1%). Radiological classes 1 to 4 were given to 11 (23.9%), 22 (47.8%), 11 (23.9%), and 2 (4.3%) patients, respectively. DAS28 was 5.22 ± 1.09 , VAS pain 47.4 ± 21.0 , VAS activity 49.0 ± 20.6 , VAS physical 44.3 ± 17.4 . The number of painful joints was 14.5 (IQR 15) and that of swollen joints was 6 (IQR 5). None of the patients had evidence of extra-articular manifestation of RA. The RA patients had lower hemoglobin concentrations (12.8 ± 1.1 vs 14.0 ± 1.4 g/dl, $p < 0.001$) and tended to have higher platelet count (269 ± 61 vs 246 ± 41 $10^3/\mu\text{l}$, $p = 0.06$). RA patients had significantly higher fibrinogen (4.18 [IQR 1.09] vs 2.56 [0.41] g/l, $p < 0.0001$) and CRP (7.82 [IQR 11.75] vs 2.13 [1.02] mg/l, $p < 0.0001$).

Coagulation factors and inhibitors

Compared to healthy controls, RA patients had significantly higher FVIII, PC and free TFPI levels, while other coagulation factors and antithrombin were similar (Table). In RA patients FVIII correlated with FIX ($r = 0.42$, $p = 0.003$), FX ($r = 0.41$, $p = 0.005$), antithrombin ($r = 0.34$, $p = 0.02$), free TFPI ($r = 0.48$, $p = 0.001$), and PC ($r = 0.48$, $p < 0.001$). None of the coagulation variables was associated with age, RA duration or CRP levels in the RA group. DAS28 was associated with FV ($r = 0.31$, $p = 0.037$), FVIII ($r = 0.27$, $p = 0.05$), FIX ($r = 0.48$, $p = 0.001$), FX ($r = 0.44$, $p = 0.002$), antithrombin ($r = 0.34$, $p = 0.022$), and free TFPI ($r = 0.44$, $p = 0.002$). Similar

associations were observed for all the 3 VAS variables (not shown). The RF positive patients did not differ from the remainder with regard to plasma factor composition (not shown). Patients receiving methotrexate had lower prothrombin levels (101 ± 10 vs $107 \pm 11\%$, $p=0.05$). Interestingly, patients treated with steroids displayed higher FVIII (232 ± 37 vs $204 \pm 45\%$, $p=0.04$) and free TFPI ($22.7 [2.0]$ vs $20.9 [3.5]$ ng/ml, $p=0.01$). No other significant treatment-related differences in coagulation factors or inhibitors were noted (not shown).

Thrombin generation

Figure 1 presents thrombin generation curves as the mean and standard deviation for the RA group and controls. The two groups are evaluated for thrombin parameters that describe the dynamics of thrombin generation; the results are shown in the Table. Significantly higher maximum levels and rates of thrombin formation were observed when the RA patients were compared to the control group, while AUC did not differ between the two populations. There were also longer clot times and the time to maximum levels of thrombin in RA patients (both $p < 0.0001$).

In RA patients the major determinants of the clot time, time to maximum rate and that to maximum level of thrombin formation were antithrombin (r from 0.49 to 0.57, $p < 0.001$) and free TFPI (r from 0.53 to 0.73, $p < 0.001$). The maximum level of thrombin generated and AUC are potentially determined by prothrombin ($r=0.82$ for both, $p < 0.001$) and antithrombin ($r=-0.70$ and $r=-0.65$, respectively, $p < 0.001$). DAS28 correlated only with clot time ($r=0.31$, $p=0.037$). Time to maximum rate of thrombin formation showed correlations with VAS pain ($r=0.35$, $p=0.018$), VAS activity ($r=0.36$, $p=0.013$), and VAS physical ($r=0.36$, $p=0.015$). None of the coagulation variables correlated with maximum levels or thrombin formation or AUC.

When we analyzed whether there was a potential influence of treatment on thrombin generation profiles, we observed a tendency to lower maximum thrombin levels in patients taking methotrexate (367 ± 51 vs 402 ± 78 nM, $p=0.07$), while those on corticosteroid therapy had longer clot times (202 ± 17 vs 190 ± 20 s, $p=0.05$).

To validate the mathematical modeling, the mean procoagulant and anticoagulant protein composition for RA patients and the controls were used in empirical coagulation proteome experiments with a 5 pM TF stimulus in the presence of a saturating phospholipid surface. Empirical thrombin generation profiles mimic the pattern that is observed in the numerical simulations (Figure 1, panel A and B) with thrombin generation being greater in RA patients.

Influence of plasma composition on RA

When the contribution of plasma factor composition on simulated thrombin generation profiles was evaluated, the results showed that FVIII and TPFI account for the increased thrombin generation in the RA population versus the controls (Figure 2, panel A). When the plasma composition for FVIII and TFPI are normalized in the empirical thrombin generation model, the resulting thrombin generation curves for the RA patients and controls are overlapping (Figure 2, panel B).

Discussion

This study shows that there are changes in the composition of plasma coagulation factors and inhibitors in the RA patients with relatively active disease ($\text{DAS28} > 5$ in $>50\%$ of cases) who have not experienced thrombotic cardiovascular events in the past. We are the

first to show that blood plasma composition, *i.e.* a marked increase in FVIII, accompanied by a higher free TFPI, coagulation inhibitor, contributes to thrombotic phenotype observed in these RA patients. Of note, RA is associated not only with an increase in a procoagulant FVIII, a crucial cofactor of the intrinsic tenase complex which leads to more efficient thrombin formation than the extrinsic tenase complex, but also with a higher level of a coagulation inhibitor (TFPI) that can blunt the prothrombotic effects of FVIII.

Moreover, simulations of thrombin generation, performed as previously described in several papers[16–18], indicate that in RA a burst of thrombin formation is significantly delayed mainly due to increased free TFPI levels (Figure 1, insert), however maximum levels of thrombin generation are significantly augmented likely due to increased FVIII levels. Importantly, total thrombin formation in our model does not differ in RA patients from that observed in well matched healthy controls, because total thrombin generation is predominantly determined by prothrombin and antithrombin levels which have been similar in both groups (Table). However, the net effect of all the alterations in plasma factor composition appears to facilitate thrombus formation by faster thrombin formation in the propagation phase. This indicates that circulating blood thrombogenicity in RA patients with the background of accelerated atherogenic vascular disease might contribute to clinically overt thrombotic cardiovascular episodes. This potentially suggests that an increased risk of venous thromboembolism reported in the RA population is at least in part determined by highly elevated FVIII in circulating blood that is known as a risk factor for venous thrombosis.[21]

A novel and unexpected finding is the presence of elevated free TFPI in the plasma of RA patients compared to healthy controls. Its anticoagulant activity of TFPI, which blocks the TF/FVIIa complex, is associated with the free form of TFPI in plasma and not lipid-bound TFPI.[22] Elevated levels of TFPI could be attributed to TFPI release from endothelial cells upon injury. [22] To our knowledge, this study is the first to quantitate free TFPI concentrations in the plasma obtained from RA patients. This antithrombotic mechanism via increased free TFPI may to some degree protect RA patients from thrombosis stemming from markedly increased levels of FVIII.

Analyses aimed at assessing the impact of blood composition on arterial thrombosis, in individuals without identified abnormalities in coagulation factor levels, have most notably identified FVIII[23] and fibrinogen.[24] Elevated FVIII, usually in the range of 150 to 250% of normal, are detected in acute myocardial ischemia, but not stable cardiovascular disease. [18] Mechanisms underlying such a marked increase in FVIII observed in RA remain to be elucidated. Lack of associations with CRP levels suggests that a link between FVIII levels and the disease activity is complex and most likely not driven by an interleukin-6-mediated pathway. Of note, corticosteroid therapy appears to be associated with higher FVIII and free TFPI, however this observation likely reflects higher activity of RA in this subgroup compared to the remainder.

The production of thrombin appears to be an individual phenotypic property[25]. In the Leiden Thrombophilia Study, thrombin generation based upon individual protein concentrations appeared to reflect an individual's prothrombotic state.[17] These techniques provide extra information regarding an individual's blood composition influence on thrombin generation relative to that available with conventional tests for the evaluation of prothrombotic states. Recently, we have shown that the prothrombotic thrombin generation profile of individuals with acute coronary syndromes appears to be driven at least in part by alterations in FVIII, antithrombin and prothrombin levels.[18] In this study, altered kinetics of thrombin formation were observed in the RA patients versus controls derived from significant differences in only one coagulation factor and two coagulation inhibitors. Given

the data on prothrombotic phenotype in patients with coronary disease, [18] it is likely that alterations in blood factor composition and kinetics of thrombin generation observed in RA patients with no previous cardiovascular events would become more pronounced following myocardial infarction. Interestingly, methotrexate therapy appears to reduce thrombin generation through its effect on prothrombin levels. This intriguing finding merits further investigation.

Phenotypic blood composition at any time reflects ongoing systemic events (*e.g.* inflammation) as well as genetic predisposition. Most likely, the changes reported in this study are related with RA, being the chronic disabling disease itself, with a negligible contribution of genetically determined variability in FVIII, free TFPI levels or PC. However, it is unclear which inflammatory pathway modulates these alterations in RA patients. Given a relatively low number of the RA patients studied, this observation should be validated in a larger study.

Overall, we demonstrated that RA with no concomitant documented cardiovascular disease alters kinetics of thrombin generation and the changes are largely dependent on plasma factors composition, namely 100% higher FVIII counteracted to some extent by increased free TFPI. These observations yield new insights into the pathogenesis of thrombotic events in RA patients.

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Figure 1. Computational and empirical thrombin generation profiles of individuals with rheumatoid arthritis

Panel A. Computationally derived thrombin generation curves shown as the mean for rheumatoid arthritis (n=46) and healthy control (n=46) populations. The positive standard deviation is shown in grey for individuals with rheumatoid arthritis and a negative standard deviation for the controls. **Insert.** Shows the first 200 seconds of the thrombin generation reaction. **Panel B.** Empirical synthetic coagulation proteome was reconstructed on a lipid surface (PCPS) using plasma purified FII, FV, FVII, FVIII, FIX, FX, antithrombin and TFPI. The concentration of the factor levels was determined from the mean of these factors in the RA and control populations seen in the Table.

Figure 2. Plasma factor composition effect on computational and empirical thrombin generation Within the rheumatoid arthritis population, FVIII and AT were adjusted to a mean physiologic concentration and compared to healthy controls. **Insert.** Shows the first 200 seconds of the thrombin generation reaction. **Panel B.** Empirical synthetic coagulation proteome was reconstructed on a lipid surface (PCPS) using plasma purified FII, FV, FVII, FVIII, FIX, FX, antithrombin and TFPI. FVIII and TFPI were set at their mean physiologic concentration while all others remained at the population mean of either the RA patients or the controls.

Table

Plasma composition and stimulated thrombin parameters

	RA (n = 46)			Healthy controls (n = 46)			P
	Mean $\pm$ SD or median [IQR]	Range	Mean $\pm$ SD or median [IQR]	Range	Range		
Factor II, %	103 $\pm$ 10	84–124	102 $\pm$ 8	84–117	84–117	NS	
Factor V, %	106 $\pm$ 10	89–125	103 $\pm$ 10	83–121	83–121	NS	
Factor VII, %	103 $\pm$ 9	89–124	104 $\pm$ 9	86–120	86–120	NS	
Factor VIII, %	226 $\pm$ 40	141–296	113 $\pm$ 15	88–143	88–143	<0.001	
Factor IX, %	106 $\pm$ 11	88–129	103 $\pm$ 9	83–122	83–122	NS	
Factor X, %	107 $\pm$ 12	84–132	104 $\pm$ 11	82–123	82–123	NS	
Antithrombin, %	102 $\pm$ 9	84–122	105 $\pm$ 9	87–119	87–119	NS	
Free TFP1, ng $\cdot$ mL ⁻¹	22.3 [2.2]	16.5–25.9	14.7 [2.1]	12.8–17.9	12.8–17.9	<0.001	
Protein C, %	107 [16]	90–140	100 [14]	76–121	76–121	<0.001	
Time to 10 nM, s	199 $\pm$ 18	153–234	164 $\pm$ 14	135–190	135–190	<0.001	
Maximum rate of thrombin, nM $\cdot$ s ⁻¹	2.82 $\pm$ 0.44	2.06–4.10	2.52 $\pm$ 0.29	1.94–3.10	1.94–3.10	<0.001	
Time to maximum rate of thrombin, s	346 $\pm$ 15	317–387	326 $\pm$ 14	296–359	296–359	<0.001	
Maximum level of thrombin, nM	378 $\pm$ 62	283–556	350 $\pm$ 41	276–452	276–452	0.01	
Time to maximum level of thrombin, s	431 $\pm$ 18	395–483	413 $\pm$ 18	376–458	376–458	<0.001	
Total thrombin (area under the curve), μ M $\cdot$ s	78 [17]	54–132	77 [15]	53–116	53–116	NS	

RA = rheumatoid arthritis; NS = not significant; TFP1 = tissue factor pathway inhibitor.