Thrombin generation assay: interactions between chronic inflammation and haemostasis in patients with autoimmune diseases

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ABSTRACT

Objective. *Growing evidences show a direct link between inflammation and activation of haemostasis. That could increase thrombotic and cardiovascular risk in patients with active autoimmune diseases such as rheumatoid arthritis (RA) and systemic sclerosis (SSc). The aim of this study was to evaluate a possible hypercoagulable condition in RA and SSc patients, using the thrombin generation assay (TGA).*

Methods. *TGA was assessed in 44 RA [33 with active disease (actRA) and 11 inactive (non-actRA)], 25 SSc patients and 41 healthy controls using a fluorimetric technique and the TGA RB Low reagent. The Lag time (tLag), the time to thrombin peak (tPeak), the maximal concentration of formed thrombin (Peak), the velocity of thrombin generation (velocity) and the total amount of thrombin generated (AUC) were determined.*

Results. *As compared to the control group, tLag was found to be significantly reduced both in patients with actRA (p=0.0001) and non-actRA (p=0.01); tPeak was found to be reduced in actRA patients (p=0.0002). Similarly, as compared to healthy subjects, Peak and AUC were found to be increased in actRA patients (p=0.01; p=0.002), as well as D-dimer (p=0.01). Analysing SSc vs RA, a higher Peak and AUC were detected in RA patients.*

Conclusion. *The TGA profile identified in actRA patients (decreased tLag and tPeak combined with higher thrombin peak and greater AUC) reflects a hypercoagulable state that could make patients more susceptible to develop a cardiovascular disease.*

Introduction

Several studies have characterised the mechanisms of the coagulation process that involves the activation of a cascade of plasma proteins leading to thrombin activation. Multiple regulatory molecules are involved in this process, including a wide spectrum of cytokines able to activate the haemostatic cascade. This is at least in part supported by an increased prevalence of deep vein thrombosis (DVT) and pulmonary thromboembolism (TEP) in patients with inflammatory diseases (1). Aim of this study is to explore the thrombin generation potential in two different autoimmune diseases, namely, rheumatoid arthritis and systemic sclerosis. Both diseases are characterised by an inflammatory pathophysiology with a different pattern of cytokine activation. RA is a chronic inflammatory disease which mainly hits the diarthrodial joints, but can also affect internal organs, and has been reported to increase the risk of thromboembolic events. SSc is an immune-mediated connective tissue disorder characterised by an excessive production of collagen, fibronectin and other matrix proteins which accumulate in the skin and organs. Routine coagulation assessment based on prothrombin time (PT) and activated partial thromboplastin time (aPTT) is not sensitive enough to reveal a hypercoagulable condition. Thrombin generation assay has been proposed as a parameter of plasma-based hypercoagulability. TGA measured by a fluorescent substrate has been claimed to be a robust method to estimate the risk of thrombotic events under appropriate conditions (2). This method measures the amount of thrombin formed upon re-calcification of citrated plasma and initiation of the cascade reaction by means of exogenous activators such as human recombinant tissue factors (TF) and phospholipids.

In the present preliminary study we use the TGA as a global haemostasis test in order to reveal hypercoagulable changes in plasma of patients with RA.

Materials and methods

Forty-four RA patients and twenty-five SSc patients as a control diseased sample who fulfilled the American College of Rheumatology criteria (3, 4) were included in the study. Patients ongoing anticoagulant therapy were excluded. Patients were also excluded if they had a history of cancer, hepatic or renal dysfunction, diabetes mellitus, previous myocardial infarction, stroke or any other acute vascular event including venous thromboembolism. Patients with anti-phospholipid antibodies (aPL) profile were not enrolled. Personal and clinical data were collected,

including indexes of disease activity (*i.e*. DAS28), rheumatoid factor (RF), antibody anti-cyclic citrullinated peptide (aCCP), interleukin 6 (IL-6) and autoantibody profile when appropriate. D-dimer, and standard inflammatory markers [C-reactive protein (CRP), erythrocyte sedimentation rate (ESR)] were also measured.

RA patients were divided into two groups according to the disease activity. Thirty-three patients were classified as having an active disease (actRA group) because of a DAS28 ≥2.6 while eleven patients (with a DAS28 <2.6) were classified as inactive (non-actRA group) (Table I). At the time of the blood collection active RA patients were given small doses of corticosteroids (45%), DMARDs, including methotrexate (54%), and TNF-inhibitors (55%), while inactive patients were taken corticosteroids (45%), DMARDs (72%), TNF-inhibitors (36%) and IL6-inhibitors (45%). SSc patients given small doses corticosteroids (40%), DMARDs (12%), iloprost (76%), bosentan (16%) and calcium channel blockers (56%).

At the time of the blood collection, 6 SSc patients were classified as limited cutaneous disease (L-SSc), 15 as diffuse disease (D-SSc) with involvement of lung and/or oesophagus, and 4 as an overlap syndrome (O-SSc). Forty-one healthy subjects were recruited as normal controls. Blood samples were collected from the antecubital vein into 0.129 mM trisodium citrate and in a test tube containing EDTA for routine test. Platelet-poor plasma (PPP) was separated within an hour from samples collection, aliquoted and stored at -80°C till analysis. TGA was performed by a commercially available assay kit (Technothrombin TGA kit, Techonoclone, Vienna, Austria) on a fully automated, computer-controlled micro-plate-reader and a specially adapted software (Technothrombin TGA, Vienna, Austria) using fluorogenic substrate Z-Gly-Gly-Arg-AMC (Bachem, Bubendorf, Switzerland). In the Technoclone assay, 40 μL of plasma was added to 10 μL of activation reagent (RB Low reagent) followed by 50 μL of calcium-fluorescent substrate reagent (7.5 mmol/L calcium and 0.5 mmol/L substrate final reaction concentrations) to start the reaction. The concentration of generated thrombin has been recorded over time resulting in a thrombin generation curve, allowing estimation of several parameters including the Lag time (tLag), time to thrombin Peak (tPeak), peak amount of thrombin generation (Peak), velocity of thrombin generation (vel), and the total amount of thrombin generated (AUC).

ESR (VES-MATIC 30, DIESSE Diagnostica Senese S.p.A.), D-dimer (Liatest D-Di Plus, Diagnostica Stago sas, Asnières sur Seine, France), antinuclear antibody (ANAFLUOR™ HEp-2, DiaSorin), IL-6 (Quantikine ELISA, R&D Systems), anti-CCP (Axis-Shield) were assayed using a commercially available assays. All samples were analysed in duplicates.

Statistics

As data distribution allowed parametric analyses, Student's test was used to reveal differences, and a *p*-value <0.05 was considered significant. To investigate a potential relationship between all TGA parameters and disease duration the Pearson correlation coefficient was used, and an r-value higher than 0.5 was evaluated significant.

Table I. Demographic features of patients with RA and SSc and control subjects.

	Controls	RA		SSc		
		actRA	non-actRA	L-SSc	D-SSc	O-SSc
Patients, number	41	33	11	6	15	4
Female/Male	19/22	27/17		24/1		
Average age years (range)	56 (33-82)	57 (29-83)	63 (33-87)			
Disease duration (mean±SD) years	-	11.8 ± 9.6	12.3 ± 8.2	8.8 ± 7.1		
CRP (mean±SD) mg/l	-	2.3 ± 1.7	0.4 ± 0.2	0.5 ± 0.1		
ESR (mean±SD) mm	8 ± 4	36 ± 30	26 ± 18			
DAS28 <2.6	n/a	10 (23%)		NA		
DAS28 ≥2.6	n/a	34 (77%)		NA		
aCCP antibody (mean±SD) U/ml	-	227.8 ± 176.6	52.0 ± 32.5	-		
IL-6 mean (range) pg/ml	-	26.8 (0-99)	2.6 (0-10.3)	-		
ANA positive high titer	-	-		17 (68%)		
ANA positive medium titer	-	-		6 (24%)		
ANA negative	-	-		2 (8%)		
RNP positive	-	-		11 (44%)		
RNP negative	-	-		14 (56%)		
Scl-70 positive	-	-		11 (44%)		
Scl-70 negative	-	-		14 (56%)		

RA: rheumatoid arthritis; actRA: active rheumatoid arthritis; non-actRA: inactive rheumatoid arthritis; SSc: systemic sclerosis; L-SSc: limited systemic sclerosis; D-SSc: diffuse systemic sclerosis; O-SSc: overlap systemic sclerosis; CRP: C-reactive protein; ESR: erythrocyte sedimentation rate; DAS28: disease activity score; aCCP: anti-cyclic citrullinated peptide; IL-6: interleukin 6; ANA: anti-nuclear antibodies; RNP: ribonucleoprotein; Scl-70: anti-topoisomerase I; SD: standard deviation; NA: not applicable.

Results

Demographic features of patients with RA and SSc are listed in Table I. An increase of CRP was found in patients with actRA compared to non-actRA (2.3±1.7 mg/l *vs*. 0.4±0.2 mg/l, respectively, p=0.0004). On the other hand, a lower CRP was found in patients with SSc compared to actRA (0.5±0.1 mg/l *vs*. 2.3±1.7 mg/l, respectively, p=0.000003). Also, anti-CCP antibody levels in actRA were higher than in the non-actRA patients (227.8±176.6 U/ml *vs*. 52.0±32.5 U/ml, p=0.01). No correlation was found comparing all TGA parameters and disease duration in RA, actRA, non-actRA and SSc patients (-0.27<r<0.45). TLag and tPeak were found to be significantly reduced in RA patients, while peak and AUC were found to be increased (Fig. 1).

Unlike the non-actRA patients, the actRA patients showed significant abnormalities of TGA parameters when compared to the control group; actRA and RA patients also showed a significant increase in all TGA parameters as compared to SSc patients (Fig. 1). Moreover, AUC was lower in the non-act RA compared to the actRA patients (p=0.04).

Table II summarises the main differ-

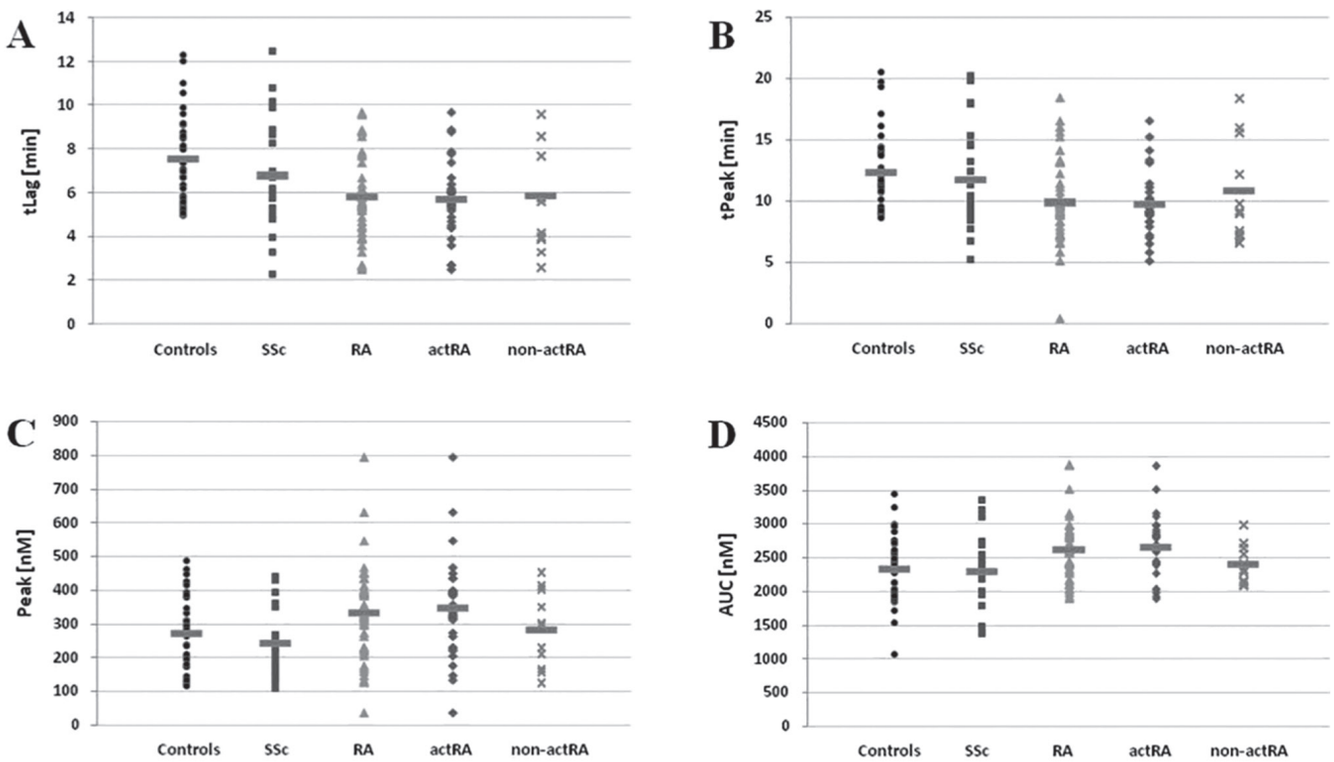

Fig. 1. SSc: systemic sclerosis; RA: rheumatoid arthritis; actRA: active rheumatoid arthritis; non-actRA: inactive rheumatoid arthritis; tLag: lag time; tPeak: time to peak thrombin; Peak: peak amount of thrombin generation; AUC: area under the curve.

Table II. Summary of TGA data obtained from diseased subjects and controls.

	Controls *vs*. SSc	Controls *vs*. RA	Controls *vs*. actRA	Controls *vs*. non-actRA	RA *vs*. SSc	actRA *vs*. SSc	non-actRA *vs*. SSc	actRA *vs*. non-actRA
tLag	NS	0.0001	0.0001	0.01	0.04	0.03	NS	NS
tPeak	NS	0.0007	0.0002	NS	0.02	0.01	NS	NS
Peak	NS	0.02	0.01	NS	0.003	0.001	NS	NS
vel	NS	0.004	0.001	NS	0.003	0.001	NS	NS
AUC	NS	0.003	0.002	NS	0.004	0.003	NS	0.04
D-dimer	0.004	0.03	0.01	NS	NS	NS	NS	NS

SSc: systemic sclerosis; RA: rheumatoid arthritis; actRA: active rheumatoid arthritis; non-actRA: inactive rheumatoid arthritis; tLag:lag time; tPeak: time to peak thrombin; Peak: peak amount of thrombin generation; vel: velocity; AUC: area under curve; NS: not significant.

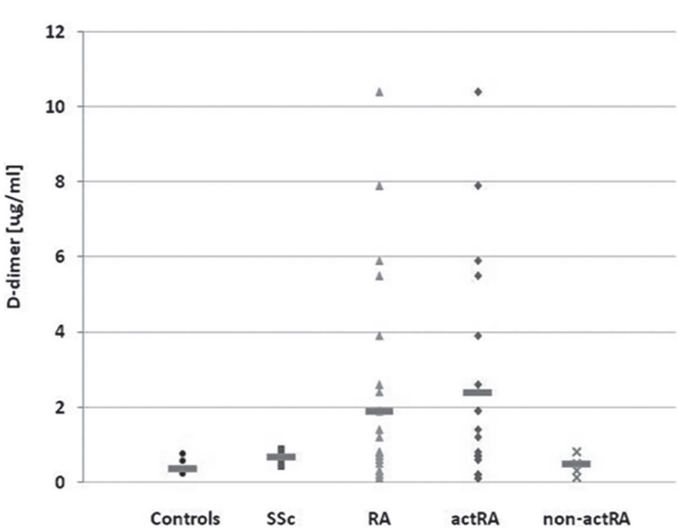

Fig. 2. SSc: systemic sclerosis; RA: rheumatoid arthritis; actRA: active rheumatoid arthritis; non-actRA: inactive rheumatoid arthritis.

ences in TGA parameters. An increase of D-dimer was found in patients with SSc (0.7±0.2 µg/ml, p=0.004) as compared to controls. RA patients showed a greater variability (1.9±2.6 µg/ml). D-dimer was significantly increased in actRA patients (p=0.01) compared to the control subjects (Fig. 2).

Discussion

In the present report we used the TGA assay, a global haemostatic test, in order to characterise the thrombin generation potential in plasma of patients with RA and SSc. Several studies have linked inflammation to increased blood clotting (5) and RA patients were found

to have an increased risk of thromboembolic cardiovascular events (6).

We found significant abnormalities in TGA parameters. These abnormalities were independent on disease duration. These findings could be related to a potential hypercoagulable condition in RA patients. TGA evaluation shows that actRA patients are definitely predisposed to prothrombotic conditions, while nonactRA show milder abnormalities.

Prati *et al*. (7) found an increase of tLag and tPeak in association with a decrease of AUC and Peak in RA patients when compared to healthy controls. Undas *et al*. (8) showed an increase of tPeak and tLag, together with a significant increase of peak in RA patients, using a computational model. Different results could be attributed to the different method used. Our data also show that SSc patients did not differ, as regard to kinetics of thrombin generation, from healthy people. D-dimer increases in SSc and RA patients, possibly due to the inflammatory condition, but the D-dimer test is less accurate than TGA to identify patients with a hypercoagulable state. Several studies suggested that an abnormal TGA could be a risk factor for venous thromboembolism, useful to predict thrombosis (9). We established that a high Peak and a great AUC combined with decreased tLag and tPeak identify a hypercoagulable state and a potential cardiovascular risk in actRA patients.

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