Blood Cells, Molecules, and Diseases 46 (2011) 235–238

Contents lists available at ScienceDirect

Blood Cells, Molecules, and Diseases

journal homepage: www.elsevier.com/locate/ybcmd

Polymorphonuclear neutrophils from JAK2^{V617F} positive MPD patients do not support hypercoagulability: A study with calibrated automated thrombography (CAT)

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ARTICLE INFO

Article history:
Submitted 5 November 2010
Revised 17 December 2010

(Communicated by B. Coller, M.D.,
14 January 2011)

Keywords:
Myeloproliferative disorders
Polymorphonuclear neutrophils
Thrombin generation
Hypercoagulability

ABSTRACT

Essential thrombocythemia and polycythemia vera are myeloproliferative disorders (MPD) with an elevated thrombotic risk. Leukocytosis has recently emerged as a new risk factor and there is increasing evidence that polymorphonuclear neutrophils (PMN) are involved. Procoagulant activity (PCA) of PMN in MPD has not yet been investigated. PCA of PMN from 22 patients with JAK2^{V617F} positive MPD and 26 healthy subjects was studied using calibrated automated thrombography: *in vitro* thrombin generation induced with 1 pM tissue factor in the presence of added procoagulant phospholipids. There were no differences between patients and controls regarding the ability of PMN to increase thrombin generation. More surprisingly, basal thrombin generation in acellular MPD-plasma was found decreased for as yet unknown reasons. The presence of an active protein C pathway or platelets might provide a better insight into the coagulation phenotype in MPD.

Introduction

Polycythemia vera (PV) and essential thrombocythemia (ET) are myeloproliferative disorders (MPD) sharing some characteristics: the JAK2^{V617F} mutation has been described in both but with different frequencies (i.e. 95% and around 50%, respectively); the clinical course is characterised by thrombotic complications [1,2]. Even if the origin of the postulated hypercoagulability is not yet understood, two clinical risk factors are well-defined: age >60 years and a history of thrombosis. But, the JAK2^{V617F} mutation has been also related to such complications [3]. For the last years, PV and ET have been shown to share another interesting feature: Leukocytosis, due to an increased polymorphonuclear neutrophils (PMN) count, has recently emerged as a potential new risk factor [4–7]. Nevertheless, the hypothesis of the involvement of PMN in the hypercoagulable state in such a context is not recent (reviewed in [8]). Falanga et al. showed for instance that markers of *in vivo* PMN activation correlated well and positively with *in vivo* coagulation activation [9]. We have previously reported that resting PMN from healthy volunteers display *in vitro* a substantial procoagulant activity (PCA) as evidenced with thromboelastometry–fibrin polymerization process [10]. There are three main possible mechanisms involved in the PMN-derived PCA: tissue factor expression (controversial); exposure of procoagulant phospholipids; and modifications of coagulation proteins due to the release of granular enzymes (cathepsin G or elastase for example). Our working hypothesis is that the procoagulant effect of PMN relies on the impairment of natural anticoagulants by released enzymes [10,11]. Thus we hypothesise that, besides a quantitative phenomenon (number of circulating cells in excess), PMN from ET or PV patients could have a greater PCA than PMN from healthy subjects. To our knowledge, no study has addressed this issue.

In the present work, we compared the effect on thrombin generation of PMN from ET or PV patients with that from healthy subjects, using calibrated automated thrombography (CAT). As we have some data suggesting that TF is not involved in PMN-associated PCA [10], we chose to trigger coagulation by TF 1 pM (final concentration).

Patients, materials and methods

The study was approved by the local ethics committee, and all patients and healthy subjects signed an informed consent. Patients

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doi:10.1016/j.bcmd.2011.01.003

(12 PV and 10 ET, diagnosed according to the criteria available at the time of diagnosis) were prospectively included after a routine visit. All patients carried the V617F JAK2 mutation. Patients treated with anticoagulants (vitamin K antagonists, heparins or fondaparinux), infected, or unstable were not included. In parallel, 26 healthy blood donors were enrolled as controls, of age and with a sex ratio close to that of patients (Table 1).

Blood was withdrawn by antecubital venipuncture and anticoagulated with trisodium citrate 0.106 M, using the S-Monovette® system (Sarstedt, Nümbrecht, Germany). The first 5 mL were discarded to limit tissue factor contamination of the blood samples. PMN-rich plasma (PMN-RP) 10×10^9/L (containing no more than 5×10^9 platelets per liter) and plasma containing 5×10^9 platelets per liter (PPP) were then immediately prepared as described [10]. Chemiluminescence and flow cytometry assays showed that PMN were not altered by the isolation procedure.

Thrombin generation was performed using the CAT method [12,13]. Briefly, 80 μL plasma samples were mixed with 20 μL PPP LOW® (Diagnostica Stago, Asnières, France; tissue factor (TF) 1 pM; phospholipids 4 μM, final concentrations in the well) in a 96-well plate (Immulon, 2HB clear U-bottom, Thermo Electron, Villebon-sur-Yvette, France). Coagulation was triggered by adding 100 mM calcium chloride (20 μL) in a BSA buffer containing 2.4 mM Z-Gly-Gly-Arg-AMC (the fluorogenic substrate purchased from Bachem, Bubendorf, Switzerland). Fluorescence was measured in an Ascent Fluoroscan® (ThermoLabsystems, Helsinki, Finland). All samples were run in triplicate. For each plasma sample, the fluorescence signal was corrected by running in parallel 2 duplicate calibrating wells where 80 μL plasma was mixed with 20 μL Thrombin Calibrator® from Thrombinoscope BV (Maastricht, The Netherlands). Fluorescence data were then analyzed with the Thrombinoscope® software version 3.0.0.29 (Thrombinoscope BV, Maastricht, The Netherlands). The following parameters were recorded:

- Endogenous thrombin potential (ETP): area under the curve, which reflects the global coagulation potential, identifying hypocoagulability or hypercoagulability [14–18].
- Lag time: time to burst of thrombin generation, which roughly corresponds to the clotting time.
- Peak: the highest thrombin concentration reached during the time course of thrombin formation and inhibition.

Regarding the experimental conditions, first we chose to trigger thrombin generation with added TF in the presence of procoagulant phospholipids (final concentrations: 1 pM and 4 μM, respectively), as our previously published data have shown that the PCA of PMN is probably not related with TF or procoagulant phospholipids expression [10]. Furthermore, preliminary assays, using the above-mentioned experimental set-up for thrombin generation, have revealed that the presence of PMN (prepared from healthy volunteers) in plasma induces significant modifications, which found expression in decreased lag time associated with increased ETP and peak. Those results indicate that PMN facilitate coagulation despite the presence of TF as trigger and of optimal amounts of phospholipids as defined by Hemker et al. [13]. Moreover, this enhancement was more pronounced when cells were activated *in vitro* with N-formyl-Met-Leu-Phe – fMLP – a synthetic peptide that mimics the activity of bacterial-derived peptides. Taking into account all these data, our working hypothesis is that the procoagulant activity of PMN relies on the impairment by released enzymes of natural anticoagulants, such as TFPI (as we demonstrated with Cath G especially [11]) or AT.

Second, we did not use CTI (corn trypsin inhibitor) to prevent contact phase activation as recommended by some authors [19]: we feared an effect on PMN-released enzymes [20] and thrombin generation is much less likely to be affected when the TF concentration is ≥1 pM [21].

In the present work, the effect of (resting) PMN on thrombin generation was assessed by comparing PPP profiles with those obtained in the presence of PMN 10×10^9/L (PMN-RP). PCA was quantified by calculating the difference for each of the above-mentioned parameters between PMN-RP and PPP and then compared between MPD patients and controls. Because of the presence of a few platelets (less than 5×10^9/L) in the PMN suspension, profiles of PMN-RP were compared with that of PPP, as justified elsewhere [10]. It should be stressed, however, that no difference was found between acellular plasma and PPP, indicating that the few remnant platelets are not the main support of PMN-associated PCA.

Table 1
Description of the populations.

		Patients			Controls
		Total	PV	ET	
N		22	12	10	26
Males/females		13/9	8/4	5/5	16/10
Mean age, y [range]		64* [27–83]	61 [27–78]	68 [55–83]	57 [52–63]
Blood cell count at diagnosis	Hb (g/dL)	16.7 ± 2.4	18.3 ± 1.9	14.7 ± 1.4	
	Ht (%)	51.0 ± 8.0	55.9 ± 6.2	45.0 ± 4.5	
	Plt ($\times10^9$/L)	689 ± 300	567 ± 171	836 ± 361	
	WBC ($\times10^9$/L)	11.8 ± 4.0	11.7 ± 4.1	11.7 ± 4.1	
	Neutrophils ($\times10^9$/L)	8.9 ± 3.4	9.2 ± 3.2	8.6 ± 3.7	
Blood cell count at the time of the study	Hb (g/dL)	14.4 ± 1.6	15.3 ± 1.3	13.2 ± 1.4	14.8 ± 1.4
	Ht (%)	43.3 ± 5.4	46.0 ± 4.0	40.0 ± 5.1	44.0 ± 3.6
	Plt ($\times10^9$/L)	455** ± 251	376 ± 113	549 ± 336	254 ± 42
	WBC ($\times10^9$/L)	9.1** ± 4.7	9.5 ± 5.2	8.6 ± 4.4	5.9 ± 1.5
	Neutrophils ($\times10^9$/L)	6.7 ± 4.3	7.1 ± 4.7	6.2 ± 3.9	ND
History of vascular events	Total	8	3	5	
	Arterial	*1*	*1*	/	
	Venous	*3*	*1*	*2*	
	Ischaemic	*4*	*1*	3	
Treatment	Cytoreductive therapy (CRT)	14	7	7	
	Antiplatelet drugs (APD)	17	8	9	
	CRT + APD	12	5	7	
	Anagrelide	1	1	/	
	Phlebotomy	2	2	/	
	INF-α	1	1	/	
	None	2	1	1	

Mean ± SD values unless otherwise stated. *$p<0.05$ vs. controls; **$p<0.01$ vs. controls.