

The Role of Chromogenic and Immunoturbidimetry Assays in Diagnostic of Thrombotic State in Myeloproliferative Disorders

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Polycythaemia vera (PV) is a monoclonal myeloproliferative disorder characterized by a haemostatic imbalance resulting in increased risk for thrombotic events. In PV there is an important affection of the vascular endothelium, so we analyzed in our study three markers of endothelium dysfunction- von Willbrand factor (vWF), Protein C (PC) and the inhibitor plasminogen activator type-1. (PAI-1) The study comprised 40 patients and the objective of the study was to investigate the level of the mentioned parameters in two groups of patients-the group of polycytemic patients without cardiovascular diseases (PV) and the group of polycytemic patients with cardiovascular pathology associated (PV+CVD). The levels of the studied parameters were found significantly modified in the PV group ($p < 0.05$); for the PV+CVD group the level of vWF was higher ($p < 0.001$), but the levels of the other parameters, didn't show a significant statistic difference compared with the first group ($p > 0.05$). In accordance with the presented results, the conclusions of our study was that in PV the risk of thrombotic accidents is related by the increase of the vWF level, as the vascular endothelium plays a key role in this pathology.

Keywords: chromogenic assay; immunoturbidimetry assay, thrombosis; Protein C, enzymatic immunoassay

After many years of careful clinical and laboratory investigation, the etiology of polycythemia vera remains unknown and there is no consensus as to the optimal therapy for the disorder. A typical feature of PV is a haemostatic imbalance resulting in increased risk for both thrombotic and hemorrhagic events [1]. Arterial and venous thromboembolism are the major causes of morbidity and mortality in these patients (approximately 40%) [2]. In the majority of PV patients, loss of homeostatic regulation results from an unique V617F mutation in the gene that encodes Janus kinase 2 (JAK2). This mutation mimics the constitutive activation of the erythropoietin receptor in erythroid precursors [3-5]. Multiple factors are likely to contribute to the pathogenesis of thrombosis, including increased cell mass, platelet number, activation of platelets and leukocytes and their interaction to form platelet leukocyte aggregates, in addition to prothrombotic circulating and endothelial factors. Endothelial activation may also have been due to elevated levels of vascular endothelial growth factor, which has also been reported to be elevated in PV. Activated leukocytes (neutrophils and monocytes) promote coagulation by releasing granule contents and formation of aggregates with platelets. Subsequent effects include platelet leukocyte aggregates playing a pathogenic role in triggering monocyte tissue factor expression as well as superoxide anion and inflammatory cytokine release causing endothelial activation and damage [6]. Thrombosis affects large arteries, especially cerebrovascular, cardiovascular systems and peripheral arterial circulations. The most common thrombotic complications in the large vessel are represented by myocardial infarction [7], superior vena cava syndrome [8], cerebral venous thrombosis [9], arteriosclerosis or pulmonary embolism [10].

Microcirculatory disturbances such as erythromelalgia, transient neurological events and even disseminated intravascular coagulation are frequent complications in patients with polycythemia vera. In terms of fluid-coagulant changes as a mechanism involved in the aetiology of thrombotic status in patients with polycythemia vera, several widespread study demonstrated the activation of coagulation proteins, the reduction of the levels of physiologic anticoagulants (antithrombin III, proteins C and S), and decreased fibrinolytic activity secondary to increased plasma levels of plasminogen activator inhibitor [11]. Besides these procoagulants mechanisms, the thrombotic risk is amplified by the association of the other risk factors for thrombosis represented by advanced age, history of thrombotic vascular diseases, repeated phlebotomies, hypercholesterolemia and smoking. Thus, in asymptomatic patients that link vascular risk factors and systematically in patients over 70 years, the main vessels have to be explored to prevent thrombotic accidents [12, 13].

The objective of the study was to investigate the differences between the groups of polycytemic patients, with (PV+CVD) and without associated cardiovascular affections (PV) regarding the behaviour of three parameters of the haemostasis: vWF, PC si PAI-1; identification of these patients with haemostasis disorders require their regular monitoring and setting their appropriate anticoagulant treatment to prevent the acute ischemic events [14].

Experimental part

Material and methods

The study comprises 20 adult patients, 10 males and 10 females, diagnosed according to Polycythemia Vera Study Group (PVSG) criteria [15] divided in 2 groups: 10 patients

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with PV syndrome (PV) and 10 patients with PV with CVD associated (PV+CVD).

The selection criteria of the patients with cardiovascular diseases were the following:

-The presence of the signals and symptoms of the cardiovascular disease under each manifestation form:

- effort stable pectoral angina;
- aggravated pectoral angina;
- chronic myocardial infarct;
- the values of TAs > 145 mmHg and of TAd > 95mmHg;
- the existence of extrasystolic atrial and ventricular irregularities;

- the existence of supra-ventricular rhythm disorders (TSV): atrial fibrillation or atrial flutter of ischemic cause;

- the existence of leading disorders (TC).

The presence in the pathological personal antecedents of the patients of one among the following affections:

- coronary disease under each manifestation form;
- non-rheumatismal valvulopathy;
- ischemic-type or hypertensive dilatative cardiomyopathy;
- ischemic vascular accident.

The patients were tested by determining three haemostasis components: von Willebrand factor (vWF), protein C (PC) and Plasminogen activator inhibitor-1 (PAI-1).

None of the patients received antiplatelet or anticoagulant therapy at the time of testing.

To determine these parameters, the venous blood, collected in 0, 105M sodium citrate vacutainer (report sodium citrate/blood=1/9), was centrifuged 15 minutes at 2500 rpm followed immediately by plasma separation and its freezing. Plasma obtained by centrifugation was frozen (no longer than 4 weeks) at -20 fC until the tests were performed.

Von Willebrand Factor

For the determination of vWF it was performed the immunoturbidimetry assay (*vWF:Ag*) on automatic analyzer, using reaction agents from the Biomnis Laboratory, France.

Reference values=50-160% (Biomnis Laboratory, France, 2010)

Protein C

The Protein C was determined by chromogenic assay on automatic analyzer, using reaction agents from the Biomnis Laboratory, France, and an extract of the snake venom (*Agkistrodon contortrix*) for direct activation of Protein C in tested plasma.

Reference values: 65-140% (Biomnis Laboratory, France, 2010)

PAI-1

For this determination, the plasma must be very low in platelets platelets-poor plasma (Platelet count <10000/ μ L), so that double centrifugation was performed.

The PAI-1 level was determined by using enzymatic immunoassay test (EIA) and reaction agents from the Biomnis Laboratory, France.

Reference values: <10 Ku/L (Biomnis Laboratory, France, 2010)

Results and discussions

The level of the three studied parameters were found significantly modified in the first group (PV) ($p < 0.05$); for the second group (PV+CVD) the level of vWF was higher than in the first group ($p < 0.001$), but the levels of the other

two parameters, although modified, do not show a significant statistic difference compared with the first group ($p > 0.05$).

Thrombosis and bleedings are the major causes of morbidity and mortality in PV [17]. This hypercoagulable state in the group of patients with PV could be partially explained by the known hypercoagulable condition associated with malignancy, by increases in white blood cells (WBC) count and platelet dysfunction. However there is the possibility of having polymorphonuclear (PMN) enzyme involvement.

Our study demonstrates that the von Willebrand factor is implicates in the physiopathology of the PV associated with the cardio - vascular diseases. In our opinion, there is a connection between the platelet function and the vascular endothelium. Platelet dysfunction in PV is typically characterized by a missing second-wave adrenaline aggregation, an increased adenosine diphosphate aggregation threshold, and reduced secretion products, but a normal arachidonic acid or collagen-induced aggregation. The proposed concept is that platelets in PV are hypersensitive. Due to the existing high shear stress in the microvasculature (end-arterial circulation), platelets spontaneously activate, secrete their products, form aggregates mediated by von Willebrand factor that transiently plug the microcirculation, deaggregate, and then recirculate as exhausted defective platelets with secondary storage pool disease on ex vivo analysis [18].

These microvascular circulation ischemic disturbances in thrombocythemia vera already occur at platelet counts in excess of $400 \times 10^9/\mu\text{L}$ [19] and are a pathognomonic feature of thrombocythaemia associated with polycythaemia vera as a consequence of platelet-mediated increased proteolysis of the large von Willebrand factor multimers leading to a type 2 acquired von Willebrand syndrome [20].

An increasing platelet count to above $1000 \times 10^9/\mu\text{L}$ is accompanied by the acquisition of a von Willebrand factor deficiency due to the loss of intermediate and large von Willebrand factor multimers. The PV patients with microvascular disturbances have shortened platelet survival, increased beta-thromboglobulin, platelet factor 4, and thrombomodulin levels, and increased urinary thromboxane B2 excretion indicating platelet-mediated processes in vivo. The transient ischemic attacks and thrombotic complications in thrombocythemia are very likely caused by hypersensitive platelets produced by spontaneously proliferating enlarged megakaryocytes in the bone marrow of PV patients [21].

The JAK2 mutation also results in platelet hypersensitivity to these prothrombotic signals, as they undergo spontaneous activation, product secretion (thromboxane A2), and aggregation mediated by the von Willebrand factor. The quantitative and qualitative dysfunctions of platelets in polycythemia vera, as well as the hypersensitivity to cytokines at the level of the endothelial wall, explain the hypercoagulable state of the disease.

Activated PMN release the reactive oxygen species and intracellular proteases which can act on the endothelial cells and platelets and may modify the hemostatic balance towards a prothrombotic state [22-24]. Endothelial activation may also have been due to elevated levels of vascular endothelial growth factor, which has also been reported to be elevated in PV. Subsequent effects include platelet leukocyte aggregates playing a pathogenic role in triggering monocyte tissue factor expression, as well as superoxide anion and inflammatory cytokine release causing endothelial activation and damage. Leukocyte

elastase and cathepsin G can induce detachment or lysis of endothelial cells and can modify endothelial cell functions involved in thromboregulation that prevent thrombin-induced prostacyclin production, induce plasminogen activator inhibitor release and proteolyze endothelial surface components such as thrombomodulin. Furthermore, the potential thrombogenic effects of PMN-derived proteases include the direct potent platelet activation elicited by cathepsin G. Finally, elastase can directly proteolyze and inactivate natural inhibitors of blood coagulation, including protein C, protein S, tissue factor pathway inhibitor, antithrombin, and heparin cofactor II. This inactivation may contribute to local progression of coagulation reactions at the inflammation sites.

Conclusions

Although about half of patients with PV display either thrombocytosis or leukocytosis, the risk of thrombosis in such patients has not been correlated with either platelet or WBC count. Furthermore, previous reports of widespread activation in coagulation proteins, reduced levels of physiologic anticoagulants (antithrombin III, proteins C and S), and decreased fibrinolytic activity that may be partially secondary to increased plasma levels of plasminogen activator inhibitor suggest a baseline pro-thrombotic state in PV. Patients with associated cardiovascular disease present a more severe endothelial dysfunction, but regarding the disorder of coagulation and fibrinolysis factors, there are no major differences compared with patients with PV syndrome without cardiovascular pathology. We may hypothesize that hypercoagulable state associated with malignancy, the increases in WBC count, endothelial dysfunction, reduced levels of physiologic anticoagulants (antithrombin III, proteins C and S) and decreased fibrinolytic activity that may be partially secondary to increased plasma levels of plasminogen activator inhibitor and the connection between the platelet hypersensitivity and von Willebrand factor could explain the pathogenesis of the thrombophilic state in PV. The quantitative and qualitative dysfunctions of platelets in polycythemia vera, as well as the hypersensitivity to cytokines at the level of the endothelial wall, explain the hypercoagulable state of the disease, leading to a type 2 acquired von Willebrand syndrome. In PV, we can see modifications of the fluid-coagulant equilibrium, the risk of thrombotic accidents being directly proportional with the increase of the vWF level, as the vascular endothelium plays a key role in this pathologic process.

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