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Parametry angiogenezy a czynniki ryzyka zakrzepicy w czerwienicy prawdziwej

Angiogenic parameters and the risk factors for thrombosis in polycythemia vera

Authors' Contribution:
A Study Design
B Data Collection
C Statistical Analysis
D Data Interpretation
E Manuscript Preparation
F Literature Search
G Funds Collection

Joanna Boinska[1 B C D F], Grażyna Gadomska[2 A D E], Katarzyna Ziołkowska[1, C D], Karolina Woźniak[3 B F], Alicja Bartoszewska-Kubiak[4, B], Danuta Rość[1 D E]

[1]Department of Pathophysiology, Faculty of Pharmacy, Collegium Medicum in Bydgoszcz, Nicolaus Copernicus University in Toruń, Poland
[2]Department of Hematology and Malignant Diseases of Hematopoietic System, Faculty of Medicine, Collegium Medicum in Bydgoszcz, Nicolaus Copernicus University in Toruń, Poland
[3]Student of the Faculty of Pharmacy, Collegium Medicum in Bydgoszcz, Nicolaus Copernicus University in Toruń, Poland
[4]Department of Genetics, Faculty of Medicine, Collegium Medicum in Bydgoszcz, Nicolaus Copernicus University in Toruń, Poland

Summary

Aim: The assessment of angiogenic parameters in so-called "liquid tumors", such as myeloproliferative neoplasms, remains an open clinical issue. The aim of the study is to evaluate the concentration of vascular endothelial growth factor (VEGF-A) and soluble receptors sVEGFR-1 and sVEGFR-2 in relations to risk factors of thrombosis in patients with polycythemia vera (PV).

Material/Methods: A total of 45 patients suffering from newly diagnosed PV and 30 healthy volunteers were enrolled into the study. Polycythemia vera was diagnosed according to the WHO (2008) criteria. In the citrated plasma samples VEGF-A, sVEGFR-1 and sVEGFR-2 were measured using ELISA tests.

Results: VEGF-A concentration was three-fold higher and sVEGFR-2 significantly lower in PV patients as compared to the control group. VEGF-A concentration was significantly higher in PV patients with JAK2V617F mutation, as compared to patients without this mutation. SVEGFR-1 and sVEGFR-2 concentrations were similar in the analyzed subgroups. In PV patients with an increased number of white blood cells (WBCs), the above upper reference value (≥10 G/l), VEGF-A concentration was two-fold higher than in patients with WBCs number <10 G/l. However, sVEGFR-1 and sVEGFR-2 concentrations did not differ between the analyzed subgroups. Analysis of correlations revealed only one relation between VEGF-A and WBCs number.

Conclusions: Increased VEGF-A and decreased sVEGFR-2 concentrations in polycythemia vera patients as compared to the control group indicate an intensification of the process of angiogenesis. A higher concentration of VEGF-A in PV patients with leukocytosis and a positive correlation between WBCs number and VEGF-A reflect the potential role of VEGF-A in the pathogenesis of thrombotic complications in hypercoagulable state in PV patients.

Keywords: **polycythemia vera • vascular endothelial growth factor • angiogenesis**

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Author's address: Joanna Boinska, PhD Department of Pathophysiology, Nicolaus Copernicus University in Toruń, Colegium Medicum in Bydgoszcz, Skłodowskiej-Curie, Street No 9, 85-094 Bydgoszcz, Poland; e-mail: joanna_boinska@o2.pl

INTRODUCTION

The clinical course of meyloproliferative neoplasms, expecially essenthial thrombocythemia and polycythemia vera, is complicated by various thrombotic events, which occur in both the arterial and venous circulations. The etiopathogenesis of these disorders is multifactoral and vascular-platelet and plasma hemostasis as well as rheological properties of blood are equally involved in blood clotting [19]. In addition to well established and common risk factors of thrombosis such as age (above 60 years) and past history of thrombosis, clinical studies indicate the role of novel risk factors influencing thrombotic risk in, e.g. Janus kinase 2 (JAK2) V617 F mutation, increased leukocytosis, elevated red blood cells (RBCs) mass (in PV), thrombocythosis and platelet dysfunction (in essential thrombocythemia). Over the last ten years, the interplay between blood coagulation and angiogenesis have been extensively examined, especially since new antiangiogenic drugs (such as bevazizumab) have become available [3].

Angiogenesis, defined as the formation of new blood vessels which sprout from existing ones, is not only an essential physiological process for embryologic development, growth, and tissue repair but is closely linked to the development of cancer. The natural consequences of tumor angiogenesis include nutrients and oxygen supply, tumor growth and metastasis to adjacent or distant organs [12]. The imbalance of pro- and anti-angiogenic factors promotes vascular network formation that is characterized by dilated, tortuous, and hyperpermeable vessels. Taking into consideration that several interactions between tissue factor, main activator of blood clotting and angiogenesis factors have been described, disturbances between hemostasis and angiogenesis may promote thrombotic or hemorrhagic events [8].

Various methods have been used to quantitate tubule formation; however, *microvessel density* (MVD) has been used extensively in many clinical studies to evaluate angiogenic activity and predict treatment outcome [8]. Although the prognostic importance of MVD in some types of cancers such as breast cancer has been well established, many clinical studies have found no association between MVD and tumor stage or metastasis [4, 5]. For this reason, new non-invasive laboratory parameters of angiogenesis are sought after with the aim of substituting invasive procedures and developing a more precise means of reflecting angiogenesis in various types of malignancies.

VEGF is a potent mitogen for endothelial cells in vitro and it affects the migration, proliferation and inhibition of their apoptosis. VEGF-A fulfills its biological functions by directly acting on three types of receptors for VEGF, located on the surface of the endothelial cells, i.e. VEGFR1, VEGFR-2 and VEGFR-3, which exhibit tyrosine kinase activity [2, 17]. In addition to membrane receptors, several studies have demonstrated the existence of soluble receptors for VEGF (sVEGFR-1 and sVEGFR-2), which appear to act as natural angiogenesis inhibitors. SVEGFR-1 and sVEGFR-2 have the ability to bind VEGF and prevent its connection with membrane receptors [1, 6, 16].

The aim of the study was to evaluate the concentration of VEGF-A and soluble receptors sVEGFR-1 and sVEGFR-2 in relation to the risk factors of thrombosis in patients with polycythemia vera.

MATERIAL AND METHODS

The study comprised 42 patients with a confirmed diagnosis of polycythemia vera (22 females and 20 males) in the age range of 47-76 years (mean age 61.2 ± 13.9). All the patients were diagnosed at the Department of Hematology and Malignant Diseases of Hematopoietic System, University Hospital No. 2 in Bydgoszcz, Poland and were untreated by phlebotomy or cytoreductive drugs at the time of blood sampling.

Polycythemia vera was diagnosed according to the World Health Organization (2008) criteria. Exclusion criteria included secondary causes of polycythemia, pregnancy, diabetes type 1 or 2, stage III of hypertension, malignant hypertension as well as thrombotic or hemorrhagic complications at the time of diagnosis.

Among 42 PV patients, 35 patients were JAK2V617F - positive (83%) and 7 were JAK2V617F - negative (17%). Past history of thromboembolism, such as myocardial infarction, cerebral ischemia and pulmonary embolism have been reported in 10 patients with PV (24%). 32 of

PV patients (76%) had no history of thrombosis. The control group consisted of 30 healthy volunteers age (mean age 47.2 ± 6.1 years) and sex (16 females and 14 males) matched.

The study was approved by the Bioethics Committee of Collegium Medicum in Bydgoszcz, the Nicolaus Copernicus University in Toruń (KB 396/2010). Written informed consent was obtained from all participants.

Blood samples were taken from the elbow vein, after overnight fasting, into 2 tubes containing EDTA 2K (plasma) and clot activator (serum). Samples were centrifuged at 3000 rev/min for 20 minutes at 4°C. The obtained plasma and serum were divided into aliquots and stored at -80°C until analysis, but not longer than for 6 months. Peripheral blood counts were performed with the Advia 120 hematology analyzer. The following tests were performed on plasma using the immunoenzymatic method (ELISA): concentration of VEGF-A, sVEGFR-1, sVEGFR-2 (R&D Systems, Inc., Minneapolis).

STATYSTICAL ANALYSIS

A statistical analysis was performed with the use of Statistica 13.0 software (StatStoft® Cracow, Poland). The Shapiro-Wilk test was applied to assess the normality of distribution. Arithmetic mean (X) and standard deviation (SD) were determined for parameters with a normal distribution, and parameters with abnormal distribution in relation to normal ones were presented as a median (Me) and quartiles: lower (Q1) and upper (Q3). The student's t-test or Mann Whitney U test were used to compare the differences between the groups. Correlation coefficients were determined using the Spearman's test. The p-values lower than 0.05 were considered statistically significant ($p<0.05$).

RESULTS

As shown in Table 1, red blood cells number, hemoglobin concentration and hematocrit were significantly increased in polycythemia vera patients as compared to the control group. White blood cells and platelet number were also higher in PV patients than in healthy volunteers.

VEGF-A concentration was three-fold higher in PV patients as compared to the control group (Me=80.59 pg/ml vs Me=25.22 pg/ml) ($p<0,000001$); however, sVEGFR-2 concentration was significantly lower in PV patients, compared to healthy controls (Me=7839.55 pg/ml vs Me=11678.16 pg/ml, $p<0.000001$). There was no statistical difference in sVEGFR-1 concentration between the studied groups.

Due to the association between angiogenesis and the occurrence of thromboembolic events in the course of myeloproliferative neoplamss, further statistical analysis assessed the level of VEGF-A and its receptors depen-

Table 1. Laboratory parameters in PV patients and healthy volunteers

Parameter	Polycythemia vera N=42			Control group N=30			p
	Me/ X	Q1;Q3/ ±SD	Min-Max	Me/ X	Q1;Q3/ ±SD	Min-Max	
RBC [T/l]	7.03	6.43; 7.58	5.22-9.67	5.21	4.86; 5.42	4.01-5.61	**<0.000001**
HGB [g/dl]	18.84	±1.41	16.80-23.10	13.65	±1.20	12.10-16.20	**<0.000001**
HCT [%]	56.70	53.91; 60.60	49.00-68.10	44.55	40.60; 46.40	36.00-47.80	**<0.000001**
WBC [G/l]	9.90	7.55; 12.60	5.40-21.56	6.10	4.90; 7.30	4.03-10.00	**<0.000001**
PLT [G/l]	470.00	280.00; 604.00	151.00-1172.00	250.00	213.00; 287.00	156.00-345.00	**0.000020**
VEGF-A [pg/ml]	80.59	54.38; 168.41	14.90-486.19	25.22	14.79; 27.12	7.22-39.84	**<0.000001**
sVEGFR-1 [pg/ml]	114.88	87.66; 166.15	43.76-487.87	115.90	71.30; 158.08	8.90-4104.83	0.416224
sVEGFR-2 [pg/ml]	7839.55	±3253.72	11.99; 14156.20	11678.16	±2342.96	7902.07-17488.96	**<0.000001**

Table 2. VEGF-A, sVEGFR-1 and sVEGFR-2 in PV patients depending on *JAK2 V617F* mutation

Parameter	JAK2V617F (+) N=35			JAK2V617F (-) N=7			p
	Me/ X	Q1;Q3/ ±SD	Min- Max	Me/ X	Q1;Q3/ ±SD	Min-Max	
VEGF-A [pg/ml]	101.06	55.99; 195.32	16.42-486.19	34.75	16.58; 69.25	14.90-86.36	**0.028109**
sVEGFR-1 [pg/ml]	114.88	92.47; 184.16	55.05-487.87	91.46	51.01; 124.29	43.76-166.15	0.417941
sVEGFR-2 [pg/ml]	7672.83	±3348.10	11.99-14156.20	8673.19	±2801.32	3047.36-11613.00	0.464557

ding on the risk factors of thrombotic events such as age, history of thrombosis, JAK2 V617F mutation and number of leukocytes. However, statistically significant differences were only found between groups in terms of Janus kinase mutation and the number of WBC (Table 2 - 3).

VEGF-A concentration was significantly higher in PV patients with JAK2V617F mutation, as compared to patients without this mutation (Me=101.06 pg/ml vs Me=34.75 pg/ml). SVEGFR-1 and sVEGFR-2 concentrations were similar in the analyzed subgroups (Table 2).

In PV patients with increased number of WBCs, above upper reference value (≥10 G/l), VEGF-Aconcentration was two-fold higher than in patients with WBC number <10 G/l (Me=119.77 pg/ml vs Me=58.56 pg/ml), however sVEGFR-1 and sVEGFR-2 concentrations did not differ between the analyzed subgroups.

An overall analysis of correlations revealed only one statistically significant relation between VEGF-A and number of WBCs (R=0.47, p=0.003) (Figure 1).

DISCUSSION

In the present study, concentration of VEGF-A was 3-fold higher in PV patients as compared to the control group. This result was similar to results of Musolino C. et al. (2002), Alonci A. et al. (2008) and Maktouf C. et al. (2011), concerning the intensity of angiogenesis in polycytemia vera patients [1, 11, 16]. Moreover, VEGF-A concentration was increased regardless of the implementation of treatment. Increased VEGF-A was observed in non--treated patients (present study) as well as in patients undergoing phlebotomy (Murphy et al. 2002) and finally in those who were treated with cytoreductive drugs ((Murphy P. et al. [15].

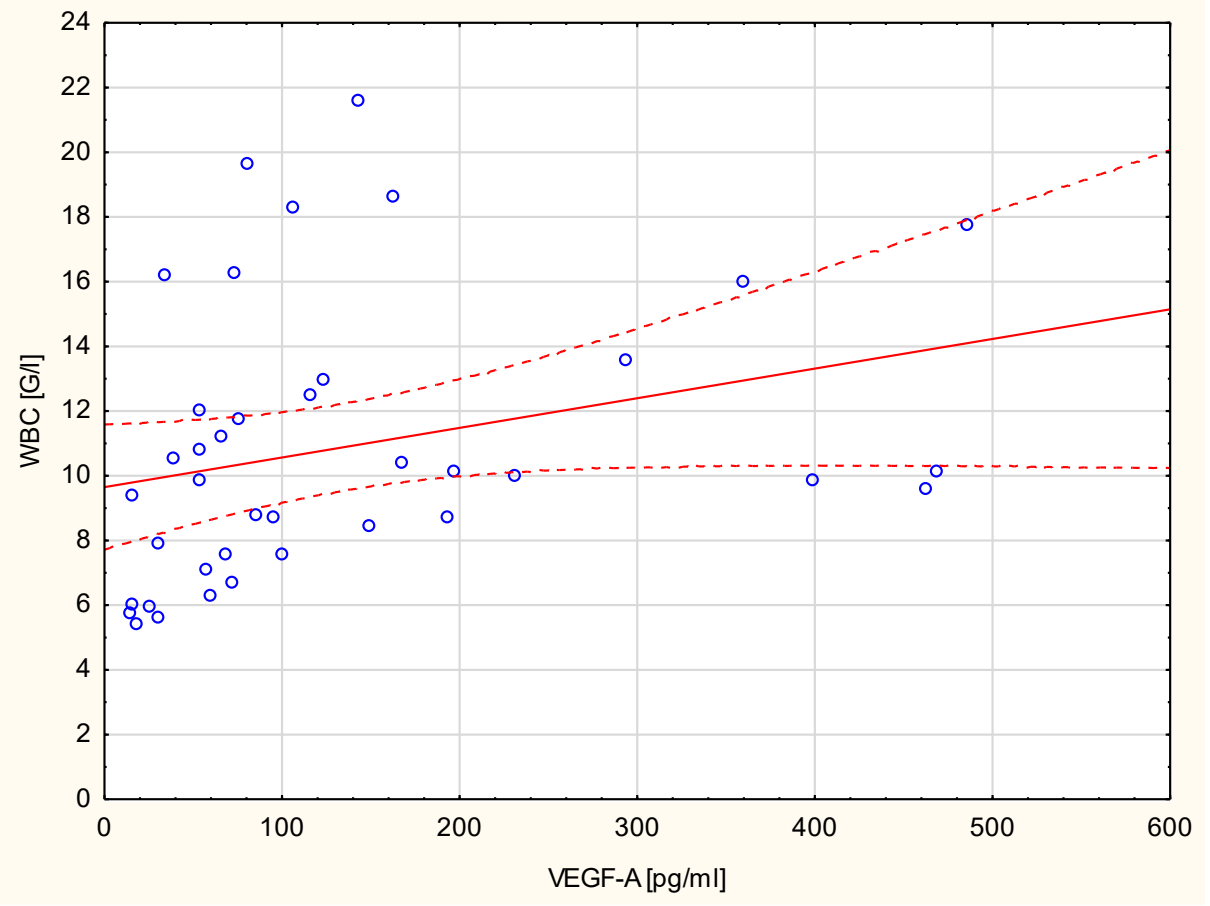
In the present study, next to the high VEGF-a concentration, we observed a significantly decreased VEGFR-2 concentration in PV patients, as compared to the control group. However, sVEGFR-1 did not differ between the study and the control groups.

Due to the fact that extra membrane VEGF-a receptors on different cell types perform inverse functions than

Table 3. VEGF-A, sVEGFR-1 and sVEGFR-2 in PV patients depending on WBCs number

Parameter	WBC≥10 G/l N=20			WBC<10 G/l N=22			p
	Me/ X	Q1;Q3/ ±SD	Min-Max	Me/ X	Q1;Q3/ ±SD	Min-Max	
VEGF-A [pg/ml]	119.77	69.55; 245.31	34.72-486.19	58.56	25.75; 96.00	14.90-399,62	**0.015676**
sVEGFR-1 [pg/ml]	120.78	103.68; 184.16	70.48-487.87	114.88	72.11; 148.47	43.76-248.42	0.172940
sVEGFR-2 [pg/ml]	8475.00	±3493.21	11.99-14156.20	7272.49	±2888.44	1479.62- 11613.00	0.209558

Fig. 1. Analysis of correlation between VEGF-A and WBCs number in PV patients



soluble receptors (angiogenesis inhibitors), the results of the present study cannot be compared with most available publications.

There is only one piece of clinical data evaluating soluble receptors for VEGF in PV patients presented by Treliński et al. (2010). The authors observed an increased sVEGFR-1 concentration in the study group as compared to healthy controls, and sVEGFR-2 concentration was similar in PV patients, when compared to the control group [20]. The results of our research are thus in contradiction to Treliński's observations, despite the similarity of the including criteria (newly diagnosed and untreated patients) and the methodology of VEGF-A and its receptors [20]. Taking into account more than 2 times higher number of patients in the present study (45 vs 16), our results seems to be more reliable. The significantly lower levels of the sVEGFR-2 receptor combined with a high concentration of VEGF-A in PV patients may form a common mechanism, leading to increased intensity of angiogenesis. Under physiological conditions, sVEGFR-2 binding to VEGF-A reduces availability of vascular endothelial growth factor for membrane receptors. The reduced concentration of sVEGFR-2 is, therefore, an additional proangiogenic stimulus.

There is a correlation between the angiogenesis and the occurrence of thromboembolic complications in myeloproliferative neoplasms, including polycythemia vera [4]. VEGF-A not only stimulates vascular endothelial cells but alsocan activate leukocytes and stimulate blood coagulation in the tissue factor dependent pathway [4, 9].

Two main factors, such as age (>60 yrs) and history of thrombotic events, are taken into account when assessing the risk of thrombotic complications in PV patients [10]. Recent studies indicate that the presence of JAK2 V617F mutation and leukocytosis may be additional risk factors for thrombosis in PV patients [7, 10].

In the present study, VEGF-A concentration in PV patients with the mean age > 60 years was almost two times higher in comparison with patients under the age of 60; however, the difference was not statistically significant. There was no relationship between age and soluble sVEGFR-1 and sVEGFR-2 receptors. The obtained results are similar to those described by other authors, who also did not observe increased angiogenesis in older patients [19]. Increased throm-

botic risk in patients >60 years of age is probably associated with increased levels of coagulation factors, increased platelet activity, endothelial dysfunction, and impaired fibrinolytic activity [18].

In the present study, the past history of thrombosis was reported in 10 patients with PV, but VEGF-A concentration and its soluble receptors were similar in divided subgroups (with and without the history of thrombosis). Our results are in close agreement with studies by Trelińskiet al. (2010), who also did not observe differences in angiogenesis parameters between PV patients with and without past thrombotis complications [20].

There are controversies surrounding the impact of JAK V617F mutation on thrombotic risk in PV patients. According to some investigators, an increased expression of platelet P-selectin and leukocyte activation markers due to the presence of JAK2 V617F mutation increases coagulation [20]. In the current study, VEGF--A concentration was almost 3 times higher in PV JAK2 (+) patients when compared to JAK2 (-) patients. However, we did not observe any changes in soluble receptor concentrations. Medinger M. et al. (2009) concluded that angiogenesis is generally independent of the JAK2 status in MPN patients, despite increased microvessel density in JAK2 (+) patients [13].

An important new aspect of our study is the significant difference found in VEGF-A concentration in PV patients with lekucytosis compared to PV patients with reference number of white blood cells. Moreover, a positive correlation was found between WBC number and VEGF-A. Analysis of available literature indicates the relationship between elevated leukocytes number and the risk of thrombotic complications [10]. Patients with PV have an increase in blood viscosity caused by, among others, an increase in the number and mass of erythrocytes, an increase in the number activated platelets and leukocytes, an increase in the percentage of platelet-leukocyte aggregates and activation of vascular endothelial cells [9].

Some authors indicate that the thrombotic risk is significantly increased when the WBC number is greater than 8.7 G/l [7, 20]. Many others consider the upper range of reference values for WBC (Mehta Ö. et al., present study) to be the cut off value, and some even up to 15 G/l [3, 14]. Studies by Carrobio et al. in patients with myeloproliferative neoplasms indicate that leukocytosis is considered a potential risk factor for thrombosis. The increase in WBC activity leads to the initiation of coagulation and vascular endothelial cells activation [7].

In the present study, we found a higher concentration of VEGF-A and a significantly lower serum sVEGFR-2 concentration in patients with polycythemia vera, which may indicate an increase in the intensity of bone marrow angiogenesis in polycythemia vera. Evaluation of VEGF concentration may be an additional, now invasive laboratory parameter, which indirectly assesses the intensity of the angiogenesis process. Vascular endothelial growth factor not only promotes angiogenesis, but also may contribute to the development of thrombotic complications especially in patients with WBC number above 10 G/l, probably through direct interaction with tissue factor.

CONCLUSIONS

Increased VEGF-A and significantly decreased sVEGFR-2 concentrations in polycythemia vera patients as compared to the control group indicate an intensification of angiogenesis. Higher concentrations of VEGF-A in PV patients with leukocytosis (>10 G/l) and a positive correlation between WBC number and VEGF-A reflect the potential role of VEGF-A in the pathogenesis of thrombotic complications in hypercoagulable state in PV patients.

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