Angiogenic and coagulation-fibrinolysis factors in non Hodgkin's lymphoma

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High serum VEGF and bFGF levels are independent prognostic factors of poor prognosis in NHL patients. There is growing evidence that both angiogenesis and haemostatic aberrancies are integral parts of the pathobiology of cancer growth and dissemination. The purpose of the study was: (a) to analyze relations of VEGF and bFGF serum levels, fibrinogen and D-dimer plasma levels with lymphoma Ann Arbor Staging System (AASS) and International Prognostic Index (IPI) and, (b) to evaluate correlations between serum levels of angiogenic cytokines and plasma levels of coagulation-fibrinolysis factors in 52 previously untreated NHL patients included to the study.

The control group consisted of 23 healthy volunteers. Serum VEGF, bFGF and plasma D-dimer levels were measured by enzyme-linked immunosorbent assay (ELISA). Plasma levels of fibrinogen were determined on Behring Coagulation System (BCS) equipment.

In lymphoma group serum VEGF and bFGF leves were significantly higher than in the control. Differences in concentrations of VEGF, bFGF between II, III and IV stage of disease acc. AASS were not statistically significant. Plasma levels of fibrinogen and D-dimer were elevated in lymphoma patients when compared with the control. Fibrinogen plasma levels were similar in all stages. The D-dimer level was significantly higher in patients with IV stage in comparison to stage II and III. Statistically significant differences of VEGF and bFGF serum levels were observed only between intermediate/high and high risk groups acc. IPI. Fibrinogen plasma levels were significantly higher in high risk group than in low risk group. D-dimer plasma levels were significantly higher in high risk group than in low risk group and low/intermediate group. We observed positive correlation between serum level of VEGF and plasma level of fibrinogen, and between serum level of bFGF and plasma level of fibrinogen. There was also negative correlation between serum level of VEGF and plasma level of D-dimer, and between serum level of bFGF and plasma level of D-dimer.

Our study indicates that D-dimer level, but not VEGF, bFGF and fibrinogen correlates with AASS and IPI in NHL patients. Significant correlations between levels of VEGF/bFGF and fibrinogen/D-dimer suggest specific interactions between angiogenic and coagulation-fibrinolysis system.

Key words: VEGF, bFGF, D-dimer, fibrinogen, non-Hodgkin's lymphoma

Non-Hodgkin's lymphoma (NHL) is a heterogenous group of lymphoproliferative malignancies. There is still a need of finding specific patterns of disease activeness and prognostic factors to improve individualization in therapy approach. The Ann Arbor Staging System (AASS) and International Prognostic Index (IPI) are the most popular clinical staging for classifying NHL patients and are used to determine the course of disease [1, 2]. Angiogenesis and activation of coagulation system are common in cancer patients and are thought to be unfavorable clinical signs. Vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) are multifunctional cytokines that are mitogens for endothelial cells and well-known stimulators of angiogenesis. There is evidence that high serum VEGF and bFGF levels are independent prognostic factors of poor prognosis in NHL patients [3]. Although the association of prothrombotic state with cancer have been recognized since more than a century, the etiology underlying this association is still poorly understood. Cancer hemostatic aberrancies are of different nature and magnitude, ranging from subtle laboratory abnormalities to massive thromboembolism or disseminated intravascular coagulation. They are found at both the systemic and locally at the tumor site. Markers of coagulation activation are strong predictors of cancer survival

[4, 5]. Plasma fibrinogen level may be a marker of coagulation activation but fibrinogen is also one of the major acute phase protein [6]. Plasma D-dimer level is a sensitive indicator of fibrinolysis. There is growing evidence that both angiogenesis and hemostatic aberrancies are integral parts of the pathobiology of cancer growth and dissemination. Although viewed as independent entities, in fact, angiogenesis and activation of coagulation system are functionally inseparable. There are complex links between angiogenic cytokines and various components of coagulation-fibrinolysis system [7, 8]. Simultaneous determination of angiogenic and hemostatic markers may be informative in the distinction of NHL patients with poor prognosis.

In an effort to determine prognostic role of angiogenesis and hemostatic regulators in lymphoma patients the aim of our study was: (a) to analyze relations of VEGF and bFGF serum levels, fibrinogen and D-dimer plasma levels with Ann Arbor Staging System and International Prognostic Index and, (b) to evaluate correlations between serum levels of angiogenic cytokines and plasma levels of coagulation-fibrinolysis factors in NHL patients.

Material and methods

Fifty-two previously untreated NHL patients (31 men, 21 women; median age 52 years), hospitalized in the Department of Hematology, Blood Neoplasms and Bone Marrow Transplantation at Wroclaw Medical University, were included to the study. In stage II according to AASS were 15 persons, in stage III – 10 persons and in stage IV – 27 persons. Moreover patients were divided into risk group according IPI: low risk – 9, low/intermediate – 5, intermediate/high – 12 and high – 26 persons.

Histological characteristic of the patients group according to World Health Organization Classification is presented in the Table 1 [9]. The control group consisted of 23 healthy volunteers (15 men and 8 women, median age 48 years).

Samples of 10 cm^3 of peripheral blood were collected by punctures of brachial veins. Blood was centrifuged with 1000 G during 10 minutes in 4 °C. Serum and plasma samples were stored at –70 °C. Serum VEGF, bFGF and plasma D-dimer levels were determined by enzyme-linked immunoabsorbent assay (ELISA). VEGF and bFGF levels were estimated with Quantikine test (R&D System, Minneapolis, USA). Plasma levels of fibrinogen were determined using Behring Coagulation System (BCS) equipment. All tests were performed in accordance with manufacturer specifications. The obtained results of VEGF and bFGF were shown as pg/ml, D-dimer as ng/ml and fibrinogen as mg%.

Statistical analysis was performed with a commercially available package STATISTICA, version 6.0. The results were presented as a mean (X) ± standard deviation (SD). The distribution of data was examined using the Shapiro-Wilk test. Differences between analyzed parameters were examined using non-parametric ANOVA Kruskal-Wallis test. Correlation between variables were tested using the Spearman correlation coefficient "r". For all tests "p" value of 0.05 and less was considered statistically significant.

Results

In lymphoma group serum VEGF and bFGF levels were significantly higher than in the control (405.37±353.38 pg/mL vs 151.03±147.30 pg/ml; $p<0.05$) and (4.53±4.24 pg/ml vs 0.36±0.26 pg/ml; $p<0.01$), respectively, (Fig. 1 and 2).

The mean serum VEGF levels in different stages of the disease were similar: 387.4±219.8 pg/ml in stage II, 399.2±235 pg/ml in stage III, and 406.9±308.8 pg/ml in stage IV (ns.). The mean serum bFGF levels were also similar: 4.3±2.9 pg/ml in stage II, 4.6±2.5 pg/ml in stage III, and 4.4±3.4 pg/ml in stage IV (ns.). Differences in concentrations of VEGF, bFGF between II, III and IV stage of disease were not statistically significant.

Plasma levels of fibrinogen and D-dimer were elevated in lymphoma patients when compared with the control: 490.31±182.87 mg% vs 325±125 mg% ($p<0.01$) and 2750.17±2308.67 ng/ml vs 280±200 ng/ml ($p<0.001$), respectively. Fibrinogen plasma levels were similar in all stages: in stage II was 514.3±198.7 mg%, in stage III: 518±209.2 mg%, and in stage IV: 459.4±155.3 mg% (ns.) (Fig. 3). Mean plasma D-dimer level were in stage II: 1654.3±1301.5 ng/ml, in stage III: 1816.6±1370.7 ng/ml, and in stage IV: 2747.1±1410.8 ng/ml. The D-dimer level was significantly higher in patients with IV stage in comparison to stage II and III ($p<0.01$) (Fig. 4).

The results of VEGF, bFGF, fibrinogen and D-dimer in IPI lymphoma risk groups are shown in Tables 2 and 3. Statistically significant differences of VEGF and bFGF serum levels were observed only between intermediate/high and high risk groups ($p<0.05$).

Fibrinogen plasma levels were significantly higher in high risk group than in low risk group ($p<0.05$). D-dimer plasma levels were significantly higher in high risk group than in low risk group ($p<0.05$) and low/intermediate group ($p<0.01$).

We observed positive linear correlation between serum level of VEGF and plasma level of fibrinogen ($r=0.45$; $p<0.01$), and between serum level of bFGF and plasma level of fibrinogen ($r=0.52$; $p<0.05$). There was also negative linear correlation between serum level of VEGF and plasma level of D-dimer ($r= –0.69$; $p<0.001$), and between serum level of bFGF and plasma level of D-dimer ($r=–0.72$; $p<0.001$), Figures 4–7.

Discussion

Chronic activation of hemostatic system has been widely recognized to be present in malignancy and its intensity is thought to be a strong predictor of overall cancer survival [6]. The pathogenesis of cancer-related coagulation-fibrinolysis activation is complex and multifactorial. Genetic changes in

Table 1. Histological characteristic of NHL patients according to World Health Organization Classification

Type of malignant lymphoma	n=52
Anaplastic Large T cell	5
Burkitt	2
Follicular	12
Lymphoblastic preB cell	6
Lymphoplasmocytic	2
Mantle cell	4
Diffuse Large B-cell	21

Table 2. Statistically significant differences of VEGF and bFGF serum levels were observed between intermediate/high and high risk groups (p<0.05)

IPI risk group	VEGF [pg/ml]		bFGF [pg/ml]	
	X	SD	X	SD
Low (n=9)	511.83	328.71	3.99	2.86
Low/intermediate (n=5)	492.42	118.14	5.75	4.62
Intermediate/high (n=12)	816.97	765.15	7.48	4.08
High (n=26)	352.98	344.69	2.57	2.01

IPI – International Prognostic Index

Table 3. Fibrinogen plasma levels were significantly higher in high risk group than in low risk group (p<0.05). D-dimer plasma levels were significantly higher in high risk group than in low risk group (p<0.05) and low/intermediate group (p<0.01).

IPI risk group	Fibrinogen		D-dimer	
	X	SD	X	SD
Low (n=9)	424.34	207.27	1469.00	1264.54
Low/intermediate (n=5)	468.40	214.76	1200.00	900.51
Intermediate/high (n=12)	471.00	154.29	2364.00	2190.54
High (n=26)	526.25	181.49	3670.00	3049.27

IPI – International Prognostic Index

malignant cells play a key role in deregulation of the hemostatic system. Cancer cells may have capacity to produce and release molecules with procoagulant or fibrinolytic activity (i.e. tissue factor (TF), plasminogen activators (PA), plasminogen activator inhibitor-1). In additio‘n, tumor cells may interact with host cells, mainly with endothelial cells, platelets, monocytes, neutrophils and stromal cells within tumors, inducing activation and release of different procoagulant mediators [10, 11]. Tumors are frequently surrounded by fibrin and fibrinogen. Fibrinolytic activity is largely attributed to plasminogen, tissue-type plasminogen activator (t-PA) and urokinase-type plasminogen activator (u-PA). Fibrinolysis facilitates tumor growth and metastasis. Through pericellular fibrinolysis tumor and endothelial cells invade into fibrin matrix [12]. There is increasing evidence that angiogenesis is related with activation of the hemostatic

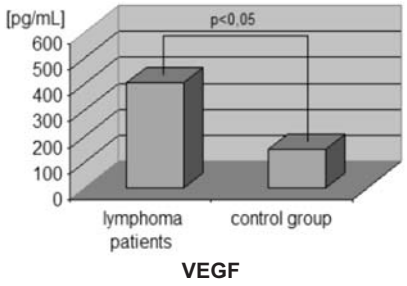

Figure 1. Plasma level of VEGF in NHL patients in comparison to control group.

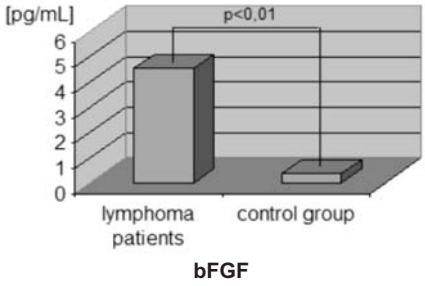

Figure 2. Plasma level of bFGF in NHL patients in comparison to control group.

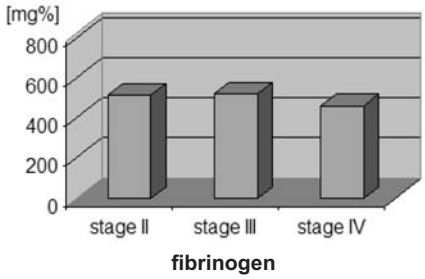

Figure 3. Plasma level of fibrinogen in patients with NHL in different stages of disease according to AASS.

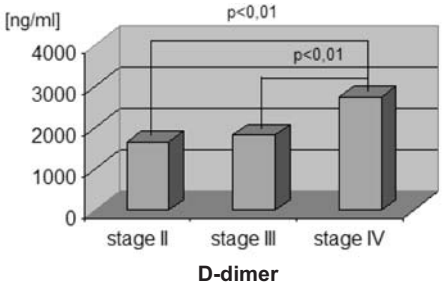

Figure 4. Plasma level of D-dimer in patients with non Hodgkin's lymphoma in different stages of disease according to AASS.

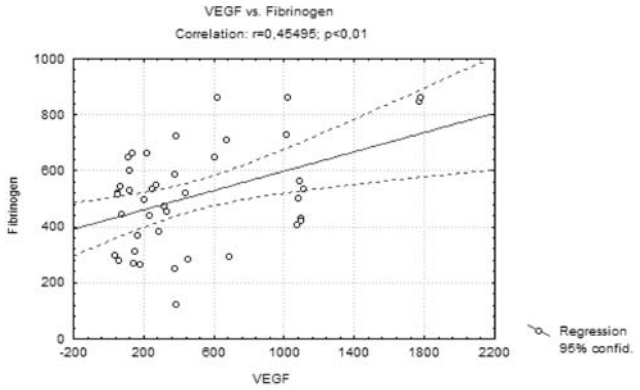

Figure 5. Positive linear correlation between serum level of VEGF and plasma level of fibrinogen in NHL patients (r=0.45; p<0.01).

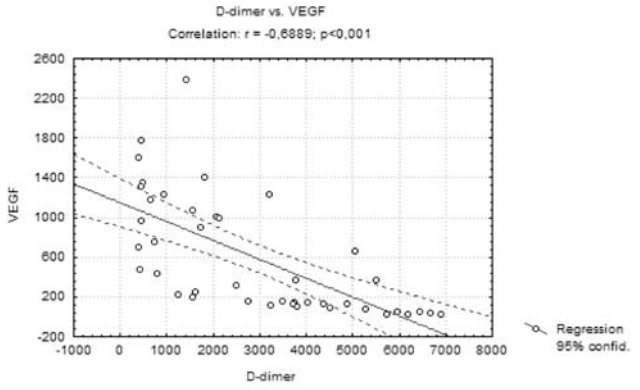

Figure 7. Negative linear correlation between serum level of VEGF and plasma level of D-dimer in NHL patients (r=–0.69; p<0.01).

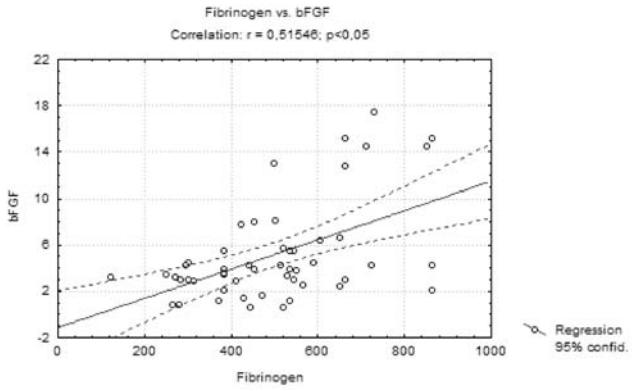

Figure 6. Positive linear correlation between serum level of bFGF and plasma level of fibrinogen in NHL patients (r=0.52; p<0.01).

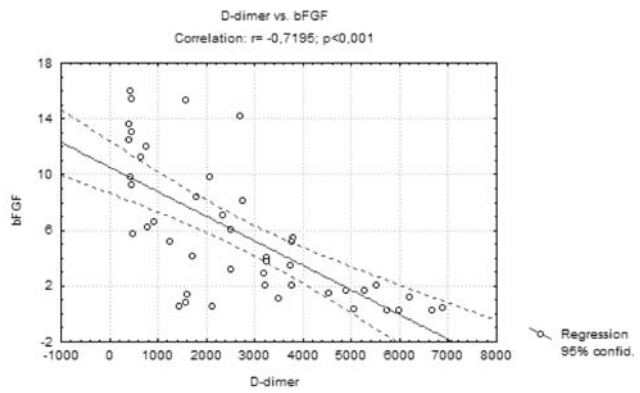

Figure 8. Negative linear correlation between serum level of bFGF and plasma level of D-dimer in NHL patients (r=–0.72; p<0.01).

system. Vascular hyperpermeability and stimulation of endothelial cells induced by VEGF ‘and bFGF results in leakage of protrombin and fibrinogen into extracelluar matrix and activation of coagulation/fibrinolysis cascade. Proteolytic properties of plasmin, t-PA, u-PA by activation of various proangiogenic soluble growth factors and cytokines intensify process of angiogenesis. There is also evidence that fibrin degradation products, mainly fragment E, are stimulators of angiogenesis [13]. Angiogenesis in NHL increases with tumor malignancy grade and that high serum VEGF and bFGF content reflect active tumor neovascularization [3, 14]. Published data show that serum VEGF and bFGF levels are independent prognostic factors of survival in NHL patients. There have been found significantly higher serum VEGF and bFGF content in high-grade NHL then in low-grade one. High serum VEGF and bFGF levels are thought to correlate also with the extent of lymphoma growth [3, 15]. However, the results of current study show that although the serum levels of the angiogenic factors VEGF and bFGF are significantly higher in NHL patients then in control group, there were no statistical differences between their level in different stages of disease according to AASS and IPI. This finding suggests that the extent of NHL determined by AASS and IPI does not correlate with angiogenesis intensity within tumors sites. In our study, stage of disease according to AASS and prognosis according to IPI were correlated with plasma level of D-dimer. There was significantly higher D-dimer level in stage IV in comparison to stage II and III. Additionally D-dimer were highest in IPI high risk group. D-dimer is a fibrin degradation product. It is produced when crosslinked fibrin is degraded by plasmin. D-dimer level is a sensitive indicator of fibrinolysis but also, because fibrinolysis is the consequence of coagulation, it may also be an indirect indicator of coagulation activation. Similar positive correlation between D-dimer and stage of lymphoma was observed by SASE et al [16].

The etiology of observed correlations between levels of angiogenic and coagulation-fibrinolysis may be the consequence of cancer development which is associated with influence on both angiogenesis and hemostatic system. But, this causative role of cancer progression can be greatly influenced by mutual impact of angiogenic and coagulation fac-

tors. There are many links between angiogenic and coagulation-fibrinolysis factors.

It has been shown that components of the hemostatic system have impact on angiogenesis and this influence cancer growth and dissemination [17]. Tissue factor, fibrin, plasminogen activation system, as well as platelets, all are able to promote tumor vessel development [18, 19]. Angiogenesis is influenced by coagulation system in both: dependent and independent manner [20]. Coagulation-dependent mechanism is caused by TF-induced generation of thrombin and, consequently, by deposition of cross-linked fibrin in the extracellular matrix. VEGF and bFGF bound to soluble fibrinogen in medium and to surface immobilized fibrinogen or fibrin with high affinity. A cross-linked fibrin network provides a scaffold for both angiogenic peptides for maximum effect at sites of angiogenesis and promotes the adhesion, proliferation, and migration of endothelial cells during angiogenesis. Coagulation-independent mechanism including upregulation of proangiogenic proteins such as VEGF or bFGF, which are released from cells activated during process of coagulation [18, 19, 21].

Clear distinction between cancer influence and impact links between angiogenic and coagulation system on VEGF, bFGF, D-dimer and fibrinogen levels are difficult. However, there are some known facts which may explain correlations determined in our study. The positive linear correlation between VEGF and fibrinogen may show indirect procoagulant VEGF action. VEGF may affect hemostasis by increasing vascular permeability and increasing TF expression on endothelial cells, which in turn activates the coagulation cascade and promotes platelets adhesion and activation [22, 23]. Tumor angiogenesis results in the formation of abnormal, with irregular endothelium blood vessels, which may be the area of activation of the coagulation system. Therefore, the more VEGF, the greatest neovascularization, coagulation activation, and higher plasma fibrinogen level. On the other hand initiation of coagulation cascade by tumor cells may in turn results in increased release of VEGF and bFGF by activated platelets and endothelial cells.

VEGF and bFGF stimulates expression of activators of fibrinolysis, such as t-PA and u-PA. Simultaneously VEGF and bFGF stimulate also plasminogen activator inhibitor-1 (PAI-1) [24–26]. Appropriately balanced regulation of PA and PAI-1 by VEGF providing exact extracellular proteolysis is required for accurate neovascularization [26]. Data concerning the correlations between D-dimer and VEGF are confused. TSENG et al found no correlation between D-dimer and VEGF in patients with hepatocellular carcinoma [27]. MATSUYAMA et al observed positive correlation between D-dimer an VEGF in lung cancer [28]. The negative linear correlation that we observed between VEGF/bFGF and D-dimer level may be not only the effect of balanced influence of VEGF on fibrinolysis activators and inhibitors but also of another VEGF property, which is a maintenance and protection for endothelium cells [29]. VEGF protects vascularity through enhancement of endothelial cell survival, induction of nitric oxide (NO) and prostacyclin production in endothelium cells, suppression of thrombosis and anti-inflammatory effects and inhibition of vascular smooth muscle cell proliferation [32, 33]. VEGF is supposed to maintain integrity of the endothelium, not only in quiescent state, but also in situations of damage or injury. In the event of local endothelium damage, endothelium cells become activated and form a prothrombotic surface. Locally produced VEGF plays a key role in endothelium repairing [32, 33]. High concentration of VEGF may protect from activation of coagulation-fibrinolysis system by endothelium, therefore, may be correlated with lower D-dimer level. Moreover in NHL patients D-dimer levels correspond very well with tumor burden but intensity of angiogenesis measured by VEGF and bFGF levels is correlated rather with tumor proliferation rate. Finally such different results concerning correlation between D-dimer and VEGF in cancer patients observed by many authors may be also depending on tumor type. Similar correlation of bFGF and VEGF with D-dimer and fibrinogen levels may be explained by the fact that bFGF elevation is usually concomitant with VEGF increase.

Taking into account complexity of angiogenesis and hemostatic system, variations in cancer genetic changes and influences of tumor microenvironment, it may be presumed that although there are some commonly observed events in cancer angiogenesis-hemostasis etiology, there are also some specific ones, not only in the context of the kind of cancer, but also in the context of individual cases. Our study indicates that D-dimer level, but not VEGF, bFGF and fibrinogen correlates with AASS and IPI in NHL patients. Significant correlations between levels of VEGF/bFGF and fibrinogen/D-dimer suggest specific interactions between angiogenic and coagulation-fibrinolysis system.

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