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ELEVATED PLASMA LEVELS OF VASCULAR ENDOTHELIAL GROWTH FACTOR AND HEPATOCYTE GROWTH FACTOR: CLINICAL SIGNIFICANCE AND CORRELATION WITH TUMOR BURDEN IN PATIENTS WITH CHRONIC MYELOID LEUKEMIA

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ABSTRACT

Angiogenesis is an established indispensable process in the development and metastasis of solid tumors. Its significance in the pathogenesis and progression of hematological malignancies is still to be elucidated. Data have recently been accumulated about its role in chronic myeloid leukemia (CML). The aims of our study were: (I) to assess vascular endothelial growth factor (VEGF) and hepatocyte growth factor (HGF) plasma levels in patients with newly diagnosed, untreated and treated CML; (II) to analyze the differences of their levels in varying phases of the disease; (III) to analyze VEGF and HGF correlation with some markers reflecting the tumor burden as well as the progression of the disease. Plasma levels of VEGF and HGF were determined by ELISA in 16 patients with CML and in 21 healthy individuals. VEGF and HGF levels were followed-up in 6 patients according to the progression of the disease or the treatment regimen. All the patients with CML showed significantly higher levels of VEGF and HGF when compared to the control group. We found a significant correlation between HGF and leukocytes, LDH, splenomegaly and blast percentage in the peripheral blood. Significant correlation was found between VEGF and platelets, LDH and leukocytes. Our data indicate that CML are highly associated with elevated plasma levels of VEGF and HGF, which corroborates the thesis of its angiogenic dependency. Likewise, the present study suggests that measurements of VEGF and HGF may be useful for assessing disease activity and progression as well.

Key words: angiogenesis,VEGF, HGF, chronic myeloid leukemia, diagnosis

INTRODUCTION

Angiogenesis is a process of forming a new blood network from a preexisting vasculature. It involves degradation of extracellular matrix proteins, activation, proliferation and migration of endothelial cells and pericytes. Except its physiologic role in ovulation, placentation and embryogenesis, angiogenesis is an integral step in the growth, dissemination and metastasis of solid tumors (1). Many positive and negative regulatory molecules are involved in the angiogenic process. The analysis of the degree of intratumoral neovascularization as well as the expression of some proangiogenic factors may provide prognostic information in patients with certain solid tumors (2,3). Substantial research data have recently been accumulated about the role of angiogenesis in hematological malignancies. Increased micro-vessel density and high serum levels of proangiogenic factors have been reported in patients with myelodysplastic syndromes (MDS), acute myeloid leukemias (AML), chronic lymphoproliferative disorders (4), myeloproliferative disorders (MPD), (4,5,6) and multiple myeloma (MM) (7). The possible prognostic significance of increased angiogenesis has so far been found in patients with MM that provides a rationale for clinical investigations of the anti-angiogenic agent Thalidomide in this disease as a part of the salvage therapeutic regimens.

During embryonic development, hematopoietic stem cells (HSCs) and early endothelial cells (EC) originate from a common precursor cell known as hemangioblast. Given this common origin, it is suggested that HSCs has a significant role in angiogenesis during the embryogenesis (8). With the identification of the hemangioblast in adult individuals (9), we can speculate that the tight relationship between hematopoiesis and angiogenesis are preserved during the postnatal life.

VEGF and HGF are among the most specific and potent inducers of angiogenesis. Their elevated plasmatic levels serve as indirect markers of increased angiogenic activity. VEGF is a multifunctional protein which activates its endothelial receptors and leads to an increased capillary permeability, endothelial cell proliferation and migration. The autocrine and paracrine actions of VEGF are studied in leukemias and MDS by some authors. Fiedler *et al.* (10) demonstrate that the bone marrow endothelium releases leukemic growth factors (GM-CSF, IL-6,

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Fas-ligand) and, in turn, the leukemic cells secrete VEGF which means that VEGF receptors on leukemic cells are functional, and thus they initiate endothelial proliferation and expansion of the bone marrow capillary net. The growing endothelial mass supports the leukemic progression in a paracrine manner with an increased production of growth factors (10). On the other hand, the expression of VEGF receptors by leukemic cells leads to an establishment of an autocrine loop and thus, supporting their migration and survival (10,11). HGF (also known as "scatter" factor) is produced by mesenchyme cells as well as by bone marrow stromal cells. It regulates the cell growth, motility and morphogenesis of various cell types. Its significance for the normal and malignant hematopoiesis has been determined (12,13). HGF induces proliferation and migration of HSCs in patients with AML and MDS *in vitro*, therefore it could contribute to the malignant potential of these cells (13).

Since MPD, and particularly CML, serve as a model of deregulation of HSCs that results in an excessive myeloid proliferation, it is reasonable to expect an increased angiogenesis in these disorders as well. The data about the clinical significance of angiogenesis in CML are contradictory and insufficient. For this reason we give our attention to investigate angiogenic activity in CML and other MPD. Some data published by now (4,5), as well as our own (14), noticed that the levels of VEGF and HGF are increased in patients with chronic MPD. In this article we analyze in detail the differences in cytokine levels in various phases of CML, the correlation with some markers reflecting the tumor burden and progression of the disease as well as the clinical significance of VEGF and HGF.

MATERIAL AND METHODS

1. The study group is classified and presented on Table 1. The control group consists of 21 volunteers with no clinical or laboratory signs of malignancy or active disease.

Table 1. Characteristics of the study group

	CML			Control group
	Chronic phase	Accelerated phase	Blast crises	
N of cases	8	3	5	N=21
	N=16			
N of plasma probes	11	4	6	
	N=22			
Age (years)	46 (18-71)			40 (23-66)
Male/female ratio	10/6			8/13

2. Plasma samples. According to the published data,15 there are differences between the plasma and serum concentrations of VEGF which may be due to its release from the platelets during the process of blood-clotting. Following the recommendations of working with plasma not with serum probes, we fixed on methods as follows: the blood probes were collected through venepunction in EDTA anticoagulant tubes in order to prevent the activation of blood-clotting systems; so-processed materials were centrifuged immediately at 3000 rpm for 7-8 min and 1ml plasma samples were separated, frozen and stored at -32°C.

3. Cytokine levels. Using enzyme-linked immunosorbent assay (ELISA), the plasma levels of VEGF and HGF were determined according to the instructions of the given protocols (Quantikine human VEGF and Quantikine human HGF ELISA kits, from R&D Systems, MN). The assay sensitivity for VEGF and HGF are 9pg/ml and 40pg/ml, respectively.

4. Statistical analyses. We performed the analyses with SPSS 10.0 statistical program for Windows. The following non-parametrical tests were used: Mann-Whitney U and Wilcoxon W tests (to analyze the cytokine differences among the study groups), and Spearman's Rho test (to assess the correlations between cytokine levels and some markers reflecting the tumor burden and progression of the disease).

RESULTS AND DISCUSSION

1. Plasmatic concentrations of VEGF and HGF

1.1. Elevated plasmatic concentrations of VEGF and HGF in patients with CML in chronic, accelerated and blast crisis phases of the disease. The summary of the results is given in Table 2, Fig. 1 and Fig. 2.

Initially, we tested the differences in VEGF and HGF levels between all of the CML patients and the control group. VEGF and HGF levels were significantly higher in the plasma samples of CML group as compared with the control group ($p<0,0001$, Mann-Whitney U test). The median VEGF in plasma samples of CML was 305pg/ml, and respectively 45pg/ml in the control group. Higher levels of HGF were found in CML patients with median level of 2563,5pg/ml than in the control group (744pg/ml). With respect to these findings, threshold values were found for both VEGF and HGF plasma levels between the 2 groups (CML patients and controls). Additionally, we divided the CML plasma samples in accordance with the three phases of the disease to test the differences among their cytokine levels. Intriguingly, we found no increase of VEGF plasma levels during the progression from chronic CML to blast crisis ($p<0,6$, Mann-Whitney U, Wilcoxon W tests). Conversely, the increase of HGF plasma levels were of great significance and correspondence with the progression to blast transformation ($p<0,001$, Mann-Whitney U, Wilcoxon W tests).

1.2. Comparison of VEGF and HGF levels in untreated and treated CML patients and controls.

We tested 22 plasma samples from CML patients according to the treatment, as we divided the patients in treated and untreated groups. We made no difference among the various treatment regimens for the main goal of the evaluation was not to be analyzed the angiogenic properties of the drugs but the activity of the disease.

Table 2. Descriptive and comparative analyses of VEGF (pg/ml) and HGF (pg/ml) in the study group

VEGF pg/ml	p (controls)	N	Mean	SD	Min.	Max.	Percentiles		
							25th	50th (Median)	75th
Control group	-	21	48	22,19	10	83	32	45	68
CML (all cases)	p<0.001	16	746	888,73	25	3064	172	305	1127
CML (Chronic phase)	p<0.001	8	1012,25	1170,89	25	3064	172	367,5	2192,5
CML (Acceleration)	p<0.01	3	427,66	351,52	180	830	180	273	830
CML (Blast crisis)	p<0.001	5	513	464,01	116	1226	143	330	974,5
HGF pg/ml									
Control group		21	749,9	244	270	1198	609,5	744	902
CML (all cases)	p<0.0001	16	5009,5	5565,69	601	18000	1103,5	2563,5	7818
CML (Chronic phase)	p<0.01	8	2412,38	2366,89	601	6752	875,5	1184,5	4688,75
CML (Acceleration)	p<0.01	3	2343	1080,44	1100	3057	1100	2872	3057
CML (Blast crisis)	p<0.001	5	10756,8	6754,88	2068	18000	5114	8326	17615

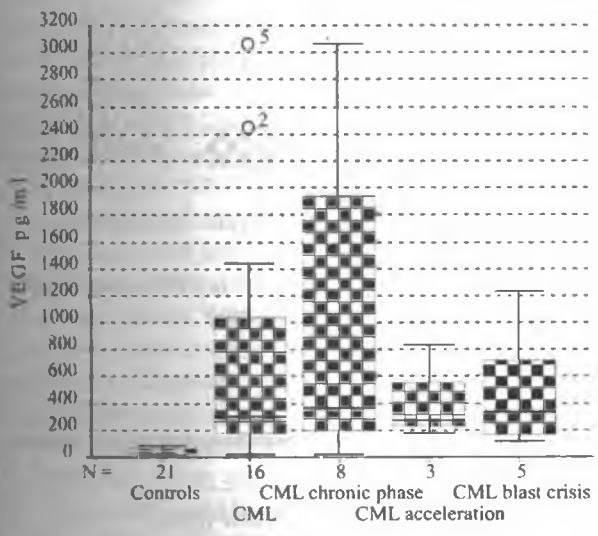

Fig. 1. Comparison of VEGF levels in CML, different phases of CML and control group

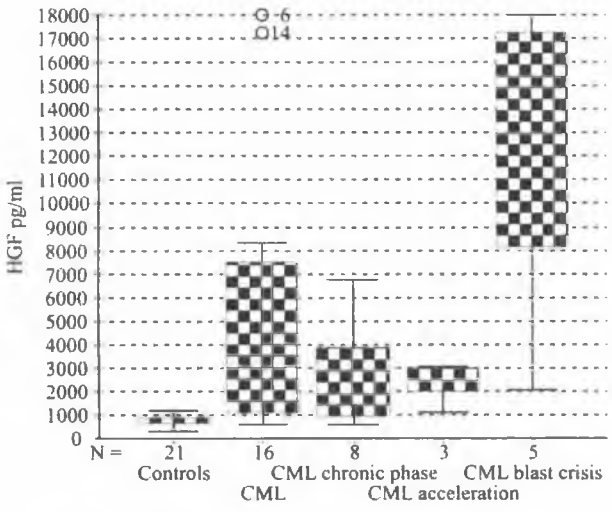

Fig. 2. Comparison of HGF levels in CML, different phases of CML and control group

As it is seen in Table 3, Fig. 3 and Fig. 4, the levels of VEGF and HGF are significantly higher in untreated CML patients than those treated with chemotherapy and the healthy individuals from the control group. The median levels of VEGF and HGF in treated patients were slightly higher than those in the controls and there was no significant difference for HGF when we performed the statistical test.

2. Correlation analyses between cytokine levels and some markers reflecting the tumor burden as well as the progression of CML. The results are presented on Table 4.

According to our data the most significant correlation was found between HGF plasma levels and leukocytes (WBC), blasts percentage in peripheral blood (PB), lactate dehydrogenase enzyme activity (LDH) and splenomegaly. VEGF levels correlated mostly with WBC and LDH, moderately with platelets but, in contrast to HGF, there was no correlation with blast cells in PB. Having in mind that the levels of VEGF do not increase significantly during the blast crisis in CML patients (Table 2 and Fig. 1), we can suspect that VEGF cannot predict the blast transformation of CML as contrasted with the higher HGF levels in this phase of the disease.

Table 3: Descriptive and comparative analysis of the untreated/treated and control groups

VEGF pg/ml	p (controls)	N	Mean	SD	Min	Max	Percentiles 25th	50th (median)	75th
Controls	*	21	48	22,19	10	83	32	45	68
Treated	p<0,03	8	108,5	81,28	25	278	44	92	144,75
Untreated	p<0,0001	14	841	913,27	116	3064	230,25	392,5	1278,25
HGF pg/ml									
Controls	*	21	749,9	244	270,00	1198	609,5	744	902
Treated	NS/p<0,06	8	1811,25	1591,98	601,00	5270	692,25	1089,5	2492,75
Untreated	p<0,002	14	5622,21	5701,4	982,00	18000	1219,75	2964,5	8201,5

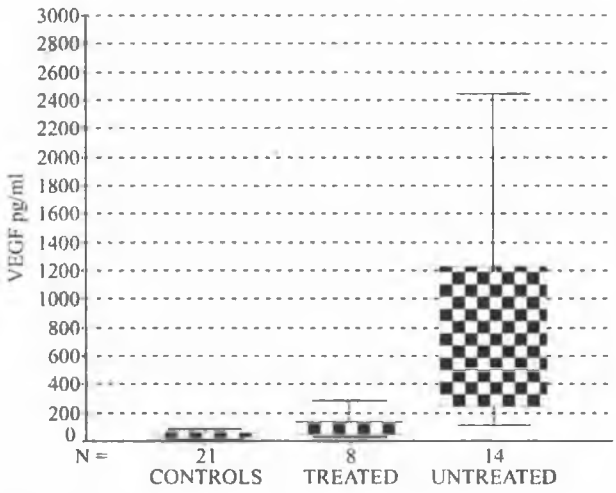

Fig. 3. Comparison of VEGF levels in CML treated and untreated patients and control group

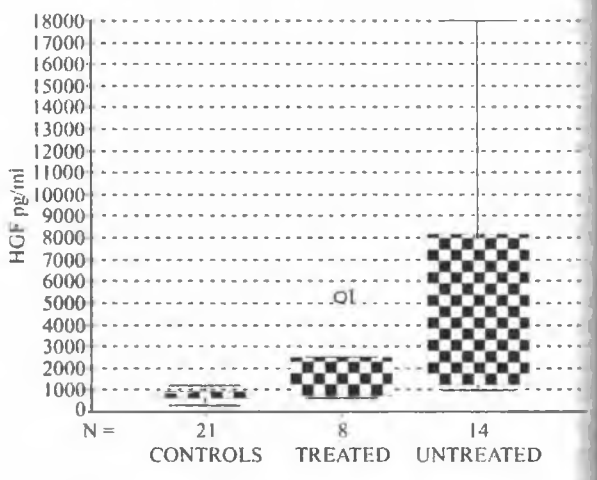

Fig. 4. Comparison of HGF levels in CML treated and untreated patients and control group

Table 4. Correlation analysis (Spearmen's Rho correlation test)

	CML	
	HGF (pg/ml)	VEGF (pg/ml)
WBC (G/L)	r=0.798; p<0.01	r=0.698; p<0.01
Blasts percentage in PB (%)	r=0.728; p<0.01	No significance
Platelets (G/L)	No significance	r=0.484; p<0.05
LDH (IU/L)	r=0.767; p<0.01	r=0.569; p<0.01
Splenomegaly (cm)	r=0.581; p<0.01	r=0.456; p<0.05

Our data indicate that CML is a disease highly associated with elevated plasma levels of VEGF and HGF, which corroborates the thesis of its angiogenic dependency.

The most significant increase of VEGF level is found in patients in chronic phase of CML as well as in the untreated group. Likewise, given its strong correlation with WBC, LDH and splenomegaly (Table 4), VEGF plasma levels reflect the tumor burden in all phases of CML.

It is notable that with the acceleration of the disease to blast crisis, VEGF level does not increase but slightly decreases which is contrary to some other results obtained by now (16), and this could be due to the following reasons:

- Platelets and megakaryocytes are one of the main sources of VEGF release. To reduce this dependency we used plasma instead of serum probes. Although this reinsurance, we found a significant correlation with platelets but no correlation was demonstrated with the blast percentage in PB;
- Having in mind that with the progression of CML the main criterion is the increase of blasts over 25% and the thrombocytopenia is a usual event, we can speculate that VEGF could not serve as a predictor for blast transformation;
- Insufficient number of patients both in acceleration and blast transformation of CML. Further study by our group is imminent in order to assess a significant number of patients as well as the cellular VEGF protein expression in bone marrow myeloid precursor cells and neovascularization.

The present study demonstrates that HGF could serve as a reliable marker reflecting the tumor burden as well as the activity and the progression of the disease. The high correlation of HGF levels with WBC, blast percentage in PB, LDH and splenomegaly suggests its possible clinical significance as a method to follow-up patients with CML. In this respect, our results are in conformity with others (4,5), as the differences and correlations are more significant in our study, probably because of the precise division of the groups. As it is seen in Table 2 and Fig. 3, HGF level significantly increases in blast crisis of CML patients. It suggests its possible predictive value to blast transformation and, respectively, it could serve as a prognostic factor.

One of the intriguing findings emerged from our study is that, with respect to both VEGF and HGF, there is evidence for the existence of a threshold value to distinguish between healthy individuals and CML patients. Threshold values of 90pg/ml and 1150pg/ml appear to exist for VEGF and HGF, respectively. With regard to clinical value, these results have demonstrated the potential usefulness of VEGF and HGF as diagnostic and prognostic parameters.

Both VEGF and HGF levels decrease during the treatment of CML patients regardless of the phases of the disease. So, it is rationale that an establishment of such an angiogenic cytokine profile could be a reliable method of assessing not only the angiogenic and disease activity but its suppression during the treatment as well.

In summary, our present data lend support to previous studies suggesting that angiogenic factors play a functional role in CML. Obviously, VEGF and HGF have a great clinical significance which must be confirmed with further investigations, and including of more cases will allow a performance of more accurate and extended analyses and conclusions.

An extended prospective study is started by our group in order to analyze a sufficient number of patients with CML and other chronic MPD as well as to assess the correlation of plasma levels of VEGF and HGF to their cellular expressions in bone marrow and neovascularization.

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