Angiogenic Activity in Chronic Myeloid Leukemia

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Abstract

Objectives: Assessment of angiogenic activity through evaluation of Vascular endothelial growth factor concentration and determination of endothelial cells percentage in the peripheral blood of patients with Chronic myeloid leukemia compared to healthy subjects in order to investigate their role in the pathogenesis and for early detection of the disease progression.

Subjects and Methods: Twenty patients with Chronic myeloid leukemia and 15 healthy controls were studied. Evaluation of Vascular endothelial growth factor level in serum was measured by enzyme-linked immunosorbent assay. Determination of circulating endothelial cells percentage expressing CD133 and/or CD34 by flow cytometry in the peripheral blood was also done.

Results: The level of Vascular endothelial growth factor was significantly elevated in all groups when compared to controls (p=<0.001). A significant increase of endothelial cells was observed in Chronic myeloid leukemia patients with blast crisis phase compared to other phases (p=<0.001). In patients with chronic phase and accelerated phase the number of endothelial cells was slightly increased compared to the control group but the differences were not statistically significant.

Conclusion: The level of Vascular endothelial growth factor was highly elevated in all phases of Chronic myeloid leukemia. While the flow cytometric evaluation of endothelial cell surface markers in the blood of Chronic myeloid leukemia patients can identify a subset of patients with a more aggressive disease course.

Keywords: Chronic myeloid leukemia; Vascular endothelial growth factor

Introduction

Chronic myeloid leukemia (CML) is a myeloproliferative disorder originating from the hematopoietic stem cell. It results from the clonal expansion of pluripotent hematopoietic stem cells containing the active BCR/ABL fusion gene produced by a reciprocal translocation of the ABL1 gene to the BCR gene.

The BCR/ABL protein displays a constitutive tyrosine kinase activity and confers on leukemic cells growth and proliferation advantage and resistance to apoptosis [1]. The clinical staging of CML is divided into chronic phase, accelerated phase and blast crisis phase. In chronic phase bone marrow is predominantly myeloid and hyper cellular. Blasts are less than 5% of all cells and maturing granulocytes are readily identified [2].

Once the disease is progressed to blast crisis phase, patients are not sensitive to treatment and result in shortened survival. Therefore, study on the pathogenesis of CML can help impeding the initiation and development of the disease as well as discovering novel therapeutic strategies [3].

Vascular endothelial growth factor (VEGF) is involved in tumor angiogenesis which is a multistep process important in tumor growth and metastasis formation. Recently there are increasing data linking VEGF with pathogenesis of different haematological malignancy including CML [4].

The assessment of angiogenesis process is based primarily on measurement of microvessel density (MVD) in bone marrow and on the evaluation of VEGF concentration. However, for the assessment of bone marrow microvessel density, invasive diagnostic procedures such as biopsy of the tumor or bone marrow are necessary [5].

Although circulating endothelial cells (CECs) are proposed to be a noninvasive marker of angiogenesis recently there has been an increased interest in the role of endothelial progenitor cells (EPCs) in the context of angiogenesis, especially in hematological malignancies. EPCs represent a heterogeneous group of blasts capable of self-renewal, colony-forming and differentiation 'on demand' into functional cells which release from bone marrow into circulation and migrate to the site of injury in vascular system [6,7].

The present study aims to assess angiogenic activity through evaluation of VEGF concentration and determination of endothelial cells (ECs) percentage in the peripheral blood of CML patients compared to healthy subjects in order to investigate their role in the pathogenesis of the disease and for early detection of the disease progression.

Subjects and Methods

Subjects

This study was conducted at clinical pathology, medical oncology and hematology departments, Faculty of medicine, Al Hussein University and Zagazig University hospitals in the period from 2016-2017.

This study included two groups

Group I: comprised of 15 aged and sex matched apparently healthy volunteers as control. The mean age of healthy volunteers was 53.7 ± 10.65 (9 males and 6 females) with a range of (32-68).

Group II: comprised of 20 clinically diagnosed CML patients, they were sub classified into 9 patients in chronic phase (45%), 3 patients in accelerated phase (15%) and 8 patients in blastic crisis (40%). The mean age of patients was 55.5 ± 8.3 (12 males and 8 females) with a range of (30-67). All the subjects gave informed consent and clearance was obtained from the hospital ethics committee.

All subjects were subjected to the following

Detailed history: with special emphasis on age, sex and presence of leukemia associated symptoms.

Clinical examination: Clinical examination for physical signs as (splenomegaly, hepatomegaly, lymphadenopathy, pallor, purpura, CNS manifestation and sternal tenderness).

Laboratory investigations

Basic investigations for CML diagnosis: Complete blood count (CBC) by automated cell counter "Sysmex KX-21" with examination of leishman stained peripheral blood. Bone marrow aspiration (for patients only) if indicated for blastic crisis and accelerated phase with examination of leishman stained smears. Conventional cytogenetic for detection of Philadelphia chromosome (for patients only).

Specific investigations: VEGF level in serum by ELISA. Determination of circulating endothelial cells percentage expressing CD133 and/or CD34 by flow cytometry (FCM).

Sample collection and storage

Four ml of venous blood was obtained from each patient and healthy volunteers by sterile syringes under aseptic conditions and were divided into 2 tubes:

Two mL of venous blood were aseptically collected from each patient, dispensed into a tube containing K-Ethylene Diamine Tetra Acetate (K-EDTA) at a concentration of 1.2 mg/ml, to be used for the flow cytometry. These samples processed as soon as possible within 6 hours of collection. When this was not possible, the samples were stored at a constant temperature of 4°C and transported at room temperature (20-25 °C) then stained and lysed within 24 hrs after collection.

Two ml were left in a tube to clot to obtain serum for VEGF measurement. These Samples were collected in serum separator tubes (SST) and allowed to clot for 30 minutes before centrifugation for 15 minutes at 1000 × g. Removed serum were either assayed immediately or stored at ≤ -20°C for about 1 year and once thawed should be used with avoiding repeated freeze-thaw cycles.

Methodology

Measurement of VEGF level in serum

Human VEGF ELISA kit Catalog Number DVE00 (Clinilab company) was an in vitro enzyme-linked immunosorbent assay (ELISA) for quantitative measurement of human VEGF in serum. This assay employed the quantitative sandwich enzyme immunoassay technique in which an antibody specific for human VEGF coated onto a 96-well plate.

Standards, samples and biotinylated anti-human VEGF were pipetted into the wells and VEGF present in a sample was captured by the antibody immobilized to the wells and by the biotinylated VEGF-specific detection antibody. After washing away unbound biotinylated antibody, HRP (horseradish peroxidase) conjugated streptavidin was pipetted into the wells.

The wells were again washed. Following this second wash step, TMB (tetramethylbenzedine) substrate solution was added to the wells, resulting in blue color development proportional to the amount of VEGF bound. The stop solution changed the color from blue to yellow, and the intensity of the color was measured at 450 nm.

Immunophenotyping of circulating endothelial cells

CECs and EPCs were evaluated using a panel of mouse antihuman monoclonal antibodies at a 1:1,000 dilution: Fluorescein isothiocyanate (FITC) labeled anti-CD34 (NU-C-kit, Cympass Biotechnology Company), phycoerythrin (PE) conjugated anti-CD133, peridinin chlorophyll protein (PerCP) anti-CD45 (Clinilab company).

Briefly, blood samples (50 μl) were incubated with 5 μl anti-CD34, anti-CD133 and anti-CD45 antibodies for 15 min at room temperature in the dark. Subsequently, red blood cells were lysed, and resuspended in phosphate buffered saline. Flow cytometric analysis was performed using a FACScan (Becton-Dickinson, San Jose, California, USA).

Analysis of endothelial cells

We first selected all living cells in a side-scatter (SSC)/CD45 plot to exclude platelets, dead cells, and debris.

Then, we gated around a region corresponding to CD45– negative and low SSC in order to exclude hematopoietic cells. Subsequently, we looked for CD34 and CD133 expression in other 2D fluorescence plots. EPCs were considered as (CD45–/dim/CD133+/CD34+) and mature CECs as (CD45–/dim/CD133–/CD34+).

CECs were reported as a percentage of the total events after the exclusion of debris. To prevent bias, only percentage values were used in statistical analyses because the leukocyte count was highly variable in each group [7] Figure 1.
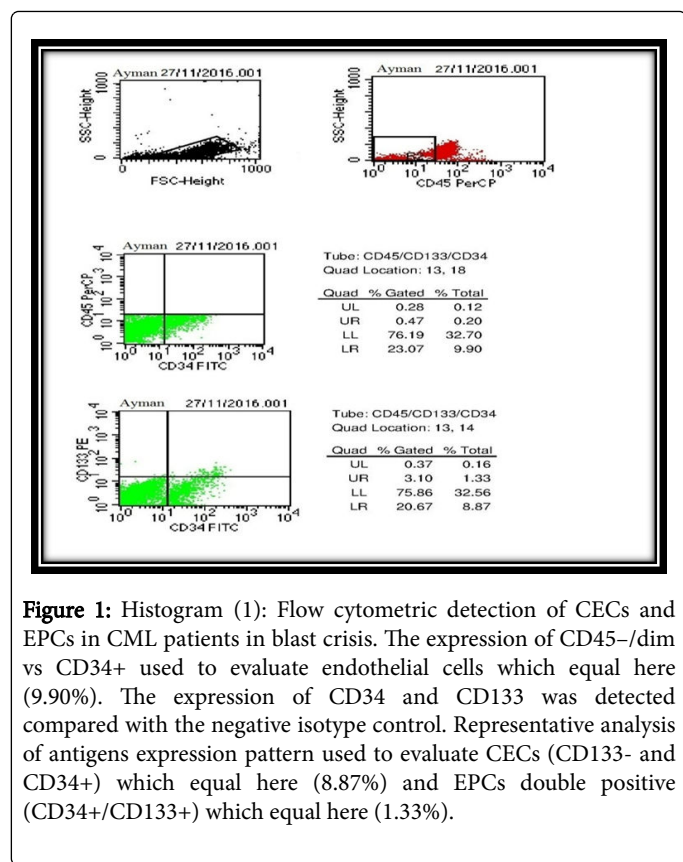
Figure 1: Histogram (1): Flow cytometric detection of CECs and EPCs in CML patients in blast crisis. The expression of CD45–/dim vs CD34+ used to evaluate endothelial cells which equal here (9.90%). The expression of CD34 and CD133 was detected compared with the negative isotype control. Representative analysis of antigens expression pattern used to evaluate CECs (CD133- and CD34+) which equal here (8.87%) and EPCs double positive (CD34+/CD133+) which equal here (1.33%).

Statistical Analysis

The data were tabulated and statistically analyzed using Microsoft Excel software and Statistical Package for the Social Sciences (SPSS version 20.0) software. According to the type of data, the following tests were used to test differences for significance; Differences between means (quantitative variables) in parametric two groups by t test, in non-parametric by Mann Whitney U test and correlation by Pearson's correlation scale. P value was set at <0.05 for significant results & <0.001 for high significant result, the smaller the P-value obtained the more significant are the results.

Results

This study included two groups: group I (15 normal individuals as control), group II (20 CML patients, they were subclassified into chronic phase, accelerated phase and blastic crisis).

	Case (n=20)		Control (n=15)		T	P
Age (years) Mean± SD Range	55.5 ± 8.3 30-67		53.7 ± 10.65 32 - 68		0.57	0.57
Gender	N	%	N	%	X2	P
Male	12	60	9	60	0.0	1.0
Female	8	40	6	40		

Table 1: Demographic data of CML patients and control group.

The demographic data of CML patients and control groups showed that, male to female ratio in both groups was (1.7:1) with no statistical difference between two groups (p=1.0) Table 1.

The level of VEGF was significantly higher in the cases than in the controls of the present study. In control group the level of VEGF was found to be 87.73 ± 44.47 pg/ml. In study group its level was 1809.85 ± 1183.67 pg/ml Table 2.

Major Groups	Number	Mean ± SD (pg/ml)	Median(range)	U Test	p - value
Control	15	87.73 ± 44.47	75(32-191)	0	<0.0001**
Cases	20	1809.85 ± 1183.67	1557(27-4000)		

Table 2: Serum VEGF values in major groups (Mann-Whitney U test).

In the current study, both CECs with mean value (5.15 ± 7.03) and EPCs with mean value (0.87 ± 1.26) were significantly increased in CML patients in comparison to the control group with mean values (0.017 ± 0.015) and (0.008 ± 0.008) respectively Table 3.

Characteristics	CML patients (n=20)	Controls (n=15)	U Test value	p - value
CEC (%)				
Mean ± SD	5.15 ± 7.03	0.017 ± 0.015	4.5	0.0001*
Median (Range)	0.18(0.03-18.63)	0.01(0.00-.05)		
EPC (%)				
Mean ± SD	0.87 ± 1.26	0.008 ± 0.0086	41.5	0.0001*
Median (Range)	0.03(0.00-3.52)	0.01(0.00-0.02)		

Table 3: CECs and EPCs in CML patients vs. controls.

On subgroup analysis of the present study, it was found that serum VEGF value was significantly elevated in all groups when compared to controls. Mean VEGF level 775.56 ± 369.23 pg/ml in chronic group while it was 1597 ± 85.44 pg/ml in accelerated phase and 3053.25 ± 695.19 pg/ml in crisis phase patients.

Significant increase of ECs was observed in CML patients with blast crisis phase with mean value of CECs was 12.71 ± 5.05 and of EPCs was 2.15 ± 1.1 compared to other phases.

In chronic phase the mean value of CECs was 0.14 ± 0.04 and of EPCs was 0.019 ± 0.01 but in accelerated phase the mean value of CECs was 0.04 ± 0.015 and of EPCs was 0.02 ± 0.02 while in control group the mean value of CECs was 0.017 ± 0.015 and of EPCs was 0.008 ± 0.008 Table 4.

	Cases			control	F	P value
	Chronic **N=(9)**	**Accelerated** **N=(3)**	**Blastic** **N=(8)**			
VEGF(pg/ml) Mean ± SD Range	775.56 ± 369.23 274-1327	1597 ± 85.44 1507-1677	3053.25 ± 695.19 1985-4000	87.73 ± 44.47 32-191	108	<0.001**
CEC (%) Mean ± SD Range	0.14 ± 0.04 0.09-0.21	0.04 ± 0.015 0.03-0.06	12.71 ± 5.05 3.15-18.63	0.017 ± 0.015 0.0-0.05	57.2	<0.001**
EPC (%) Mean ± SD Range	0.019 ± 0.01 0.0-0.03	0.02 ± 0.02 0.01-0.05	2.15 ± 1.1 0.33-3.52	0.008 ± 0.008 0.0-0.02	35.5	<0.001**

Table 4: Comparison between different phases of CML patients group and control group as regard VEGF, CEC and EPC.

In the present study there were highly significant positive correlations between VEGF and CECs in the three phases (chronic, accelerated and blastic crisis) (r=0.77, p=0.001), (r=0.96, p=<0.001) and (r=0.9, p=<0.001) respectively. The results of present study also showed highly significant positive correlations between EPCs and VEGF in blastic crisis (r=0.94, p<0.001) but there was no significant correlation in chronic or accelerated phases Table 5.

		Chronic phase	Accelerated phase	Blastic crisis phase
CEC	r	0.77	0.96	0.9
	P	<0.001**	<0.001**	<0.001**
EPC	r	0.04	0.81	0.94
	P	>0.05 NS	>0.05 NS	<0.001**

Table 5: Correlation between VEGF and the endothelial cells in different phases of CML

Discussion

Chronic myeloid leukemia (CML) is a form of leukemia characterized by unregulated growth of predominantly myeloid cells in the bone marrow and accumulation of these cells in the blood. CML is a clonal bone marrow stem cell disorder in which a proliferation of mature granulocytes and their precursors is found. It is a type of myeloproliferative disease associated with a characteristic chromosomal translocation called the Philadelphia chromosome [8].

In cases of CML the percentage of males was found to be 60% against 40% of females with a ratio of 1.7:1. This agrees with Martin Höglund et al. who published that CML is more common in males than in females with male to female ratio varying between 1.2 and 1.7 in different studies [9].

In the present study the mean of age was (55.5 ± 8.3) ranging from 30 to 67. This is in concordance with a study done by Smith et al. in which the incidence of CML increased by age, at least up to 75-80 years and in Europe, the median age at diagnosis of CML, as estimated from population-based registries, is 57-60 years [10].

The level of VEGF was significantly higher in the cases than in the controls of the present study. These results are similar to those reported by Lundberg et al. and Alvaro et al. who demonstrated that the chronic myeloproliferative diseases associated with an increased vascular density in the bone marrow compared to the bone marrow of healthy subjects and there were association between increased bone marrow vascularity and high plasma levels of VEGF as well as other angiogenic factors in patients with leukemias including CML [11-12].

On subgroup analysis of the present study, it was found that serum VEGF value was significantly elevated in all groups when compared to controls. Explanation of that found in recent investigations showed that the production of VEGF in CML cells may be directly triggered by disease specific oncogene BCR/ABL. Moreover, the tyrosine kinase inhibitor imatinib that targets the kinase activity of BCR/ABL, down-regulates the mRNA expression of VEGF in BCR/ABL+ cells [13].

To corroborate the VEGF findings in this study, analysis of the CECs and EPCs was carried out using Flow cytometers. That in accordance with Almici et al. findings in which CECs and EPCs quantitation in the peripheral blood has been proposed as a valid surrogate marker of angiogenesis, whose golden standard is considered MVD assessment [14]. These findings suggest that, even if not perfectly superimposable to MVD determination, CECs quantitation may be considered at least as a useful tool for clinical studies concerning angiogenesis assessment, especially in those cases where multiple sampling of the same patient is requested.

In the current study, both CECs and EPCs were significantly increased in CML patients in comparison to the control group. These findings are in accordance with those by Wierzbowska et al. who observed that the levels of CECs and EPCs were significantly higher in CML patients than in the control group by a 17 and 18 fold respectively [15].

Analysis of the results obtained in the present study showed that a significant increase of ECs was observed in CML patients with blast crisis phase compared to other phases. However, in patients with chronic phase and accelerated phase the number of ECs was slightly increased compared to the control group but the differences were not statistically significant that agreed with Godoy et al. [16].

That also matched with Ozdogu et al. [7] who demonstrated that the flow cytometric evaluation of cells with the CEC phenotype may identify a subset of patients with a more aggressive disease course. These observations may represent a rationale for the development of new treatment strategies targeting the angiogenic process.

In the present study there were highly significant positive correlations between VEGF and CECs in the three phases. This is in agree with Zhang et al. who demonsterated that VEGF is the only one among the tested cytokines that directly correlates with the amount of total and activated CECs [17].

The results of present study also showed highly significant positive correlations between EPCs and VEGF in blastic crisis but there was no significant correlation in chronic or accelerated phases. That in accordance with Rafat et al. results showed significant correlations between EPCs number and serum level of VEGF as well as between EPCs and vessel density [18].

In conclusion, the present study revealed that the level of VEGF was highly elevated in all phases of CML. While the flow cytometric evaluation of endothelial cell surface markers in the blood of CML patients can identify a subset of patients with a more aggressive disease course. So quantification of CECs and EPCs in peripheral blood was useful for predicting and identifying early progression of CML to blastic crisis.

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