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Angiogenesis factor pattern differs in acute lymphoblastic leukemia and chronic lymphocytic leukemia

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Abstract

Angiogenesis is an important event in the survival and progression of solid tumors. The angiogenic status and the exact role of the angiogenic cytokines in lymphoid leukemia has not been fully elucidated.

We have investigated the profile of the systemic components of angiogenic regulation in B-lineage acute lymphoblastic leukemia (B-ALL) and B-chronic lymphocytic leukemia (B-CLL), namely vascular endothelial growth factor (VEGF), tumor necrosis factor-alpha (TNF-α), endostatin and matrix metalloproteinase-9 (MMP-9) using enzyme-linked immunosorbent assay (ELISA).

In B-ALL patients, sVEGF, and MMP-9 were significantly lower than control levels at diagnosis ($p < 0.001$) and increased to near control levels in remission ($p > 0.05$). Both serum TNF-α and endostatin levels showed no significant difference at diagnosis ($p > 0.05$) and in remission ($p > 0.05$) compared to control levels. VEGF, TNF-α, MMP-9 and endostatin levels were not significantly correlated with peripheral white cell count or bone marrow blast cell count, but were positively correlated with platelet count.

In B-CLL patients, serum VEGF, MMP-9 and TNF-α were significantly higher ($p < 0.001 = 0.009, 0.007$, respectively) and decreased to near control levels in remission ($p > 0.05$ for all). Serum endostatin levels showed no significant difference at diagnosis and in remission compared to control levels ($p > 0.05$). A significant positive correlation between VEGF, TNF-α, MMP-9 and peripheral white cell counts, bone marrow lymphocytic count and platelets count were found.

In conclusion, our data suggest that the driving forces of angiogenic factors (VEGF, TNF-α and MMP-9) in adult B-ALL appears different from that in B-CLL patients.

Keywords: *Angiogenesis, ALL, CLL, VEGF*

Introduction

Angiogenesis is an absolute requirement for the establishment and maintenance of a vascular supply in both normal and neoplastic tissues. Angiogenesis and the development of metastasis are intrinsically connected. Experimental data suggest that the establishment and growth of metastases are influenced by soluble factors secreted from the originating solid tumor. Among these factors are the so-called endogenous inhibitors of angiogenesis which maintain metastases in a non-proliferating quiescent state. For a number of tumors, it has been shown that this dormant state is mediated through inhibition of angiogenesis. This dormant state is characterized by normal proliferation, increased apoptosis and insufficient neovascularization. Removal of inhibiting anti-angiogenic factors leads to growth of dormant metastases [1].

The shift in balance between production of anti-angiogenic and pro-angiogenic factors is a process that has been referred to as an angiogenic switch. The new vessel formation is a critical step initiating the process leading to increased vascular permeability, vascular wall

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disassembly, basement membrane degradation and endothelial cell migration and invasion [2].

Although the role of angiogenesis and angiogenic factors are becoming clear in acute myeloid leukemia (AML) [3,4,5], their role in B-lineage acute lymphoblastic leukemia (B-ALL) and B-chronic lymphocytic leukemia (B-CLL) is less clear.

The aim of this work was to determine the serum levels of pro-angiogenic (VEGF, MMP-9 and TNF-α) and anti-angiogenic (endostatin) cytokines in B-ALL and B-CLL patients at diagnosis, in remission and to correlate their level with clinco-pathological parameters.

Subjects and methods

The present study was carried out on 40 newly diagnosed patients with B-ALL ($n = 25$) and B-CLL ($n = 15$) (Table I). In addition, 13 healthy individuals of matched age and sex served as a control group. Twenty-three (14 B-ALL and 9 B-CLL) of the forty patients were reassessed at remission. The patients' characteristics is shown in Table I. The diagnosis was based on both morphological examination of air dried Leishman stained peripheral blood (PB) and bone marrow (BM) films and immunophenotyping by flow cytometry. FAB classification for ALL and Rai staging for B-CLL were performed.

Table I. Descriptive data of the patients and control group.

	B-ALL	B-CLL	Control
Number	25	15	13
Age (years)			
Mean	30.8	47.4	34.3
±SD	10.9	6.2	11.0
Range	20–44	40–64	18–61
Sex			
Male (*n*)	16	8	9
%	64.0	53.3	69.2
Female (*n*)	9	7	4
%	36.0	46.7	30.8
FAB/Rai stages	L1 9	II 4	–
	L2 16	III 8	–
		IV 3	–
Splenomegaly (%)	36.0	86.7	–
Hepatology (%)	36.0	73.3	–
Lymphadenopathy (%)	36.0	60.0	–
Pallor (%)	46.0	33.3	–
Fever (%)	24.0	13.3	–
Jaundice (%)	0	0	–
Follow up period (month)	1–3	7–16	–
Hb (g/dl)			
Mean	8.4	9.5	12.8
±SD	1.6	1.2	1.7
TLC ($\times 10^9$/l)			
Mean	34.0	70.3	9.2
±SD	18.6	46.0	3.1
Platelets ($\times 10^9$/l)			
Mean	66.6	117.5	212.0
±SD	46.8	46.4	90.0

Complete remission (CR) was defined as the presence of less than 5% blast cells in the bone marrow aspirates for ALL cases, no detectable PB and BM B-CLL cells which was determined by immunophenotyping using flow cytometry.

Serum samples from patients and controls were obtained for determination of vascular endothelial growth factor (VEGF), tumor necrosis factor-alpha (TNF-α), endostatin, matrix metalloproteinase-9 (MMP-9). The samples were stored at −70°C until analysis.

Determination of serum levels of VEGF, TNF-α, MMP-9 and endostatin

For assessment of serum levels of VEGF, TNF-α, MMP-9 and endostatin, venous blood samples were collected from each case by clean vein puncture, were delivered into plastic tubes containing no anticoagulant. This sample was left to clot and then centrifuged to obtain serum which was stored at −70°C. For the quantitative determination of VEGF, TNF-α, MMP-9 and endostatin, we used competitive enzyme immunoassay (ELISA), which measures the natural and recombinant forms of the cytokine (Cytimmune Science Inc., MD). For each individual sample, 100 μl of sample were added to their designated wells. This assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for cytokine was pre-coated onto a microplate. Standards and samples were pipetted into the wells, and cytokine bound by the immobilized antibody. After washing away unbound substances, an enzyme-linked polyclonal antibody specific for cytokine is added to the wells. Following a wash to remove unbound antibody-enzyme reagent, a substrate solution is added to the wells and color develops in proportion to the amount of total cytokine (pro and/or active) bound in the initial step. The color development is stopped and the intensity of the color is measured.

Statistical analysis

Statistical analysis was performed using Microsoft Excel and SPSS version 10 (Statistical Package for Social Sciences). The first part of the data was descriptive in the form of median and range, and the second part was analytic. Kolmogron–Smirnov test (K–S test) was done to determine normality of data distribution. The data were shown to be non-normally distributed. For this reason, a non-parametric test was used. Mann–Whitney test was used to diagnose statistical difference between two groups. The Spearman correlation coefficient test was used to determine the association strength between variables. Significance was considered when P value < 0.05.

Table II. Levels of soluble VEGF, TNF-α, MMP-9 and Endostatin at diagnosis and in remission of B-ALL patients and healthy control.

Group parameter	Diagnosis ($n = 25$)	Remission ($n = 14$)	Control ($n = 13$)	P1	P2
VEGF (ng/ml)					
Median Range	10.0 0–61.6	32.2 7.2–68	40 5 26–75.8	<0.001	$P > 0.05$
TNF-α (ng/ml)					
Median Range	11.0 1.2–56.8	5.4 1.2–11.2	4.0 1.2–6.8	$P > 0.05$	$P > 0.05$
MMP-9 (ng/ml)					
Median Range	8.0 1.2–22.0	48 2.5–77.0	52.1 2.0–74.0	0.004	$P > 0.05$
Endostatin (ng/ml)					
Median Range	73.2 28–151.6	78.0 40–152.8	51.6 40.8–134.4	$P > 0.05$	$P > 0.05$

P1 Diagnosis vs. control, P2 Remission vs. control.

Results

Patients with B-ALL elicited a highly significant reduction of serum VEGF, and MMP-9 levels at diagnosis ($P < 0.001$; $P = 0.004$) which increased to near the control levels in remission ($P > 0.05$). Also, there were insignificant differences in TNF-α levels at diagnosis and in remission as compared to control group ($P > 0.05$ for both). The same results were obtained as regard endostatin levels ($P > 0.05$ for both) (Table II). sVEGF, sTNF-α, s-MMP-9 and endostatin level were not significantly correlated to PB blast cell count and bone marrow blast cell count. However, only the sVEGF level was positively correlated to the platelet count in B-ALL (Table III).

In B-CLL patients VEGF, MMP-9 and TNF-α levels were significantly increased at diagnosis ($P = 0.001$, 0.009 and 0.007, respectively) and decreased to near the control level in remission ($P > 0.05$ for both). There were statistically insignificant differences in endostatin levels at diagnosis and in remission as compared to control group ($P > 0.05$ for both; Table IV).

There was a significant positive correlation between sVEGF, sTNF-α, sMMP-9 and PB and BM (CD5+ and CD19 +) lymphocyte counts, but a negative correlation with platelet count were detected in B-CLL (Table V).

Discussion

Mounting evidence suggests that angiogenesis is regulated by the net balance between positive (angiogenic) and negative (angiostatic) regulators of blood vessel growth. A balance shifted towards predominantly positive regulators is an angiogenic-phenotype, whereas, a shift favoring negative regulators is an angiostatic phenotype. Therefore, the impaired regulation of angiogenesis is often associated with the development of angiogenesis-dependent diseases [6].

Angiogenesis plays a fundamental role in the neoplastic process and metastasis of solid tumors [7]. Although the angiogenic factor levels and role in AML are becoming clearer, their role in lymphocytic leukemia is not fully elucidated.

In the present study, patients with B-ALL showed a highly significant reduction of serum VEGF as compared to the control group ($P < 0.001$) and increased to nearly control level when hematological remission was obtained. Similar findings were reported by Yetgin et al. [8] who analyzed serum levels of bFGF and VEGF in ten healthy controls and 31 children with ALL at the time of diagnosis and remission. Faderal et al. [9] and Kim et al. [10] reported similar findings. This unexpected finding could be explained on the following basis:

Table III. Correlation between investigated angiogenic factors and PB white cell count, BM blast cell count, FAB subtypes, platelets count in B-ALL.

	PB blast cell count	BM Blast cell count	Platelets count	FAB subtypes
VEGF	$R = 0.29$ $P > 0.05$	$R = 0.08$ $P > 0.05$	$R = 0.51$ $P = 0.009$	$R = 0.19$ $P > 0.05$
TNF-α	$R = 0.13$ $P > 0.05$	$R = 0.06$ $P > 0.05$	$R = -0.13$ $P > 0.05$	$R = 0.19$ $P > 0.05$
MMP-9	$R = 0.27$ $P > 0.05$	$R = -0.2$ $P > 0.05$	$R = -0.20$ >0.05	$R = 0.10$ $P > 0.05$
Endostatin	$R = -0.08$ >0.05	$R = 0.01$ $P > 0.05$	$R = 0.14$ $P > 0.05$	$R = 0.11$ $P > 0.05$

Table IV. Levels of soluble VEGF, TNF α, MMP-9 and Endostatin at diagnosis and in remission of B-CLL patients and healthy control.

Group parameter	Diagnosis ($n = 15$)	Remission ($n = 9$)	Control ($n = 13$)	$P1$	$P2$
VEGF (ng/ml)					
Median	100.0	53.5	40.5	<0.001	$P > 0.05$
Range	75.6–128.4	21.0–82.2	26–75.8		
TNF-α (ng/ml)					
Median	45.2	6.6	4.0	0.007	$P > 0.05$
Range	20–122	2.8–16.4	1.2–6.8		
MMP-9 (ng/ml)					
Median	89.0	50	52.1	0.009	$P > 0.05$
Range	20.0–250.2	4–70	2.0–98.0		
Endostatin (ng/ml)					
Median	74.4	76.2	51.6	$P > 0.05$	$P > 0.05$
Range	46.4–160	40.4–127.2	40.8–134.4		

$P1$ Diagnosis vs. control, $P2$ Remission vs. control.

At diagnosis, with active angiogenesis, cell-associated VEGF may have an increased expression, which is observed in ALL cell lines [11]. Thus locally present VEGF, may not be reflected in the serum as much as in bone marrow due to increased consumption of VEGF by increased angiogenesis. Perez-Atayde et al. [12] revealed higher microvessel density in the bone marrow of ALL patients as compared to that found in normal controls. In remission with renewal of normal hematopoiesis, the activation of angiogenesis in the bone marrow may return to the normal rate with decreased consumption of VEGF resulting in its elevation to normal levels in the serum [4,8]. The second possibility is that VEGF expression may be minimal in lymphoid system.

In B-ALL patients, there was and insignificant increase in the serum TNF-α level at the time of diagnosis compared to the control level ($P > 0.05$). Also, there was no significant difference between B-ALL patients in remission and the control group ($P > 0.05$). This result is in agreement with that of Athanassiadou et al. [13] who explained immunoregulatory abnormalities and increased susceptibility to infection in patients with ALL by the decreased ability of PB monocytes in ALL patients to secrete TNF-α upon stimulation with lipopolysaccharides, since activation of the monocyte/macrophage system with subsequent secretion of so-called "acute phase proteins" including TNF-α is needed to promote immune responses. However, our data do not agree completely with that of Schulz et al. [14] who demonstrated that ALL cells express mRNA for TNF-α, and Bont et al. [15]) who found that the monocytes from ALL patients have an intrinsic capacity to produce TNF-α. These conflicting results may be due to the fact that secretion of TNF-α by leukemic ALL cells depends on immunophenotype [16].

Besides VEGF, MMP-9 plays a critical role in angiogenesis, tumor invasion and metastasis. It has been evident that the new vessel formation is accompanied by excessive matrix degradation. This is a critical step initiating the process leading to increased vascular permeability, vascular wall disassembly, basement membrane degradation and endothelial cell migration and invasion [17]. In the present study, the man serum MMP-9 level showed a highly significant reduction in the newly diagnosed B-ALL patients as compared to control group ($P = 0.004$) and increased during remission reaching the control level. A similar finding was reported by Lin et al. [18]. Hendrix et al. [19] have shown that the ability of the lymphoblast cells to invade through the matrigel correlates with the MMP-2 expression, while Ivanoff et al. [20] found MMP-9 expression to be more important.

In B-ALL patients, VEGF levels were found to be significantly correlated to the platelet count prior to treatment. Since this factor has been shown in platelets, this finding seems to be acceptable [8,11,21,22].

In B-CLL patients, the mean serum VEGF level was significantly higher at diagnosis ($P < 0.001$) and decreased to nearly control level in remission

Table V. Correlation between investigated angiogenic factors and PB and BM lymphocytes (CD5 + and CD19 +) counts, Rai Stages, platelets count in B-CLL.

	PB lymphocyte (CD5 + , CD19 +) count	BM(CD5 + , CD19 +) lymphocyte count	Platelets count	Rai stages
VEGF	$R = 0.05$, > 0.05	$R = 0.78$, $P < 0.01$	$R = 0.51$, $P < = 0.01$	$R = 0.95$, $P < 0.001$
TNF-α	$R = 0.87$, $P < 0.001$	$R = 0.78$, $P = 0.002$	$R = -0.82$, $P < 0.001$	$R = 0.83$, $P < 0.001$
MMP-9	$R = 0.16$, > 0.05	$R = 0.01$, $P > 0.05$	$R = 0.0.08$, > 0.05	$R = 0.05$, $P > 0.05$
Endostatin	$R = 0.17$, > 0.05	$R = 0.08$, $P > 0.05$	$R = 0.04$, $P > 0.05$	$R = 0.1$, $P > 0.05$

($P > 0.05$). Chen et al. [23] found, by ELISA and immunocytochemical staining, that B-CLL cells already constitutively produce measurable amounts of VEGF under *in vitro* culture conditions and the rate of VEGF secretion was indeed increased up to 7-fold in response to hypoxic stimulation. Interestingly, the same results were obtained by different methods [23]. Molica et al. [24], on the basis of flow cytometric analysis, showed that B-CLL patients displayed a positive reaction for VEGF and mean fluorescence intensity (MFI) of cases with a progressive pattern of disease was higher than MFI of patients with stable disease. Peterson and Kini [25] found that bone marrow sections from CLL patients had a mean microvessel density and mean hot-spot microvessel density significantly higher than in the control biopsy sections.

In the current study, there was a highly significant increase of serum TNF-α at diagnosis comparing to control level ($P = 0.007$) and decreased nearly to control level in remission ($P > 0.05$). Foa et al. [26] found that TNF-α is constitutively produced by cells of B-CLL and hairy cell leukemia and this was further confirmed by the presence of the mRNA for TNF-α in primary or in pre-activated cells from B-CLL patients.

In the present study there was no significant difference between serum endostatin level in either B-ALL and B-CLL patients compared to the control group ($P > 0.05$ for both). Gora-Tybor et al. [27] found that the levels of Endostatin were not significantly different in CLL and control groups. The same results were obtained by Kay et al. [28] who demonstrated that only leukemic cells from one out of 35 patients secreted endostatin.

The levels of MMP-9 in the serum of untreated B-CLL patients was significantly higher as compared to the control group ($P = 0.009$) and decreased to control levels at remission. Kamiguti et al. [29] found that CLL cells secrete variable amounts of pro-MMP-9 as in monomeric, dimeric or complexed forms and the specific MMP-9 inhibitor, RO31-9790 can inhibit the transmigration of CLL cells across type IV collagen-coated membranes and endothelial monolayer suggesting that the enzyme may be involved in CLL cell egress and infiltration. Bauvois et al. [30] found that the production of proMMP-9 in B-CLL cells is much higher than in normal B cells. Moreover, high levels of VEGF and TNF-α have also been detected in the B-CLL culture supernatant. B-CLL derived TNF-α and VEGF seem sufficient to up regulate MMP-9. Exogenous TNF-α type I and II can inhibit the expression of MMP-9 at transcriptional levels, whilst having no effect on the production of other angiogenic factors, which may explain the lack of efficacy of IFN treatment in B-CLL patients. P38 MAPK can constitutively activate and regulate MMP-9 secretion in B-CLL cells, and are both important for the survival of B-CLL cells on the stromal cells [31].

Serum levels of VEGF and MMP-9 were positively correlated with Rai stages and bone marrow lymphocytosis. Molica et al. [24], Gora-tybor et al. [32] stated that serum levels of VEGF reflect B-CLL clinicohematological features such as advanced Rai clinical substages, high PB lymphocytosis and diffuse bone marrow histology.

Serum TNF-α levels were positively correlated with PB WBCs, bone marrow lymphocytosis and Rai substages whereas negatively correlated platelet count in CLL patients. Accordingly, Gora-tybor et al. [32] and Ferrajoli et al. [33] stated that TNF-α levels in patients with CLL is correlated with disease characteristic and serves as a prognostic factor as high levels of TNF-α is associated with lower hemoglobin and platelets level, greater percentage cells expressing CD38, more advanced Rai and Binet stage of disease and shorting of the survival of patients. Therefore, inhibition of TNF-α in patients with CLL may have a therapeutic importance.

In conclusion, our data suggest that the driving forces of angiogenic factors (VEGF, TNF-α and MMP-9) in adult B-ALL appears different from that in B-CLL patients. Further investigation on the biology of angiogenesis in ALL is required. The final target of these research should be to establish the individualization of specific based therapeutic approach to patients based on more thorough understanding of disease related pathophysiologic pathways.

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