Vascular Endothelial Growth Factor Levels in Childhood Acute Lymphoblastic and Myeloblastic Leukemia

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Abstract Angiogenesis has been associated with the growth, dissemination and metastasis and has been shown to be a prognostic. Although there are some data suggesting that angiogenesis may have a role in the pathophysiology of leukemia, its role in patient prognosis is yet to be defined. We analyzed the expression level of vascular endothelial growth factor (VEGF), an angiogenesis promoter and its possible- prognostic value in bone marrow samples at the time of diagnosis and remission of acute childhood leukemia patients. Besides 46 patients diagnosed as ALL or AML, 16 children were also included as a control group in the study. Our data have demonstrated that VEGF levels of AML patients were found higher than the control group statistically ($P = 0.022$). However we could not find any significant difference between VEGF levels of diagnosis and remission in both AML and ALL groups by blastic VEGF expression ($P > 0.05$). In this study the higher levels of VEGF in AML patients is one of the main findings although we were not able to assess any role of VEGF in predicting prognosis in pediatric leukemia patients by evaluating blastic cell VEGF expression. These results have demonstrated that the relationship between

angiogenesis or angiogenesis promoters and hematological malignancies is not clear and simple as different methods or different cells beside different angiogenesis promoters are involved to these studies. So that not only tumor cells and their cytokines but also surrounding cells and their cytokines must be taken into consideration with the standardized study methods in the further studies to obtain a promising treatment approach.

Keywords Childhood leukemia · Angiogenesis · Vascular endothelial growth factor

Introduction

Angiogenesis is the formation of new capillaries from established blood vessels, and is an essential process in growth and development [1], wound healing [2] and reproduction [3]. However, it provides malignant cells with a survival advantage over their normal counterparts and confers potential of metastasis. One of the major positive regulators of tumor-related angiogenesis is vascular endothelial growth factor (VEGF) [4]. It has been reported that VEGF levels within tumor tissue [5, 6], urine [7], serum [8], and plasma [9], are elevated and are of prognostic significance in a wide variety of patients with solid tumors. VEGF binds with high affinity to the receptor tyrosine kinases (RTKs) called VEGFR-1 (Flt-1) and VEGFR-2 (Flk-1, KDR), which upon ligand binding become tyrosine phosphorylated and activate cellular substrates [10, 11]. VEGF and VEGFR-2 were shown to play an important role in neoangiogenesis and during the growth of solid tumors [12].

Recent evidences suggest that angiogenesis is critical in the pathogenesis of numerous different hematological

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malignancies, including acute lymphoblastic leukemia [13] acute myelogenous leukemia [14], chronic lymphocytic leukemia [15], chronic myeloid leukemia [16], and multiple myeloma [17]. In this study, we have measured the VEGF mRNA expression levels of blastic cells isolated from bone marrow aspirations of patient groups and compared the results with a control group in order to assess the relationship between angiogenesis and leukemia.

Materials and Methods

Forty-six leukemic children diagnosed as ALL or AML by cytomorphological, immunohistochemical and immunocytometric methods and a control group of 16 children included in this study conducted in Çukurova University Faculty of Medicine, Pediatric Hematology Department. Control group was formed by cases selected as they have indication for bone marrow aspirations and results demonstrate normal bone marrow morphology with non leukemia disease.

Disease subgroups were formed according to FAB classification. Bone marrow aspiration smears revealed that blastic ratios of all of the patients were over 95%. All of the patients were treated with BFM chemotherapy protocols according to B and T immunophenotyping. After the treatment, bone marrow sampling of the patients in remission and mononuclear cell isolation were performed again.

We searched the expression of VEGF, an angiogenesis factor, at blastic cells of bone marrow of the patients diagnosed as ALL and AML. We studied the VEGF expression by PCR as VEGF mRNA level at individual cellular levels and the level of the expression as absolute countification of total VEGF level by real-time RT-PCR using LightCycler technology, a specific and sensitive method. Blastic VEGF expressions of patients were measured by PCR technique on cellular isolation from the diagnostic bone marrow aspirations before treatment. The expressions of the VEGF levels were detected by Light-Cycler technique, a Real-Time fast PCR equipment. VEGF primers were; VEGF Forward 5'-AAC TTT CTG CTG TCT TGG GTG-3' and VEGF Reverse 5'-ACA AAT GCT TTC TCC GCT CT-3'.

We compared the expressions of VEGF levels of ALL and AML patients with control groups at the time of diagnosis, remissions, and relapses. The results were evaluated with SPSS ver. 11 (Statistical Package for Social Sciences), for windows statistical program with One way ANOVA, Univariate analysis of Variance and for the relations between characteristic findings, spearman

rank test and for relations between groups Kruskal–Wallis test and for survival relations Cox regression tests were used.

Results

In the patient group, 28 (60%) and 18 (40%) of 46 children were diagnosed as ALL and AML, respectively. The control group comprised 16 children and there was no statistically significant difference between patient groups and control group by means of age and gender distribution ($P > 0.05$).

According to FAB classification, in ALL group, 13 patients (46%) were L1, 9 were L2 (32%) and 6 were L3 (22%) morphology. In AML group 8 of the total 18 patients were in M1 (44%), 6 were in M2 (34%), 3 were in M5 (16%), 1 were in M7 (6%) subgroup. Immunophenotypic classification revealed that 19 patients in the ALL group (68%) were CALLA (+) B ALL, 3 was (10%) CALLA (-) B ALL and 6 were (22%) T ALL. Immunophenotypic classification was also performed in AML group with surface antigens specific to AML. After the induction chemotherapy in ALL patients, remission was achieved in 21 (75%) patients at day 33 of the BFM protocol. Remission was achieved in 12 (68%) of AML patients after induction treatment of BFM AML protokol. There was no statistically significant difference between both groups in remission achievement ($P > 0.05$). The levels of VEGF expressions in ALL, AML and control groups are presented in Table 1. In the follow-up of the patients with achieved remission, relapse rate was found to be 32% ($n = 9$) in ALL and 33% ($n = 6$) in AML.

In AML an ALL groups, there was no statistically significant difference between diagnosis, relapse and remission levels of VEGF ($P > 0.05$). When VEGF expression levels at the time of diagnosis and relapse of patient groups were compared to control group, there was no difference between ALL patients and controls ($P > 0.05$) however AML patients had significantly higher levels of VEGF expression than the control group (One Way Anova, $P = 0.022$). But VEGF expression levels were not different in ALL and AML patient groups by means of being in remission or not, and being relapsed or not.

When the upper level of the control group taken as cut off point and VEGF expression levels above this level presumed as VEGF (+) in the patient group, in the ALL group 6 patients (% 21) and in the AML group 7 patients were VEGF (+). The cut off point generally determined as median + 2SD of the controls or upper limit of the control may be regarded as cut of point as we and some other studies done [18, 19]. The analysis of remission

Table 1 Clinical characteristics and VEGF expression levels of the ALL and AML patients and the control group

	FAB	Immunophenotype	Remission achievement after induction	VEGF expression at diagnosis (mRNA copy/10 ⁴ cell)	VEGF expression in remission (mRNA copy/10 ⁴ cell)
ALL (<i>n</i> = 28)	L1 (<i>n</i> = 13)	Calla (+) B ALL (<i>n</i> = 19)	21 (75%)	54569 ± 11304 (<i>P</i> > 0.05)	29187 ± 23203 (<i>P</i> > 0.05)
	L2 (<i>n</i> = 9)	Calla (−) B ALL (<i>n</i> = 3)			
	L3 (<i>n</i> = 6)	TALL (<i>n</i> = 6)			
AML (<i>n</i> = 18)	M1 (<i>n</i> = 8)		12 (68%)	160537 ± 22701 (<i>P</i> = 0.022, One Way Anova)	23363 ± 24210 (<i>P</i> > 0.05)
	M2 (<i>n</i> = 6)				
	M5 (<i>n</i> = 3)				
	M7 (<i>n</i> = 1)				
Control (<i>n</i> = 16)				32297 ± 29664

Fig. 1 **a** The relation of VEGF and Mortality in ALL group. **b** The relation of VEGF and Mortality in AML group

achievement, relapses and even mortality were not statistically different in both VEGF (+) and (−) groups (Fig. 1a, b).

Discussion

Researches about angiogenesis in hematological malignancies are performed by different methods in different studies. Bone marrow micro vessel density detection by histological or measuring angiogenesis promoter levels in serum or expressions are some of them. One of the angiogenesis promoters is VEGF and its level may be measured by Elisa in the serum or its cellular expressions by PCR as we did. Huge diverse results coming from the differences of the studies on different cells, cytokines and techniques.

A bone marrow blastic cellular VEGF expression is a method for assessment of angiogenesis. VEGF levels were found higher during recurrences compared to the ones at the time of diagnosis, but without any effects on prognosis

in the study made by VEGF expression like our study [20]. We also found that patients diagnosed as AML had increased levels of VEGF expression but we were not able to find any statistically significant relation between cellular VEGF expression and characteristic findings of disease, FAB classification, immunophenotypic properties, cytogenetic abnormalities, age, sex, remission achievement, or relapses. Our findings are similar to some of the previous studies [16]. Pule and friends studied angiogenesis by bone marrow biopsies found that micro vessel density is increased at presentation of the children with leukemia. But increased level of micro vessel density was not related with the disease status or phenotype [21]. This result is similar with our increased levels in AML patients without any relation with prognosis. There are also some results contrary to ours, showing an increased level of angiogenesis at active phases of disease [22, 23]. In one of these studies, Koomagi et al. [20] had studied VEGF expression by Real Time PCR at ALL patients at diagnosis and recurrences and found that VEGF expression levels during relapses

were significantly higher than those at diagnosis. They concluded that there was a relation between VEGF levels and disease activation. But recent pediatric study is inconclusive about the same point that the VEGF levels of pediatric leukemia patients at diagnosis were lower than remissions by measuring serum soluble VEGF with Elisa [24].

Pruneri et al. [22] have found that micro vessel density of MDS patients were lower than AML patients' were, but higher than controls. As an advanced investigation, they have studied VEGF mRNA expressions immunohistochemically at biopsy specimens. Expressions at fields having megakaryocytes abundantly were found to be significantly higher than others having blasts abundantly. In addition, Litvin et al. [25] have founded that micro vessel density of AML patients were 4 times higher at diagnosis and 7.8 times higher than the control group. However, VEGF expressions of mononuclear cells of the patient and control groups were not statistically different. Depending on those results, they have concluded that micro vessel density and tumor cell VEGF expressions could not be correlated and angiogenesis could be induced not only by tumoral cell cytokines but also by other angiogenesis promoters produced by cells of microenvironment.

There are some studies about the role of stroma cells in angiogenesis in human body. In one of the studies investigating the relationship between bone marrow stroma and angiogenesis, Sensebe et al. [26] studied on myoid cell groups found in normal bone marrow stroma. They studied cell lines and their cytokines and intended to find expressions of interleukins. They found that these cell lines express many cytokines, growth factors, and negative regulators (VEGF, FGF, IL1-6-7-8, TGF, and PDGF). Also in a recent study it is found that leukemia cell derived VEGF is not effective as bone marrow stromal cell derived ones on the VEGF receptor for apoptotic resistance of the leukemic cells [27]. VEGF expression is affected from other factors as RUNX1 negatively in the AML patients although VEGF is found to be independent prognostic importance in this patient group [28].

In the literature most of the studies were performed in adult group that is different in the physiologic aspects from children in whom only 10% of the studies performed about VEGF and leukemia. Adult studies reveal more trust on VEGF and its prognostic value although studies on children are not [24]. Also there is a different method as PCR, Elisa, and Histopathology that may result in variations in the disease prognosis analyses. Angiogenesis affects from different cytokines other than VEGF and VEGF is also affected from different cytokines. As a result, the relationship between angiogenesis and hematological malignancies is not clear and not only tumor cells and their

cytokines but also other cells of the bone marrow and cytokines secreted by these cells must be taken into consideration in the future investigations.

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