Circulating VEGF and its soluble receptors sVEGFR-1 and sVEGFR-2 in patients with acute leukemia

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ABSTRACT. Angiogenesis plays an important role in the pathogenesis of acute leukemia, and vascular endothelial growth factor (VEGF) is a crucial, positive regulator of this process. The biological activity of VEGF is mediated by two different receptor tyrosine kinases: VEGFR-2 and VEGFR-1. The soluble form of VEGFR-1 is likely to be a negative regulator of VEGF availability, but the physiological role of sVEGFR-2 is still unclear. The plasma levels of sVEGFR-1 and sVEGFR-2 in patients with acute leukemia have not been investigated. We measured the plasma concentrations of VEGF and its two soluble receptors in 39 AML and 15 ALL patients as well as in the control group, using the ELISA assay. We also correlated the plasma levels of these proteins with disease status and known prognostic factors. The sVEGFR-1 level was significantly higher in patients with AML and ALL than in the healthy subjects ($p < 0.002$ and $p < 0.03$ respectively). The sVEGFR-2 level was significantly higher in AML patients compared with the control group ($p < 0.03$). The VEGF levels in AML and ALL patients and in healthy subjects did not differ significantly. The sVEGFR-1 level was higher in AML patients with > 50% of blasts in the bone marrow (BM), WBC > 20 G/L and elevated LDH level, than in the group with BM blasts < 50% ($p < 0.01$), WBC < 20 G/L ($p < 0.02$) and a normal LDH level ($p < 0.05$). Positive correlations between sVEGFR-1 level and WBC ($p < 0.02$),% of BM blasts ($p < 0.05$), the absolute blast count in peripheral blood (ABC) ($p < 0.009$) and LDH ($p < 0.000001$) were found. The sVEGFR-1/VEGF ratio (R1) was calculated, and a positive correlation between R1 and ABC in AML ($p < 0.03$) was determined. A higher (above median) sVEGFR-1/VEGF ratio correlated with a lower CR rate and a shorter survival ($p < 0.03$ and $p = 0.0007$ respectively). In conclusion, the plasma concentration of sVEGFR-1 is higher in leukemia patients than in healthy subjects and correlates with tumour burden and poor prognosis. The sVEGFR-1/VEGF ratio may be of greater prognostic value than VEGF alone. Further investigation is recommended to better determine their function.

Keywords: angiogenesis, VEGF, soluble VEGR receptor, acute leukemia, prognosis

INTRODUCTION

Angiogenesis is the formation of new blood vessels from an existing vasculature [1]. Increased angiogenesis contributes to the pathophysiology of solid tumours and other non-malignant diseases (rheumatoid arthritis, diabetic retinopathia, psoriasis, etc) [2]. Recent studies show that angiogenesis and angiogenic factors play an important role in hematological malignancies [3, 4].

In the recent years, there has been significant progress in the understanding of angiogenesis in acute leukemia. Both acute myeloid leukemia (AML) and acute lymphoblastic leukemia (ALL) are associated with a substantial increase in bone marrow vascularity, as well as an increase in the levels of various angiogenic factors [4-6].

Vascular endothelial growth factor (VEGF) is an important, positive regulator of angiogenesis [7]. The biological activity of VEGF is mediated by two different receptor tyrosine kinases: VEGFR-2 (kinase domain receptor – KDR) and VEGFR-1 (fms-like tyrosine kinase1 - FLT-1) [8, 9]. Both receptors have a high affinity for VEGF, and are expressed on vascular endothelial cells as well as on leukemic cells [10, 11]. Recently, a naturally occurring soluble form of VEGFR-1 (sVEGFR-1) has been identified, but no naturally occurring secreted forms of sVEGFR-2 have been reported to date [12]. The physiological role of sVEGFR-1 and sVEGFR-2 is still undetermined. It has been shown that mRNA for a soluble form of VEGFR-1 was generated by alternative splicing in human umbilical vein endothelial cells (HUVEC) [12]. sVEGFR-1 retains its high-affinity binding to VEGF [13] and it is likely to be a negative regulator of VEGF availability by sequestrating the ligand and by forming inactive heterodimers with membrane-bound VEGF receptors, or it may prolong the different VEGF activities associated with this protein [14]. *In vitro* studies have revealed that sVEGFR-2 is also able to bind VEGF, but that receptor-ligand complex formation, in contrast to sVEGFR-1, is heparin-dependent [13]. Based on these findings, the soluble VEGF receptors may be involved in the pathophysiology of acute leukemia, and their interaction with

VEGF may influence the clinical course of the leukemia and the prognosis.

In some reports it has been shown that VEGF is expressed in leukemic cells from most patients with AML [15], and that the high cellular and plasma VEGF levels correlate with poor prognosis [16, 17]. To the best of our knowledge, the plasma levels of sVEGFR-1 and sVEGFR-2 in patients with acute leukemia have not been investigated to date. In the present study, the plasma concentrations of VEGF and its two soluble receptors, in patients with acute leukemia (39 with AML and 15 with ALL), were measured, as well as in the control group, using the enzyme-linked (ELISA) assay. The plasma levels of these proteins were also correlated with disease activity and known prognostic factors, such as cytogenetic group, white blood cell (WBC) count, lactate dehydrogenase (LDH) activity and performance status.

METHODS

Patients. The study involved 54 patients with newly diagnosed, acute leukemia (39 with AML, 15 with ALL). The median age of the patients was 57 years (range 19 to 81 years). There were 32 males and 35 females. The diagnosis of acute leukemia was based on standard morphological, cytochemical, immunophenotypic and molecular criteria [18, 19]. Cytogenetic analysis was also performed. Each patient underwent a thorough physical examination, performed by one of the authors, on the day of blood sample collection. All of the patients eligible for intensive chemotherapy were treated according to the Polish Adult Leukemia Group clinical research protocols, in the Hematological Department of Medical University in Lódz. The patients diagnosed with poor performance status (> 2 WHO), and in whom the expected early mortality rate exceeded 50%, were not eligible for intensive chemotherapy and were given palliative care with hydroxyurea. All patients were monitored regularly in the Hematological Department's outpatient clinic.

The analysis of the plasma levels of VEGF and its soluble receptors were also performed in 10/39 AML patients at the time of complete remission (CR) after chemotherapy. A CR was defined as normal bone marrow morphology with < 5% blasts, with no evidence of extramedullary leukemia, absolute neutrophil count (ANC) > 1.0 G/L, and platelets > 100 G/L.

A control group of 14 healthy volunteers was also studied. The group consisted of 8 men and 6 women, aged from 22 to 83 years (median 35 years).

Laboratory tests. On the day of blood sampling for VEGF and soluble VEGF receptors, the following laboratory parameters were analysed: WBC and absolute blasts counts in the peripheral blood (PB), haemoglobin level, platelet count, urea nitrogen and creatinine levels, and serum LDH activity. Bone marrow smear examination was also performed.

Serum sampling and cytokine determination. Venous blood samples were collected at the time of clinical assessment in pyrogen-free, heparinised tubes. Plasma was separated by centrifuging at 2.000 g for 10 min in a refrigerated centrifuge. The plasma obtained was divided into aliquots and stored at – 70°C until assayed for VEGF and its soluble receptors. The cytokine plasma concentrations were assayed by specific, commercially available, ELISA assay kits (Quantikine, R & D Systems Inc., Minneapolis, MN USA), in accordance with the manufacturers instructions, and analysed with an ELISA reader at 492 mm. In each assay the appropriate recombinant human cytokine or receptor was used to generate the standard curve. The procedure has previously been described in detail [20]. The sensitivity of the assay for VEGF and sVEGFR-1 was 5.0 pg/ml. Plasma for the sVEGFR-2 concentration measurement was diluted 5 fold, and the minimum detectable dose was typically less than 4.6 pg/ml. The concentrations of VEGF and soluble VEGF receptors in the samples were determined by interpolation from the standard curve.

Statistical analysis. The medians were compared using the Mann-Whitney U test and the Kruskal-Wallis test. The means were calculated using all of the observations, including the zero levels. The linear correlations between the plasma cytokine or receptors levels with each other or with the other factors (WBC,% of blasts in bone marrow etc.) were evaluated using the Sperman rank-sum correlation coefficient. Survival was plotted using Kaplan-Meier plots and compared using the log-rank test. The chi-squared and the Fisher tests were used to compare proportions where appropriate. Comparison and correlation were considered significant when $p < 0.05$.

RESULTS

The results of the measurement of sVEGFR-1, sVEGFR-2 and VEGF are shown in table I. sVEGFR-1 and sVEGFR-2 were detectable in all patients with AML and ALL as well as in all healthy control subjects. The sVEGFR-1 plasma level was found to be significantly higher in AML (Me 23.6 pg/ml) and ALL patients (Me 28.5 pg/ml) at diagnosis, when compared to those in healthy subjects (Me 11.2 pg/ml) ($p < 0.002$ and $p < 0.03$ respectively). The sVEGFR-1 level in newly diagnosed AML was also higher than in AML patients in CR (Me 14.2 pg/ml), however the difference was determined not to be significant ($p > 0.05$). No differences in sVEGFR1 plasma concentrations in AML patients in CR compared with normal individuals were observed ($p > 0.05$). The sVEGFR-2 levels were significantly higher in AML patients at diagnosis (Me 11.28 ng/ml) and those in CR (Me 12.09 ng/ml) than in the control group (Me 8.33 ng/ml) ($p < 0.03$ and $p < 0.008$ respectively). There was no difference in sVEGFR-2 plasma concentrations in ALL patients (Me 8.2 ng/ml) when compared to normal individuals ($p > 0.05$). VEGF was measurable in 38/39 patients with AML, in 14/15 patients with ALL and in 12/14 control subjects. The median VEGF plasma levels present in AML and ALL patients were 32.6 pg/ml and 23.6 pg/ml respectively. These measurements did not differ significantly from those of the control group (34.9 pg/ml) ($p > 0.05$). The sVEGFR-1/VEGF (R1) and sVEGFR-2/VEGF (R2) ratios were also calculated and it was found that R1 was higher in AML patients (Me 0.62) and ALL patients (Me 0.84) than that in healthy control subjects (Me 0.29). However, the differences were not significant ($p > 0.05$) (Table I).

In our study, only the sVEGFR-1 level was higher in AML patients with > 50% of blasts in bone marrow (BM) (33.2 pg/ml), WBC > 20 G/L (36.3 pg/ml) and elevated LDH level (26.85 pg/ml) than in the group with BM

Table 1
The plasma levels of VEGF, soluble VEGF receptors and the sVEGFR1/VEGF ratio in AML and ALL patients and the control group.

Cytokine/receptor	AML n = 39 (1)	AML (CR) n = 10 (2)	ALL n = 15 (3)	Control group n = 14 (0)	p
sVEGFR-1 pg/ml					Gr 1 vs 0 p = 0.002*
(Me)	23.6	14.2	28.5	11.2	Gr 2 vs 0 NS
Range	5.3-158.3	1.6-40.4	5.3-199.7	7.5-27.4	Gr 3 vs 0 p = 0.03*
					Gr 1 vs 2 NS
sVEGFR-2 ng/ml					Gr 1 vs 0 p = 0.03*
(Me)	11.28	12.09	8.20	8.33	Gr 2 vs 0 p = 0.0008*
Range	6.47-18.18	9.45-19-42	4.97-11.42	5.17-16.61	Gr 3 vs 0 NS
					Gr 1 vs 2 p = 0.0005*
VEGF pg/ml					Gr 1 vs 0 NS
(Me)	32.6	43.2	23.6	34.9	Gr 2 vs 0 NS
Range	0.0-2604.8	10.3-80.9	0.0-144.2	0.0-107.8	Gr 3 vs 0 NS
					Gr 1 vs 2 NS
sVEGFR-1/VEGF					Gr 1 vs 0 NS
(Me)	0.62	0.39	0.84	0.29	Gr 2 vs 0 NS
Range	0.02-33.68	0.15-1.08	0.05-30.26	0.087-3.07	Gr 3 vs 0 NS
					Gr 1 vs 2 NS

*statistically significant difference; NS – non-significant difference; Me – median, n = number of investigated patients; AML – acute myeloid leukemia; CR – complete remission, Gr – group.

blasts < 50% (20.4 pg/ml, $p < 0.01$), WBC < 20 G/L (20.15 pg/ml, $p < 0.02$) and a normal LDH level (17.2 pg/ml; $p < 0.05$) respectively. The sVEGFR-1/VEGF ratio was also higher in patients with > 50% of blasts BM and with WBC > 20 G/L, but the difference was determined as not significant. The relationship between plasma concentrations of VEGF and its soluble receptors with known prognostic factors was analysed in the AML subjects. A significant positive correlation between sVEGFR-1 level and WBC count ($\rho = 0.389$, $p < 0.02$), percentage blasts in BM ($\rho = 0.323$, $p < 0.05$), absolute blast count (ABC) in peripheral blood (PB) ($\rho = 0.418$, $p < 0.009$) and LDH level ($\rho = 0.707$, $p < 0.000001$) (Figure 1) was found. A significant positive correlation between R1 and absolute blast count in PB was similarly observed ($\rho = 0.374$, $p < 0.03$). There were no significant differences in the levels of VEGF, sVEGFR-1, sVEGFR-2 and R1 compared with other prognostic factors (e.g.: cytogenetic group, age, antecedent hematological disorder, platelet count).

A cut-off point of the median value (for VEGF, sVEGFR-1, sVEGFR-2 and R1) was used to divide patients into 'high-expressers' and 'low-expressers' of investigated protein. It was found that AML patients with an sVEGFR-1/VEGF ratio lower than the median (0.62) had significantly longer survival times than patients with R1 $\geqslant 0.62$ ($p = 0.0013$). No significant differences in survival times were observed in high and low-expressers of VEGF, sVEGFR-1 and sVEGFR-2. In this study, 23/39 (59%) AML patients received intensive chemotherapy. Similarly, this intensively treated group displayed significantly longer survival times in patients with R1 < 0.62 when compared to patients with R1 $\geqslant 0.62$ ($p = 0.0007$). A significantly higher complete remission rate was found in low-expressers of R1 ($p < 0.03$) than in high-expressers; but similar results were not determined for VEGF, sVEGFR-1 and sVEGFR-2.

The analysis in ALL was not performed because of the small number of patients.

DISCUSSION

This study is the first to address the sVEGFR-1 and sVEGFR-2 plasma types, as well as VEGF levels and their clinical significance in acute leukemia. We have found that the plasma levels of sVEGFR-1 were significantly higher in AML and ALL patients than those in the healthy control subjects. Significant increases in sVEGFR-1 levels have been observed in AML patients diagnosed with poor performance status, and with more than 50% blasts in the bone marrow, WBC > 20 G/L and elevated serum LDH activity; factors known to be associated with large tumour mass and poor survival times. To our knowledge, the concentration of sVEGFR-1 has not been determined in leukemia patients. However, the elevated levels of this receptor have been reported in patients with other neoplasmas [21, 22].

Recent studies have shown that in healthy people the main source of sVEGFR-1 are endothelial cells (EC) and monocytes [23]. However, in neoplastic diseases sVEGFR-1 may also be generated by tumour cells, although the regulation of switching the transcription from the transmembrane to the soluble form is still not understood [24]. Inoue *et al.* has reported that sVEGFR-1 can be produced by malignant haematopoietic cells. They have found sVEGFR-1 protein in culture supernatants of leukemic cell lines. Moreover, they have shown sVEGFR-1 mRNA expression in 17 out of 52 cell lines representing the main haematopoietic lineages, suggesting that this production may play some role in the regulation of VEGF activity in normal and malignant haematopoietic cell proliferation [25]. A significant positive correlation observed in this study between sVEGFR-1 levels and the WBC count, the percentage blasts in BM, the absolute blast count in PB and the LDH level, may indicate that the sVEGFR plasma level reflects, at least in part, a tumour mass in AML patients, and that this soluble receptor is also produced by leukemic blasts alone, or EC stimulated by leukemia cell-derived angiogenic factors [26].

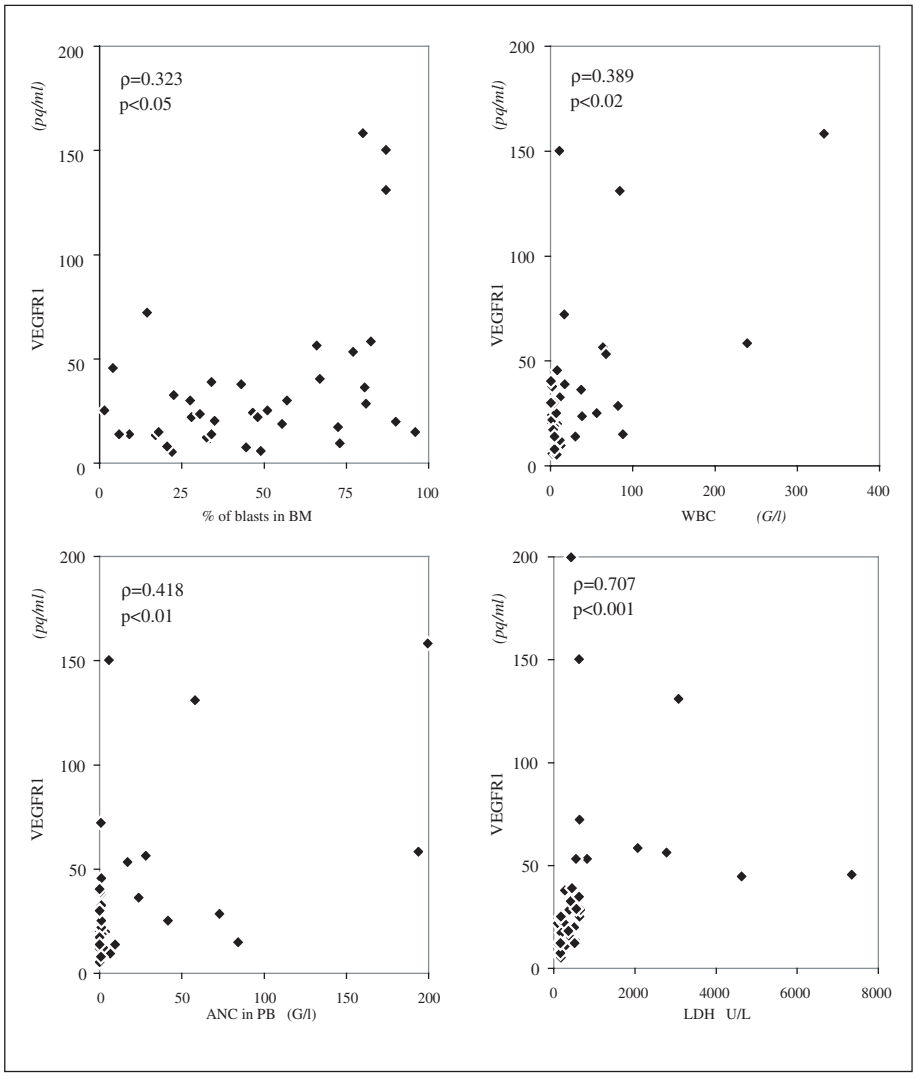

Figure 1
Relationship between plasma sVEGFR-1 concentration and percentage blasts in bone marrow (BM), WBC, absolute blasts count (ABC) in peripheral blood (PB) and LDH level.

In our study, sVEGFR-2 plasma concentrations were significantly higher in AML patients in comparison to healthy control subjects. However, no correlations between sVEGFR-2 level and tumour mass or other prognostic factors were found. Little is known about the activity of sVEGFR-2. Recombinant sVEGFR-2 has been examined for its anti-tumour activity and has been shown to effectively block tumour growth *in vitro* and *in vivo*, by functioning as a dominant-negative inhibitor for VEGF-induced angiogenesis [27]. It can be stated that in some AML patients, sVEGFR-2 levels may be elevated in the plasma, but its role *in vivo* remains uncertain and further investigation should be undertaken to better determine its exact function.

When the VEGF levels in plasma samples obtained from previously untreated AML and ALL subjects were analysed, and compared with samples from healthy individuals, no significant difference was found. Similarly, no significant correlation between the VEGF plasma level, tumour burden and prognostic factors has been established. Our observations contrast with those of the Aguayo *et al.*, who have reported increased levels of this cytokine in plasma from patients with AML and MDS, when compared to a control group. In their study, elevated plasma levels of VEGF were associated with a reduced survival time and lower CR rates [17]. However, it should be emphasized that the median VEGF levels in the plasma of AML patients in our study (Me 32.6 pg/ml) were similar to those obtained by Aguayo *et al.* (Me 30.63 pg/ml). The lack of significant difference may be due to the smaller numbers of subjects in this study's groups.

It should be reiterated that the molecular interaction of sVEGFR-1 with VEGF might modify its biological function and might influence the clinical course and prognosis of leukemia. In our study, evidence of a high (above median value) sVEGFR-1/VEGF ratio correlated with a lower CR rate and shorter survival time. It is difficult to explain this surprising result. However, as discussed above, a significant sVEGFR-1 increase was observed in AML patients diagnosed with poor performance status and with large tumour mass — factors known to be associated with poor survival times. Another possibility is that

sVEGFR-1 complexed to VEGF may protect VEGF from proteases resulting in its longer half-life, and in this way may prolong the different VEGF activities [14].

In conclusion, it can be stated that the plasma concentration of sVEGFR-1 has been found to be higher in leukemia patients than in healthy subjects, and that this correlates with tumour burden and poor prognosis. The elevated level of soluble VEGF receptors may play an important role in the pathophysiology and the course of acute leukemia, and the sVEGFR-1/VEGF ratio may provide a more significant prognostic value than VEGF alone. The mechanism by which a high sVEGFR-1/VEGF ratio confers shorter survival times in AML is unclear. Similarly unclear is the complicated and still uncertain relationship to angiogenic factors and their soluble receptors *in vivo*. Further investigation is recommended to more accurately determine their function.

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