Vascular Endothelial Growth Factor Secretion Is an Independent Prognostic Factor for Relapse-free Survival in Pediatric Acute Myeloid Leukemia Patients

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ABSTRACT

Substantial improvements in long-term survival have been made with acute myeloid leukemia (AML). However, the overall success rate in treatment of AML is around 50%, despite intensive chemotherapeutic regimens. AML cell survival seems to be related to vascular endothelial growth factor (VEGF). The purpose of this study was to investigate whether VEGF production by AML cells is a prognostic factor for therapeutic outcome and whether this is independent of known prognostic factors such as WBC count, French-American-British (FAB) classification, and risk assessment in which the presence of t(8;21), t(15;17), and inv(16) or FAB M3 defines a low-risk group. Pretreatment levels were measured in the supernatant of AML cells obtained from 47 children with newly diagnosed AML treated between 1988 and 1998. All patients were treated with intensive chemotherapeutic protocols from the Dutch Childhood Leukemia Study Group [DCLSG (DCLSG-ANLL87, DCLSG-ANLL92/94, and DCLSG-ANLL97)]. VEGF was measured at the mRNA level with reverse transcription-PCR and at the protein level using a VEGF immunoassay. VEGF in the supernatant from AML cells was highly variable and in concordance with reverse transcription-PCR results. The low-risk group had significantly lower VEGF levels compared with all others ($P = 0.002$). VEGF levels were significantly increased in AML FAB M4/M5 *versus* AML patients with FAB M1/M2/M3/M4eo ($P = 0.011$), who are reported to have a longer remission duration. Subsequently, the influence of different variables on therapeutic outcome was analyzed. No differences were found in overall survival. But within the limits of the small patient population, VEGF levels as well as age at diagnosis had an independent significant effect on relapse-free survival ($P = 0.032$ and $P = 0.029$, respectively) in multivariate analysis.

INTRODUCTION

Despite the fact that the outcome of AML[3] in young patients has improved substantially, only 45–55% of the children with AML are long-term survivors using intensive chemotherapy protocols (1, 2). Years ago, WBC count, age at diagnosis, and FAB classifications were described as prognostic factors in AML (3). Recently, it was shown that a risk assessment consisting of the combination of cytogenetic abnormalities, FAB-type cytomorphology, and treatment response (CR) could identify pediatric AML patients with good prognosis more adequately (1, 2, 4). Low-risk children with AML were defined by favorable cytogenetic abnormalities [t(8;21), t(15;17), and inv(16)] or FAB type M3 (28%), whereas high-risk AML patients were defined by adverse cytogenetic abnormalities (abnormalities of chromosomes 5 or 7, abnormalities in 3q or complex karyotype) or by not achieving remission after one or two courses (20%). The remainder of the group was composed of standard-risk children with AML (52%). Low-risk pediatric AML patients with recurrent disease still have an OS of 61% at 3 years. In contrast to low-risk children, high-risk and standard-risk patients showed a survival of 0% and 17%, respectively, at 3 years after relapse (2, 4). The need to explore other therapeutic options, especially for standard- and high-risk patients, remains important.

Increased angiogenesis is shown in bone marrow biopsies of patients at diagnosis of AML (5–7). This is normalized in complete hematological remission. Aguayo *et al.* (8) showed that cellular VEGF levels appeared to be of prognostic value in a subgroup of newly diagnosed adult AML cases presenting with high WBC and high blast counts. Leukemic cell cultures produce significantly higher VEGF levels than CD34-enriched

Received 11/12/01; revised 4/15/02; accepted 4/30/02.
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[3] The abbreviations used are: AML, acute myeloid leukemia; VEGF, vascular endothelial growth factor; FAB, French-American-British; RT-PCR, reverse transcription-PCR; DCLSG, Dutch Childhood Leukemia Study Group; RFS, relapse-free survival; OS, overall survival; CR, complete remission; VEGFR, VEGF receptor; Ara-C, 1-β-D-arabinofuranosylcytosine; Hb, hemoglobin.

Table 1 Characteristics of pediatric AML patients

Characteristics[a]	Total group	Low-risk group	High-risk group	No detectable VEGF	Detectable VEGF
No.	47	22	25	20	27
Age (range) (yrs)	7 (0–14)	8.5 (1–14)	5 (0–14)	9 (1–14)	3 (0–14)
Sex (male/female)	26/21	14/8	12/13	9/11	17/10
Karyotype					
t(8;21), or t(15;17), or inv(16)	21	21	0	15	6
−5/−7	1	0	1	0	1
Other karyotype	24	0	24	5	19
APL[b]	3[c]	3[c]	0	2[c]	1
Secondary AML	0	0	0	0	0
Peripheral blood cells at diagnosis					
WBC ($\times 10^9$/liter)	40.2 (1.6–355)	35.2 (8.2–355)	42.8 (1.6–345)	55.4 (8.6–355)	39.9 (1.6–345)
Patient WBC $> 100 \times 10^9$/liter	8	2	6	4	4
% Circulating blasts	62 (8–95)	57 (8–95)	68.5 (17–92)	71 (8–95)	57.5 (17–91)
Hb (mmol/liter)	5.1 (2.8–7.8)	4.9 (2.8–7.8)	5.1 (2.9–7.6)	5 (2.9–7.8)	5.2 (2.8–7.6)
Platelets ($\times 10^9$/liter)	40 (13–254)	35.5 (13–184)	41 (16–254)	32 (13–254)	51 (16–192)
% blasts in bone marrow	74 (23–97)	70 (23–97)	79 (42–97)	76 (23–97)	73 (35–97)
Initial testis involvement	1	1	0	0	1
Initial central nervous system involvement	6	4	2	2	4
FAB classification:					
M1	5	2	3	2	3
M2	14	9	5	9	5
M3	2	2	0	1	1
M3V	1	1	0	1	0
M4	11	6	5	5	6
M4eo	2	2	0	1	1
M5	12	0	12	1	11
Treatment					
DCLSG-ANLL87	14	4	10	5	9
DCLSG-ANLL92/94	27	12	15	9	18
DCLSG-ANLL97	6	6	0	6	0
Death during induction	2	1	1	1	1
% CR	45/47	20/21	25/26	19/20	26/27
Duration 1 CR (weeks)	140 (2–520)	130 (11–364)	170.5 (2–520)	124 (11–520)	223 (2–520)
Relapse (no.)	15	5	10	5	10
Death after CR					
Leukemic (*n*)	9	1	8	2	7
Toxic/infection (*n*)	6	3	3	2	4
Survival time (weeks)	144 (4–520)	130 (0–451)	156 (0–520)	130 (0–520)	250 (0–520)
Follow-up time (weeks)	250 (50–520)	162.5 (50–451)	364 (140–520)	140 (50–520)	312 (156–520)

[a] When not mentioned otherwise, all characteristics are given as median (range).
[b] APL, acute prohyelocytic leukemia.
[c] Two APL patients have t(15;17).

cell cultures of normal volunteer donors (9). Recently, we showed that the increased vessel density at diagnosis of AML was correlated with VEGF expression in leukemic cells (7). Together, these findings suggest a direct link between malignant cell proliferation, angiogenesis, and VEGF expression.

VEGF was originally cloned from the HL-60 cell line and appeared to be a potent stimulator of endothelial cell migration and proliferation (10, 11). Differential splicing of exon 6 and/or 7 leads to various isoforms; the two shortest isoforms (121 and 165 amino acids) have the most potent activity on endothelial cells (12). VEGF, recently called VEGF-A, can bind to VEGFR-1 (also called Flt-1) or VEGFR-2 (also called Flk-1), both of which belong to the class of tyrosine kinase receptors (13, 14). VEGFR-2 is an important receptor to transduce angiogenic signals, and it is highly expressed on endothelial cells but expressed in a low number of AML patients (9, 15). Signaling via VEGFR-2 enhances AML cell survival (15).

The purpose of this study was to investigate whether VEGF production by AML cells is predictive for OS and/or RFS in pediatric AML and is related to the recently used risk assessment. The prognostic significance of VEGF levels in newly diagnosed pediatric AML cases was examined, and its relation with other well-known prognostic factors such as FAB classification, WBC count, and risk assessment profiles was investigated.

MATERIALS AND METHODS

Patients and Treatment Protocols. After informed consent was obtained, pretreatment VEGF levels were measured in the supernatant from AML cells obtained from 47 children with newly diagnosed AML treated between 1988 and 1998. Diagnosis of AML and its FAB type were confirmed at the laboratory of the DCLSG. Table 1 summarizes patient characteristics obtained from the data files of the DCLSG. Patients with known cytogenetic abnormalities were divided and stratified by risk profile. Low-risk children with AML were defined by favorable

cytogenetic abnormalities [t(8;21), t(15;17), and inv(16)] or FAB type M3. High-risk AML patients were defined by adverse cytogenetic abnormalities (abnormalities of chromosomes 5 or 7, abnormalities in 3q or complex karyotype) or by not achieving complete hematological remission after one or two courses, whereas standard-risk children with AML contained all other cytogenetic abnormalities (2, 4). Patients were divided into a low-risk group and a combined group of standard- and high-risk patients, hereafter called the high-risk group, to investigate the relation of VEGF with low-risk profiles. All patients were treated with intensive combination chemotherapy protocols. DCLSG ANLL-87 combined Ara-C, daunorubicin, etoposide, and intrathecal Ara-C (16). DCLSG-ANLL92/94 consisted of a combination of Ara-C, idarubicin, etoposide, mitoxantrone, and intrathecal Ara-C, like the first intensive part of the BFM-AML 91. DCLSG-ANLL97 combined Ara-C, intrathecal methotrexate/Ara-C, and etoposide with daunorubicin or mitoxantrone, identical to the United Kingdom Medical Research Council AML 12 trial.

Leukemic Cells. Samples were obtained at presentation, and mononuclear cells were separated by Ficoll density gradients, cryopreserved in liquid nitrogen, and stored at the laboratory of the DCLSG. Cryopreserved AML cells were thawed rapidly at 37°C, diluted in a 5× volume of normal calf serum as described previously (17). After centrifugation, the remaining pellet was T-cell-depleted by sheep RBCs and separated over Ficoll density gradient. The remaining blast cell population contained >95% AML cells and is referred to hereafter as AML cells. AML cells (at least 2×10^6 cells/2 ml) were cultured in serum-free medium (X-vivo 10; Biowhittaker, Brussels, Belgium) overnight. Cell-free supernatant was collected for VEGF ELISA, and the remaining cells (5×10^6) were used for RNA extraction.

RNA Extraction and RT-PCR. Total RNA was extracted by the Trizol method following the manufacturer's description (Life Technologies, Inc., Grand Island, NY). cDNAs were prepared by reverse transcription at 37°C for at least 1 h in a 20-μl reaction mixture containing 2 μg of total RNA, random hexamers (Pharmacia), 5× first-strand buffer, RNasin, and 2 μl of reverse transcriptase (Life Technologies, Inc.). cDNA was amplified in the presence of primers, 10× buffer, 1.5 mM $MgCl_2$, deoxynucleotide triphosphates, and Taq (Life Technologies, Inc.). The mixture was amplified in a Perkin-Elmer apparatus with PCR cycle conditions specific for the PCRs tested. The PCR product was analyzed by electrophoresis in a 1.5% agarose gel. Gels were stained with ethidium bromide and photographed. The specific primers used for β_2-microglobulin were CCAGCAGAGAATGGAAAGTC (sense) and GATGCTGCTTACATGTCTCG (antisense), and the PCR product was 260 bp (22 cycles, annealing temperature, 55°C). For VEGF, the primers used were GAGTGTGTGCCCACTGAGGAGTCCAAC (sense) and CTCCTGCCCGGCTCACCGCCTCGGCTT (antisense), and the PCR product was 177, 312, and 384 bp (32 cycles; annealing temperature, 60°C; Ref. 18). The primers for VEGF span the splice junctions, allowing the amplified product of each splice variant to be separated electrophoretically. Nested RT-PCR primers for VEGFR-2 were CTTCAACCAGTCTGGGAGTGAGA (sense) and CTCTCCTGCTCAGTGGGCTGCATGT (antisense), and the PCR product was 860 bp (25 cycles; annealing temperature, 60°C; 1 mM $MgCl_2$ and 5% DMSO). For nested PCR, the primers used were TGGAAGTGGCATGGAATCTC (sense) and TTGCCGCTTGATAACAAGG (antisense), and the PCR product was 571 bp (35 cycles; annealing temperature, 53°C; 1.25 mM $MgCl_2$, using 4 μl of the first PCR product). To control for the addition of cDNA in the first PCR reaction, β_2-microglobulin PCR was performed from the same first PCR product as used for the second VEGFR-2 (nested). The result was always positive in the samples shown.

VEGF ELISA. Secretion of human VEGF by AML cells was detected by Quantikine human VEGF immunoassay (R&D, Minneapolis, MN) following the manufacturer's instructions. The detection limit for VEGF was 3 ng/liter.

Statistical Analysis. Nonparametric tests (Mann Whitney *t* test and Kruskal-Wallis test) and Fisher's exact test were used to compare different variables in different groups. Associations between quantitative covariates were assessed by Spearman rank correlation. Outcome events were death during induction therapy, failure to achieve CR, death during remission, and relapse. RFS was the time from CR to the first relapse. OS was the time from start of treatment to death from any cause. Survival analysis (Kaplan-Meier plots, log-rank tests, and Cox regression) was used to study the effect of explanatory variables on OS and RFS. Statistical tests were performed at the 5% level of significance.

RESULTS

The group with high-risk profiles and the group with low-risk profiles were comparable with regard to age, hematopoietic cells, and follow-up time. However, significant differences were found for FAB classification; M5 type was restricted to the high-risk group. A difference was found between the groups for the treatment protocol used; 6 low-risk patients were treated with the latest ANLL-97 DCLSG protocol, whereas none of the high-risk patients was treated with this protocol.

In all 47 patients AML cell-free supernatant was harvested and used for assessment of VEGF level. Expression of VEGF mRNA was examined in 26 available RNA samples.

Expression and Secretion of VEGF by AML Cells. VEGF mRNA was demonstrated in 21 of the 26 mRNA tested samples and showed wide variation (Fig. 1). The amount of VEGF secreted was detected by ELISA in the supernatant of AML cells. The levels of VEGF are in line with the results obtained by semiquantitative RT-PCR (Fig. 1). Coinciding expression of VEGFR-2 may result in the generation of an autocrine loop that might enhance leukemic cell survival (15). Fig. 1 shows the results of RT-PCR for VEGFR-2; we demonstrated VEGFR-2 mRNA in only 8 of the 21 (38%) tested samples.

Variable levels of VEGF were found in the supernatant of AML cells (median, 11.5 ng/liter; range, 3–142.5 ng/liter; Fig. 2). In 20 of the 47 tested supernatants, the VEGF level was below the detection limit of 3 ng/liter. To compare the clinical characteristics of patients with and without detectable VEGF, we added in Table 1 the patient characteristics for patients with no detectable VEGF levels ($n = 20$) *versus* patients with detectable VEGF levels ($n = 27$). The groups were comparable with respect to hematopoietic cells. Significant differences were

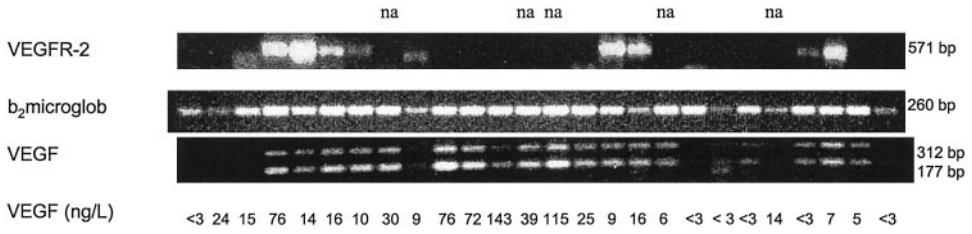

Fig. 1 Expression of various angiogenic factors at the mRNA level in AML cells. Individual RT-PCR results of VEGFR-2, β_2-microglobulin, and VEGF were assayed in AML cells of different patients from whom RNA was available ($n = 26$). VEGFR-2 nested RT-PCR is demonstrated for 21 patients. *na*, no available cDNA left ($n = 5$). Two bands for VEGF PCR products identify the two shortest splice variants of the VEGF mRNA level (see "Materials and Methods"). Beneath the VEGF RT-PCR, VEGF ELISA results are given for individual patients, confirming the concordance between VEGF mRNA and VEGF protein level.

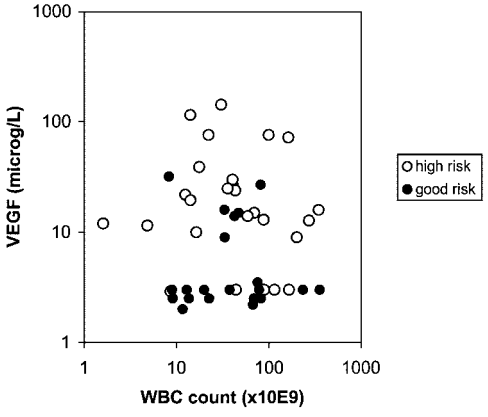

Fig. 2 Relations among VEGF level of AML cells, WBC count, and risk assessment in individual AML patients. Individual VEGF levels were measured in the supernatant of AML cells (ng/liter) with a threshold of VEGF determination of 3 ng/liter. WBC count is given at $\times 10^9$/liter. Risk assessment: ●, AML patients with a low-risk profile defined by favorable cytogenetic abnormalities [t(8;21), t(15;17), and inv(16)] or FAB type M3; and ○, all remaining AML patients.

Table 2 The results of various variables when the patients are divided by FAB classification (A) or by risk assessment (B)

A. FAB M1/M2/M3/M4eo *versus* M4/M5

	FAB M1/M2/M3/M4eo ($n = 24$)	FAB M4/M5 ($n = 23$)	P^a
VEGF (pg/ml)	3 (3–32)[b]	14 (3–142.5)	0.011
WBC ($\times 10^9$/liter)	27.7 (4.8–115)	77.8 (1.6–355)	0.004
Hb (mmol/liter)	4.9 (2.8–7.5)	5.5 (3–7.8)	0.154
Platelets ($\times 10^9$/liter)	36 (13–254)	45 (16–188)	0.831
Age at diagnosis (mo)	8.5 (0–14)	2 (0–13)	0.052

B. Low-risk *versus* high-risk group

	Low-risk group ($n = 22$)	High-risk group ($n = 25$)	P^a
VEGF (pg/ml)	3 (3–32)[b]	15 (3–142.5)	0.002
WBC ($\times 10^9$/liter)	35.2 (8.2–355)	42.8 (1.6–345)	0.348
Hb (mmol/liter)	4.9 (2.8–7.8)	5.1 (2.9–7.6)	0.709
Platelets ($\times 10^9$/liter)	36 (13–184)	41 (16–254)	0.468
Age at diagnosis (mo)	8.5 (1–14)	5 (0–14)	0.143

[a] P in Mann-Whitney U test.
[b] Values shown are the median (minimum-maximum).

found again for FAB classification; M5 was overexpressed in the group with detectable VEGF levels. The same differences were found between the VEGF groups for the treatment protocol used; the latest ANLL-97 DCLSG protocol was restricted to the group in which no VEGF was detectable.

VEGF and Prognostic Factors. There was no correlation between VEGF levels, initial WBC, and Hb and platelet count, whereas the VEGF levels of AML cells were inversely correlated to age at diagnosis ($r = -0.471$; $P = 0.001$). Years ago, FAB classification was reported to be of prognostic value; M1, M2, M3, and M4eo are associated with a longer remission duration time than M4 and M5 (3). Table 2A shows the results when the patients were divided according to their FAB classification into two groups. The children with AML FAB M4/M5 were younger ($P = 0.052$) and have higher leukocyte counts ($P = 0.004$) compared with children with M1, M2, M3, and M4eo. Moreover, VEGF was significantly higher in FAB type M4 and M5 *versus* FAB type M1, M2, M3, and M4eo ($P = 0.011$).

Subsequently, to investigate whether VEGF levels are directly related to morphological or cytogenetic favorable or less favorable abnormalities, as recently used more commonly, patients were divided by risk profile; the low-risk group was defined by favorable cytogenetic abnormalities [t(8;21), t(15;17), and inv(16)] or FAB type M3, and the high-risk group contained all remaining patients. Between low- and high-risk groups, no differences were found for standard prognostic factors such as WBC and age at diagnosis (Table 2B). However, a significant difference was found between the VEGF level in the low-risk group (median, 3 ng/liter; range, 3–32 ng/liter) and that in the high-risk group [median, 15 ng/liter; range, 3–142.5 ng/liter ($P = 0.002$); Fig. 2]. In summary, VEGF levels were inversely correlated to age at diagnosis and were significantly increased in FAB classification M4/M5 as well as in high-risk patients.

As shown in Table 1, patients with a low-risk profile based on the previously described cytogenetic profile were treated more often with DCLSG-ANLL97 (6 of 22), in contrast to patients with high-risk profiles (0 of 25; Fisher's exact test, $P = 0.012$). When patients are divided into groups with or without VEGF expression, 6 patients treated with DCLSG-ANLL97 were restricted to the group with no detectable VEGF levels (Fisher's exact test, $P = 0.009$). Statistical analysis of VEGF

Table 3 RFS in relation to different factors: results of Cox regression analysis

	Relative risk	95% Confidence interval of RR	*P*
Age at diagnosis (yrs)	1.19	1.02–1.4	0.029
VEGF (pg/ml)	1.03	1.00–1.05	0.032
Risk group	1.04	0.3–3.7	0.94
Treatment protocol			0.24
ANLL 97	2.54	0.26–25	
ANLL 92/94	1.02	0.1–10	
ANLL 87	1		

levels in relation to treatment protocol showed a significant difference (Kruskal-Wallis test, $P = 0.027$). The treatment protocol used might influence the conclusions on the relationship between VEGF and therapeutic outcome, and hence there is a need to correct for this selection bias with multivariate analysis.

VEGF and Outcome. First, we tested whether the defined risk profiles, FAB classification, and treatment protocol had an impact on OS. No difference was found in OS when the patients were divided by low-risk *versus* high-risk profiles (log-rank, 1.72; $P = 0.19$). No relation was found between FAB classification and OS (log-rank, 0.29; $P = 0.60$). As mentioned above, a difference in treatment protocols was observed between the low-risk group and the high-risk group. However, no difference in OS was found when stratifying for the different treatment protocols (log-rank test, $P = 0.36$). Most importantly, the patients studied had similar outcomes for OS with different treatment protocols.

Next, we examined the influence of different variables on RFS. Table 3 shows the result of Cox regression analysis with age at diagnosis, treatment protocol (three groups), risk profiles (two groups), and VEGF levels on RFS. The results suggest that as VEGF continued to increase with each 10 pg/ml, RFS continued to decrease $(1.03)^{10} = 1.34$-fold. Within the limits of small numbers, we find that independent prognostic factors for duration of first CR (RFS) are VEGF levels ($P = 0.032$) and age at diagnosis ($P = 0.029$) after correction for treatment protocol and risk group.

DISCUSSION

Recent data suggest that neoangiogenesis is not only important in solid tumors but also in hematological malignancies and that VEGF plays a major role in neovascularization and leukemic cell survival (6, 7, 15, 19, 20). Correlations have been described between VEGF levels and poor prognosis in several solid tumors such as lung, bladder, colon, and breast cancers and melanoma (21–25).

The aim of this study was to relate VEGF to risk assessment stratification upon favorable cytogenetics and to examine the possible prognostic significance of VEGF production by AML cells for therapeutic outcome.

In the present study, VEGF mRNA expression and VEGF secretion by AML cells varied widely in newly diagnosed pediatric AML patients. Several prognostic factors were studied in this group of 47 children with AML, such as FAB classification, WBC count, age at diagnosis, and risk assessment. VEGF in AML cell-free supernatant was significantly lower in the prognostic favorable FAB types (M1, M2, M3, and M4eo) compared with the less favorable FAB types (M4 and M5; Refs. 3 and 26). A leukocyte count of less than 100×10^9/liter was also shown to be of prognostic value (3). However, in this group, the number of patients with a leukocyte count above 100×10^9/liter was too low to analyze the data in this way (Table 1). VEGF levels were significantly increased in high-risk patients as compared with low-risk patients.

Our results show that VEGF levels in the supernatant of AML cells and age at diagnosis have prognostic value for RFS in children with AML. A possible confounding factor is the variation in treatment protocol. Multivariate analysis revealed that VEGF secretion by AML cells affects prognosis irrespective of cytogenetic abnormalities, WBC, FAB classification, risk assessment, and age at diagnosis.

Our results suggest that as VEGF continued to increase, RFS continued to shorten. VEGF as a continuous variable underscores the previous results of Aguayo *et al.* (8). Moreover, effects of VEGF on tumor cells seem to be dose dependent (27).

The mechanism by which VEGF affects prognosis is not completely clear. A direct effect of VEGF on AML cells can be hypothesized in the patients with VEGFR-2 expression by RT-PCR. In our study population, 38% of the patients expressed VEGFR-2. Recently, it was demonstrated that VEGF was able to support AML cell survival *in vitro* (15). Thus, signaling via the VEGFR-2 on AML cells might be of great functional importance. A more indirect way in which VEGF can support AML growth is by increasing neovascularization. Besides the fact that VEGF is able to lead to endothelial cell proliferation and migration necessary in the process of angiogenesis, VEGF stimulates endothelial cells to produce several growth factors, such as granulocyte colony-stimulating factor, granulocyte macrophage colony-stimulating factor, and interleukin 6, that have been shown to enhance AML cell survival (28). Contact between AML cells and endothelial cells can result in AML growth advantage.

In conclusion, in multivariate analysis, VEGF was an independent prognostic factor for RFS; however, the number of AML patients was small. A high VEGF level in the supernatant of AML cells seems to be related to AML growth and worse survival. In view of these results, one can hypothesize that intervention with VEGF inhibitors in AML patients can improve outcome.

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