Endogenous Vascular Endothelial Growth Factor-C Expression Is Associated with Decreased Drug Responsiveness in Childhood Acute Myeloid Leukemia

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Abstract

Purpose: We hypothesized that downstream effects of endogenous vascular endothelial growth factor (VEGF)/VEGF receptor signaling on acute myelogenous leukemia (AML) cell survival resulted in increased *in vitro* cellular drug resistance and a longer time to kill most leukemic cells *in vivo* upon drug exposure.

Experimental Design: In primary AML cells from pediatric patients, VEGFA and VEGFC mRNA expression and *in vitro* cellular resistance to nine cytotoxic drugs were studied. As *in vivo* equivalents for *in vitro* drug resistance, *in vivo* AML blast reduction upon drug exposure, measured as blast cell reduction on day 15 in the bone marrow and as time in days from diagnosis to complete remission (CR) were used.

Results: Increased endogenous VEGFC levels significantly correlated with increased *in vitro* resistance for six typical AML drugs in primary AML cells from pediatric patients. Patients with >5% blasts on day 15 showed a 12.9-fold increase in the median VEGFC level compared with patients with ≤5% blasts ($P = 0.002$). Time to reach CR was studied using linear regression analysis with VEGFC, age at diagnosis, sex, treatment protocol, FAB type, cytogenetic risk profile, and WBC counts as variables. There was a significant positive independent association between VEGFC levels and time to CR ($b = 6.02$, SE = 1.58, $P \leq 0.0001$, $n = 72$).

Conclusions: These results suggest for the first time that higher endogenous VEGFC levels of AML cells are related to decreased *in vitro* and *in vivo* drug responsiveness.

Children with acute myelogenous leukemia (AML) have a poor prognosis; only 50% to 60% are long-term survivors using intensive chemotherapy protocols (1–4). To increase survival rates in the future, it will be necessary to develop additional treatment strategies.

In general, by vascular endothelial growth factor (VEGF) stimulation, VEGF receptors (VEGFR) become phosphorylated and transmit intracellular signals leading to cell proliferation and survival (5). There are three known types of VEGFRs; FLT1 (VEGFR-1), KDR (VEGFR-2), and FLT4 (VEGFR-3), which all belong to the class of tyrosine kinase receptors (6). In addition, six VEGFs (A-F) are known (7, 8). Among these six structurally related VEGF proteins, VEGFA and VEGFC are expressed by AML cells (9, 10). VEGFA exerts its effects by binding to FLT1 and KDR (11, 12). The downstream effects of VEGFA are mainly executed by KDR binding, resulting in increased AML cell survival and proliferation (via mitogen-activated protein kinase and phosphoinositide 3-kinase/AKT signaling) and protection against apoptosis (via bcl2 and mcl1; refs. 13–16). VEGFC is a lymphangiogenic and angiogenic growth factor and signals through KDR and FLT4 receptors (17, 18). It was previously shown that exogenously added VEGFC increased AML cell survival and proliferation *in vitro* (19).

AML blast disappearance upon drug exposure can be measured *in vitro* and *in vivo*. Good *in vivo* equivalents, in terms of *in vivo* AML blast reduction upon drug exposure, for *in vitro* drug resistance are blast counts on day 15 in the bone marrow (>5%/≤5%) and the time in days from diagnosis to complete remission (CR). Time from diagnosis to CR is the time in which the bulk of AML cells decreases upon drug exposure to at least <5% blasts in a regenerated bone marrow. Moreover, the blast count on day 15 in the bone marrow is a variable of well-defined prognostic value (20, 21).

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We hypothesized that downstream effects of endogenous VEGF/VEGFR signaling resulted in decreased drug responsiveness in AML. The aim of this study was to investigate endogenous VEGFA and VEGFC mRNA expression of AML cells in relation to *in vitro* cellular drug resistance, blast counts on day 15, and the time to reach CR. In this study, we show for the first time that increased endogenous VEGFC mRNA levels are related to increased *in vitro* drug resistance and a longer time to kill most leukemic cells *in vivo* upon drug exposure.

Materials and Methods

Patient samples. In this study, the AML-BFM Study Group (Münster, Germany), VU University Medical Center, and University Medical Center Groningen participated. The initial diagnosis of AML and its subtypes were determined accordingly to the French-American-British classification (22). AML smears were routinely investigated at the three university hospitals. When necessary subtyping was confirmed by immunologic methods. This study was approved by the local ethical committees. Consecutive AML samples collected between 1998 and 2002 were included (n = 74). The clinical data of these patients reflect the normal distribution of published AML data with regard to age, WBC count, sex, and FAB type distribution (Table 1; refs. 1, 4, 20). The patients were divided in three cytogenetic subgroups, i.e., favorable, including t(15;17), t(8;21), inv*16*, t(16;16), t(9;11) in the pediatric population; intermediate, including normal karyotype; and unfavorable, including -5/del(5q), -7/del(7q), inv*3*/t(3;3), +8, and complex karyotype (23–26). Given the heterogeneity of the treatment protocols and the relatively small study population, we did not study the relation between VEGFC and long-term clinical outcome. Bone marrow blast counts on day 15 underwent central review (available for 33 of the 74 patients). Leukemic cells were distinguished from nonleukemic precursor cells in the generally hypocellular bone marrow by morphology. Patients were all treated with intensive cytarabine/anthracycline-based induction protocols.

Table 1. Characteristics of pediatric AML patients

Characteristics	
No. patients	74
Age at diagnosis (y)	9.6 (0.2-19.9)
Sex (male/female)	46/28
Karyotype	
Favorable	18
Intermediate	27
Unfavorable	14
Unclassified	15
WBC ($\times 10^9$/L)	54.1 (2.1-388)
Patient WBC > 100 $\times$ 10^9/L	24
French-American-British classification (22)	
M0	4
M1	7
M2	19
M3	4
M4	25
M5	11
M7	2
Unknown	2
Death before CR	2
No. patients in CR	72/72
Time from diagnosis to CR (d)	42.5 (27-228)
Day 15 blasts (≤5%/>5%)	24/9

NOTE: The characteristics (age, WBC, and time to CR) are given as median (range). Karyotype: favorable, including t(15;17), t(8;21), inv*16*, t(16;16), t(9;11) in the pediatric population; intermediate, including normal karyotype; and unfavorable, including -5/del(5q), -7/del(7q), inv*3*/t(3;3), +8, and complex karyotype (23–26). Patients were all treated with intensive cytarabine/anthracycline-based induction protocols.

Isolation of acute myeloid leukemic cells. AML cells were isolated as described previously (27). We removed contaminating lymphocytes using immunomagnetic beads as described earlier (28). Isolated cells were collected in the culture medium. We only included samples which contained >80% leukemic cells, as determined by cytospin preparations stained with May-Grünwald-Giemsa (Merck). Not in all samples could all study variables be measured, mainly due to a limited number of cells.

Total cell kill 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. To determine the *in vitro* drug resistance of the AML samples, we used a 4-day cell culture assay based on the principle that only viable cells are able to reduce 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide to a colored formazan product, which we determined spectrophotometrically at 562 nm, as described earlier in great detail (28, 29). We evaluated data from bone marrow and peripheral blood samples together and data from fresh and cryopreserved samples, because this does not influence the results of *in vitro* drug resistance testing (30, 31). We tested a panel of nine drugs, each at six different concentrations in duplicate in 96-well microcultured plates. The absorbance (A) is linearly related to the number of viable cells (29). We considered the results evaluable only if the control wells contained >70% leukemic cells (as determined by morphology after May-Grunwald-Giemsa staining) after 4 days of culture. Also, the mean control absorbance after correction for the background at day 4 must have exceeded 0.05 arbitrary units for valid results. The LC_{50} value, which is the drug concentration needed to kill 50% of the leukemic cells, was used as a measure of resistance.

Cytotoxic drugs. We tested the following nine drugs at the given concentrations (brand names in parentheses): 0.002 to 10.0 μg/mL cytarabine (Cytosar, Pharmacia & Upjohn), 0.012 to 400 μg/mL gemcitabine (Gemzar, Eli Lilly), 0.016 to 16 μg/mL fludarabine (Fludara, Schering Nederland B.V.), 1.56 to 50 μg/mL 6-thioguanine (Lanvis, Glaxo Wellcome), 0.002 to 2 μg/mL daunorubicin (Cerubidine, Rhône-Poulenc Rorer), 0.05 to 50 μg/mL etoposide (Vepesid, Bristol-Myers Squibb), 0.05 to 50 μg/mL vincristine (Oncovin, Eli Lilly), 0.003 to 10 IU/mL L-asparaginase (Paronal, Christiaens), and 0.0004 to 40 μg/mL cladribine (Leustatin, Ortho Biotech).

RNA extraction and PCR. We extracted total RNA using Trizol reagent according to the manufacturers' description (Life Technologies, Inc.). cDNAs were prepared as described previously (27). PCRs were done for VEGFA, VEGFC, KDR, as well as FLT4. We amplified the mixture using the specific PCR cycle conditions summarized in Table 2. VEGFA cDNA was amplified with primers designed to pick up all of the VEGFA isoforms. Quantitative LightCycler PCR was done using the LightCycler-FastStart DNA Master SYBR Green I System using 1 μL of a 10-fold diluted cDNA in each PCR reaction in a final volume of 10 μL (Roche Molecular Biochemicals). The specificity of the PCR reactions was verified by generation of a melting curve and by agarose gel electrophoresis of the amplified products. Serial cDNA dilutions of a mixture of all patient samples were used to generate standard curves. The expression of each gene in each sample was analyzed in duplicate. β-Actin (ACTB) mRNA expression levels were used to calculate relative expression levels. All data are presented as ratio of the target gene/ACTB. Because KDR and FLT4 mRNA expression could not be detected in most of the cases with a quantitative PCR, we used a nonquantitative PCR for detecting the expression of KDR (nested PCR) and FLT4. Non-diluted cDNA in each PCR reaction in a final volume of 24 μL was used. Details about the PCR conditions are given in Table 2. To control for the addition of cDNA in the first PCR reaction, ACTB PCR was done from the first PCR product as used for the second KDR (nested). The results were always positive in the samples tested. PCR products were analyzed by electrophoresis in a 1.5% agarose gel.

Table 2. Characteristics of the PCRs

Gene	Forward	Reverse	C	*T*
VEGFA	GAGTGTGTGCCCACTGAGGAGTCCAAC	CTCCTGCCCGGCTCACCGCCTCGGCTT	40	55
VEGFC	GATCTGGAGGAGCAGTTAGG	GAGTTGAGGTTGGCCTGTTC	40	55
KDR (exon)	CGGAGTGACCAAGGATTGTA	CTCTCCTGCTCAGTGGGCTGCATGT	25	56
KDR (nested)	TGGAAGTGGCATGGA ATCTC	TTGCCGCTTGGATAACAAGG	32	53
FLT4	CAAGCCATCCGAGGAGCTAC	GTCTTGCACTTCGCACACATAGTGG	40	58
β-Actin	GCTGTGCTACGTCGCCCTG	GGAGGAGCTGGAAGCAGCC	40	61

NOTE: VEGFA, VEGFC, and β-actin mRNA expression were detected with a quantitative PCR. A nonquantitative PCR was done to detect KDR (using a nested PCR) and FLT4 mRNA expression.
Abbreviations: Forward and reverse, specific primers; *T*, annealing temperature given as °C; C, cycles.

VEGFC protein determination. AML patient cells were seeded at a density of 1.0×10^6/mL in serum-free medium (X-vivo 10; Biowhittaker). After 8 h of culture, supernatants were harvested for determination of VEGFC protein by ELISA (R&D Systems).

Statistical analysis. Differences in the distribution of VEGFC levels between samples were tested using a Mann-Whitney *U* test. Correlations between VEGFA, VEGFC mRNA, the LC_{50} values of the different drugs, and time to CR were calculated with the Spearman rank correlation coefficient (ρ). Additionally, the associations of LC_{50} values for each of the nine drugs and VEGFC mRNA expression were studied using stepwise forward linear regression analyses with each of these drugs separately into the model and with adjustment for the following potential confounders: sex, age at diagnosis, WBC counts, cytogenetic risk profile, FAB type, and treatment protocol. Likewise, we analyzed this for VEGFA mRNA expression. We checked normal distribution using visual inspection of the probability plots of the residuals.

Blast counts on day 15 in the bone marrow are defined as >5% or ≤5%. CR is defined according to the Cancer and Leukemia Group B criteria as <5% blasts in a normocellular bone marrow and normal hematopoiesis characterized as at least 1,000/μL neutrophils and 100,000/μL platelets in the peripheral blood (32). *In vivo* AML blast reduction upon drug exposure was defined as blast counts on day 15 in the bone marrow and time from diagnosis to CR in days. Stepwise forward linear regression analysis was done to study the association between VEGFC and blast counts on day 15 and time to CR with adjustment for the following potential confounders: sex, age at diagnosis, WBC counts, cytogenetic risk profile, FAB type, and treatment protocol. Statistical tests done were two-sided; *P* values of ≤0.05 were considered to be statistically significant, whereas *P* values of 0.05 to 0.10 were considered to indicate a trend for significance.

Results

VEGF/VEGFR expression in AML. VEGFA mRNA levels were above the detection limit in all samples, and VEGFC levels were detectable in 69 of 74 patient samples (VEGFA: median 0.74, range 0.06-5.09 and VEGFC: median 0.18, range 0-18.45, given as arbitrary units; Supplementary Fig. S1). To investigate the relation between VEGFC mRNA levels and protein secretion, we determined concomitantly the expression of VEGFC of five randomly selected AML patient samples by qPCR and by ELISA in AML cell culture supernatants. A significant correlation ($P = 0.04$) between mRNA and protein levels was found (Supplementary Fig. S2). KDR mRNA expression was detected in 55% of the samples. Samples with detectable KDR showed significantly higher VEGFA mRNA levels (median 0.99, range 0.11-5.09) compared with samples with undetectable KDR expression (median 0.39, range 0.06-3.42; $P = 0.04$, Mann-Whitney *U* test). FLT4 mRNA expression was detected in 48% of the samples. Samples with detectable FLT4 showed significantly higher VEGFC mRNA expression levels (median 1.08, range 0-18.45) compared with samples with undetectable FLT4 expression (median 0.15, range 0-2.76; $P = 0.04$, Mann-Whitney *U* test). In sum, varying VEGFA and VEGFC mRNA expression levels were detectable in the vast majority of AML samples. VEGFA levels were higher in patients with detectable KDR expression, whereas VEGFC levels were higher in patients with detectable FLT4 expression levels compared with patients with undetectable KDR and FLT4 levels, respectively.

Table 3. Correlations between VEGFA, VEGFC mRNA expression and LC_{50} values of the drugs

Drug		VEGFA	VEGFC	LC_{50} values	
Cytarabine	ρ	-0.132	0.279	Median	0.38
	P	0.417	*0.082*	Range	0.02 to 3.85
	n	40	40		
Gemcitabine	ρ	0.099	0.326	Median	2.60
	P	0.544	**0.040**	Range	<0.01 to 205.1
	n	40	40		
Fludarabine	ρ	0.143	0.311	Median	0.44
	P	0.380	**0.051**	Range	0.03 to 12.84
	n	40	40		
Cladribine	ρ	0.089	0.452	Median	0.02
	P	0.584	**0.003**	Range	<0.0004 to 0.21
	n	40	40		
6-Thioguanine	ρ	-0.168	0.396	Median	5.86
	P	0.315	**0.014**	Range	<1.56 to 21.48
	n	38	38		
Daunorubicin	ρ	0.353	0.461	Median	0.14
	P	*0.065*	**0.005**	Range	0.02 to 0.84
	n	36	36		
Etoposide	ρ	0.007	0.428	Median	3.60
	P	0.966	**0.008**	Range	0.16 to 29.81
	n	37	37		
Vincristine	ρ	0.245	0.279	Median	1.17
	P	0.170	0.115	Range	<0.05 to 48.83
	n	33	33		
L-Asparaginase	ρ	0.079	0.219	Median	0.35
	P	0.658	0.212	Range	<0.003 to >10.0
	n	34	34		

NOTE: LC_{50} value is the drug concentration needed to kill 50% of the leukemic cells and is given as μg/mL except for L-asparaginase (IU/mL). Using the two-tailed test, *P* of ≤0.05 was considered statistically significant (in bold) and *P* values of 0.05 to 0.10 were considered to indicate a trend for significance (in italics).

VEGFC mRNA expression is related to increased* in vitro *drug resistance. First, we found that VEGFC mRNA levels did not correlate with age, WBC counts, FAB type, or cytogenetic risk profile. Next, we investigated more specifically the relation with cellular *in vitro* drug resistance. In 40 of 74 patients, we were able to test not only VEGF/VEGFR mRNA expression levels but also the cellular resistance for several different cytotoxic drugs; seven AML and two acute lymphoblastic leukemia drugs. LC_{50} values of the tested drugs are given in Table 3. There were clear differences in LC_{50} values between individual samples for each drug. LC_{50} values of the tested drugs were comparable with other studies examining *in vitro* drug resistance in AML (33, 34). VEGFA mRNA levels showed no significant correlation with the LC_{50} values of the tested cytotoxic drugs (Table 3).

Increased continuous VEGFC mRNA expression significantly correlated with increased resistance for six of nine drugs: gemcitabine, fludarabine, 6-thioguanine, daunorubicin, etoposide, and cladribine (Table 3). For cytarabine, such a trend was seen. The distribution of LC_{50} values of etoposide, daunorubicin, cladribine, and 6-thioguanine between samples with high VEGFC (above the median) versus low VEGFC (below the median) mRNA levels are shown (Fig. 1). Additional linear regression analysis confirmed the significance of the independent associations of increased VEGFC mRNA expression with increased resistance for four drugs: 6-thioguanine (b = 0.006, SE = 0.003, P = 0.045), daunorubicin (b = 0.133, SE = 0.050, P = 0.012), etoposide (b = 0.003, SE = 0.001, P = 0.016), and cladribine (b = 0.858, SE = 0.218, P = ≤0.001). These four drugs also showed the strongest correlations with VEGFC using Spearman rank correlation coefficient.

***Increased VEGFC levels are related to a significantly higher number of AML blasts in the bone marrow on day 15* in vivo.** Because we aimed to study the kinetics of *in vivo* reduction of the bulk of AML blasts upon drug exposure in relation to VEGFC, relevant variables for drug responsiveness are blast counts on day 15 and the time to reach CR. VEGFC mRNA levels were compared between patients with >5% AML

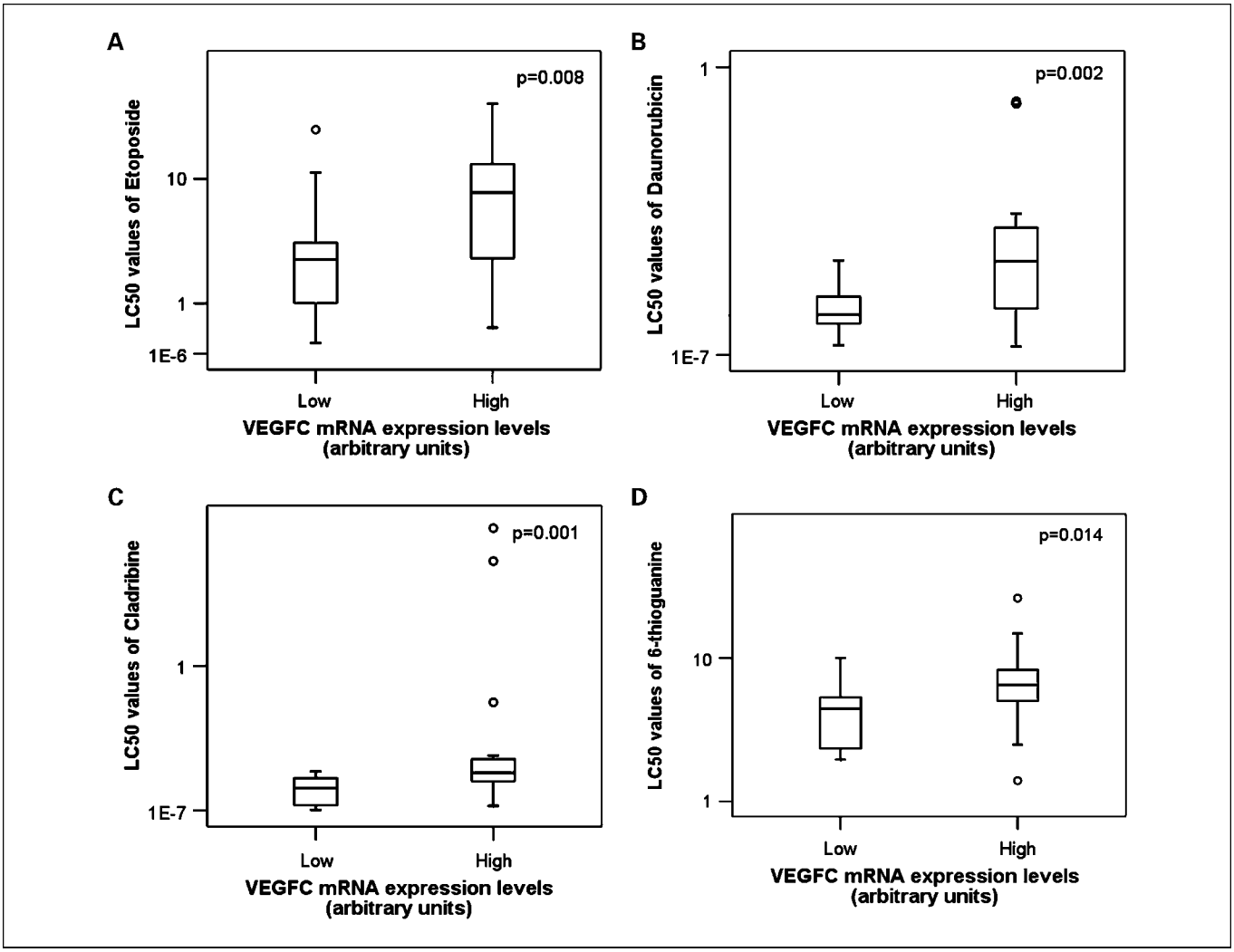

Fig. 1. Distribution of LC_{50} values of etoposide (*A*), daunorubicin (*B*), cladribine (*C*), and 6-thioguanine (*D*) between samples with high versus low VEGFC mRNA expression levels. Dividing the group by the median VEGFC mRNA expression level, the samples with high VEGFC (i.e., above the median) were significantly more resistant to etoposide (n = 37), daunorubicin (n = 36), cladribine (n = 40), and 6-thioguanine (n = 38) compared with samples with low VEGFC mRNA expression levels (i.e., below the median). *P*, *P* value of the Mann-Whitney *U* test; *boxes,* LC_{50} values showing the 5%, 25%, 50%, 75%, and 95% cumulative relative frequencies. °, outliers.

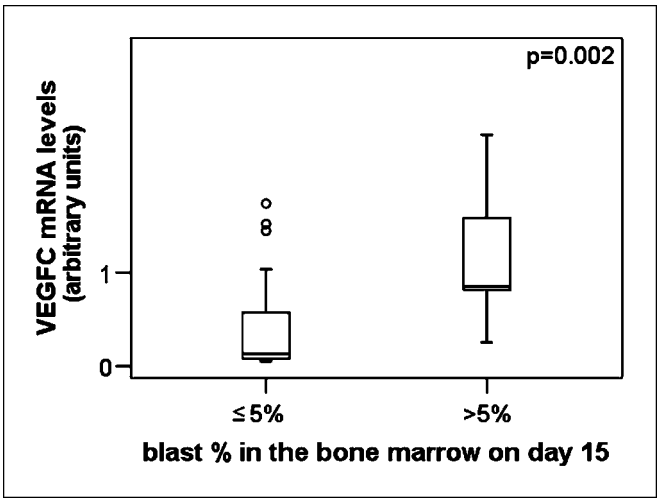

Fig. 2. Distribution of VEGFC mRNA expression levels between patients with >5% versus ≤5% AML blasts on day 15 in the bone marrow. A Mann-Whitney *U* test was used to study the differences in distribution of VEGFC levels between patients with ≤5% AML blasts (n = 24) versus >5% AML blasts (n = 9). *Boxes,* VEGFC levels showing the 5%, 25%, 50%, 75%, and 95% cumulative relative frequencies. °, outliers.

blasts (n = 9) versus ≤5% AML blasts (n = 24) on day 15 in the bone marrow. Patients with >5% AML blasts on day 15 showed a 12.9-fold increase in the median VEGFC level compared with the result of patients with ≤5% AML blasts (P = 0.002; Fig. 2). Linear regression analysis showed a significant independent positive association between VEGFC mRNA levels and the AML blast count on day 15 (i.e., ≤5%/>5%; b = 1.40, SE = 0.30, P = 0.001).

***Increased VEGFC levels are related to a longer time to reach CR* in vivo.** VEGFA mRNA expression was not related to the time to reach CR but increased continuous VEGFC mRNA levels significantly correlated to a longer time to CR (ρ = 0.280, P = 0.017, n = 72; Fig. 3A). Dividing the group by the median VEGFC mRNA level, patients with high VEGFC showed a significantly longer time to reach CR compared with patients with low VEGFC levels (P = 0.037; Fig. 3B). Moreover, we found that VEGFC is an independent prognostic factor for the time to reach CR after correction for treatment protocol, cytogenetic risk group, age at diagnosis, and WBC count. Linear regression analysis showed a significant independent positive association between VEGFC mRNA levels and time to CR (b = 6.02, SE = 1.58, P = <0.0001).

Discussion

In this study, we show that an increased *endogenous* VEGFC mRNA level of pediatric AML cells is correlated with decreased drug responsiveness based on *in vitro* data and *in vivo* variables, i.e., blast percentage on day 15 of induction chemotherapy and time to achieve CR. The strength of this study is that (although groups were relatively small), with the above-mentioned three independent different *in vitro* and/or *in vivo* assays coherent, results were obtained, strongly pointing out a relation between VEGFC and drug responsiveness in AML.

A limitation of our study is that mRNA expression levels were measured. However, no posttranscriptional regulation of VEGFA or VEGFC has been described, and in a previous study, we showed that VEGFA mRNA levels correlated with protein levels (35, 36). Furthermore, a positive correlation was found between VEGFC mRNA and protein levels, supporting the validity of the use of cDNA for our investigation.

Exogenous levels of VEGFA had only a marginal effect at protecting one of two tested leukemia cell lines from chemotherapy-induced apoptosis *in vitro* (19). Also, in our study, no role for VEGFA mRNA expression in drug resistance of AML cells was shown. Exogenously added VEGFC protected AML cell lines from *in vitro* chemotherapy-induced apoptosis for three drugs (daunorubicin, etoposide, and cytarabine; ref. 19). This protective *paracrine* effect of VEGFC shown *in vitro*, after FLT4 binding, was associated with an increased bcl2/bax ratio (19).

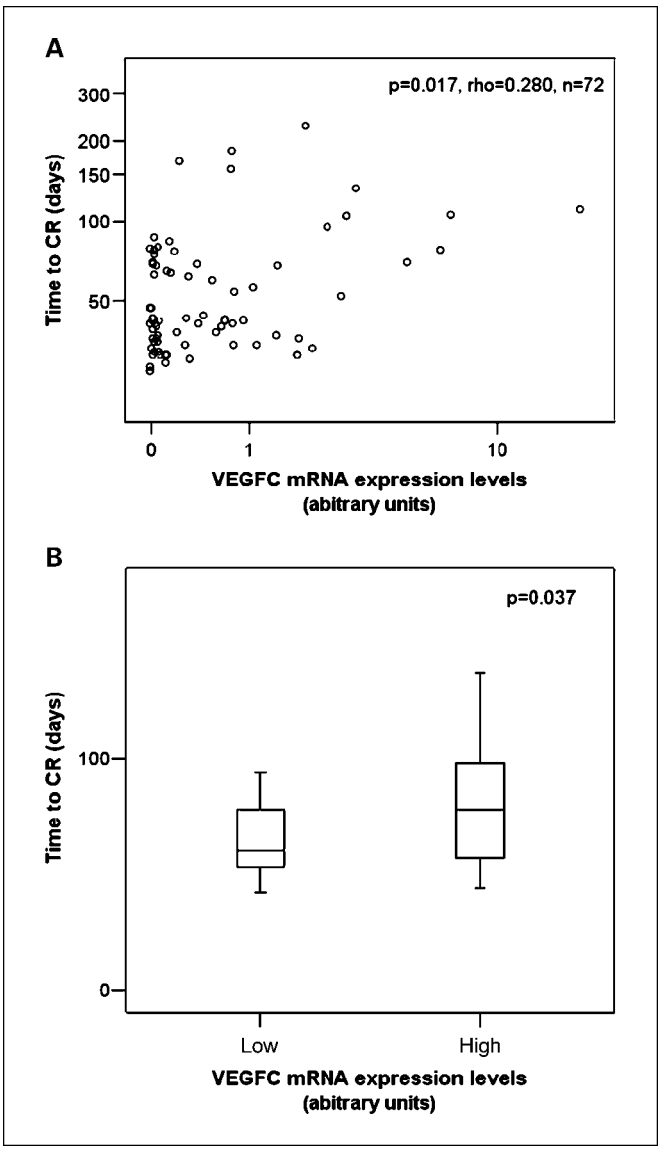

Fig. 3. Relation between VEGFC mRNA expression levels and the time to reach CR. *A,* correlation between the continuous variables VEGFC and time to CR. Time to CR is the time between diagnosis and CR in days. *P, P* value of the Spearman rank correlation coefficient (ρ); 72 patients were included. *B,* the samples were divided by the median VEGFC level in a group with high (above the median) versus low (below the median) VEGFC levels mRNA expression levels. *P, P* value of the Mann-Whitney *U* test; *boxes,* time to CR showing the 5%, 25%, 50%, 75%, and 95% cumulative relative frequencies.

Here, we took a step further by showing that high endogenously expressed VEGFC levels of primary AML cells are related to increased *in vitro* resistance for six drugs. It is noteworthy that, as one might expect, endogenous VEGFC levels correlated with *in vitro* resistance for typical AML drugs and not for acute lymphoblastic leukemia drugs, such as vincristine and L-asparaginase.

The measurement of drug resistance in cell cultures shows the ability of AML cells to be rescued from cell death induced by cytotoxic drugs (37). In 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assays, it is likely that AML cells are not growing, so the conditions may not truly represent what is going on in the patients when exposed to drugs. Therefore, we used in this study not only *in vitro* variables but also *in vivo* variables. Given the heterogeneity of the treatment protocols, the relation between VEGFC and long-term clinical outcome was not studied. Moreover, in our view, end points as overall survival and/or DFS/EFS are not the optimal variables to study the kinetics of *in vivo* reduction of the bulk of AML blasts upon drug exposure. As a first *in vivo* variable for drug responsiveness blast counts on day 15 in the bone were used. Blast counts on day 15 (≤5%/>5%) reflect the disappearance of AML blasts upon drug exposure.

A second *in vivo* equivalent for *in vitro* drug resistance is the time to reach CR. There are several potential flaws in the time to CR analysis. Blood counts were not measured daily in all patients, and bone marrow punctures were not always done on the first date when blood counts indicated a CR. The date on which CR was established, therefore, was not necessarily the date on which CR occurred. A longer time to CR might also reflect a reduced number or poorer quality of residual normal stem cells in view of the requirement of regenerated normal cells for CR. However, this seems highly unlikely in a pediatric population after only one or two courses of chemotherapy. Moreover, until now, high VEGFC levels have not been linked to delayed recovery of normal hematopoietic cells. In contrast, some studies support the idea that outgrowth of normal hematopoietic stem cells might be VEGF dependent (38).

In the present study, we show the relation of VEGFC and blast disappearance during induction *in vivo* by showing that endogenous VEGFC is independently related to higher blast counts on day 15 in the bone marrow and a longer time to reach CR.

FLT4 and VEGFC are known as factors promoting lymphangiogenesis and metastasis in a mouse solid tumor model (39). Beside direct effects of VEGFC on AML cells (described in the present study and in the study of Dias et al.), it is unclear whether additional effects of VEGFC on the neovasculature (e.g., on endothelial cells) play a role in AML malignant progression. Our results might be related to the observation that angiopoietin 2, another important angiogenic factor, was found to be an independent prognostic factor in AML (36). In more detail, AML patients with high angiopoietin 2 and low VEGFC levels had a good long-term prognosis. In contrast, the prognosis of patients with high VEGFC levels was much less influenced by angiopoietin 2.

In conclusion, the present report shows for the first time that increased endogenous VEGFC mRNA levels of pediatric AML cells are related to a slower disappearance of AML blasts *in vitro* and *in vivo*. The results presented here suggest that further studies exploring the exact mechanism of VEGFC induced drug responsiveness and the role of anti-VEGFC therapy in the treatment of AML patients are desirable.

References

1. Burnett AK, Goldstone AH, Stevens RM, et al.; UK Medical Research Council Adult and Children's Leukaemia Working Parties. Randomised comparison of addition of autologous bone-marrow transplantation to intensive chemotherapy for acute myeloid leukaemia in first remission: results of MRC AML 10 trial. Lancet 1998;351:700–8.
2. Creutzig U, Ritter J, Zimmermann M, et al. Improved treatment results in high-risk pediatric acute myeloid leukemia patients after intensification with high-dose cytarabine and mitoxantrone: results of Study Acute Myeloid Leukemia-Berlin-Frankfurt-Munster 93. J Clin Oncol 2001;19:2705–13.
3. Kaspers GJ, Creutzig U. Pediatric acute myeloid leukemia: international progress and future directions. Leukemia 2005;19:2025–9.
4. Stevens RF, Hann IM, Wheatley K, Gray RG; MRC Childhood Leukaemia Working Party. Marked improvements in outcome with chemotherapy alone in paediatric acute myeloid leukemia: results of the United Kingdom Medical Research Council's 10th AML trial. Br J Haematol 1998;101:130–40.
5. Petrova TV, Makinen T, Alitalo K. Signaling via vascular endothelial growth factor receptors. Exp Cell Res 1999;253:117–30.
6. Veikkola T, Karkkainen M, Claesson-Welsh L, Alitalo K. Regulation of angiogenesis via vascular endothelial growth factor receptors. Cancer Res 2000;60:203–12.
7. Clauss M, Molecular biology of the VEGF and the VEGF receptor family. Semin Thromb Hemost 2000;26:561–9.
8. Suto K, Yamazaki Y, Morita T, Mizuno H. Crystal structures of novel vascular endothelial growth factors (VEGF) from snake venoms: insight into selective VEGF binding to kinase insert domain-containing receptor but not to fms-like tyrosine kinase-1. J Biol Chem 2005;280:2126–31.
9. Bellamy WT, Richter L, Frutiger Y, Grogan TM. Expression of vascular endothelial growth factor and its receptors in hematopoietic malignancies. Cancer Res 1999;59:728–33.
10. Fielder W, Graeven U, Ergun S, et al. Expression of FLT4 and its ligand VEGF-C in acute myeloid leukemia. Leukemia 1997;11:1234–7.
11. de Vries C, Escobedo JA, Ueno H, Houck K, Ferrara N, Williams LT. The fms-like tyrosine kinase, a receptor for vascular endothelial growth factor. Science 1992;255:989–91.
12. Dougher AM, Wasserstrom H, Torley L, et al. Identification of a heparin binding peptide on the extracellular domain of the KDR VEGF receptor. Growth Factors 1997;14:257–68.
13. Igarashi K, Isohara T, Kato T, et al. 8-(3-oxo-4,5,6-trihydroxy-3h-xanthen-9-yl)-1-naphthoic acid inhibits MAPK phosphorylation in endothelial cells induced by VEGF and bFGF. Int J Mol Med 1998;2:211–5.
14. Karsan A, Yee E, Poirier GG, Zhou P, Craig R, Harlan JM. Fibroblast growth factor-2 inhibits endothelial cell apoptosis by Bcl-2-dependent and independent mechanisms. Am J Pathol 1997;151:1775–84.
15. Santos SC, Dias S. Internal and external autocrine VEGF/KDR loops regulate survival of subsets of acute leukemia through distinct signaling pathways. Blood 2004;103:3883–9.
16. Xia P, Aiello LP, Ishii H, et al. Characterization of vascular endothelial growth factor's effect on the activation of protein kinase C, its isoforms, and endothelial cell growth. J Clin Invest 1996;98:2018–26.
17. Cao Y, Linden P, Farnebo J, et al. Vascular endothelial growth factor C induces angiogenesis *in vivo*. Proc Natl Acad Sci U S A 1998;95:14389–94.
18. Joukov V, Pajusola K, Kaipainen A, et al. A novel vascular endothelial growth factor, VEGF-C, is a ligand for the Flt4 (VEGFR-3) and KDR (VEGFR-2) receptor tyrosine kinases. EMBO J 1996;15:290–8.
19. Dias S, Choy M, Alitalo K, Rafii S. Vascular endothelial growth factor (VEGF)-C signaling through FLT-4 (VEGFR-3) mediates leukemic cell proliferation, survival, and resistance to chemotherapy. Blood 2002;99:2179–84.
20. Creutzig U, Zimmermann M, Ritter J, et al. Definition of a standard-risk group in children with AML. Br J Haematol 1999;104:630–9.
21. Kern W, Haferlach T, Schoch C, et al. Early blast clearance by remission induction therapy is a major independent prognostic factor for both achievement of complete remission and long-term outcome in acute myeloid leukemia: data from the German AML Cooperative Group (AMLCG) 1992 Trial. Blood 2003;101:64–70.
22. Bennett JM, Catovsky D, Daniel MT, et al. Proposed revised criteria for the classification of acute myeloid leukemia. A report of the French-American-British Cooperative Group. Ann Intern Med 1985;103:620–5.
23. Byrd JC, Mrozek K, Dodge RK, et al. Pretreatment cytogenetic abnormalities are predictive of induction success, cumulative incidence of relapse, and overall survival in adult patients with *de novo* acute myeloid leukemia: results from Cancer and Leukemia Group B (CALGB 8461). Blood 2002;100:4325–36.
24. Grimwade D, Walker H, Oliver F, et al.; The Medical Research Council Adult and Children's Leukaemia Working Parties. The importance of diagnostic cytogenetics on outcome in AML: analysis of 1,612

patients entered into the MRC AML 10 trial. Blood 1998;92:2322–33.

25. Lowenberg B, Downing JR, Burnett A. Acute myeloid leukemia. N Engl J Med 1999;341:1051–62.

26. Rubnitz JE, Raimondi SC, Tong X, et al. Favorable impact of the t(9;11) in childhood acute myeloid leukemia. J Clin Oncol 2002;20:2302–9.

27. de Bont ES, Fidler V, Meeuwsen T, Scherpen F, Hahlen K, Kamps WA. Vascular endothelial growth factor secretion is an independent prognostic factor for relapse-free survival in pediatric acute myeloid leukemia patients. Clin Cancer Res 2002;8:2856–61.

28. Kaspers GJ, Veerman AJ, Pieters R, et al. Mononuclear cells contaminating acute lymphoblastic leukaemic samples tested for cellular drug resistance using the methyl-thiazol-tetrazolium assay. Br J Cancer 1994;70:1047–52.

29. Pieters R, Loonen AH, Huismans DR, et al. *In vitro* drug sensitivity of cells from children with leukemia using the MTT assay with improved culture conditions. Blood 1990;76:2327–36.

30. Klumper E, Pieters R, Kaspers GJ, et al. *In vitro* chemosensitivity assessed with the MTT assay in childhood acute non-lymphoblastic leukemia. Leukemia 1995;9:1864–9.

31. Pieters R, Huismans DR, Leyva A, Veerman AJ. Comparison of the rapid automated MTT-assay with a dye exclusion assay for chemosensitivity testing in childhood leukaemia. Br J Cancer 1989;59:217–20.

32. Cheson BD, Cassileth PA, Head DR, et al. Report of the National Cancer Institute-sponsored workshop on definitions of diagnosis and response in acute myeloid leukemia. J Clin Oncol 1990;8:813–9.

33. Hubeek I, Peters GJ, Broekhuizen R, et al. *In vitro* sensitivity and cross-resistance to deoxynucleoside analogs in childhood acute leukemia. Haematologica 2006;91:17–23.

34. Zwaan CM, Kaspers GJ, Pieters R, et al. Cellular drug resistance in childhood acute myeloid leukemia is related to chromosomal abnormalities. Blood 2002;100:3352–60.

35. de Bont ES, Rosati S, Jacobs S, Kamps WA, Vellenga E. Increased bone marrow vascularization in patients with acute myeloid leukaemia: a possible role for vascular endothelial growth factor. Br J Haematol 2001;113:296–304.

36. Loges S, Heil G, Bruweleit M, et al. Analysis of concerted expression of angiogenic growth factors in acute myeloid leukemia: expression of angiopoietin-2 represents an independent prognostic factor for overall survival. J Clin Oncol 2005;23:1109–17.

37. Kaspers GJ, Veerman AJ. Clinical significance of cellular drug resistance in childhood leukemia. Recent Results Cancer Res 2003;161:196–220.

38. Martin R, Lahlil R, Damert A, et al. SCL initiates with VEGF to suppress apoptosis at the onset of hematopoiesis. Development 2004;131:693–702.

39. Skobe M, Hawighorst T, Jackson DG, et al. Induction of tumor lymphangiogenesis by VEGF-C promotes breast cancer metastasis. Nat Med 2001;7:192–8.

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