Vascular Endothelial Growth Factor in Newly Diagnosed and Recurrent Childhood Acute Lymphoblastic Leukemia as Measured by Real-Time Quantitative Polymerase Chain Reaction

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ABSTRACT

Purpose: Overexpression of vascular endothelial growth factor (VEGF) is associated with increased angiogenesis, growth, and metastasis in solid tumors, but to date the significance of VEGF in leukemia has received only limited attention. Therefore, this study examined the cellular VEGF levels in 31 newly diagnosed and 22 recurrent cases of childhood acute lymphoblastic leukemia (ALL).

Experimental Design: VEGF was determined with real-time quantitative PCR methods. Kaplan-Meier statistical analyses were conducted for the relapse-free intervals and the overall survival times. The groups were compared by log-rank and rank-sum tests.

Results: The VEGF levels were significantly higher in recurrent ALL compared with newly diagnosed ALL (28.0 versus 3.1 units; \( P = 0.001 \)). Kaplan-Meier estimates were conducted to analyze the prognostic value of VEGF levels in newly diagnosed ALL with regard to the relapse-free intervals and the overall survival times. In this analysis, the median relapse-free interval of patients with low VEGF levels was more than 10 years, whereas the relapse-free interval of patients with high VEGF expression was only 1.2 years. The median overall survival time for the collective of patients with low VEGF levels was >10 years, whereas the survival of the group of patients with high VEGF levels was 3.9 years. This difference was not statistically significant. This may be attributable to the small number of patients involved.

Conclusion: Our data suggest that VEGF may play an important role in the pathophysiology of ALL. The expression of VEGF raises the possibility of using angiogenesis inhibitors as a novel therapeutic strategy in childhood ALL.

INTRODUCTION

VEGF,² also called vascular permeability factor, is a multifunctional protein that is active in endothelial cell growth and in increasing blood vessel permeability (1). VEGF plays an important role in organ development, wound healing, and tissue regeneration as well as in abnormal events such as neoplastic proliferation, rheumatoid arthritis, and retinopathies (2). Overexpression of VEGF is associated with increased angiogenesis, growth, and metastasis in solid tumors (3–5). VEGF is also secreted by a variety of tumor cell lines, including hematopoietic cells (1). Until now, the significance of VEGF in leukemia has received only limited attention despite the fact that VEGF was originally cloned from the leukemic cell line HL60 (6). Fiedler et al. (7) investigated the expression of VEGF in fresh leukemic blasts with PCR and found a specific transcript in 20 of 28 patients with newly diagnosed AML and in 3 of 5 patients with secondary AML. Aguayo et al. (8) used a RIA to evaluate VEGF levels and their prognostic significance in 99 newly diagnosed cases of AML and found that a relationship existed between increasing VEGF levels and shorter survival times as well as shorter disease-free survival times.

In the present study, we use standard RT-PCR and the real-time quantitative PCR methods to examine the cellular VEGF levels in newly diagnosed (de novo) and recurrent childhood ALL.

MATERIALS AND METHODS

Patients. Thirty-one children (13 boys and 18 girls) with initial ALL were analyzed for the expression of VEGF. The median age of the patients was 6 years (range, 1–15 years). Sixteen patients had initial peripheral blast cell counts >50 × 10⁹/liter and 15 patients had <50 × 10⁹/liter. The diagnosis of leukemia was made according to the French-American-British classification (Ref. 9; L1 = 23 cases; L2 = 8 cases). According to their immunological subtypes, the patients were grouped as precursor B-ALL (n = 4 cases), common ALL (n = 14 cases), and T-ALL (n = 10 cases; 3 patients were not available for immunophenotyping). In addition, 22 patients with relapsed ALL were investigated.

Leukemic blast cells were isolated from the bone marrow before chemotherapy. Cell samples were collected in heparinized flasks, and mononuclear cells were isolated by Ficoll-Hypaque density gradient centrifugation. After separation, the

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²The abbreviations used are: VEGF, vascular endothelial growth factor; AML, acute myeloid leukemia; ALL, acute lymphoblastic leukemia; RT-PCR, reverse transcription-PCR.
samples contained >95% blast cells. The cells were cryopreserved in liquid nitrogen with 10% DMSO and 5% FCS in a programmable freezer.

Treatment of ALL. All patients with newly diagnosed ALL were treated according to the two modified BFM protocols, ALL-VII/81 and ALL-VIII/87 (10). The treatment protocols consist of an induction therapy with prednisone, vincristine, daunorubicin, and l-asparaginase followed by a consolidation therapy with cyclophosphamide, cytarabine, 6-mercaptopurine, and methotrexate.

RT-PCR. Total RNA was extracted using the single-step, guanidinium thiocyanate-phenol/chloroform procedure (11). The quality of each total RNA sample was checked and controlled by measuring the absorbance. The samples were examined by gel electrophoresis to ensure that the RNA was not degraded and evaluated according to the integrity and intensity of the 18S rRNA signals. Reverse transcription to generate cDNA was performed only with samples without RNA degradation. Finally, we conducted a semiquantitative RT-PCR for the 18S rRNA at low cycle numbers. Reverse transcription was conducted as follows: 50 ng of random hexamers were added to 2 μg of RNA, which then underwent reverse transcription according the instructions of the Superscript Preamplification System (Life Technologies, Inc., Eggenstein, Germany). A 2-μl aliquot of the first strand of cDNA was then amplified by PCR. PCR was performed with the Redtaq PCR-Reaction Mix (Sigma Chemical Co.-Aldrich, Munich, Germany) according to a protocol provided by the manufacturer, with 10 pmol of primer per each reaction. Specific human primers were used to detect VEGF-165 (541 bp): forward, 5'-GAA GTG GTG AAG TTC ATG CTT TCC-3'; reverse, 5'-CGA TCG TTC TGT ATC AGT GAT GTC-3'; housekeeping gene (611 bp): forward, 5'-GAA GTG GTG AAG TTC ATG CTT TCC-3'; reverse, 5'-CGA TCG TTC TGT ATC AGT GAT GTC-3' (1). PCR conditions were 95°C for 5 min followed by amplification at 95°C for 30 s, 58°C for 30 s, and 72°C for 30 s (35 cycles). Amplification of the 18S RNA as a "housekeeping gene" was conducted in parallel with VEGF.

Real-Time Quantitative PCR. Real-time quantitation was based on the LightCycler assay, using a fluorogenic SYBR Green I reaction mixture for PCR with the LightCycler Instrument (Roche, Mannheim, Germany). The PCR reactions were carried out in a total volume of 20 μl, which included SYBR Green I with a Taq DNA polymerase reaction buffer, deoxynucleotide triphosphate mixture, 10 mM MgCl2, PCR-grade water, 2 μl of template DNA, and primers. To generate the standard curve, we used a standard control kit DNA, which included serial dilutions of the human DNA 110-bp fragment from the β-globulin gene (LightCycler Control Kit DNA; Roche), and serial dilutions of the positive controls for VEGF in the cell line. All dilutions varied from 10^2 to 10^7 copies of the target molecule/μl. The reaction conditions were as follows: preincubation at 95°C for 5 min, followed by 45 cycles (amplification) of 95°C for 15 s, 55°C for 5 s, and 72°C for 10 s. All experiments were conducted three times, and negative and positive controls were included. As a negative control, the template DNA was replaced with PCR-grade water. Fluorescence emission spectra were monitored and analyzed. PCR products were measured by the threshold cycles (C_T), at which specific fluorescence became detectable. The C_T was used for kinetic analysis and was proportional to the initial number of target copies in the sample. A melting curve analysis was performed to assess the specificity of the amplified PCR products. The quantity of the samples was calculated after the C_T of the serial dilutions were compared with a control.

Statistical Analysis. Kaplan-Meier life table analyses were conducted for the relapse-free intervals and for the overall survival times. The groups were compared by log-rank tests and rank-sum tests. Differences in the groups were evaluated by the Wilcoxon rank-sum test. P < 0.05 was considered significant.

RESULTS
Leukemic cells from 31 children with newly diagnosed, untreated ALL and from 22 children with treated and recurrent ALL were analyzed for the expression of VEGF by standard
PCR and real-time quantitative PCR. In most cases, the newly diagnosed and recurrent cases of ALL came from different patients.

A typical standard PCR experiment and a typical real-time PCR experiment are represented in Figs. 1 and 2, respectively. Both methods exhibit obvious differences in the expression of VEGF for the various ALL samples. In the following, we used the data obtained by real-time quantitative PCR.

The median value of VEGF was 3.1 units for the newly diagnosed cases of ALL and 28.0 units for the recurrent cases of ALL. VEGF levels were significantly higher in recurrent ALL (P < 0.001, Wilcoxon test; Fig. 3). The variability within the groups was quite large. The newly diagnosed and recurrent ALL cells originated from the same patient in only five cases; the data for these five patients as measured by real-time PCR are presented in Table 1. In all five cases, the VEGF levels were higher in recurrent ALL than in newly diagnosed ALL.

Kaplan-Meier estimates were conducted to analyze the prognostic value of the VEGF levels for both the relapse-free intervals and the overall survival times of children with initial ALL. In this analysis, we determined that children with newly diagnosed ALL and with higher VEGF levels tended to have a poorer prognosis (Fig. 4). The median relapse-free interval of the group with low VEGF expression was >10 years, whereas the relapse-free interval of the group with high VEGF levels was 1.2 years (Fig. 4). The relative risk for the group with high VEGF expression was 1.3 compared with the group with low VEGF expression. The overall survival time was also shorter for patients with high-VEGF leukemic cells (Fig. 5). The median overall survival time for the collective with low VEGF levels was >10 years, whereas the survival time of the group of patients with high VEGF levels was 3.9 years. The relative risk for the group with high VEGF levels was 1.9 compared with the group with low VEGF levels. This result could be confirmed by standard PCR and Northern blot hybridization analysis (data not shown).

In the present study, no significant relationship was detected between VEGF levels and age, sex, French-American-British type, immunological subtype, and blast cell counts (Fig. 6).

Table 1  VEGF levels in newly diagnosed and recurrent ALL as determined by real-time quantitative PCR

<table>
<thead>
<tr>
<th>Patient</th>
<th>Newly diagnosed ALL</th>
<th>Recurrent ALL</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>15.9</td>
<td>19.8</td>
</tr>
<tr>
<td>2</td>
<td>6.7</td>
<td>8.4</td>
</tr>
<tr>
<td>3</td>
<td>10.0</td>
<td>34.9</td>
</tr>
<tr>
<td>4</td>
<td>10.5</td>
<td>20.2</td>
</tr>
<tr>
<td>5</td>
<td>5.6</td>
<td>17.6</td>
</tr>
</tbody>
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*P = 0.02 (Wilcoxon test).

DISCUSSION

During the past few years, it has been proposed that angiogenesis may play a role not only in solid tumors, but also in
hematopoietic malignancies. Previous studies of hematopoietic malignancies have demonstrated a close relationship between the bone marrow and angiogenesis (12, 13). In the present study, we detected VEGF expression in human ALL cases and showed that VEGF levels were significantly higher in recurrent ALL than in newly diagnosed ALL. Additionally, we found that VEGF is a prognostic indicator for the survival of children. However, this difference did not reach statistical significance. This may have been attributable to the small number of patients evaluated. Nevertheless, these results were confirmed by both standard PCR and Northern blot hybridization, which showed a similar tendency (data not shown).

The prognostic significance of neovascularization has been demonstrated for solid tumors, but only a few reports are available on hematopoietic malignancies. Peres-Atayde et al. (12) found that microvessel density in the bone marrow of children with ALL was significantly higher than in controls. These findings suggested that leukemia cells induce angiogenesis in the bone marrow and that leukemia could be dependent on angiogenesis. Several studies have indicated that VEGF production exhibits a relationship to tumor proliferation and apoptosis and support a possible role for VEGF in the prognosis of leukemia. Fiedler et al. (7) detected VEGF-specific transcripts in 20 of 28 patients with newly diagnosed AML and in 3 of 5 patients with secondary AML.

Bellamy et al. (1) examined the expression of mRNA and protein for VEGF in 12 human hematopoietic tumor cell lines and discovered that VEGF was expressed in these cell lines. Similar results were obtained by Hussong et al. (13). Aguayo et al. (8) found a relationship between increasing VEGF levels in AML and shorter survival times. Fiedler et al. (7) demonstrated that AML cells express VEGF as well as VEGF receptors. This result suggests that VEGF may assume the role of an autocrine growth factor for leukemic cells. Katoh et al. (14) reported that VEGF inhibits apoptotic cell death induced by exposure to the chemotherapeutic drugs etoposide and doxorubicin. Furthermore, they were able to show that the expression level of MCL1, a member of the BCL2 family, was increased by VEGF. This agrees with our earlier findings. We determined that children with newly diagnosed ALL and Fas (Apo 1) ligand expression, which functions as a rapid inducer of cell death, exhibit longer relapse-free and overall survival times than patients who do not express the Fas ligand (15).

Our data and the findings of other authors suggest that VEGF may play an important role in the pathophysiology of hematopoietic malignancies and the progression of leukemia. Furthermore, our data show that VEGF may have clinical relevance and raise the possibility of using angiogenesis inhibitors as a novel therapeutic strategy in childhood ALL.

ACKNOWLEDGMENTS

We thank Dr. M. Hollstein and A. Wittmann for helpful assistance.

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