Vascular endothelial growth factor (VEGF) is a multifunctional, secreted cytokine that stimulates endothelial cells to proliferate, migrate, and to increase their permeability to plasma proteins, as has recently been reviewed. VEGF has been purified from conditioned media and has been molecularly cloned. It shows sequence homology to the A and B chain of PDGF and occurs naturally as a homodimer. Because of differential splicing, various isoforms exist. The most abundant have 121, 165, or 189 amino acids, respectively. VEGF expression is physiologically induced by hypoxia and plays a role in inflammatory diseases such as rheumatoid arthritis, wound healing, and diabetic retinopathy. Furthermore, VEGF is secreted by a variety of malignant tumors, including Kaposi's sarcoma, melanoma, glioma, and renal cell cancer, contributing to neoangiogenesis. Activation of oncogenes such as H-Ras, K-Ras, and Raf or overexpression of the Src oncogene may result in upregulation of VEGF secretion. On the other hand, wild-type p53 may suppress VEGF expression and angiogenesis.

Two receptors for VEGF have been identified, FMS-like tyrosine kinase-1 (FLT1) and fetal liver kinase-1 (FLK-1/KDR). Both belong to the class III of receptor tyrosine kinase (RTK). They show sequence homology to each other and to other members of this class of RTK such as FMS and KIT. During hypoxia, at least one of the receptors, KDR, is upregulated.

Because VEGF was originally cloned from a human leukemic cell line HL60, we investigated spontaneous expression of VEGF and its receptor by leukemic blasts from patients with acute myeloid leukemia (AML). Furthermore, we studied paracrine provision of granulocyte-macrophage colony-stimulating factor (GM-CSF) by endothelial cells after stimulation with VEGF.

MATERIALS AND METHODS

Isolation of peripheral blood or bone marrow mononuclear cells. Peripheral blood or bone marrow samples from 28 consecutive patients with newly diagnosed or relapsed AML were obtained before chemotherapy. Mononuclear cells were separated by density gradient centrifugation on Ficoll-Hypaque (Pharmacia, Uppsala, Sweden). These preparations contained at least 90% myeloblasts as judged by morphologic criteria on Papenheim smears. A control group of 9 healthy volunteer bone marrow donors was included.

Preparation of CD34+ cells. Mononuclear cells from about 30 mL of bone marrow from normal human volunteers were recovered after a Ficoll-Hypaque gradient, washed twice, and counted. To select for CD34+ cells, about 106 mononuclear cells were applied to the Mini Macs column (Miltenyi Biotech, Bergisch Gladbach, Germany) according to the directions of the supplier. Recovered cells were evaluated for purity by fluorescence-activated cell sorter (FACS) analysis using a CD34 monoclonal antibody that recognizes an epitope different from the one used for the Mini Macs column (Anti HPCA-2; Becton Dickinson, San Jose, CA). The purity of obtained cells was at least 93% (not shown).

Preparation of c-DNA and reverse transcription-polymerase chain reaction (RT-PCR). Mononuclear cells were washed twice in phosphate-buffered saline (PBS) and collected by centrifugation. Total cellular RNA was prepared using Quagen minicolumns (Quagen, Hilden, Germany) as described by the manufacturer. One microgram of RNA was used for c-DNA synthesis, employing avian myeloblastosis virus (AMV) reverse transcriptase and oligo dT as primer.

Different aliquots of c-DNA were amplified with specific primers for VEGF, KDR, FLT1, and actin as control for successful c-DNA synthesis. For KDR and FLT1, two rounds were performed and for
VEGF and actin one round was performed of 35 cycles of PCR in a programmable heat block at 94°C for 1.5 minutes, at 60°C for 3 minutes, and at 72°C for 4 minutes. PCR products were separated on 1% agarose gels, stained with ethidium bromide, and visualized under UV light. Primer sequences were as follows: VEGF sense primer, 5'-TCAAAAATTGTTTCTGGG-3'; FLT1 outer sense primer, 5'-ATTTGTGATTTTGGCCTTGC-3'; FLT1a outer antisense primer, 5'-CAGGCTCATGAACCTTTAGC-3'; KDR outer antisense primer, 5'-GTCAAGGGAAGACTACGTTGG-3'; KDR inner antisense primer, 5'-CAGCTTCAAGTGGCTAAGG-3'; KDR inner sense primer, 5'-CCTGGTGAGAGATCTGGTTG-3'; KDR outer antisense primer, 5'-TCAAAAATTGTTTCTGGG-3'; FLT1 outer antisense primer, 5'-CTGCTGAGAGATCTGGTTG-3'; FLT1 inner sense primer, 5'-CAACAGAGCGAGCCTG-3'; and actin antisense primer, 5'-GTCAAGGGAAGACTACGTTGG-3'. The VEGF primers correspond to sequences in the untranslated region resulting in amplification of four different splice variants of a size of 516, 648, 720, and 771 bp. Primers specific for KDR, FLT1, and actin recognize coding sequences. PCR product sizes are, for KDR outer primer pair, 591 bp; for KDR inner primer pair, 213 bp; for FLT1 outer primer pair, 555 bp; for FLT1 inner primer pair, 196 bp; and for actin, 619 bp, respectively. To avoid cross-contamination, the set up of PCR reactions and gel electrophoresis was performed in different rooms using different sets of pipettes. Appropriate control reactions remained always negative. For each primer pair, PCR products were subcloned using the TA cloning kit (Invitrogen, San Diego, CA). Single bacterial colonies were picked and cultured overnight. Bacteria were diluted in destilled water and boiled for 10 minutes. PCR reactions were performed with primers recognizing sites flanking the PCR product (Dynal A and B; Dynal, Hamburg, Germany). Because one of the primers is biotinylated, strand separation was performed using streptavidin-coated Dynabeads (Dynal). DNA sequencing was performed with the T7 cycle sequencing kit (Applied Biosystems, Foster City, CA) and the automatic DNA sequencer 373A (Applied Biosystems). The identified DNA sequences corresponded to the published ones for all primer pairs.

**Immunocytochemistry.** Cells were transferred from culture into the chamber slides, where they were washed twice with PBS for 10 minutes. They were then fixed for 15 minutes with fresh paraformaldehyde (4%) at room temperature. Afterwards, they were washed in...
Fig 2. (Left side) Immunocytochemistry with a polyclonal antibody against VEGF. (Right side) Control reactions with the VEGF antibody after preabsorption with specific peptide. (Upper panel) Cell line U 937. (Middle panel) Cell line TF-1. (Lower panel) Cells from a representative patient with AML. VEGF expression in all three cases. (Original magnification × 100.)
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RESULTS

VEGF expression was studied by PCR analysis in leukemic blasts of 28 patients with de novo AML at diagnosis or relapse. Five additional patients with secondary AML were analyzed. Seventy-one percent of patients with de novo AML and 60% of patients with secondary AML were found to express VEGF transcripts. A representative example of a RT-PCR analysis is shown in Fig. 1. Table 1 gives the rates of VEGF expression according to French-American-British (FAB) type. In normal bone marrow, VEGF m-RNA was detected in 2 of 7 volunteer donors, possibly due to expression in macrophages.

The cell lines U 937 and TF-1 and leukemic blasts from 8 patients were investigated for VEGF protein expression with immunocytochemistry using a modified APAP method. In concordance with the PCR analysis, U937 and TF-1 cells were positive for VEGF protein expression. In Fig 2, photomicrographs of immunocytochemistry of cell lines and representative AML patients are shown. A good correlation existed between PCR and immunocytochemistry results.

To obtain quantitative data on VEGF protein expression, we performed a VEGF ELISA of cell culture supernatants. One million fresh leukemic cells, normal bone marrow cells, or CD34+ cells per milliliter were cultured for 3 days. Supernatants were harvested and kept at −70°C until further use.

Preparation of supernatants from leukemic cells, normal bone marrow, and CD34+ cells. Mononuclear cells from 24 AML patients and from 9 normal bone marrow donors and CD34+ cells from 3 normal bone marrow donors were incubated at a density of 1 million cells per milliliter in RPMI 1640 medium with 10% fetal calf serum (FCS). After 3 days, supernatants were removed and stored at −70°C until further use.

Preparation and stimulation of human umbilical vein endothelial cells (HUVEC) with VEGF. HUVEC were isolated from umbilical cords as described. Cells were grown to confluency on fibronectin (GIBCO, Eggenstein, Germany) -coated plasticware in medium 199 (GIBCO) supplemented with 20% FCS and endothelial cell growth supplement (EGCS; Sigma) as indicated by the supplier. 50 µg/mL heparin, and glutamine. Cells were starved for 24 hours in medium 199 supplemented with 5% FCS. HUVEC were stimulated with VEGF (R&D Systems, Abingdon, UK) at a concentration of 0, 2, 10, or 50 ng/mL in starvation medium and incubated for 24.48, or 72 hours. Supernatants were removed and kept at −70°C until further use. HUVEC were trypsinized and c-DNA was prepared as indicated above.

VEGF and GM-CSF enzyme-linked immunosorbent assay (ELISA). VEGF and GM-CSF ELISA kits were purchased from R&D Systems. VEGF and GM-CSF determinations were performed as indicated by the supplier. All samples were run in duplicate.

Statistical analysis. The nonparametric, signed rank Wilcoxon test was used for statistical analysis. The level of significance was set to 5%.

Fig 3. VEGF concentrations in supernatants from fresh leukemic cells of 24 individual AML patients (first column), of bone marrow cells from 9 normal donors (second column), and of CD34+ cells from bone marrow from 3 donors (third column) after 3 days of culture determined by ELISA. Median concentrations are indicated by a horizontal line.
endothelial cells with and without VEGF stimulation were also performed. Induction of GM-CSF mRNA was found, indicating that GM-CSF induction occurred mainly on the transcriptional level.

**DISCUSSION**

VEGF expression of fresh leukemic blasts from patients with AML was studied on the transcriptional and protein level using PCR analysis and immunocytochemistry. Twenty-three of 32 patients with de novo or secondary AML expressed VEGF transcripts. Results of immunocytochemistry performed in 8 patients and two cell lines were compatible with the PCR analysis. Using the ELISA technique, VEGF concentrations of supernatants of fresh leukemic blasts of 24 AML patients were significantly higher than VEGF levels of supernatants of low-density bone marrow cells of 9 normal donors or of CD34-enriched cells of 3 additional volunteers.

Because leukemic blasts of about 50% of the AML patients express FLT1 receptors and 20% express KDR receptors, autocrine effects of VEGF on leukemic blasts are possible. We demonstrated binding of biotinylated VEGF to CD34+ TF-1 cells using a double-labeling FACS technique (data not shown). This indicates that VEGF receptors on leukemic cells are functional. VEGF has been evaluated for its capacity to induce colony formation on normal hematopoietic progenitor cells. It has been shown that VEGF enhances colony formation of more mature progenitor cells in concert with colony-stimulating factors, but suppresses growth of immature progenitors that depend on Steel factor or FLT3 ligand.24 We did not find colony formation of leukemic blasts of 3 AML patients with VEGF alone. The addition of VEGF to submaximal concentrations of granulocyte colony-stimulating factor and GM-CSF reduced leukemic colony formation slightly (own unpublished observation). But VEGF may exert effects on leukemic cells that do not result in proliferation. It has been shown that VEGF reduces apoptotic cell death of normal hematopoietic stem cells and CMK86 cells after ionizing radiation.25

Endothelial cells are found in the stroma layer of long-term bone marrow cultures in close contact to hematopoietic cells. Porcine brain microvascular endothelial cells have been shown to support expansion of human progenitor cells in concert with hematopoietic growth factors.26 In vivo, increased amounts of von Willebrand factor-positive endothelial cells have been detected in histologic sections of bone marrow biopsies from patients with AML.27 In multiple myeloma, increased microvessel density in bone marrow biopsies was significantly correlated with labeling index and prognosis.28 Therefore, paracrine exchange of growth factors may contribute to growth stimulation of AML cells. Because we could establish a dose-response curve between VEGF concentration in the culture medium and secretion of GM-CSF by HUVEC, such a paracrine loop may exist between AML cells and bone marrow endothelial cells. Similar paracrine mechanisms have been described for interleukin-1 and tumor necrosis factor-α secreted by leukemic blasts.29,30 Work in our laboratory is in progress to isolate human bone marrow endothelial cells and to study paracrine growth mechanisms between these cells and normal and leukemic hematopoietic cells.

Paracrine growth stimulation may not only be restricted to the bone marrow microenvironment, but may also take place at extramedullary sites. Circulating AML blasts may profit from paracrine provision of growth factors in various capillary beds. This may result in their expansion in peripheral blood. Under favorable conditions, extramedullary manifestations of AML, such as chloromas, gingiva hyperplasia, or organ infiltration, may be initiated by this mechanism.

**REFERENCES**

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Vascular Endothelial Growth Factor, a Possible Paracrine Growth Factor in Human Acute Myeloid Leukemia

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