

*Featured Article*

# Targeting Vascular Endothelial Growth Factor for Relapsed and Refractory Adult Acute Myelogenous Leukemias: Therapy with Sequential 1- $\beta$ -D-Arabinofuranosylcytosine, Mitoxantrone, and Bevacizumab

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**ABSTRACT**

**Purpose:** Vascular endothelial growth factor (VEGF) promotes acute myelogenous leukemia (AML) cell growth and survival and may contribute to drug resistance. bevacizumab, an anti-VEGF monoclonal antibody, exhibits clinical activity against diverse malignancies when administered with cytotoxic chemotherapy. We conducted a Phase II clinical trial of bevacizumab administered after chemotherapy to adults with refractory or relapsed AML, using a timed sequential therapy (TST) approach.

**Experimental Design:** bevacizumab 10 mg/kg was administered on day 8 after 1- $\beta$ -D-arabinofuranosylcytosine 2 g/m<sup>2</sup>/72 h beginning day 1 and mitoxantrone 40 mg/m<sup>2</sup> beginning day 4. *In vivo* laboratory correlates included AML cell VEGF receptor-1 (FLT-1) expression, marrow microvessel density, and free serum VEGF before and during TST with bevacizumab.

**Results:** Forty-eight adults received induction therapy. Myelosuppression occurred in all of the patients similar to other TST regimens. Toxicities were decreased ejection fraction (6%), cerebrovascular bleed (4%), and mortality of 15%. Overall response was 23 of 48 (48%), with complete response (CR) in 16 (33%). Eighteen (14 CR and 4 partial

response) underwent one consolidation cycle and 5 (3 CR and 2 partial response) underwent allogeneic transplant. Median overall and disease-free survivals for CR patients were 16.2 months (64%, 1 year) and 7 months (35%, 1 year). Marrow blasts demonstrated FLT-1 staining before bevacizumab and marked decrease in microvessel density after bevacizumab. VEGF was detected in pretreatment serum in 67% of patients tested, increased by day 8 in 52%, and decreased in 93% (67% undetectable) 2 h after bevacizumab.

**Conclusions:** In this single arm study, cytotoxic chemotherapy followed by bevacizumab yields a favorable CR rate and duration in adults with AML that is resistant to traditional treatment approaches. The clearance of marrow blasts in some patients after bevacizumab suggests that VEGF neutralization might result directly in leukemic cell death. The potential biological and clinical activity of bevacizumab in AML warrants additional clinical and laboratory study.

**INTRODUCTION**

The process of angiogenesis may contribute to leukemic cell survival and a resultant resistance to chemotherapy-triggered cell death. A major determinant in this process is vascular endothelial growth factor (VEGF), a heparin-binding cytokine that promotes the proliferation and survival of endothelial cells (1, 2) and multipotential hematopoietic stem cells (3, 4). The compartment of pluripotent hematopoietic stem cells that gives rise to lineage-restricted precursors expresses CD34, VEGF receptor-1 (FLT-1), and VEGF receptor-2 (KDR; Ref. 4). An internal autocrine loop between VEGF and KDR in this stem cell compartment appears to be critical to net cell survival (5–7) and may operate in leukemic as well as normal stem cells. In this regard, a significant proportion of *de novo* and secondary AML blast populations express VEGF mRNA and/or produce and secrete VEGF protein, with the amount of VEGF secreted by cultured leukemic blasts exceeding that secreted by cultured normal bone marrow counterparts (8–10). Moreover, blasts from some patients with newly diagnosed AML exhibit relative overexpression of KDR (10). In addition to autocrine stimulation, secreted VEGF may provoke marrow stromal production and release of inflammatory cytokines that drive leukemic cell (8, 11, 12) and endothelial cell proliferation (13–15) in paracrine fashion. In turn, stromal cytokines may trigger a self-sustaining stimulatory loop by inducing endothelial cell release of VEGF-C, which interacts with the recently described FLT-4 (VEGF receptor-3) and protects AML cells against apoptosis induced by antileukemic drugs including 1- $\beta$ -D-arabinofuranosylcytosine (ara-C), anthracyclines, and VP-16 (16).

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Studies comparing bone marrow microvasculature in acute myelogenous leukemia (AML) and normal marrows demonstrate increased amounts of angiogenesis in leukemia (12, 17, 18) with a striking reduction in microvessel density (MVD) observed at the time of chemotherapy-induced marrow aplasia in patients who ultimately achieve complete response (CR) but not in those who fail to respond to therapy (17). Furthermore, at least in patients who present with high blast counts, the amount of VEGF produced by AML cells is inversely related to the duration of CR and survival (18). Thus, it is reasonable to postulate that VEGF may act as a growth and survival factor in AML and may contribute to net drug resistance.

bevacizumab is a recombinant humanized IgG monoclonal antibody directed against VEGF that blocks the binding of VEGF to its cognate receptor(s). Phase I clinical trials demonstrated linear pharmacokinetics for doses  $\geq 1$  mg/kg with a  $t_{1/2}$  of roughly 15 days, no impact on cytotoxic drug pharmacokinetics, and no anti-bevacizumab antibody formation. Toxicities in Phase II studies confirm the Phase I findings of predisposition to serious hemorrhagic and thrombotic events, proteinuria, and hypertension when administered with cytotoxic agents (19). A Phase II, randomized trial of fluorouracil and leucovorin with/without bevacizumab for patients with metastatic colon cancer demonstrated that patients receiving bevacizumab enjoyed higher overall response rates with prolonged time to disease progression and longer median survival (19). A Phase III trial where bevacizumab 5 mg/kg every 2 weeks added to irinotecan, fluorouracil, and leucovorin as first line therapy for metastatic colorectal cancer has confirmed the increases in CR and overall response rates, duration of response, and progression-free and median survival (20).

Historically, there have been many attempts to improve the durable antileukemic effects of ara-C-based treatments. Our group has focused on timed sequential therapy (TST), a treatment strategy that attempts to exploit drug-induced changes in residual leukemia cell cycle kinetics to increase the sensitivity of those leukemic cells to cycle-dependent antileukemic agents (21–23). Whereas TST has been shown to induce prolonged disease-free survival in certain groups of adults and children with AML (21, 24–29), it is also associated with toxicities to other cells that are actively cycling. Such toxicities are especially pronounced for oral and gastrointestinal mucosal cells, which are similar to hematopoietic cells in terms of growth kinetic profiles and the recovery kinetics after cytotoxic injury (21, 24–28, 30). Because bevacizumab does not appear to have direct antimucosal effects, the use of this agent on day 8 instead of chemotherapy might spare mucosal cells and avoid some of the mucosal destruction and attendant inflammatory and infectious complications seen after the administration of traditional cytotoxic drugs given at the predicted peak of residual leukemic cell and mucosal cell proliferation.

In addition to the practical impact that bevacizumab might have by sparing mucosal cells, there is a theoretical advantage of its administration after cytotoxic chemotherapy. Multiple cytokines, of which VEGF may be one, could drive the proliferation of hematopoietic precursors and endothelial cells in an attempt to reconstitute a bone marrow that has been damaged by cytotoxic agents.

Using our TST model as a template, we designed and

conducted a Phase II clinical trial to examine the role of bevacizumab administered at the predicted time of peak AML cell regeneration after initial chemotherapy (21–23, 25, 29) in adults with relapsed or refractory AML. We used ara-C and mitoxantrone as initial cytoreductive therapy, because these drugs have been combined to induce meaningful CRs in patients with relapsed and refractory disease (24, 33–37). In concert with the clinical trial and as potential molecular and biological correlates of clinical response, we assessed blast cell expression of FLT-1, marrow MVD, and free serum VEGF levels before and during TST with bevacizumab.

## MATERIALS AND METHODS

### Patient Eligibility and Selection

Adults age 18 or older with pathologically confirmed acute leukemia that was unlikely to be cured by existing therapies were eligible for study, including primary refractory (induction failure) AML, multirefractory AML (refractory or relapsed after  $\leq 3$  prior induction regimens), newly diagnosed AML in adults with known poor-risk features (antecedent hematological disorder, adverse cytogenetics), and AML arising from myelodysplasia or secondary AML were eligible provided they had Zubrod performance status 0–2, normal bilirubin; hepatic enzymes  $\leq 2\times$  normal, serum creatinine  $\leq 1.5\times$  normal, and left ventricular ejection fraction (LVEF)  $\geq 45\%$ . Complete history, physical examination, laboratory, imaging and cardiac evaluations (electrocardiogram and LVEF) were performed within 3 days of study entry. Recovery from toxicities of previous treatment and intervals of  $\geq 3$  weeks from prior chemotherapy and  $\geq 1$  week from any growth factor therapy were required before beginning ara-C and mitoxantrone. Patients were ineligible if they had peripheral blast count  $\geq 50,000/\text{mm}^3$ ; disseminated intravascular coagulation; active uncontrolled infection; active central nervous system leukemia; history of ara-C-related neurotoxicity; prior radiation of  $>25\%$  of bone marrow; concomitant radiotherapy, chemotherapy, or immunotherapy; or coexisting medical or psychiatric conditions that could interfere with study procedures. Pregnant or lactating women were ineligible. All of the patients provided written informed consent according to University of Maryland Baltimore Institutional Review Board guidelines.

**Treatment Schema.** Therapy was initiated on day 1 with ara-C 2 g/m<sup>2</sup> administered as a 72 h continuous infusion (667 mg/m<sup>2</sup>/24 h; Refs. 21, 23, 25, 26). Mitoxantrone 40 mg/m<sup>2</sup> was administered as a single i.v. bolus over 30–60 min on day 4, 12 h after completion of the ara-C infusion. bevacizumab was supplied by Genentech through Clinical Trials Evaluation Program, National Cancer Institute under a Cooperative Research and Development Agreement between National Cancer Institute and Genentech. bevacizumab 10 mg/kg was administered on day 8 by 90 min i.v. infusion.

**Supportive Care.** All of the patients received daily oral allopurinol 300 mg and aluminum hydroxide 30 ml every 6 h until 24 h after the completion of ara-C and mitoxantrone (day 5). Corticosteroid eye drops were used on days 1–8 to prevent ara-C-related conjunctivitis. Antiemetics were used according to standard practices. Premenopausal women were placed on hormonal therapy to suppress menstrual bleeding. Norfloxacin 400

mg twice daily for gastrointestinal decontamination and acyclovir or famvir prophylaxis against herpes simplex virus activation began day 1 and continued until achievement of an absolute neutrophil count (ANC)  $>100/\text{mm}^3$ .

**Definitions of Response.** To assess response to therapy, bone marrow aspiration and biopsy were performed before treatment and on day 8 before bevacizumab, on day 15, and at the time of hematological recovery or when leukemia regrowth was suspected. Hematological recovery was defined as an ANC of at least  $500/\text{mm}^3$  and a transfusion-independent platelet count of  $50,000/\text{mm}^3$ . CR required a normal bone marrow aspirate with absence of identifiable leukemia, ANC  $\geq 1,000/\text{mm}^3$ , platelet count  $\geq 100,000/\text{mm}^3$ , and absence of blasts in peripheral blood (38). Clearance of cytogenetic abnormalities was not required for CR but was noted and described separately. Partial response (PR) was defined as the presence of trilineage hematopoiesis in the marrow with normalization of peripheral counts but with 5–25% blasts in the marrow (38). No response was defined as persistent leukemia in marrow and/or blood without significant decrease from pretreatment levels. Patients who achieved CR or PR were eligible for a second course of bevacizumab-based TST using the same drugs in the identical dose and schedule, beginning  $30 \pm 7$  days after hospital discharge from the first cycle. Toxicity was recorded and graded according to the National Cancer Institute Common Toxicity Criteria version 2.0.

### Laboratory Correlates

**Leukemic Marrow Cell Expression of FLT-1.** Trepine bone marrow specimens were collected from patients before initiation of therapy and at intervals until recovery, normal marrow, or AML. Specimens were fixed in formalin, decalcified, and paraffin-embedded. Four- $\mu\text{m}$  sections of each sample were processed for immunohistochemistry by treatment with Dako's Target Retrieval Solution (for von Willebrand factor) or Trilogy Solution (Cell Marque, Hot Springs, AR) for FLT-1 according to the manufacturer's directions. Antigen retrieval was by incubation in a steamer for 20 min for von Willebrand factor or in a pressure cooker for 15 min for FLT-1. A monoclonal antibody to human von Willebrand factor (number A086; Dako, Glostrup, Denmark), diluted 1:200, was used to identify microvessels. A monoclonal antibody to the human VEGF receptor FLT-1, raised in rabbits (sc-316; Santa Cruz Biotechnology, Santa Cruz, CA), diluted 1:25, was used to stain blasts (8). The chromogen was 3,3'-diaminobenzidine, and reactivity was developed with the avidin-biotin complex method. Padro *et al.* (17) have indicated the difficulty of using other antibodies (anti-CD31 and anti-CD34) in MVD studies of leukemic patients.

**Bone Marrow MVD.** MVD was determined in accord with methods proposed by an international consensus report (39), as modified by Padro *et al.* (17). Due to the relatively small cross-sectional area of the marrow biopsies, all or the majority of marrow space was examined at  $\times 400$  magnification, avoiding the need to identify "hotspot" areas of the most intense neovascularization. All of the stained cells or cell clusters, with or without a lumen, separate from other microvessels, blasts, and other marrow cells were counted as microvessels. Megakaryocytes, which typically stain strongly for von Will-

ebbrand factor, were not counted. Results are expressed as the mean number of microvessels per unit area.

**Free VEGF Levels in Serum.** Peripheral blood was collected and allowed to clot overnight at  $4^\circ\text{C}$ . After centrifugation at 1000 rpm for 10 min, serum was aspirated from the supernatant and stored in aliquots at  $-80^\circ\text{C}$ . VEGF<sub>165</sub> ELISA was performed with the Quantikine human VEGF kit from R&D Systems, Inc. (Minneapolis, MN). On the day of the assay, samples were thawed, and 200  $\mu\text{l}$  of sera was diluted with 200  $\mu\text{l}$  calibrator diluent RD6U as recommended. A portion of each sample (100  $\mu\text{l}$ ) in duplicate was added to the wells of a 96-well plate coated with anti-VEGF antibody and containing 100  $\mu\text{l}$  of assay diluent RD1W. After incubating for 2 h at room temperature, the plates were washed three times, and 200  $\mu\text{l}$  of VEGF antibody conjugate was added to each well. After incubation for 2 h at room temperature, the wells were washed three times and 200  $\mu\text{l}$  of Substrate solution was added to each well for 25 min at room temperature. Stop solution (50  $\mu\text{l}$ /well) was added, and the absorbance of each well was determined at 450 nm with a reference reading at 560 nm. A VEGF standard curve was generated by serial dilution of a stock 2000 pg/ml solution of human VEGF using the calibrator diluent. The detection limit was 31.2 pg/ml, and the sensitivity was 9 pg/ml.

## RESULTS

**Patient Characteristics.** A total of 48 adults (median age, 49; range, 19–80) with poor-risk, refractory, or relapsed AML were entered on this Phase II study of ara-C, mitoxantrone, and bevacizumab (Table 1). Three patients had newly diagnosed, previously untreated AML (therapy-related M5, germ cell-associated M7, and M0 with complex cytogenetics), 27 had refractory AML (15 primary induction failure, 12 refractory to  $\geq 2$  induction regimens, or to reinduction after first relapse), and 18 had AML in first relapse (median first CR, 5.5 months; range, 3–19). Fourteen patients (29%) had secondary AML (AML evolved from antecedent myelodysplasia and/or cytotoxic therapy for a previous malignancy). Nine (19%) had extensive myelofibrosis. Cytogenetics were abnormal in 30 (62.5%), including abnormalities of chromosome 7 in 10 (21%) and complex cytogenetics (with or without chromosome 7 abnormalities) in 17 (35%). The vast majority (92%) had received anthracycline or mitoxantrone as therapy for AML or a prior malignancy. Similarly, 90% had received dose-intensive infusional ara-C and/or high-dose bolus ara-C during initial antileukemia induction and/or consolidation regimens. Twenty-five (52%) had received previous TST with ara-C and anthracycline, including 8 patients with primary refractory AML and 5 patients in first relapse with CR 1 durations  $<6$  months.

**Toxicities.** One patient with primary refractory leukemia suffered overwhelming tumor lysis with multiorgan failure on day 3 of ara-C and mitoxantrone and did not receive bevacizumab. Drug-induced myelosuppression occurred in all of the patients, with similar depth and duration of myelosuppression to other TST regimens using cytotoxic drugs (25, 26). For cycle 1, median day of ANC recovery to  $>100/\text{mm}^3$  was day 27 (range, 19–39) and to  $>500/\text{mm}^3$  day 30 (range, 20–46). ANC recovery during cycle 2 was similar (median, 29 days; range, 19–35). Platelet recovery to  $\geq 50,000/\text{mm}^3$  occurred on median day 31

Table 1 Characteristics of 48 patients undergoing TST<sup>a</sup> with bevacizumab

Males/females	23 (48%)/25 (52%)
Median age (range)	49 (19–80)
Prior drug exposure	
Ara-C	43 (90%)
HiDAC <sup>b</sup>	13
2 g/m <sup>2</sup> /72 h <sup>c</sup>	25
Anthracyclines	44 (92%)
Adriamycin	5
Mitoxantrone	22
Stage of disease	
New diagnosis (poor risk) <sup>d</sup>	3 (6%)
First relapse	18 (37.5%)
Median CR1 duration	5.5 months (3–19)
Refractory	27 (56%)
Primary refractory	15
Multi-refractory <sup>e</sup>	12
Biologic features	
Secondary AML	14 (29%)
MDS/AML	8
Treatment-related AML	6
Adverse cytogenetics <sup>f</sup>	30 (62.5%)
Myelofibrosis	9 (19%)

<sup>a</sup> TST, timed sequential therapy; ara-C, 1-β-D-arabinofuranosylcytosine; AML, acute myelogenous leukemia; MDS, myelodysplasia.

<sup>b</sup> HiDAC, ara-C 2–3 g/m<sup>2</sup> over 2 h every 12 h × 4–12 doses.

<sup>c</sup> Continuous infusion ara-C given over the first 72 h of TST in combination with anthracycline.

<sup>d</sup> Treatment-related acute monocytic leukemia (M5, post-breast cancer chemotherapy), 46XX, t(9;11)(p21;q23); acute megakaryocytic leukemia (M7) associated with mediastinal germ cell tumor, 83–90XY,+1(pter),+ring; M0 AML, 45XX, -7, -11, -13, -17, -17, +2(q31), +3(q27), +12(p11.2), +4 markers.

<sup>e</sup> Refractory to >2 induction regimens or to reinduction after first relapse.

<sup>f</sup> inv (3)(q21q26), -5/5q, -7/7q, +8,11q23 translocations, -20q, complex karyotype.

(range, 20–48) for cycle 1 and median day 40 (range, 18–56) for cycle 2. As depicted in Table 2, the predominant nonhematologic toxicities were cardiovascular in nature. Three of 48 (6%) in cycle 1 and 2 of 18 (11%) in cycle 2 had decreases in LVEF from ≥50% to 30–35% beginning 10–14 days after bevacizumab administration. Of the 3 patients who had a decrease in LVEF during cycle 1, 2 had treatment-related AML with prior exposure to Adriamycin at totals of 240–400 mg/m<sup>2</sup> (2 of 5, or 40%, of those who received prior Adriamycin), and 1 had experienced transient LVEF decrease with previous mitoxantrone therapy (total dose, 40 mg/m<sup>2</sup>). Both patients whose LVEF decreased during cycle 2 had preexisting hypertension and had received prior mitoxantrone (40–80 mg/m<sup>2</sup> total). LVEF returned to normal in 3 of the 5 patients after 2–4 weeks. Hypertension (≤grade 2) occurred in 7 patients during cycle 1 and 2 patients during cycle 2. Each of these patients had preexisting hypertension, and blood pressure was readily controlled with adjustment of antihypertensives and without cardiovascular complications. Two (4%) suffered central nervous system bleeds. Other toxicities included nondestructive (≤grade 2) oral and gastrointestinal mucositis occurring in a relatively small proportion of patients with a lesser intensity than commonly seen in other TST regimens (21, 24–28, 30, 32).

Death occurred in 7 of 48 patients [15%; 95% confidence

interval (CI), 6–28%] in cycle 1 and 2 of 18 (11%; 95% CI, 1.4–35%) patients in cycle 2. Causes of death during cycle 1 were related cardiac failure (2), central nervous system bleed (2), multiorgan failure (1), overwhelming tumor lysis (1), and irreversible marrow aplasia after achieving complete tumor clearance (1). Both deaths in cycle 2 were caused by overwhelming fungal infections.

**Clinical Outcome.** Table 3 summarizes the clinical outcome of one cycle of ara-C, mitoxantrone, and bevacizumab. Pretreatment marrow cellularity and blast percentage showed significant interindividual variation, with cellularity ranging from 20% to 100% and blast percentage ranging from 10% to >90%. Likewise, overall cellularity and blast percentages varied widely among the day 8 marrow aspirates and biopsies, with virtually all of the patients exhibiting some decrement relative to pretreatment values. Nonetheless, residual leukemia was detected morphologically in 38 of 48 (79%) patients on day 8 pre-bevacizumab. Marrow aspirates and biopsies on day 15

Table 2 Nonhematologic toxicities of TST<sup>a</sup> with ara-C, mitoxantrone, and bevacizumab

	Cycle 1 (n = 48)	Cycle 2 (n = 18)
Cardiovascular	14 (29%)	3 (17%)
Decrease in ejection fraction	3 (2 grade 2, 1 grade 4)	2 (< grade 2)
Hypertension	7 (grade 1–2)	2 (grade 1)
Frontal headache	3 (grade 1–2)	1 (grade 2)
Cerebrovascular bleed	2 (grade 5)	0
Mucositis	6 (12.5%)	2 (11%)
Oropharyngeal	5 (1 grade 3, 1 grade 2)	1 (grade 3)
Gastrointestinal	5 (1 grade 3, 2 grade 2)	1 (grade 2)
Death	7 (15%)	2 (11%)
Cardiovascular	2	0
Cerebrovascular bleed	2	0
Multiorgan failure	1	0
Irreversible marrow aplasia	1	0
Tumor lysis syndrome	1	0
Fungal sepsis	0	2

<sup>a</sup> TST, timed sequential therapy; ara-C, 1-β-D-arabinofuranosylcytosine.

Table 3 Clinical outcome of TST<sup>a</sup> with ara-C, mitoxantrone, and bevacizumab

Stage of disease (No. of patients)	CR (%)	PR (%)	NR (%)	NE (%)
New diagnosis (3)	2 (67)		1 (33)	
First relapse (18)	9 (50)	3 (16)	3 (16)	3 (16)
Refractory (27)				
Primary refractory (15)	4 (27)	4 (27)	6 (40)	1 (7)
Multirefractory <sup>b</sup> (12)	1 (8)		7 (58)	4 (33)
Total (48)	16 (33)	7 (15)	17 (35)	8 (17)

<sup>a</sup> TST, timed sequential therapy; ara-C, 1-β-D-arabinofuranosylcytosine; CR, complete response; PR, partial response; NR, no response; NE, nonevaluable.

<sup>b</sup> Refractory to >2 induction regimens or to reinduction after first relapse.

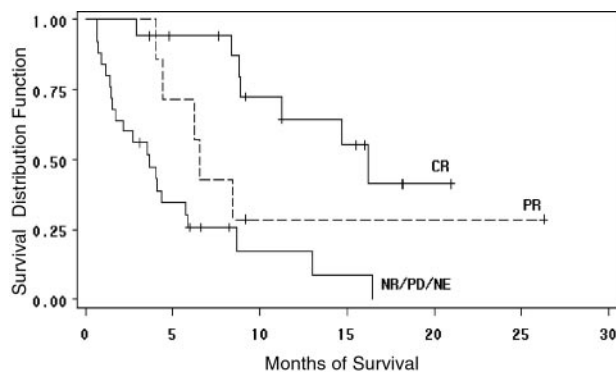


Fig. 1 Overall survival for 48 patients receiving timed sequential therapy with bevacizumab, according to clinical response to induction therapy. CR, complete remission, 16 patients; PR, partial remission, 7 patients; NR, no response; PD, progressive disease; NE, nonevaluable, total 25 patients.

showed complete tumor clearance in 28 of 48 (58%) patients. The regimen induced CRs in 16 (33%; 95% CI, 20–48%) of the total cohort. CR was achieved in 2 of 3 patients with newly diagnosed, poor-risk AML and in 9 of 18 (50%) AML in first relapse, but only in 5 of 27 (19%) with refractory disease (4 of 15 primary refractory and 1 of 12 multirefractory). An additional 7 patients (15%; 95% CI, 6–28%) achieved PR (3 of 18 first relapse and 4 of 14 primary refractory).

Thus, the overall response rate (CR+PR) achieved with one cycle of TST with bevacizumab was 23 of 48, or 48% (95% CI, 33–63%). Patients who achieved CR or PR were eligible to go on for additional therapy. Of 16 CR patients, 14 received a second cycle of TST, and 3 underwent matched unrelated donor marrow transplantation (2 at 2 months after TST cycle 1 and 1 at 3 months after 2 TST cycles). Of 7 PR patients, 4 received a second cycle of TST, 2 underwent matched unrelated donor marrow transplantation after cycle 1, and 1 received no additional therapy due to poor performance status. Thus, a total of 18 patients (14 CR and 4 PR) underwent a second cycle of TST with Bevacizumab and 5 patients (3 CR and 2 PR) underwent matched unrelated donor transplantation.

As of July 1, 2003, the median follow-up for the entire group is 16 months (475 days; range, 18–800). OS for this group of refractory and relapsed patients relates directly to clinical response to the first cycle of TST with bevacizumab (log-rank test,  $P < 0.0001$ ; Fig. 1). Median OS for the entire group is 8.4 months (95% CI, 4.1–11.2 months), with 35% survival at 1 year (95% CI, 19.5–38.4%) and 18% survival at 2 years (95% CI, 4.1–32.1%). For the 16 CR patients, median OS is 16.2 months (95% CI, 8.9–29.6 months) with 64% alive at 1 year (95% CI, 39.8–89.8%) and 41% at 18 months (95% CI, 10.2–72.5%). Median OS for the 7 PR patients is 6.5 months (95% CI, 4.4–9.7 months) with 2 patients alive at 9+ and 25+ months in CR after matched unrelated donor transplant. In contrast, median OS for the 25 nonresponders was 3.7 months (95% CI, 1.5–5.8 months) with a 1-year survival of 17% (95% CI, 0–35%) and no 18-month survival. Median disease-free survival for the entire group of 16 CR patients (Fig. 2) is 7 months, with 35% (95% CI, 10.3–58.8%) remaining in CR at 1

year and 18% remaining in CR at 21 months (95% CI, 4.8–50.5%).

**Expression of the VEGF Receptor FLT-1 by AML Marrow Blasts.** The impact of bevacizumab on AML cell growth and survival relates to the expression of VEGF receptors on the target cell as well as the ability of the monoclonal antibody to neutralize the activating ligand. For this reason, we measured FLT-1 expression on AML blasts in marrow biopsy specimens obtained from 13 patients before treatment (day 0) and again on day 8 after ara-C and mitoxantrone and before bevacizumab using immunohistochemical staining and comparing the intensity of the AML marrows with that seen in biopsy specimens of normal bone marrows. We were unable to measure blast cell KDR expression immunohistochemically. As demonstrated in Fig. 3, pretreatment (day 0) AML marrow cells from 12 of the 13 patients exhibited significantly greater staining in terms of both cell numbers and intensity relative to normal marrow counterparts. Repeat immunohistochemical assessment of FLT-1 expression was evaluable in 10 of the 13 day 8 marrow samples. FLT-1 positivity was detected in 8 of the 10 patients, with cellular staining that was at least as intense as pretreatment biopsy specimens in 7 of the 8 patients. There was no clear relationship observed between the detection of day 8 FLT-1 positivity and clinical outcome in the few patients studied.

**Changes in Bone Marrow MVD during TST with bevacizumab.** One functional measurement of VEGF suppression may be the detection of a relative decrease in overall tissue MVD as a marker of neovascularization. To examine this potential biological marker of bevacizumab effect, we assessed MVD in bone marrow biopsy specimens obtained longitudinally from a total of 13 patients (7 CR, 2 PR, 3 no response, and 1 nonevaluable) before treatment (day 0), day 8 after ara-C and Mitoxantrone, and day 15 at the time of predicted maximal TST-induced bone marrow aplasia (21, 24–26). Day 0 MVD ranged from 38–193 vessels per field (mean, 88; median, 92). Serial samples were evaluable from 11 patients (7 CR, 2 PR, and 2 no response). MVD in day 8 marrows demonstrated a broad range of effects, with an increase of at least 20% (range, 20–

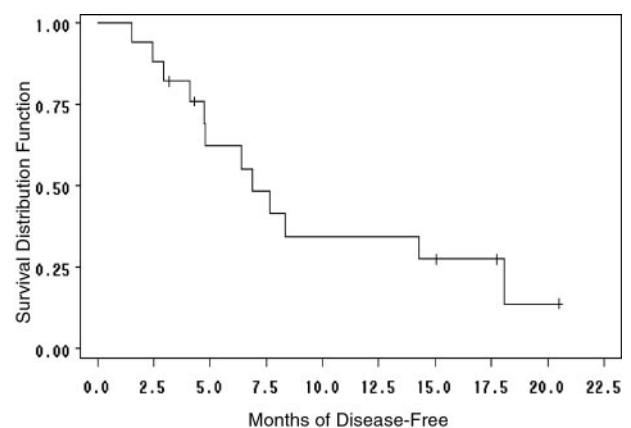


Fig. 2 Disease-free survival (DFS) for the 16 patients who achieved complete remission in response to induction timed sequential therapy with bevacizumab. Median DFS, 7 months; 12 month DFS, 35%; 18 month DFS, 28%.

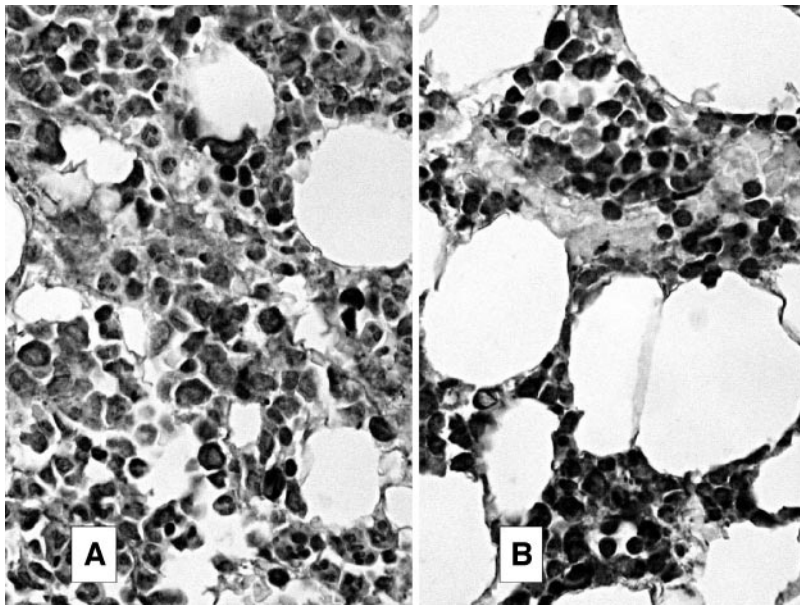


Fig. 3 Immunohistochemical staining of the vascular endothelial growth factor receptor 1 in biopsy specimens of normal bone marrow (A) and acute myelogenous leukemia bone marrow obtained before timed sequential therapy with bevacizumab (B). Immunohistochemistry demonstrates intense cytoplasmic positivity, with some nuclear positivity, in leukemic blasts (B) as compared with the more restricted expression in normal marrow cells (A). Antivascular endothelial growth factor receptor-1,  $\times 400$ .

300%) in 5 of 11 patients (45%), of whom 4 achieved CR of 5.5, 6, 20, and 21+ months of duration. Two additional CR patients (durations 4, 8, and 16 months), however, evinced decrements in day 8 marrow MVD. Nonetheless, day 15 marrow MVD decreased significantly in 8 of 11 patients (73%), ranging from <1% to 58% of day 0 and/or day 8 values. Of these 8 patients, 5 achieved CR (duration, 5.5–21+ months), 1 achieved PR, and 2 were no response.

**Serum VEGF Levels.** To additionally evaluate the basis for the decrease in MVD seen in the majority of day 15 marrow biopsies, we measured serum levels of free VEGF<sub>165</sub> before treatment (day 0) and at the time of predicted increased activity on day 8 before bevacizumab. In addition, we measured free VEGF levels 2 h after the end of the bevacizumab infusion to determine whether monoclonal antibody administration neutralized circulating VEGF activity *in vivo*. As depicted in Fig. 4, free VEGF was detectable in 14 (67%) of 21 patients before therapy, with a median level of 12 pg/ml (range, 0–139). Day 8 VEGF levels (median, 19 pg/ml; range, 0–124) increased over day 0 levels in 11 (52%) including 4 patients in whom VEGF was initially undetectable, and decreased in 6 (29%). Post-bevacizumab VEGF serum levels were determined in 15 patients, 10 (67%) of whom had complete suppression of detectable VEGF to 0 pg/ml and an additional 4 whose VEGF levels were suppressed to 24–72% of day 8 levels. Thus, 14 of the 15 patients whose VEGF levels were determined serially exhibited neutralization of VEGF levels.

## DISCUSSION

The results of this single arm, Phase II clinical laboratory trial indicate that chemotherapy followed by bevacizumab yields a favorable CR rate and duration of CR in relapsed and refractory adult AMLs that are, by definition, resistant to classical antileukemic cytotoxic agents. At least part of the clinical effects are due to the initial chemotherapy. Indeed, a Phase I

dose-escalation study of single high-dose mitoxantrone (40–80 mg/m<sup>2</sup>) in combination with high-dose ara-C (3 g/m<sup>2</sup> daily  $\times 5$ ) yielded CRs in 7 of 18 (38%) adults with relapsed AML, with the median duration of those remissions being 4 months (36). Nonetheless, based on prior studies of cell growth kinetics accompanying TST regimens where residual tumor cells on day 8 are viable and cycling and persist if no additional therapy is given (21–23), the clearance of marrow blasts after bevacizumab in some patients raise the possibility that VEGF neutralization might result directly in leukemic cell death. In future clinical trials, direct *in vitro* testing of bevacizumab cytotoxicity toward leukemic marrow blasts needs to be done to confirm or refute this hypothesis.

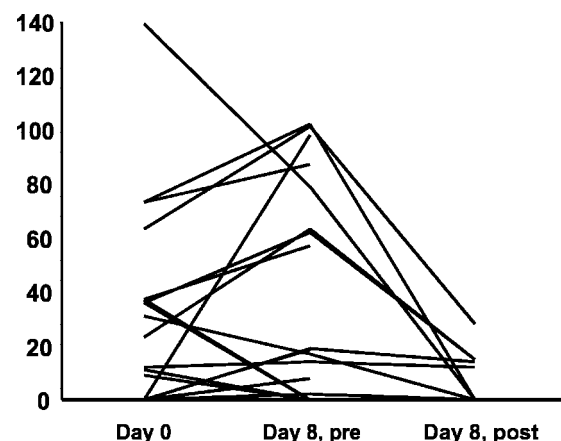


Fig. 4 Changes in ELISA detection of free VEGF<sub>165</sub> in serum during timed sequential therapy with bevacizumab. Sera were obtained before treatment (Day 0), at the time of predicted peak serum stimulatory activity after initial cytoreduction and before bevacizumab administration (Day 8, pre) and 2 h after the completion of bevacizumab infusion (Day 8, post).

Whereas the clinical results are promising with regard to CR rate and durability and avoidance of mucosal toxicities, our trial is neither randomized nor controlled. Nonetheless, the results can be viewed in the context of historical data where initial ara-C with or without anthracycline was administered without additional drug on day 8 (30). In a Phase I study of continuous infusion ara-C in doses ranging from 2 g/m<sup>2</sup>/72 h to 18 g/m<sup>2</sup>/72 h in adults with relapsed or refractory AML, only 1 of 12 (8%) of patients receiving 2–6 g/m<sup>2</sup>/72 h achieved complete tumor clearance on day 8, and no patients achieved CR (30). Even with ara-C at 10–14 g/m<sup>2</sup>/72 h, only 2 of 8 (25%) achieved complete tumor clearance on day 8, whereas 3 of 8 (37.5%) achieved transient CR. These data support the notion that the initial ara-C-based cytoreduction by itself is not adequate to induce remission. Given that the addition of bevacizumab on Day 8 resulted in a sufficiently high number of durable CRs in refractory disease, it is reasonable to suggest that the use of bevacizumab in TST or other combinations warrants additional clinical and laboratory study in AML. In this context, it may be appropriate to develop randomized studies of chemotherapy alone, TST alone *versus* chemotherapy, or TST with bevacizumab to define the optimal role of bevacizumab in the treatment of acute leukemias.

The clinical outcome with chemotherapy followed by bevacizumab is comparable with the results from other TST chemotherapy regimens in adults with relapsed or refractory disease. In patients with primary refractory disease after a single cycle of TST induction therapy (26), the CR rate attained with a second cycle of TST was 17% (3 of 18 patients; 2 of 11 with *de novo* AML and 1 of 7 with secondary AML). TST using ara-C, mitoxantrone, and VP-16 in patients with previously treated AML who had not received prior TST regimens yielded CR in 40–45% of refractory patients (primary induction failure, multirefractory, or CR ≤6 months) with median disease-free survival roughly 6 months, with the majority of patients receiving a second cycle of TST, autologous or allogeneic stem cell transplant as consolidation in CR (24). Similarly, a TST regimen of sequential high-dose ara-C (1–3 g/m<sup>2</sup> every 12 h) days 1, 2, 8, and 9 and mitoxantrone 10 mg/m<sup>2</sup> days 3, 4, 10, and 11 (S-HAM) induced CRs in 8 of 15 (53%) with relapsed AML with first CR <6 months duration or in more than or equal to second relapse, with a median CR duration of 4 months (37).

The serial immunohistochemical studies of changes in AML cell FLT-1 expression and bone marrow MVD during TST with bevacizumab support the notion that the ability to interfere with the interactions between VEGF and its cognate receptors may be relevant to the clinical activity of bevacizumab. The intense FLT-1 staining of AML blasts relative to normal marrow before therapy and again on day 8 (pre-bevacizumab) suggests the possibility that AML cells may be preferentially responsive to VEGF in terms of proliferation and survival, particularly after initial cytoreduction on day 8. It would be instructive, as well, to obtain serial measurements of leukemic cell KDR expression in future studies. The detection of a decrease in MVD after bevacizumab may, in fact, be a functional assay of *in vivo* antibody blockade of VEGF activity on endothelial cells and may, thus, be a surrogate marker of clinical response. Similar effects have been noted with cytotoxic chemotherapy, particularly in patients who achieve clinical responses

(17). Nonetheless, these findings support the notion that bevacizumab may contribute to leukemic cell death indirectly through its antiangiogenic effects on marrow vascularity as well as through a potential direct effect on leukemic cell survival.

The longitudinal measurements of serum VEGF levels substantiate two concepts on which the trial was built, namely that VEGF is detectable in the circulation before therapy and after initial cytoreduction, and that bevacizumab effectively abrogates the ability to detect free VEGF in the subset of patients in whom such measurements were made. We measured VEGF in serum rather than plasma, because our previous studies have examined the effects of serum on marrow cell proliferation (21–23). Many investigators have used plasma rather than serum to avoid the possibility that release of VEGF from platelets during the clotting process could confound interpretation of ELISA assay results (12, 40–42). In our cohort of patients, however, the concern over VEGF variation that might relate to differences in platelet count is likely mitigated by the marked thrombocytopenia that is present throughout the assay period.

Mucosal cell damage is a common devastating toxicity of TST regimens, because oral and gastrointestinal mucosal cells have growth kinetic profiles that are similar to hematopoietic cells. This specific toxicity was largely averted with the use of bevacizumab, perhaps because VEGF may not play a primary role in gastrointestinal mucosal cell survival. Additionally, the toxicity profile of TST with bevacizumab in this cohort of AML patients differed from that seen in patients with colorectal cancers, particularly with respect to the occurrence of deep venous thromboses in the latter trials (19, 20). It is likely that patients with colorectal cancers have a greater baseline predisposition to thrombotic events, in part related to hypercoagulability. Moreover, patients in our study may have been protected from thrombotic events by the profound thrombocytopenia present throughout therapy.

Instead, the toxicity profile of bevacizumab is cardiovascular, consistent with the importance of VEGF in vasculogenesis and vascular integrity (interestingly, VEGF is also known as vascular permeability factor). The cardiac effects of TST with bevacizumab were clinically significant in patients with prior exposure to cardiotoxic agents and occurred despite a normal LVEF before beginning treatment. Whether these effects are directly related to bevacizumab, high-dose mitoxantrone, or a combination of the two is not clear. In this regard, ≥10% diminutions in LVEF were detected in 6 of 26 (16%) patients enrolled in the Phase I trial of high-dose mitoxantrone in combination with high-dose ara-C, although clinical dysfunction was detected in only 1 patient who received 75 mg/m<sup>2</sup> mitoxantrone (36). Also, it is worth noting that 2 of the 5 patients in our study who had received prior Adriamycin experienced decreases in LVEF. Interestingly, the combination of Herceptin (the monoclonal antibody to erbB-2 receptor) and Adriamycin for women with advanced breast cancer is associated with synergistic cardiotoxicity (43), perhaps in part related to Herceptin-based abrogation of signaling pathways that protect against Adriamycin-induced apoptosis in myocardial cells (44–46). Whether or not bevacizumab might exert a similar down-regulation of antiapoptosis pathways in myocardial tissue requires additional study. In any case, careful clinical monitoring to detect and characterize compromised cardiac function result-

ing from treatment with bevacizumab, especially in combination with anthracycline drugs, is an important adjunct to future clinical trials.

The administration of bevacizumab targets extracellular VEGF and thereby blocks the paracrine stimulation that results from VEGF binding to diverse cognate cell surface receptor tyrosine kinases. In addition, Bellamy *et al.* (8) demonstrated that an anti-VEGF monoclonal antibody can inhibit blast cell clonogenicity in an autocrine fashion as well as via inhibition of both blast cell and stromal cell production of inflammatory cytokines including interleukin 1. Nonetheless, the internal autocrine loop may not be completely interrupted by bevacizumab. Thus, it is possible that VEGF signaling could continue to promote AML cell growth and survival, perhaps especially in those populations that are most resistant to cytotoxic chemotherapy and also to bevacizumab. New agents currently in clinical development may decrease intracellular VEGF by diverse mechanisms; for example, the cyclin-dependent kinase inhibitor flavopiridol decreases VEGF production (at least in response to a hypoxic stimulus) in part by inhibiting transcriptional elongation (reviewed in Ref. 47), whereas farnesyl-transferase inhibitors may suppress VEGF gene expression by blocking Ras-mediated activation of the phosphatidylinositol 3'-kinase /AKT pathway (again, related in part to a hypoxic stimulus; reviewed in Ref. 48). Another strategy aimed at interfering with VEGF signaling is the targeted inhibition of VEGF receptor phosphorylation by SU5416, a small molecule receptor tyrosine kinase (49, 50) that has exhibited single-agent biological activity in adults with refractory AML. The potential to combine bevacizumab with one or more of these agents raises the possibility that VEGF-driven cell proliferation and survival could be abrogated at multiple levels.

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