American Journal of Pathology, Vol. 150, No. 3, March 1997

Short Communication

Spectrum of Tumor Angiogenesis in the Bone Marrow of Children with Acute Lymphoblastic Leukemia

Antonio R. Perez-Atayde, Stephen E. Sallan, Usha Tedrow, Susan Connors, Elizabeth Allred, and Judah Folkman

From the Departments of Pathology and Surgery and the Division of Hematology and Oncology, Children's Hospital, and the Department of Pediatric Oncology, Dana Farber Cancer Institute, Harvard Medical School, Boston, Massachusetts

It has been shown that solid tumors progress in concert with an induction of tumor angiogenesis. It is not known, however, whether a similar phenomenon occurs in leukemia. Angiogenesis was characterized immunohistochemically by factor VIII staining of bone marrow biopsies and quantified by assessment of microvessel density using previously described techniques. We evaluated bone marrow biopsies from 40 children with newly diagnosed, untreated acute lymphoblastic leukemia. In 22 of the patients, we also evaluated angiogenesis after the completion of remission induction chemotherapy. Control specimens were obtained from children undergoing staging evaluations at the time of diagnosis of solid tumors and lymphomas. Microvessels were counted throughout the entire core specimen in consecutive ×200 fields, and a median count per field (cpf) was calculated. In addition, the number of microvessels in the single ×200 field with the highest microvessel density was designated as the "hot spot." Biopsies from children with leukemia and from controls showed median microvessel densities of 42 and 6 counts per field, respectively ($P \leq 0.0001$). Microvessel density of the hot spots of leukemia specimens and controls were also significantly different, 51 and 8, respectively ($P \leq 0.0001$). Three-dimensional reconstruction model of bone marrow vascularity showed a complex, arborizing branching of microvessels in leukemic specimens compared with single, straight microvessels without branching in controls. Urinary basic fibroblast growth factor, a potent angiogenic factor, was measured in 22 of the children with newly diagnosed leukemia and in 39 normal, age-matched controls. Urinary basic fibroblast growth factor levels were increased in all 22 patients before treatment, were variable during induction chemotherapy, and demonstrated statistically insignificant decreases at the time of complete remission. These findings suggest that leukemia cells induce angiogenesis in the bone marrow and that leukemia might be angiogenesis dependent and raise the possibility for a role of antiangiogenic drugs in the treatment of leukemia. (Am J Pathol 1997, 150:815–821)

It is well established that the viability and growth of solid tumors is dependent on angiogenesis (neovascularization).[1,2] Expansion of solid tumors beyond the size of a few millimeters in diameter requires the induction of new capillaries, a phenomenon mediated by angiogenic molecules released by tumor cells and activated macrophages. It has also been shown that the degree of angiogenic activity in a given tumor directly correlates with its growth, bleeding tendency, and potential for metastases; the more

Supported in part by grant CA68484 from the National Institutes of Health.

Accepted for publication November 18, 1996.

Address reprint requests to Dr. Antonio R. Perez-Atayde, Department of Pathology, Children's Hospital, 300 [illegible]

active the angiogenesis, the greater the probability of metastases.[1–3]

The angiogenic activity of hematopoietic neoplasms (so-called liquid tumors) has not yet been demonstrated. It is not known whether leukemic cells, which usually grow in the absence of a visible connective tissue stroma and circulate in the blood and body fluids, are also dependent on neovascularization to proliferate and expand.

We therefore studied the extent of angiogenesis in acute lymphoblastic leukemia, the most common type of childhood leukemia, its modulation with early and late chemotherapy, and its correlation with prognosis. In addition, we investigated the urine levels of the angiogenic peptide basic fibroblast growth factor (bFGF) in these children and the changes with chemotherapy.

Materials and Methods

To study the response of angiogenesis to treatment, we evaluated 61 bone marrow biopsies from 40 children with acute lymphoblastic leukemia. All biopsies were obtained for histological diagnosis at presentation (day 0). In 15 of these children additional serial biopsies were available for study; those were usually obtained on days 3 (15 biopsies) and 31 (3 biopsies) after the initiation of chemotherapy. Seven additional children had bone marrow biopsies only on day 31, so that comparison of microvessel counts with day 0 biopsies could not be done. Of these 10 children with day 31 biopsies, 6 were in complete and 4 in partial remission. Twenty seven of the 40 children with acute lymphoblastic leukemia were retrospectively selected, because they had long follow-up periods of more than 10 years, to investigate a possible correlation of the degree of angiogenesis with prognosis. Of these children, 18 had relapsed and were either dead with disease or were rescued with bone marrow transplantation, and 9 had no evidence of relapse and were currently alive and well without evidence of leukemia.

To establish controls, we studied 10 bone marrow biopsies of children with various neoplastic disorders that were obtained as part of their clinical staging. Four of the 10 had solid tumors without bone marrow involvement (malignant peripheral nerve sheath tumor, neuroblastoma, rhabdomyosarcoma, and Wilms' tumor), and 6 had lymphoma without bone marrow involvement (4 Hodgkin's disease and 2 lymphoblastic lymphoma).

One 4-μm-thick section from each paraffin-embedded bone marrow biopsy was immunostained with factor VIII-related antigen to highlight endothelial cells (Dako Polyclonal; Dako, Santa Barbara, CA) using a standard immunoperoxidase technique. Control bone marrow biopsies were also immunohistochemically stained with CD31 (BioGenex, San Ramon, CA) and CD34 (Signet Laboratories, Dedham, MA), antibodies highly sensitive for endothelium. The number of vessels per ×20 high-power field was counted in the entire bone marrow core (each field representing an area of 0.74 mm^2), and the median was calculated. The area with the highest microvessel count in each bone marrow core was termed the "hot spot." The microvessel counts of control marrows using factor VIII antibody, CD31, or CD34 were similar.

For the three-dimensional reconstruction of the normal and leukemic bone marrow, serial 4-μm-thick sections were cut and stained with factor VIII-related antigen. Representative areas of 500 × 400 μm were identified on 21 and 29 consecutive sections from the normal and leukemic marrow, respectively. These specific areas were photographed at ×250 using 35-mm film and a Zeiss (Oberkochen, Germany) Axiophot light microscope; serial 8 × 12-inch prints were made. The photographs were positioned under a video camera, and each image was captured by a Vision 8 frame grabber(InSync Technologies, Inc., Oakland, CA), which was interfaced with a personal computer. Images of adjacent sections were microaligned while switching between the stored image and the live image to minimize motion of structures in the selected area.[4–6] Using the Vision 8 system of tracing programs developed at the Children's Hospital Image Graphic Laboratory, the stained vessel contours as well as the easily visualized borders of trabecular bone were outlined on each digitized image. These contours, along with an assumed 4-μm thickness between slice surfaces, were used as input to the Interactive Computerized Analysis Reconstruction system program (ISG Technologies, Mississauga, Ontario, Canada)[7] to generate a three-dimensional construct. Nonoverlapping serial contours were left disconnected so that no data were added to the histological information. The Interactive Computerized Analysis Reconstruction system was used to display, rotate, and photograph the reconstructed images.

For the quantitative determination of human bFGF in the urine we used the Quantikine FGF immunoassay (R & D systems, Inc., Minneapolis, MN), a solid-phase enzyme-linked immunosorbent assay method that gives a rapid, sensitive, and specific measurement of bFGF in the urine or any other biological

Table 1. *Vessel Count on Day 0*

Vessel count	Leukemia (n = 40)	Controls (n = 10)	*P*	Nonrelapsed (n = 9)	Relapsed (n = 18)	*P**
Median	42	6	≤0.0001	38	40	0.74
Hot spot	51	8	≤0.0001	48	49	0.28

*Wilcoxon sign rank test.

zyme immunoassay technique, using antibodies specific for bFGF and a chromogen and measuring the color intensity by optical density.

Results

The bone marrow microvessel density was markedly and significantly increased in children with acute lymphoblastic leukemia compared with bone marrow controls (Figure 1). The median and highest (hot spot) microvessel counts at day 0 (biopsy performed at presentation for diagnosis) were markedly high in all biopsies with leukemia. The median and highest microvessel counts in children with leukemia were 42 and 51, respectively, versus 6 and 8, respectively, in controls ($P \leq 0.0001$; Table 1). The numerous microvessels seen in leukemic marrow were tortuous, often without visible lumina, and appeared packed with leukemic lymphoblasts. Small endothelial "sprouts" without discernible lumina were frequently present, which probably represented newly formed microvessels.

Although the median and highest microvessel counts at day 0 bone marrow biopsies were slightly lower in the specimens from patients who never relapsed, compared with those who did relapse, this difference was not statistically significant, 38 and 48, respectively, in nonrelapsed leukemias *versus* 40 and 49, respectively, in leukemias that relapsed ($P \leq 0.74$ and 0.28, respectively; Table 1). The microvessel counts did not change significantly during chemotherapy in children in whom serial biopsies on days 0, 3, and 31 were performed. Between days 0 and 3, there was no difference at all in microvessel density, with a hot spot median difference of 0 ($P \leq 0.97$). Between days 0 and 31 of therapy there was a nonsignificant decrease in microvessel density, with a hot spot median difference of −10 ($P \leq 0.6$; Table 2). Although a total of 10 children had biopsies on day 31, only for 3 of these children were day 0 biopsies also available for comparison of microvessel counts. For the other 7 children the day 0 biopsies were either not done (diagnosis made in the aspirate) or not suitable for immunohistochemical stains. In these 7 children, however, the median respectively) were similar to those of the other 3 patients. Although numerically the microvessels did not change significantly during chemotherapy, there was a morphological change that was observed early during treatment (from day 3). Microvessels became open, empty, and focally dilated, acquiring a sinusoidal appearance (Figure 1C). These changes were the result of unpacking of tumor cells and decreasing tumor burden in the marrow from the cytolytic chemotherapy.

The computer-aided three-dimensional reconstruction model revealed a complex arborizing branching of microvessels in bone marrow with leukemia, compared with few simple, straight microvessels without branching in controls (Figure 2). The shape, size, and caliber of microvessels in leukemia were variable and nonuniform, with focal areas of narrowings and dilatations, areas of complex loopings, and formation of microsaccules or diverticuli.

Urine levels of bFGF at day 0 were markedly high in children with leukemia compared with controls. The median value in 22 leukemic children was 9075 pg/g creatinine, compared with 1210 pg/g creatinine in 39 age-matched control children ($P \leq 0.0001$; Table 3).

Discussion

Previous studies of normal bone marrow have demonstrated a close association between the bone marrow vessels and the islands of developing blood cells, suggesting an important interdependence between the bone marrow hematopoietic function and its vascular bed.[9,10] Indeed, the circulation within the bone marrow has been compared with that of major organs, such as the spleen or liver.[10] Using angiographic methods in a variety of experimental animal models, it has been shown that the nutrient artery and vein penetrate the bone together. The artery then coils helically around the venous stem, which runs along the central axis of the bone. The arterial vessels arborize into arterioles and capillaries, which open conically into widening sinusoids. These sinusoids are elongated fusiform structures forming a complex anastomosing hexagonal network. Direct

Table 2. *Effects of Chemotherapy on Vessel Count*

Treatment days	Hot spot median difference	*P**
0 and 3 (n = 15)	0	0.97
0 and 31 (n = 6)	−10	0.6

*Wilcoxon matched-pairs sign rank test.

the blood to bypass this sinusoidal system. Studies in animals have shown the existence of an extensive network of capillaries encircling fat cells. Many of these capillaries are nonpatent and functionally dormant. When the activity of the marrow increases, some of these collapsed capillaries become patent.[10]

In the normal bone marrow specimens used as controls for this study, the capillaries and sinusoidal network were difficult to visualize in routinely stained hematoxylin and eosin or Giemsa preparations. With the aid of immunohistochemical stains using factor VIII-related antigen, CD31, or CD34, the microvessel density appeared to be very low (Figure 1A) in comparison with that reported in angiographic or injection studies.[9,10] This sparse microvessel density may be related to the nonfunctionality or dormancy of the endothelium in most capillaries and sinusoids or perhaps to nonreactivity to these antibodies in normal bone marrow capillaries and sinusoids. Nonreactivity to factor VIII-related antigen has been reported in normal hepatic sinusoids.[11–15] Hepatic sinusoids become reactive to factor VIII, acquiring a capillary-type basal lamina (capillarization) in certain disorders of the liver, including chronic hepatitis, alcohol liver disease, and nodular regeneration.[11–15]

Both routine immunohistochemical studies and a computer-generated three-dimensional reconstruction model revealed a remarkable increase in the number of visualized vessels in bone marrow replaced with leukemia cells (Figures 1 and 2). This marked increase in vessel counts might be related to reactivation of marrow sinusoids for factor VIII, as occurs in the hepatic sinusoid, or alternatively to a true neoangiogenic phenomenon. The highly variable morphology of the microvessels, however, with arborizing branching, the presence of endothelial sprouts without discernible lumina, the presence of hot spots, and the fact that microvessels were exceedingly numerous, all suggest a truly neoangiogenic phenomenon.

The microvessel counts at diagnosis remained high in biopsies taken after 3 days of single-agent antileukemia treatment when tumor cells were markedly decreased. At this time microvessels appeared a mild, statistically insignificant decrease in microvessel counts after 1 month of chemotherapy when the leukemia was in complete remission. The involution of microvessels thus lagged behind the killing of leukemia cells. The time necessary for the microvessels to regress to a normal number after the child is cured of the leukemia remains to be known. No biopsies were performed after the first month of treatment in children with favorable outcomes.

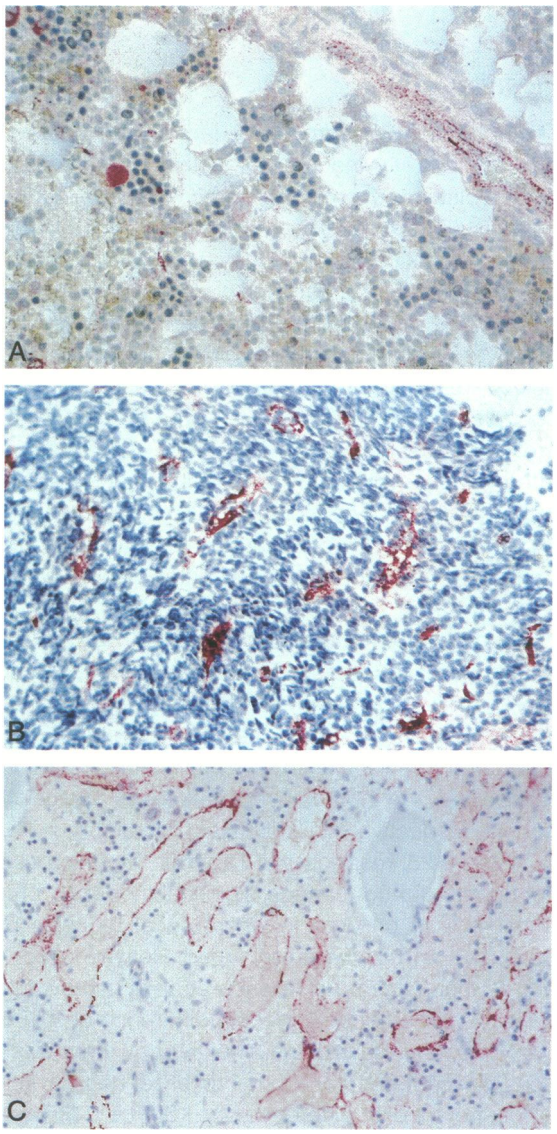

Figure 1. A: *Factor VIII-stained control bone marrow showing very rare capillaries. The endothelium of an arteriole is highlighted in the* upper right corner. *Occasional megakaryocyte appear strongly stained.* B: *Leukemic bone marrow at day 0 showing replacement of hematopoietic cells by a monotonous neoplastic cell population and numerous microvessels highlighted in red by factor VIII immunohistochemical stain. Some capillaries are small without lumen, probably representing newly formed endothelial sprouts.* C: *Leukemic bone marrow after treatment (day 3) showing marked decrease in tumor cell*

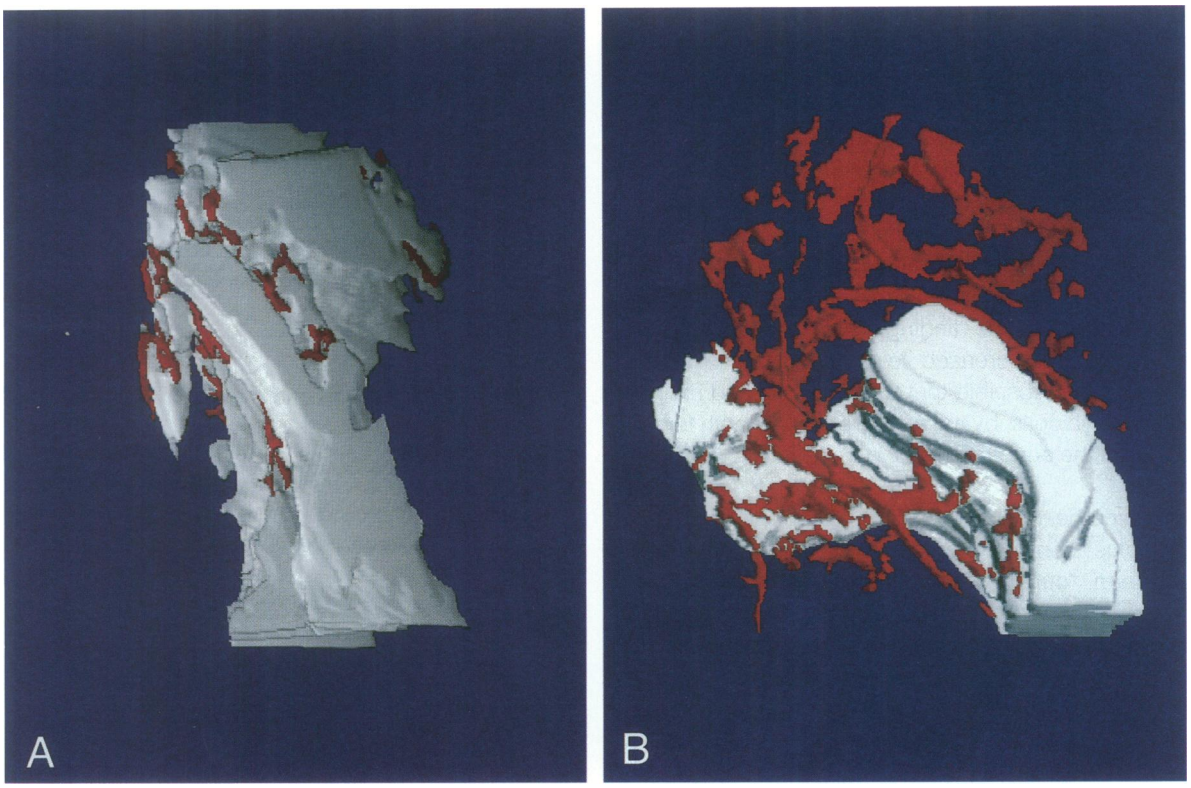

Figure 2. A: *Computer-aided three-dimensional reconstruction of control bone marrow after immunohistochemical staining for factor VIII. Few straight microvessels (in red) are seen surrounding a fragment of trabecular bone.* **B:** *In contrast, the three-dimensional model in this leukemic marrow reveals numerous arborizing and branching microvessels with irregular contours, with formation of saccular dilations and diverticuli. The areas depicted in* (**A**) *and* (**B**) *have identical dimensions representing similar amounts of marrow.*

The prognostic significance of neovascularization in solid neoplasms was first shown in cutaneous melanoma. Two reports correlated increased angiogenesis with a higher rate of metastases in thin and intermediate thickness melanomas.[16,17] This contention, however, has been recently disputed.[18] The biological phenomenon of tumor angiogenesis as an indication of higher malignant potential, however, has been shown on multiple occasions with other solid neoplasms.[1,19,20] In invasive breast carcinoma, microvessel density in the area of most intense angiogenesis was found to be an independent and highly significant prognostic indicator of survival even in patients without evidence of lymph node metastases.[19]

In our study there was no statistically significant difference in microvessel density at day 0 between relapsed and nonrelapsed leukemias. This lack of correlation may be related to the current high survival rate of children with acute lymphoblastic leukemia. This is, however, a preliminary study with a small sample of cases. Prospective studies should be performed to further clarify this issue.

Urine bFGF levels at day 0 were markedly increased in all children with acute lymphoblastic leukemia, and there was a mild decrease in levels during the first month of treatment. It has been shown that bFGF is chemotactic and mitogenic for endothelial cells *in vitro*.[21,22] It also induces breakdown of basement membrane proteins and increases colla-

Table 3. *Urine Levels of bFGF (pg/g Creatinine)*

	Leukemia (n = 22)	Controls (n = 39)	*P*	Effects of chemotherapy	
				Days 0 and 31	*P*
Median*	9075 pg/g	1210 pg/g	≤0.0001	NA	NA
Median difference†	NA	NA	NA	−3354 pg/g	0.46

NA, not applicable.

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