

9 TUMOR ANGIOGENESIS

JUDAH FOLKMAN, MD

Angiogenesis, the growth of new capillary blood vessels, is central to the growth of cancer. An understanding of the cellular and molecular basis of tumor angiogenesis is therefore important for clinicians who diagnose and treat cancer by whatever modalities. This chapter is focused on certain general principles of tumor angiogenesis that are intrinsic to the behavior of human cancer.* Almost every week, the biomedical literature contains elegant reports on the cause and development of cancer at the cellular and molecular levels. This rapid progress in understanding the molecular and genetic events that underlie the transformation of a normal cell to a cancer cell is reflected in many chapters in this book. These studies provide strong evidence for the hope that, someday, there will be molecular solutions to the cancer problem. However, in virtually all scenarios of current or future therapy, the target is the cancer cell.

Experimental evidence indicates that it is prudent to develop cancer therapies against another target, the vascular endothelial cell, without implying that the two targets are mutually exclusive.

Consider a cancer cell that has progressed through a series of mutations so that by activation of certain oncogenes and by loss of specific suppressor genes, it has become self-sufficient in growth signals, insensitive to antigrowth signals, unresponsive to apoptotic signals, capable of limitless replicative potential, and tumorigenic.¹ Are these neoplastic properties necessary and sufficient for such a cell to expand into a population that is clinically detectable, symptomatic, or lethal? Current evidence argues that the answer may be no. These neoplastic properties may only be necessary but not sufficient for the cancer cell to be lethal. The reported studies suggest that the microvascular endothelial cell dictates to a cancer cell whether it can grow a tumor to a clinically detectable size, metastasize, or kill its host. For a tumor to develop a metastatic or a lethal phenotype, it must first recruit and sustain¹ its own private blood supply, a process called tumor angiogenesis. Tumors unable to induce angiogenesis remain dormant at a microscopic in situ size. Such nonangiogenic lesions are usually not detectable unless they are on external surfaces such as skin, oral mucosa, or cervix. In a mouse, a nonangiogenic tumor is often called “no-take.”

Angiogenesis is fundamental to reproduction, development, and repair. In the adult, repair and reproductive angiogenesis occur mainly as brief bursts of capillary blood vessel growth that usually last only days or weeks. Such physiologic angiogenesis, including neovascularization in exercised muscle, is tightly regulated.²⁻⁴

A variety of circulating and sequestered inhibitors suppress proliferation of vascular endothelium under normal conditions. As a result, endothelial cells are among the most quiescent cells of the body. Turnover times of endothelial cells are measured in hundreds of days in contrast to bone marrow cells, which maintain an average turnover time of 5 days and proliferate at approximately 6 billion cell divisions per hour. During angiogenesis, microvascular endothelial cells can proliferate as rapidly as bone marrow cells. Furthermore, endothelial proliferation is not the only event necessary for development of a new capillary blood vessel. Endothelial cells must degrade their own basement membrane, develop sprouts from preexisting microvessels, invade the extracellular matrix, form tubes, and connect the tips of these tubes to create loops capable of handling blood flow.^{5,6} Even in the absence of endothelial DNA synthesis in tissue that has been heavily

irradiated, new capillary blood vessels and their branches still develop for a few days.⁷

A hallmark of pathologic angiogenesis is persistent growth of blood vessels (i.e., sustained neovascularization). Angiogenesis that continues for months or years supports the progression of many neoplastic and non-neoplastic diseases.⁸ However, both physiologic and pathologic angiogenesis are usually focal. An angiogenic focus appears as only a tiny fraction or a small “hot spot” of proliferating and migrating endothelial cells that arise from a monolayer of resting endothelium of approximately 1000 m², an area the size of a tennis court. A cubic millimeter of human cardiac muscle contains approximately 2500 millimeters of capillary blood vessels, (as determined by stereologic methods).³ The fundamental objective of all antiangiogenic therapy is to return a pathologic neovascular focus to its normal resting state or to prevent its initiation.

HISTORIC BACKGROUND

For more than 100 years, tumors had been observed to be more vascular than normal tissues.⁹ This tumor hyperemia observed during surgery was explained by simple dilation of existing host blood vessels.¹⁰ Vasodilation was generally thought to be a side effect of metabolites or of necrotic tumor products escaping from the tumor. Three reports, although largely overlooked, suggested that tumor hyperemia could be related to new blood vessel growth—that is, to neovascularization—and not solely to vasodilation. A 1939 paper showed that whereas neovascularization of a wound in a transparent chamber in a rabbit ear regressed completely after the wound healed,¹¹ a tumor implant in the chamber was associated with accelerated growth of new capillary blood vessels. The other reports, in 1945 and 1947, demonstrated that new vessels in the neighborhood of a tumor implant arose from host vessels and not from the tumor itself.^{12,13} These papers notwithstanding, debate continued in the literature for two more decades about whether a tumor could expand to a large size (centimeters) by simply living on preexisting vessels.¹⁴ Even among the few investigators who accepted the concept of tumor-induced neovascularization, it was generally assumed that this vascular response was an inflammatory reaction, a side effect of tumor growth, not a requirement for tumor growth.¹⁵

DEPENDENCE OF TUMORS ON ANGIOGENESIS: THE BEGINNING OF THE FIELD OF ANGIOGENESIS RESEARCH

A HYPOTHESIS IS ADVANCED THAT TUMOR GROWTH IS ANGIOGENESIS DEPENDENT In 1971, I proposed a new view of the role of blood vessels in tumor growth in the form of a hypothesis that tumor growth is angiogenesis dependent.¹⁶ I suggested that tumor cells and vascular endothelial cells within a neoplasm may constitute a highly integrated ecosystem and that endothelial cells may be switched from a resting state to a rapid growth phase by a “diffusible” chemical signal from tumor cells. An additional speculation was that angiogenesis could be a relevant target for tumor therapy (i.e., antiangiogenic therapy). Because of the existing confusion between inflammation and angiogenesis, I attempted to distinguish between the two processes. These ideas arose from experiments in my laboratory in the early 1960s, which revealed that tumor growth in isolated perfused organs was severely restricted in the absence of vascularization of the tumors.¹⁷⁻²²

These concepts were not accepted at the time. Although a few investigators in the early 1970s perceived that tumors could actually induce neovascularization, the belief persisted that such neovascularization was an inflammatory host response to necrotic tumor cells and likely even a host defense response detrimental to the tumor.²³ Another obstacle to research on tumor angiogenesis was the conventional wisdom at that time that any new vessels induced by a tumor, like new vessels in a wound, would become “established” and thus could not involute. From this assumption, scientists concluded that antiangiogenic therapy could never regress a tumor; therefore, it would be fruitless to try to discover angiogenesis inhibitors. In this pessimistic atmosphere, it was not an easy task to produce compelling evidence that tumor growth depended on neovascularization. Eventual acceptance of the

*Because the field of angiogenesis research is growing so rapidly (1500 papers from January to December 1999), it is not possible to include all pertinent references. For some well-established subjects in this field that have hundreds of citations since the last edition of this book in 1996 (e.g., vascular endothelial growth factor), I have included selected primary references and important reviews. For newly emerging subjects (e.g., endothelial precursors), all of the primary references are cited here. I apologize in advance to any colleagues in this field whose work I have been unable to discuss.

1971 hypothesis was slow because it would be 2 more years before the first vascular endothelial cells were successfully cultured *in vitro*,^{24,25} 8 years before it was possible to grow capillary endothelial cells *in vitro*,²⁶ 11 years before the discovery of the first angiogenesis inhibitor,²⁷ and 13 years before the purification of the first angiogenic protein.²⁸

Throughout the 1970s, laboratory studies were devoted to demonstrating that: tumor vessels were new proliferating capillaries; the sequential steps of the angiogenic process; that qualitative and quantitative bioassays for angiogenesis could be developed²⁹; that viable tumor cells released diffusible angiogenic factors which stimulated new capillary growth and endothelial mitosis *in vivo*,^{30–32} despite the arrest of tumor cell proliferation by irradiation³³; that necrotic tumor products were not angiogenic *per se* (reviewed in Folkman and Cotran³⁴); and whether angiogenesis could be inhibited, if at all. These efforts were designed to provide supporting evidence that tumor growth was angiogenesis dependent. The field of angiogenesis research thus began as a laboratory effort to understand tumor angiogenesis. Today, however, the field has broadened to include a wide spectrum of basic science disciplines, from developmental biology to molecular genetics, as well as a variety of clinical specialties, from cardiology to ophthalmology.

EXPERIMENTAL EVIDENCE THAT TUMOR GROWTH IS ANGIOGENESIS DEPENDENT Indirect Evidence. By the mid-1980s, considerable experimental evidence had been assembled to support the hypothesis that tumor growth is angiogenesis dependent. The idea could now be stated in its simplest terms: “Once tumor take has occurred, every further increase in tumor cell population must be preceded by an increase in new capillaries which converge upon the tumor”.³¹ The hypothesis predicted that if angiogenesis could be completely inhibited, tumors would become dormant at a small, possibly microscopic size.²⁰ It forecast that whereas the presence of neovascularization would be necessary but not sufficient for expansion of a tumor, the absence of neovascularization would prevent expansion of a primary tumor mass beyond 1 to 2 mm³ and restrict a metastasis to a microscopic dormant lesion. Most non-neovascularized tumors are not clinically detectable, with the exception of surface lesions on the skin or the external mucous membranes.

The evidence for these predictions was mostly indirect because it was based on *in vitro* studies of tumor spheroids, measurements of the prevascular stage of tumors *in vivo*, and mechanical separation of tumors from their vascular bed. Direct experimental evidence did not become available until the late 1980s. Both types of evidence are summarized below because they provide, in part, the scientific basis for current clinical trials of different types of angiogenesis inhibitors (for review see Folkman³⁵):

1. In two-dimensional flat cultures, a population of tumor cells expands indefinitely as long as fresh medium is added and unlimited cell-free surface is provided (i.e., passage of cells to a new flask). In contrast, three-dimensional spheroids of the same cells, suspended in soft agar or methylcellulose, stop enlarging at a diameter of a few millimeters, despite repeated passage of the spheroids to fresh media.³⁶ In these “steady-state” spheroids, cell proliferation is balanced by cell death.^{37–39} This *in vitro* model is analogous to a dormant micrometastasis in which angiogenesis is blocked.⁴⁰
2. Tumors implanted into subcutaneous transparent chambers grow slowly before vascularization, and tumor volume increases linearly. After vascularization, tumor growth is rapid and tumor volume may increase exponentially.¹³
3. Tumor growth in the avascular rabbit cornea proceeds slowly and at a linear rate but converts to exponential growth after vascularization.⁴¹
4. Tumors suspended in the aqueous fluid of the anterior chamber of the rabbit eye remain in a dormant state: viable, avascular, and limited in size (<1 mm³). These tumors induce neovascularization of iris vessels, but the new vessels are out of reach of the tumors floating in the aqueous fluid. Once a tumor spheroid is pushed against the proliferating iris vessels, the tumor is neovascularized and can enlarge up to 16,000 times its original volume within 2 weeks.⁴²

5. Tumors grown in the vitreous of the rabbit eye remain viable but are restricted to diameters of less than 0.50 mm for as long as 100 days. Once such a tumor reaches the retinal surface, it becomes neovascularized and within 2 weeks can undergo a 19,000-fold increase in volume over the avascular tumor.⁴³ Cross-sectional histology of the avascular tumors reveals proliferating cells at the periphery of the tumor and necrotic tumor cells in its center.
6. Human retinoblastomas that have metastasized to the vitreous are viable and avascular and tumor expansion is restricted.⁴⁴
7. Within a solid tumor, the H³-thymidine labeling index of tumor cells decreases with increasing distance from the nearest open capillary vessel.⁴⁵ The tissue oxygen tension also decreases with increasing distance of a tumor cell from the center of a capillary vessel. Because the oxygen diffusion distance is 100 to 200 microns, tumor cells that exceed these distances from a capillary vessel become anoxic (as determined by infrared spectroscopy of tumors in transparent skin chambers in mice).⁴⁶
8. Tumors implanted into the chorioallantoic membrane of the chick embryo remain restricted in growth during the avascular phase but enlarge rapidly once they are vascularized.⁴⁷
9. Vascular casts of metastases in the rabbit liver reveal that tumors of up to 1 mm in diameter are usually avascular, but beyond that size are vascularized.⁴⁸
10. Human ovarian carcinoma may metastasize to the peritoneal membrane as tiny avascular seeds. These implants rarely grow beyond a limited diameter of a few millimeters, until after vascularization. This “avascular” state of peritoneal implants has also been demonstrated in mice with four different tumor types and is independent of whether the mice are immunocompetent or immunodeficient. The avascular peritoneal implants are less than 0.5 mm diameter and are of uniform size (Catherine Chen, unpublished data and personal communication).
11. In transgenic mice that develop carcinomas of the beta cells in the pancreatic islets, large tumors arise from a subset of preneoplastic hyperplastic islets, but only after they have become vascularized.⁴⁹
12. Neoplastic cells injected subcutaneously develop into tumors, which become vascularized at about 0.4 mm³. As tumor size increases, blood vessels continue to proliferate and are enveloped by tumor cells that appear to grow toward and around the new vessels. The vessels eventually occupy up to 1.5% of the tumor volume. This is a 400% increase in vascular density over normal subcutaneous tissue.⁵⁰ In this model, new microvascular sprouts that converge on a tumor are enveloped by tumor cells, giving the appearance that the tumor has been penetrated by vessels.
13. In colon carcinomas arising in rats after carcinogen exposure, there is an early phase (tumor diameter < 3.5 mm) during which the tumor is temporarily supplied by preexisting host microvessels that are dilated and widened.⁵¹ This stage is similar to “cooption” of blood vessels recently reported.⁵² Subsequently, new capillary vessels sprout and proliferate (angiogenesis), which leads to increasing microvessel density and rapid tumor growth.

Direct Evidence. It did not become possible to inhibit tumor angiogenesis by biochemical and molecular methods until the late 1980s and early 1990s. These experiments were based on (1) administration of molecules that inhibited angiogenesis specifically or selectively, (2) blockade of tumor-derived angiogenic factors, (3) transfection of dominant-negative receptors for an angiogenic factor into endothelial cells in the tumor bed, (4) transfection of angiogenesis inhibitor proteins into tumor cells, and (5) the development of spontaneous tumors in transgenic mice. Examples of such are listed below.

1. An angiogenesis inhibitor, TNP-470 (AGM-1470), a synthetic analogue of fumagillin, selectively inhibited proliferating endothelial cells *in vitro* and *in vivo*.⁵³ It potently inhibited tumor growth *in vivo* but did not inhibit tumor cells *in vitro*. At this writing, there are more than 60 reports from different laboratories of inhibition of 33 different types of human, mouse, rat, hamster, and rabbit pri-

- mary tumors in animals and of 23 different types of metastatic tumors including human tumor metastases in mice and rat and hamster tumor metastases. Tumor growth was inhibited at an average of 65% with a range of 43 to 100% (i.e., complete regression). Complete regression was observed in seven tumor types in mice. These included human neurofibrosarcoma, neurofibroma, breast cancer, gastric cancer, and choriocarcinoma, as well as mouse reticulum sarcoma and gastric carcinoma.⁵⁴ Because TNP-470 inhibited microvascular endothelial cells and not tumor cells, these studies provided the first clue that angiogenesis inhibitors could be broad spectrum anticancer agents that were not dependent on tumor types. The appearance of different therapeutic responses by different tumor types (e.g., 43 to 100%) was more adequately explained by the total angiogenic output of each tumor type matched against a fixed dose of angiogenesis inhibitor.
2. In another experiment, the cDNA for human basic fibroblast growth factor (bFGF) hybridized to a signal sequence was transfected into normal mouse fibroblasts.⁵⁵ The transfected fibroblasts became tumorigenic, exported bFGF, and were also highly angiogenic. They formed large lethal tumors when implanted into mice. The angiogenesis was mediated solely by the bFGF released from these tumors. Furthermore, the structure of the bFGF had been modified by site-specific mutagenesis so that two serines had been substituted for cysteines. Thus, the bFGF released by the tumor could be neutralized by a specific antibody that had no effect on natural bFGF. When this antibody was administered to the tumor-bearing mice, there was dramatic reduction in neovascularization and in tumor volume.
 3. In an analogous experiment, a neutralizing antibody to another angiogenic protein, vascular endothelial growth factor (VEGF), was administered to mice bearing tumors that induced angiogenesis solely by VEGF.⁵⁶ Tumor growth was inhibited by more than 90%. The antibody had no effect on the tumor cells in vitro. With but few exceptions, VEGF is considered to be a specific mitogen for vascular endothelial cells.⁵⁷
 4. The growth of brain tumors in nude mice was significantly inhibited or prevented when tumor angiogenesis was suppressed by a strategy in which a dominant-negative mutant of the receptor (Flk-1) for the angiogenic protein VEGF was introduced into host endothelial cells (carried by a retrovirus). This signaling-defective receptor mutant formed an inactive dimer with the native Flk-1 receptor on endothelial cells and prevented the formation of new capillary blood vessels in response to VEGF released by the tumor.⁵⁸
 5. Transformed cells were not tumorigenic until after they had become angiogenic.⁵⁹
 6. Specific immunologic inhibition of overexpression of the integrin $\alpha_v\beta_3$ on capillary endothelial cells resulted in apoptosis of proliferating endothelial cells, blocked neovascularization, and induction of tumor regression.⁶⁰
 7. Angiostatin, a 38 kD internal fragment of plasminogen (generated by Lewis lung carcinoma),^{61,62} endostatin, a 20 kD internal fragment of collagen XVIII⁶³ (generated from a murine hemangioma),⁶³⁻⁶⁵ and a 53 kD conformationally changed fragment of antithrombin III (generated from human small cell lung cancer),⁶⁶ are the first specific inhibitors of endothelial proliferation and of angiogenesis. They do not inhibit proliferation of resting confluent endothelial cells, epithelial cells, smooth-muscle cells, fibroblasts, or tumor cells in vitro. These proteins inhibit angiogenesis in the chick chorioallantoic membrane or in the mouse cornea. Both primary and metastatic tumors are markedly inhibited and tumor regression is achieved without toxicity or drug resistance.⁶⁵
 8. When tumor cells were transfected with the cDNA for angiostatin and implanted into mice, the higher the secretion of angiostatin, the slower the growth of tumors. However, tumor cell proliferation in vitro was not affected by angiostatin transfection.⁶⁷
 9. When tumors were transfected with the secretable antiangiogenic protein thrombospondin-2 (TSP-2), tumor growth was directly

proportional to suppression of angiogenesis. Tumor growth was inhibited up to 100% (i.e., microscopic dormant tumor or no tumor) when angiogenesis was suppressed completely. Tumor cell proliferation was independent of level of TSP-1 or TSP-2 production.⁶⁸ (Fig. 9.1).

10. Id1 and Id3 are helix-loop-helix proteins that may control differentiation by interfering with DNA binding of transcription factors. After targeted disruption of one allele of Id1 and two alleles of Id3, three different types of implanted tumors failed to induce angiogenesis, their growth was severely restricted, and they did not metastasize.⁶⁹ These mice, genetically engineered so that tumors cannot induce angiogenesis, provide compelling evidence that tumors are angiogenesis dependent.

METASTASIS IS ALSO ANGIOGENESIS DEPENDENT Experimental and clinical evidence suggests that the process of metastasis is also angiogenesis dependent. For a tumor cell to metastasize successfully, it must breach several barriers and be able to respond to specific growth factors.⁷⁰⁻⁷⁴ Thus, tumor cells must gain access to the vasculature in the primary tumor, survive the circulation, arrest in the microvasculature of the target organ,^{72,75} exit from this vasculature,⁷⁶ grow in the target organ, and induce angiogenesis.^{77,78} Therefore, angiogenesis appears to be necessary at the beginning as well as at the completion of the metastatic cascade.

In experimental animals, tumor cells are rarely shed into the circulation before a primary tumor is vascularized, but they can appear in the circulation continuously after neovascularization.^{79,80} The number of cells shed from the primary tumor correlates with the density of tumor blood vessels as well as with the number of lung metastases observed later. Tumor cells can enter the circulation by penetrating through proliferating capillaries that have fragmented basement membranes and are leaky.^{80,81} Further, angiogenic factors from tumors such as bFGF and VPF/VEGF induce increased production of plasminogen activator and collagenases in proliferating endothelial cells, thus further contributing to degradation of basement membranes.⁸²⁻⁸⁴ These degradative enzymes may facilitate the entry of tumor cells into the circulation. Tumor cells that have successfully metastasized may not immediately become neovascularized after reaching the target organ. Such a metastasis lacking angiogenic activity for any of a variety of reasons may remain as a microscopic tumor of 100 to 200 μm diameter indefinitely.^{61,85,86} It is generally assumed that in human dormant micrometastases (e.g., metastases appearing 5-10 years after removal of a breast cancer), tumor cells are not cycling or are in G_0 . Increasing experimental evidence, however, indicates that micrometastases can be held dormant by blocked angiogenesis that results in a balance of tumor cell proliferation and apoptosis.^{40,85} Finally, experimental metastases are as susceptible as primary tumors to control by specific angiogenesis inhibitors.^{62,87,88} For these inhibitors, the microvascular endothelial cell is the only target; tumor cells are not directly affected in vitro.

In many types of human cancer, microvessel density in a histologic section of the tumor is an independent prognostic indicator of the risk of future metastases⁷⁷ (see Clinical Applications, below). Since the report of Weidner and colleagues in 1991,⁷⁷ there have been 25 different reports (up to 1998) that in human breast cancer there is a positive association between tumor angiogenesis and metastatic risk in a total of 3475 patients.⁸⁹ Because of the clonal origin of metastases,^{78,87} a primary tumor containing a high proportion of angiogenic malignant cells is more likely to generate metastases that are already angiogenic when they arrive at the target tissue. In contrast, there were seven reports of no association between tumor angiogenesis and metastatic risk in 771 patients (for review see Folkman⁸⁹ and Gasparini and Harris⁹⁰). Several reports that show that microvessel density is not a prognostic marker for metastatic risk may be due to technical problems or to subjective variation because counting vessels that have been highlighted in a histologic section introduces subjective error. However, there is also the possibility that a primary tumor whose tumor cells generate high levels of a circulating angiogenesis inhibitor⁴⁰ could produce metastases that inhibit their own angiogenesis long after the primary tumor is controlled.

THE PREVASCULAR PHASE LIMITS TUMOR EXPANSION During the prevascular phase, when angiogenic activity is absent or insufficient, tumors remain small, with volumes measured in a few cubic millimeters. Growth of the whole tumor is slow, and doubling times for the whole tumor may be years. However, this does not mean that the tumor cells are proliferating slowly. Experimental studies show that tumor cells in a prevascular neoplasm may have a ^3H -thymidine labeling index as high as that of a large vascularized tumor. However, the prevascular tumor reaches a steady state in which generation of new tumor cells is balanced by a high rate of tumor cell death, or apoptosis.⁴⁹

When the prevascular phase of bladder cancer,⁹¹ cervical cancer,^{92,93} or cutaneous melanoma⁹⁴ is first detected, these lesions are usually thin, slowly growing, stable for months to years, asymptomatic, and rarely metastatic. For the majority of tumors, however, the prevascular stage is clinically undetectable and can only be observed microscopically. For example, in breast and prostate cancer, carcinomas *in situ* can be observed before and after neovascularization in the same specimen.^{77,95,96}

The size limits of experimental tumors when angiogenesis is blocked or absent are between approximately 0.2 mm diameter (e.g., for lung metastases in mice⁴⁰) and 2 mm (e.g., for vascular chondrosarcomas in rats,⁹⁷ having a tumor population of 10^5 – 10^6 cells). The differences in size of avascular preangiogenic tumors may be due in part to the capacity of tumor cells to survive under differing degrees of hypoxia.⁴⁶ However, if cancer cells are already angiogenic at the time of implantation, they can initiate angiogenesis before the tumor population would have reached the limiting size of a non-neovascularized tumor (i.e., 0.2–2 mm). The evidence for this conclusion is from a recent experiment in which only 20 to 30 angiogenic mammary tumor cells were implanted into a transparent skin chamber in a mouse.^{98,99} The cells elongated by day 2, migrated toward the nearest microvessels in a parallel orientation by day 4, divided unidirectionally, induced vascular dilation and tortuosity in the neighboring microvessels by day 6, and stimulated new vascular sprouts with intermittent blood flow by day 8. By this time, a microscopic colony of up to 300 to 400 cells was visible. Tumor cells preferentially grew contiguous to the microvessel sprouts, and by day 20 the tumor was filled with a newly formed vasculature. When a truncated soluble receptor (ex-Flk1) for vascular endothelial growth factor (VEGF) was injected into the chamber, some tumor cells underwent apoptosis and microscopic tumors regressed or became dormant within 5 days (i.e., before the appearance of neovascular sprouts). Whereas the soluble receptor had no effect on tumor cells *in vitro*, it potentially inhibited endothelial cell proliferation. This result provides compelling evidence for the operation of a two-way paracrine exchange of growth factors and survival factors between tumor cells and neighboring vascular endothelial cells (see below). In this model, tumor cells secrete angiogenic proteins that activate endothelial cells to elaborate chemoattractants for tumor cells. Simultaneously, the activated endothelium is preparing to send new sprouts toward the tumor. Vascular endothelial cells can produce at least 20 mitogens and antiapoptotic factors.¹⁰⁰ Many of these proteins, such as basic fibroblast growth factor (bFGF) and heparin-binding epithelial growth factor (HB-EGF), are stored in the extracellular matrix and could be mobilized by VEGF stimulation of endothelial cells to secrete proteases.¹⁰¹

It is also possible that VEGF elaborated from the tumor cells increases the permeability of local microvessels so that the tumor microcolony is bathed in nutrients even before neovascularization begins. The diffusion of nutrients (and oxygen) into the tumor bed would also be facilitated by loss of pericytes from microvessels due to endothelial elaboration of angiopoietin-2.¹⁰² Endothelial cells upregulate expression of angiopoietin-2 in the presence of tumor cells by an unknown mechanism. The discovery of a tumor-derived “angiopoietin-2 inducing factor,” would have important clinical implications. Further, plasma and fibrin leakage from the microvessels could facilitate chemotaxis and alignment of tumor cells,¹⁰³ as well as subsequent migration of endothelial cells.¹⁰⁴ VEGF also up-regulates synthesis of nitric oxide in vascular endothelium.^{105–107} Vasodilation and vessel tortuosity permit endothelial elongation and thus may be a prerequisite for endothelial cells to undergo mitosis and migration in

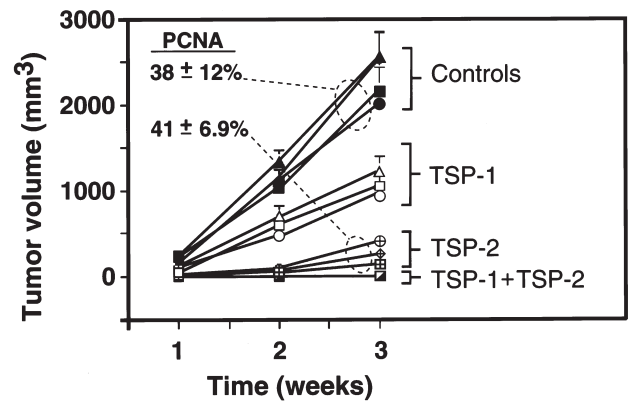


Figure 9.1. Squamous cell carcinoma cells transfected with thrombospondin-1 and or thrombospondin-2 and implanted into syngeneic mice. Different tumor growth rates are proportional to the extent of neovascularization of the tumor and are independent of tumor cell proliferation rate. (Modified from Streit et al.⁶⁸ with permission of the publisher.)

response to angiogenic factors like VEGF.¹⁰⁷ Even highly transformed neoplastic cells respond more efficiently to mitogens when the cells are elongated or spread than when they are rounded.^{108,109} Furthermore, when well-vascularized tumors in mice were treated with anti-VEGF monoclonal antibodies, there was a dramatic reduction in diameter, tortuosity, and vascular permeability in the tumor vessels.¹¹⁰

In summary, when a microscopic population of tumor cells emerges in avascular epidermis or mucosa—before becoming angiogenic—it may remain dormant as an *in situ* carcinoma separated from its vascular bed by a basement membrane¹¹¹ (Plate 3, Figs. 9.2A and 9.2B). If a nonangiogenic tumor emerges in vascularized tissue (i.e., an islet cell carcinoma¹¹¹) or a metastasis,^{40,86} it may form an *in situ* dormant microcylinder of tumor cells around capillary vessels (also called cooption⁵²). In all of these situations, the preangiogenic tumor is restricted in size to a range of approximately 0.2 mm⁴⁰ to 2 mm diameter.⁹⁷ In contrast, a microscopic population of *angiogenic* tumor cells will begin to modify microvessels in the neighborhood by inducing an increase in vascular permeability, dilation, and tortuosity even before the induction of new vascular sprouts.¹⁰⁰

HUMAN TUMORS AND SPONTANEOUS ANIMAL TUMORS SWITCH TO AN ANGIOGENIC PHENOTYPE Mechanisms of the Angiogenic Switch.

Normal cells that have been transformed to neoplastic cells are not usually angiogenic at the outset. Experimental studies of spontaneous tumors in transgenic mice reveal that the angiogenic switch is a discrete event that develops during progressive stages of tumorigenesis, beginning with the premalignant stage in these mouse models.^{111–113} By the time most human tumors are detected, for example, by a positive mammogram, neovascularization has already occurred. However, most human tumors arise without angiogenic activity, exist *in situ* without neovascularization for months to years, and then switch to an angiogenic phenotype.¹¹³ Therefore, the angiogenic phenotype appears after the expression of the malignant phenotype in the majority of primary tumors. However, for certain human tumors such as carcinoma of the cervix, the preneoplastic stage of dysplasia becomes neovascularized before the malignant tumor appears.⁹³ This sequence of events also occurs in certain spontaneously arising tumors in animals.¹¹⁵ At least four mechanisms of the angiogenic switch have been identified in both human tumors and spontaneous tumors in mice (Table 9.1).

Avascular Tumors Recruit Their Own Blood Supply. This is the most common mechanism of the angiogenic switch. Approximately 95% of human cancers are carcinomas that originate as microscopic *in situ* lesions in an avascular epidermal layer or in a mucosal layer, separated by a basement membrane from underlying vasculature in the dermis or submucosa, respectively. Once neovascularization has been stimulated by tumor cells, the basement membrane may not be immediately breached. In microscopic ductules of breast or prostate con-

Table 9.1. Types of Angiogenesis Switching*

1. Avascular tumors recruit their own blood supply.^{16,113}
2. Circulating endothelial stem cells can incorporate into an angiogenic focus.^{118–124}
3. Tumors can stimulate host cells.¹²⁵
4. Preexisting vessel can be coopted by tumor cells.⁵²

*A recent publication raises the question of whether certain tumors can make their own vascular channels for a short distance before they attach these to host vessels (e.g., “vascular mimicry”).¹¹⁶ This suggestion has provoked considerable controversy¹¹⁷ and remains unresolved.

taining in situ carcinoma that has switched to the angiogenic phenotype, but before the basement membrane has been breached, one can observe a ring of new microvessels encircling the ductule. However, these vessels remain temporarily separated from the tumor cells by intact basement membrane (see Figure 9.2A and 9.2B). After the basement membrane has been breached, new microvessels converge toward the tumor and become enveloped by tumor cells.

Circulating Endothelial Stem Cells. Recently, circulating endothelial cells, apparently derived from bone marrow, have been found to incorporate themselves into microvessels of neovascular foci. Thus, once a tumor angiogenic focus has been established, circulating endothelial precursors may home to it and enhance neovascularization at the site.^{118–124} Furthermore, circulating endothelial cells may be a target of angiostatin.¹²⁵

Stimulation of Host Cells. In addition to stimulating vascular endothelium in the host, certain tumors may also trigger host fibroblasts in the tumor bed to overexpress the angiogenic protein VEGF. This is another mechanism of amplification of the angiogenic phenotype once it has been initiated.¹²⁶

Vessel Cooption. In certain metastases, for example, in the mouse brain, it has been shown that tumor cells exit from microvessels in the target organ, begin to grow around these vessels, cause the endothelial cells to undergo apoptosis, and finally induce neovascular sprouts from neighboring vessels. This process, called “cooption,” may represent an intermediate or alternative step in the switch to the angiogenic phenotype.⁵²

The general hypothesis that tumors are angiogenesis dependent and that antiangiogenic therapy is a method of controlling tumor growth operates for any or all of these mechanisms of angiogenic switching.

Molecular Components of the Angiogenic Switch. The first proteins that positively or negatively regulate angiogenesis were discovered in the early 1980s.

Promoters of Angiogenesis. The observation in the 1970s that tumors implanted into the avascular cornea or onto the vascularized chick chorioallantoic membrane induced an ingrowth of new capillaries indicated that tumors released diffusible angiogenic factors.¹¹¹ This result motivated the development of in vitro and in vivo bioassays to guide the search for tumor-derived angiogenic factors.^{127,128}

Fibroblast Growth Factors. Basic fibroblast growth factor (bFGF or FGF-2) was the first angiogenic protein to be isolated and purified from a tumor (1982),²⁸ followed shortly by acidic FGF (aFGF or FGF-1) (for review see Christofori,¹²⁹ Friesel and Maciag,¹³⁰ and Folkman and Shing).¹³¹ There is extensive literature on the FGFs and their receptors (reviewed in Almaric and colleagues¹³²). Acidic and bFGFs stimulate endothelial cell mitosis and migration in vitro and are among the most potent angiogenic proteins in vivo. They have high affinity for heparin and heparan sulfate, are stored in extracellular matrix,¹³³ but lack a signal sequence for secretion. The expression of bFGF receptors is very low. Although many different cells synthesize bFGF, including tumor cells of the central nervous system, sarcomas,^{37,134–136} genitourinary tumors,¹³⁷ and even endothelial cells in the tumor vasculature,¹¹⁴ it is not clear how bFGFs released or mobilized, unless proteinases or heparanases mediate release of FGF from extracellular matrix.^{133,138} Furthermore, some tumors recruit macrophages¹³⁹ and activate them to secrete bFGF,^{114,140} whereas others attract mast cells, which, because of their high content of heparin, could sequester bFGF.¹⁴⁰ An unsolved

problem is how bFGF is exported from tumor cells in the absence of a signal sequence. In spontaneous tumors that arise in transgenic mice, aFGF and bFGF are exported into conditioned medium by angiogenic tumor cells but not by preangiogenic cells in earlier stages of tumor progression.^{49,112} Recent work on identification of an FGF-binding protein secreted by tumors into the extracellular matrix may illuminate a mechanism of tumor mobilization of stored FGFs.^{141,142} bFGF is not a specific endothelial mitogen but has several cell targets including fibroblasts, smooth-muscle cells, and neurons. Therefore, it is puzzling why experimental tumors transfected with bFGF containing an engineered signal sequence^{55,143} stimulate mainly endothelial proliferation almost to the exclusion of smooth muscle and fibroblast proliferation.⁵⁵ Could this be explained by the ability of endothelial-derived angiopoietin-2 to repel smooth muscle?¹⁴⁴ (See Angiopoietins below.)

bFGF interferes with adhesion of leukocytes to endothelium, and it has been suggested that tumors that elaborate bFGF may produce a form of local immunologic tolerance.^{145–147}

Abnormally elevated levels of bFGF are found in the serum and urine of cancer patients¹⁴⁸ and in the cerebrospinal fluid of patients with different types of brain tumors.¹⁴⁹ High bFGF levels in renal carcinoma correlated with a poor outcome.¹⁵⁰ Also, bFGF levels in the urine of children with Wilms’ tumor correlated with stage of disease and tumor grade.¹⁵¹

Vascular Endothelial Growth Factor/Vascular Permeability Factor. Dvorak first proposed that tumor angiogenesis is associated with increased microvascular permeability.¹⁵² This led to the identification of vascular permeability factor (VPF).¹⁵³ VPF was subsequently sequenced by Ferrara and shown to be a specific inducer of angiogenesis called vascular endothelial growth factor.^{154–156} Since then, at least 15 angiogenic inducers have been identified, most of them as tumor products (Table 9.2).¹⁵⁷

VEGF is an endothelial cell mitogen and motogen that is angiogenic in vivo.^{158–160} Its expression correlates with blood vessel growth during embryogenesis,^{161–164} with angiogenesis in the female reproductive tract,¹⁶⁵ and in tumors.¹⁶⁶ VEGF is a 40 to 45 kDa homodimeric protein with a signal sequence secreted by a wide variety of cells and the majority of tumor cells. VEGF exists as five different isoforms of 121, 145, 165, 189, and 206 amino acids, of which (VEGF₁₆₅) is the predominant molecular species produced by a variety of normal and neoplastic cells. Two receptors for VEGF are found mainly on vascular endothelial cells, the 180 kDa fms-like tyrosine kinase (Flt-1)¹⁶⁷ and the 200 kDa human kinase insert domain-containing receptor (KDR) and its mouse homolog, Flk-1.¹⁶⁸ VEGF binds to both receptors, but KDR/Flk-1 transduces the signals for endothelial proliferation and chemotaxis (reviewed in Klagsbrun and Moses⁶; See also references^{169–172}). Other structural homologues of the VEGF family have recently been identified, including VEGF-B, VEGF-C, VEGF-D, and VEGF-E.^{172,173} VEGF-C binds to Flt-4, which is preferentially expressed on lymphatic endothelium.¹⁷⁴ Neuropilin-1, a neuronal guidance molecule, is a recently discovered receptor for VEGF₁₆₅, but not for VEGF₁₂₁.¹⁷⁵ Neuropilin is not a tyrosine kinase receptor and is expressed on nonendothelial cells, including tumor cells. This allows VEGF that is synthesized by tumor cells to bind to their surface. Surface-bound VEGF could make endothelial cells chemotactic to endothelial cells, or it could act in a juxtacrine manner to mediate cooption⁵² of microvessels by tumor cells (see Fig. 9.2f, g, h).

VEGF is up-regulated by hypoxia^{176–178} and is often elevated near areas of tumor necrosis.^{179,180} Hypoxia activates a hypoxia inducible factor-1 (HIF-1)-binding sequence in the VEGF promoter, which leads to VEGF mRNA transcription and stability¹⁷⁸ (reviewed in Klagsbrun and Moses⁶).

VEGF expression is also up-regulated by the *ras* oncogene. The farnesyl transferase inhibitors inhibit *ras* expression. It has been suggested that at least one mechanism of their antitumor effect may be to inhibit angiogenesis by inhibiting VEGF expression.¹⁸¹

VEGF expression is inhibited by the von Hippel Landau (VHL) protein.¹⁸² The VHL tumor suppressor gene is inactivated in patients with VHL disease and in most sporadic clear-cell renal carcinomas.^{183,184} The VHL gene negatively regulates a series of hypoxia-inducible genes, including the VEGF, platelet-derived growth factor B,

and the glucose transporter GLUT1 genes¹⁸² (reviewed in Ferrara¹⁸⁵). When VHL is mutated or deleted, these genes are overexpressed even under normoxic conditions.

VEGF also induces fenestrations in endothelium of small venules and capillaries and even in tissues where microvessels are not normally fenestrated (reviewed in Ferrara¹⁸⁵).¹⁸⁶ The increased permeability of tumor vessels as revealed, for example, by the edema of brain tumors, may be partly mediated by VEGF-induced fenestrations.

It is possible that certain other positive regulators of angiogenesis may operate through VEGF or be VEGF dependent. It remains to be seen whether the high angiogenic activity of bFGF is somehow acting indirectly through another endothelial mitogen such as VEGF (see below), because of the following observations. First, bFGF induces the expression of VEGF.¹⁸⁷ Second, the two endothelial mitogens act synergistically,¹⁸⁷⁻¹⁹⁰ to stimulate capillary tube formation *in vitro*. Third, systemic administration of a soluble receptor for VEGF (Flk-1) completely blocks cornea angiogenesis induced by implanted bFGF (Robert D'Amato and Calvin Kuo; personal communication). Another positive regulator of angiogenesis, transforming growth factor beta (TGF β), may also be dependent on VEGF. VEGF mRNA and protein are induced in fibroblasts and epithelial cells by (TGF β).¹⁹¹ In contrast, mRNA for placental growth factor, an angiogenic protein related to VEGF, is not induced by TGF β . These recent findings suggest that the angiogenic effect of TGF β is mediated partly by its induction of VEGF in tissues. A clinical implication of these studies is that angiogenesis inhibitors that block VEGF may inhibit other angiogenic promoters as well.

Angiopoietins. Angiopoietin-1 is a 70 kD ligand that binds to a specific tyrosine kinase expressed only on endothelial cells, called Tie2 (also called Tek).¹⁹²⁻¹⁹⁶ (A ligand for Tie1 has not been elucidated.) Like VEGF, angiopoietin-1 is an endothelial cell specific growth factor. Angiopoietin-1 however, is not a direct endothelial mitogen *in vitro*. Rather, it induces endothelial cells to recruit pericytes and smooth-muscle cells to become incorporated in the vessel wall. Pericyte and smooth muscle recruitment are mediated by endothelial production of PDGF-BB (and probably other factors) when Tie2 is activated by angiopoietin-1.¹⁹⁷ In mice that overexpress angiopoietin-1 in the skin there is increased vascularization.¹⁹⁵ The vessels are significantly larger than normal and the skin is reddened. The vessels are not leaky and there is no skin edema, in contrast to dermal vessels of mice overexpressing VEGF. In double transgenic mice expressing both angiopoietin-1 and VEGF in the skin, dermal angiogenesis is increased in an additive manner, but the vessels do not leak.¹⁹⁸ This model closely approximates angiogenesis in healing wounds (i.e., relatively nonleaky vessels with pericytes and some perivascular smooth-muscle cells contained in the vascular wall). In contrast, tumor vessels are leaky and thin-walled with a paucity of pericytes. Angiopoietin-2 blocks the Tie-2 receptor¹⁹⁶ and acts to repel pericytes and smooth muscle. It is produced by vascular endothelium in a tumor bed, but it is unclear how tumor cells mediate this. Nevertheless, tumor vessels remain thin "endothelial-lined tubes" even though some of these microvessels reach the diameter of venules (see Fig. 9.2 f, g, h). A key point is that angiopoietin-2 and VEGF together increase angiogenesis. However, if VEGF is antagonized or withdrawn at this point, endothelial cells may undergo apoptosis and new microvessels can regress.¹⁹⁸

These provocative results have important clinical implications. Angiogenesis inhibitors that block VEGF or its receptor may be capable of regressing a tumor if administered at an optimum schedule. The differences between wound healing angiogenesis and tumor angiogenesis suggest that tumor angiogenesis may be more vulnerable to certain angiogenesis inhibitors than wound angiogenesis. In a recent study, endostatin (see below) inhibited tumor growth, but did not delay wound healing in mice (J. Marler, personal communication).

Angiogenesis Inhibitors. At least three general categories of angiogenesis inhibitors have been reported to date: (1) synthetic inhibitors, peptides, or antibodies designed to interfere with various steps in the angiogenic process (e.g., antagonists of the integrin $\alpha_v\beta_3$)⁶⁰; inhibitors of metalloproteinases,^{215,216} or inhibitors of VEGF and other angiogenic promoters; (2) low molecular weight compounds (e.g., TNP-470,⁵³) thalidomide,²¹⁷⁻²¹⁹ angiostatic steroids including

tetrahydrocortisol,²²⁰ 2-methoxyestradiol^{217,221} squalamine²²²; and (3) endogenous (natural) proteins that generally prevent vascular endothelial cells from responding to a wide spectrum of angiogenic promoters (e.g., interferon-alpha,²²³ interleukin-12,²²⁴ platelet factor 4,^{27,225} thrombospondin-1,²²⁶ angiostatin,⁶¹ endostatin,⁶³ PEX,²²⁷ pigment epithelium-derived factor,²²⁸ and antiangiogenic antithrombin (an internal fragment of antithrombin III, named aaAT).⁶⁶

Angiostatin, endostatin, and aaAT are specific angiogenesis inhibitors. The rationale that led to their discovery is discussed here in detail because (1) these proteins have profoundly changed our thinking about how primary tumors and metastases regulate their own growth, (2) the method of their discovery provides a unique strategy that is leading to the elucidation of an enlarging family of endothelial inhibitor proteins that normally prevent angiogenesis or limit physiologic angiogenesis, and (3) endostatin is currently in Phase I clinical trial and angiostatin and aaAT are candidates for future clinical trials.

The first clue to the existence of endogenous angiogenesis inhibitors came from the discovery that interferon-alpha/beta inhibited endothelial cell migration^{229,230} and that platelet factor-4 inhibited endothelial proliferation.^{27,231,232} Both were subsequently shown to inhibit angiogenesis.^{233,234} However, Rastinejad and colleagues were the first to show that a tumor could generate an angiogenesis inhibitor.²²⁶ They subsequently proposed that the angiogenic phenotype was the result of a net balance of endogenous inhibitors and stimulators of angiogenesis.^{235,236} A nontumorigenic hamster cell line became tumorigenic in association with the loss of a suppressor gene and concomitant with the onset of angiogenic activity. The nontumorigenic line secreted high levels of an angiogenesis inhibitor. The truncated form of thrombospondin-1 (TSP-1), that decreased by about 96% in the tumorigenic cells.²³⁷ TSP-1 was shown to be regulated by the wild-type tumor suppressor p53 in fibroblasts⁵⁹ and in mammary epithelial cells.²³⁸ Loss of p53 function in the transformed derivatives of these cells dramatically decreased the level of angiogenesis inhibitor. Restoration of p53 up-regulated TSP-1 and raised the antiangiogenic activity of the tumor cells.

The demonstration by Rastinejad et al. that the switch to an angiogenic phenotype involved a negative regulator of angiogenesis generated by the tumor per se immediately suggested to me a unifying angiogenic mechanism to explain a well-recognized but unsolved clinical phenomenon that had previously been mysterious: the inhibition of tumor growth by tumor mass. A description of this phenomenon is quoted from the introduction of our paper⁶¹: "The removal of certain tumors e.g., breast carcinomas, colon carcinomas, and osteogenic sarcomas can be followed by rapid growth of distant metastases."²³⁹⁻²⁴² Postoperative chemotherapy was introduced mainly to prevent or delay the growth of secondary metastases. Several studies in terminally ill

Table 9.2. Endogenous Angiogenesis Promoters

Protein	Molecular weight (kDa)	Year	Reference
Basic fibroblast growth factor (FGF-2)	18	1984	28
Acidic fibroblast growth factor (FGF-1)	16.4	1984	199
		1985	200
Angiogenin	14.1	1985	201
Transforming growth factor alpha	5.5	1986	202
Transforming growth factor beta	25	1986	203
Tumor necrosis factor alpha	17	1987	189, 204
Vascular endothelial growth factor (VPF/VEGF)	40-45	1983	153
		1989	154, 205, 206
Platelet-derived endothelial growth factor	45	1989	207
Granulocyte colony-stimulating factor	17	1991	208
Placental growth factor	25	1991	209
Interleukin-8	40	1992	210
Hepatocyte growth factor	92	1993	211, 212
Proliferin	35	1994	213
Angiopoietin-1	70	1996	192
Leptin	16	1998	214

patients demonstrate the suppression of a secondary tumor by a primary tumor.^{136,243–245} A primary tumor can suppress metastasis from a different type of tumor (e.g., a breast cancer can inhibit melanoma metastases). In melanoma, partial spontaneous regression of the primary tumor may be followed by rapid growth of metastases. Regression of small-cell lung cancer by ionizing radiation may be followed by rapid growth of distant metastases.⁶⁶ If one portion of a primary tumor is removed (e.g., cytoreductive surgery for testicular cancer), the residual tumor increases its rate of expansion.²⁴⁶ The same phenomenon is observed in several different animal tumors, that is, that some primary tumors may inhibit the growth but not the number of their metastases.^{247–254} Partial removal of a primary tumor increases growth rate of the residual tumors similar to humans.^{255,256} Furthermore, metastatic growth can suppress the growth of a primary tumor²⁵⁷ (analogous to the occult primary in a cancer patient). Many primary tumors can suppress the growth of a second tumor inoculation.^{258–265} This “resistance” to a second tumor challenge is inversely proportional to the size of the tumor inoculum and directly proportional to the size of the first tumor. A threshold size is necessary for the inhibitory effect to occur, and some primary tumors can inhibit a secondary tumor of a different type.^{263,264,266}

At least three hypotheses have been advanced to explain these diverse observations and experiments: (1) “concomitant immunity”—a primary tumor induces an immunologic response against a secondary tumor or a metastasis in the same host,^{259,264,267} (2) depletion of nutrients by the primary tumor, or (3) production of antimitotic factors from the primary tumor that directly inhibit the proliferation of the secondary tumor.^{268–274} None of these concepts, however, offered a molecular mechanism to explain all of the experiments cited above, in which tumor growth is suppressed by tumor mass. Once it was demonstrated that a tumor could generate a negative regulator of angiogenesis,²²⁶ then it became clear that a primary tumor, while stimulating

angiogenesis in its own vascular bed, could possibly inhibit angiogenesis in the vascular bed of a distant metastasis. However, at least two conditions would be necessary: first, the primary tumor (i.e., the first tumor to grow) would need to generate an angiogenic promoter in excess of an inhibitor in its own vascular bed and, second, the putative inhibitor would need to have a longer half-life in the circulation than the angiogenic promoter. After arriving in my laboratory in the summer of 1991, Michael O’Reilly validated this hypothesis²⁷⁵ by discovering angiostatin, endostatin, and antiangiogenic antithrombin over the next 8 years.^{61–63,65,66}

Angiostatin. Angiostatin is a 38 kD internal fragment of plasminogen (Fig. 9.3A) that was purified from serum and urine of mice bearing a subcutaneous Lewis lung carcinoma that suppressed growth of its lung metastases by inhibiting their angiogenesis⁶¹ (Plate 4, Fig. 9.4A). Lung metastases remained microscopic and did not grow beyond approximately 200 μm diameter. They were not neovascularized and usually formed a microcylinder around a single microvessel. In these dormant metastases, 38% of the tumor cells were proliferating (DNA synthesis determined by bromodeoxyuridine) and 7 to 8% were undergoing apoptosis.⁴⁰ The presence of a circulating angiogenesis inhibitor was evidenced by the almost complete inhibition of bFGF-stimulated corneal neovascularization in these mice (see example in Fig. 9.4D). Further, serum from these tumor-bearing mice specifically inhibited capillary endothelial cell proliferation in vitro by >70% (but not fibroblast, smooth-muscle cell, epithelial cell or tumor cell proliferation). The tumor-bearing serum also inhibited angiogenesis on the chick chorioallantoic membrane compared to serum from mice without tumors (which stimulated endothelial cells). After removal of the primary tumor, serum endothelial inhibitory activity disappear by 6 days (half-maximal 2.5 days), lung metastases were neovascularized by 5 days (see Fig. 9.4C), and by 15 days the mice were dying of large vascularized metastases (see Fig. 9.4B, bottom panel). There was no change in tumor cell proliferation (approximately 38% bromodeoxyuridine [BrdU] labelled cells), but tumor cell apop-

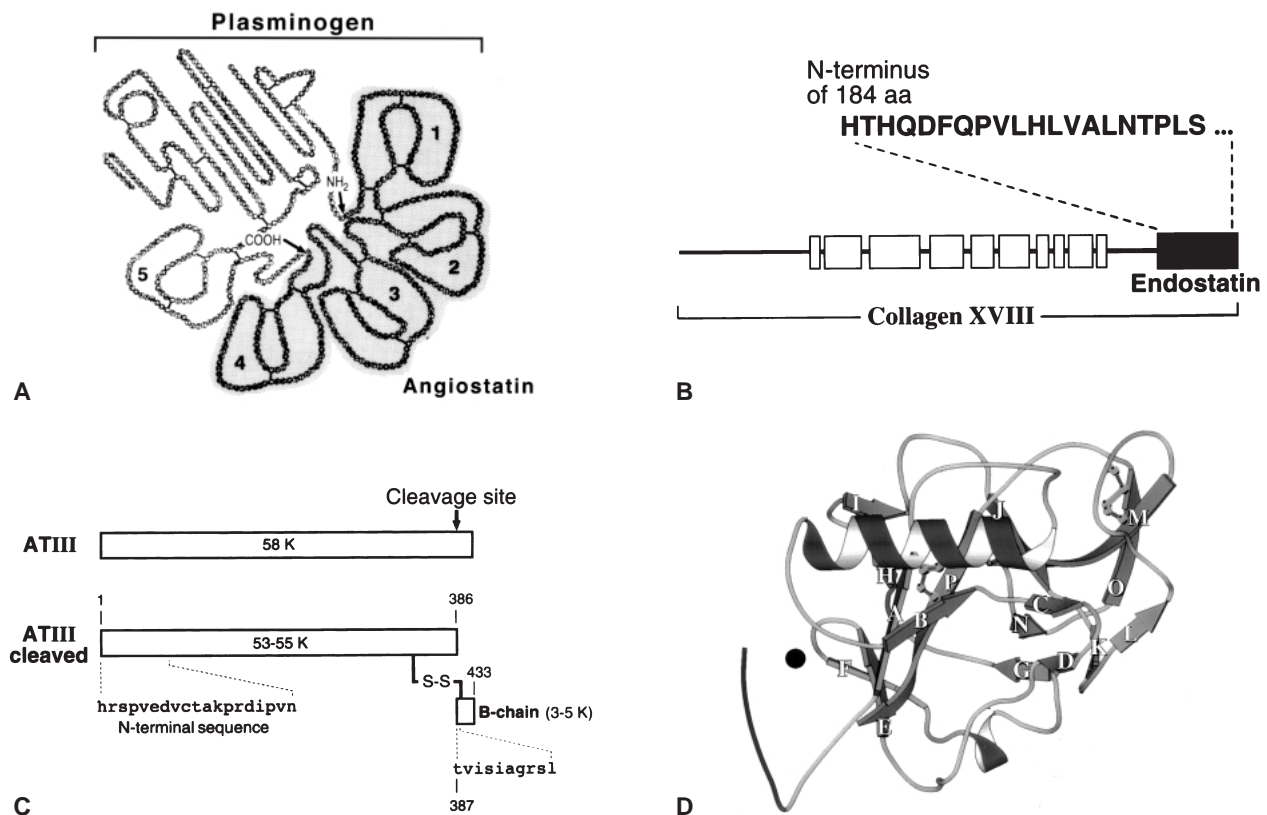


Figure 9.3. Amino acid sequences of **A**, angiostatin, **B**, endostatin, and **C**, 53 kD fragment of antithrombin III. **D**, Crystal structure of human endostatin. (References are in text.)

tosis fell to 2%. Systemic administration of angiostatin purified from mouse urine significantly inhibited angiogenesis in lung metastases and restricted their growth to a microscopic dormant size,⁶¹ and recombinant angiostatin potently inhibited growth of other tumor types.⁶² Again, tumor cell proliferation remained as high as in the tumor-bearing mice, and apoptosis was significantly reduced.⁴⁰ Angiogenesis inhibitor in the urine of tumor-bearing mice was completely removed by angiostatin neutralizing antibodies. A subline of Lewis lung carcinoma incapable of inhibiting metastatic growth did not generate angiostatin or inhibit corneal angiogenesis, nor did its serum inhibit endothelial cell growth. VEGF, the major angiogenic promoter in Lewis lung carcinoma, has a half-life of approximately 3 minutes in the circulation.

Angiostatin is not secreted by tumor cells but is generated through proteolytic cleavage of circulating plasminogen by a series of enzymes released from the tumor cells. At least one of these tumor-derived enzymes, uPA, converts plasminogen to plasmin, while a phosphoglycerate kinase²⁷⁶ from hypoxic tumor cells then reduces the plasmin so that it can be converted to angiostatin by one of several different metalloproteinases (Fig. 9.5). Other types of tumors have since been reported to generate angiostatin (e.g., human prostate cancer).^{277–279} Human prostate carcinoma cells express enzymatic activity that converts human plasminogen to the angiogenesis inhibitor angiostatin.²⁷⁶ Prostate-specific antigen generates angiostatin-like fragments from plasminogen.²⁸⁰

Furthermore, when murine fibrosarcoma cells were transfected with angiostatin, primary subcutaneous tumors formed whose growth was slowed in proportion to increased levels of angiostatin production by the tumor cells. In these tumors, the total angiogenic output of the primary tumor was decreased by transfected angiostatin, which opposed in a dose-dependent manner the activity of the tumor's secreted angiogenic promoter but never completely counteracted it.⁶⁷ It should be emphasized that the rate of tumor growth (expansion of tumor mass) was directly proportional to total angiogenic output of the tumor, inversely proportional to angiostatin production and to tumor cell apoptosis, and virtually independent of tumor cell proliferation. In a similar recent experiment, growth of squamous carcinomas in mice was dramatically inhibited when tumor cells were transfected with the angiogenesis inhibitors thrombospondin-1 and/or thrombospondin-2.⁶⁸ Tumor growth was directly proportional to angiogenic output of the tumor, as quantified by microvessel density, inversely proportional to production of the angiogenesis inhibitor, and independent of proliferation rate (as determined by staining for the proliferating cell nuclear antigen [PCNA]) (see Fig. 9.1). In these studies, transfected thrombospondin was secreted by the tumor cells.

Angiostatin and its isoforms induce cell arrest and apoptosis of endothelial cells^{281–286}, inhibit endothelial migration^{287,288}, inhibit angiogenesis *in vitro*²⁸⁹, inhibit angiogenesis in the quail chorioallantoic membrane, which provides a quantitative bioassay²⁹⁰, can be generated by different enzymes and by other cell types^{276–278,291–297}; can inhibit other tumor types^{298–303}; decrease activity of the mitogen-activated protein kinase ERK-1 and ERK-2 in endothelial cells³⁰⁴; up-regulate E-selectin in proliferating endothelial cells³⁰⁵; can be delivered *in vivo* by gene therapy^{278,306}; bind specifically to ATP-synthase, a transmembrane protein expressed by vascular endothelial cells³⁰⁷; bind to a fragment of vitronectin³⁰⁸; and potentiate radiation therapy of experimental tumors.^{309,310}

A potential molecular mechanism of angiostatin has been demonstrated most recently in my laboratory by Debra Chao. In the presence of angiostatin, hyperphosphorylation of retinoblastoma (Rb) protein and the kinase activity of cyclin-dependent kinase CDK2 were suppressed after cells were released from quiescence by adding bFGF and serum. Importantly, angiostatin-treated endothelial cells had increased levels of the CDK inhibitor p21Cip1 but not p27Kip1, suggesting that angiostatin may regulate specific cell-cycle mediators(s) to control the growth of endothelium.³¹¹

A provocative recent finding is that proliferation of circulating precursor endothelial cells are inhibited at significantly lower concentrations of angiostatin than are endothelial cells isolated from tissues.¹²⁵ Because precursor endothelial cells from bone marrow can home to angiogenic sites and participate in new vessel formation in a

tumor (see above), it has been speculated that at least one mechanism of angiostatin is to inhibit this subpopulation of endothelium, and that these cells may be employed as a sensitive bioassay for identification of novel antiangiogenic molecules.¹²⁵

When a strategy similar to the one that uncovered angiostatin (e.g., suppression of a tumor growth by tumor mass), was employed by O'Reilly with murine hemangioendothelioma and human small-cell lung cancer, endostatin⁶³ and antiangiogenic antithrombin (aaAT)⁶⁶ were discovered. Both endostatin and aaAT are generated from larger parent proteins by enzymes released by the tumor cells.

Endostatin. Endostatin^{63,65} is a 20–22 kDa internal fragment of collagen XVIII^{312,313} (see Fig. 9.3B). It was isolated and sequenced from conditioned medium of murine hemangioendothelioma³¹⁴ based on the same strategy employed for the discovery of angiostatin (i.e., hemangioendothelioma suppressed secondary tumors). It is a specific inhibitor of endothelial cell proliferation and migration like angiostatin. The crystal structures for mouse endostatin³¹⁵ and for human (see Fig. 9.3D) endostatin have been elucidated.³¹⁶ At this writing, one of the enzymes produced from medium conditioned by hemangioendothelioma cells has been identified as an elastase.³¹⁷ Endostatin is present in basement membranes and vessel walls and is especially rich in elastic fibers of the aorta and sparse elastic fibers of veins.³¹⁸ Some, but not all, capillaries or arterioles show weak labelling for endostatin. Within the elastic fibers, endostatin is co-localized with fibulin-2, fibulin-1, and nidogen-2 and binds to these components of the elastic fibers. Tumors rarely invade or grow in the walls of large arteries. This could possibly be related to the high endostatin content of these vessels. It remains to be demonstrated whether endostatin plays a role in limiting plaque angiogenesis in coronary or carotid arteries under normal conditions.³¹⁹

Systemic administration of endostatin can inhibit or regress different tumors. When endostatin was administered for prolonged periods of time in mice (185 days for Lewis lung carcinoma, 160 days for T241 fibrosarcoma, and 80 days for B-16 melanoma), there was no drug resistance. After discontinuation of endostatin at these time periods, tumors did not recur. They remained dormant at a microscopic size.⁶⁵ This was not due to an immunologic mechanism but to some type of unknown modification of the local tumor bed that rendered the tumor unable to mount an angiogenesis response. Wounding the tumor site or moving the tumor to another site in the same mouse initiated tumor neovascularization and growth. We have observed this same dormancy after 40 days of endostatin therapy of rat mammary cancer induced by oral carcinogen. Yamaguchi and colleagues reported that endostatin administered into a tumor will inhibit tumor growth at significantly lower doses than systemic administration.³²⁰ In our experience, complete removal of zinc from endostatin almost completely inactivates its tumor-inhibiting activity when administered systemically,³²¹ but zinc appears to be unnecessary for intratumoral administration. The reason for this difference is unclear but may possibly

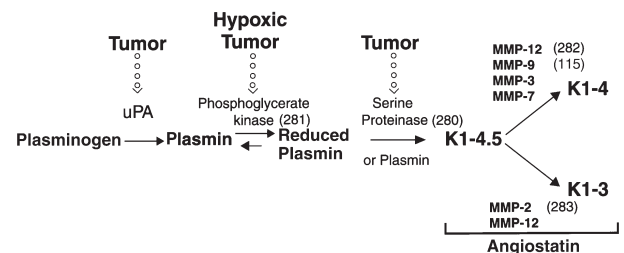


Figure 9.5. Current model of angiostatin formation. Urokinase plasminogen activator from a tumor cleaves plasminogen to plasmin. Plasmin in this state is still relatively resistant to further proteolysis until it is subsequently reduced by a phosphoglycerate kinase that is generated by hypoxic tumor cells.²⁷⁶ Reduced plasmin can be further cleaved to angiostatin (kringle 1–4.5) either by a tumor-derived serine proteinase,²⁷⁷ or by autolysis. Smaller forms of angiostatin (e.g., Kringle 1–3) can then be generated by metalloproteinases, such as metalloproteinases 7 and 9,¹¹⁵ metalloproteinase-12,²⁷⁸ or metalloproteinase-2.²⁷⁹

depend on stability of the protein in the circulation. For recent reports on methods of production of endostatin and studies of its mechanism of action on endothelial cells and its storage in the vessel wall, see references 318, 320, and 322 to 331. Endostatin gene therapy has been used in tumor-bearing mice.³³² Although endostatin is an internal fragment of collagen XVIII, a novel angiogenesis inhibitor has recently been isolated from collagen XV called restin.³³³ Taken together, these findings suggest that extracellular matrix may be a storehouse of antiangiogenic peptides.

Human endostatin produced in yeast entered Phase I clinical trial in October 1999, for patients with advanced cancer, just as this chapter was being finalized.

Antiangiogenic Conformation of Antithrombin III. A human small-cell lung carcinoma suppressed angiogenesis and tumor growth at remote sites in immunodeficient mice (see Fig. 9.4, *F, G*). These cells generated an enzyme in vitro that converted the 58 kD conformation of circulating antithrombin III to a 53 kD form of the protein in which the externally configured stressed loop of antithrombin was retracted into the body of the molecule⁶⁶ (see Fig. 9.3 *C*). The 53 kD “cleaved” form is a specific endothelial inhibitor and a potent angiogenesis inhibitor and has no thrombin binding activity. Antithrombin III has no antiendothelial or antiangiogenic activity. The enzyme(s) that induce this conformational change have not yet been elucidated. Human pancreatic cancer also generates the 53 kD cleaved antiangiogenic antithrombin (Steven Pirie-Shepherd, Oliver Kisker, and J. Folkman, unpublished data).

Tropinin I. A novel protein, tropinin I, was purified from cartilage during an attempt to find the inhibitors responsible for the avascularity of cartilage.³³⁴ It is a 22 kD subunit of the tropinin complex that, along with tropomyosin, is responsible for the calcium-dependent regulation of striated muscle contraction. It is a specific inhibitor of endothelial cell proliferation and of angiogenesis in the mouse cornea and chick embryo. It inhibits growth of primary tumor and metastases.

Other Endogenous Angiogenesis Inhibitors. In addition to the specific angiogenesis inhibitors discussed above, the plasma and tissues contain other endogenous angiogenesis inhibitors that, although not specific, appear to preferentially inhibit endothelial proliferation and/or migration. These inhibitors have quite different structures. However, because so many inhibitors have been identified to date suggests the existence of a machinery dedicated to the physiologic suppression of angiogenesis. An analogy may be drawn to the more than 40 proteins of the clotting system, many of which function to prevent coagulation under normal conditions (see below). Endogenous angiogenesis inhibitors include interferon beta,³³⁵ platelet factor 4,^{225,336} fibronectin,^{337,338} thrombospondin-1,^{226,235,237,339–341} tissue inhibitors of metalloproteinases (TIMPs),^{215,216,342,343} certain angiostatic steroids such as tetrahydrocortisol,²²⁰ a nonglucocorticoid, nonmineralocorticoid metabolite of cortisol, and 2-methoxyestradiol,³⁴⁴ and interleukin-12.²²⁴ (In addition to its cytotoxic properties, mediated by T-cell activation, interleukin-12 also induces the up-regulation of interferon gamma, which itself up-regulates inducible protein 10 (IP-10), recently shown to be an angiogenesis inhibitor.³⁴⁵ Certain cryptic antiangiogenic protein fragments are contained within larger proteins of the hemostatic system, in addition to angiostatin and the cleaved conformation of antithrombin III. These include an internal fragment (domain 5) of high molecular weight kininogen in plasma³⁴⁶ and the first kringle domain (NK1), or the first two kringle domains (NK2) of hepatocyte growth factor (HGF), itself a stimulator of angiogenesis in platelets. The hemostatic system, like the extracellular matrix, also appears to store certain angiogenesis inhibitors, which may be needed during wound healing angiogenesis (for review see Browder and colleagues³⁴⁷).

CLINICAL PATTERNS OF METASTASIS MAY BE GOVERNED BY ANGIOGENIC MECHANISMS Cancer metastases may present at least four common clinical patterns (Table 9.3): (I) a primary tumor such as a colon carcinoma is removed, but within a few months metastases appear; (II) metastases are already present when the primary tumor is first detected; (III) metastases appear first, and the primary remains occult; and (IV) the primary is removed (or treated by other therapy),

and metastases do not appear until years later (e.g., 5–10 years). A fifth and rare pattern is that metastases disappear after removal of the primary tumor (e.g., a few cases of renal cell carcinoma). These patterns of metastatic presentation are well recognized, but their biologic basis has been poorly understood.

New experimental evidence suggests that the majority of the presenting patterns of metastases may be dictated by the intensity of angiogenesis in their vascular bed. The essential role that angiogenesis plays in the metastatic cascade can be appreciated by examining animal models that have been developed for each of the common presenting patterns of metastases in cancer patients. The patient whose metastases appear within a few months after removal of the primary tumor (metastatic pattern I in Table 9.3) is analogous to a mouse model of Lewis lung carcinoma in which lung metastases remain microscopic while the primary is present but grow rapidly a few days after the primary tumor is removed. In this model, the primary tumor directly inhibits angiogenesis in the bed of the lung metastases. The metastases remain unvascularized and restricted to a radius of approximately 150 μm .⁶¹ Angiogenesis in the primary tumor is mediated mainly by VEGF, which is presumably present at higher concentrations than local angiostatin. Because of up-regulation of VEGF receptors on endothelial cells in hypoxic areas,^{57,177} it is also possible that VEGF is retained in the vascular bed of the primary tumor. The half-life of VEGF in the circulation is approximately 3 minutes (N. Ferrara, personal communication). This rapid clearance would prevent VEGF from accumulating in the plasma. In contrast, the half-life of the anti-mitogenic activity (against endothelial cells in vitro) of serum from mice bearing an angiostatin-generating tumor is approximately 2.5 days.⁶¹ Also, it appears to accumulate in the serum with increasing size of the primary tumor.⁶¹

The patient whose metastases are already present when the primary tumor is first diagnosed (metastatic pattern II in Table 9.3) is analogous to a mouse model of a subclone of Lewis lung carcinoma in which the primary tumor does not suppress its lung metastases and does not generate detectable levels of angiostatin in the circulation.⁶¹

The patient who presents with metastases in the absence of a detectable primary tumor (metastatic pattern III in Table 9.3 called the “occult primary”) is similar to a mouse model in which metastatic cells inhibit the growth of the primary tumor (although it has not been ascertained whether the inhibition was mediated by a circulating angiostatic protein).²⁵⁷ We speculate that if metastases in a patient are shed from a small primary tumor soon after it becomes neovascularized, the tumor may not be large enough to suppress angiogenesis in remote metastases. In mice with angiostatin-generating tumors, the primary tumor had to be at least 0.6 to 1.0 cm^3 before angiostatin could be detected in the circulation.⁶¹ Further, if the metastases have a slightly faster proliferation rate than the primary tumor, they could increase their total mass faster than the primary tumor. At this point they produce sufficient quantities of circulating angiogenesis inhibitor and suppress the primary tumor—an example of a secondary tumor inhibiting its primary lesion.

The patient whose metastases do not appear until years after removal of the primary tumor (metastatic pattern IV in Table 9.3) is analogous to a mouse model of B-16 melanoma in our laboratory in

Table 9.3. Metastatic Patterns in Cancer Patients

<i>At first diagnosis:</i>		Recurrence of metastases
Primary Tumor	Metastases	
I	+	○
II	+	+
III	○	+
IV	+	○
V	+	+
(Metastases regress when primary tumor removed: renal carcinoma, rare.)		

which dormant but viable nonangiogenic lung metastases of less than 0.1 to 0.2 mm diameter were found months to a year after removal of the primary tumor (M. O'Reilly, unpublished data). The mice were healthy.

There are rare reports of regression of metastases after removal of a primary renal cell carcinoma (metastatic pattern V in Table 9.3). The animal model that most closely resembles this clinical pattern is V2 carcinoma in the rabbit. Lung metastases grow as long as a primary tumor is growing in the thigh. However, surgical removal of the thigh tumor is followed by regression of the metastases (H. Verheul and R. D'Amato, unpublished data). This does not appear to be an immune reaction because fresh tumor can be successfully grown in the same rabbit. One can speculate that the metastases may have been dependent on high production of circulating angiogenic factors and possibly other growth factors from the primary tumor.⁸⁵ In human renal cell carcinomas, high tissue levels of bFGF correlate with high mortality.¹⁵⁰ In fact, in our own study of bFGF in serum and urine, 10% of a group of patients with a wide spectrum of malignancies had abnormally elevated levels of the angiogenic polypeptide bFGF in their serum and 37% of 950 patients had abnormally elevated levels of bFGF in their urine.¹⁴⁸

The similarity of such animal models to human patterns of metastatic presentation does not prove that angiogenic control of metastatic growth is a central mechanism of dormancy, nor does it mean that the human patterns are all based on angiogenic mechanisms. These models are described here because they offer a plausible unifying mechanism to explain the different patterns of metastasis presentation in cancer patients. The detailed experimental evidence is developed elsewhere by Holmgren and colleagues.⁴⁰ Further attempts to uncover evidence that supports or refutes the hypothesis may be fruitful. Finally, to the extent that angiogenic processes are operating in human primary tumors and metastases, then it may be prudent to include this in thinking about the design of clinical trials of angiogenesis inhibitors.

A fundamental principle underlying these clinical patterns is that the dormant tumor in all of these patients may depend on blocked angiogenesis leading to a microscopic tumor with high replication and high death rate of its tumor cells. This is an alternative hypothesis to the widely held assumption that tumor cells in a dormant microscopic tumor are not cycling and remain in G₀.

Leukemia is Angiogenesis Dependent. Because free floating leukemic cells appear in the peripheral circulation, leukemia and other malignant hematologic diseases have traditionally been regarded as "liquid tumors." Therefore, it has been widely assumed that leukemia does not require angiogenesis for its growth. However, when bone marrow biopsies from children with newly diagnosed untreated acute lymphoblastic leukemia were evaluated for angiogenesis by immunohistochemical staining of microvessels with antibody to von Willebrand factor,³⁴⁸ there was a six- to seven-fold increase in microvessel density in the leukemic marrows in contrast to control bone marrows from children undergoing staging evaluations at the time of diagnosis of solid tumor³⁴⁹ (Plate 5, Fig. 9.6). When three-dimensional 50- μ m-thick confocal microscopic sections of the bone marrows are compared with thin 4- μ m histologic cross-sections, the leukemic cells are observed to be clustered around new vessels like grapes on a vine. This configuration of neoplastic cells and vascular endothelial cells is similar to solid tumors. Urinary levels of the angiogenic protein bFGF were also approximately seven-fold higher in the leukemic children than in controls. In adults with acute myeloid leukemia, cellular levels of the angiogenic factor VEGF are abnormally elevated and provide an independent predictor of outcome.³⁵⁰ The bone marrow in multiple myeloma is also highly neovascularized.^{352-354,382}

The myeloproliferative diseases, polycythemia vera, chronic myelocytic leukemia, and myelofibrosis, also have significantly increased neovascularity (Jan Palmblad, unpublished data, personal communication). Further, human hematopoietic cells express high levels of bFGF, a potent endothelial cell mitogen.³⁵⁵ Release of bFGF from bone marrow cultures has also been reported.³⁵⁶ Moreover, vascular endothelial cells can release G-CSF (a mitogen for marrow cells).³⁵⁷ However, to demonstrate that leukemia is angiogenesis dependent, Timothy Browder in my laboratory has shown that endostatin, a specific angiogene-

sis inhibitor, can significantly prolong survival or cure either B-cell, T-cell, or myelogenous leukemias in mice (T. Browder, personal communication, unpublished data). These observations provide a conceptual basis for the future use of angiogenesis inhibitors in leukemia, perhaps first in patients in whom all conventional therapy has failed and later as an adjunct to conventional therapy.

The Host Angiogenic Response May be Genetically Controlled.

A recent finding is that different strains of inbred mice have an approximately 10-fold range of response to a constant dose of angiogenic stimulation (bFGF) in the corneal micropocket assay.³⁵⁸ Furthermore, the *in vitro* migratory activity of endothelial cells from aortic rings of selected strains correlates with the *in vivo* responsiveness. Also, high angiogenesis responders require higher doses of an angiogenesis inhibitor to achieve the same suppression of angiogenesis as a low dose of inhibitor in a low angiogenesis responder. If this early work translates to humans, one can speculate that a low angiogenic host response would decrease the probability of *in situ* carcinomas switching to the angiogenic phenotype, that tumors that did become angiogenic would grow slowly or be indolent (e.g., indolent prostate cancer), and that relatively low doses of angiogenesis inhibitors would be necessary to achieve effective therapy. In contrast, a high angiogenic host response would predict a higher frequency of *in situ* switching to the angiogenic phenotype, faster growing tumors, and significantly higher doses of antiangiogenic therapy to achieve efficacy.

CLINICAL TRANSLATION OF ANGIOGENESIS RESEARCH Clinical Trials of Angiogenesis Inhibitors. At this writing, 19 angiogenesis inhibitors produced by the biotechnology and pharmaceutical industry are in clinical trial for patients with advanced metastatic cancer—5 are in Phase III (Table 9.4). These inhibitors have been developed by the following strategies: (1) identification of a target molecule and development of an antibody or a synthetic inhibitor to counteract it (e.g., an antibody to VEGF), (2) discovery that an old drug has antiangiogenic activity (e.g., thalidomide, interferon alpha), and (3) discovery of specific endogenous angiogenesis inhibitors (e.g., endostatin). The inhibitors operate by quite different mechanisms, which reveal that multiple pathways in the angiogenic process are vulnerable to attack. Metalloproteinase inhibitors can block proteolytic activity initiated by endothelial cells as they invade tissue (e.g., Marimastat). Blockade of endothelial adhesion to specific integrins (e.g., $\alpha_v\beta_3$) in the extracellular matrix leads to apoptosis of proliferating endothelial cells (e.g., Vitaxin). Direct antagonists of angiogenic stimulators usually block one angiogenic factor such as VEGF. However, SU6668 is an example of a small molecule that blocks receptor signalling for VEGF, FGF, and EGF. Certain inhibitors operate by an indirect mechanism that up-regulates an endogenous angiogenesis inhibitor. For example, interleukin-12 up-regulates inducible protein 10 [IP10], which is a direct angiogenesis inhibitor. Angiostatin and endostatin block endothelial cells from responding to a wide variety of endothelial mitogens and angiogenic inducers. For other angiogenesis inhibitors, there is as yet no known mechanism (e.g., thalidomide and TNP-470).³⁵⁹ It is not yet possible to predict which, if any, of the angiogenesis inhibitors currently in clinical trial will eventually receive FDA approval for clinical use. Nevertheless, it is becoming clear that there will be a continuing need for a variety of different angiogenesis inhibitors. In the coming years, it may be important to develop clinical protocols in which angiogenesis inhibitors are added to conventional chemotherapy or to radiotherapy, or to other modalities such as immunotherapy or gene therapy. Furthermore, it may be possible to treat cancer at earlier stages with combinations of angiogenesis inhibitors and also to use antiangiogenic therapy as maintenance therapy to prevent recurrences.

Antiangiogenic Scheduling of Cytotoxic Chemotherapy.

If conventional cytotoxic chemotherapy must traverse vascular endothelium before reaching tumor cells, why don't cytotoxic agents behave as angiogenesis inhibitors? One possibility is that the usual dose-schedule regimen for chemotherapeutic agents is not conducive to sustained blockade of angiogenesis. Conventional chemotherapy is usually administered at maximum tolerated doses up-front followed by an extended treatment-free interval to permit recovery of normal host

Table 9.4. Angiogenesis Inhibitors in Clinical Trials

Phase I		
Drug	Sponsor	Mechanism
COL-3	Collagenex, NCI	Synthetic MMP inhibitor; tetracycline derivative
Combretastatin	Oxigene	Apoptosis in proliferating endothelium
BMS-275291	Bristol-Myers Squibb	Synthetic MMP inhibitor
SU6668	Sugen	Blocks VEGF, FGF & EGF receptor signaling
Endostatin	EntreMed	Inhibits endothelial proliferation
Phase II		
Squalamine	Magainin	Inhibits Na/H exchanger
PTK787/ZK22584	Novartis	Blocks VEGF receptor signaling
TNP-470	TAP Pharm.	Fumagillin analogue; inhibits endothelial proliferation
Thalidomide	Celgene	Unknown
SU5416	Sugen	Blocks VEGF receptor signaling
Vitaxin	Ixsys	Antibody to integrin on endothelial surface
Interleukin-12	Genetics Inst.	Induces IFN-gamma and IP-10
CAI	NCI	Inhibits calcium influx
Anti-VEGF Ab	Genentech	Monoclonal antibody to VEGF
Phase III		
Marimastat	British Biotech	Synthetic MMP inhibitor
AG3340	Agouron	Synthetic MMP inhibitor
Neovastat	Aeterna	Natural MMP inhibitor
Interferon-alfa	Commercially available	Inhibition of bFGF production
IM862	Cytran	Endothelial inhibitor

From National Cancer Institute Database (Updated November 23, 1999).

cells such as rapidly growing hematopoietic progenitors and gastrointestinal tract mucosa. However, during this treatment-free interval, microvascular endothelial cells in the tumor bed may also resume growth and support regrowth of tumor cells. This could increase the risk of emergence of drug-resistance tumor cells. Timothy Browder in my laboratory has demonstrated that a standard cytotoxic agent, cyclophosphamide, can be administered at a dose and schedule that is optimized for more sustained apoptosis of endothelial cells but not of tumor cells, called the antiangiogenic schedule. It consists of a lower dose administered every 6 days in contrast to the conventional schedule, which is a maximum tolerated dose administered every 21 days. A drug-sensitive murine tumor, Lewis lung carcinoma, became drug resistant on the conventional schedule and killed all mice but was eradicated on the antiangiogenic schedule. When the tumor was made drug resistant before therapy, the antiangiogenic schedule suppressed tumor growth three-fold more effectively than the conventional schedule, and when combined with TNP-470, an angiogenesis inhibitor, eradicated drug-resistant tumors (Browder, personal communication, submitted for publication). This is a provocative finding because TNP-470 alone

cannot regress Lewis lung carcinoma. Furthermore, both the cyclophosphamide and the TNP-470 are targeting only endothelial cells; the tumor cells are completely resistant to cyclophosphamide (and continue to proliferate at rates similar to untreated tumor cells), and TNP-470 has no effect on tumor cells. Thus, a cytotoxic chemotherapeutic agent administered in an antiangiogenic dose-schedule can more effectively control tumor growth in mice, whether or not its tumor cells are drug resistant—an advance based on using new logic for an old drug.

There are certain important implications of this work. It confirms an earlier hypothesis by Kerbel that antiangiogenic therapy would be a strategy to circumvent acquired drug resistance.³⁶⁰ Furthermore, certain cytotoxic agents such as paclitaxel already have significant antiangiogenic activity,^{361,362} and it may be possible to take advantage of this property in patients who have become “paclitaxel resistant.” These results in mice may help to explain why some patients who are receiving long-term maintenance or even palliative chemotherapy continue to have stable disease beyond the time that the tumor cells would have been expected to develop drug resistance. These data also suggest that other modifications in delivery or schedule of cytotoxic chemotherapeutic agents may increase antiangiogenic activity. For example, extremely low concentrations of doxorubicin conjugated to vascular integrin-binding peptides can be targeted to the angiogenic vessels in a tumor, leading to significant tumor suppression without side effects on host tissues.^{363–365}

Antiangiogenic Activity of Interferon Alpha: Lessons for Other Angiogenesis Inhibitors. During the 1980s, it was demonstrated that interferon alpha was an angiogenesis inhibitor,^{229,230,366} although relatively weak and nonspecific compared to the inhibitors available today (e.g., angiostatin or endostatin). Interferon alpha appears to act by inhibiting overproduction of bFGF by tumor cells.³³⁵ Nevertheless, over the past 10 years, interferon alpha has been useful for the treatment of sight-threatening or life-threatening hemangiomas of infancy that have failed to respond to all other therapy (i.e., hemangiomas in the brain, airway, heart, and liver, which have failed to respond to all conventional therapy, e.g., corticosteroids [for review see Folkman and colleagues²²³]). Hemangiomas are tumors of host blood vessels and represent a form of relatively pure angiogenesis. The first use of interferon alpha as an angiogenesis inhibitor was in 1988 in a child with pulmonary hemangiomas.^{367,368} The disease underwent accelerated regression. This was the first antiangiogenic therapy in a human. Such accelerated regression of life-threatening hemangiomas was also observed in subsequent patients,^{190,369–377} and it has been especially useful for airway hemangiomas beyond the reach of laser therapy.³⁷⁴ Interferon alfa-2a (or 2b) has been used at doses of 3 million units/m² and treatment has been for 6 to 12 months. Urine levels of bFGF have been a useful guideline to determine when to discontinue therapy. In most normal infants, urine bFGF is up to 4000 picograms per liter. In children with proliferating hemangioma, urine bFGF may range from 25,000 to 50,000 picograms per liter. Unless the hemangioma is beginning to involute and the urine bFGF is decreasing toward normal, discontinuation of interferon alfa-2a may be premature and the hemangioma will resume growth. A few patients remain refractory to either steroid or interferon alpha, and the majority of these refractory lesions are Kaposi's hemangioendothelioma (KHE). We advise neurologic-developmental evaluation before beginning interferon therapy and periodic assessments during and after therapy because infants under 1 year are susceptible to neurotoxic side effects (increased motor tone of the lower extremities [spastic diplegia] and delayed walking,^{333,378} although this is almost always reversible if the interferon is discontinued at the first sign of spasticity and is rare in older children.³⁷⁹)

Following a 10-year experience of treating 74 infants with interferon alfa whose life-threatening hemangiomas had failed to respond to all conventional therapy including high-dose steroids, we were presented with a 5-year-old girl with a baseball-sized giant cell tumor of the mandible that recurred three times after surgical resection (including radical mandibulectomy). Radiotherapy was recommended, but because of the potentially severe complications of stunted facial growth, the possibility of antiangiogenic therapy was suggested. A urinary bFGF level was abnormally elevated. Therefore, a trial of interferon alpha was begun at 1 million units per m² and increased to 4.4

million units per m² at 6 months, and briefly to 6.16 units per m². After 1 year of therapy, the tumor had completely regressed. Surprisingly, the mandible healed completely. Tumor regression coincided with a decrease in urine bFGF to normal. She has been tumor-free 3 years off therapy.³⁸⁰ We have seen similar results in (1) a 2-month-old boy with an angioblastoma of the hand that destroyed the 5th metacarpal bone and that completely regressed after 11 months on interferon alpha-2a (again with bone healing); (2) in an angioblastoma of the palate that recurred after radical resection in a 10-month-old boy, which completely regressed after 1 year on interferon alpha-2a. The drug was then continued for a total of 3.5 years before discontinuing therapy; and (3) a giant cell tumor that filled the pelvis of a 17-year-old girl and recurred after radiotherapy but at this writing has undergone more than a 90% regression on interferon alpha-2a at 3 million units/day (J. Folkman and J. Marler, unpublished data).

From this experience, it is clear that long-term antiangiogenic therapy can be administered successfully even with a single relatively weak angiogenesis inhibitor, if the tumor under treatment employs bFGF as its major or sole angiogenic mediator and does not undergo mutations that up-regulate additional angiogenic proteins over time (as, for example, occurs with breast cancer).³⁸¹ Second, the dose of angiogenesis inhibitor was titrated to the output of angiogenic activity (in this case bFGF) by the tumor. Third, stable disease occurs for a prolonged period before tumor regression takes place. Therefore, this pattern of tumor response resembles tamoxifen more than it does cytotoxic chemotherapy. No patients had any evidence of toxicity or other side effects, except for the usual elevation of liver enzymes seen in all patients on interferon alpha-2a.

Thalidomide. Thalidomide, a sedative with anti-inflammatory activity, was recently discovered to inhibit angiogenesis when administered orally to rabbits.²¹⁷ In this study, the antiangiogenic activity appeared to be independent of the TNF-alpha suppressing activity of thalidomide. The antiangiogenic activity suggests a possible explanation for thalidomide's teratogenic effects in early pregnancy. Thalidomide is currently in Phase II clinical trials for the treatment of recurrent brain tumors, other solid tumors, and multiple myeloma. In a recent study, "84 previously treated patients with refractory myeloma (76 with relapse after high-dose chemotherapy), received oral thalidomide as a single agent for a median of 80 days (2–465).³⁸² The starting dose was 200 mg daily, and the dose was increased every 2 weeks until it reached 800 mg per day. Response was assessed on the basis of a reduction of the myeloma protein in serum or Bence Jones protein in urine that lasted for at least 6 weeks. The serum or urine levels of paraprotein were reduced by at least 90% in eight patients (two had complete remission), at least 75% in six patients, at least 50% in seven patients, and at least 25% in six patients, for a total rate of response of 32%. Reductions in the paraprotein levels were apparent within 2 months in 78% of the patients with a response and were associated with decreased numbers of plasma cells in bone marrow and increased hemoglobin levels. At least one-third of patients had mild or moderate constipation, weakness or fatigue, or somnolence. After 12 months of follow-up, Kaplan-Meier estimates of the mean (\pm SE) rates of event-free survival and overall survival for all patients were $22 \pm 5\%$ and $58 \pm 5\%$, respectively. Thus, thalidomide can induce marked and durable responses in some patients with multiple myeloma, including those who relapse after high-dose chemotherapy."

Antitumor Effect of Ionizing Radiation is Potentiated by Angiogenesis Inhibitors. Experimental studies in mice demonstrate that angiogenesis inhibitors combined with ionizing radiation produce a synergistic antitumor effect without increasing collateral damage to normal host tissues. When TNP-470 was administered together with radiotherapy, intratumoral oxygen, tumor response, and cure rates all significantly increased.³⁸³ When angiostatin was combined with radiotherapy, one-tenth of the effective antitumor dose of angiostatin reduced tumor volumes in mice by 16%, radiotherapy alone by 18%, but radiotherapy plus angiostatin by 64% ($p = .001$).^{310,384} Angiostatin behaves as a radiosensitizer that focuses radiotherapy on the microvascular endothelial compartment in the tumor bed but does not sensitize other tissues. Angiostatin may specifically prevent repair of radiation damage in proliferating endothelial cells. For example, ionizing irra-

diation of a tumor increases production of VEGF, which protects tumor vessels from radiation-mediated cytotoxicity. However, blockade of VEGF by neutralizing antibody increased the antitumor effects of ionizing radiation.³⁸⁴ Because approximately 50% of cancer patients are treated with radiotherapy, it will be important to determine in future clinical trials whether angiogenesis inhibitors will enhance radiotherapy of human tumors as it does in animal tumors.

Misperceptions About Tumor Angiogenesis in The Clinical Setting. Because the field of angiogenesis research is so extensive, it is difficult for clinicians to keep up with the rapidly moving basic science literature. Certain misperceptions persist. These are discussed below because they may otherwise interfere with design of clinical protocols.

The presence of angiogenesis does not distinguish between a benign and a malignant tumor.³⁸⁵ Adrenal adenomas are benign tumors that are highly neovascularized but appear to lack the growth potential to take advantage of the new blood vessels they have induced. Thus, the onset of angiogenesis permits expansion of a tumor mass but does not guarantee it. In fact, the switch to the angiogenic phenotype occurs independently of other events in tumorigenesis. In most tumors, angiogenesis appears after the expression of the malignant phenotype. However, in carcinoma of the cervix, the preneoplastic stage of dysplasia becomes neovascularized before the malignant tumor appears.⁹³ This sequence of events also occurs in certain spontaneously arising tumors in animals.^{113,385}

Angiogenesis may not be necessary for certain tumor cells that can grow as a flat sheet between membranes (e.g., gliomatosis in the meninges).

It is still assumed by some oncologists that the blood vessels of a large tumor are "established." Proponents of this idea argue, therefore, that antiangiogenic therapy could never reduce tumor size or cause tumor regression because "established" vessels would by definition be refractory to such treatment. Antiangiogenic therapy, however, can cause growing blood vessels to involute³⁶⁹ and can bring about regression of growing tumors.^{65,387,388} Further, the replication rate of endothelial cells in tumor capillary vessels is significantly greater than in the endothelial cells of normal tissue.³⁸⁹ A few feeder vessels, usually arteries, may be observed in the midst of a histologic cross-section of a tumor and could be considered as established. However, tumor cells depend on *thin-walled microvessels* for diffusion of nutrients, growth factors, and oxygen, and these vessels continue to undergo high turnover rates even in a large, slowly growing, or indolent tumor. These microvessels require the continuous presence of endothelial growth factors such as VEGF. Withdrawal or blockade of VEGF leads to endothelial apoptosis and regression of microvessels (see section on angiopoietins above).

It is commonly stated that tumors "outgrow their blood supply." This is inaccurate. Growing tumors can gradually compress their blood supply because of increasing interstitial pressure. These compressed areas become ischemic, but they are not avascular. Necrosis follows. Vessel compression also interferes with the optimal delivery of therapeutic agents.³⁹⁰ Paradoxically, antiangiogenic therapy can decrease ischemia, apparently because of its effect of decreasing interstitial pressure.

Rapidly growing tumors in mice are more responsive to conventional cytotoxic agents than slowly growing tumors are, and adult human tumors are less responsive to cytotoxic agents than mouse tumors are. Therefore, it has been assumed by many colleagues that angiogenesis inhibitors will follow the same pattern and be less effective against more slowly growing human tumors than against rapidly growing mouse tumors.³⁹¹ In fact, we have found just the opposite. Slowly growing mouse tumors respond more effectively to angiogenesis inhibitors (TNP-470 or angiostatin) than rapidly growing tumors (Wolf Dietrich Becken, unpublished data, personal communication). Rapidly growing tumors require higher doses of angiogenesis inhibitors to suppress their growth to the same extent as slowly growing tumors.

Patients are often told by their physicians that they would not be a candidate for antiangiogenic therapy because their particular tumor is not highly vascularized. This misperception comes from trying to estimate the angiogenic activity of a tumor by looking at an angiogram or a

gross tumor specimen. An angiogram of a tumor can have a large, dark, unstained area in which vessels do not fill with radiopaque dye. This is often interpreted as “avascular” tumor, but at the microscopic level, histologic sections reveal high microvessel density. The “avascular” angiogram is likely the result of poor perfusion due to high interstitial tissue pressure. A large tumor observed at the operating table, such as a neurofibrosarcoma, may be a hard white mass and assumed to be “poorly vascularized” when in fact the histologic microsections show high neovascularization. At a recent international meeting, a distinguished surgeon predicted that pancreatic cancer would never respond to antiangiogenic therapy because it is a poorly vascularized tumor.

Prognostic Significance of Tumor Vascularity. In 1972, Steven Brem in my laboratory reported the first quantitative method for histologic grading of tumor angiogenesis. He correlated neovascularization in human brain tumors with tumor grade.³⁹² In the 1980s, there was an additional report of a method for quantitating grade of tumor vascularization³⁹³ followed by the first report of the use of tumor vascularity as a prognostic marker (cutaneous melanoma).⁹⁴ In 1991, Noel Weidner and I used specific antiendothelial antibodies to highlight tumor vasculature to demonstrate that microvessel density was a prognostic marker for human breast cancer.⁷⁷ Since then, the majority of reports have confirmed that microvessel density is a powerful and often an independent prognostic indicator for many different types of human cancer. However, a few other reports fail to show that microvessel density is a prognostic indicator, especially for certain types of tumors. Many of the negative reports may be due to critical differences in methodologies, whereas others may be due to important biologic differences that are as yet unclear, for example, the coexistence of angiogenesis inhibitors and stimulators in certain tumors. Gasparini and Harris have analyzed the variables in quantitation of tumor angiogenesis in histologic sections and have summarized the reports up to 1999.⁹⁰ These reports are assembled in Table 9.5.^{23,95,96,394-444}

Clinical Signs and Symptoms in Cancer Patients That May Be Based on Angiogenesis. Certain clinical signs and symptoms from tumor neovascularization are associated with specific tumor types. For example, retinoblastomas in the posterior eye induce iris neovascularization in the anterior chamber. Certain brain tumors induce angiogenesis in remote areas of the brain. Bone pain in metastatic prostate cancer may be related in part to neovascularization. A problem in the diagnosis of a primary bone tumor is that if the biopsy specimen contains only the neovascular response at the periphery of the tumor, it may be mistaken for granulation tissue or inflammation. A variety of cancer syndromes, such as inappropriate hormonal activity, hypercoagulation, and cachexia, are secondary to the presence of biologically active peptides released into the circulation from vascularized tumors. Therefore, it might be predicted that an early therapeutic effect of antiangiogenic therapy would be increased appetite, weight gain, and disappearance of certain cancer syndromes. This early therapeutic effect would be most apparent with those angiogenesis inhibitors that had the least side effects. The angiogenesis induced by cervical cancer may be observed by colposcopy⁹²; the appearance of telangiectasia or “vascular spiders” in a mastectomy scar may herald local recurrence of tumor; color Doppler imaging can demonstrate neovascularization in breast cancer⁴⁴⁵ and other tumors; bladder carcinoma is detected by cystoscopy based, in part, on its neovascularization; and mammography often reveals the vascularized rim of a breast tumor. In fact, a wide range of radiologic signs of cancer are based on “enhancement” of lesions by radiopaque dyes trapped in the neovascularity of a tumor. Moreover, in some tumors, large central areas cannot be penetrated by radiopaque dyes because of vascular compression, a situation that is unusual in prevascular tumors.

Antiangiogenic Therapy Requires Different Management Than Cytotoxic Chemotherapy. Preclinical studies in mice, rats, rabbits, and monkeys, early clinical trials of antiangiogenic therapy, and 10 years of clinical experience with interferon treatment for life-threatening hemangioma point to important principles of antiangiogenic therapy that may be useful in the future management of patients with cancer (for review see Eckhardt).⁴⁴⁶

First, antiangiogenic therapy is directed mainly at a small focus of migrating and proliferating capillary endothelial cells in a tumor bed or in metastatic sites. Therefore, a specific angiogenesis inhibitor is not likely to cause bone marrow suppression, gastrointestinal symptoms, or hair loss. This is not to say that such drugs would have no other actions and would not produce side effects.

Second, optimal antiangiogenic therapy appears to require treatment for months to a year or more, without a break. Angiogenesis inhibitors generally down-regulate neovascularization by inhibiting endothelial cell proliferation and migration, not by a cytotoxic effect on endothelial cells. Regression or involution of a vigorously growing capillary bed is a slower process than lysis of tumor cells. Thus, in the design of clinical trials, antiangiogenic therapy may need to be administered over longer periods (without a break) than conventional cytotoxic agents. Conventional chemotherapy usually requires treatment-free periods to permit recovery of normal host cells in the bone marrow and gastrointestinal tract. Treatment-free periods are not only unnecessary during antiangiogenic therapy but may be counterproductive. Experimental studies with endostatin in tumor-bearing mice reveal an accumulative effect, so that the longer the inhibitor is administered, the more likely it is that tumors will not recur after discontinuation of the drug.⁶⁵ Premature discontinuation of the inhibitor may be followed by rapid tumor growth. This phenomenon may not be limited to endostatin but was observed in tumor-bearing mice treated by Sugen’s SU6668, a synthetic inhibitor of the receptors for FGF, VEGF, and EGF (Julie Cherrington, personal communication).

Third, resistance to angiogenesis inhibitors has not been a major problem in long-term animal studies⁴⁴⁷ or in clinical trials to date. Babies with large hemangiomas of the mediastinum or liver who were treated with interferon alfa-2a daily for up to a year did not develop drug resistance. Antiangiogenic therapy was proposed as a strategy to circumvent acquired resistance to anticancer agents³⁶⁰ and this idea has been validated by endostatin-treated mice.⁶⁵

Fourth, a combination of antiangiogenic and cytotoxic therapy may be more effective than either type of therapy alone. In tumor-bearing animals, such combinations can be curative, whereas either agent alone is only inhibitory.^{88,448,449} An angiogenesis inhibitor such as AGM-1470 (TNP-470) can significantly decrease DNA synthesis in endothelial cells in a tumor bed, whereas cytotoxic agents such as adriamycin and cisplatin do not.⁴⁵⁰ These results suggest that therapy directed against both the endothelial cell population and the tumor cell population of a tumor is more effective than therapy directed only against its tumor cells. Radiotherapy is also potentiated by antiangiogenic therapy in tumor-bearing animals, in part by decreasing tumor hypoxia.^{310,448} Furthermore, antiangiogenic therapy could be used in combination with other anticancer modalities including immunotherapy or gene therapy. Finally, combinations of angiogenesis inhibitors may provide a form of cancer therapy that is the least toxic and carries the least risk of acquired drug resistance.

SUMMARY AND FUTURE DIRECTIONS

An important lesson from angiogenesis research is to think about a tumor as containing two cell compartments that stimulate each other: the endothelial cell compartment and the tumor cell compartment. Anticancer therapy may be more efficacious if each compartment is treated by drugs that selectively target each cell type. The mutational rate is high in the tumor cell compartment and low in the endothelial cell compartment. This is why it may be possible to employ antiangiogenic therapy for the long term, either alone or together with conventional chemotherapy, and subsequently in the postchemotherapy period.

The heuristic value of a tumor model in which the endothelial cell and tumor cell compartments interact with each other is best summarized by Hahnfeldt and colleagues.⁴⁵¹

Conventional cancer treatment includes many modalities, all having the same basic intent: to directly kill tumor cells or prevent their proliferation. Accordingly, kinetic understanding of tumor control has focused on the elucidation of tumor cell proliferation and sensitivity characteristics. However, a tumor population is far from stable, manifesting with its genetic, epige-

Table 9.5. Tumor Vascularity as a Prognostic Indicator

Breast Cancer-Intratumor Vascularization and Prognosis			
Authors	Number of Patients	Median Follow-up (yr)	Relapse-free Survival
Weidner et al. ⁹⁵	165	4.0	<0.001
Bosari et al. ³⁹⁴	180	9.0	<0.03
Visscher et al. ³⁹⁵	58	5.1	NS
Obermair et al. ³⁹⁶	64	4.1	<0.01
Ogawa et al. ³⁹⁷	155	7.0	<0.002
Fox et al. ³⁹⁸	211	3.5	ND
Toi et al. ³⁹⁹	125	5.1	<0.01
Toi et al. ⁴⁰⁰	328	4.6	<0.0001
Simpson et al. ⁴⁰¹	178	6.0	0.002
Gasparini et al. ⁴⁰²	531	6.3	<0.001
Bevilacqua et al. ⁴⁰³	211	6.6	<0.0001
Obermair et al. ⁴⁰⁴	230	4.6	ND
Fox et al. ⁴⁰⁵	109	2.0	0.04
Heimann et al. ⁴⁰⁶	167	20.0	0.04
Barbareschi et al. ⁴⁰⁷	91	5.5	0.006
Gasparini et al. ⁴⁰⁸	191	5.5	<0.01
Gasparini et al. ⁴⁰⁹	178	5.2	<0.01
Hall et al. ⁴¹⁰	87	9.5; 1.5	NS
Axelsson et al. ⁴¹¹	220	11.5	NS
Van Hoef et al. ⁴¹²	93	13.0	NS

Published positive studies. ND = not done; NS = not significant.

Prognostic Value of Microvessel Density in Breast Cancer Patients Treated with Adjuvant Therapy

Authors	Number of Patients	Median Follow-up (yr)	Adjuvant Treatment	Relapse-free Survival
Weidner et al. ⁹⁵	82	4.0	Heterogeneous	<0.001
Toi et al. ⁴⁰⁰	198	4.6	Heterogeneous	<0.001
Gasparini et al. ⁴⁰⁸	191	5.5	CD31	<0.001
Gasparini et al. ⁴⁰⁹	178	5.2	CD31	<0.001
Macaulay et al. ⁴¹³	88	2.5	Tamoxifen	ND

ND = not done.

Prognostic Value of Microvessel Density in Non-Small Cell Lung Cancers

Authors	Number of Patients	Median Follow-up (mo)	Prognostic Value
Macchiarini et al. ⁴¹⁴	87	60	Yes
Yamazaki et al. ⁴¹⁵	42	71	Yes
Fontanini et al. ⁴¹⁶	253	24	Yes
Giatromanolaki et al. ⁴¹⁷	107	36	Yes
Angeletti et al. ⁴¹⁸	96	24	Yes
Apolinario et al. ⁴¹⁹	116	60	Yes
(stage II only)			
Fontanini et al. ⁴²⁰	470	29	Yes
Fontanini et al. ⁴²¹	73	47	Yes

Prognostic Value of Microvessel Density in Genitourinary Cancers

Authors	Number of Patients	Prognostic Value
Testicular germinal cell tumor		
Olivarex et al. ⁴²²	65	Yes
Prostatic cancer		
Wakui et al. ⁴²³	101	Yes
Fregene et al. ⁴²⁴	34	Yes
Weidner ⁹⁶	74	Yes
Vesalainen et al. ⁴²⁵	88	Yes
Brawer et al. ⁴²⁶	37	Yes
Silberman et al. ⁴²⁷	109	Yes
Barth et al. ⁴²⁸	41	Yes
Rogatsch et al. ⁴²⁹	46	Yes
Bladder cancer		
Dickensen et al. ⁴³⁰	45	Yes
Bochner et al. ⁴³¹	164	Yes
Grossfeld et al. ⁴³²	163	Yes

ND = not done.

Prognostic Value of Microvessel Density in Esophageal and Gastrointestinal Tumors

Authors	Number of Patients	Median Follow-up (mo)	Prognostic Value
Esophageal cancer			
Tanigawa et al. ⁴³³	43	3.1	Yes
Gastric cancer			
Maeda et al. ⁴³⁴	124	>5	Yes
Tanigawa et al. ⁴³⁵	181	4.1	Yes
Colorectal cancer			
Saclarides et al. ⁴³⁶	48	4.4	Yes
Tomisaki et al. ⁴³⁷	175	5.0	Yes
Takebayashi et al. ⁴³⁸	166	6.3	Yes
Bossi ⁴³⁹	178	5.0	No
Lindmark et al. ⁴⁴⁰	212	4.5	Yes

High vascularity associated with good prognosis.

Prognostic Value of Microvessel Density in Malignant Melanoma

Authors	Number of Patients	Median Follow-up (mo)	Thickness (mm)	Prognostic Value
Srivastava et al. ⁹⁴	20	>5	0.76–4.00	Yes
Fallowfield and Cook ²³	64	ND	0.48–18.5	Yes
Carnochan et al. ⁴⁴¹	107	>5	0.85–1.25	No
Busam et al. ⁴⁴²	120	8.9	Invasive	No
Graham et al. ⁴⁴³	37	>10	0.76	Yes
Vlaykova et al. ⁴⁴⁴	31	>3	0.76	Yes

ND = not done.

netic and microenvironmental heterogeneity a constantly evolving spectrum of tumor cell expressions and behaviors. This raises the concern that current therapeutic attempts to target the expanding array of tumor expressions with customized molecular attacks may be overascribing durable and exploitable mechanistic bases to what are, in fact, largely temporal and hyper-variable events. By contrast, therapy directed against tumor vasculature [i.e., the microvascular endothelial cell population in the tumor bed] does not exploit tumor cell sensitivities, relying instead on tumor suppression consequent to inhibition of associated vasculature. By providing a means to control an exceptionally heterogeneous, unconstrained tumor population via a relatively homogenous and constrained endothelial population, antiangiogenic therapy allows one to disregard a vast array of spatial and temporal details of tumor expression.

The field of angiogenesis research, which began as an inquiry into the mechanisms by which tumors induce a new blood supply, has now broadened to include a diverse group of disciplines. For example, the development of the vascular system itself is being explored. Genes that turn angiogenesis on or that suppress angiogenic activity are being elucidated. Angiogenic molecules are being employed to accelerate the cardiac angiogenesis in ischemic heart disease. Angiogenesis inhibitors intended for eventual anticancer therapy are also being studied for their potential use in ocular angiogenesis, arthritis, and other non-neoplastic diseases.

These developments in fields parallel to oncology may bring new information to bear on the problem of tumor angiogenesis. We need to understand how tumors become angiogenic and what angiogenic molecules they employ. It will be important to know how these molecules are released, whether specific angiogenic molecules are produced by certain types of tumors, and how angiogenesis suppressor activity is down-regulated during tumor progression. It is still not clear what percentage of tumor-induced angiogenesis must be blocked before tumor growth is inhibited, nor is it known if endothelial cells can become "resistant" to angiogenesis inhibitors. Beyond these considerations lie more fundamental questions. Can the onset of angiogenic activity be detected in the blood or other body fluids for use in

diagnosis? Can the process of angiogenesis itself be manipulated by genetic therapies? These questions and others provide the basis for continuing excitement in the field of angiogenesis research.

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