Tumor Angiogenesis

Judah Folkman, MD
Raghu Kalluri, PhD

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, eventually, 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 microvascular 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 these neoplastic properties may only be necessary but not sufficient for the cancer cell to become metastatic and 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 sustain its own private blood supply, a process called tumor angiogenesis.[1] 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 immunodeficient mouse models, heterotransplanted malignant cells sometimes fail to form grossly-identifiable tumor nodules but nonetheless persist as small nonangiogenic tumors called "no-takes."[2]

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.[3–5]

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.[6,7] 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.[8]

A hallmark of pathologic angiogenesis is persistent growth of blood vessels (ie, sustained neovascularization). Angiogenesis that continues for months or years supports the progression of many neoplastic and nonneoplastic diseases.[9,10] 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 m^2, an area the size of a tennis court. A cubic millimeter of human cardiac muscle contains approximately 2,500 millimeters of capillary blood vessels, (as determined by stereologic methods).[4] 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.[11] This tumor hyperemia observed during surgery was explained by simple dilation of existing host blood vessels.[12] 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,[13] a tumor implant in the chamber was associated with accelerated growth of new capillary blood vessels. The other two 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.[14,15] These papers not withstanding, 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.[16] 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.[17]

BEGINNING OF ANGIOGENESIS RESEARCH

HYPOTHESIS: TUMOR GROWTH DEPENDS ON ANGIOGENESIS In 1971, Folkman 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.[18] This hypothesis 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 (ie, antiangiogenic therapy). Folkman proposed these ideas from experiments he performed with Frederick Becker in the early 1960s, which revealed that tumor growth in isolated perfused organs was severely restricted in the absence of vascularization of the tumors.[19–24]

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 or possibly a host defense detrimental to the tumor. 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 be made to 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 1971 hypothesis was slow because it would be 2 more years before the first vascular endothelial cells were successfully cultured in vitro, 8 years before it was possible to grow capillary endothelial cells in vitro, 11 years before the discovery of the first angiogenesis inhibitor, and 13 years before the purification of the first angiogenic protein.[25–29]

Throughout the 1970s, laboratory studies were devoted to demonstrating that: tumor vessels were new proliferating capillaries; the sequential steps of the angiogenic process could be identified; qualitative and quantitative bioassays for angiogenesis could be developed; viable tumor cells released diffusible angiogenic factors that stimulated new capillary growth and endothelial mitosis in vivo, despite the arrest of tumor cell proliferation by irradiation; necrotic tumor products were not angiogenic per se (reviewed in Folkman and Cotran); and that angiogenesis itself could be inhibited.[30–35] Because of these efforts to provide supporting evidence that tumor growth was angiogenesis dependent, the field of angiogenesis research began. Today 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, which include in addition to oncology, cardiology, dermatology, gynecology, ophthalmology, and rheumatology. Approximately 40 publications with angiogenesis in the title, appear every week.

Experimental 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 that converge upon the tumor."[32] The hypothesis predicted that if angiogenesis could be completely inhibited, tumors would become dormant at a small, possibly microscopic, size.[22] 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 mm^3 and restrict a metastasis to a microscopic dormant lesion. Most nonneovascularized tumors are not clinically detectable, with the exception of surface lesions on the skin or the external mucous membranes.

The hypothesis that tumors are angiogenesis dependent is supported by biologic and pharmacologic evidence and proved by genetic evidence. Both types of evidence are summarized below because they provide a scientific basis for current clinical trials of angiogenesis inhibitors.[36,37]

Biologic evidence

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 (ie, 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.[38] In these "steady-state" spheroids, cell proliferation is balanced by cell death.[39–41] This in vitro model is analogous to a dormant micrometastasis in which angiogenesis is blocked.[42]
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.[15,43]
3. Tumor growth in the avascular rabbit cornea proceeds slowly at a linear rate, but converts to exponential growth after neovascularization.[44]
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 mm^3). 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 apposed to the proliferating iris vessels, the tumor becomes neovascularized and can enlarge up to 16,000 times its original volume within 2 weeks.[45] A current interpretation of this experiment is that the tumor spheroid had already switched to the angiogenic phenotype, but that angiogenesis was blocked by separation of the floating tumor from the nearest vascular bed.[46]
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.[47] 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.[48]
7. Within a solid tumor, the [H^3]thymidine labeling index of tumor cells decreases with increasing distance from the nearest open capillary vessel.[49] The tissue oxygen tension also decreases with increasing distance of a tumor cell from the center of a capillary vessel.[50] Because the limit of oxygen diffusion is 100 to 200 μm, 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).[51]
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.[52]
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.[53]
10. Human ovarian carcinomas 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 (C. Chen, personal communication, 1998).
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.[54]
12. Neoplastic cells injected subcutaneously develop into tumors that become vascularized at about 0.4 mm^3.[55] 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.[55] 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.[56] This stage is similar to "cooption" of blood vessels recently reported.[57] Subsequently, new capillary vessels sprout and proliferate (angiogenesis), which leads to increasing microvessel density and rapid tumor growth.

Pharmacologic evidence By the late 1980s it became possible to inhibit tumor angiogenesis by biochemical and molecular methods. These experiments were based on (1) administration of molecules that inhibited angiogenesis specifically or selectively, (2) blockade of tumor-derived angiogenic factors, and (3) the development of spontaneous

tumors in transgenic mice. Some 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.[59] It potently inhibited tumor growth in vivo but did not inhibit tumor cells in vitro. In pre-clinical studies it has a broader spectrum of antitumor activity than any known anticancer drug. There are more than 60 reports from different laboratories of inhibition of 33 different types of human, mouse, rat, hamster, and rabbit primary 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% (ie, complete regression). All of these reports employed the same dose, 30 mg/kg every other day. Complete regression was observed in seven tumor types in mice, including neurofibrosarcoma, neurofibroma, breast cancer, gastric cancer, and choriocarcinoma, as well as mouse reticulum sarcoma and gastric carcinoma.[60] Because TNP-470 inhibits microvascular endothelial cells at a concentration 3 logs lower than tumor cells, this angiogenesis inhibitor is virtually a specific inhibitor of endothelial proliferation and migration. These studies provided the first clue that angiogenesis inhibitors could be broad spectrum anticancer agents that are relatively independent of tumor type. The appearance of different therapeutic responses by different tumor types (eg, 43 to 100%) was more adequately explained by the total angiogenic output of each tumor type matched against a fixed dose of angiogenesis inhibitor. (See below for improvements in TNP-470.)
2. In another experiment, antibody against bFGF (basic fibroblast growth factor) secreted by a tumor administered to mice bearing the tumor, resulted in dramatic reduction in neovascularization and in tumor volume.[61] To engineer bFGF that was secreted from the tumor, the cDNA for human bFGF hybridized to a signal sequence was transfected into normal mouse fibroblasts.[61] Furthermore, the structure of the bFGF was modified by site-specific mutagenesis in which two serines were substituted for cysteines. Thus, the bFGF released by the tumor could be neutralized by a specific antibody that had no effect on natural bFGF. The transfected fibroblasts became tumorigenic, exported bFGF, and were highly angiogenic. They formed large lethal tumors when implanted into mice. Tumor angiogenesis was mediated solely by bFGF released from these tumors.
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.[62] 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.[63]
4. 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 induced tumor regression.[64]
5. Endogenous specific inhibitors of endothelial proliferation and of angiogenesis include: angiostatin, a 38 kDa internal fragment of plasminogen (generated by Lewis lung carcinoma), endostatin, a 20 kDa internal fragment of collagen XVIII (generated from a murine hemangioendothelioma), a 53 kDa conformationally changed fragment of antithrombin III (generated from human small cell lung cancer), and tumstatin, a 28 kDa internal fragment of collagen IV (α 3NC1 domain).[65–71] 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 has been achieved without toxicity or drug resistance.[69]
6. In SCID mice bearing human pancreatic cancer, human recombinant endostatin was injected into the peritoneal cavity once daily, or delivered continuously by an implanted micro-osmotic pump that released 1 microliter/hour.[72] Continuous dosing inhibited tumor growth 10-fold more effectively than once a day bolus dosing and also induced tumor regression, whereas bolus dosing did not (Figure 11-1).
7. An antibody to two receptors of VEGF called VEGF-Trap, inhibits growth of VEGF producing tumors in mice.[73]

Genetic evidence Genetic evidence that tumors are angiogenesis dependent began to be reported by the mid-1990s and provides the most compelling proof that tumors and their metastases cannot not grow beyond a microscopic in situ size without recruiting new microvessels. These experiments are based on (1) transfection of dominant-negative receptors for a proangiogenic protein into endothelial cells in the tumor bed, (2) transfection of genes for antiangiogenic proteins into tumor cells, (3) regulation of the expression of angiogenic oncogenes, and (4) manipulation of developmental genes. Some examples are listed below.

1. The growth of brain tumors in nude mice was significantly inhibited or prevented when tumor angiogenesis was suppressed by a

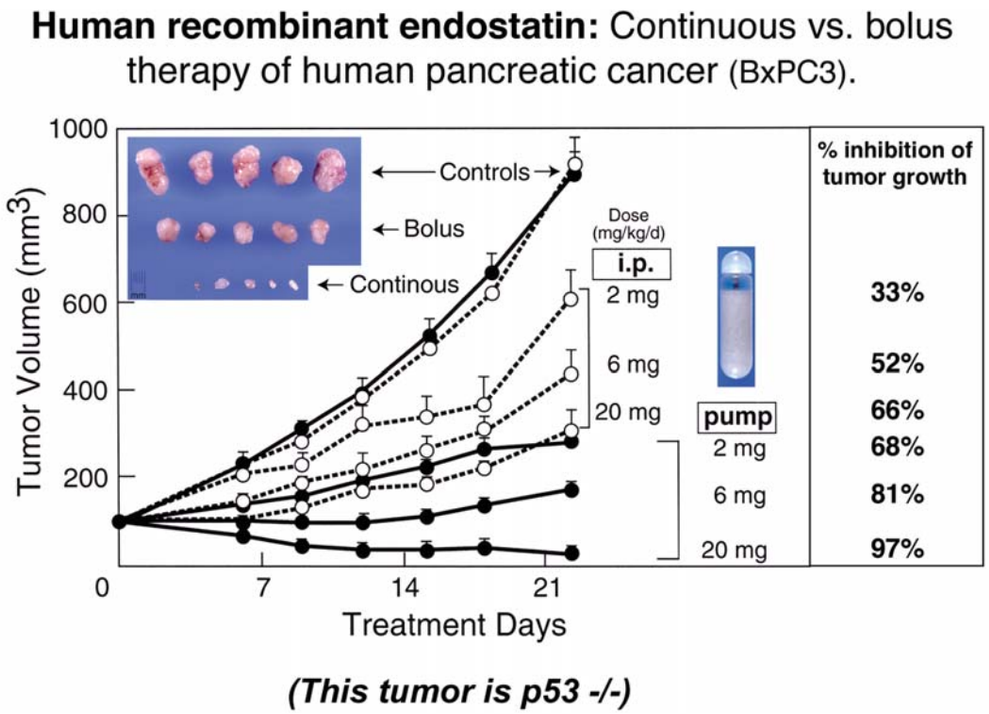

Figure 11-1 Continuous administration of endostatin is ten times more effective than the same dose given once a day as a bolus. Continuous dosing can regress tumors whereas bolus dosing does not. In this paper the pharmacokinetics show that bolus dosing gives a high peak of endostatin with a rapid fall below effective theraputic levels. In contrast, continuous dosing provides constant therapeutic levels of 200 ng/ml up to 250 ng/ml. The implication of this study is that in order to stop tumor growth or to induce tumor regression, endothelial cells in the tumor bed must be continuously exposed to therapeutic levels of inhibitor to counteract stimulators of endothelial growth which constantly bathe the endothelium.[72] (Four-color version of figure on CD-ROM)

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.[74]

2. Transformed cells were not tumorigenic until after they had become angiogenic by downregulating expression of thrombospondin-1 a process that was p53-dependent.[75]
3. Transfection of normal human cells with the SV40 large T oncogene, generated immortalized cells in vitro. However, when these cells were inoculated into mice they formed microscopic sized nonangiogenic tumors that did not grow beyond approximately 1 mm diameter.[76] A subsequent transfection with the *ras* oncogene switched the cells to the angiogenic phenotype, upregulated VEGF production, downregulated production of tissue inhibitors of metalloproteinases, and generated highly neovascularized, rapidly growing tumors.
4. Downregulation of the *ras* oncogene in a melanoma driven by doxycycline inducible ras, led to massive apoptosis of microvascular endothelium in the tumor bed starting within 6 hours. Tumor cells began to die days later, and large tumors had completely disappeared by 12 days.[77]
5. 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.[78]
6. When tumors were transfected with the secretable antiangiogenic protein thrombospondin-1 (TSP-1) and/or thrombospondin-2 (TSP-2), tumor growth was directly proportional to suppression of angiogenesis. Tumor growth was inhibited up to 100% (ie, 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.[79]
7. 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.[80] However, when these mice were injected with bone marrow from wild-type mice containing normal Id1 and Id3 markers, progenitor endothelial cells expressing Id1 and Id3 circulated from the bone marrow to the tumor vascular bed and permitted the tumors to undergo neovascularization and to grow.[81]

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 respond to specific growth factors.[82–86] Thus, tumor cells must gain access to the vasculature in the primary tumor, survive the circulation, arrest in the microvasculature of the target organ exit from this vasculature, grow in the target organ, and induce angiogenesis.[84–90] 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.[91,92] The number of cells shed from the primary tumor appears to correlate 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.[92,93] 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.[94–96] These degradative enzymes may facilitate the entry of tumor cells into the circulation. Tumor cells may not immediately become neovascularized after reaching the target organ. Such a metastasis lacking angiogenic activity for a variety of reasons may remain as a microscopic tumor of 100 to 200 μm diameter indefinitely.[42,97,98] It has been the conventional wisdom that in human dormant micrometastases (eg, metastases appearing 5 to 10 years after removal of a breast cancer), tumor cells are not cycling or are in G_0. However, increasing experimental evidence, indicates that micrometastases can be held dormant by blocked angiogenesis that results in a balance of tumor cell proliferation and apoptosis.[42,97] Finally, experimental metastases are as susceptible as primary tumors to control by specific angiogenesis inhibitors.[65,66] However, the relative efficacy of a given angiogenesis inhibitor can be affected by differences in metastatic sites and by the presence or absence of the primary tumor.[65]

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 [^{3}H]thymidine labeling index as high as that of a large vascularized tumor. The difference between the two is that the prevascular tumor reaches a steady state in which generation of new tumor cells is balanced by tumor cell apoptosis.[99]

When the prevascular phase of bladder cancer, cervical cancer, or cutaneous melanoma is first detected, these lesions are usually thin, slowly growing, stable for months to years, asymptomatic, and rarely metastatic.[100–103] 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.[89,104,105]

The size limits of experimental tumors when angiogenesis is blocked or absent are between approximately 0.2 mm diameter (eg, for lung metastases in mice) and 2 mm (eg, for chondrosarcomas in rats,[106] having a tumor population of 10^5–10^6 cells).[42,106] The differences in size of prevascular (nonangiogenic) tumors may be due in part to the capacity of tumor cells to survive under differing degrees of hypoxia (Figure 11-2).[51,107] However, if cancer cells are already angiogenic at the time of implantation, they can initiate angiogenesis before the tumor population

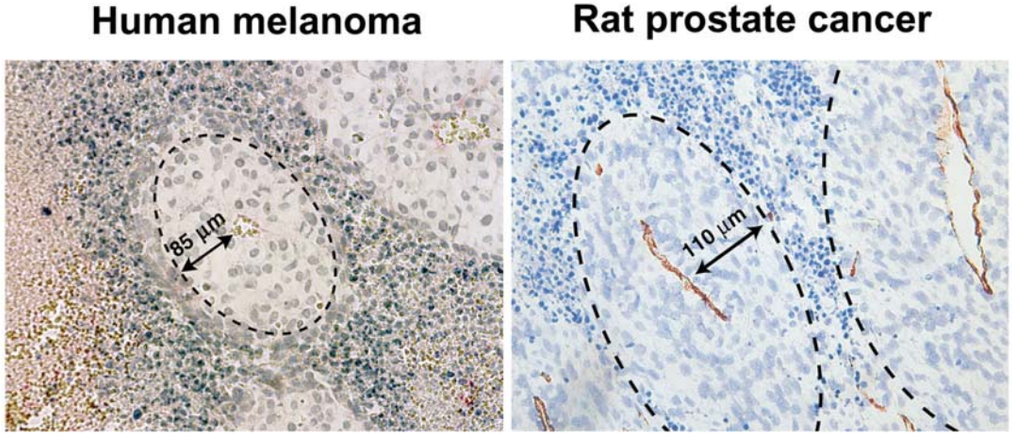

Figure 11-2 Left Panel: A cuff of live tumor cells around a microvessel in a human melanoma growing in a SCID mouse has an average radius of 85 microns. The appearance of an ellipsoid is due to the way the section is cut. Right Panel: A cuff of rat prostate cancer cells around a microvessel has an average radius of 110 microns.[37] (Four-color version of figure on CD-ROM)

would have reached the limiting size of a non-neovascularized tumor (ie, 0.2 to 2 mm). The evidence for this conclusion is from an experiment in which only 20 to 30 angiogenic mammary tumor cells were implanted into a transparent skin chamber in a mouse.[108,109] The tumor cells elongated by day 2, migrated toward the nearest microvessels in a parallel orientation by day 4, divided undirectionally, induced vascular dilation and tortuosity in the neighboring microvessels by day 6, and stimulated new vascular sprouts and loops 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 angiogenesis was prevented by injection into the chamber of a truncated soluble receptor (Flk 1) for vascular endothelial growth factor (VEGF), some tumor cells underwent apoptosis and microscopic tumors regressed or became dormant within 5 days (ie, before the appearance of neovascular sprouts). The soluble receptor potently inhibited endothelial cell proliferation in vitro, but had no effect on tumor cells. This result provides 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 forms new vascular sprouts directed toward the tumor. Vascular endothelial cells can produce at least 20 mitogens and anti-apoptotic factors.[110] 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.[106]

It is also possible that VEGF elaborated from 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.[111] Endothelial cells up-regulate expression of angiopoietin-2 in the presence of tumor cells. Further, plasma and fibrin leakage from the microvessels could facilitate chemotaxis and alignment of tumor cells, as well as subsequent migration of endothelial cells.[112,113] VEGF also up-regulates synthesis of nitric oxide in vascular endothelium.[114–116] Vasodilation and vessel tortuosity permit endothelial elongation and thus may be a prerequisite for endothelial cells to undergo mitosis and migration in response to angiogenic factors like VEGF.[117] Even highly transformed neoplastic cells respond more efficiently to mitogens when the cells are elongated or spread than when they are rounded.[118,119] Furthermore, when well-vascularized tumors in mice were treated with antiVEGF monoclonal antibodies, there was a dramatic reduction in diameter, tortuosity, and vascular permeability in the tumor vessels.[58]

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 membrane.[46] If a nonangiogenic tumor emerges in vascularized tissue (ie, an islet cell carcinoma) or as a micrometastasis, it may form a microcylinder of tumor cells around preexisting capillary vessels (also called cooption).[42,46,57,58,98] In all of these situations, the pre-angiogenic tumor is restricted in size to a range of approximately 0.2 mm to 2 mm diameter.[42,106] In contrast, a microscopic population of angiogenic tumor cells may 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.[56]

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.[46,120,121] By the time most human tumors are detected, for example, by a positive mammogram, neovascularization has usually 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.[121] 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.[102] This sequence of events also occurs in certain spontaneously arising tumors in animals.

At least four mechanisms of the angiogenic switch have been identified in both human tumors and spontaneous tumors in mice (Table 11-1).

Prevascular 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 epithelial layer separated by a basement membrane from underlying vasculature in the dermis or submucosa, respectively. In microscopic ductules of breast or prostate containing in situ carcinoma that has switched to the angiogenic phenotype, 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. The basement membrane is a physical and molecular barrier to migrating endothelial cells. For example, tumstatin, a potent inhibitor of endothelial cell migration and proliferation is found in collagen IV.[71] After the basement membrane has been breached by new vessel sprouts, tumor cells form multiple cell layers around each new capillary blood vessel.[122] The radius of these microcylinders is restricted to the oxygen diffusion limit for a given tumor type as originally defined by Thomlinson and Gray.[37,50] For example, for a human melanoma, the oxygen diffusion limit is approximately 85 μm. Beyond that distance from a capillary blood vessel, virtually all tumor cells are apoptotic (or necrotic). Within that radius, most tumor cells are viable (see Figure 11-2). For a prostate carcinoma the oxygen diffusion limit is approximately 110 μm, and it may be greater for certain tumors, such as for a chondrosarcoma or a tumor in which p53 is mutated or absent, but rarely would 200 μm be exceeded.

Table 11-1 Metastatic Patterns in Cancer Patients

At first diagnosis			
	Primary Tumor	Metastases	Recurrence of Metastases
I	+	0	months
II	+	+	
III	0	+	
IV	+	0	years
V	+	+	

Metastases regress when primary tumor removed; renal carcinoma, rare.

Clinical patterns of metastasis may be governed by angiogenic mechanisms. Roman numerals define a patient class.

Circulating endothelial stem cells in tumor angiogenesis Recent experimental and clinical evidence reveals that circulating endothelial progenitor cells derived from stem cell reservoirs in the postnatal bone marrow can be recruited to the vascular bed of tumors and contribute to tumor growth.[81,123–135] Functional vascular endothelial growth factor receptor-1 (VEGFR-1) is expressed on hematopoietic stem cells ($CD34^+$ in humans and $Lin^-Sca\text{-}1^+c\text{-}Kit^+$ in mice).[133] Functional VEGFR-2 is expressed on $CD34^+$ $AC133^+$ progenitor human endothelial cells. VEGF elaborated by a variety of tumor signals through both VEGFR-1 (Flt-1) and VEGFR-2 (Flk-1), can mobilize progenitor endothelial cells into the circulation where they are recruited into the vascular bed of certain tumor types, but not others.[81]

Before endothelial progenitor cells can leave the bone marrow, they must transfer (along with hematopoietic stem cells) from the quiescent to the proliferative niche within the marrow. Mobilization of hematopoietic stem cells and progenitor endothelial cells necessary for their transfer to the proliferative niche is dependent upon release of soluble Kit-ligand, which itself depends upon upregulation of metalloproteinase-9.[134] Thus, the microvascular endothelial cells in the vascular bed of a tumor may be

recruited both from the local neighborhood as well as from the bone marrow. Rafii has shown that the ratio of these endothelial cells from different sources may differ by tumor type (S. Rafii, personal communication,). For example, in lymphomas the majority (>90%) of endothelial cells are derived from the bone marrow. In breast cancer, bone marrow-derived endothelial cells may approximate the number of endothelial cells from the local neighborhood. In contrast, in prostate cancer the majority of endothelial cells are recruited locally and very few come from the bone marrow. At this writing, the ratios of bone marrow-derived endothelium and local endothelium have not been elucidated for a wide variety of tumors. Nevertheless, the possible implications of these studies are provocative.

First, the efficacy of conventional chemotherapy may turn out to correlate with the percent of bone marrow-derived endothelial cells in a tumor. Second, during conventional chemotherapy, the off therapy intervals necessary to rescue bone marrow may result in a surge of progenitor endothelial cells that could traffic to the tumor (S. Rafii, personal communication, 2002). Third, certain angiogenesis inhibitors may suppress the release of bone marrow-derived progenitor endothelial cells. Angiostatin targets progenitor endothelial cells; endostatin induces apoptosis in circulating endothelial cells in tumor-bearing mice, and thalidomide decreases circulating endothelial cells by 10-fold in multiple myeloma.[136–138] Endothelial cells (P1H1-12) in the circulation may be shed from the vascular bed of a tumor, especially after therapy when apoptotic endothelial cells may appear in the circulation. Some of these cells may be apoptotic (J. Heymach and S. Soker, unpublished data). Quantification of these two populations of circulating endothelial cells, bone marrow-derived progenitor endothelial cells and endothelial cells shed from the vascular bed of a tumor, is currently being evaluated as a potential surrogate marker for efficacy of antiangiogenic therapy, or perhaps for chemotherapy.

Circulating VEGF may be one of the angiogenic signals by which tumors can recruit endothelial cells from bone marrow. Subcutaneously implanted collagen gels embedded with VEGF are invaded by endothelial cells from bone marrow as well as from the local neighborhood.[81] VEGF serum concentrations closely correlate with platelet counts in cancer patients. VEGF is stored, transported, and released from platelets.[355,356] Furthermore, Pinedo and colleagues report that platelet counts have prognostic significance for cancer patients: higher platelet counts correlate with a worse prognosis.[357–359] Therefore, it is possible that for those types of tumors that recruit bone marrow-derived endothelial cells, communication from tumor to bone marrow may be mediated in part by the VEGF in circulating platelets. At this writing, these results have as yet no direct impact on clinical cancer therapy. However, oncologists should be aware of a potential role for platelets in cancer growth, and in tumor angiogenesis. For example, bone marrow-supportive agents, currently used in high-dose chemotherapy, contribute to platelet production and thereby may influence response to therapy.[357]

Nonendothelial host cells may amplify tumor angiogenesis In addition to recruiting vascular endothelium from the host, certain tumors may also attract mast cells, macrophages, and inflammatory cells.[139–142] These cells can amplify tumor angiogenesis by releasing proangiogenic molecules such as bFGF, or by releasing metalloproteinases that can mobilize VEGF and other angiogenic proteins.[106,113,139,143] Tumor angiogenesis and tumor growth are significantly diminished in mice deficient in metalloproteinase-9.[141] Certain tumor cells may also trigger host stromal cells in the tumor bed to overexpress the angiogenic protein VEGF.[144] This is another mechanism of amplification of the angiogenic phenotype once it has been initiated.

Vessel cooption In certain metastases (eg, 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.[57,58]

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.

Persistence of nonangiogenic tumor cells in the vascularized tumor After a nonangiogenic microscopic in situ carcinoma has become neovascularized, a significant percentage of tumor cells remain nonangiogenic.[2] The vascularized human primary tumors that we have studied in the Folkman lab so far, generally appear to contain a mixture of angiogenic and nonangiogenic tumor cells. The nonangiogenic tumor cells can be isolated from a human tumor removed at surgery by implanting numerous tiny pieces (1 mm^3) of tumor into the subcutaneous dorsum of immunodeficient SCID mice, or by implanting cultured human tumor cells.[2] While a few tumors grow to a palpable and visible size (100 mm^3 to >1,000 mm^3) within a few weeks, and a few others appear after several months, most human tumor transplants remain invisible in the majority of mice, a phenomenon called "no take." However, upon opening the skin of these mice, one invariably finds a tiny (<0.5 to 1 mm^3) whitish avascular or poorly vascularized tumor that is transplantable, contains proliferating and apoptotic tumor cells, but does not expand nor metastasize. A few of these nonangiogenic dormant tumors have spontaneously become angiogenic after 1 or more months, depending on the tumor type and have then grown. Others (eg, osteosarcoma) have remained nonangiogenic and dormant up to 3 years after being transplanted to new mice every 8 months (Figure 11-3).[2,99] Some tumor types that remain nonangiogenic can be rapidly switched to the angiogenic phenotype by transfection with the ras oncogene, which significantly increases tumor cell production of VEGF and decreases thrombosponsin-1 production.[99] Labeling the nonangiogenic tumor cells by transfection with green fluorescent protein (GFP), permits them to be visualized beneath the skin. When the green nonangiogenic tumor cells are mixed 1:1 with angiogenic tumor cells that are not labeled, or mixed with angiogenic tumor cells transfected with red fluorescent protein (T. Udagawa et al unpublished data), the resulting large neovascularized tumor, which grows up rapidly, contains tiny (<0.5 mm) green nonangiogenic colonies dispersed throughout.[99] When the nonangiogenic green tumor cells are mixed with a decreasing fraction of angiogenic tumor cells, there is a long latent period of one or more months before neovascularized tumors arise. The latent period appears to be the time required for the angiogenic tumor cells to accumulate to a threshold population sufficient to recruit new blood vessels for the whole tumor. Once neovascularization has occurred, spontaneous metastases may appear in the lung (depending on tumor type). Non-angiogenic tumor cells form microscopic nonangiogenic metastases (T. Udagawa et al, unpublished personal communication). These studies indicate that a nonangiogenic, dormant primary tumor may not shed tumor cells into the circulation. Once it switches to the angiogenic phenotype, it may then shed both angiogenic and nonangiogenic tumor cells into the circulation. The nonangiogenic tumor cells may be the source of microscopic metastases that are capable of remaining dormant for prolonged periods. It has also been reported that in some instances, tumor cells may remain dormant as solitary cells.[145,146]

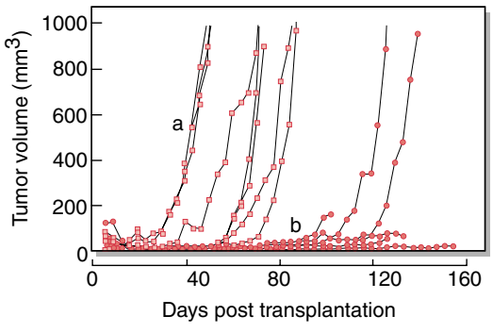

Figure 11-3 Tumor growth curves of subcutaneous xenotransplants of human liposarcoma in severe combined immunodeficient (SCID mice). Tumor cells were transplanted into groups of 28 mice. A majority of tumors remained as non-angiogenic in situ tumors of less than one cubic millimeter beyond 150 days. Some tumors switched to the angiogenic phenotype by approximately 30 days, while others did not become angiogenic until 100 days or more. Once the tumors were angiogenic, the growth rates of the angiogenic tumors were similar to each other. This experiment reveals that human tumors that are angiogenic contain subclones of non-angiogenic tumor cells mixed with angiogenic tumor cells.[2]

Molecular components of the angiogenic switch The first proteins that 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.[46] This result motivated the development of in vitro and in vivo bioassays to guide the search for tumor-derived angiogenic factors.[147–149]

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), followed shortly by acidic FGF (aFGF or FGF-1).[29,150–152] There is extensive literature on the FGFs and their receptors (reviewed in Amalric and colleagues).[153] 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, but lack a signal sequence for secretion.[154] The expression of bFGF receptors is very low. Although many different cells synthesize bFGF, including tumor cells of the central nervous system, sarcomas, genitourinary tumors, and even endothelial cells in the tumor vasculature, it is not clear how bFGFs, in the absence of a signal sequence, are exported from tumors, unless proteinases or heparanases mediate release of FGF from extracellular matrix.[143,154–158] 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.[159,160,161] Furthermore, some tumors recruit macrophages[140] and activate them to secrete bFGF,[143] whereas others attract mast cells, which, because of their high content of heparin, could sequester bFGF. In spontaneous tumors that arise in transgenic mice, aFGF and bFGF are exported into conditioned medium by angiogenic tumor cells but not by pre-angiogenic cells in earlier stages of tumor progression.[54,120] 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 stimulate mainly endothelial proliferation almost to the exclusion of smooth muscle and fibroblast proliferation.[61,163] However, the ability of endothelial-derived angiopoietin-2 to repel smooth muscle or to prevent smooth muscle or pericytes from intimate contact with endothelial cells may explain this phenomenon in part (see Angiopoietins below).[164–166]

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.[167–169]

Abnormally elevated levels of bFGF are found in the serum and urine of cancer patients and in the cerebrospinal fluid of patients with different types of brain tumors.[170,171] High bFGF levels in renal carcinoma correlate with a poor outcome.[172] Also, bFGF levels in the urine of children with Wilms' tumor correlate with stage of disease and tumor grade.[173]

VEGF/VPF Dvorak first proposed that tumor angiogenesis is associated with increased microvascular permeability.[174] This led to the identification of vascular permeability factor (VPF).[175–177] VPF was subsequently sequenced by Ferrara and shown to be a specific inducer of angiogenesis called vascular endothelial growth factor (VEGF).[176–179] Since then, more than 15 angiogenic inducers have been identified, most of them as tumor products (Table 11-2).[180]

VEGF is an endothelial cell mitogen and motogen that is angiogenic in vivo.[181–183] Its expression correlates with blood vessel growth during embryogenesis and is essential for development of the embryonic vascular system.[184–186] VEGF expression also correlates with angiogenesis in the female reproductive tract, and in tumors.[187–190] 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 ($VEGF_{165}$ 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)[191] and the 200 kDa human kinase insert domain-containing receptor (KDR) and its mouse homolog, Flk-1.[192] VEGF binds to both receptors, but KDR/Flk-1 transducers the signals for endothelial proliferation and chemotaxis .[7,193–196] Other structural homologues of the VEGF family have recently been identified, including VEGF-B, VEGF-C, VEGF-D, and VEGF-E.[197,198] VEGF-C binds to Flt-4, which is preferentially expressed on lymphatic endothelium.[199,200] Neuropilin-1, a neuronal guidance molecule, is a recently discovered receptor for $VEGF_{165}$, but not for $VEGF_{121}$.[201] 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 tumor cells, or it could act in a juxtacrine manner to mediate cooption of microvessels by tumor cells. Neuropilin also binds placenta growth factor-2 (PlGF-2) and heparin is essential for the binding of $VEGF_{165}$ and PlGF-2 to neuropilin-1.[57,202] The natural cell surface polysaccharide in vivo is heparan sulfate, not heparin. Heparan sulfate may act as a template to accelerate the interaction of VEGF or PLGF-2 with VEGF. VEGF expression by tumors is up regulated by hypoxia and is often elevated near areas of tumor necrosis.[203–207] Hypoxia activates a hypoxia inducible factor-1 (HIF-1)-binding sequence in the VEGF promoter, which leads to VEGF mRNA transcription and stability.[7,205]

VEGF expression is also upregulated 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.[208]

VEGF expression is inhibited by the von Hippel Lindau (VHL) protein.[209] The VHL tumor suppressor gene is inactivated in patients with VHL disease and in most sporadic clear-cell renal carcinomas.[210,211] The VHL gene negatively regulates a series of hypoxia-inducible genes, including the VEGF, platelet-derived growth factor (PDGF) B, and the glucose transporter GLUT1 genes.[209,212] When VHL is mutated or deleted, these genes are overexpressed even under normoxic conditions. A recent important therapeutic advance employed an inhibitor (SU5416) of the VEGF receptor (VEGFR2) to restore sight in a patient with von Hippel Lindau syndrome who had a hemangioblastoma in the retina of her only eye.[213]

VEGF also induces fenestrations in endothelium of small venules and capillaries and even in

Table 11-2 Endogenous Angiogenesis Promoters

Protein	Molecular Mass (kDa)	Year
FGF-β	18	1984
FGF-α	16.4	1984
Angiogenin	14.1	1985
Transforming growth factor-α	5.5	1986
Transforming growth factor-β	25	1986
Tumor necrosis factor-α	17	1987
Vascular endothelial growth factor	40–45	1983
(VPF/VEGF)		1989
Platelet-derived endothelial growth factor	45	1989
Granulocyte colony-stimulating factor	17	1991
Placental growth factor	25	1991
Interleukin-8	40	1992
Hepatocyte growth factor	92	1993
Proliferin	35	1994
Angiopoietin-1	70	1996
Leptin	16	1998

FGF-β = basic fibroblast growth factor; FGF-α = acidic FGF; VPF = vascular permeability factor.

tissues where microvessels are not normally fenestrated.[212,214] The increased permeability of tumor vessels as revealed, for example, by the edema of brain tumors, may be partly mediated by VEGF-induced fenestrations.[215]

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.[216] Second, the two endothelial mitogens act synergistically[216,217,218,219] to stimulate capillary tube formation in vitro. Third, systemic administration of a soluble receptor for VEGF (Flk-1) partially blocks cornea angiogenesis induced by implanted bFGF (R. D'Amato and C. Kuo, unpublished data). Another positive regulator of angiogenesis, transforming growth factor (TGF)-β, may also be dependent on VEGF. VEGF mRNA and protein are induced in fibroblasts and epithelial cells by TGF-β.[220] 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 kDa ligand that binds to a specific tyrosine kinase expressed only on endothelial cells, called Tie2 (also called Tek). A ligand for Tie1 has not been elucidated.[221–225] 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.[226] There is increased vascularization in mice that overexpress angiopoietin-1 in the skin.[224] 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.[227] This model closely approximates angiogenesis in healing wounds (ie, 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, produced by vascular endothelium in a tumor bed, blocks the Tie-2 receptor and acts to repel pericytes and smooth muscle.[225] Nevertheless, tumor vessels remain thin "endothelium-lined tubes" even though some of these microvessels reach the diameter of venules (Figure 11-4). 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.[227]

It is useful for clinicians to think of two classes of angiogenesis inhibitors: direct and indirect (Figure 11-5).[37]

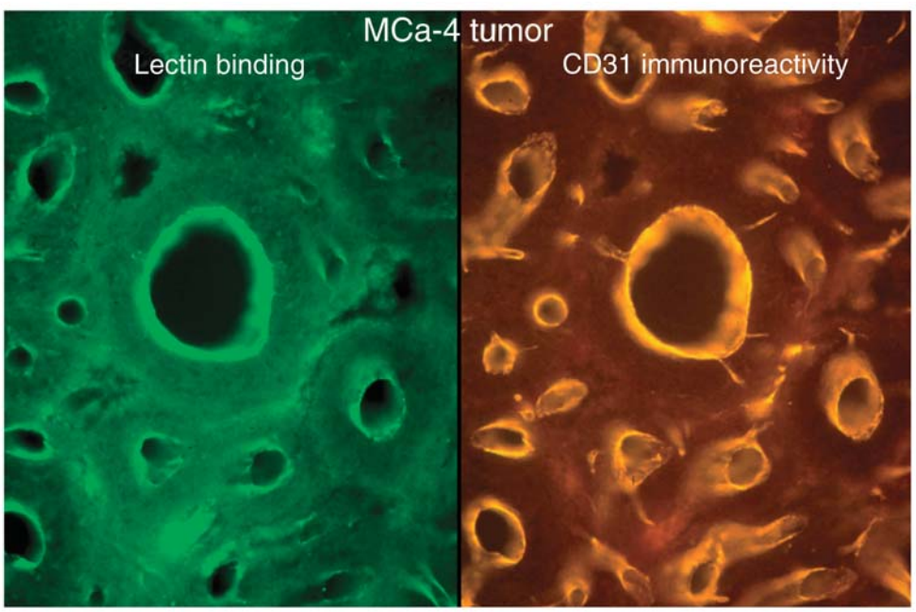

Figure 11-4 Blood vessels in a MCa-IV breast cancer. Tumors were transplanted into mice and subsequently the tumors were perfused with fixative so that the microvessels would not be compressed. From Professor Donald McDonald, University of California, San Francisco.[122] (Four-color version of figure on CD-ROM)

Indirect angiogenesis inhibitors These inhibitors generally decrease or block expression of a tumor cell product, neutralize the tumor product itself, or block its receptor on endothelial cells (Figure 11-6). Many of these tumor cell proteins are the products of oncogenes that drive the angiogenic switch. Rak and Kerbel reviewed the impact of 15 different oncogenes on tumor angiogenesis (Table 11-3).[37,228–230] In vitro assays were originally employed to elucidate the activities of oncogene and tumor suppressor gene products, including, proliferation of cancer cells, resistance to apoptosis, immortalization, and anchorage independence.[228,229] In these in vitro assays, increased cancer cell proliferation and decreased apoptosis associated with oncogene mutations correlated so well with tumor growth in vivo, there was at first no indication that oncogenes could also upregulate angiogenic activity in tumor cells.[231] Nor was there any basis to predict that novel anticancer drugs, which inhibit oncogene activity by inhibition of its signal transduction, (eg, signal transduction inhibitors such as Herceptin) could also block proangiogenic products of tumor cells.[231] Only recently has it been recognized that many oncogenes are proangiogenic, and that this may be a major component of their tumorigenic activity. The proangiogenic oncogenes (in addition to bcl-2) listed in Table 11-3 upregulate expression by tumor cells of angiogenic proteins and/or downregulate inhibitors of angiogenesis.[228,232]

Other experimental studies in which transfection with oncogenes increase tumor cell output of proangiogenic proteins and/or decrease expression of negative regulators of angiogenesis, support this concept.[76,77,99,230] For example, when the bcl-2 oncogene was transfected into prostate cancer cells that were already capable of developing large neovascularized tumors, VEGF expression and microvessel density both increased significantly.[232] Therefore, drugs that target oncogenes, or their products, or the receptors of those products, not only inhibit proliferation of tumor cells and increase their susceptibility to apoptosis, but, most importantly, these drugs disrupt production of tumor angiogenesis. Even re-activation of the tumor suppressor p53, can inhibit angiogenesis by at least four different mechanisms discussed below.[75,161,233,234] It is not surprising then that certain anticancer drugs developed for their capacity to block an oncogene product (for example, inhibitors of the EGF receptor tyrosine kinase), may have significant antiangiogenic activity, and that it will be "indirect" (Tables 11-3 and 11-5).[228,231,235] For example, ras farnesyl transferase inhibitors block oncogene signaling pathways which upregulate tumor cell production of VEGF and downregulate production of the angiogenesis inhibitor, thrombospondin-1.[208]

Why emphasize in this chapter that anticancer drugs that target an oncogene product may inhibit tumor angiogenesis? Because a clinician armed with this knowledge should be better able to optimize the dose and schedule of an

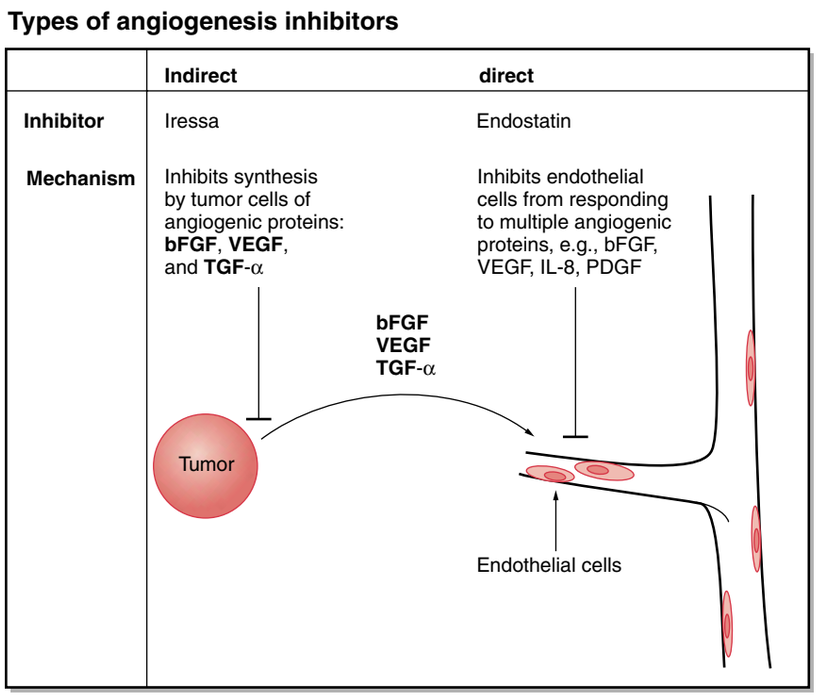

Figure 11-5 Direct angiogenesis inhibitors are less likely to induce acquired drug resistance because they inhibit endothelial cells in the tumor bed from responding to a wide spectrum of pro-angiogenic proteins from tumor or from stroma. In contrast, indirect angiogenesis inhibitors, block an oncogene expressed by the tumor, or a tumor cell product, or the receptor for that product. This permits the emergence of mutants producing pro-angiogenic proteins that might not be antagonized by the indirect angiogenesis inhibitor.

"antioncogene drug" or a "signal transduction inhibitor" by following antiangiogenic guidelines.[37] For example, if mutant tumor cells that express an angiogenic protein different from the one blocked by an "indirect" angiogenesis inhibitor arise, the indirect angiogenesis inhibitor may be discontinued prematurely, on the assumption that the tumor had become drug resistant, and because conventional chemotherapy is traditionally discontinued in the presence of acquired drug resistance (see Figure11-6). However, it may be prudent to continue a drug with significant antiangiogenic activity, especially if it is not toxic, and add a second angiogenesis inhibitor. For example, trastuzumab (Herceptin), an antibody that blocks HER-2/neu receptor tyrosine kinase signaling, suppresses cancer cell production of angiogenic factors such as TGF-β, angiopoietin-1, and plasminogen activator inhibitor-1 (PAI-1) and possibly VEGF.[231,235] Herceptin also up-regulates expression of an endogenous angiogenesis inhibitor, thrombospondin, which may be the important basis of its antiangiogenic activity.[235] If tumor cells begin to express a different angiogenic protein such as bFGF, or interleukin-8 the tumor under treatment may appear to have become "resistant" to Herceptin.[236,237] A similar speculation may apply to certain epidermal growth factor (EGF) receptor inhibitors such as Iressa (gefitinib; ZD 1839), or to other tumor cell products which regulate angiogenesis (see Table 11-3).[237,238]

Of interest is that certain tumors such as giant cell bone tumors and angioblastomas, generate only or mainly bFGF, without usually expressing other angiogenic proteins. When patients with these tumors were treated with interferon-α at low daily doses, drug resistance was not observed with therapy of 1 to 3.5 years duration.[239–242] Interferon-α at low doses inhibits bFGF production by tumor cells and would therefore be considered an indirect angiogenesis inhibitor.[243] However, interferon also inhibits endothelial cell motility directly, and therefore can be considered to have both direct and indirect antiangiogenic activity.[244]

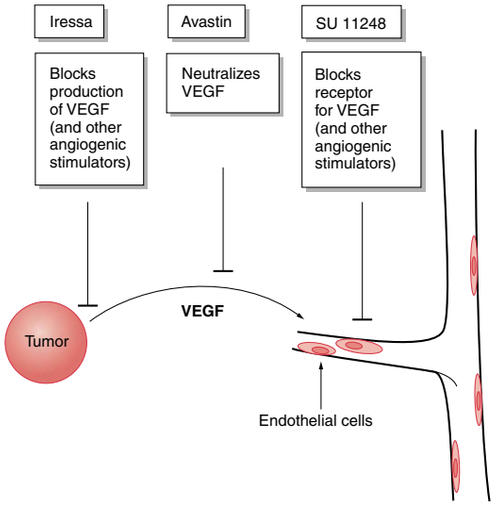

Figure 11-6 Examples of indirect angiogenesis inhibitors that can block production of a tumor cell angiogenic protein (Iressa), or neutralize a pro-angiogenic factor in the blood (Avastin), or block a receptor for a tumor cell produced angiogenic factor (SU11248).

Direct angiogenesis inhibitors These inhibitors directly block vascular endothelial cells from proliferating, migrating or increasing their survival in response to a spectrum of proangiogenic proteins, including VEGF, bFGF, IL-8, PDGF, and PD-ECGF and others (Table 11-4). Direct angiogenesis inhibitors are less likely to induce acquired drug resistance, because they target genetically stable endothelial cells, rather than unstable mutating tumor cells.[245] Tumors in mice treated with antiangiogenic therapy did not develop drug resistance.[69] This does not mean that angiogenesis inhibitors are less susceptible to metabolic degradation, increased clearance from the blood stream, or development of neutralizing antibodies, than any other drug that is administered systemically.

Direct angiogenesis inhibitors include (1) certain synthetic inhibitors or peptides designed to interfere with various steps in the angiogenic process (eg, antagonists of the integrin $\alpha_v\beta_3$); or inhibitors of metalloproteinases; (2) low molecular weight compounds (eg, TNP-470), thalidomide, angiostatic steroids such as tetrahydrocortisol, 2-methoxyestradiol, and squalamine; and (3) endogenous (natural) proteins, which generally prevent vascular endothelial cells from responding to a wide spectrum of angiogenic promoters (eg, interferon-α, interleukin-12, platelet factor 4, thrombospondin-1, angiostatin, endostatin, arresten, canstatin, tumstatin, PEX, pigment epithelium-derived factor, and antiangiogenic antithrombin III (an internal fragment of antithrombin III, named aaAT).[28,59,64,65,67,70,246,247–266]

Table 11-3 Impact of Oncogenes or Potential Oncogenes on Tumor Angiogenesis

Oncogene	Implicated Proangiogenic Activity
K-ras, H-ras	VEGF upregulation, TSP-1 downregulation
v-src	VEGF upregulation, TSP-1 downregulation
c-myb	TSP-2 downregulation
N-myc	angiogenic properties in neuroblastoma
c-myc	angiogenic properties in epidermis
HER-2	VEGF upregulation
EGFR	VEGF, bFGF, IL-8 upregulation
PyMT	TSP-1 downregulation
c-fos	VEGF expression
trkB	VEGF downregulation
HPV-16	secretion of VEGF and IFN-α
v-p3k	VEGF production and angiogenesis
ODC	novel angiogenic factor
PTTG1	VEGF and bFGF upregulation
E2a-Pbx1	induction of mouse angiogenin-3
bcl-2	VEGF upregulation

bFGF = basic fibroblast growth factor; IFN = interferon; TSP = thrombospondin; VEGF = vascular endothelial growth factor.

Table 11-4 Direct Angiogenesis Inhibitors

Drug	Endothelial Cell Target	Clinical Trial
Angiostatin	Binds to ATP synthase, angiomotin, and annexin II on endothelial cells to inhibit endothelial cell proliferation and migration	Phase I
Bevacizumab (Avastin)	Recombinant humanized monoclonal antibody against VEGF	Phase II and III
Arresten	Believed to bind integrin-$\alpha_1\beta_1$ to inhibit endothelial cell proliferation, migration, tube formation, and neovascularization	No
Canstatin	Believed to bind integrin-$\alpha_v\beta_3$ to inhibit endothelial cell proliferation, migration, and tube formation	No
Combretastatin	Microtubules: induces reorganization of the actin cytoskeleton and early membrane blebbing in human endothelial cells	Completed phase I
Endostatin	Inhibits VEGF receptor. Binds and inhibits MMP2. Blocks endothelial motility by binding to α5 and αv integrins. Interferes with phosphorylation of eNOS at Ser 1177. Inhibits cyclin D1 in endothelial cells. Prevents bFGF-induced and VEGF-induced loss of adhesion of endothelial cells to basement membrane	Phase I and II
NM-3	An isocoumarin small-molecule that selectively inhibits endothelial cell proliferation, sprouting, and tube formation in vitro	Phase I
Thalidomide	Inhibits bFGF-induced angiogenesis. Decreases VEGF and bFGF in plasma of multiple myeloma patients. Decreases bone marrow-derived circulating endothelial cells by 10-fold toward normal in multiple myeloma. Its antiangiogenic activity is not its relatively weak TNF-α activity or its very weak immunosuppressive activity.	Phase II
Thrombospondin	Blocks endothelial cell migration and neovascularization in the cornea, but might not be specific for endothelial cells	No
Tumstatin	Binds to integrin-$\alpha_v\beta_3$ on endothelial cells; inhibits endothelial cell proliferation and neovascularization	No
2-Methoxyestradiol	Inhibits microtubule function in proliferating endothelial cells, resulting in endothelial cell apoptosis	Phase I and II
Vitaxin	A humanized monoclonal antibody against $\alpha_v\beta_3$	Phase I and II

eNOS= endothelial nitric oxide synthase; MMP = matrix metalloproteinase; TNF = tumor necrosis factor.

Endogenous Inhibitors of Angiogenesis

Angiostatin, endostatin, tumstatin, arresten, canstatin and aaAT are specific angiogenesis inhibitors that are endogenous. 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 which, under physiologic conditions, may act to limit or prevent angiogenesis; and (3) endostatin and angiostatin are currently in Phase II clinical trials.[267] Tumstatin is a candidate for future clinical trials (see Clinical Trials below).

The first clue to the existence of endogenous angiogenesis inhibitors came from the discovery that interferon-α/-β inhibited endothelial cell migration and that platelet factor-4 inhibited endothelial proliferation.[244,260,268] Both were subsequently shown to inhibit angiogenesis.[244,260,268–270]

However, Rastinejad and colleagues were the first to show that a tumor could generate an angiogenesis inhibitor. They subsequently proposed that the angiogenic phenotype was the result of a net balance of endogenous inhibitors and stimulators of angiogenesis.[271] 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, a truncated form of thrombospondin-1 (TSP-1), that decreased by about 96% in the tumorigenic cells.[261] TSP-1 was shown to be regulated by the wild-type tumor suppressor p53 in fibroblasts and in mammary epithelial cells.[75,272] Loss of p53 function in the transformed derivatives of these cells dramatically decreased the level of angiogenesis inhibitor. Restoration of p53 upregulated TSP-1 and raised the antiangiogenic activity of the tumor cells. Deletion of thrombospondin-1 leads to accelerated growth of breast cancers that arise spontaneously in neu-transgenic mice.[273] The demonstration by Rastinejad and coworkers that the switch to an angiogenic phenotype involved a negative regulator of angiogenesis generated by the tumor per se suggested to Folkman a unifying angiogenic mechanism to explain a well-recognized but previously unsolved clinical and experimental phenomenon: the inhibition of tumor growth by tumor mass. A description of this phenomenon is quoted from the introduction of Folkman's paper.[65] "The removal of certain tumors, eg, breast carcinomas, colon carcinomas, and osteogenic sarcomas can be followed by rapid growth of distant metastases."[274–277] Postoperative chemotherapy was introduced mainly to prevent or delay the growth of secondary metastases. Several studies in terminally ill patients demonstrate the suppression of a secondary tumor by a primary tumor.[278,279] A primary tumor can suppress metastases originating from a different type of tumor (eg, a breast cancer can inhibit melanoma metastases). In melanoma, partial spontaneous regression of the primary tumor may be followed by rapid growth of metastases. When ionizing radiation is employed to regress a small-cell lung cancer, distant metastases may undergo rapid growth.[70,280] If one portion of a primary tumor is removed (eg, cytoreductive surgery for testicular cancer), the residual tumor increases its rate of expansion.[281] 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.[282–286] Partial removal of a primary tumor increases growth rate of the residual tumors similar to humans.[287,288] Furthermore, metastatic growth can suppress the growth of a primary tumor (analogous to the occult primary in a cancer patient).[289] Many primary tumors can suppress the growth of a second tumor inoculation.[290–297] 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.[295,297]

At least three hypotheses have been advanced to explain these diverse observations and experiments: (1) "concomitant immunity," in which a

Table 11-5 Antiangiogenic Activity of Drugs Targeting an Oncogene, Its Product, Or Product Receptor

Oncogene Product or Its Receptor	Proangiogenic Protein(s)	Inhibited By:
EGF tyrosine kinase receptor	VEGF, bFGF, TGF-α	ZD 1839 (Iressa), ZD 6474, OSI 774 (Tarceva), CI 1033, PKI 1666, IMC 225 (Erbitux)
VEGF receptor	VEGF receptor on endothelium	PTK 787, ZD 6474, SU 5416, SU 6668, SU 11248
VEGF	VEGF	Avastin
PDGF receptor	PDGF receptor	PTK 787, SU 11248, STI 571 (Gleevec)
HER-2 receptor	VEGF, Angiopoietin-1, TGF-α, PAI-1, (Thrombospondin = endothelial inhibitor) Upregulated by Herceptin	Herceptin
bFGF	bFGF	Interferon-α

EGF = epidermal growth factor; PDGF = platelet-derived growth factor.

primary tumor induces an immunologic response against a secondary tumor or a metastasis in the same host, (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.[291,295,298–300] 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, 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.[261] However, at least two conditions would be necessary: first, the primary tumor (ie, 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 hypothesis by discovering angiostatin, endostatin, and antiangiogenic antithrombin over the next 8 years.[65–67,69,70,301]

Angiostatin Angiostatin is a 38 kDa internal fragment of plasminogen 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 angiogenesis (Figure 11-7).[65] Lung metastases remained microscopic and did not grow beyond approximately 200 μm in 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 was determined by bromodeoxyuridine [BrdU] labeling) and 7 to 8% were undergoing apoptosis.[45] The presence of a circulating angiogenesis inhibitor was evidenced by the almost complete inhibition of bFGF-stimulated corneal neovascularization in these mice. Further, serum from these tumor-bearing mice specifically inhibited capillary endothelial cell proliferation in vitro by more than 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 disappeared by 6 days (half-maximal 2.5 days), lung metastases were neovascularized by 5 days, and by 15 days the mice were dying of large vascularized metastases. There was no change in tumor cell proliferation (approximately 38% BrdU-labeled cells), but tumor cell apoptosis 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,[65] and recombinant angiostatin potently inhibited growth of other tumor types.[66] Again, tumor cell proliferation remained as high as in the tumor-bearing mice, and apoptosis was significantly reduced.[45,48] 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 cir-

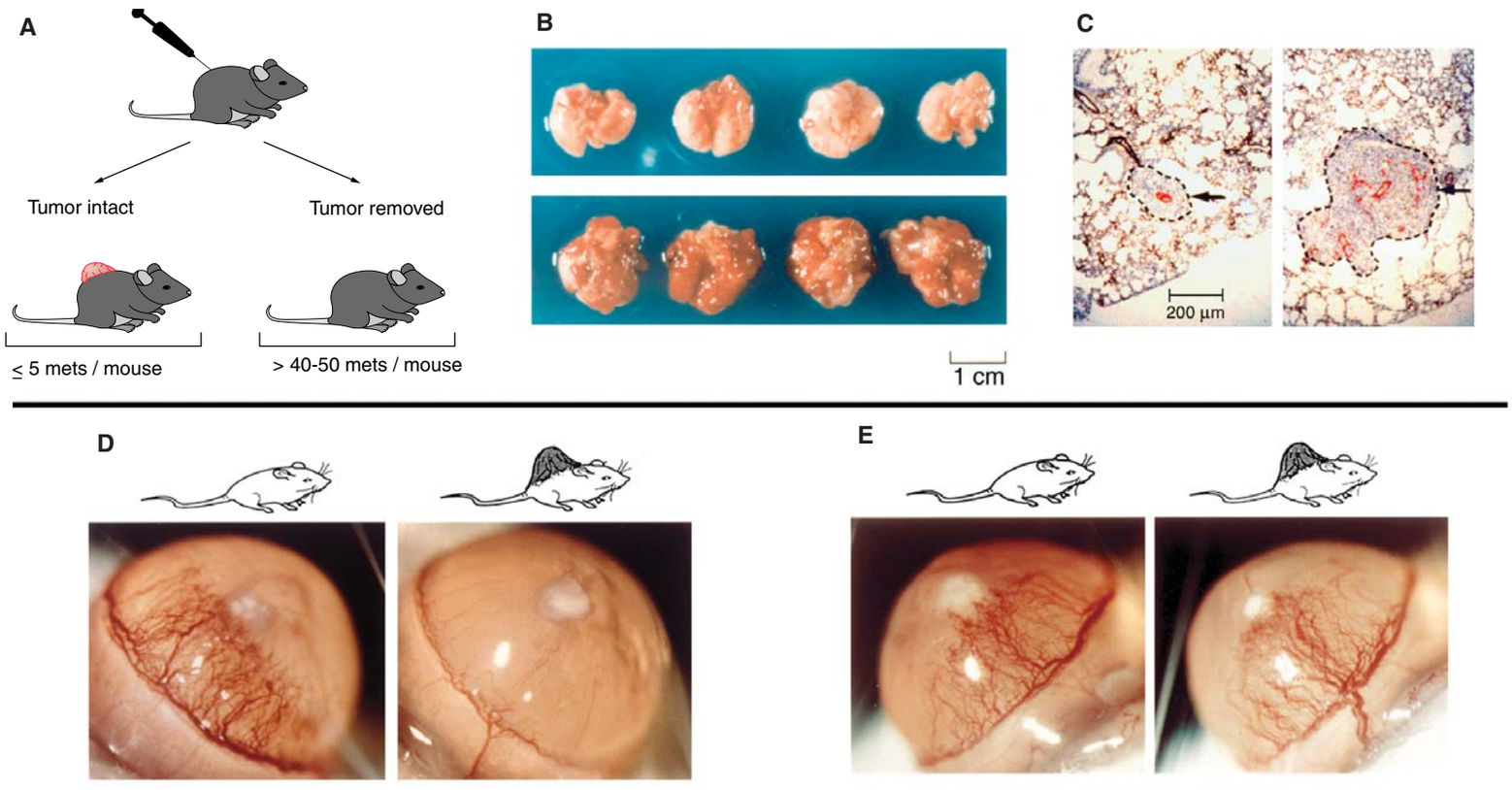

Figure 11-7 **A,** Animals bearing Lewis Lung carcinoma. Tumors were surgically removed when tumor size reached 1.5 - 2 cm^2 and the animals were killed 5 days or 15 days later. **B**, Upper panel: lungs removed from animals still bearing the primary tumor. Lower panel: lungs removed at the same time from animals in which the tumor had been removed and the animals killed 15 days later. **C,** Left panel: microscopic metastasis in the lung of an animal in which a primary tumor is in place at the same time as the right panel. This shows no angiogenesis and only a single central microvessel stained with antibody to von Willebrand factor. This dormant metastasis is approximately 200 microns in its longest diameter. Right panel: lung metastasis from an animal killed 5 days after removal of the primary tumor, showing 8 or 9 new vessels in an enlarging metastasis. **D,** A human prostate carcinoma (LNCaP) growing on the dorsum of a SCID immunodeficient mouse almost completely inhibits cornea neovascularization induced by an implanted sustained release pellet of bFGF (80 ng) (right panel). Left panel shows bFGF-induced corneal neovascularization at 5 days in the absence of a primary tumor. LNCaP prostate cancer generates angiostatin. **E,** A human colon cancer that does not generate an angiogenesis inhibitor, growing on the dorsum of a SCID mouse as a control for **D**. (Four-color version of figure on CD-ROM)

culating plasminogen by a series of enzymes released from the tumor cells. At least one of these tumor-derived enzymes, urokinase plasminogen activator (uPA), converts plasminogen to plasmin, while a phosphoglycerate kinase from hypoxic tumor cells then reduces the plasmin so that it can be converted to angiostatin by one of several different metalloproteinases (Figure 11-8).[302] Other types of tumors have since been reported to generate angiostatin (eg, human prostate cancer).[303–305] Human prostate carcinoma cells express enzymatic activity that converts human plasminogen to the angiogenesis inhibitor angiostatin.[302] Prostate-specific antigen generates angiostatin-like fragments from plasminogen.[307]

Tranfection of Angiostatin. 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.[78] 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. This result was confirmed in a different tumor model, Kaposi's sarcoma subjected to angiostatin gene transfer, which resulted in sustained angiostatin expression, delayed tumor growth and reduced tumor vascularization.[360] Gene transfer with a different angiogenesis inhibitor, thrombospondin, gave similar results. Streit and colleagues reported (in an experiment discussed above) that when tumor cells were transfected with the angiogenesis inhibitors thrombospondin-1 and/or thrombospondin-2, proliferation rate (as determined by staining for the proliferating cell nuclear antigen [PCNA]) (see Figure 11-9), also remained relatively constant, despite the decreased angiogenesis and suppressed tumor growth.[79]

Mechanisms of Action. Angiostatin and its isoforms induce cell arrest and apoptosis of endothelial cells and inhibit endothelial migration, angiogenesis in vitro, and angiogenesis in the quail chorioallantoic membrane, which provides a quantitative bioassay.[308–317] They can also be generated by different enzymes and by other cell types; can inhibit other tumor types; decrease activity of the mitogen-activated protein kinase ERK-1 and ERK-2 in endothelial cells; up-regulate E-selectin in proliferating endothelial cells; and can be delivered in vivo by gene therapy.[302–304,313–333] In addition, they bind specifically to ATP-synthase, a transmembrane protein expressed by vascular endothelial cells; bind to a fragment of vitronectin; and can potentiate radiation therapy of experimental tumors.[334–337]

A provocative recent finding is that proliferation of circulating precursor endothelial cells derived from bone marrow is inhibited at significantly lower concentrations of angiostatin than is proliferation of endothelial cells isolated from tissues.[136] 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.[136] Angiostatin effectively blocks endothelial cell migration induced by plasmin binding to $\alpha_v\beta_3$ integrin.[346,362] Angiostatin may act at least in part by transiently increasing ceramide, a sphingolipid second messenger implicated in a proapoptotic pathway, and by increasing RhoA, an effector of cytoskeletal structure.[361]

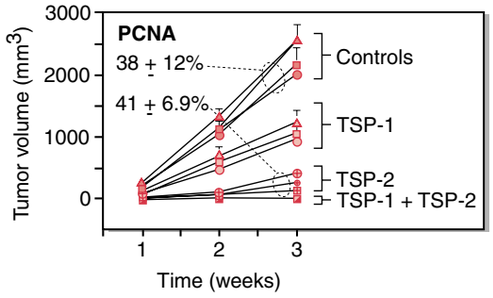

Figure 11-9 Squamous cell carcinoma cells transfected with the angiogenesis inhibitor thrombospondin-1 and or thrombospondin-2. Tumors producing the most antiangiogenic activity (TSP-1 and TSP-2) have the lowest growth rate and remained dormant. (Modified from Streit et al.[79]) Tumors with low or no release of antiangiogenic activity have the highest growth rate and produce large tumors. The proliferation rate of tumor cells (PCNA) is not significantly different between large and small tumors.

Numerous additional recent studies have been reported on the mechanism of angiostatin and on its biological and pharmacological activities. A few of these new findings are discussed here. Angiostatin, but not plasminogen, specifically binds to tyrosine kinase substrate annexin II through the lysine-binding domain in endothelial cells.[338] This affinity appears to be specific for endothelial cells and does not occur with fibroblasts. Annexin II on endothelial cells organizes assembly of plasminogen on its surface and produces plasmin at maximum efficiency in the vascular bed.[338–341] Plasmin is a serine protease that degrades fibrin and several endothelial cell matrix proteins, such as laminin, thrombospondin, and collagens, and facilitates capillary sprouting during angiogenesis as well as endothelial migration.[338,346,362] In fact, angiostatin has been shown to inhibit endothelial and melanoma cellular invasion by blocking matrix-enhanced plasminogen activation (see Figure 11-8).[326] However, because hypoxic tumor cells produce a phosphoglycerate kinase that enzymatically reduces plasmin, this mechanism contributes to the generation of angiostatin, which can then block plasmin organization on the endothelial surface and interfere with angiogenesis.[302,363] The gene for annexin II is also upregulated in endothelial cells during angiogenesis. Annexin II is a central molecule in signal transduction mechanisms, and disruption of its function affects calcium signaling, tyrosine phosphorylation, cell proliferation and apoptosis.[342–344] Angiostatin mediation of endothelial cell death appears to be through a calcium signaling mechanism.[345] Therefore, annexin II may act as

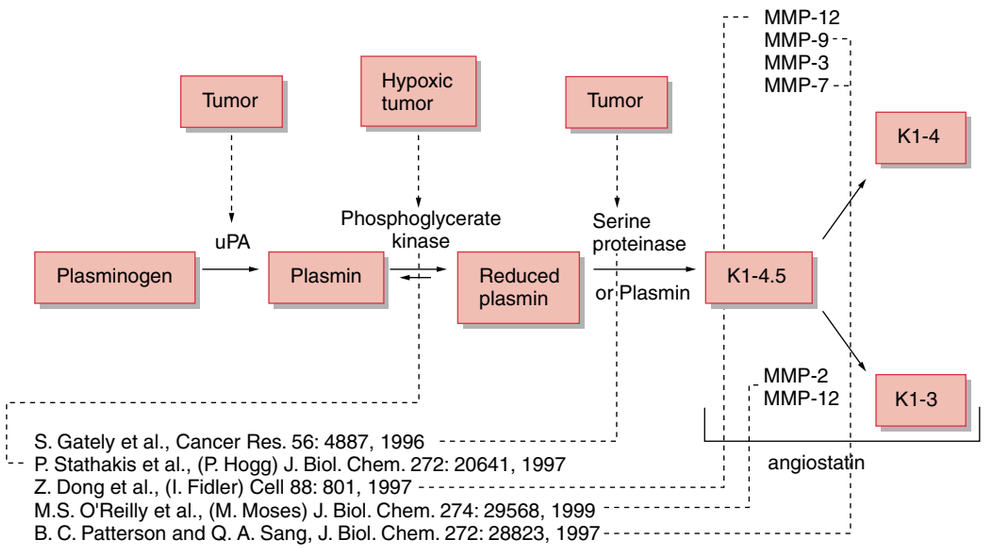

Figure 11-8 Model of angiostatin formation. Urokinase plasminogen activator from tumors converts plasminogen to plasmin. A phosphoglycerate kinase from hypoxic tumors converts plasmin to reduced plasmin which is then susceptible to further cleavage by a serine proteinase from tumors or by plasmin itself, to kringle fragments 1-4.5 or lower depending upon which metalloproteinase is present. From Stathakis et al.[302]

an endogenous angiogenic switch, and its blockade may be at least one potential mechanism for angiostatin.

Binding proteins for angiostatin. Three other binding proteins have been suggested as putative receptors for angiostatin: (1) ATP synthase on the endothelial surface, (2) aVb3, and (3) angiomotin, which belong to a novel protein family with conserved coiled-coil and PDZ binding domains.[334,346–348] Taken together with annexin II, Tuszynski and colleagues suggest that angiostatin may bind to multiple receptors and could mediate a cascade of events leading to inhibition of angiogenesis.[338] Angiostatin also inhibits signaling of hepatocyte growth factor (HGF) in endothelial cells (and in smooth muscle cells), by blocking HGF-induced signaling of c-met, Akt, and ERK1/2.[349] However, angiostatin does not block VEGF or bFGF signaling events in endothelial cells. Angiostatin also induces intracellular acidosis in endothelial cells and anoikis (loss of cell attachment) in endothelial cells that are incubated under conditions resembling the low pH in a tumor.[350] Angiostatin downregulates expression of VEGF expression in tumor cells.[351] This implies that angiostatin may act not only as a direct inhibitor of angiogenesis, but also as an indirect angiogenesis inhibitor. Analysis of the x-ray crystallographic structure of angiostatin reveals that kringles 1, 2, and 3 produce a central cavity suggesting a unique domain where they may function in concert.[352,364] The enzymatic mobilization of angiostatin is facilitated by tumor-derived matrix-metalloproteinase.[353] Other labs confirm the report that circulating angiostatin generated by a primary tumor decreases significantly after the primary tumor is removed and that this is followed by growth of remote metastases.[35,65]

Angiostatin gene therapy. Angiostatin gene therapy shows efficacy in tumor-bearing mice against a variety of tumors and their metastases including, breast cancer, renal cell cancer, gliomas, melanoma, Kaposi's sarcoma, squamous cell carcinoma, and leukemia.[310,360,365–375] Of interest was that viral vector-mediated antiangiogenic therapy using an angiostatin-endostatin fusion protein demonstrated significant downregulation of ascites, tumor growth, vascularity and prolongation of animal survival in three different ascites tumor models.[376] Angiostatin has been transfected into canine eyes as a preclinical model for the treatment of human uveal melanoma.[377] Long-term expression of angiostatin and endostatin from lentivirus-transduced human bladder carcinoma cells inhibited proliferation of endothelial cells co-cultured with the tumor cells, the first use of a lentiviral vector for antiangiogenic gene delivery.[378] Angiostatin gene therapy has also shown efficacy in nonneoplastic mouse models of disease such as arthritis, retinal neovascularization, choroidal neovascularization, and endometriosis.[379–382]

Angiostatin protein therapy. Angiostatin protein therapy has shown significant efficacy when delivered systemically to mice bearing a variety of different tumors, and also in combination with endostatin where it has shown a synergistic effect or as a fusion protein of angiostatin and endostatin, or in combination with interleukin-12.[351,383–387] When three different types of murine tumors were engineered to produce granulocyte colony-stimulating factor, (GM-CSF), the level of angiostatin in the serum increased 4-fold above controls and directly correlated with GM-CSF production.[388] Angiostatin levels directly correlated with macrophage metalloelastase production, which appeared to mediate cleavage of angiostatin from plasminogen. Metastases were suppressed in all three tumor systems.

Replacement of angiostatin after radiotherapy. Radiotherapy of primary Lewis lung carcinomas in mice induced regression of the primary tumor, but led to disappearance of angiostatin from the blood and rapid growth of lung metastases.[280] Angiostatin replacement therapy prevented growth of lung metastases after regression of the primary tumor by radiotherapy. In some patients successful radiotherapy of a primary tumor is followed by growth of distant metastases, for example, nonsmall cell lung cancer. It may eventually be possible to determine if a tumor is generating an endogenous angiogenesis inhibitor prior to radiotherapy and then administer that inhibitor in combination with the radiotherapy.

Angiostatin protein also inhibits angiogenesis in nonneoplastic states. For example, angiostatin inhibits corneal neovascularization, corpus luteum development in the pre-ovulatory follicle, and as pathological retinal angiogenesis, but not physiological retinal neovascularization.[389–392] Although the original report of 38 kDa angiostatin included kringles 1-4 of plasminogen, a recent report reveals the generation of a novel plasminogen fragment of 22 kDa generated by enzymatic activity from tumor cells.[65,393] The p22 plasminogen fragment includes kringle 1 plus additional N- and C- terminal residues, but circular dichroism and intrinsic fluorescence analysis define structural differences between it and recombinant kringle 1. Angiogenesis and tumor growth are potently inhibited by p22.

When a strategy similar to the one that uncovered angiostatin (eg, suppression of a tumor growth by tumor mass) was employed by O'Reilly and colleagues with murine hemangioendothelioma and human small-cell lung cancer, endostatin and antiangiogenic antithrombin (aaAT) were discovered.[67,69,70] Both endostatin and aaAT are generated from larger parent proteins by enzymes released by the tumor cells. Angiostatin is currently in phase I/II clinical trials.

Endostatin Endostatin is a 20-22 kDa internal fragment of collagen XVIII.[67,69,394,395] It is the first of a group of endogenous angiogenesis inhibitors which are predominately extracellular proteins, which generally require proteolytic processing to become active.[396,397] More than 350 reports on endostatin have been published in the 5 years since its isolation and identification as an angiogenesis inhibitor.[67] These range from mechanistic studies to experimental therapeutics. A variety of new findings have been reported on mechanism of action, physiologic function, structure-activity relationships, experimental therapeutics including gene therapy, and early clinical studies. A few of these studies are reviewed here.

Structure-activity relationships. Endostatin was first isolated and sequenced from conditioned medium of murine hemangioendothelioma based on the same strategy employed for the discovery of angiostatin (ie, hemangioendothelioma suppressed secondary tumors).[398] It is a specific inhibitor of endothelial cell proliferation and migration like angiostatin. High-resolution radiographic structures have been determined for mouse and for human endostatin (Figure 11-10).[399,400] At least two enzymes produced by tumor cells are necessary to cleave endostatin from collagen XVIII, an elastase and a cathepsin.[401,402] Endostatin is derived from the nontriple helical C-terminal NCI domain of collagen XVIII. Subsequently collagen XV has also been found to share a C-terminal NC1 domain containing the endostatin molecule, which has not been found to date in any other proteins.[403] Endostatin is released proetolytically in trimeric form and further converted to monomeric forms of about 20 kDa. Both endostatin isoforms share a compact globular fold, but differ in binding properties for proteins and cells.[403] Tissue distributions are also different. Differences in activity have been found between NCI domains and

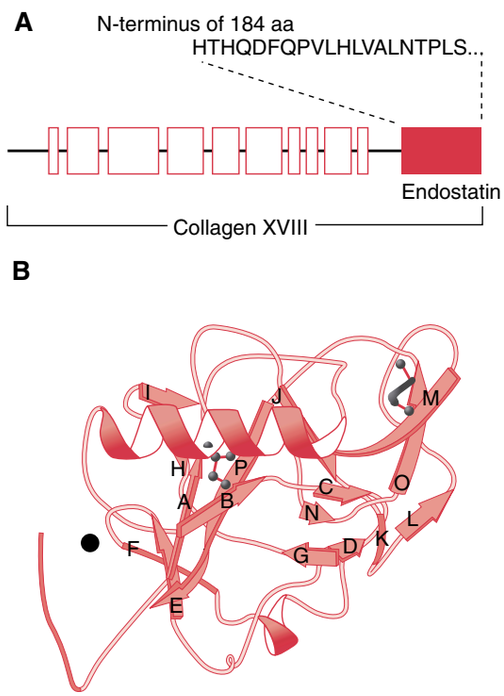

Figure 11-10 **A,** Amino acid sequence of human endostatin.[67] **B**, Crystal structure of human endostatin resolved to 3.5 angstroms. [400]

endostatin activity may be related to its oligomerization state.

Endostatin in basement membrane. 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.[404] Some, but not all, capillaries or arterioles show weak labeling for endostatin. Within the elastic fibers, endostatin is co-localized and has been reported to bind to fibulin-1 and -2, nidogen-1 and -2, laminin-1 and perlecan.[405] Recombinant mouse endostatin binds to heparin with a K_d of 0.3 μM.[406] A major site of four clustered arginines, 155, 158, 184 and 270 and a second site (R193, R 194) is essential for binding to heparin as well as to heparan sulfate and sulfatides, but not to fibulin-1 and fibulin-2. A minimum heparin size of 12-mer (dodecasaccharide) is necessary for efficient binding and there is a crucial role for 2-O and 6-O sulfation. A synthetic arginine-rich dendrimer that mimicked the surface of endostatin and had high affinity for heparin, had similar antiangiogenic activity in the chick embryo. This experiment demonstrates the important role of heparin affinity for the inhibitory activity of endostatin.[407] A decreased inhibitory activity after mutation of the zinc-binding site was reported for insoluble recombinant endostatin from *E. coli,*[408] but elimination of zinc binding had no effect on antiangiogenic activity in mouse soluble recombinant endostatin.[406] Also, zinc ligand-disrupted recombinant soluble human endostatin from yeast showed potent antitumor activity in mice.[409] It is unclear why insoluble recombinant endostatin required zinc for inhibitory activity,[408] but that soluble endostatin did not. However, it should not be overlooked, that the original insoluble recombinant zinc-containing endostatin induced more rapid tumor regression in different tumor types than has been obtained to date with soluble forms with or without zinc.[67,69] One possibility is that zinc is required to maintain folding of the insoluble protein.[403] Alternatively we have preliminary data that peptides of endostatin released from insoluble endostatin inhibit angiogenesis (K. Javaherian, personal communication). Furthermore, under physiological conditions endostatin exists mainly as a fragment of an insoluble matrix protein, collagen XVIII. It appears that only a small fraction of endostatin circulates in the soluble form (ie, in the range of 30 ng/mL).

Of the 20 collagen isoforms that have been identified so far in mammalian species, collagens IV, XV and XVIII (which possess C-terminal globular domains) have been implicated in regulation of angiogenesis.[410] Of interest is that homologues of these three collagens have also been identified in the worm *C. elegans*, where the endostatin domain of type XVIII collagen controls migration of neuronal cells and axon guidance instead of vascular endothelial cells.[411] The evolutionary age of endostatin of approximately 600 million years, may be responsible in part, for its lack of toxicity in animals and patients.[67,69,267] It is also fascinating from an evolutionary perspective that certain regulators of endothelial cell migration have (or had) control of axon migration, eg, endostatin, neuropilin semaphorins and ephrins, among others.[412–415] This relationship provides insight into how development of nerves and blood vessels may be coordinated.

In the chick embryo angiogenesis induced by bFGF was inhibited by endostatin, but not by an endostatin mutant that does not bind to heparin.

Mechanisms of action. Novel mechanisms have recently been reported for the antiangiogenic action of endostatin, although we are a long way from a complete picture.

Inhibition of VEGF receptor. Endostatin blocks the binding of $VEGF_{121}$ and $VEGF_{165}$ to the KDR/Flk-1 receptor, blocks tyrosine phosphorylation of this receptor, and blocks activation of its intracellular signaling events, ERK, p38 MAPK and p125FAK.[416] This receptor mediates endothelial cell motility and proliferation. Endostatin also blocks VEGF.

Downregulation of VEGF expression. While endostatin does not bind to VEGF,[416] it does downregulate VEGF expression in tumor cells (similar to the effect of angiostatin).[351] Endostatin can therefore be considered to act as both a 'direct' and an 'indirect' angiogenesis inhibitor. In bFGF-treated endothelial cells, endostatin induces endothelial cell apoptosis, in part by activating tyrosine kinase signaling of the Shb adaptor protein.[417] However, endostatin does not compete with binding of bFGF to tissues, and it does not affect bFGF receptor signaling.[418] This result is consistent with the observation that bFGF (FGF-2) stimulation of endothelial cell proliferation in vitro and angiogenesis in vivo is mediated by VEGF.[419]

Prevention of bFGF-induced or VEGF-induced loss of endothelial cell–cell adhesion or endothelial cell adhesion to basement membrane. Endothelial cell migration, which is critical for new sprout formation during angiogenesis, requires continuous turnover of cell-cell interactions and of cell-matrix interactions.[420] Cell-cell interactions between endothelial cells are maintained in part by β-catenin localized at cell-cell junctions in cadherin complexes of capillary endothelial cells in vitro. Treatment of these cells with bFGF shifts β-catenin to the cytoplasm, which loosens cell-cell adhesion and facilitates endothelial migration.[421] However, co-treatment with endostatin transiently blocks this shift.[421] Cell-matrix interactions are maintained in part by formation of focal adhesions and actin stress fibers. Either bFGF or endostatin alone, induced tyrosine phosphorylation of focal adhesion kinase and paxillin, which promoted formation of focal adhesions and actin stress fibers. However, when microvascular endothelial cells were co-treated with bFGF and endostatin, focal adhesion and actin stress fibers were decreased, which decreased migration of endothelial cells. In an in vivo situation, the entire vascular bed would be exposed to circulating endostatin. But its antiangiogenic effect would only become evident in areas of high growth factor stimulation, such as those present in the tumor endothelium.[421] Taken together, these results provide a plausible mechanism to explain clinical trial data (see below) in which antiangiogenic effects of endostatin are observed in the absence of systemic toxicity.[421]

Vessel stabilization: reversal of bFGF-induced or VEGF-induced loss of endothelial cell–cell adhesion or endothelial cell adhesion to basement membrane. Another antiangiogenic mechanism of endostatin in addition to its antiproliferative and antimotility effect on angiogenic endothelial cells is the stabilization of newly formed endothelial tubes. Endostatin decreases formation of VEGF-induced microvessels sprouting from aortic rings in vitro. By immunohistochemistry, administered endostatin localizes to endothelial cell-cell junctions as well as to adhesion sites between endothelial cells and their basement membrane, thus anchoring endothelial cells to each other and to their basement membrane (Figure 11-11).[422] Endostatin does not localize to quiescent vasculature. High concentrations of endostatin were detected by immunohistology in rat and human brain tumors in and around endothelial cells, in tumors cells, and in macrophages and leukocytes in the tumor's vascular bed, but normal brain showed no endostatin reactivity.[447] This unique localization of endostatin only in newly formed angiogenic microvessels, appears to reverse the loosening of endothelial cells to each other and to their basement membrane, which was necessary for them to form angiogenic sprouts in the first place. Similar "stabilizing" effects of endostatin were observed in tumors in vivo, where administered endostatin was more abundant in association with angiogenic vessels undergoing maturation, mainly at the tumor marginal zone where angiogenesis was highly active. Endostatin immunostaining is present during vascular formation up to the stage exhibiting one layer of peri-endothelial cells, but disappears in further stabilized vessels that have more than one layer of these cells in their wall.[422] This suggests that the vascular stabilizing effect of endostatin occurs earlier than the integration of peri-endothelial cells into the vascular wall. A subsequent step of vascular stabilization by recruitment and integration of peri-endothelial cells (pericytes and smooth muscle cells) is mediated by angiopoietin-1, TGF-β, and PDGF.[226] Of interest is that during endothelial tube formation induced by VEGF, endostatin plus VEGF-treated tubes became so stable that after cessation of endostatin, the survival time of these tubes was approximately doubled in comparison to VEGF alone. Furthermore, incubation of endothelial cells with an antibody to endostatin prevented formation of stable VEGF-induced tubes and disrupted existing tubes. The role of endostatin in modulating endothelial cell interaction with extracellular matrix is also revealed by experiments in which oligomeric endostatin (from the NC1 domain of collagen XVIII), induces motility and morphogenesis of tube

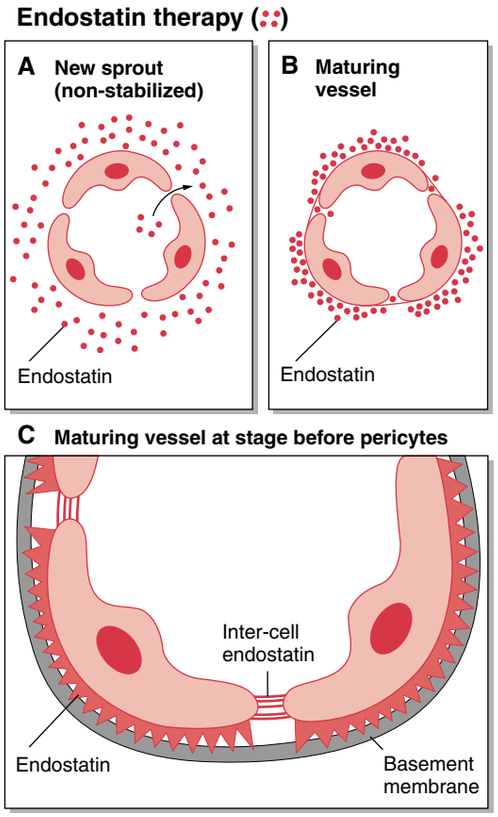

Figure 11-11 Model for the antiangiogenic mechanism of endostatin. During vascular stabilization and maturation endostatin immunostaining (red dots) changes from a diffuse distribution around endothelial cells of non-stabilized blood vessels to a stronger staining in the inter-endothelial junctions and in the contact zone between endothelial cells and their basement membrane. **(A)** It can be postulated that endostatin (ES) stabilizes the wall of newly formed blood vessels via anchoring of endothelial tubes to the basement membrane (BM) and of endothelial cells (green) to each other which leads to vascular maturation **(B)**. This process converts the angiogenic blood vessels into the quiescent phenotype, thereby inhibiting tumor vascularization. One of the potential consequences of the angiogenic switch may be the reduction of vascular pemeability leading to tumor regression. Beside the demonstrated anti-proliferative effect of endostatin on endothelial cells, these effects point to an additional antiangiogenic mechanism of endostatin, e.g. tumor regression via stabilization of newly formed blood vessels. Therapeutic strategies accelerating vascular stabilization and maintenance promise to be effective in tumor treatment.[422]

forming endothelial cells in Matrigel, whereas monomeric endostatin inhibits these effects.[423] Endostatin downregulates the uPA system on the endothelial cell surface.[424] Pro-angiogenic proteins such as bFGF increase the secretion of uPA) and plasminogen activator inhibitor (PAI-1) leading to an overall increase in proteolytic activity prerequisite for endothelial cell sprouting and tube formation. Endostatin treatment of endothelial cells markedly decreases their production of uPA and PAI-1.

Inhibition of metalloproteinase. Endostatin also binds and inhibits the catalytic activity of MMP-2.[425,426] As a result, endothelial cell invasion is inhibited and tumor cell invasion may also be decreased. Tumor progression and angiogenesis are reduced in MMP-2 deficient mice and MMP-2 is required for the switch to the angiogenic phenotype.[106,427]

Binding of endostatin to endothelial cell surface integrins and other proteins that modulate motility and morphogenesis. Endostatin also inhibits integrin-dependent endothelial cell migration because it binds to α_5- and α_v-integrins on the endothelial cell surface, in particular $\alpha_5\beta_1$.[428] Kalluri and colleagues have also proposed that $\alpha_5\beta_1$-integrin may be a functional receptor for endostatin (R. Kalluri, unpublished data).[37] High concentrations of endostatin also interact with tropomyosin to interrupt microfilament integrity, which inhibits endothelial motility and may participate in part in endothelial apoptosis.[429] Endostatin also binds to glypicans on the endothelial cell surface at concentrations which suggest that glypicans may be low affinity endostatin receptors.[430]

Inhibition of cyclin D1 causing G1 arrest of endothelial cells. Endostatin causes G1 arrest in endothelial cells, by decreasing the hyperphosphorylated retinoblastoma gene product and downregulating cyclin D1 mRNA and protein.[431] Transcription through the LEF1 site in the cyclin D1 promoter is essential for endostatin's inhibitory activity. Others report that endostatin arrests proliferation and causes apoptosis of endothelial cells.[432,433]

Dephosphorylation of endothelial nitric oxide. Human recombinant endostatin dose-dependently blocked VEGF-induced endothelial cell migration, with complete inhibition at 100 ng/mL.[434] VEGF stimulates synthesis of endothelial nitric oxide (eNOS). In endothelial cells sphingosine-1 phosphate activates the serine/threonine kinase Akt and stimulates phosphorylation of eNOS to release nitric oxide.[435] VEGF-stimulated endothelial nitric oxide synthesis (eNOS) was abolished by endostatin, (although VEGF-induced formation of prostacyclin was not significantly affected by endostatin). Endostatin interfered with activation of eNOS downstream of Akt by specifically inhibiting phosphorylation of eNOS at Ser 1177 by stimulating activation of the phosphatase PP2A (Figure 11-12).[434] Nitric oxide synthesis also appears to be critical for the mitogenic effect of VEGF on endothelial cells.[114–116] Inhibition of nitric oxide synthesis also blocks tumor angiogenesis.[436] We have previously proposed another role for nitric oxide synthesis in angiogenesis: early vasodilation of pre-existing quiescent vessels as a prerequisite for sprout formation.[116]

Hypoxic downregulation of endostatin production by endothelial cells and pericytes. Collagen XVIII is converted to endostatin, which is secreted from endothelial cells and pericytes in vitro at a constant rate.[437] The rate of conversion is higher in pericytes. During hypoxia, production of endostatin is significantly decreased. (30-40% of this hypoxia-induced 43% degradation of endostatin occurs in pericytes and approximately 10% in endothelial cells.) Exogenous endostatin

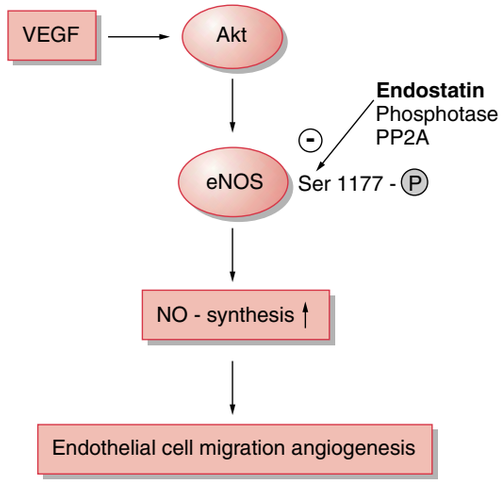

Figure 11-12 Endostatin specifically interferes with eNOS phosphorylation at Ser 1177. The upstream signaling pathway including VEGF-induced Akt activation is not affected. [434]

is more rapidly degraded when incubated with hypoxic conditioned media of endothelial cells or pericytes. The mechanism of endostatin degradation by the conditioned medium is unknown. These results imply that: (1) decreased endostatin production by hypoxic pericytes and/or endothelial cells may be another mechanism to facilitate the angiogenic switch in a tumor; (2) pericytes may employ endostatin as one of the strategies to inhibit endothelial proliferation under normal conditions; and (3) the treatment of a patient with a large tumor burden or with a tumor with a large hypoxic center (eg, glioblastoma), may require high doses of endostatin at the beginning of therapy, because of endostatin degradation.

Physiologic Functions Physiologic functions for endostatin are gradually being elucidated.

Regulation of development of retinal vasculature. Endogenous endostatin in the eye may contribute in part to the normal development of vasculature in the retina. During development of the eye, hyaloid vessels supply the vitreous and the lens. Subsequently, these vessels regress leaving a transparent vitreous and lens. Endostatin is thought to play a key role in regression of the hyaloid vessels because they fail to regress in endostatin's absence. In a rare disease called Knobloch syndrome, the hyaloid vessels fail to regress, retinal vasculature fails to develop, and patients suffer retinal degeneration and blindness, among other abnormalities.[438,439] In this syndrome, a splice mutation in human collagen XVIII leads to a truncated protein lacking the endostatin fragment. A proposed mechanism of retinal degeneration is that persistent hyaloid vessels increase oxygen in the retina, which suppresses hypoxia-inducible factor-1 (HIF-1α) and VEGF, both of which drive normal vascular growth in the normal developing retina. Retinal vasculature also fails to develop in a collagen XVIII-deficient mouse model.[440] Thus, a pathological deficiency of endostatin in humans and mice reveals that endostatin is an endogenous

inhibitor of angiogenesis under normal physiological conditions.

Endostatin in platelets. Platelets contain at least 14 positive regulators and approximately 12 negative regulators of angiogenesis.[441] The positive regulators include VEGF-A,VEGF-C, bFGF, HGF, angiopoietin-1, PDGF, heparanse and sphingosine-1 phosphate, among others. The negative regulators include endostatin, platelet factor 4, thrombospondin, plasminogen (angiostatin), high molecular weight kininogen (domain 5), and α-2 antiplasmin, among others. These stimulators and inhibitors of endothelial cell migration and proliferation may be released into a blood clot in a wound in an orchestrated sequence to regulate neovascularization.[442] For example, thrombin induces the release of the proangiogenic protein VEGF from platelets.[356] Thrombin also induces the release of endostatin from platelets.[443]

Thrombin induction of endostatin release is mediated by proteinase-activated receptor-4 (PAR-4), a mechanism that is independent of platelet aggregation and of the action of ADP. When a clot forms in a wound, invasion by microvascular endothelial cells is initially inhibited, which avoids premature clot lysis.[442] Subsequently, endothelial cells are recruited into the clot during its neovascularization ("granulation tissue"). It is not known how the release of negative and positive regulators of endothelial migration from platelets is orchestrated in a wound. Recent evidence, however, reveals that if a thrombus in a large vessel is recanalized, the neovasculature is made up mainly of bone marrow-derived endothelial cells.[444] This is analgous to certain tumors (eg, lymphomas) that recruit the majority of their endothelial cells from bone marrow. Endostatin is also released from platelets by certain drugs, such as ticlopidine, which inhibits platelet aggregation by blocking the action of ADP with its receptor.[445]

The nonsteroidal antiinflammatory drugs celecoxib and flurbiprofen or HCT-1026 (a nitric oxide-releasing derivative of flurbiprofen), all of which are cyclooxygenase inhibitors, also release endostatin from platelets and elevate the plasma level of endostatin.[446] From these findings, one can ask whether the antiangiogenic activity of celecoxib (Celebrex) operates in part by releasing endostatin from its stores and making it available to endothelial cells in an angiogenic site. Also, future research will be necessary to answer other questions. What is the turnover rate of endostatin in platelets? Do cancer patients on long-term celecoxib therapy deplete their platelet stores of endostatin? Can platelets be loaded with endostatin that is administered systemically or in vitro? Would cancer patients treated with celecoxib benefit from periodic endostatin therapy? Would patients on long-term endostatin benefit from celecoxib therapy? Taken together these findings indicate that endostatin is normally distributed among three compartments: (1) stored as a cryptic fragment in collagen XVIII in basement membranes; (2) stored in platelets; and (3) circulating as low levels of free endostatin. A fourth compartment can be considered as the concentration of endostatin in and around angiogenic vessels in tumors, and possibly at other angiogenic sites.

CONTROL OF VASCULAR PERMEABILITY Endostatin decreases vascular permeability. bFGF treatment increases permeability of [^{3}H]inulin through confluent monolayers of capillary endothelial cells in a transwell assay, an effect that is counteracted by co-treatment with endostatin.[421] In mice that have received an intravenous injection of Evans blue dye, a large blue stain appears at the site of a subcutaneous injection of VEGF or platelet-activating factor (PAF) (Miles test). Pretreatment with endostatin blocks the permeability induced by these proteins (S. Soker, J. Heymach, and J. Folkman, unpublished data). These results imply that systemic administration of endostatin to patients may decrease edema in brain tumors, but this speculation has not yet been tested.

Experimental therapeutics including gene therapy ***Insoluble protein administered systemically*** Systemic administration of endostatin can inhibit or regress different tumors. The first recombinant endostatin was from *Escherichia coli* in an insoluble form, because this had previously been successful with angiostatin, and because refolding of the inclusion body to a soluble form by urea, guanidine-HCL and glutathione gave a low yield (1%).[65,67,69] When this endostatin preparation was administered subcutaneously every day 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. Wounding the tumor site or moving the tumor to another site in the same mouse initiated tumor neovascularization and growth. Another possibility is preferential storage of endostatin in the matrix at the site of the regressed tumor.[448] We have observed this same dormancy after 40 days of endostatin therapy of rat mammary cancer induced by an oral carcinogen.[449] Other investigators have reported successful tumor inhibition with insoluble endostatin from *E. coli*.[450] Also, soluble endostatin from *E. coli* has inhibited tumor growth.[451]

Soluble recombinant endostatin A wide variety of other tumors in many different laboratories have been inhibited by endostatin in mice and rats without evidence of toxicity or drug resistance. These include lung adenocarcinoma, thyroid carcinoma, colon carcinoma, leukemia, human nonsmall cell lung cancer, human pancreatic cancer, human neuroblastoma, mammary cancer (soluble endostatin from *E. coli*), colon cancer metastases to liver, spontaneous mouse mammary carcinoma (delayed onset, decreased tumor burden, and prolonged survival), and spontaneous pancreatic islet carcinomas.[72,452–460] Mammary carcinomas were more potently inhibited by a mutated form of endostatin in which an alanine residue was substituted for a proline at position 125 (P125A), than by the wild-type endostatin.[458] Gliosarcoma both orthotopic and ectopic in rats was markedly inhibited (~50%) after only 10 days of treatment by a relatively low dose of murine endostatin (0.3 mg/kg).[461] Inhibition of tumor growth persisted after cessation of therapy. Yamaguchi and colleagues reported that endostatin administered into a tumor will inhibit tumor growth at significantly lower doses than systemic administration.[461a]

Bolus versus continuous administration A general characteristic of optimal antiangiogenic therapy is that angiogenesis inhibitors should be at effective levels in the blood continuously. Endothelial cells in the vascular bed of a tumor are constantly exposed to a proangiogenic stimulus from the tumor cells and local stroma. This stimulus needs to be continuously opposed by inhibitors in the blood. An analogy would be titration of blood glucose by insulin, where endothelium is glucose. In a compelling experiment, 24-hour continuous parenteral administration of human endostatin was 10-fold more effective than the same dose administered as a bolus once every day, against human pancreatic cancer in SCID immunodeficient mice (se Figure 11-1).[72] Tumor growth was inhibited in a dose-dependent manner, but only continuous administration caused tumor regression. It should be noted that continuous administration of endostatin in vitro (by a floating microosmotic pump in 24-well plates) inhibited endothelial proliferation more significantly than once daily addition of endostatin.

Combinations of endostatin with angiostatin Reports of endostatin administered in combination with angiostatin, whether as a separate proteins, separate genes, or as fusion protein of angiostatin and endostatin in tumor-bearing animals, all show a synergistic increase in efficacy.[69,386,375,462] While the mechanism of synergy is unclear at this writing, one possibility is that endostatin and angiostatin each up-regulate thrombospondin expression or protect it from enzymatic destruction (Figure 11-13).

Combination of endostatin with chemotherapy, radiotherapy, or immunotherapy Endostatin (and angiostatin) potentiate radiotherapy in tumor-bearing mice, when the inhibitor is administered at the same time as the ionizing radiation.[463] Tumors underwent regression when treated with a combination of ionizing radiation and endostatin, but not with either agent alone, even when the endostatin dose was lowered below the effective dose and the tumor cells were radioresistant. It can be speculated that radiation damage to active endothelium in a tumor bed may not be repaired in the presence of an angiogenesis inhibitor. This experiment also suggests that radiotherapy may be in part endothelial-dependent. The optimal schedule of radiotherapy and antiangiogenic therapy for cancer patients is unclear.

Endostatin may also potentiate immunotherapy. In an elegant experiment by Davidoff et al

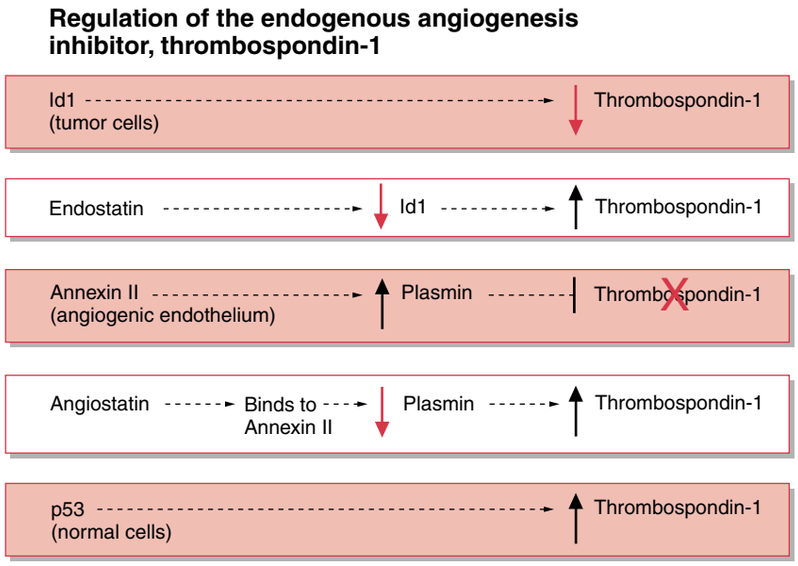

Figure 11-13 Endostatin, angiostatin, and wild type p53 all act to up-regulate thrombospondin-1, a potent endogenous angiogenesis inhibitor. Endostatin down-regulates Id1 which leads to the up-regulation of thrombospondin-1. Angiogenic endothelium overexpresses annexin II which mobilizes plasmin at the surface of endothelial cells. Plasmin degrades thrombospondin-1. Angiostatin binds to annexin II to decrease plamin which can increase available thrombospondin-1.

the gene for the potent immunogen, green fluorescent protein (GFP) and the gene for endostatin were transferred together to murine neuroblastoma cells prior to inoculation into syngeneic immunocompetent mice.[464] Prevention of formation of appreciable tumors was effected in 63% of mice, while tumor cells expressing endostatin alone or GFP alone yielded only a modest delay in tumor growth. Mice treated with the combination were protected against subsequent tumor challenge with unmodified cells. Endostatin also potentiates conventional chemotherapy in tumor-bearing animals.[465] However, it is not clear how combinations of antiangiogenic therapy and chemotherapy at maximum tolerated dose schedules will translate to patients (see below).

Endostatin gene therapy Endostatin gene therapy in tumor-bearing mice has generally been successful when administered locally, but it has been problematic when administered systemically.

Local gene therapy. In an example of local gene therapy, mouse liver tumor cells transfected with a retroviral vector containing the murine endostatin gene were injected subcutaneously or into the peritoneal cavity.[467] After 63 days, control tumors (vector without endostatin) reached 2400 mm^3 compared to 30 mm^3 for endostatin transduced tumor cells ($p < 0.001$). After 123 days all 16 mice given an intraperitoneal injection of control tumor cells had died, compared to only 3 of 32 mice given an injection of the endostatin tumor cells (91% survival) (Figure 11-14).[467] Increased levels of endostatin were detected in tumor lysates, but not in serum of mice with subcutaneous tumors. Other reports of local gene therapy with endostatin show effective tumor inhibition in animals. Examples are inhibition of human liver carcinoma, renal cell carcinoma, mouse melanoma.[468–471] A human breast carcinoma, MDA-MB435, was markedly inhibited when the cells were transduced with endostatin, but another breast cancer MCF7 was not inhibited.[365] (However, angiostatin gene transfer inhibited MCF7, but not MDA MB435).[365] Gliomas were inhibited by gene transfers to tumor cells that were then implanted into rat brains.[472] Gliomas were also inhibited by intratumoral implantation of alginate-encapsulated beads containing packaging cells transfected with endostatin.[473–475]

Of interest is that intratumoral gene transfer of endostatin inhibited mouse mammary tumors (MidT2-1), but had a minimal effect in human breast MDA-MD-231 in SCID mice.[476] Despite many successes of endostatin gene transfer into tumor cells, unsolved problems remain. The fact that one author can report marked and sustained tumor inhibition with endostatin transduction of a mouse breast cancer, but minimal effect in a human breast cancer MDA-MD-231, highlights the problem.[476] A third author reports marked inhibition by endostatin gene transfer into a human breast cancer MDA-MBA335, but minimal effect in MCF7, yet angiostatin gene transfer into MCF7 was very inhibitory, but had only minimal effect on MDA-MB435 breast cancer cells.[365] For unknown reasons, the inhibitor may lose activity when produced by one type of tumor cell, but not by another. Whether this is a protein-folding problem, or results from excess local levels of endostatin at the endothelial receptor site is not clear (O. Kisker, et al., unpublished data). A fourth author reports that breast cancer (MCA-4) transduced with endostatin is significantly inhibited.[477]

Systemically administered gene therapy. Systemically administered gene therapy (by an intravenous or intramuscular vector in mice or rats) significantly inhibited tumor growth in

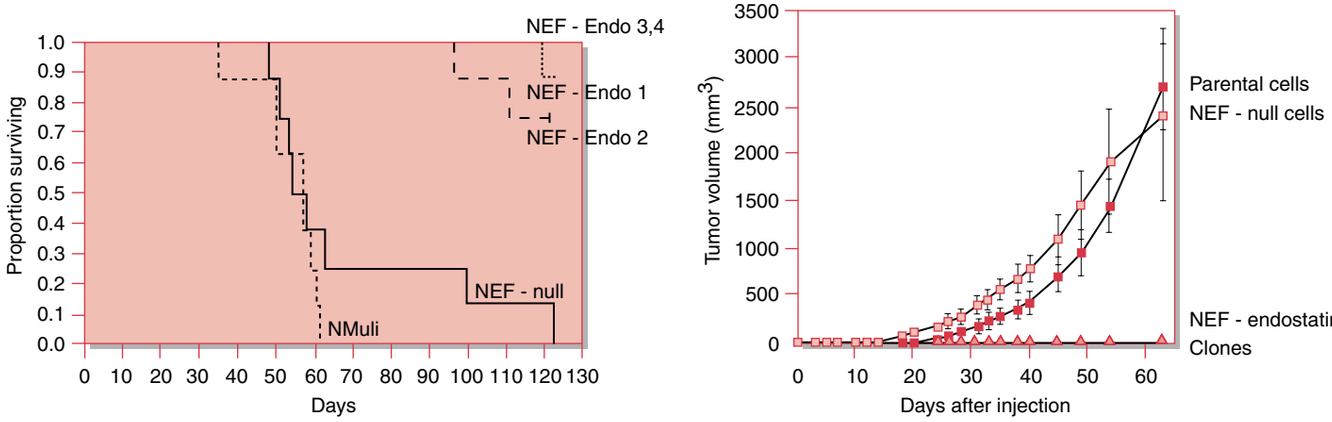

Figure 11-14 Left panel: Mouse survival after intraperitoneal inoculation of parental tumor cells and tumor cells transduced with endostatin. n = 8. Controls are NEF-null and NMmuLi. Mice bearing NEF-endostatin transduced tumor cells survive up to 120 days. Right panel: Growth curves of endostatin transduced tumor cells which remain dormant and microscopic vs. control tumor cells which grow to over 2 grams by 60 days.[467]

renal cell carcinoma, Lewis lung carcinoma adenocarcinoma MC38, hemangioendothelioma, spontaneous breast cancer and its metastases, and colon cancer (when the gene was injected intramuscularly and endostatin serum levels were only moderately elevated, [35-40 ng/mL]).[367,469,478–481] Adenoviral gene transfer of endostatin delayed growth of breast cancer implants and retarded their growth by 67% after the tumors appeared.[478a]

Problems with systemic gene therapy. However, other reports of systemically administered endostatin gene therapy failed to inhibit tumor growth. For example, either local or systemic endostatin gene therapy failed to inhibit rat tumors despite the achievement of serum levels more than 10 to 200 times above normal levels of endostatin (eg, 500 ng/mL up to 10 μm/mL).[482] However, these tumors were significantly suppressed when an inhibitor of plasmin was administered together with endostatin by gene therapy, but not when the plasmin inhibitor was administered alone.[482] This result suggests that very high levels of plasmin in a tumor bed may interfere with endostatin activity. It is interesting that angiostatin, by binding to annexin II when it is overexpressed by endothelial cells in an angiogenic site, decreases plasmin production on the endothelial surface.[326,338–341]

It remains to be demonstrated if this mechanism plays a role in the synergy of angiostatin–endostatin combinations.[69,375,386,462] We also were unable to demonstrate significant inhibition of tumor growth and only 23% to 33% ($p < 0.002$ and 0.0001 respectively) inhibition of corneal angiogenesis, when viruses encoding angiostatin or endostatin were administered as a single intravenous injection, despite the fact that peak serum levels of endostatin reached 400 to 599 times above the normal level.[485] In contrast, viruses encoding soluble forms of Flk1 or Flt1, the receptors for VEGF, resulted in approximately 80% growth inhibition of the same tumor and strong inhibition of corneal neovascularization (74% to 80%) ($p < 0.001$). A strong correlation was observed between the effects of the different viruses on tumor growth and the activity of the viruses in the inhibition of corneal micropocket angiogenesis. It is unclear why angiostatin and endostatin delivered by systemically administered gene therapy should be so ineffective as tumor inhibitors, when soluble VEGF receptors administered by the same viruses and in the same tumor models should inhibit tumor growth so effectively. However, three speculations may be considered as a basis for future research. Antiangiogenic and antitumor activity of endostatin are dose-dependent, but recent experiments reveal a U-shaped curve (O. Kisker, et al., unpublished data), analogous to interferon-α and also TGF-β.[485a,485b] In one tumor system human recombinant endostatin protein administered at 50 mg/kg/day inhibited tumor growth by 47%; 100 mg/kg by 84% and 1000 mg/kg by 37%. Therefore, it is possible that super high levels of endostatin above an optimum level lose activity in vivo. A second speculation is that endostatin may aggregate at very high serum levels, or that its folding may be affected. A third speculation is that serum plasmin levels may be increased by certain viruses or by high viral titers. Other authors subsequently have reported failure of endostatin gene transfer to significantly inhibit a murine leukemia, when endostatin was transduced into hematopoietic stem cells and serum endostatin levels of 300 ng/mL were reached, or to inhibit metastases from a fibrosarcoma when serum endostatin levels of up to 700 ng/mL were obtained.[483,484] Of interest is that these authors previously reported in collaboration with Folkman and O'Reilly, enhanced antitumor activity with an endostatin-angiostatin fusion protein engineered in hematopoietic stem cells.[484,484a]

Endostatin signaling network To determine how the endothelial cell genome and proteome react to endostatin treatment, we used a genome-wide expression profile covering 95% of the human genome and phosphorylation analysis on antibody arrays (Abdollahi A, Maercker C, Rastert R, et al. Endostatin signaling network. [In preparation]) Briefly, we generated the Human Unigene Chip II containing 74,834 elements, which represents one of the largest gene sets interrogated in array-based gene expression studies to date. Alteration in gene expression was analyzed after four-hour treatment human microvascular endothelial cells with human recombinant endostatin (*Pichia Pastoris,* endotoxin-free EntreMed/Calbiochem). Using 12 reusable chips more than 1,800,000 gene expression measurements in total were performed on endostatin-treated endothelium and compared to control samples of untreated endothelium from two different human subjects. The identities of 50,000 gene clusters (spotted DNA clones) have been sequence verified. By these criteria, 429 anigene clusters were more than 5-fold upregulated. In contrast, 421 anigene clusters were downregulated by 80%.

Overexpressed genes during endostatin treatment included apoptosis genes, cardiovascular and stress response genes, (heat shock, etc.), and genes associated with angiogenesis inhibition. Underexpressed genes during endostatin treatment included gene families associated with cancer and angiogenesis induction (the angiogenic switch), as well as transcripts encoding cytokines, cytokine receptors, cellular adhesion molecules, G proteins, G protein-coupled-receptors and transcription factors.

Endostatin downregulated expression of hypoxia-inducible transcription factor-1 α (HIF-1α) by 2.5-fold.[365,427] Endostatin also downregulated many upstream activators of HIF-lα, including c-jun/Fos, Ets, and EGFR. On the other side, HIF-1α normally helps to trigger a coordinated angiogenic response by inducing the expression of genes involved in blood vessel growth including, VEGF and its receptors, VEGFR2, and neuropilin, as well as nitric oxide synthase and cyclooxygenase-2.

Endostatin also downregulated the Id transcription factors. These genes are proangiogenic. Endostatin substantially downregulated MMP-2 and $\alpha_v\beta_3$-integrin.[425] Furthermore, Id-1 regulates angiogenesis through transcriptional repression of thrombospondin-1.[528] Therefore, endostatin, by downregulating Id-1, up-regulates expression of thrombospondin-1, a powerful angiogenesis inhibitor on its own (see Figure 11-13).

Ephrin signaling is downregulated by endostatin. The ephrin/Eph family has recently been shown to participate in the regulation of angiogenesis. Both ephrin B1 and ephrin B2 induce sprouting angiogenesis in vitro to an extent similar to the angiogenic activity of VEGF or angiopoietin 1.[529, 530]

Endostatin inhibited tumor necrosis factor-α-induced angiogenic signaling by downregulating the TNF-α receptor. Additionally, NF-κB (nuclear factor of kappa light chain gene enhancer in B-cells) was downregulated by endostatin. It is thought that activation of NF-kappaB by TNF-α, ionizing radiation or chemotherapeutic agents protects cells from apoptosis.[532,533] Therefore, endostatin inhibition of NF-kappaB in these settings should enhance apoptosis of endothelial cells. STAT1 and STAT3 were downregulated in endothelial cells treated by endostatin. STATs (signal transducers and activators of transcription factors) up-regulate genes that encode inhibitors of apoptosis (Bcl-family), inducers of angiogenesis (VEGF), and cell cycle regulators (cyclins D1/D2 and c-myc).[534] After endostatin treatment of endothelial cells, most of the genes downstream of STAT were strongly downregulated. These include the apoptosis inhibitor Bcl-2, and the cell cycle regulators, cyclins D1/D2 and c-myc.

Thrombin receptors (PAR-1 and PAR-2) were also significantly downregulated by endostatin. Thrombin is a potent stimulus for release of VEGF by transcriptional regulation activation of HIF-1α.[535] Thrombin also up-regulates expression of VEGF receptors on endothelial cells.[536] In a positive loop VEGF accelerates thrombin generation, another proangiogenic event.[537] This loop has implications for cancer patients. The majority of cancer deaths are directly attributable to thrombosis or bleeding. This observation emphasizes that endostatin downregulates proangiogenic genes and simultaneously up-regulates genes that lead to inhibition of angiogenesis.

Endostatin directly downregulated Ets-1 in endothelial cells in a time-dependent manner and decreased the mRNA level of most of the Ets-1 target genes like MMP-1 and integrin-β3. Ets positively regulates angiogenesis by inducing several target genes including uPA, MMP-1, MMP-3, MMP-9 and integrin-β3, and the elimination of the Ets-1 activity by a dominant negative molecule inhibits angiogenesis in vivo.[538–542] A striking finding is that several pathways thought to be distinct, in fact intercommunicate to bring about a focused antiangiogenic response in the tumor bed, directly as a result of endostatin treat-

ment. It is not clear whether many of the small molecular angiogenesis inhibitors produce such a orchestrated antiangiogenic response at the genetic level as endostatin does.

Clinical applications of endostatin There are several interesting characteristics of endostatin that make it advantageous for clinical use in cancer patients. First, it is not toxic.[267] At this writing, endostatin is in phase I/II clinical trials, and to date it has only been used in not more than approximately 120 patients. However, all centers report that there are virtually no side effects, even in patients who have been on endostatin for up to one year (Endostatin Banbury Conference. A critical review of endostatin and its biology; 2002 Mar 10–13; Cold Spring Harbor Laboratories, New York). Patients who receive the subcutaneous preparation of endostatin take their own injections each day at home, like insulin.

Wound Healing. Endostatin does not delay wound healing in patients, nor does it delay wound healing in animals.[486] We have confirmed that wound healing in mice is not delayed by endostatin doses sufficient to completely inhibit growth of tumors in the same animal (J. Marler, et al., unpublished data).

Pregnancy. Endostatin has no effect on pregnancy in mice. Pregnant mice treated with tumor-regressing doses of endostatin deliver normal babies, even if the mothers bear a tumor that is then inhibited by the endostatin (Robert D'Amato, et al, unpublished data).

Down Syndrome. Serum endostatin levels are significantly elevated in Down syndrome patients.[492] Endostatin serum levels in normal individuals range from 4 to 40 ng/mL (20.3 ± 11.5, mean and SD). For Down syndrome individuals, it is 38.6 ± 20.1, a significant elevation. The collagen XVIII gene is on chromosome 21q22.3. Because Down syndrome is most commonly caused by a duplication of one of the copies of chromosome 21, these individuals have an extra copy of collagen XVIII in which endostatin is a cleavage product. Down syndrome individuals have a low incidence of solid tumors.

A recent study in the United States of 17,987 people with Down syndrome revealed that they have approximately 0.1 the incidence of the control age-matched population for virtually all malignancies except testicular cancer and leukemia.[493] This low incidence of cancer is associated with a large reduction in cancer associated deaths among Down syndrome patients. It has been proposed that an increase of about one-third of normal endostatin serum levels may represent an effective therapeutic dose to significantly inhibit many solid tumors.[492] When this observation is taken together with endostatin's lack of toxicity, endostatin may someday be used chronically in a preventive mode for patients with a high cancer risk for breast cancer or other tumors.

Clinical Trial. A polymorphism in endostatin predisposes for the development of prostate cancer.[494] A single missense substitution of asparagine for aspartic acid at D104N located in the C-terminal globular domain NCI of collagen XVIII, yields endostatin with this mutation at N104. Men who are heterozygous for this mutation have a 2.5 times increased risk of prostate cancer. The authors propose that (1) the DNA segment containing this residue might contain a novel interaction site to an as yet unknown receptor; (2) the presence of N104 may impair the angiogenesis inhibitory function of endostatin; and (3) this mutation could possibly be used for a prostate cancer screening test.[494]

Prostate Cancer. In a phase I trial of endostatin 25 patients who had failed all previous therapy were treated with endostatin.[267] They received a daily intravenous bolus dose of endostatin over a period of 20 minutes. Endostatin was safe and had a linear pharmacokinetic profile up to 599 mg/m^2/day. Tumors regressed in two patients, although not sufficiently to satisfy the criteria for the terms 'partial' or 'complete' response used for chemotherapy. In one patient with metastatic melanoma there was prolonged stabilization for 426 days with a much improved quality of life and he returned to work.

The limited antitumor effect of bolus dosing in these patients is similar to the weak antitumor effect observed in mice that received bolus dosing.[72] In ongoing phase II trials, patients in Boston are receiving subcutaneous injections of a slow-release preparation of endostatin, which provides a steady state blood level of endostatin similar to that obtained by continuous intravenous dosing in patients and continuous intraperitoneal pump dosing in mice.[72] In these patients endostatin is showing more anticancer activity and more prolonged stable disease up to 1 year or more.

Production and manufacture of endostatin Recombinant endostatin can be produced in large scale in *E. coli*, *Drosophila* cells, *P. pastoris* and in mammalian cells.[487–491] Current clinical trials in the United States and The Netherlands employ recombinant human endostatin produced in yeast (*P. pastoris*).

A family of angiogenesis inhibitors in matrix proteins The discovery of endostatin, a specific and potent angiogenesis inhibitor residing as an internal fragment of collagen XVIII, further implicated the potential role of basement membranes in the regulation of angiogenesis. In 1996, three independent lines of evidence led Kalluri and his colleagues to launch a new line of investigation into the role of vascular basement membrane in the regulation of angiogenesis: (1) the observation that vascular basement membrane proteins are associated with resting and growth arrested capillary endothelial cells, while proliferating and migrating endothelial cells are under the influence of provisional matrix molecules such as vitronectin and fibronectin; (2) studies by Madri and also by Furcht that suggest that sub-domains of type IV collagen exhibit different properties as compared to the intact full-length molecule; and (3) the demonstration by Furcht that the noncollagenous-1 domain (NC1 domain) can disrupt the assembly of type IV collagen.[498–500,670–673] Kalluri hypothesized that during the inductive phase of angiogenesis involving the degradation of vascular basement membrane, proteolytic degradation fragments of basement membrane are liberated with the potential for positive and negative regulation of capillary endothelial proliferation and migration. Such a potential role for matrix fragments could provide the regulatory balance for an organized formation of new capillaries.

A biochemical approach was designed to extract vascular basement membranes and perform degradation with tumor microenvironment associated enzymes. The isolated vascular basement membrane per se, did not reveal any antiangiogenic activity in its solid state. But, degradation by various basement membrane-degrading enzymes liberated cryptic domains of collagen molecules from the vascular basement membrane with novel antiangiogenic activity.[500] Interestingly, while these fragments are present in the intact molecules, in this form they do not exhibit antiangiogenic activity. The initial screen of fragments from vascular basement membrane identified three antiangiogenic fragments from type IV collagen, known as arresten, canstatin and tumstatin. Endostatin was also identified in this collection of basement membrane fragments. The discovery of arresten, canstatin, tumstatin and α6 chain NCI domain of type IV collagen was later also independently published by Petitclerc and coworkers.[504]

Arresten In vitro antiangiogenic activity Arresten (26 kDa) is the NC1 domain fragment of α1 type IV collagen.[262] Arrestin exhibits antiangiogenic activity in various in vitro and in vivo assays and is found circulating in normal individuals. Recombinant arresten inhibited the growth of small and large renal cell carcinoma xenografts and prostate carcinoma-xenografts in nude mice at doses as low as 4 mg/kg/day/intraperitoneally. The decrease in tumor size was consistent with a decrease in CD31 positive vasculature.[262]

Mechanism of action. The antiangiogenic and antitumor action of arresten is potentially mediated by heparan sulfate proteoglycan and $\alpha_1\beta_1$-integrin on proliferating endothelial cells.[262] Two independent studies support the contention that arresten may function via $\alpha_1\beta_1$-integrin. Senger and colleagues demonstrate that α_1-integrin-neutralizing antibodies can inhibit VEGF driven angiogenesis without detectable effect on pre-existing vasculature, and VEGF can induce expression of $\alpha_1\beta_1$- and $\alpha_2\beta_1$-integrins.[501] Additionally, Pozzi and colleagues demonstrate that tumors grow slower in α_1 integrin null mice, when compared to the wild-type mice.[502,503] Collectively, all of these studies suggest that arresten can potentially act as an endogenous inhibitor of $\alpha_1\beta_1$-induced tumor angiogenesis.

Canstatin Canstatin (24 kDa) is the NCI domain fragment of α2 type IV collagen.[263] Canstatin exhibits antiangiogenic activity in several in vitro and in vivo assays.[263] Recombinant canstatin inhibits the growth of small and large

renal cell carcinoma tumors by four- or threefold with respect to placebo-treated mice. Established human prostate carcinoma (PC-3) in severe combined immunodeficient (SCID) mice or athymic (nu/nu) mice exhibited fractional tumor volumes of about threefold less than placebo-treated mice when treated with 3 mg/kg canstatin. This decrease in tumor size was consistent with a decrease in CD31 positive vasculature.[263] Recent studies using syngeneic mouse Lewis lung tumors (LLC) in C57BL/6 mice and orthotopic MDA-MB 435 breast tumors in nude mice also reveal significant tumor inhibition with recombinant canstatin. The antiangiogenic activity of canstatin was also independently validated subsequently by Petitclerc and coworkers.[504] Petitclerc and colleagues demonstrate that recombinant canstatin, tumstatin, and the α6(IV)NC1 domain can inhibit angiogenesis in a CAM assay. Petitclerc and colleagues also show that canstatin can bind to $\alpha_v\beta_3$-integrin on endothelial cells.

Mechanism of action. The mechanism of how canstatin inhibits proliferation and migration of endothelial cells was further investigated. Kamphaus and colleagues investigated the effect of canstatin on ERK activation induced by 20% fetal bovine serum and endothelial mitogens. Canstatin does not act primarily by inhibiting VEGF or bFGF activation of ERK.[263] Instead, canstatin specifically induces apoptosis of endothelial cells with insignificant effect on nonendothelial cells. Apoptosis of endothelial cells by canstatin is mediated by induction of a steady decrease in FLIP protein (an antiapoptotic protein associated with FAS-mediated apoptosis pathway) levels in the presence of 20% fetal calf serum, bFGF and VEGF. Interestingly, canstatin did not affect FLIP levels in the absence of growth factors. Canstatin's lack of effect on endothelial cells in the absence of growth factors may indicate that only proliferative endothelium is targeted.[262] Recent studies suggest that canstatin binds to $\alpha_v\beta_3$-integrin and also $\alpha_3\beta_1$-integrin on proliferating endothelial cells.[504] The importance of $\alpha_v\beta_3$ and $\alpha_3\beta_1$-integrins for the antiangiogenic activity of canstatin is yet to be determined.

Tumstatin Tumstatin (28 kDa) is the NC1 domain fragment of α3 collagen molecule and exhibits antiangiogenic activity in both the in vitro and in vivo assays.[71,264,505,506,507] The antiangiogenic activity has been narrowed down to a 25 amino acid stretch in the middle of the molecule. At equal molar concentrations, the full-length tumstatin exhibits similar activity as the 25 amino acid peptide termed as T7 peptide[71,264,505,506,507] Recombinant tumstatin and T7 peptide exhibit significant inhibition of human tumor xenografts in nude and SCID mice, syngeneic LLC and B16F10 tumors in C57BL/6 mice, and orthotopic MDA-MB 435-breast tumor in nude mice at a dosage of 1 mg/kg/day/ip. or iv.

Tumstatin (α3(IV)NC1) binds to endothelial cells via $\alpha_v\beta_3$-integrin and $\alpha_6\beta_1$-integrin.[71,264,505,506] The $\alpha_3\beta_1$-binding activity for tumstatin was also demonstrated by Petitclerc and colleagues.[504] We show that the binding to $\alpha_v\beta_3$ is pivotal for the antiangiogenic activity associated with tumstatin and that the activity is restricted to amino acids 54-132 (tum-5) within the 244 amino acid tumstatin using deletion mutagenesis.[71,264,506] Additionally, $\alpha_v\beta_3$ binding to tumstatin is mediated via a mechanism independent of the RGD-containing amino acid sequence and vitronectin/fibronectin binding of proliferating endothelial cells. The antiangiogenic activity of tumstatin can be further localized to a 25 amino acid region within tum-5 and a synthetic peptide representing this region preserves 'full' antiangiogenic activity and binds to $\alpha_v\beta_3$-integrin.[71,264,506] Cell biological experiments demonstrate that the antiangiogenic activity of tumstatin is dependent on $\alpha_v\beta_3$-binding on proliferating endothelial cells.[71,506] These experiments support the notion that through the action of endogenous inhibitors such as tumstatin, $\alpha_v\beta_3$-integrin could also function as a negative regulator of angiogenesis.[71,508–511]

Mechanism of Action. Tumstatin induces apoptosis of proliferating endothelial cells.[507] Maeshima and colleagues demonstrate that tumstatin is an $\alpha_v\beta_3$-integrin-dependent inhibitor of cap-dependent translation mediated through negative regulation of mTOR signaling. In essence, tumstatin is an endothelial cell specific inhibitor of cap-dependent translation. The mTOR inhibitory property of tumstatin mimics that of rapamycin, except unlike rapamycin, which is pan-specific, tumstatin is specific for only endothelial cells.[507] The action on mTOR is mediated by the FAK-PI3K-Akt pathway downstream of $\alpha_v\beta_3$-integrin.

Endogenous angiogenesis inhibitors as cryptic tumor suppressor proteins With the discovery of angiostatin, endostatin, arresten, canstatin, and tumstatin, a new paradigm has emerged that puts forward a novel notion that some proteins such as plasminogen and basement membrane collagens, harbor unique properties that are cryptic and become exposed only upon proteolytic degradation. Thus, in their intact form (full-length) plasminogen and basement membrane collagen do not exhibit antiangiogenic activity or antitumor activity; but, when plasminogen and basement membrane collagens undergo degradation, they now expose or liberate novel cryptic fragments, which possess antiangiogenic activity.

All of these inhibitors are found in blood of normal individuals. A decrease in the physiologic levels of tumstatin is speculated to result in an acceleration of tumor growth. A change in amino acid sequence of endostatin due to a polymorphism correlates with an increased susceptibility to prostate cancer. Conversely, an increase in physiological levels of endostatin results in less propensity for emergence of solid tumor in Down syndrome patients (due to an extra copy of type XVIII collagen) (see above).[492] Collectively, these results argue that angiostatin, endostatin, tumstatin, canstatin and arresten may function as cryptic tumor suppressor proteins, offering an additional line of defense against tumor progression by blocking angiogenesis switching.

Antiangiogenic conformation of antithrombin III A human small-cell lung carcinoma suppressed angiogenesis and tumor growth at remote sites in immunodeficient mice. These cells generated an enzyme in vitro that converted the 58 kDa conformation of circulating antithrombin III to a 53 kDa form of the protein in which the externally configured stressed loop of antithrombin was retracted into the body of the molecule.[70] The 53 kDa "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 kDa cleaved antiangiogenic antithrombin.[512]

Tropinin I A novel protein, troponin I, was purified from cartilage during an attempt to find the inhibitors responsible for the avascularity of cartilage.[513] It is a 22 kDa subunit of the troponin complex that, along with tropomysin, 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 angiogenesis inhibitors that are endogenous In addition to the specific angiogenesis inhibitors, endostatin, tumstatin, canstatin and arresten 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. The increasing number of endogenous inhibitors of angiogenesis being discovered indicates the existence of a family of molecules that provide 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). Examples of endogenous angiogenesis inhibitors include interferon-β, platelet factor 4, fibronectin fragments, fragments of SPARC, thrombospondin-1, tissue inhibitors of metalloproteinases (TIMPS), certain angiostatic steroids such as tetrahydrocortisol, a , nonmineralocorticoid metabolite of cortisol, and 2-methoxyestradiol, and interleukin-12.[243,246,247,259–261,514–525] Thrombospondin also inhibits lumen formation, a rather unique property of an angiogenesis inhibitor.[521] In addition to its cytotoxic properties, mediated by T-cell activation, interleukin-12 also induces the upregulation of interferon-γ, which itself upregulates inducible protein 10 (IP-10), reported to be an angiogenesis inhibitor.[526] Certain cryptic antiangiogenic protein fragments are con-

tained 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 plasma 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.[527] The hemostatic system, like the extracellular matrix, also appears to store certain angiogenesis inhibitors that may be needed during wound healing angiogenesis (for review see Browder, et al.).[442]

Synthetic angiogenesis inhibitors ***TNP-470*** Takeda neoplastic product-470 (TNP-470) is a synthetic analogue of fumagillin, a compound secreted by the fungus *Aspergillus fumigatus fresenius*, which selectively inhibits methionine aminopeptidase-2 and blocks cdk2, inhibits cdc, and inhibits phosphorylation of Rb.[59,534,544] Against animal models of human, rabbit, hamster, rat and mouse tumors, both primary and metastatic, reports from more than 100 laboratories show that TNP-470 has the broadest anticancer spectrum of any known agent.[60] TNP-470 at 30 mg/kg subcutaneously every other day inhibited the growth of human glioblastomas, embryonal rhabdosarcoma, meduloblastoma, meningioma, fibrosarcoma, neurofibrosarcoma, hemangioendothelioma, and gastric, colon, breast, prostate and esophageal carcinomas, as well as murine Lewis lung carcinoma, melanoma, osteosarcoma, reticulum cell sarcoma, glioma, hepatoma, fibrosarcoma and mammary, bladder, tongue and pancreatic cancer by 50 to 95% with complete regression in a variety of tumors especially those of the central nervous system.

In early clinical trials with TNP-470 used as a single agent, best results were that 18% of patients had a 50% regression of tumor. Furthermore, 5 patients had "complete or dramatic and durable tumor regression, despite having failed all conventional therapy," including patients with carcinoma of the cervix metastatic to lung, high grade sarcoma of the kidney, renal cell carcinoma, androgen-independent prostate cancer, and Kaposi's sarcoma.[545–549] TNP-470 also showed promise when used in combination with chemotherapy.[550–552] However, at effective therapeutic doses, some patients have experienced neurocognitive toxicity and minor seizures have been reported. Recently, Ronit Satchi-Fainaro in the Folkman laboratory has determined that TNP-470 rapidly enters the cerebrospinal fluid. She has conjugated TNP-470 to a water-soluble synthetic polymer, (2-hydroxypropyl)methacrylamide (HPMA). Lysosomal enzymes (cathepsin B) in activated endothelial cells cleave the linker between the polymer and the drug, releasing free TNP-470. As a result, TNP-470 does not enter the cerebrospinal fluid, the efficacy of the conjugated HPMA-TNP-470 is significantly greater than free TNP-470 in tumor-bearing animals, and a maximum tolerated dose has not yet been found even at 7 times the previous maximum tolerated dose (R. Satchi-Fainaro, Targeting angiogenesis with an HPMA-copolymer-TNP-470 conjugate.

Thalidomide In 1994 Robert D'Amato and colleagues reported that the antiinflammatory drug thalidomide could inhibit angiogenesis induced by bFGF or VEGF in a rabbit cornea micropocket assay.[249] Thalidomide inhibited new blood vessel formation in rabbits and mice independently of its ability to suppress infiltrating host inflammatory cells.[249] Thalidomide reduced the growth of carcinomas in rabbits and Lewis lung carcinoma in mice.[554] In 1999, thalidomide therapy was shown to be active in humans against advanced multiple myeloma.[555] Thirty-two percent of patients treated with thalidomide had a positive response, as assessed by reduction of the serum levels of myeloma protein and urine levels of Bence Jones protein. These findings have been confirmed by other studies.[556] Thalidomide is now being tested in numerous clinical trials in the United States (>160 clinical trials in >70 medical centers in the United States [Celgene Corp., Warren, NJ, January 2001]), and also in Europe for the treatment of a variety of solid tumors and has become one of the most effective drugs for multiple myeloma, either as first line therapy or for the treatment of disease resistant to conventional chemotherapy.

Mechanism of Action. Thalidomide treatment suppresses production of tumor necrosis factor (TNF)-α, which has been reported to be angiogenic. However, other, more potent inhibitors of TNF-α such as pentoxifylline or dexamethasone have little or no activity against corneal angiogenesis.[252] Ibuprofen, which inhibits angiogenesis, actually increases serum levels of TNF-α in mice.[252] Furthermore, TNF-α inhibitors are not effective in animal models of myeloma or in patients. TNF-α suppression therefore does not appear to be a major part of thalidomide's antiangiogenic activity.

Thalidomide does have a direct antiproliferative effect on multiple myeloma cells in vitro, although very high concentrations of thalidomide (up to 100 μM) are required.[556] Nevertheless, this drug might be able to target both cancer cells and vascular endothelial cells. In patients with multiple myeloma, or myelodysplastic syndromes, plasma levels of the proangiogenic proteins, VEGF and bFGF were significantly decreased compared to pretreatment levels. This decrease correlated with efficacy of thalidomide therapy.[138]

Surrogate markers of efficacy in patients. In the original clinical reports of the antitumor effects of thalidomide against multiple myeloma, decreased microvessel density was not associated with disease remission.[555] This created a question of whether the clinical efficacy of thalidomide was mediated entirely or even partly by its antiangiogenic features.[557] The problem is an assumption that because microvessel density (measured by the method of Weidner and colleagues) can determine cancer prognosis, it should also be useful for determining therapeutic efficacy.[558] While increased microvessel density in bone marrow is associated with relapse in multiple myeloma or with untreated multiple myeloma in virtually all reports to date, microvessel density has not been an effective measure of therapeutic response in many patients with multiple myeloma. For example, some studies reported that increased microvessel density persists in patients who have undergone a complete response to thalidomide or to stem cell transplantation.[555,559] Microvessel density also remains elevated in patients who have undergone remission after chemotherapy for acute lymphoblastic leukemia.[560] Therefore, microvessel density may not be a useful indicator of efficacy in solid tumors, even though it has continued to be a valid prognostic indicator for predicting future mortality and metastasis.[37,252]

As a substitute for microvessel density, other surrogate markers of efficacy of antiangiogenic therapy are being studied. Circulating bone marrow-derived endothelial cells showed a 10-fold reduction and returned to normal values after thalidomide treatment.[138] Decreased circulating levels of VEGF correlated with thalidomide's therapeutic efficacy in multiple myeloma and other hematologic diseases.[138] However, serum VEGF alone has not been a predictable marker of angiogenesis in other tumors, in part because of the high VEGF content of platelets, and because circulating angiogenesis inhibitors are not taken into account.[358]

Principles of angiogenesis important for clinical translation **Antiangiogenic chemotherapy** In some clinical trials angiogenesis inhibitors are also being administered in combination with conventional chemotherapy. Chemotherapeutic agents have in fact been shown to have antiangiogenic properties in animal models, in addition to their ability to induce direct cancer cell death. Paclitaxel, which inhibits microtubule polymerization, inhibits vascular endothelial cell proliferation, motility and invasiveness in a dose-dependent manner in vitro and tumor angiogenesis in vivo.[562] Secondary effects such as these could contribute to the antitumor efficacy of chemotherapy in vivo and might delay or prevent the acquisition of drug-resistance by cancer cells.

Antiangiogenic chemotherapy Browder and colleagues proposed that the traditional dose schedule regimen for chemotherapeutic agents cannot, however, provide the sustained blockade of angiogenesis achieved by angiogenesis inhibitors.[563] This could be because chemotherapy is usually administered at the maximum tolerated dose, followed by a treatment-free interval to allow recovery of bone marrow and gastrointestinal tract cells. During the treatment-free interval, microvascular endothelial cells in the tumor bed can resume their proliferation and support tumor regrowth.[563] Browder and colleagues experimented with changing scheduling and dose of a cytotoxic agent to augment its antien-

dothelial activity, an approach called 'antiangiogenic chemotherapy' (metronomic therapy).

Antiangiogenic chemotherapy was first shown to be effective in tumor-bearing mice.[563] Administration of cyclophosphamide at more frequent intervals at an overall lower dose with a brief treatment-free interval induced sustained apoptosis of endothelial cells in the vascular bed of the tumors, and more effectively controlled growth of drug-resistant tumors. This protocol also reduced side-effects and avoided bone marrow suppression, These results indicated that antitumor efficacy of cytotoxic drugs may be improved by changing the schedule and dose to provide optimum cytotoxic targeting of the microvascular endothelial cells in the tumor bed.

These results might also help to explain why some patients who receive long-term maintenance or even palliative chemotherapy have stable disease beyond the time that the tumor would have been expected to develop drug resistance. Patients with slow-growing cancers who are on antiangiogenic scheduling of chemotherapy involving continuous infusion 5-fluorouracil, weekly paclitaxel, or daily oral etoposide have shown an improved outcome despite the fact that in some of these patients the tumors had already become drug resistant to conventional chemotherapy.[564–571]

Antiangiogenic chemotherapy has also been called 'metronomic' therapy, but the two terms do not have precisely the same meaning. Antiangiogenic chemotherapy signifies that the target of the chemotherapy is microvascular endothelium in the tumor bed.[572] Metronomic therapy indicates that the schedule of administration is at very regular intervals.

Combinations of antiangiogenic therapy and standard chemotherapy Clinical trials of antiangiogenic chemotherapy are underway at present.[573,574] These trials usually combine very low-dose chemotherapy at frequent intervals with one or more angiogenesis inhibitors. For example, a current clinical trial at the Dana Farber Cancer Center, developed by Mark Kieran, combines celecoxib, very low-dose cyclophosphamide, and thalidomide. Every 3 weeks, low-dose etoposide is substituted for cyclophosphamide. In other clinical trials, angiogenesis inhibitors have been added to high-dose conventional chemotherapy. Millar however, has cautioned about possible limitations of combining standard chemotherapy with antiangiogenic therapy or, alternatively, with an oncogene signaling inhibitor that has antiangiogenic activity.[466] Unexpected toxicities may occur, or at the least, lack of increased efficacy. However, we and subsequently others have reported that angiogenesis inhibitors can be combined with chemotherapeutic drugs which are administered on an 'antiangiogenic' or 'metronomic' dose and schedule; ie, that is more frequently than most conventional schedules, and at significantly lower than maximum tolerated doses.[37,563,572,575–577] Administration of chemotherapeutic drugs in this manner is designed to maximize their antiangiogenic activity while reducing the severe and acute toxicities often associated with conventional regimens of maximum tolerated dose.[578] Clinical trials of antiangiogenic chemotherapy (metronomic) are currently underway.[573,574]

Clinical patterns of metastasis may be governed by angiogenic mechanisms Metastases may present at least four common clinical patterns (see Table 11-1): (1) a primary tumor such as a colon carcinoma is removed, but within a few months metastases appear; (2) metastases are already present when the primary tumor is first detected; (3) metastases appear first, and the primary remains occult; and (4) the primary is removed (or treated by other therapy), and metastases do not appear until years later (eg, 5–10 years). A fifth and rare pattern is that metastases disappear after removal of the primary tumor (eg, 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 (see Table 11-1). These five patterns can be explained by a unifying angiogenic mechanism and will depend mainly upon whether or not a primary tumor generates an angiogenesis inhibitor(s) (eg, angiostatin, endostatin), which circulate and suppress angiogenesis in remote metastases.[97]

Hematologic malignancies are angiogenic Although solid tumors are known to be angiogenesis dependent, it was assumed until 1993 that leukemias and other hematologic malignancies did not induce angiogenesis to promote their own survival.[579] In 1993, Brunner and colleagues observed that bFGF was expressed by human bone marrow and peripheral blood cells, and the following year Nguyen and coworkers reported that bFGF was elevated in the urine of newly diagnosed leukemic patients to higher levels than most other malignancies.[170,580] Bone marrow angiogenesis was subsequently found to correlate with multiple myeloma progression, and was also observed in lymph nodes of B cell non-Hodgkin lymphoma patients and in bone marrow biopsies from children with newly diagnosed untreated acute lymphoblastic leukemia.[561,581–584] By 1999, cellular levels of another angiogenic protein, VEGF, were reported to predict outcome of patients with multiple myeloma (Figure 11-15).[585]

Angiogenesis inhibitors might therefore be useful in treating hematologic malignancies. A recent study reports that the retroviral gene transfer of a vector encoding the direct angiogenesis inhibitors angiostatin and endostatin inhibits bone marrow angiogenesis and tumor growth in a mouse model of leukemia.[375] This therapy was shown to directly inhibit endothelial proliferation in vitro, but to have no effect on leukemia cell proliferation. Mice inoculated with B-cell, T-cell or myelogenous leukemias and treated with recombinant endostatin have also been observed to live significantly longer and experience fewer toxic side effects than with conventional chemotherapy (T. Browder, et al., unpublished observation).

Host angiogenic response may be genetically controlled A recent finding is that differ-

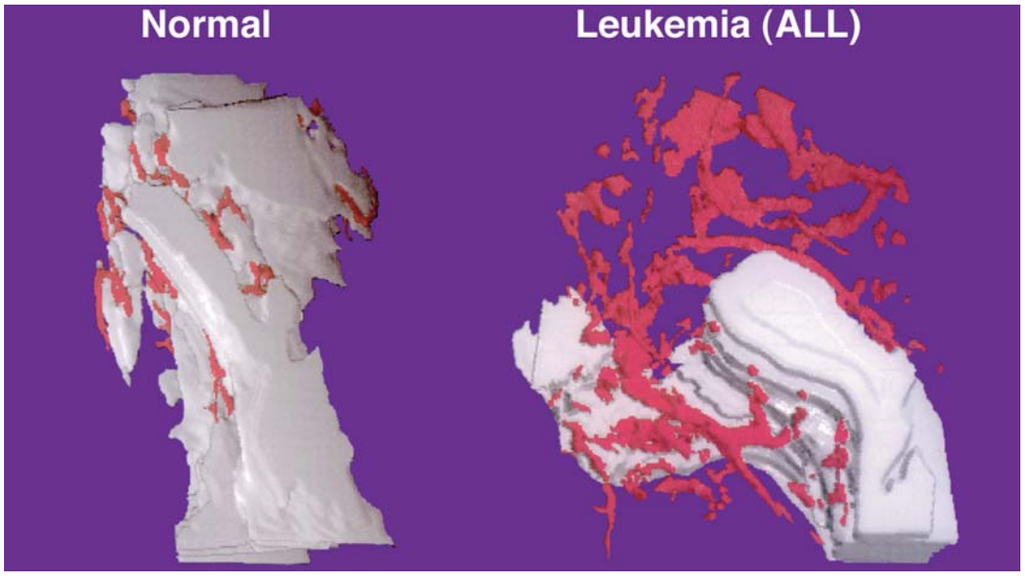

Figure 11-15 Leukemia in human bone marrow. Comparison of normal vs. leukemic bone marrow, with blood vessels shown in red. Confocal microscopic sections of bone marrow biopsies stained with antibody to von Willebrand factor to highlight blood vessels. In the left panel, normal bone marrow (from a child with a non-neoplastic disease) shows normal microvasculature of uniform sized vessels. In the right panel, a bone marrow biopsy from a child with newly diagnosed acute lymphoblastic leukemia reveals intense neovascularization, with microvessels of variable diameter.[560] (Four-color version of figure on CD-ROM)

ent 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.[586] 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 (eg, 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 would be required.

p53 normally suppresses angiogenesis p53 mutations also allow tumor cells to become resistant to apoptosis under conditions of hypoxia.[587] In mice, p53 mutations decrease the response of cancer cells to antiangiogenic therapies.[588] Wild-type p53 normally suppresses tumor angiogenesis by upregulating thrombospondin-1, inducing degradation of HIF-1α, suppressing transcription of VEGF, and downregulating bFGF-binding protein expression.[75,161,233] The increased neovascularization that occurs following loss of p53 function, however, can be overcome by increasing the dose of antiangiogenic therapy. For example, in mice bearing a mutant p53-associated human pancreatic tumor, there was a dose-dependent response to a single angiogenesis inhibitor, varying from 33% inhibition to 97% inhibition of tumor growth with tumor regression.[72,589]

It remains to be seen whether this result can be translated to those patients with tumors that possess p53 mutations. Therefore, in contrast to the maximum tolerated doses of chemotherapy, antiangiogenic therapy dosing may be optimized by titrating against the total angiogenic output of a tumor, analogous to the titration of insulin against blood sugar or Coumadin against prothrombin levels. There is currently, however, no quantitative method for determining the total angiogenic output of a patient's tumor burden, so surrogate markers such as circulating progenitor endothelial cells are being studied.[137]

Slowly growing indolent tumors Rapidly growing tumors are generally more sensitive to conventional cytotoxic chemotherapy than slowly growing, indolent tumors. In fact, the most slowly growing tumors (such as neurofibromas and indolent prostate carcinomas) are virtually unresponsive to chemotherapy. It has been assumed by some oncologists that slowly growing tumors would be as unresponsive to antiangiogenic therapy as they are to chemotherapy.[590] However, when two types of human bladder cancers, a rapidly growing highly vascularized tumor and a slowly growing poorly vascularized tumor were implanted into immunodeficient mice, growth of both tumor types was inhibited by angiogenesis inhibitors. For a given dose of angiogenesis inhibitor, the slower the tumor was growing, the more effective the inhibitor.[590] In carcinogen-treated mice, breast cancers that arose spontaneously and grew very slowly were inhibited and/or regressed by treatment with mouse endostatin.[285] Spontaneously arising carcinomas of the pancreatic beta cells in transgenic mice grew very slowly compared to most transplantable tumors, but retained a high degree of sensitivity to angiogenesis inhibitors.[449]

Poorly vascularized tumors Another common misconception about antiangiogenic therapy is that it is only effective in highly vascularized tumors. Some cancer patients who have failed conventional therapy lament that their physician told them they were not candidates for antiangiogenic therapy because their tumor was not vascularized. It is sometimes assumed by surgeons that a tumor that appears to be white and avascular (eg, pancreatic cancer) is not a candidate for antiangiogenic therapy. Based on these assumptions, some angiogenesis inhibitor clinical trials currently require a pretreatment biopsy for microvessel density to exclude patients whose tumor microvessel density is too low. Another clinical trial for a new angiogenesis inhibitor is restricted to renal cell carcinoma and other highly vascularized tumors.

There are several flaws in these assumptions. First, virtually any tumor that is large enough to be visible or palpable has already undergone neovascularization to attain that size. Second, intensity of neovascularization cannot be determined by gross inspection of a tumor. A white neurofibrosarcoma of one or more cubic centimeters may have a microvessel density by microscopy that is similar to the microvessel density of a reddish hepatic carcinoma of similar size, except in the whitish tumor, the vessels are more compressed. Third, the lower the vascularity of a tumor, the more susceptible it appears to be to antiangiogenic therapy.[590] Highly vascularized tumors may require higher doses of an angiogenesis inhibitor or combinations of angiogenesis inhibitors to achieve a tumor response.

Predicting efficacy of antiangiogenic therapy: is microvessel density helpful? In 1972, a quantitative method for histologic grading of tumor angiogenesis was developed that correlated the amount of neovascularization with tumor grade in human brain tumors.[591] Additional methods for quantifying grade of tumor vascularization were developed, and followed by the first report of the use of tumor vascularity as a prognostic marker for cutaneous melanoma.[103,592] In 1991, Weidner and colleagues used antiendothelial cell antibodies to identify tumor vasculature and showed that microvessel density was a useful prognostic marker for human breast cancer.[89] Microvessel density measures the relative intensity of angiogenesis in angiogenic clones from one tumor to another. In the past 10 years, microvessel density quantification has become a reproducible prognostic factor for risk of metastasis. The most plausible explanation for this correlation is that most human tumors contain angiogenic and nonangiogenic cells, both of which enter the circulation, but only the angiogenic cells can form detectable metastases.[2] Although there are a few reports in which microvessel density did not correlate with risk of metastasis or mortality, this could be due to technical or biologic causes. For example, if a primary tumor that was the source of a biopsy for quantification of microvessel density, generated a high level of circulating angiogenesis inhibitor(s), which suppressed growth of distant metastases, the prognostic value of this test could be negated. For a review of more than 50 publications of microvessel density quantified in more than 8,000 patients as a prognostic indicator of future metastasis or mortality, see Gasparini et al.[561,558,593]

Although microvessel density is a useful prognostic marker of metastatic risk, it is not a good indicator of therapeutic efficacy for several reasons. In normal tissues, degree of vascularization and oxygen/nutrient demand are tightly coupled. In tumors, however, degree of vascularization and tumor growth are loosely coupled, or even uncoupled. This may be because in tumor cells, expression of angiogenic factors, such as VEGF, are no longer regulated by oxygen concentration. During tumor regression under antiangiogenic therapy, microvessel density may decrease if capillary dropout exceeds tumor cell dropout (autolysis), increase if tumor cell dropout exceeds capillary dropout, or, remain the same if disappearance of capillaries and tumor cells parallel each other (Figure 11-16). Figure 11-16 shows osteosarcomas taken from mice treated with endostatin until there was more than a 50% inhibition of growth.[252] Despite the fact that this drug inhibited growth of both treated tumors depicted, the intensity of vascularization after treatment differed significantly between the tumors. Microvascular density was quantified over the entire histological section rather than over vascular hotspots, in order to avoid the effects of heterogeneity of vascularization in the tissue sample. Microvascular density dropped sharply in one treated tumor but rose slightly in the second, yet both tumors were equivalently reduced in size by the treatment relative to control. Thus, detection of a decrease in microvessel density during treatment with an angiogenesis inhibitor, suggests that the agent is active. However, the absence of a decrease in microvessel density does not indicate that the agent is ineffective.[558]

Antiangiogenic activity of interferon-a: lessons for other angiogenesis inhibitors Interferon-α has been widely used as an antiviral agent to treat chronic hepatitis, but also as a cytotoxic agent to treat certain leukemias and some bladder cancers.[258] The first evidence that inter-

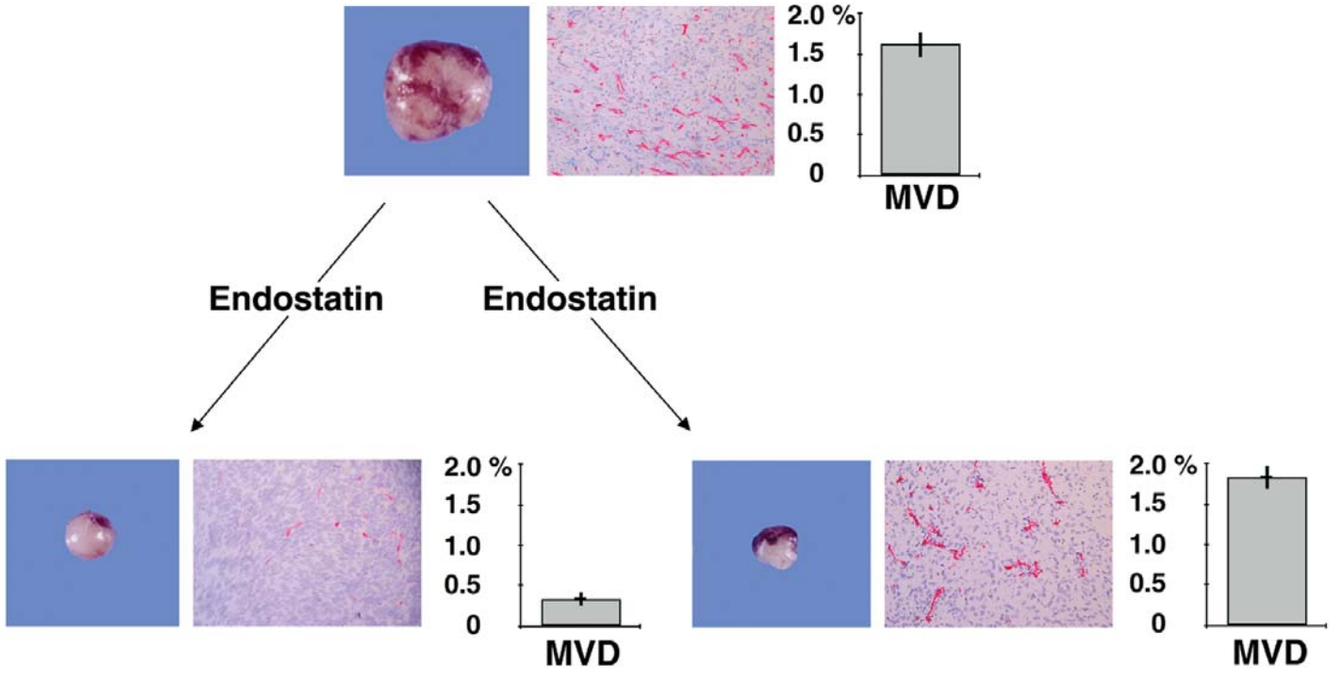

Figure 11-16 Microvessel density may not predict efficacy of an angiogenesis inhibitor. Human osteosarcoma xenografts were treated with the angiogenic inhibitor endostatin. Shown are tumor size, vascularization as detected by an antibody to CD34, and vascular density quantified by digitized imaging for a control and two endostatin-treated tumors. Endostatin significantly inhibited tumor growth in treated tumors. Despite the fact that endostatin inhibited both treated tumors, the post-treatment level of vascularization of the two tumors varies considerably. Entire tumor sections were scored for microvascular density rather than just vascular hotspots, thereby avoiding effects of heterogeneity in vascularization over the sample. Sections were scored by imaging as many microscope fields at 200X as it took to cover the entire sections. Vascular density was found to drop sharply in one treated tumor (left), yet actually rose slightly in the second (right). The mechanism of this disparity, based on the relative rates of capillary dropout vs. dropout of tumor cells in the perivascular cuff, is explained in the text.[252] (Four-color version of figure on CD-ROM)

feron-α had antiangiogenic activity was reported in 1980 when it was found to inhibit motility of vascular endothelial cells in vitro in a dose-dependent and reversible manner, and subsequently found to inhibit angiogenesis in vivo.[244,269,270] Experimental studies in mice demonstrate that the antiangiogenic efficacy of interferon-α is optimal at low doses and declines at higher doses.[485a] New blood vessel growth in proliferating hemangiomas has been associated with increased expression of bFGF, and interferon-α has been shown to down-regulate bFGF expression in human cancer cells.[239,240,243,594,595]

The first use of antiangiogenic therapy in a human was in 1988, when pulmonary hemangiomatosis in a 12-year old boy was successfully treated with low dose interferon-α therapy (3 million units/m^2).[596,597] Subsequently, infants with life-threatening or sight-threatening hemangiomas were successfully treated with the same low-dose interferon-α.[598,599] Durable complete regressions have been achieved with low-dose interferon-α therapy in patients with solid tumors such as angioblastomas and giant cell tumors, where tumor angiogenesis was mediated mainly by bFGF.[239–241] Interferon-α at low doses has also been used to successfully treat hemangioendothelioma with or without metastases.[600–602] In a recent report, Kaban and colleagues treated recurrent high-grade giant cell tumors of the maxilla or mandible by simple surgical enucleation to spare nerves and teeth, and after 48 hours, patients received interferon-α, 3 million units/day subcutaneously for up to 8 months.[241,242] All 8 of 8 patients had complete and durable regressions without recurrence for follow-up for up to 6 years.

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 colposcopy; 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 cancer 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.[92,445] In fact, a wide range of radiologic signs of cancer are based on "enhancement" of lesions by radiopaque dyes trapped in the neovasculature 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.

Future directions There are currently so many clinical trials of angiogenesis inhibitors in the United States and other countries that space does not allow a summary of these trials. However, two recent reports validate the efficacy of antiangiogenic therapy for recurrent tumors when conventional therapy has failed. Kaban and colleagues report complete and durable regression of recurrent high grade giant cell tumors of the maxilla and mandible in adults and children by low-dose daily α-interferon for eight months, initiated 48 hours after simple enucleation surgery.[241] Aiello and colleagues report the first case of treatment of an ocular hemangioblastoma with an angiogenesis inhibitor that blocks the receptor for VEGF.[213] In this patient there was rapid and durable return of vision. The rapidity

of return of eyesight was at the outset due to inhibition of increased permeability of the tumor by the antiVEGF regimen.

Need for surrogate markers of anitangiogenic efficacy As translation of antiangiogenic therapy for cancer continues in clinical trials, there are several important challenges ahead. There is a need for surrogate markers of antiangiogenic efficacy. Maximum tolerated dose is not a useful indicator for antiangiogenic therapy. Administration of antiangiogenic therapy may be more analogous to insulin therapy than to chemotherapy. However, there is as yet no way to titrate dosing of angiogenesis inhibitors to the output of proangiogenic activity of the patient's tumor burden. Microvessel density, although useful as a prognostic indicator, is not as useful to determine efficacy of therapy. Quantification of proangiogenic factors such as VEGF, although correlative with prognosis in a few reports, may not measure all of the positive and negative regulators of angiogenesis generated in a tumor bed. Preliminary data indicates that quantification of circulating endothelial cells may become a promising surrogate marker of efficacy of antiangiogenic therapy, but much work remains to be done. Detection of novel circulating molecular markers of ongoing angiogenesis is a wide open opportunity for molecular biologists.

Combinations of angiogenesis inhibitors Combinations of angiogenesis inhibitors will need to be tested in the future, as single angiogenesis inhibitors are approved for use in cancer. For example, it will be important to know whether bisphosphonates are synergistic with certain natural angiogenesis inhibitors such as angiostatin, endostatin, thrombospondin, or tumstatin.

Preventative antiangiogenic therapy Preventive antiangiogenic therapy may also be possible in the future. Because antiangiogenic therapy is generally less toxic and less susceptible to induction of acquired drug resistance, it is possible that angiogenesis inhibitors could be used as prophylactic therapy in patients who have a high risk for cancer or for recurrence of cancer. An experimental study of spontaneous carcinogen-induced breast cancer in rats revealed that endostatin prevented the onset of breast cancer and also prolonged survival, compared with untreated controls.[449] Another example of preventive antiangiogenic therapy would be therapy guided by a molecular marker even before a tumor is visible. For example, in medullary carcinoma of the thyroid after surgical removal of the primary tumor, secondary tumors sometimes appear in the mediastinum and other sites. Because tumor recurrence can be preceded by rising serum calcitonin as early as 1 year or more before the recurrent tumor itself is detectable, it may in the future be feasible to give antiangiogenic therapy and follow the decline in calitonin level analgous to the treatment of high grade giant cell tumors with low-dose interferon, where bFGF is the marker that is followed.[239]

Antiangiogenic foods It is also possible that in the future, foods that have high levels of antiangiogenic substances may be studied in clinical trials for prevention of cancer or its recurrence. Recently, there has been a summary of foods reported to contain antiangiogenic substances.[602]

Antiangiogenic gene therapy Finally, in the long-term, antiangiogenic gene therapy may be useful, especially for the endogenous angiogenesis inhibitors that are proteins. For example, would it be possible to achieve slightly elevated levels of endostatin in the blood similar to the levels in patients with Down syndrome? The question could then be addressed as to whether this would benefit patients at high risk of new tumors such as women with the breast cancer gene, or with a high risk of tumor recurrence after treatment of the initial primary tumor.

ACKNOWLEDGMENTS

This work was supported by the National Institutes of Health grants P01 CA45548 and R37 CA36395 and R01 CA64481, a grant from the Breast Cancer Research Foundation, a grant to Children's Hospital from EntreMed, Inc., Rockville, MD, and a gift to Children's Hospital from the Sylvetsky and Goodman families to Dr. Folkman. Dr. Folkman thanks Alison Clapp, Wendy Foss, and Sarah Schmidt for administrative support and help with the references.

REFERENCES

1. Hanahan D, Weinberg RA. The hallmarks of cancer. Cell 2000;100:57–70.
2. Achilles E-G, Fernandez A, Allred EN, et al. Heterogeneity of angiogenic activity in a human liposarcoma: a proposed mechanism for 'no take' of human tumors in mice. J Natl Cancer Inst 2001;93:1075–81.
3. Hudlická O. Growth of capillaries in skeletal and cardiac muscle. Circ Res 1982;50:451–61.
4. Hoppeler H, Kayar SR. Capillarity and oxidative capacity of muscles. News Physiol Sci 1988;3:113–6.
5. Adair TH, Gay WJ, Montani J-P. Growth regulation of the vascular system: evidence for a metabolic hypothesis. Am J Physiol 1990;259:R393–R404.
6. Ausprunk DH, Folkman J. Migration and proliferation of endothelial cells in preformed and newly formed blood vessels during tumor angiogenesis. Microvasc Res 1977;15:53–65.
7. Klagsbrun M, Moses M. Molecular angiogenesis. Chem Biol 1999;6:R217–R224.
8. Sholley MM, Ferguson GP, Seibel HR, et al. Mechanisms of neovascularization: vascular sprouting can occur without proliferation of endothelial cells. Lab Invest 1984;51:624–34.
9. Folkman J, Brem H. Angiogenesis and Inflammation. In: Gallin JI, Goldstein IM, Snyderman R, editors. Inflammation: basic principles and clinical correlates. 2nd ed. New York: Raven Press; 1992. p. 821–39.
10. Folkman J. Angiogenesis in arthritis. In: Smolen J, Lipsky P, editors. Targeted therapies in rheumatology. London: Martin Dunitz; 2002. p. 111–31.
11. Warren BA. The vascular morphology of tumors. In: Peterson H-I, editor. Tumor blood circulation: angiogenesis, vascular morphology and blood flow of experimental human tumors. Florida: CRC Press; 1979. p. 1–47.
12. Coman DR, Sheldon WF. The significance of hyperemia around tumor implants. Am J Pathol 1946;22:821–31.
13. Ide AG, Baker NH, Warren SL. Vascularization of the Brown-Pearce rabbit epithelioma transplant as seen in the transparent ear chamber. AJR Am J Roentgenol 1939;42:891–9.
14. Algire GH, Legallais FY. Growth rate of transplanted tumors in relation to latent period and host vascular reaction. Cancer Res 1947;7:724.
15. Algire GH, Chalkely HW, Legallais FY, Park H. Vascular reactions of normal and malignant tumors in vivo: I. Vascular reactions of mice to wounds and to normal and neoplastic transplants. J Natl Cancer Inst 1945;6:73–85.
16. Day ED. Vascular relationships of tumor and host. Prog Exp Tumor Res 1964;4:57–97.
17. Folkman J. Toward an understanding of angiogenesis: search and discovery. Perspect Biol Med 1985; 29:10–36.
18. Folkman J. Tumor angiogenesis: therapeutic implications. N Engl J Med 1971;285:1182–6.
19. Folkman J, Long DM, Becker FF. Growth and metastasis of tumor in organ culture. Cancer 1963;16:453–67.
20. Folkman J, Cole P, Zimmerman S. Tumor behavior in isolated perfused organs: in vitro growth and metastases of biopsy material in rabbit thyroid and canine intestinal segment. Ann Surg 1966;164:491–502.
21. Folkman J. Anti-angiogenesis: new concept for therapy of solid tumors. Ann Surg 1972;175:408–16.
22. Folkman J. The vascularization of tumors. Sci Am 1976;234:58–73.
23. Folkman J, Gimbrone MA Jr. Perfusion of the thyroid. In: Karolinska Symposia on Research Methods in Reproduction Endocrinology, 4th Symposium: Perfusion Techniques. Edited by E. Diczfalusy. Stockholm: Karolinska Institutet, 1971, p. 237–248.
24. Folkman J. The intestine as an organ culture. In: Burdette WJ, editor. Carcinoma of the colon and antecedent epithelium. Springfield, IL: CC Thomas; 1970. p. 113–27.
25. Gimbrone MA Jr, Cotran RS, Folkman J. Endothelial regeneration and turnover. Studies with human endothelial cell cultures. Series Haematol 1973;6:453–5.
26. Jaffe EA, Nachman RL, Becker CG, Minick CR. Culture of human endothelial cells derived from umbilical veins: identification by morphologic and immunologic criteria. J Clin Invest 1972;52:2745–56.
27. Folkman J, Haudenschild CC, Zetter BR. Long-term culture of capillary endothelial cells. Proc Natl Acad Sci USA 1979;76:5217–21.
28. Taylor S, Folkman J. Protamine is an inhibitor of angiogenesis. Nature 1982;297:307–12.
29. Shing Y, Folkman J, Sullivan R, et al. Heparin affinity: purification of a tumor-derived capillary endothelial cell growth factor. Science 1984;223:1296–8.
30. Folkman J. Antiangiogenesis. In: DeVita VT Jr, Hellman S, Rosenberg SA. Biologic therapy of cancer. Philadelphia: Lippincott, 1991. p. 743–53.
31. Folkman J, Merler E, Abernathy C, Williams G. Isolation of a tumor factor responsible for angiogenesis. J Exp Med 1971;133:275–88.
32. Folkman J. Angiogenesis. In: Jaffe EA, editor. Biology of endothelial cells. Boston, MA: Nijhoff; 1984. p. 412–28.
33. Klagsbrun M, Knighton D, Folkman J. Tumor angiogenesis activity in cell grown in tissue culture. Cancer Res 1976;36:110–4.
34. Auerbach R, Arensman R, Kubai L, Folkman J. Tumor-induced angiogenesis: lack of inhibitor by irradiation. Int J Cancer 1975;15:241–5.
35. Folkman J, Cotran RS. Relation of vascular proliferation to tumor growth. Int Rev Exp Pathol 1976;16:207–48.
36. Folkman J. What is the evidence that tumors are angiogenesis dependent? J Natl Cancer Inst 1990;82:4–6.
37. Kerbel R, Folkman J. Clinical translation of angiogenesis inhibitors. Nat Rev Cancer 2002;2:727–39.
38. Folkman J, Hochberg M. Self-regulation of growth in three dimensions. J Exp Med 1973;138:745–53.
39. Adam JA, Maggelakis AA. Diffusion of regulated growth characteristics of a spherical prevascular carcinoma. Bull Math Biol 1990;52:549–82.
40. Sutherland RM. Cell and environment interactions in tumor microregions: the multicell spheroid model. Science 1988;240:177–84.
41. Sutherland RM, McCredie JA, Inch WR. Growth of multicell spheroids in tissue culture as a model of nodular carcinomas. J Natl Cancer Inst 1971;46:113–20.
42. Holmgren L, O'Reilly MS, Folkman J. Dormancy of micrometastases: balanced proliferation and apoptosis in the presence of angiogenesis suppression. Nat Med 1995;1:149–53.
43. Jain RK. Vascular and interstitial barriers to delivery of therapeutic agents in tumors. Cancer Metastasis Rev 1990;9:253–66.
44. Gimbrone MA Jr, Cotran R, Leapman S, Folkman J. Tumor growth and neovascularization: an experimental model using rabbit cornea. J Natl Cancer Inst 1974;52:413–27.
45. Gimbrone MA Jr, Leapman S, Cotran RS, Folkman J. Tumor dormancy in vivo by prevention of neovascularization. J Exp Med 1972;136:261–76.
46. Hanahan D, Folkman J. Patterns and emerging mechanisms

of the angiogenic switch during tumorigenesis. Cell 1996;86:353–64.
47. Brem S, Brem H, Folkman J, et al. Prolonged tumor dormancy by prevention of neovascularization in the vitreous. Cancer Res 1976;36:2807–12.
48. Folkman J. Tumor angiogenesis factor. Cancer Res 1974;34:2109–13.
49. Tannock IF. Population kinetics of carcinoma cells, capillary endothelial cells, and fibroblasts in a transplanted mouse mammary tumor. Cancer Res 1970;30:2470–6.
50. Thomlinson R, Gray L. The histological structure of some human lung cancers and the possible implications for radiotherapy. Br J Cancer 1955;9:539–49.
51. Helmlinger G, Yuan F, Dellian M, Jain RK. Interstitial pH and PO2 gradients in solid tumors in vivo: high-resolution measurements reveal a lack of correlation. Nat Med 1997;3:177–82.
52. Knighton D, Ausprunk D, Tapper D, Folkman J. Avascular and vascular phases of tumour growth in the chick embryo. Br J Cancer 1977;35:347–56.
53. Lien W, Ackerman NB. The blood supply of experimental liver metastases. II. A microcirculatory study of normal and tumor vessels of the liver with the use of perfused silicone rubber. Surgery 1970;68:334–40.
54. Folkman J, Watson K, Ingber D, Hanahan D. Induction of angiogenesis during the transition from hyperplasia to neoplasia. Nature 1989;339:58–61.
55. Thompson WD, Shiach KJ, Fraser RA, et al. Tumors acquire their vasculature by vessel incorporation, not vessel ingrowth. J Pathol 1987;151:323–32.
56. Skinner SA, Tutton PJ, O'Brien PE. Microvascular architecture of experimental colon tumors in the rat. Cancer Res 1990;50:2411–7.
57. Holash J, Maisonpierre PC, Compton D, et al. Vessel cooption, regression, and growth in tumors mediated by angiopoietins and VEGF. Science 1999;284:1994–8.
58. Kim ES, Serur A, Huang J, et al. Potent VEGF blockade causes regression of coopted vessels in a model of neuroblastoma. Proc Natl Acad Sci USA 2002;99:11399–404.
59. Ingber DM, Fujita T, Kishimoto S, et al. Synthetic analogues of fumagillin that inhibit angiogenesis and suppress tumour growth. Nature 1990;348:555–7.
60. Folkman J. Tumor angiogenesis. In: Wells SA Jr, Sharp PA. Accomplishments in cancer research. Philadelphia, PA: JB Lippincott Williams and Wilkins; 1998. p. 32–44.
61. Hori A, Sasada R, Matsutani E, et al. Suppression of solid tumor growth by immuno-neutralizing monoclonal antibody against human basic fibroblast growth factor. Cancer Res 1991;51:6180–4.
62. Kim KJ, Li B, Winer J, et al. Inhibition of vascular endothelial growth factor-induced angiogenesis suppresses tumour growth in vivo. Nature 1993;362:841–4.
63. Plate KH, Breier G, Millauer B, et al. Up-regulation of vascular endothelial growth factor and its cognate receptors in a rat glioma model of tumor angiogenesis. Cancer Res 1993;53:5822-7.
64. Brooks PC, Montgomery AMP, Rosenfeld M, et al. Integrin αvβ3 antagonists promote tumor regression by inducing apoptosis of angiogenic blood vessels. Cell 1994;79:1157–64.
65. O'Reilly MS, Holmgren L, Shing Y, et al. Angiostatin: a novel angiogenesis inhibitor that mediates the suppression of metastases by a Lewis lung carcinoma. Cell 1994;79:315–28.
66. O'Reilly MS, Holmgren L, Chen C, Folkman J. Angiostatin induces and sustains dormancy of human primary tumors in mice. Nature Med 1996;2:689–92.
67. O'Reilly MS, Boehm T, Shing Y, et al. Endostatin: an endogenous inhibitor of angiogenesis and tumor growth. Cell 1997;88:277–85.
68. O'Reilly MS, Brem H, Folkman J. Treatment of murine hemangioendotheliomas with the angiogenesis inhibitor AGM-1470. J Pediatr Surg 1994;30:325–30.
69. Boehm T, Folkman J, Browder T, O'Reilly MS. Antiangiogenic therapy of experimental cancer does not induce acquired drug resistance. Nature 1997;390:404–7.
70. O'Reilly MS, Pirie-Shepherd S, Lane WS, Folkman J. Antiangiogenic activity of the cleaved conformation of the serpin antithrombin. Science 1999;285:1926–8.
71. Maeshima Y, Manfredi M, Reimer C, et al. Identification of the anti-angiogenic site within vascular basement membrane-derived tumstatin. J Biol Chem 2001;276:15240–8.
72. Kisker O, Becker CM, Prox D, et al. Continuous administration of endostatin by intraperitoneally implanted osmotic pump improves the efficacy and potency of therapy in a mouse xenograft tumor model. Cancer Res 2001;61:7669–74.
73. Holash J, Davis S, Papadopoulos N, et al. VEGF-Trap; a VEGF blocker with potent antitumor effects. Proc Natl Acad Sci USA 2002;99:11393–8.
74. Millauer B, Shawver LK, Plate KH, et al. Glioblastoma growth inhibited in vivo by a dominant-negative Flk-1 mutant. Nature 1994;367:576–9.
75. Dameron KM, Volpert OV, Tainsky MA, Bouck N. Control of angiogenesis in fibroblasts by p53 regulation of thrombospondin-1. Science 1994;265:1582–4.
76. Arbiser JL, Moses MA, Fernandez CA, et al. Oncogenic H-ras stimulates tumor angiogenesis by two distinct pathways. Proc Natl Acad Sci USA 1997;94:861-6.
77. Chin L, Tam A, Pomerantz J, et al. Essential role for oncogenic Ras in tumour maintenance. Nature 1999;400:468–72.
78. Cao Y, O'Reilly MS, Marshall B, et al. Expression of angiostatin cDNA in a murine fibrosarcoma suppresses primary tumor growth and produces long-term dormancy of metastases. J Clin Invest 1998;101:1055–63.
79. Streit M, Riccardi L, Velasco P, et al. Thrombospondin-2: a potent endogenous inhibitor of tumor growth and angiogenesis. Proc Natl Acad Sci USA 1999;96:14888–93.
80. Lyden D, Young AZ, Zagzag D, et al. Id1 and ID3 are required for neurogenesis, angiogenesis and vascularization of tumour xenografts. Nature 1999;401:670–7.
81. Lyden D, Hattori K, Dias S, et al. Impaired recruitment of bone-marrow-derived endothelial and hematopoietic precursor cells blocks tumor angiogenesis and growth. Nature Med 2001;7:1194-201.
82. Fidler IJ, Gersten DM, Hart IR. The biology of cancer invasion and metastasis. Adv Cancer Res 1978;28:149–250.
83. Nicolson GL. Cancer metastasis. Sci Am 1979;240:66–76.
84. Nicolson GL. Organ specificity of tumor metastasis: role of preferential adhesion, invasion and growth of malignant cells at specific secondary sites. Cancer Metastasis Rev 1988;7:143–188.
85. Watanabe H, Hori A, Seno M, et al. A sensitive enzyme immunoassay for human basic fibroblast growth factor. Biochem Biophys Res Commun 1991;175:229–35.
86. Zetter BR. Angiogenesis and tumor metastasis. Annu Rev Med 1998;49:407–24.
87. Netland PA, Zetter BR. Organ-specific adhesion of metastatic tumor cells in vitro. Science 1984;224:1113–5.
88. Boxberger HJ, Paweletz N, Spiess E, Kriehuber R. An in vitro model study of BSp73 rat tumour cell invasion into endothelial monolayer. Anticancer Res 1989;9:1777–86.
89. Weidner N, Semple JP, Welch WR, Folkman J. Tumor angiogenesis correlates with metastasis in invasive breast carcinoma. N Engl J Med 1991;324:1–8.
90. Weinstat-Saslow D, Steeg PS. Angiogenesis and colonization in the tumor metastatic process: basic and applied advances. FASEB J 1994;8:401–7.
91. Liotta LA, Tryggvason K, Garbisa S, et al. Metastatic potential correlates with enzymatic degradation of basement membrane collagen. Nature 1980;284:67–8.
92. Liotta LA, Saidel MG, Kleinerman J. The significance of hematogenous tumor cell clumps in the metastatic process. Cancer Res 1976;36:889–94.
93. Dvorak HF, Nagy JA, Dvorak JT, Dvorak AM. Identification and characterization of the blood vessels of solid tumors that are leaky to circulating macromolecules. Am J Pathol 1988;133:95–109.
94. Kalebic T, Garbisa S, Glaser B, Liotta LA. Basement membrane collagen: degradation by migrating endothelial cells. Science 1983;221:281–3.
95. Moscatelli D, Gross JL, Rifkin DB. Angiogenic factors stimulate plasminogen activator and collagenase production by capillary endothelial cells [abstract]. J Cell Biol 1981;91:201a.
96. Nagy JA, Brown LF, Senger DR, et al. Pathogenesis of tumor stroma generation: a critical role for leaky blood vessels and fibrin deposition. Biochem Biophys Acta 1989;948:305–26.
97. Folkman J. Angiogenesis in cancer, vascular, rheumatoid and other disease. Nature Med 1995;1:27–31.
98. Chambers AF. The metastatic process: basic research and clinical implications. Oncol Res 1999;11:161–8.
99. Udagawa T, Fernandez A, Achilles E-G, et al. Persistence of microscopic human cancers in mice: alterations in the angiogenic balance accompanies loss of tumor dormancy. FASEB J 2002;16:1361-70.
100. Hicks RM, Chowaniec J. Experimental induction, histology, and ultrastructure of hyperplasia and neoplasia of the urinary bladder epithelium. Int Rev Exp Pathol 1978;18:199–280.
101. Sillman F, Boyce J, Fruchter R. The significance of atypical vessels and neovascularization in cervical neoplasia. Am J Obstet Gynecol 1981;139:154–9.
102. Smith-McCune KK, Weidner N. Demonstration and characterization of the angiogenic properties of cervical dysplasia. Cancer Res 1994;54:800–4.
103. Srivastava A, Laidler P, Davies RP, Horfan K. The prognostic significance of tumor vascularity in intermediate-thickness (0.76–4.0 mm thick) skin melanoma. A quantitative histologic study. Am J Pathol 1988;133:419–23.
104. Weidner N, Folkman J, Pozza F, et al. Tumor angiogenesis: a new significant and independent prognostic indicator in early stage breast carcinoma. J Natl Cancer Inst 1992;84:1875–87.
105. Weidner N, Carroll PR, Flax J, et al. Tumor angiogenesis correlates with metastasis in invasive prostate carcinoma. Am J Pathol 1993;143:401–9.
106. Fang J, Shing Y, Wiederschain D, et al. Matrix metalloproteinase-2 (MMP-2) is required for the switch to the angiogenic phenotype in a novel tumor model. Proc Natl Acad Sci USA 2000.;97:3884-9.
107. Carmeliet P, Dor Y, Herbert JM, et al. Role of HIF-1α in hypoxia-mediated apoptosis, cell proliferation and tumor angiogenesis. Nature 1998;394:485–90.
108. Li C-Y, Shan S, Huang Q, et al. Initial stages of tumor cell-induced angiogenesis: evaluation via skin window chambers in rodent models. J Natl Cancer Inst 2000;92:143–7.
109. Folkman J. Incipient angiogenesis. J Natl Cancer Inst 2000;92:94–5.
110. Rak J, Filmus J, Finkenzeller G, et al. Oncogenes as inducers of tumor angiogenesis. Cancer Metastasis Rev 1995;14:263–77.
111. Holash J, Wiegand SJ, Yancopoulos GD. New model of tumor angiogenesis: dynamic balance between vessel regression and growth mediated by angiopoietins and VEGF. Oncogene 1999;18:5356–62.
112. Senger DR, Brown LF, Claffey KP, Dvorak HF. Vascular permeability factor, tumor angiogenesis and stroma generation. Invasion Metastasis 1994;14:385–94.
113. Kadish JL, Butterfield CE, Folkman J. The effect of fibrin on cultured vascular endothelial cells. Tissue Cell 1979;11:99–108.
114. Ziche M, Morbidelli L, Choudhuri R, et al. Nitric oxide synthase lies downstream from vascular endothelial growth factor-induced but not basic fibroblast growth factor-induced angiogenesis. J Clin Invest 1997; 99:2625–34.
115. Papapetropoulos A, Garcia-Cardena G, Madri JA, Sessa WC. Nitric oxide contributes to the angiogenic properties of vascular endothelial growth factor in human endothelial cells. J Clin Invest 1997;100:3131–9.
116. Garcia-Cardena G, Folkman J. Is there a role for nitric oxide in tumor angiogenesis? J Natl Cancer Inst 1998;90:560–1.
117. Ingber DE, Folkman J. How does the extracellular matrix control capillary morphogenesis? Cell 1989;58:803–5.
118. Chen CS, Mrksich M, Huan S, et al. Geometric control of cell life and death. Science 1997;276:1425–8.
119. Yuan F, Chen Y, Dellian M, et al. Time-dependent vascular regression and permeability changes in established human tumor xenografts induced by an anti-vascular endothelial growth factor/vascular permeability factor antibody. Proc Natl Acad Sci USA 1996;93:14765–70.
120. Kandel J, Bossy-Wetzel E, Radvanyi F, et al. Neovascularization is associated with a switch to the export of bFGF in the multistep development of fibrosarcoma. Cell 1991;66:1095–104.
121. Hanahan D, Christofori G, Naik P, Arbeit J. Transgenic mouse models of tumor angiogenesis: the angiogenic switch, its molecular controls, and prospects for preclinical therapeutic models. Eur J Cancer 1996;14:2386–93.
122. McDonald D, Hashizume H, Baluk P, Morikawa S, et al. Openings between defective endothelial cells explain tumor vessel leakiness. Am J Pathol 2000;156:1363-80.
123. Asahara T, Murohara T, Sullivan A, et al. Isolation of putative progenitor endothelial cells for angiogenesis. Science 1997;275:964–7.
124. Shi Q, Rafii S, Wu MH-D, et al. Evidence for circulating bone marrow-derived endothelial cells. Blood 1998;92:362–7.
125. Hatzopoulos AK, Folkman J, Vasile E, et al. Isolation and characterization of endothelial progenitor cells from mouse embryos. Development 1998;125:1457–68.
126. Asahara T, Takahashi T, Masuda h, et al. VEGF contributes to postnatal neovascularization by mobilizing bone marrow-derived endothelial progenitor cells. Embo J 1999;18:3964–72.
127. Murohara T, Ikeda H, Duan J, et al. Transplanted cord blood-

derived endothelial precursor cells augment postnatal neovascularization. J Clin Invest 2000; 105:1527–36.
128. Rafii S. Circulating endothelial precursors: mystery, reality, and promise. J Clin Invest 2000:105(1):17–9.
129. Peichev M, Naiyer AJ, Pereira D, et al. Expression of VEGFR-2 and AC133 by circulating human CD34(+) cells identifies a population of functional endothelial precursors. Blood 2000;95:952–8.
130. Gehling UM, Ergun S, Schumacher U, et al. In vitro differentiation of endothelial cells from AC133-positive progenitor cells. Blood 2000;95:3106–12.
131. Hattori K, Dias S, Heissig B, et al. Vascular endothelial growth factor and angiopoietin-1 stimulate postnatal hematopoiesis by recruitment of vasculogenic and hematopoietic stem cells. J Exp Med 2001:193:1004–14.
132. Hattori K, Heissig B, Tashiro K, et al. Plasma elevation of stromal cell-derived factor-1 induces mobilization of mature and immature hematopoietic progenitor and stem cells. Blood 2001;97:3354–60.
133. Hattori K, Heissig B, Wu Y, et al. Placental growth factor reconstitutes hematopoiesis by recruiting VEGFR1(+) stem cells from bone marrow microenvironment. Nature Med 2002;8:841–9.
134. Heissig B, Hattori K, Dias S, et al. Recruitment of stem and progenitor cells from the bone marrow niche requires MMP-9 mediated release of kit-ligand. Cell 2002;109:625–37.
135. Reyes M, Dudek A, Jahagirdar B, et al. Origin of endothelial progenitors in human postnatal bone marrow. J Clin Invest 2002;109:337–46.
136. Ito H, Rovira II, Bloom ML, et al. Endothelial progenitor cells as putative targets for angiostatin. Cancer Res 1999;59:5875–7.
137. Monestiroli S, Mancuso P, Burlini a, et al. Kinetics and viability of circulating endothelial cells as surrogate angiogenesis marker in an animal model of human lymphoma. Cancer Res 2001;61:4341–4.
138. Bertolini F, Mingrone W, Alietti A, et al. Thalidomide in multiple myeloma, myelodysplastic syndromes and histiocytosis. Analysis of clinical results and of surrogate angiogenesis markers. Ann Oncol 2001; 12:987–90.
139. Folkman J. Role of angiogenesis in tumor growth. Semin Cancer Biol 1992;3:65–71.
140. Polverini P, Leibovich S. Induction of neovascularization in vivo and endothelial proliferation in vitro by tumor-associated macrophages. Lab Invest 1984;51:635–42.
141. Bergers G, Brekken R, McMahon J, et al. Matrix metalloproteinases-9 triggers the angiogenic switch during carcinogenesis. Nature Cell Biol 2000;2:737-44.
142. Coussens LM, Tinkle CL, Hanahan D, Werb Z. MMP-9 supplied by bone marrow-derived cells contributes to skin carcinogenesis. Cell 2000;103:481–90.
143. Schulze-Osthoff K, Risau W, Vollmer E, Sorg C. In situ detection of basic fibroblast growth factor by highly specific antibodies. Am J Pathol 1990;137:85–92.
144. Fukumura D, Xavier R, Sugiura T, et al. Tumor induction of VEGF promotor activity in stromal cells. Cell 1998;94:715–25.
145. Luzzi KJ, MacDonald IC, Schmidt EE, et al. Multistep nature of metastatic inefficiency: dormancy of solitary cells after successful extravasation and limited survival of early micrometastases. Am J Pathol 1998; 153:865–73.
146. Cameron MD, Schmidt EE, Kerkvliet N, et al. Temporal progression of metastasis in lung: cell survival, dormancy, and location dependence of metastatic inefficiency. Cancer Res 2000;60:2541–6.
147. Folkman J. Angiogenesis and its inhibitors. In: DeVita VT Jr, Hellman S, Rosenberg SA, editors. Important advances in oncology. Philadelphia: JB Lippincott; 1985. p. 42–62.
148. Cockerill GW, Gamble JR, Vadas MA. Angiogenesis: models and modulators. Int Rev Cytol 1995;159:113–60.
149. Murray JC. Angiogenesis protocols. New Jersey: Humana Press; 2001.
150. Christofori G. The role of fibroblast growth factors in tumour progression and angiogenesis. In: Bicknell R, Lewis CE, Ferrara N. Tumour angiogenesis. Oxford: Oxford University Press; 1996. p. 201–37.
151. Friesel RE, Maciag T. Molecular mechanisms of angiogenesis: fibroblast growth factor signal. FASEB J 1995;9:919–25.
152. Folkman J, Shing Y. Angiogenesis. J Biol Chem 1992;267:10931–4.
153. Almaric F, Baldin V, Bosc-Bierne I, et al. Nuclear translocation of basic fibroblast growth factor. Ann NY Acad Sci 1991;638:127–38.
154. Folkman J, Klagsbrun M, Sasse J, et al. A heparin-binding angiogenic protein–basic fibroblast growth factor–is stored within basement membrane. Am J Pathol 1988;130:393–400.
155. Paulus W, Grothe C, Sensenbrenner M, et al. Localization of basic fibroblast growth factor, a mitogen and angiogenic factor, in human-brain tumors. Acta Neuropathol 1990;79:418–23.
156. Takahashi JA, Fukumoto M, Igarashi K, et al. Correlation of basic fibroblast growth factor expression levels with the degree of malignancy and vascularity in human gliomas. J Neurosurg 1992;76:792–8.
157. Chodak GW, Hospelhorn V, Judge SM, et al. Increased levels of fibroblast growth factor-like activity in urine of patients with bladder or kidney cancer. Cancer Res 1988;48:2083–8.
158. Vlodavsky I, Bashkin PK, Korner G, et al. Extracellular matrix-resident growth factors and enzymes: relevance to angiogenesis and metastasis. Proc Annu Meet Am Assoc Cancer Res 1990;31:491–3.
159. Czubayko F, Liaudet-Coopman EDE, Aigner A, et al. A secreted FGF-binding protein can serve as the angiogenic switch in human cancer. Nat Med 1997;3:1137–40.
160. Rak J, Kerbel RS. FGF-2 and tumorangiogenesis: back in the limelight. Nature Med 1997;3:1083–4.
161. Sherif ZA, Nakai S, Pirollo KF, et al. Down-modulation of bFGF-binding protein expression following restoration of p53 function. Cancer Gene Ther 2001;8:771–82.
162. Norrby K. Mast cells and angiogenesis. APMIS 2002;110:355–71.
163. Soutter AD, Nguyen M, Watanabe H, Folkman J. Basic fibroblast growth factor secreted by an animal tumor is detectable in urine. Cancer Res 1993;53:5297–9.
164. Klagsbrun M, D'Amore P. Regulators of angiogenesis. Annu Rev Physiol 1991;53:217–39.
165. Yancopoulos GD, Klagsbrun M, Folkman J. Vasculogenesis, angiogenesis and growth factors. Cell 1998;93:661–4.
166. Uemura A, Ogawa M, Hirashima M, et al. Recombinant angiopoietin-1 restores higher-order architecture of growing blood vessels in mice in the absence of mural cells. J Clin Invest 2002;110:1619–28.
167. Melder RJ, Koenig GC, Witwer BP, et al. During angiogenesis, vascular endothelial growth factor and basic fibroblast growth factor regulate natural killer cell adhesion to endothelium. Nature Med 1996;2:992–7.
168. Griffioen AW, Tromp SC, Hillen HF. Angiogenesis modulates the tumour immune response. Int J Exp Pathol 1998;76:363–8.
169. Griffioen AW, Damen CA, Mayo KH, et al. Angiogenesis inhibitors overcome tumor induced endothelial cell anergy. Intl J Cancer 1999;80:315–9.
170. Nguyen M, Watanabe H, Budson AE, et al. Elevated levels of an angiogenic peptide, basic fibroblast growth factor, in the urine of patients with a wide spectrum of cancers. J Natl Cancer Inst 1994;86:356–61.
171. Li VW, Folkerth RD, Watanabe H, et al. Microvessel count and cerebrospinal fluid basic fibroblast growth factor in children with brain tumours. Lancet 1994;344:82–6.
172. Nanus DM, Schmitz-Drager BJ, Motzer RJ, et al. Expression of basic fibroblast growth factor in primary human renal tumors: correlation with poor survival. J Natl Cancer Inst 1993;85:1597–9.
173. Lin RY, Argenta PA, Sullivan KM, Adzick NS. Diagnostic and prognostic role of basic fibroblast growth factor in Wilm's tumor patients. Clin Cancer Res 1995;1:327–31.
174. Dvorak HF. Tumors: wounds that do not heal. Similarities between tumor stroma generation and wound healing. N Engl J Med 1986;315:1650–9.
175. Senger DR, Galli SJ, Dvorak AM, et al. Tumor cells secrete a vascular permeability factor that promotes accumulation of ascites fluid. Science 1983;219:983–5.
176. Ferrara N, Henzel WJ. Pituitary follicular cells secrete a novel heparin-binding growth factor specific for vascular endothelial cells. Biochem Biophys Res Commun 1989;161:851–5.
177. Brown LF, Detmar M, Claffey K, et al. Vascular permeability factor/vascular endothelial growth factor: a multifunctional angiogenic cytokine. In: Goldberg ID, Rosen E, editors. Control of angiogenesis. Berlin: Birkhauser Verlag; 1997. p. 233–69.
178. Ferrara N. Vascular endothelial growth factor. Trends Cardiovasc Med 1993;3:244–50.
179. Rosenthal RA, Megyesi JF, Henzel WJ, et al. Contitioned medium from mouse sarcoma 180 cells contains vascular endothelial growth factor. Growth Factors 1990;4:53–9.
180. Bouck N, Stellmach V, Hsu S. How tumors become angiogenic. Adv Cancer Res 1996;69:135–74.
181. Connolly DT. Vascular permeability factor: a unique regulator of blood vessel function. J Cell Biochem 1991;47:219–23.
182. Dvorak HF, Sioussat TM, Brown LF, et al. Distribution of vascular permeability factor (vascular endothelial growth factor) in tumors: concentration in tumor blood vessels. J Exp Med 1991;174:1275–8.
183. Ferrara N, Houck K, Jakeman L, Leung DW. Molecular and biological properties of the vascular endothelial growth factor family of proteins. Endocr Rev 1992;13:18–32.
184. Breier G, Albrecht U, Sterrer S, Risau W. Expression of vascular endothelial growth factor during embryonic angiogenesis and endothelial cell differentiation. Development 1992;114:521–32.
185. Peters KG, De Vries C, Williams LT. VEGF receptor expression during embryogenesis and tissue repair suggests a role in endothelial differentiation and blood vessel growth. Proc Natl Acad Sci USA 1993;90:8915–9.
186. Gerber HP, Hillan KJ, Ryan AM, et al. VEGF is required for growth and survival in neonatal mice. Development 1999;126:1149-59.
187. Otani N, Minami S, Yamoto M, et al. The vascular endothelial growth factor/fms-like tyrosine kinase system in human ovary during the menstrual cycle and early pregnancy. J Clin Endocrinol Metab 1999;84:3845–51.
188. Brown LF, Berse B, Jackman RW, et al. Increased expression of vascular permeability factor (vascular endothelial growth factor) and its receptors in kidney and bladder carcinomas. Am J Pathol 1993;143:1255–62.
189. Inoue M, Hager JH, Ferrara N, et al. VEGF-A has a critical, nonredundant role in angiogenic switching and pancreatic beta cell carcinogenesis. Cancer Cell 2002;1:193–202.
190. Gerber HP, Kowalski J, Sherman D, et al. Complete inhibition of rhabdomyosarcoma xenografts growth and neovascularization requires blockade of both tumor and host vascular endothelial growth factor. Cancer Res 2000;60:6253-8.
191. De Vries C, Escobedo JA, Ueno H, et al. The fms-like tyrosine kinase, a receptor for vascular endothelial growth factor. Science 1992;255:989–94.
192. Terman BI, Dougher-Vermazen M, Carrion ME, et al. Identification of the KDR tyrosine kinase as a receptor for vascular endothelial cell growth factor. Biochem Biophys Res Comm 1992;187:1579–86.
193. Klagsbrun M, D'Amore P. Vascular endothelial growth factor and its receptors. Cytokine Growth Factor Rev 1996;7:259–70.
194. Neufeld G, Cohen T, Gengrinovitch S, Poltorak Z. Vascular endothelial growth factor (VEGF) and its receptors. FASEB J 1999;13:9–22.
195. Dvorak HF, Nagy JA, Feng D, et al. Vascular permeability factor/vascular endothelial growth factor and the significance of microvascular hyperpermeability in angiogenesis. Curr Top Microbiol Immunol 1999;237:97–132.
196. Ferrara N. VEGF and the quest for tumour angiogenesis factors. Nat Rev Cancer 2002;2:795–803.
197. Ferrara N. Vascular endothelial growth factor: molecular and biological aspects. Curr Top Microbiol Immunol 1999;237:1–30.
198. Korpelainen EI, Alitalo K. Signaling angiogenesis and lymphangiogenesis. Curr Opin Cell Biol 1998; 10:159–64.
199. Kaipainen A, Korhonen J, Mustonen T, et al. Expression of the fms-like tyrosine kinase 4 gene becomes restricted to lymphatic endothelium during development. Proc Natl Acad Sci USA 1995;92:3566–70.
200. Chang L, Kaipainen A, Folkman J. Lymphangiogenesis. New mechanisms. Ann NY Acad Sci 2002;979:111–9.
201. Soker S, Takashima S, Miao HQ, et al. Neuropilin-1 is expressed by endothelial and tumor cells as an isoform-specific receptor for vascular endothelial growth factor. Cell 1998;92:735–45.
202. Mamluk R, Gechtman Z, Kutcher ME, et al. Neuropilin-1 binds vascular endothelial growth factor 165, placenta growth factor-2, and heparin via its blb2 domain. J Biol Chem 2002;277:24818–25.
203. Plate K, Breier G, Weich HA, Risau W. Vascular endothelial growth factor is a potential tumour angiogenesis factor in human gliomas in vivo. Nature 1992;359:845–8.
204. Shweiki D, Itin A, Soffer D, Keshet E. Vascular endothelial growth factor induced by hypoxia may mediate hypoxia-initiated angiogenesis. Nature 1992;359:843–5.
205. Levy AP, Levy NS, Goldberg MA. Post-transcriptional regulation of vascular endothelial growth factor by hypoxia. J Biol Chem 1996;271:2746–53.

206. Dor Y, Keshet E. Ischemia-driven angiogenesis. Trends Cardiovasc Med 1997;7:289–94.
207. Shima DT, Adamis AP, Ferrara N, et al. Hypoxic induction of endothelial cell growth factors in retinal cells: identification and characterization of vascular endothelial growth factor (VEGF) as the mitogen. Mol Med 1995;1:182–93.
208. Okada F, Rak J, St. Croix B, et al. Impact of oncogenes on tumor angiogenesis: mutant K-ras upregulation of VEGF/VPF is necessary but not sufficient for tumorigenicity of human colorectal carcinoma cells. Proc Natl Acad Sci USA 1998;95:3609–14.
209. Iliopoulos O, Levy AP, Jiang C, et al. Negative regulation of hypoxia-inducible genes by the von Hippel-Lindau protein. Proc Natl Acad Sci USA 1996;93:10595–9.
210. Siemeister G, Weindel K, Mohrs K, et al. Reversion of deregulated expression of vascular endothelial growth factor in human renal carcinoma cells by von Hippel-Lindau tumor suppressor protein. Cancer Res 1996;56:2299–301.
211. Gnarra JR, Zhou S, Merrill MJ, et al. Post-transcriptional regulation of vascular endothelial growth factor mRNA by the product of the VHL tumor suppressor gene. Proc Natl Acad Sci USA 1996;93:10589–94.
212. Ferrara N. Role of vascular endothelial growth factor in regulation of angiogenesis. In: Teicher BA, editor. Antiangiogenic agents in cancer therapy. New Jersey: Humana Press; 1999. p. 119–42.
213. Aiello LP, George DJ, Cahill MT, et al. Rapid and durable recovery of visual function in a patient with von Hippel-Lindau syndrome after systemic therapy with vascular endothelial growth factor receptor inhibitor SU5416. Ophthalmology 2002;109:1745–51.
214. Roberts WG, Palade GE. Neovasculature induced by vascular endothelial growth factor is fenestrated. Cancer Res 1997;57:765–72.
215. van Bruggen N, Thibodeaux H, Palmer JT, et al. VEGF antagonism reduces edema formation and tissue damage after ischemia/reperfusion injury in the mouse brain. J Clin Invest 1999; 104:1613–20.
216. Pepper MS, Ferrara N, Orci L, Montesano R. Potent synergism between vascular endothelial growth factor and basic fibroblast growth factor in the induction of angiogenesis in vitro. Biochem Biophys Res Commun 1992;89:824–31.
217. Goto F, Goto K, Weindel K, Folkman J. Synergistic effects of vascular endothelial growth factor and basic fibroblast growth factor on the bovine capillary endothelial cells within collagen gels. Lab Invest 1993;69:508–17.
218. Stavri GT, Zachary IC, Baskerville PA, et al. Basic fibroblast growth factor upregulates the expression of vascular endothelial growth factor in vascular smooth muscle cells. Synergistic interaction with hypoxia. Circulation 1995;92:11–4.
219. Asahara T, Bauters C, Zheng LP, et al. Synergistic effect of vascular endothelial growth factor and basic fibroblast growth factor on angiogenesis in vivo. Circulation 1995;92:II365–71.
220. Houck KA, Leung DW, Rowland AM, et al. Dual regulation of vascular endothelial growth factor bioavailability by genetic and proteolytic mechanisms. J Biol Chem 1992;267:26031–7.
221. Davis S, Aldrich TH, Jones PF, et al. Isolation of angiopoietin-1, a ligand for the TIE2 receptor, by secretion-trap expression cloning. Cell 1996;87:1161–9.
222. Sato TN, Tozawa Y, Deutsch U, et al. Distinct roles of the receptor tyrosine kinases Tie-1 and Tie-2 in blood vessel formation. Nature 1995;376:70–4.
223. Koblizek TI, Weiss C, Yancopoulos GD, et al. Angiopoietin-1 induces sprouting angiogenesis in vitro. Curr Biol 1998;8:529–32.
224. Suri C, McClain J, Thurston G, et al. Increased vascularization in mice overexpressing angiopoietin-1. Science 1998;282:468–71.
225. Maisonpierre PC, Suri C, Jones PF, et al. Angiopoietin-2, a natural antagonist for Tie2 that disrupts in vivo angiogenesis. Science 1997;277:55–60.
226. Folkman J, D'Amore P. Blood vessel formation: what is its molecular basis? Cell 1996;87:1153–5.
227. Thurston G, Suri C, Smith K, et al. Leakage-resistant blood vessels in mice transgenically overexpressing angiopoietin-1. Science 1999;286:2511–4.
228. Rak J, Yu JL, Klement G, Kerbel RS. Oncogenes and angiogenesis: signaling three-dimensional tumor growth. J Invest Dermatol Symp Proc 2000;5:24–33.
229. Kerbel RS, Viloria-PetitA, Okada F, Rak J. Establishing a link between oncogenes and tumor angiogenesis. Mol Med 1998;4:286–95.
230. Rak J, Yu JL, Kerbel RS, Coomber BL. What do oncogenic mutations have to do with angiogenesis/vascular dependence of tumors? Cancer Res 2002;62:1931–4.
231. Petit AM, Rak J, Hung MC, et al. Neutralizing antibodies against epidermal growth factor and ErB-2/neu receptor tyrosine kinases down-regulate vascular endothelial growth factor production by tumor cells in vitro and in vivo: angiogenic implications for signal transduction therapy of solid tumors. Am J Pathol 1997;151:1523–30.
232. Fernandez A, Udagawa T, Schwesinger C, et al. Angiogenic potential of prostate carcinoma cells overexpressing bcl-2. J Natl Cancer Inst 2001;93:208–13.
233. Ravi R, Mookerjee B, Bhujwalla ZM, et al. Regulation of tumor angiogenesis by p53-induced degradation of hypoxia-inducible factor 1α. Genes Dev 2000;14:34–44.
234. Zhang L, Zu D, Hu M, et al. Wild-type p53 suppresses angiogenesis in human leiomyosarcoma and synovial sarcoma by transcriptional suppression of vascular endothelial growth factor expression. Cancer Res 2000;60:3655–61.
235. Izumi Y, Xu L, di Tomaso E, et al. Tumour biology: herceptin acts as an anti-angiogenic cocktail. Nature 2002;416:279–80.
236. Relf M, LeJeune S, Scott, PA, et al. Expression of the angiogenic factors vascular endothelial cell growth factor, acidic and basic fibroblast growth factor, tumor growth factor β-1, platelet-derived endothelial cell growth factor, placenta growth factor, and pleiotrophin in human primary breast cancer and its relation to angiogenesis. Cancer Res 1997;57:963-9.
237. Viloria-Petit A, Crombet T, Jothy S, et al. Acquired resistance to the antitumor effect of epidermal growth factor receptor-blocking antibodies in vivo: a role for altered tumour angiogenesis. Cancer Res 200l;61:5090–101.
238. Ciardiello F, Caputo R, Bianco R, et al. Inhibition of growth factor production and angiogenesis in human cancer cells by ZD1839 (Iressa), a selective epidermal growth factor receptor tyrosine kinase inhibitor. Clin Cancer Res 2001;7:1459–65.
239. Kaban LB, Mulliken JB, Ezekowitz RA, et al. Antiangiogenic therapy of a recurrent giant cell tumor of the mandible with interferonα-2a. Pediatrics 1999;103:1145–9.
240. Marler JJ, Rubin JB, Trede NS, et al. Successful antiangiogenic therapy of giant cell angioblastoma with interferon alfa 2b: report of 2 cases. Pediatrics 2002;109:1–5.
241. Kaban LB, Troulis MJ, Ebb D, et al. Antiangiogenic therapy with interferon alpha for giant cell lesions of the jaws. J Oral Maxillofac Surg 2002;60:1101-11.
242. Folkman J. Antiangiogenic therapy with interferon alpha for giant cell lesions of the jaws. J Oral Maxillofac Surg 2002;60:1111–3.
243. Singh RK, Gutman M, Bucana CD, et al. Interferon-α and -β down-regulate the expression of basic fibroblast growth factor in human carcinomas. Proc Natl Acad Sci USA 1995;92:4562–6.
244. Brouty Boye D, Zetter BR. Inhibition of cell motility by interferon. Science 1980;208:516–28.
245. Kerbel RS. Inhibition of tumour angiogenesis as a strategy to circumvent acquired resistance to anticancer therapeutic agents. Bioessays 1991;13:31–6.
246. Stetler-Stevenson WG, Krutzch HC, Liotta LA. Tissue inhibitor of metalloproteinases (TIMP-2). A new member of the metalloproteinases inhibitor family. J Biol Chem 1989;264:17374–8.
247. Moses MA, Sudhalter J, Langer R. Identification of an inhibitor of neovascularization from cartilage. Science 1990;248:1408–10.
248. Kruger EA, Figg WD. TNP-470: an angiogenesis inhibitor in clinical development for cancer. Exp Opin Invest Drugs 2000;9:1383–96.
249. D'Amato RJ, Loughnan MS, Flynn E, Folkman J. Thalidomide is an inhibitor of angiogenesis. Proc Natl Acad Sci USA 1994;91:4082–5.
250. Udagawa T, Verheul HMW, D'Amato RJ. Thalidomide and analogs. In: Teicher BA, editor. Antiangiogenic agents in cancer therapy. New Jersey: Humana Press; 1999. p. 263–74.
251. Figg WD, Reed E, Green S, Pluda JM. Thalidomide. A prodrug that inhibits angiogenesis. In: Teicher BA, editor. Antiangiogenic agents in cancer therapy. New Jersey: Humana Press; 1999. p. 407–22.
252. Folkman J. Angiogenesis-dependent diseases. Semin Oncol 2001;28:536–42.
253. Hideshima T, Anderson KC. Molecular mechanisms of novel therapeutic approaches for multiple myeloma. Nature Reviews Cancer 2002;2:927–37.
254. Crum R, Szabo S, Folkman J. A new class of steroids inhibits angiogenesis in the presence of heparin or a heparin fragment. Science 1985;230:1375–8.
255. D'Amato R, Lin CM, Flynn E, et al. 2-Methoxyestradiol, an endogenous mammalian metabolite inhibits tubulin polymerization by interacting at the colchicines site. Proc Natl Acad Sci USA 1994;91:3964–8.
256. Fotsis T, Zhang Y, Pepper MS, et al. The endogenous oestrogen metabolite 2-Methoxyestradiol inhibits angiogenesis and suppresses tumour growth. Nature 1994;368:237–9.
257. Williams JI. Squalamine. A new angiostatic steroid. In: Teicher BA, editor. Antiangiogenic agents in cancer therapy. New Jersey: Humana Press; 1999. p. 153–74.
258. Folkman J, Mulliken JB, Ezekowitz RAB. Antiangiogenic therapy of haemangiomas with interferon A. In: Stuart-Harris R, Penny R, editors. The clinical applications of the interferons. London: Chapman & Hall Medical; 1997. p. 255–65.
259. Voest EE, Kenyon BM, O'Reilly MS, et al. Inhibition of angiogenesis in vivo by interleukin 12. J Natl Cancer Inst 1995;87:581–6.
260. Maione TE, Gray GS, Petro J, et al. Inhibition of angiogenesis by recombinant human platelet factor-4 and related peptides. Science 1990;247:77–9.
261. Rastinejad F, Polverini P, Bouck NP. Regulation of the activity of a new inhibitor of angiogenesis by a cancer suppressor gene. Cell 1989;56:345–55.
262. Colorado PC, Torre A, Kamphaus G, et al. Anti-angiogenic cues from vascular basement membrane collagen. Cancer Res 2000;60:2520–6.
263. Kamphaus GD, Colorado PC, Panka DJ, et al. Canstatin, a novel matrix-derived inhibitor of angiogenesis and tumor growth. J Biol Chem 2000:275: 1209–15.
264. Maeshima Y, Colorado PC, Torre A, et al. Distinct antitumor properties of a type IV collagen domain derived from basement membrane. J Biol Chem 2000;275: 21340–8.
265. Brooks PC, Silletti S, von Schalscha TL, et al. Disruption of angiogenesis by PEX, a noncatalytic metalloproteinase fragment with integrin binding activity. Cell 1998;92:391–400.
266. Dawson DW, Volpert OV, Gillis P, et al. Pigment epithelium-derived factor: a potent inhibitor of angiogenesis. Science 1999;285:245–8.
267. Eder JP Jr., Supko JG, Clark JW, et al. Phase I clinical trial of recombinant human endostatin administered as a short intravenous infusion repeated daily. J Clin Oncol 2002; 18:3772–84.
268. Gengrinovitch S, Greenberg SM, Cohen T, et al. Platelet factor-4 inhibits the mitogen activity of VEGF121 and VEGF165 using several concurrent mechanisms. J Biol Chem 1995;270:15059–65.
269. Sidky YA, Borden EC. Inhibition of angiogenesis by interferons: effects on tumor- and lymphocyte-induced vascular responses. Cancer Res 1987;47:5155–61.
270. Dvorak HF, Gresser I. Microvascular injury in pathogenesis of interferon-induced necrosis of subcutaneous tumors in mice. J Natl Cancer Inst 1989;81:497–502.
271. Bouck N. Tumor angiogenesis: the role of oncogenes and tumor suppressor genes. Cancer Cells 1990;2:179–85.
272. Volpert OV, Stellmach V, Bouck N. The modulation of thrombospondin and other naturally occurring inhibitors of angiogenesis during tumor progression. Br Cancer Res Treat 1995;36:119–26.
273. Rodriguez-Manzaneque JC, Lane TF, Ortega MA, et al. Thrombospondin-1 suppresses spontaneous tumor growth and inhibits activation of matrix metalloproteinase-9 and mobilization of vascular endothelial growth factor. Proc Natl Acad Sci USA 2001;98(22):12485–90.
274. Warren BA, Chauvin WJ, Philips J. Blood-borne tumor emboli and their adherence to vessel walls. In: Day SB, Myers WPL, Stansly P, editors. Cancer invasion and metastasis: bio mechanisms and therapy. New York: Raven; 1977. p. 185–97.
275. Sugarbaker EV, Thornthwaite J, Ketcham AS. Inhibitory effect of a primary tumor on metastasis. In: Day SB, Myers WPL, Stansly P, editors. Progress in cancer research and therapy. New York: Raven; 1977. p. 227–40.
276. Clark WH Jr, Elder DE, Guerry DIV, et al. Model predicting survival in stage 1 melanoma based on tumor progression. J Natl Cancer Inst 1989;81:1893–904.
277. Woodruff M. The Walter Hubert lecture. Interaction of cancer and host. Br J Cancer 1982;46:313–22.
278. Southam CM, Brunschwig A. Quantitative studies of autotransplantation of human cancer. Cancer 1961;14:971–8.

279. Koike A, Moore GE, Mendoza CB, Watne AL. Heterologous, homologous, and autologous transplantation of human tumors. Cancer 1963;16:1065–71.
280. Camphausen K, Moses MA, Beecken W-D, et al. Radiation therapy to a primary tumor accelerates metastatic growth in mice. Cancer Res 2001;61:2207–11.
281. Lange PH, Hekmat K, Bosl G, et al. Accelerated growth of testicular cancer after cytoreductive surgery. Cancer 1980;45:1498–506.
282. Marie P, Clunet J. Fréquence des métastases viscérales chez les souris cancéreuses aprés ablation chirurgicale de leur tumeur. Bull Assoc Franç l'Etude Cancer 1910;3:19–23.
283. Greene HSN, Harvey EK. The inhibitory influence of a transplanted hamster lymphoma on metastasis. Cancer Res 1960;20:1094–103.
284. Milas L, Hunter N, Mason K, Withers HR. Immunological resistance to pulmonary metastases in C3Hf/Bu mice bearing syngeneic fibrosarcoma of different sizes. Cancer Res 1974;34:61–71.
285. Gorelik E, Segal S, Feldman M. Growth of a local tumor exerts a specific inhibitory effect on progression of lung metastases. Int J Cancer 1978;21:617–25.
286. Bonfil RD, Ruggiero RA, Bustuoabad OD, et al. Role of concomitant resistance in the development of murine lung metastases. Int J Cancer 1988;41:415–22.
287. Simpson-Herren L, Sanford AH, Holmquist JP. Effects of surgery on the cell kinetics of residual tumor. Cancer Treat Rep 1976;60:1749–60.
288. Fisher B, Gunduz N, Saffer EA. Influence of the interval between primary tumor removal and chemotherapy on kinetics and growth of metastases. Cancer Res 1983;43:1488–92.
289. Yuhas JM, Pazmino NH. Inhibition of subcutaneously growing line 1 carcinoma due to metastatic spread. Cancer Res 1974;34:2005–10.
290. Ehrlich P, Apolant H. Beobachtungen über maligne Mäusetumoren. Berl Klin Wochenschr 1905;42:871–4.
291. Bashford EF, Murray JA, Cramer W. The natural and induced resistance of mice to the growth of cancer. Proc R Soc Lond Biol Sci 1907;79:164–87.
292. Gershon RK, Carter RL, Kondo K. On concomitant immunity in tumor-bearing hamsters. Nature 1967;213:674–6.
293. Lausch RN, Rapp F. Concomitant immunity in hamsters bearing DMBA-induced tumor transplants. Int J Cancer 1969;4:226–31.
294. Gunduz N, Fisher B, Saffer EA. Effect of surgical removal on the growth and kinetics of residual tumor. Cancer Res 1979;39:3861–5.
295. Gorelik E. Concomitant tumor immunity and the resistance to a second tumor challenge. Adv Cancer Res 1983;39:71–120.
296. Ruggiero RA, Bustuoabad OD, Bonfil RD, et al. "Concomitant immunity" in murine tumors of non-detectable immunogenicity. Br J Cancer 1985;51:37–48.
297. Himmele JC, Rabenhorst B, Werner D. Inhibition of Lewis lung tumor growth and metastasis by Ehrlich ascites tumor growing in the same host. J Cancer Res Clin Oncol 1986;111:160–5.
298. Prehn RT. The inhibition of tumor growth by tumor mass. Cancer Res 1991;51:2–4.
299. Mohr U, Althoff J, Kinzel V, et al. Melanoma regression induced by "chalone": a new tumour inhibiting principle acting in vivo. Nature 1968;220:138–9.
300. DeWys WD. Studies correlating the growth rate of a tumor and its metastases and providing evidence for tumor-related systemic growth-retarding factors. Cancer Res 1972;32:374–9.
301. O'Reilly M, Rosenthal R, Sage EH, et al. The suppression of tumor metastases by a primary tumor. Surg Forum 1993;44:474–6.
302. Stathakis P, Fitzgerald M, Matthias LJ, et al. Generation of angiostatin by reduction and proteolysis of plasmin. Catalysis by a plasmin reductase secreted by cultured cells. J Biol Chem 1997;272:20641–5.
303. Gately S, Twardowski P, Stack MS, et al. Human prostate carcinoma cells express enzymatic activity that converts human plasminogen to the angiogenesis inhibitor, angiostatin. Cancer Res 1996;56:4887–90.
304. Dong Z, Kumar R, Yang X, Fidler IJ. Macrophage-derived metalloelastase is responsible for the generation of angiostatin in Lewis lung carcinoma. Cell 1997;88:801–10.
305. O'Reilly MS, Wiederschain D, Stetler-Stevenson WG, et al. Regulation of angiostatin production by matrix metalloproteinase-2 in a model of concomitant resistance. J Biol Chem 1999;274:29568–71.
306. Lay AJ, Jiang X-M, Kisker O, et al. Phosphoglycerate kinase acts in tumour angiogenesis as a disulphide reductase. Nature 2000;408:869–73.
307. Heidtmann H-H, Nettelbeck DM, Mingels A, et al. Generation of angiostatin-like fragments from plasminogen by prostate-specific antigen. Br J Cancer 1999;81:1269–73.
308. Lu H, Dhanabal M, Volk R, et al. Kringle 5 causes cell cycle arrest and apoptosis of endothelial cells. Biochem Biophys Res Commun 1999;258:668–73.
309. Lucas R, Holmgren L, Garcia I, et al. Multiple forms of angiostatin induce apoptosis in endothelial cells. Blood 1998;92:4730–41.
310. Griscelli F, Li H, Bennaceur-Griscelli A, et al. Angiostatin gene transfer: inhibition of tumor growth in vivo by blockage of endothelial cell proliferation associated with a mitosis arrest. Proc Natl Acad Sci USA 1998;95:6367–72.
311. Claesson-Welsh L, Welsh M, Ito N, et al. Angiostatin induces endothelial cell apoptosis and activation of focal adhesion kinase independently of the integrin-binding motif RGD. Proc Natl Acad Sci USA 1998;95:5579–83.
312. Wu Z, O'Reilly MS, Folkman J, Shing Y. Suppression of tumor growth with recombinant murine angiostatin. Biochem Biophys Res Commun 1997;236:651–4.
313. Cao Y, Chen A, An SSA, et al. Kringle 5 of plasminogen is a novel inhibitor of endothelial cell growth. J Biol Chem 1997;272:22924–8.
314. Ji WR, Castellino FJ, Chang Y, et al. Characterization of kringle domains of angiostatin as antagonists of endothelial cell migration, an important process in angiogenesis. FASEB J 1998;12:1731–8.
315. Ji WR, Barrientos LG, Llinas M, et al. Selective inhibition by Kringle 5 of human plasminogen on endothelial cell migration, an important process in angiogenesis. Biochem Biophys Res Commun 1998;247:414–9.
316. Barendsz-Janson AF, Griffioen AW, Muller AD, et al. In vitro tumor angiogenesis assays: plasminogen lysine binding site 1 inhibits in vitro tumor-induced angiogenesis. J Vasc Res 1998;35:109–14.
317. Parsons-Wingerter P, Lwai B, Yang MC, et al. A novel assay of angiogenesis in the quail chorioallantoic membrane: stimulation by bFGF and inhibition by angiostatin according to fractal dimension and grid intersection. Microvasc Res 1998;55:201–14.
318. Cornelius LA, Nehring LC, Harding E, et al. Matrix metalloproteinases generate angiostatin: effects on neovascularization. J Immunol 1998;161:6845–52.
319. Falcone DJ, Khan KM, Layne T, Fernandes L. Macrophage formation of angiostatin during inflammation. A byproduct of the activation of plasminogen. J Biol Chem 1998;273:31480–5.
320. O'Mahony CA, Albo D, Tuszynski GP, Berger DH. Transforming growth factor-β1 inhibits generation of angiostatin by human pancreatic cancer cells. Surgery 1998;124:388–93.
321. O'Mahony CA, Seidel A, Albo D, et al. Angiostatin generation by human pancreatic cancer. J Surg Res 1998;77:55–8.
322. Lijnen HR, Ugwu F, Bini A, Collen D. Generation of an angiostatin-like fragment from plasminogen by stromelysin-1 (MMP-3). Biochem 1998;37:4699–702.
323. Patterson BC, Sang QA. Angiostatin-converting enzyme activities of human matrilysin (MMP-7) and gelatinase B/type IV collagenase (MMP-9). J Biol Chem 1997;272:28823–5.
324. Gately S, Twardowski P, Stack MS, et al. The mechanism of cancer-mediated conversion of plasminogen to the angiogenesis inhibitor angiostatin. Proc Natl Acad Sci USA 1997;94:10868–72.
325. Cao R, Wu HL, Veitonmaki N, et al. Suppression of angiogenesis and tumor growth by the inhibitor K1-5 generated by plasmin-mediated proteolysis. Proc Natl Acad Sci USA 1999;96:5728–33.
326. Stack S, Gately S, Bafetti LM, et al. Angiostatin inhibits endothelial and melanoma cellular invasion by blocking matrix-enhanced plasminogen activation. Biochem J 1999;340(Pt 1):77–84.
327. Kirsch M, Strasser J, Allende R, et al. Angiostatin suppresses malignant glioma growth in vivo. Cancer Res 1998;58:4654–9.
328. Sim BK, O'Reilly MS, Liang H, et al. A recombinant human angiostatin protein inhibits experimental primary and metastatic cancer. Cancer Res 1997;57:1329–34.
329. Lannutti BJ, Gately ST, Quevedo ME, et al. Human angiostatin inhibits murine hemangioendothelioma tumor growth in vivo. Cancer Res 1997;57:5277–80.
330. Joe YA, Hong YK, Chung DS, et al. Inhibition of human malignant glioma growth in vivo by human recombinant plasminogen kringles 1-3. Int J Cancer 1999;82:694–9.
331. Redlitz A, Daum G, Sage EH. Angiostatin diminishes activation of the mitogen-activated protein kinases ERK-1 and ERK-2 in human dermal microvascular endothelial cells. J Vasc Res 1999;36:28–34.
332. Luo J, Lin J, Paranya G, Bischoff J. Angiostatin upregulates E-selectin in proliferating endothelial cells. Biochem Biophys Res Commun 1998;245:906–11.
333. Tanaka T, Cao Y, Folkman J, Fine HA. Viral vector-targeted anti-angiogenic gene therapy utilizing an angiostatin complementary DNA. Cancer Res 1998;58:3362–9.
334. Moser TL, Stack MS, Asplin I, et al. Angiostatin binds ATP synthase on the surface of human endothelial cells. Proc Natl Acad Sci USA 1999;96:2811–6.
335. Kost C, Benner K, Stockmann A, et al. Limited plasmin proteolysis of vitronectin. Characterization of the adhesion protein as morpho-regulatory and angiostatin-binding factor. Eur J Biochem 1996;236:682–8.
336. Gorski DH, Mauceri HJ, Salloum RM, et al. Potentiation of the antitumor effect of ionizing radiation by brief concomitant exposures to angiostatin. Cancer Res 1998;58:5686–9.
337. Mauceri HJ, Hanna NN, Beckett MA, et al. Combined effects of angiostatin and ionizing radiation in antitumour therapy. Nature 1998;394:287–91.
338. Tuszynski GP, Sharma MR, Rothman VL, Sharma MC. Angiostatin binds to the tyrosine kinase substrate annexin II through the lysine-binding domain in endothelial cells. Microvasc Res 2002;64:448–62.
339. Felez J, Miles LA, Fabregas P, et al. Characterization of cellular binding sites and interactive regions within reactants required for enhancement of plasminogen activation by tPA on the surface of leukocytic cells. Thromb Haemost 1996;76:577–84.
340. Kassam G, Choi KS, Ghuman J, et al. The role of annexin II tetramer in the activation of plasminogen. J Biol Chem 1998;273:4790–9.
341. Hajjar KA, Jacovina AT, Chacko J. An endothelial cell receptor for plasminogen/tissue plasminogen activator. I. Identity with annexin II. J Biol Chem 1994; 269:21191–7.
342. Aitkenhead M, Wang SJ, Nakatsu MN, et al. Identification of endothelial cell genes expressed in an in vitro model of angiogenesis: Induction of ESM-1, (beta)ig-h3, and NrCAM. Microvasc Res 2002;63:159–71.
343. Boyko V, Mudrak O, Svetlova M. A major cellular substrate for protein kinases, annexin II, is a DNA-binding protein. FEBS Lett 1994;345:139–42.
344. Chiang Y, Davis RG, Vishwanatha JK. Altered expression of annexin II in human B-cell lymphoma cell lines. Biochim Biophys Acta 1996;1313:295–301.
345. Jiang L, Jha V, Dhanabal M, et al. Intracellular Ca(2+) signaling in endothelial cells by the angiogenesis inhibitors endostatin and angiostatin. Am J Physiol 2001;280:C1140–C1150.
346. Tarui T, Miles LA, Takada Y. Specific interaction of Angiostatin with integrin αvβ3 in endothelial cells. J Biol Chem. 2001;276:39562-8.
347. Troyanovsky B, Levchenko T, Mansson G, et al. Angiomotin: an angiostatin binding protein that regulates endothelial cell migration and tube formation. J Cell Biol 2001;152:1247–54.
348. Bratt A, Wilson WJ, Troyanovsky B, et al. Angiomotin belongs to a novel protein family with conserved coiled-coil and PDZ binding domains. Gene 2002;298:69–77.
349. Wajih N, Sane DC. Angiostatin selectively inhibits signaling by hepatocyte growth factor in endothelial and smooth muscle cells. Blood 2002;101:1857-63.
350. Wahl ML, Owen CS, Grant DS. Angiostatin induces intracellular acidosis and anoikis in endothelial cells at a tumor-like low pH. Endothelium 2002;9:205–16.
351. Hajitou A, Grignet C, Devy L, et al. The antitumor effect of endostatin and Angiostatin is associated with a down-regulation of vascular endothelial growth factor expression in tumor cell. FASEB J 2002;16:1802–4.
352. Abad MC, Arni RK, Grella Dk, et al. The X-ray crystallographic structure of the angiogenesis inhibitor angiostatin. J Mol Biol 2002;318:1009–17.
353. Moses MA, O'Reilly MS. Regulation of Angiostatin mobilization by tumor-derived matrix metalloproteinases-2. Methods Mol Med 2003;74:375–90.
354. Apte RS, Niederkorn JY, Mayhew E, Alizadeh H. Angiostatin produced by certain primary uveal melanoma cell lines impedes the development of liver metastases. Arch Ophthalmol 2001;119:1805–9.

355. Verheul HMW, Hoekman K, Luykx-de Bakker S, et al. Platelet: transporter of vascular endothelial growth factor. Clin Cancer Res 1997;3(12 Pt 1):2187–90.
356. Möhle R, Green D, Moore MAS, et al. Constitutive production and thrombin-induced release of vascular endothelial growth factor by human megakaryocytes and platelets. Proc Natl Acad Sci USA1997;94:663–8.
357. Verheul HMW, Pinedo HM. Tumour growth: a putative role for platelets? Oncologist 1998;3:ii.
358. Pinedo HM, Verheul HMW, D'Amato RJ, Folkman J. Involvement of platelets in tumour angiogenesis? The Lancet 1998;352:1775–7.
359. Verheul HM, Jorna AS, Hoekman K, et al. Vascular endothelial growth factor-stimulated endothelial cells promote adhesion and activation of platelets. Blood 2000;96:4216–21.
360. Indraccolo S, Morini M, Gola E, et al. Effects of angiostatin gene transfer on functional properties and in vivo growth of Kaposi's sarcoma cells. Cancer Res 2001;61:5441–6.
361. Gupta N, Nodzenski E, Khodarev NN, et al. Angiostatin effects on endothelial cells mediated by ceramide and RhoA. EMBO Rep 2001;2:536–40.
362. Tarui T, Majumdar M, Miles LA, et al. Plasmin-induced migration of endothelial cells. A potential target for the anti-angiogenic action of Angiostatin. J Biol Chem 2002;277:33564–70.
363. Lay AJ, Jiang XM, Dal E, et al. Plasmin reduction by phosphoglycerate kinase is a thiol-independent process. J Biol Chem 2002;277:9062–8.
364. Abad MC, Geiger J. Crystallization and preliminary X-ray diffraction studies of human Angiostatin. Acta Crystallogr D Biol Crystallogr 2002;58(Pt 3):513–4.
365. Indraccolo S, Gola E, Rosato A, et al. Differential effects of angiostatin, endostatin and interferon-α(1) gene transfer on in vivo growth of human breast cancer cells. Gene Ther 2002;9:867–78.
366. Gyorffy S, Palmer K, Gauldie J. Adenoviral vector expressing murine Angiostatin inhibits a model of breast cancer metastatic growth in the lungs of mice. Am J Pathol 2001;159:1137–47.
367. Sacco MG, Cato EM, Ceruti R, et al. Systemic gene therapy with anti-angiogenic factors inhibits spontaneous breast tumor growth and metastasis in MMTVneu transgenic mice. Gene Ther 2001;8:67–70.
368. Fukumuri T, Nishitani M, Naroda T, et al. Expression of Angiostatin cDNA in a murine renal cell carcinoma suppresses tumor growth in vivo. Urology 2002;59:973–7.
369. Ma HI, Lin SZ, Chiang YH, et al. Intratumoral gene therapy of malignant brain tumor in a rat model with Angiostatin delivered by adeno-associated viral (AAV) vector. Gene Ther 2002;9:2–11.
370. Ma HI, Guo P, Li J, et al. Suppression of intracranial human glioma growth after intramuscular administration of an adeno-associated viral vector expressing angiostatin. Cancer Res 2002;62:756–63.
371. Zhang X, Wu j, Fei Z, et al. Angiostatin K(103) gene for treatment of human gliomas: an experimental study. Chin Med J 2000;113:996–1001.
372. Griscelli F, Li H, Cheong C, et al. Combined effects of radiotherapy and Angiostatin gene therapy in glioma tumor model. Proc Natl Acad Sci USA 2000;97:6698–703.
373. Rodolfo M, Cato EM, Soldati S, et al. Growth of human melanoma xenografts is suppressed by systemic angiostatin gene therapy. Cancer Gene Ther 2001;8:491–6.
374. Matsumoto G, Ohmi Y, Shindo J. Angiostatin gene therapy inhibits the growth of murine squamous cell carcinoma in vivo. Oral Oncol 2001;37:368–78.
375. Scappaticci FA, Smith R, Pathak A, et al. Combination angiostatin and endostatin gene transfer induces synergistic antiangiogenic activity in vitro and antitumor efficacy in leukemia and solid tumors in mice. Mol Ther 2001;3(2):186–96.
376. Hampl M, Tanaka T, Albert PS, et al. Therapeutic effects of viral vector-mediated antiangiogenic gene transfer in malignant ascites. Human Gene Ther 2001;12:1713–29.
377. Andrawiss M, Maron A, Beltran W, et al. Adeno-mediated gene transfer in canine eyes: a preclinical study for gene therapy of human uveal melanoma. J Gene Med 2001;3:228–39.
378. Shichinohe T, Bochner BH, Mizutani K, et al. Development of lentiviral vectors for antiangiogenic gene delivery. Cancer Gene Ther 2001;8:879–89.
379. Kim JM, Ho SH, Park EJ, et al. Angiostatin gene transfer as an effective treatment strategy in murine collagen-induced arthritis. Arthritis Rheum 2002;46:793–801.
380. Raisler BJ, Berns KI, Grant MI, et al. Adeno-associated virus type-2 expression of pigmented epithelium-derived factor or Kringles 1-3 of Angiostatin reduce retinal neovascularization. Proc Natl Acad Sci USA 2002;99:8909–14.
381. Lai CC, Wu WC, Chen SL, et al. Suppression of choroidal neovascularization by adeno-associated virus vector expressing Angiostatin. Invest Ophthalmol Vis Sci 2001;42:2401–7.
382. Dabrosin C, Gyorffy S, Margetts P, et al. Therapeutic effect of Angiostatin gene transfer in a murine model of endometriosis. Am J Pathol 2002;161:909–18.
383. Mauceri HJ, Seetharam S, Beckett MA, et al. Angiostatin potentiates cyclophosphamide treatment of metastatic disease. Cancer Chemother Pharmacol 2002;50:412–8.
384. Sun X, Kanwar JR, Leung E, et al. Angiostatin enhances B7.1-mediated cancer immunotherapy independently effects on vascular endothelial growth factor expression. Cancer Gene Ther 2001;8:719–27.
385. te Velde EA, Vogten JM, Gebbink MF, et al. Enhanced antitumour efficacy by combining conventional chemotherapy with Angiostatin or endostatin in a liver metastasis model. Br J Surg 2002;89:1302–9.
386. Scappaticci FA, Contreras A, Smith R, et al. Statin-AE: a novel angiostatin-endostatin fusion protein with enhanced antiangiogenic and antitumor activity. Angiogenesis. 2001;4(4):263–8.
387. Matsunaga T, Weihrauch DW, Moniz MC, et al. Angiostatin inhibits coronary angiogenesis during impaired production of nitric oxide. Circulation 2002;105:2185–91.
388. Dong Z, Yoneda J, Kumar R, Fidler IJ. Angiostatin-mediated suppression of cancer metastases by primary neoplasms engineered to produce granulocyte/macrophage colony-stimulating factor. J Exp Med 1998;188:755–63.
389. Ambati BK, Joussen AM, Ambati J, et al. Angiostatin inhibits and regresses corneal neovascularization. Arch Ophthalmol 2002;120:1063–8.
390. Hazzard TM, Rohan RM, Molskness TA, et al. Injection of antiangiogenic agents into the macaque preovulatory follicle: disruption of corpus luteum development and function. Endocrine 2002;17:199–206.
391. Drixler TA, Rinks IH, Ritchie ED, et al. Angiostatin inhibits pathological but not physiological retinal angiogenesis. Invest Ophthalmol Vis Sci 2001;42:3325–30.
392. Berns KI, Meneses P, Duvoisin R, et al. Use of recombinant angiostatin to prevent retinal neovascularization. Trans Am Clin Climatol Assoc 2001;112:68–75.
393. Kwon M, Yoon CS, Fitzpatrick S, et al. p22 Is a novel plasminogen fragment with antiangiogenic activity. Biochemistry 2001;40:13246–53.
394. Oh SP, Kamagata Y, Muragaki Y, et al. Isolation and sequencing of cDNAs for proteins with multiple domains of Gly-Xaa-Yaa repeats identify a distinct family of collagenous proteins. Proc Natl Acad Sci USA 1994;91:4229–33.
395. Rehn M, Pihlajaniemi T. Alpha1(XVIII), a collagen chain with frequent interruptions in the collagenous sequence, a distinct tissue distribution, and homology with type XV collagen. Proc Natl Acad Sci USA 1994;91:4234–8.
396. Carmeliet P, Jain RK. Angiogenesis in cancer and other diseases. Nature 2000;407:249–57.
397. Ortega N, Werb Z. New functional roles for non-collagenous domains of basement membrane collagens. J Cell Sci 2002;115:4201–14.
398. Obeso J, Weber J, Auerbach R. A hemangioendothelioma-derived cell line: its use as a model for the study of endothelial cell biology. Lab Invest 1990;63:259–69.
399. Hohenester E, Sasaki T, Olsen BR, Timpl R. Crystal structure of the angiogenesis inhibitor endostatin at 1.5. A resolution. EMBO J 1998;17:1656–64.
400. Ding Y-H, Javaherian K, Lo K-M, et al. Zinc-dependent dimers observed in crystals of human endostatin. Proc Natl Acad Sci USA 1998;95:10443–8.
401. Wen W, Moses MA, Wiederschain D, et al. The generation of endostatin is mediated by elastase. Cancer Res 1999;59:6052–6.
402. Felbor U, Dreier L, Bryant RAR, et al. Secreted cathepsin L generates endostatin from collagen XVIII. EMBO J 2000;19:1187–94.
403. Sasaki T, Hohenester E, Timpl R. Structure and function of collagen-derived endostatin inhibitors of angiogenesis. IUBMB Life. 2002;53(2):77–84.
404. Miosge N, Sasaki T, Timpl R. Angiogenesis inhibitor endostatin is a distinct component of elastic fibers in vessel walls. FASEB J 1999;13:1743–50.
405. Sasaki T, Larsson H, Tisi D, et al. Endostatins derived from collagens XV and XVIII differ in structural and binding properties, tissue distribution and anti-angiogenic activity. J Mol Biol 2000;301:1179–90.
406. Sasaki T, Larsson H, Kreuger J, et al. Structural basis and potential role of heparin/heparan sulfate binding to the angiogenesis inhibitor endostatin. EMBO J 1999;18:6240–8.
407. Kasai S, Nagasawa H, Shimamura M, et al. Design and synthesis of antiangiogenic/heparin-binding arginine dendrimer mimicking the surface of endostatin. Bioorg Med Chem Lett 2002;12:951–4.
408. Boehm T, O'Reilly MS, Keough K, et al. Zinc-binding of endostatin is essential for its antiangiogenic activity. Biochem Biophys Res Comm 1998;252:190–4.
409. Sim BKL, Fogler WE, Zhou XH, et al. Zinc ligand-disrupted recombinant human endostatin: potent inhibition of tumor growth, safety and pharmacokinetic profile. Angiogenesis 1999; 3:41–51.
410. Marneros AG, Olsen BR. The role of collagen-derived proteolytic fragments in angiogenesis. Matrix Biol 2001;20:337–45.
411. Ackley BD, Crew JR, Elamaa H, et al. The NC1/endostatin domain of Caenorhabditis elegans type XVIII collagen affects cell migration and axon guidance. J Cell Biol. 2001;152(6):1219–32.
412. Gagnon ML, Bielenberg DR, Gechtman Z, et al. Identification of a natural soluble Neuropilin-1 that binds vascular endothelial growth factor: in vivo expression and anti-tumor activity. Proc Natl Acad Sci USA 2000;97:2573–8.
413. Miao H-Q, Lee P, Lin H, et al. Neuropilin-1 expression by tumor cells promotes tumor angiogenesis and progression. FASEB J 2000;14:2532–9.
414. Shin D, Garcia-Cardena G, Hayashi S, et al. Expression of ephrinB2 identifies a stable genetic difference between arterial and venous vascular smooth muscle as well as endothelial cells, and marks subsets of microvessels at sites of adult neovascularization. Dev Biol 2001;230:139–50.
415. Wang HU, Chen ZF, Anderson DJ. Molecular distinction and angiogenic interaction between embryonic arteries and veins revealed by ephrin-b2 and its receptor Eph-B4. Cell 1998;93:741–53.
416. Kim YM, Hwang S, Kim YM, et al. Endostatin blocks vascular endothelial growth factor-mediated signaling via direct interaction with KDR/Flk-1. J Biol Chem. 2002;277(31):27872–9.
417. Dixelius J, Larsson H, Sasaki T, et al. Endostatin-induced tyrosine kinase signaling through the Shb adaptor protein regulates endothelial cell apoptosis. Blood 2000;95:3403–11.
418. Chang Z, Choon A, Friedl A. Endostatin binds to blood vessels in situ independent of heparan sulfate and does not compete for fibroblast growth factor-2 binding. Am J Pathol 1999;155:71–6.
419. Seghezzi G, Patel S, Ren CJ, et al. Fibroblast growth factor-2 (FGF-2) induces vascular endothelial growth factor (VEGF) expression in the endothelial cells of forming capillaries: an autocrine mechanism contributing to angiogenesis. J Cell Biol 1998; 141:1659–73.
420. Shuster CB, Herman IM. The mechanics of vascular cell motility. Microcirculation 1998;5:239–57.
421. Dixelius J, Cross M, Matsumoto T, Sasaki T, Timpl R, Claesson-Welsh L. Endostatin regulates endothelial cell adhesion and cytoskeletal organization. Cancer Res. 2002;62(7):1944–7.
422. Ergun S, Kilic N, Wurmbach JH, et al. Endostatin inhibits angiogenesis by stabilization of newly formed endothelial tubes. Angiogenesis 2001;4:193–206.
423. Kuo CJ, LaMontagne KR Jr, Garcia-Cardena G, et al. Oligomerization-dependent regulation of motility and morphogenesis by the collagen XVIII NC1/endostatin domain. J Cell Biol. 2001;152(6):1233–46.
424. Wickström S, Veikkola T, Rehn M, et al. Endostatin-induced modulation of plasminogen activation with concomitant loss of focal adhesions and actin stress fibers in cultured human endothelial cells. Cancer Res 2001;61:6511–6.
425. Kim Y-M, Jang J-W, Lee O-H, et al. Endostatin inhibits endothelial and tumor cellular invasion by blocking the activation and catalytic activity of matrix metalloproteinases 2. Cancer Res 2000;60:5410–3.
426. Lee SJ, Jang JW, Kim YM, et al. Endostatin binds to the catalytic domain of matrix metalloproteinase-2. FEBS Lett 2002;519(1-3):147–52.
427. Itoh T, Tanioka M, Yoshida H, et al. Reduced angiogenesis and tumor progression in gelatinase a-deficient mice. Cancer Res 1998;58:1048–51.

428. Rehn M, Veikkola T, Kukk-Valdre E, et al. Interaction of endostatin with integrins implicated in angiogenesis. Proc Natl Acad Sci USA 2001;98:1024–9.
429. MacDonald NJ, Shivers WY, Narum DL, et al. Endostatin binds tropomyosin. A potential modulator of the antitumor activity of endostatin. J Biol Chem. 2001;276(27):25190–6.
430. Karumanchi SA, Jha V, Ramchandran R, Karihaloo A, et al. Cell surface glypicans are low-affinity endostatin receptors. Mol Cell 2001;7(4):811–22.
431. Hanai J, Dhanabal M, Karumanchi SA, et al. Endostatin causes G1 arrest of endothelial cells through inhibition of cyclin D1. J Biol Chem. 2002;277(19):16464–9.
432. Dhanabal M, Ramchandran R, Waterman MJF, et al. Endostatin induces endothelial cell apoptosis. J Biol Chem 1999;274:11721–6.
433. Xu R, Yao ZY, Xin L, et al. NC1 domain of human type VIII collagen (alpha 1) inhibits bovine aortic endothelial cell proliferation and causes cell apoptosis. Biochem Biophys Res Commun. 2001;289(1):264–8.
434. Urbich C, Reissner A, Chavakis E, et al. Dephosphorylation of endothelial nitric oxide synthase contributes to the anti-angiogenic effects of endostatin. FASEB J 2002;16:706–8.
435. Morales-Ruiz M, Lee M-J, Zollner S, et al. Sphingosine 1-phosphate activates akt, nitric oxide production, and chemotaxis through a Gi protein/phosphoinositde 3-kinase pathway in endothelial cells. J Biol Chem 2001;276:19672–7.
436. Gallo O, Masini E, Morbidelli L, et al. Role of nitric oxide in angiogenesis and tumor progression in head and neck cancer. J Natl Cancer inst 1998;90:587–96.
437. Wu P, Yonekura H, Li H, et al. Hypoxia down-regulates endostatin production by human microvascular endothelial cells and pericytes. Biochem Biophys Res Commun 2001;288(5):1149–54.
438. Sertie AL, Sossi V, Camargo AA, et al. Collagen XVIII, containing an endogenous inhibitor of angiogenesis and tumor growth, plays a critical role in the maintenance of retinal structure and in neural tube closure (Knobloch syndrome). Human Mol Genet 2000;9:2051–8.
439. Suzuki OT, Sertie AL, Der Kaloustian VM, et al. Molecular analysis of collagen XVIII reveals novel mutations, presence of a third isoform, and possible genetic heterogeneity in Knobloch syndrome. Am J Hum Genet 2002;71(6):1320-1-9.
440. Fukai N, Eklund L, Marneros AG, et al. Lack of collagen XVIII/endostatin results in eye abnormalities. EMBO J 2002;21:1535–44.
441. Folkman J, Browder T, Palmblad J. Angiogenesis research: guidelines for translation to clinical application. Thromb Haemost 2001;86:23–33.
442. Browder T, Folkman J, Pirie-Shepherd S. The hemostatic system as a regulator of angiogenesis. J Biol Chem 2000;275:1521–4.
443. Ma L, Hollenberg MD, Wallace JL. Thrombin-induced platelet endostatin release is blocked by a proteinase activated receptor-4 (PAR4) antagonist. Br J Pharmacol. 2001;134(4):701–4.
444. Tepper OM, Murayama T, Kearney M, et al. Therapeutic neovascularization as a novel approach to thrombus recanalization and resolution [abstract 324]. Circulation 2002;106:II-65.
445. Ma L, Elliott SN, Cirino G, et al. Platelets modulate gastric ulcer healing: role of endostatin and vascular endothelial growth factor release. Proc Natl Acad Sci USA 2001;98(11):6470–5.
446. Ma L, del Soldato P, Wallace JL. Divergent effects of new cyclooxygenase inhibitors on gastric ulcer healing: shifting the angiogenic balance. Proc Natl Acad Sci USA. 2002;;99(20):13243–7.
447. Strik HM, Schluesener HJ, Seid K, et al. Localization of endostatin in rat and human gliomas. Cancer 2001;91:10130–9.
448. Black WR, Agner RC. Tumour regression after endostatin therapy. Nature 1998;391:4509.
449. Perletti G, Concari P, Giardini R, et al. Antitumor activity of endostatin against carcinogen-induced rat primary mammary tumors. Cancer Res 2000;60(7):1793–6.
450. Huang X, Wong MK, Zhao Q, et al. Soluble recombinant endostatin purified from *Escherichia coli*: antiangiogenic activity and antitumor effect. Cancer Res 2001;61(2):478–81.
451. He ZY, Chen ZY, Qiu CP, et al. Cloning, expression and tumor suppression of human endostatin. Sheng Wu Hua Xue Yu Sheng Wu wu Li Xue Bao 2000;32:333–6.
452. Liu DL, Wen JX, Tong WC, Ben LH. Inhibition of lung adenocarcinoma LA795 in mice by recombinant human endostatin. Di Yi Jun Yi Da Xue Xue Bao 2001;21:917–9.
453. Ye C, Feng C, Wang S, et al. Antiangiogenic and antitumor effects of endostatin of follicular thyroid carcinoma. Endocrinology 2002;143:3522–8.
454. Solaun MS, Mendoza L, De Luca M, et al. Endostatin inhibits murine colon carcinoma sinusoidal-type metastases by preferential targeting of hepatic sinusoidal endothelium. Hepatology. 2002;35(5):1104–16.
455. Iversen PO, Sorensen DR, Benestad HB. Inhibitors of angiogenesis selectively reduce the malignant cell load in rodent models of human myeloid leukemias. Leukemia. 2002;16:376–81.
456. Boehle AS, Kurdow R, Schulze M, et al. Human endostatin inhibits growth of human non–small-cell lung cancer in a murine xenotransplant model. Int J Cancer. 2001;94(3):420–8.
457. Kuroiwa M, Ikeda H, Hongo T, Tsuchida Y, et al. Effects of recombinant human endostatin on a human neuroblastoma xenograft. Int J Mol Med 2001;8:391–6.
458. Calvo A, Yokoyama Y, Smith LE, et al. Inhibition of the mammary carcinoma angiogenic switch in C3(1)/SV40 transgenic mice by a mutated form of human endostatin. Int J Cancer. 2002;101(3):224–34.
459. Yokoyama Y, Green JE, Sukhatme VP, Ramakrishnan S. Effect of endostatin on spontaneous tumorigenesis of mammary adenocarcinoma in a transgenic mouse model. Cancer Res 2000;60(16):4362–5.
460. Bergers G, Javaherian K, Lo KM, et al. Effects of angiogenesis inhibitors on multistage carcinogenesis in mice. Science. 1999;284(5415):808–12.
461. Sorensen DR, Read TA, Porwol T, et al. Endostatin reduces vascularization, blood flow, and growth in a rat gliosarcoma. Neurooncol 2002;4(1):1–8.
461a. Yamaguchi N, Anand-Apte B, Lee M, et al. Endostatin inhibits VEGF-induced endothelial cell migration and tumor growth independently of zinc binding. EMBO J 1999;18:4414–23.
462. Yokoyama Y, Dhanabal M, Griffioen AW, et al. Synergy between angiostatin and endostatin: inhibition of ovarian cancer growth. Cancer Res 2000;60(8):2190–6.
463. Hanna NN, Seetharam S, Mauceri HJ, et al. Antitumor interaction of short-course endostatin and ionizing radiation. Cancer J 2000;6:287–93.
464. Davidoff AM, Leary MA, Ng CY, et al. Autocrine expression of both endostatin and green fluorescent protein provides a synergistic antitumor effect in a murine neuroblastoma model. Cancer Gene Ther 2001;8(7):537–45.
465. Sorenson DR, Leirdal M, Iverson PO, Sioud M. Combination of endostatin and a protein kinase C α DNA enzyme improves the survival of rats with malignant glioma. Neoplasia 2002;4:474–9.
466. Millar A. Limitations of combination anti-angiogenesis and chemotherapy. Nature Reviews Cancer 2003. In press.
467. Feldman AL, Alexander HR, Hewitt SM, et al. Effect of retroviral endostatin gene transfer on subcutaneous and intraperitoneal growth of murine tumors. J Natl Cancer Inst 2001;93:1014–20.
468. Wang X, Liu F, Li X, et al. Inhibitory effect of endostatin mediated by retroviral gene transfer on human liver carcinoma SMMC7721 in vivo. Zhonghua Wai Ke Za Zhi. 2002;40:692–5.
469. Cichon T, Jamrozy L, Glogowska J, et al. Electrotransfer of gene encoding endostatin into normal and neoplastic mouse tissues: inhibition of primary tumor growth and metastatic spread. Cancer Gene Ther 2002;9(9):771–7.
470. Szary J, Szala S. Intra-tumoral administration of naked plasmid DNA encoding mouse endostatin inhibits renal carcinoma growth. Int J Cancer. 2001;91(6):835–9.
471. Yamanaka R, Zullo SA, Ramsey J, et al. Induction of therapeutic antitumor antiangiogenesis by intratumoral injection of genetically engineered endostatin-producing Semliki Forest virus. Cancer Gene Ther 2001;8(10):796–802.
472. Peroulis I, Jonas N, Saleh M. Antiangiogenic activity of endostatin inhibits C6 glioma growth. Int J Cancer 2002;97(6):839–45.
473. Read TA, Farhadi M, Bjerkvig R, et al. Intravital microscopy reveals novel antivascular and antitumor effects of endostatin delivered locally by alginate-encapsulated cells. Cancer Res 2001;61(18):6830–7.
474. Read TA, Sorensen DR, Mahesparan R, et al. Local endostatin treatment of gliomas administered by microencapsulated producer cells. Nat Biotechnol. 2001;19(1):29–34.
475. Joki T, Machluf M, Atala A, et al. Continuous release of endostatin from microencapsulated engineered cells for tumor therapy. Nat Biotechnol. 2001;19(1):35–9.
476. Jin X, Bookstein R, Wills K, et al. Evaluation of endostatin antiangiogenesis gene therapy in vitro and in vivo. Cancer Gene Ther. 2001;8(12):982–9.
477. Ding I, Sun JZ, Fenton B, et al. Intratumoral administration of endostatin plasmid inhibits vascular growth and perfusion in MCa-4 murine mammary carcinomas. Cancer Res 2001;61(2):526–31.
478. Feldman AL, Restifo NP, Alexander HR, et al. Antiangiogenic gene therapy of cancer utilizing a recombinant adenovirus to elevate systemic endostatin levels in mice. Cancer Res 2000;60:1503–6.
478a. Sauter BV, Martinet O, Zhang WJ, et al. Adenovirus-mediated gene transfer of endostatin in vivo results in high level expression and inhibition of tumor growth and metastases. Proc Natl Acad Sci USA 2000;97:4802–7.
479. Shi W, Teschendorf C, Muzyczka N, Siemann DW. Adeno-associated virus-mediated gene transfer of endostatin inhibits angiogenesis and tumor growth in vivo. Cancer Gene Ther. 2002;9(6):513–21.
480. Wen XY, Bai Y, Stewart AK. Adenovirus-mediated human endostatin gene delivery demonstrates strain-specific antitumor activity and acute dose-dependent toxicity in mice. Human Gene Ther 2001;12:347–58.
481. Blezinger P, Wang J, Gondo M, et al. systemic inhibition of tumor growth and tumor metastases by intramuscular administration of the endostatin gene therapy. Nature Biotech 1999;17:343–8.
482. Lefesvre P, Attema J, van Bekkum D. Adenoviral gene transfer of angiostatic ATF-BPTI inhibits tumour growth. BMC Cancer. 2002;2(1):17.
483. Eisterer W, Jiang X, Bachelot T, et al. Unfulfilled promise of endostatin in a gene therapy-xenotransplant model of human acute lymphocytic leukemia. Mol Ther. 2002;5(4):352–9.
484. Pawliuk R, Bachelot T, Zurkiya O, et al. Continuous intravascular secretion of endostatin in mice from transduced hematopoietic stem cells. Mol Ther. 2002; 5(4):345–51.
484a. Bachelot T, Pawliuk R, Treilleux I, et al. Retrovirus-mediated gene transfer of an Angiostatin-endostatin fusion protein with enhanced anti-tumor properties in vivo [abstract 1856]. Proc Am Assoc Cancer Res 1998;39:271–2.
485. Kuo CJ, Farnebo F, Yu EY, et al. Comparative evaluation of the antitumor activity of antiangiogenic proteins delivered by gene transfer. Proc Natl Acad Sci USA. 2001;98(8):4605–10.
485a. Slaton JW, Perotte P, Inoue K, et al. Interferon-α-mediated down-regulation of angiogenesis-related genes and therapy of bladder cancer are dependent on optimization of biological dose and schedule.Clin Cancer Res 1999;5:2726–34.
485b. Massague J. TGF-B signaling transduction. Annu Rev Biochem 1998;67:753–91.
486. Berger AC, Feldman AL, Gnant MF, et al. The angiogenesis inhibitor, endostatin, does not affect murine cutaneous wound healing. J Surg Res. 2000;91(1):26–31.
487. Xu R, Du P, Fan JJ, Zhang Q, et al. High-level expression and secretion of recombinant mouse endostatin by Escherichia coli. Protein Expr Purif 2002;24:453–9.
488. Park JH, Chang KH, Lee JM, et al. Optimal production and in vitro activity of recombinant endostatin from stably transformed Drosophila melanogaster S2 cells. In Vitro Cell Dev Biol Anim. 2001;37(1):5–9.
489. Trinh L, Noronha SB, Fannon M, Shiloach J. Recovery of mouse endostatin produced by Pichia pastoris using expanded bed adsorption. Bioseparation 2000;9:223–30.
490. Xia H, Zhang L, Wen JX, Tong WC. Expression and purification of human endostatin in Pichia pastoris and its inhibition on the growth of mouse pulmonary adenocarcinoma cell line LA795. Di Yi Jun Yi Da Xue Xue Bao 2002;22(5):393–6.
491. Rokstad AM, Holtan S, Strand B, et al. Microencapsulation of cells producing therapeutic proteins: optimizing cell growth and secretion. Cell Transplant 2002;11(4):313–24.
492. Zorick TS, Mustacchi Z, Bando SY, et al. High serum endostatin levels in Down syndrome: implications for improved treatment and prevention of solid tumours. Eur J Hum Genet 2001;9(11):811–4.
493. Yang Q, Rasmussen SA, Friedman JM. Mortality associated with Down's syndrome in the USA from 1983 to 1997: a population-based study. Lancet 2002;359:1019–25.
494. Iughetti P, Suzuki O, Godoi PH, et al. A polymorphism in endostatin, an angiogenesis inhibitor, predisposes for the development of prostatic adenocarcinoma. Cancer Res 2001;61(20):7375–8.
495. Feldman AL, Pak H, Yang JC, et al. Serum endostatin lev-

els are elevated in patients with soft tissue sarcoma. Cancer. 2001;91(8):1525–9.
496. Feldman AL, Alexander HR Jr, Yang JC, et al. Prospective analysis of circulating endostatin levels in patients with renal cell carcinoma. Cancer. 2002;95(8):1637–43.
497. Herbst RS, Hess KR, Tran HT, et al. Phase I study of recombinant human endostatin in patients with advanced solid tumors. J Clin Oncol 2002;20(18):3792–803.
498. Madri JA. Extracellular matrix modulation of vascular cell behaviour. Transpl Immunol 1997;5:179–83.
499. Furcht LT. Critical factors controlling angiogenesis: cell products, cell matrix, and growth factors. Lab Invest 1986;55:505–9.
500. Kalluri R. Discovery of type IV collagen non-collagenous domains as novel integrin ligands and endogenous inhibitors of angiogenesis. Cold Spring Harbor 67th Quantitative Symposium, New York, 2002;in press.
501. Senger DR, Claffey KP, Benes JE, et al. Angiogenesis promoted by vascular endothelial growth factor: regulation through alpha1beta1 and α2β1-integrins. Proc Natl Acad Sci USA 1997;94:13612–7.
502. Pozzi A, LeVine WF, Gardner HA. Low plasma levels of matrix metalloproteinase 9 permit increased tumor angiogenesis. Oncogene 2002;21:272–81.
503. Pozzi A, Moberg PE, Miles LA, et al. Elevated matrix metalloprotease and angiostatin levels in integrin alpha 1 knockout mice cause reduced tumor vascularization. Proc Natl Acad Sci USA 2000;97:2202–27.
504. Petitclerc E, Boutaud A, Prestayko A, et al. New functions for non-collagenous domains of human collagen type IV. Novel integrin ligands inhibiting angiogenesis and tumor growth in vivo. J Biol Chem 2000;275:8051–61.
505. Maeshima Y, Colorado PC, Kalluri R. Two RGD-independent αvβ3-integrin binding sites on tumstatin regulate distinct anti-tumor properties. J Biol Chem 2000;275:23745–50.
506. Maeshima Y, Yerramalla UL, Dhanabal M, et al. Extracellular matrix-derived peptide binds to αvβ3-integrin and inhibits angiogenesis. J Biol Chem 2001;276:31959–68.
507. Maeshima Y, Sudhakar A, Lively JC, et al. Tumstatin, an endothelial cell-specific inhibitor of protein synthesis. Science 2002;295:140–3.
508. Cheresh DA, Stupack DG. Integrin-mediated death: an explanation of the integrin-knockout phenotype? Nat Med 2002;8:193–4.
509. Hynes RO. A reevaluation of integrins as regulators of angiogenesis. Nat Med 2002;8:918–21.
510. Stupack DG, Cheresh DA. ECM remodeling regulates angiogenesis: endothelial integrins look for new ligands. Sci STKE 2002:E7.
511. Stupack DG, Puente XS, Boutsaboualoy S, et al. Apoptosis of adherent cells by recruitment of caspase-8 to unligated integrins. J Cell Biol 2001;155:459–70.
512. Kisker O, Onizuka S, Banyard J, et al. Generation of multiple angiogenesis inhibitors by human pancreatic cancer. Cancer Res 2001;61:7298–04.
513. Moses MA, Wiederschain D, Wu I, et al. Troponin I is present in human cartilage and inhibits angiogenesis. Proc Natl Acad Sci USA 1999;96:2645–50.
514. Maione TE, Sharpe RJ. Development of angiogenesis inhibitors for clinical applications. Trends Pharmacol Sci 1990;11:457–61.
515. Homandberg GA, Williams JE, Grant D, et al. Heparin-binding fragments of fibronectin are potent inhibitors of endothelial cell growth. Am J Pathol 1985;120:327–32.
516. Funk SE, Sage EH. Differential effects of SPARC and cationic SPARC peptides on DNA synthesis by endothelial cells and fibroblasts. J Cell Physiol 1993;154:53–63.
517. Good DJ, Polverini PJ, Rastinejad F, et al. A tumor suppressor-dependent inhibitor of angiogenesis is immunologically and functionally indistinguishable from a fragment of thrombospondin. Proc Natl Acad Sci USA 1990;87:6624–8.
518. Tolsma SS, Volpert OV, Good DJ, et al. Peptides derived from two separate domains of the matrix protein thrombospondin-1 have antiangiogenic activity. J Cell Biol 1993;122:497–511.
519. Murphy-Ullrich JE, Schultz-Cherry S, Hook M. Transforming growth factor-B complexes with thrombospondin. Mol Biol Cell 1992;3:181–8.
520. Sage EH, Bornstein P. Approaches for investigating matrix components produced by endothelial cells: type VIII collagen, SPARC, and thrombospondin. In: Haralson MA, Hassell JR, editors. Extracellular matrix molecules: a practical approach. Oxford: Oxford University Press; 1995. p. 131–60.
521. Tolsma SS, Stack MS, Bouck N. Lumen formation and other angiogenic activities of cultured capillary endothelial cells are inhibited by thrombospondin-1. Microvasc Res 1997;54:13–26.
522. Liotta LA, Steeg PS, Stetler-Stevenson WG. Cancer metastasis and angiogenesis: an imbalance of positive and negative regulation. Cell 1991;64:327–36.
523. Spurbeck WW, Ng CY, Strom TS, et al. Enforced expression of tissue inhibitor of matrix metalloproteinases-3 affects functional capillary morphogenesis and inhibits tumor growth in a murine tumor model. Blood 2002;100:3361–8.
524. Chang C, Werb Z. The many faces of metalloproteases: cell growth, invasion, angiogenesis and metastasis. Trends Cell Biol 2001;11:S37–S43.
525. Fassina G, Ferrari N, Brigati C, et al. Tissue inhibitors of metalloproteinases: regulation and biological activities. Clin Exp Metastasis 2000;18:111–20.
526. Angiolillo AL, Sgadari C, Taub DD, et al. Human interferon-inducible protein 10 is a potent inhibitor of angiogenesis in vivo. J Exp Med 1995;182:155–62.
527. Colman RW, Pixley RA, Najamunnisa S, et al. Binding of high molecular weight kininogen to human endothelial cells is mediated via a site within domains 2 and 3 of the urokinase receptor. J Clin Invest 1997;100:1481–7.
528. Volpert OV, Pili R, Sidker HA, et al. Id1 regulates angiogenesis through transcriptional repression of thrombospondin-1. Cancer Cell 2002;2:473–83.
529. Brantley DM, Cheng N, Thompson EJ, et al. Soluble Eph A receptors inhibit tumor angiogenesis and progression in vivo. Oncogene 2002;21:7011–26.
530. Sarma V, Wolf FW, Marks RM, et al. Cloning of a novel tumor necrosis factor-α-inducible primary response gene that is differentially expressed in development and capillary tube-like formation in vitro. J Immunol 1992;148:3303–12.
531. Daniel TO, Stein E, Cerretti DP, et al. ELK and LERK-2 in developing kidney and microvascular endothelial assembly. Kidney Int 1996;57:S73–S81.
532. Wang CY, Mayo MW, Baldwin AS Jr. TNF- and cancer therapy-induced apoptosis: potentiation by inhibition of NF-kappaB. Science 1996;274:784–7.
533. Beg AA, Baltimore D. An essential role for NF-kappaB in preventing TNF-α-induced cell death. Science 1996;274:782–4.
534. Buettner R, Mora LB, Jove R. Activated STAT signaling in human tumors provides novel molecular targets for therapeutic intervention. Clin Cancer Res 2002;8:945–54.
535. Carmeliet P. Biomedicine. Clotting factors build blood vessels. Science 2001;293:1602–4.
536. Tsopanoglou NE, Maragoudakis ME. On the mechanism of thrombin-induced angiogenesis. Potentiation of vascular endothelial growth factor activity on endothelial cells by up-regulation of its receptors. J Biol Chem 1999;274:23969–76.
537. Zucker S, Mirza H, Conner CE, et al. Vascular endothelial growth factor induces tissue factor and matrix metalloproteinases production in endothelial cells: conversion of prothrombin to thrombin results in progelatinase A activation and cell proliferation. Int J Cancer 1998;75:780–6.
538. Sharrocks AD. The ETS-domain transcription factor family. Nat Rev Mol Cell Biol 2001;2:827–37.
539. Chen Z, Fisher RJ, Riggs CW, et al. Inhibition of vascular endothelial growth factor-induced endothelial cell migration by ETS1 antisense oligonucleotides. Cancer Res 1997;57:2013–9.
540. Nakano T, Abe M, Tanaka K, et al. Angiogenesis inhibition by transdominant mutant Ets-1. J Cell Physiol 2000; 184:255–62.
541. Oda N, Abe M, Sato Y. ETS-1 converts endothelial cells to the angiogenic phenotype by inducing the expression of matrix metalloproteinases and integrin beta 3. J Cell Physiol 1999;178:121–32.
542. Iwasaka C, Tanaka K, Abe M, Sato Y. Ets-1 regulates angiogenesis by inducing the expression of urokinase-type plasminogen activator and matrix metalloproteinases-1 and the migration of vascular endothelial cells. J Cell Physiol 1996;269:522–31.
543. Griffith ED, Su Z, Turk BE, et al. Methionine aminopeptidase (type 2) is the common target for angiogenesis inhibitors AGM-1470 and ovalicin. Chem Biol 1997;4:461–71.
544. Sin N, Meng L, Want MQW, et al. The anti-angiogenic agent fumagillin covalently binds and inhibits the methionine aminopeptidase, MetAP-2. Proc Natl Acad Sci USA 1997;94:6099–103.
545. Kudelka AP, Verschraegen CF, Loyer E. Complete remission of metastatic cervical cancer with the angiogenesis inhibitor TNP-470. N Engl J Med 1998;338:991–2.
546. Bhargava P, Marshall JL, Rizvi N, et al. A Phase I and pharmacokinetic study of TNP-470 administered weekly to patients with advanced cancer. Clin Cancer Res 1999;5:1989–95.
547. Chien M, Astumian M, Stadler WM. Normal human urothelial cell (HUC) response to H2O2: senescence-like growth arrest without p16INK4A expression [abstract 1762]. Proc Amer Assoc Cancer Res 1998;39:257.
548. Zukiwiski A, Gutterman J, Bui C, et al. Phase I trial of the angiogenesis inhibitor TNP-470 (AGM-1470) in patients (Pts) with androgen independent prostate cancer (AlPCa). Proc Am Soc Clin Oncol 1994;13:252.
549. Dezube BJ, Von Roenn JH, Holden-Wiltse J, et al. Fumagillin analog in the treatment of Kaposi's sarcoma: a phase I AIDS clinical trial group study. AIDS clinical trial group no. 215 team. J Clin Oncol 1998;16:1444–9.
550. Herbst RS, Madden TL, Tran HT, et al. Safety and pharmacokinetic effects of TNP-470, an angiogenesis inhibitor, combined with paclitaxel in patients with solid tumors: evidence for activity in non–small-cell lung cancer. J Clin Oncol 2002;20:4440–7.
551. Kim ES, Herbst RS. Angiogenesis inhibitors in lung cancer. Curr Oncol Rep 2002;4:325–33.
552. Settles B, Stevenson A, Wilson K, et al. Down-regulation of cell adhesion molecules LFA-1 and ICAM-1 after in vitro treatment with the anti-TNF-α agent thalidomide. Cell Mol Biol 2001;47:1105–14.
553. Singhal S, Mehta J, Desikan R, et al. Antitumour activity of thalidomide in refractory multiple myeloma. N Engl Med 1999;341:1565-71.
554. Verheul HMW, Panigrahy D, Yuan J, D'Amato RJ. Combination oral anti-angiogenic therapy with thalidomide and sulindac inhibits tumour growth in rabbits. Br J Cancer 1999;79:114–8.
555. Bauditz J, Wedel S, Lochs H. Thalidomide reduces tumour necrosis factor-α and interleukin 12 production in patients with chronic active Crohn's disease. Gut 2002;50:196–200.
556. D'Amato RJ, Lentzsch S, Anderson KC, Rogers MS. Mechanism of action of thalidomide and 3-aminothalidomide in multiple myeloma. Semin Oncol 2001;28:597–601.
557. Raje N, Anderson K. Thalidomide—a revival story. N Engl J Med 1999;341:1606–9.
558. Hlatky L, Hahnfeldt P, Folkman J. Clinical application of antiangiogenic therapy: microvessel density, what it does and doesn't tell us. J Natl Cancer Int 2002;94:883–93.
559. Rajkumar SV, Fonseca R, Witzig TE, et al. Bone marrow angiogenesis in patients achieving complete response after stem cell transplantation for multiple myeloma. Leukemia 1999;13:469–72.
560. Perez-Atayde AR, Sallan SE, Tedrow U, et al. Spectrum of tumor angiogenesis in the bone marrow of children with acute lymphoblastic leukemia. Am J Pathol 1997;150:815–21.
561. Folkman J. Tumour angiogenesis. In: Holland JF, Frei E III, Bast RC Jr, et al., editors. Cancer medicine. 5th ed. Ontario: BC Decker; 2000. p. 132–52.
562. Belotti, D, Vergani V, Drudis T, et al. The microtubule-affecting drug paclitaxel has anti-angiogenic activity. Clin Cancer Res 1996;2:1843–9.
563. Browder T, Butterfield CE, Kraling BM, et al. Anti-angiogenic scheduling of chemotherapy improves efficacy against experimental drug-resistant cancer. Cancer Res 2000;60:1878–86.
564. Meta-analysis Group in Cancer. Efficacy of intravenous continuous infusion of fluorouracil compared with bolus administration in advanced colorectal cancer. J Clin Oncol 1998;16:301–8.
565. Gabra H, Cameron DA, Lee LE, et al. Weekly doxorubicin and continuous infusional 5-fluorouracil for advanced breast cancer. Br J Cancer 1996;74:2008–12.
566. Hansen RM, Ryan L, Anderson T, et al. Phase III study of bolus versus infusion fluorouracil with or without cisplatin in advanced colorectal cancer. J Natl Cancer Inst 1996;88:668–74.
567. Abu-Rustum NR, Aghahanian C, Barakat RR, et al. Salvage weekly paclitaxel in recurrent ovarian cancer. Semin Oncol 1997;24(5 Suppl 15):S15-62–S15-67.
568. Loffler TM, Freund W, Lipke J, Hausamen TU. Schedule- and dose-intensified paclitaxel as weekly 1-hour infusion in pretreated solid tumours: results of a phase I/II trial. Semin Oncol 1996;23(6 Suppl 16):32–4.
569. Kakolyris S, Samonis G, Koukourakis M, et al. Treatment

of non-small-cell lung cancer with prolonged oral etoposide. Am J Clin Oncol 1998;21:505–8.
570. Chamberlain MC. Recurrent supratentorial malignant gliomas in children. Long-term salvage therapy with oral etoposide. Arch Neurol 1997;54:554–8.
571. Neskovic-Konstantinovic ZB, Bosnjak SM, Radulovic SS, Mitrovic LB. Daily oral etoposide in metastatic breast cancer. Anticancer Drugs 1996;7:543–7.
572. Hanahan D, Bergers G, Bergsland E. Less is more, regularly: metronomic dosing of cytotoxic drugs can target tumour angiogenesis in mice. J Clin Invest 2000;105:1045–7.
573. Colleoni M, Rocca A, Sandri MT, et al. Low-dose oral methotrexate and clophosphamide in metastatic breast cancer: antitumor activity and correlation with vascular endothelial growth factor levels. Ann Oncol 2002;13:73–80.
574. Kerbel RS, Klement G, Pritchard KI, Kamen B. Continuous low-dose anti-angiogenic/metronomic chemotherapy: from the research laboratory into the oncology clinic. Ann Oncol 2002;13:12–5.
575. Klement G, Baruchel S, Rak J, et al. Continuous low-dose therapy with vinblastine and VEGF receptor-2 antibody induces sustained tumour regression without overt toxicity. J Clin Invest 2000;105:R15–R24.
576. Bello L, Carrabba G, Giussani C, et al. Low-dose chemotherapy combined with an antiangiogenic drug reduces human glioma growth in vivo. Cancer Res 2001;61:7501–6.
577. Kerbel RS, Folkman J. Reply. Nature Reviews Cancer 2003; in press.
578. Gately S, Kerbel R. Antiangiogenic scheduling of lower dose cancer chemotherapy. Cancer J 2002;7:427–36.
579. Folkman J. Regulation of angiogenesis. Blood 1993;82(Suppl 1):60.
580. Brunner G, Nguyen H, Gabrilove J, et al. Basic fibroblast growth factor expression in human bone marrow and peripheral blood cells. Blood 1993;81:631–8.
581. Vacca A, Ribatti D, Roncali L, et al. Bone marrow angiogenesis and progression in multiple myeloma. Br J Haematol 1994;87:503–8.
582. Ribatti D, Vacca A, Nico B, et al. Bone marrow angiogenesis and mast cell density increase simultaneously with progression of human multiple myeloma. Br J Cancer 1999;79:451–5.
583. Vacca A, Ribatti D, Presta M, et al. Bone marrow neovascularization, plasma cell angiogenic potential, and matrix metalloproteinase-2 secretion, parallel progression of human multiple myeloma. Blood 1999; 93:3064–73.
584. Vacca A, Ribatti D, Roncali L, Dammacco F. Angiogenesis B cell lymphoproliferative diseases. Biological and clinical studies. Leuk Lymphoma 1995;20:27–38.
585. Rak J, Mitsuhashi Y, Bayko L, et al. Mutant ras oncogenes upregulate VEGF/VPF expression: implications for induction and inhibition of tumor angiogenesis. Cancer Res 1995;55:4575–80.
586. Rohan RM, Fernandez A, Udagawa T, et al. Genetic heterogeneity of angiogenesis in mice. FASEB J 2000;14:871–6.
587. Soengas MS, Capodieci P, Polsky D, et al. Inactivation of the apoptosis effector Apaf-1 in malignant melanoma. Nature 2001;409:207–11.
588. Yu, JL, Rak J, Carmeliet P, Coomber BL. Heterogenous vascular dependence of tumour populations. Am J Path 2001;58:1325–34.
589. Furuwatari C, Yagi A, Yamagami O, et al. A comprehensive system to explore p53 mutations. Am J Clin Pathol 1998;110:368–73.
590. Beecken W-DC, Fernandez S, Jouddrn SM, et al. Effect of anti-angiogenic therapy on slowly growing, poorly vascularized tumours in mice. J Natl Cancer Inst 2001;93:382–7.
591. Brem S, Cotran R, Folkman J. Tumor angiogenesis: a quantitative method for histologic grading. J Natl Cancer Inst 1972;48:347–56.
592. Mlynek ML, van Beunigen D, Leder LD, Streffer C. Measurement of the grade of vascularization in histological tumour tissue sections. Br J Cancer 1985;52:945–8.
593. Gasparini G, Harris AL. Prognostic significance of tumor vascularity. In: Teicher BA, editor. Antiangiogenic agents in cancer therapy. New Jersey: Humana Press; 1999. p. 317–39.
594. Takahashi K, Mulliken JB, Kozakewich HPW, et al. Cellular markers that distinguish the phases of hemangioma during infancy and childhood. J Clin Invest 1994;93:2357–64.
595. Bielenberg DR, Bucana CD, Sanchez R, et al. Progressive growth of infantile cutaneous hemangiomas is directly correlated with hyperplasia and angiogenesis of adjacent epidermis and inversely correlated with expression of the endogenous angiogenesis inhibitor, IFN-B. Intl J Oncol 1999;14:401–8.
596. White C, Sondheimer HM, Crouch EC, et al. Treatment of pulmonary hemangiomatosis with recombinant interferon alfa-2a. N Engl J Med 1989;320:1197–200.
597. Folkman J. Successful treatment of an angiogenic disease. N Engl J Med 1989;320:1211–2.
598. Ezekowitz RA, Mulliken JB, Folkman J. Interferon α 2A therapy for "life-threatening" hemangiomas in infancy. N Engl J Med 1992;326:1456-1463. See also, N Engl J Med 1994;330:300; See also, N Engl J Med. 1995;333:595-596.
599. Mulliken JB, Boon LM, Takahashi K, et al. Pharmacologic therapy for endangering hemangiomas. Curr Opin Derm 1995;2:109–13.
600. Palmieri G, Montella L, Martignetti A, Bianco AR. Interferon-α-2b at low doses as long-term anti-angiogenic treatment of a metastatic intracranial hemangioendothelioma: a case report. Oncol Rep 2000;7:145–9.
601. Deb G, Donfrancesco A, Ilari I, et al. Hemangioendotheliomas: successful therapy with interferon-α. A study in association with the Italian Pediatric Haematology/Oncology Society (AIEOP). Med Pediatr Oncol 2002;38:118–9.
602. Losso JN. Preventing degenerative diseases by anti-angiogenic functional foods. Food Technol 2002;56:78–88.
603. Kanai T, Konno H, Tanaka T, et al. Effect of angiogenesis inhibitor TNP-470 on the progression of uman gastric cancer xenotransplanted into nude mice. Int J Cancer 1997;71:838–41.
604. Kalebic T, Tsokos M, Helman LJ. Suppression of rhabdomyosarcoma growth by fumagillin analog TNP-470. Int J Cancer 1996;68:596–9.
605. Yanase T, Tamura M, Fujita K, et al. Inhibitory effect of angiogenesis inhibitor TNP-470 on tumor growth and metastasis of human cell lines in vitro and in vivo. Cancer Res 1993;53:2566–70.
606. Konno H, Tanaka T, Kanai T, et al. Efficacy of an angiogenesis inhibitor, TNP-470, in xenotransplanted human colorectal cancer with high metastatic potential. Cancer 1996;77:1736–40.
607. Yazaki T, Takamiya Y, Costello PC, et al. Inhibition of angiogenesis and growth of human non-malignant and malignant meningiomas by TNP-470. J Neurooncol 1995;23:23–9.
608. Isobe N, Uozumi T, Kurisu K, et al. Experimental studies of the antitumor effect of TNP-470 on malignant brain tumors. Antitumor effect of TNP-470 on a human medulloblastoma xenograft line. Neuropediatrics 1996; 27:136–42.
609. Yamaoka M, Yamamoto T, Ikeyama S, et al. Angiogenesis inhibitor TNP-470 (AGM-1470) potently inhibits the tumor growth of hormone-independent human breast and prostate carcinoma cell lines. Cancer Res 1993;53:5233–6.
610. Taki T, Ohnishi T, Arita N, et al. Anti-proliferative effects of TNP-470 on human malignant glioma in vivo: potent inhibition of tumor angiogenesis. J Neurooncol 1994;19:251–8.
611. McLeskey SW, Zhang L, Trock BJ, et al. Effects of AGM-1470 and pentosan polysulphate on tumorigenicity and metastasis of FGF-transfected MCF-7 cells. Br J Cancer 1996;73:1053–62.
612. Takamiya Y, Brem H, Ojeifo J, et al. AGM-1470 inhibits the growth of human glioblastoma cells in vitro and in vivo. Neurosurgery 1994;34:869–75.
613. Kragh M, Spang-Thomsen M, Kristjansen PE. Time until initiation of tumor growth is an effective measure of the anti-angiogenic effect of TNP-470 on human glioblastoma in nude mice. Oncol Rep 1999;6:759–62.
614. Singh Y, Shikata N, Kiyozuka Y,et al. Inhibition of tumor growth and metastasis by angiogenesis inhibitor TNP-470 on breast cancer cell lines in vitro and in vivo. Breast Cancer Res Treat 1997;45:15–27.
615. Takamiya Y, Friedlander RM, Brem H,et al. Inhibition of angiogenesis and growth of human nerve-sheath tumors by AGM-1470. J Neurosurg 1993;78:470–6.
616. Arbiser JL, Panigrathy D, Klauber N,et al. The antiangiogenic agents TNP-470 and 2-methoxyestradiol inhibit the growth of angiosarcoma in mice. J Am Acad Dermatol 1999;40:925–9.
617. Katzenstein HM, Rademaker AW, Senger C, et al, Cohn SL. Effectiveness of the angiogenesis inhibitor TNP-470 in reducing the growth of human neuroblastoma in nude mice inversely correlates with tumor burden. Clin Cancer Res 1999;5:4273–8.
618. Qian CN, Min HQ, Lin HL, et al. Anti-tumor effect of angiogenesis inhibitor TNP-470 on the human nasopharyngeal carcinoma cell line NPC/HK1. Oncology 1999;57:36–41.
619. Beecken WD, Fernandez A, Panigrahy D, et al. Efficacy of antiangiogenic therapy with TNP-470 in superficial and invasive bladder cancer models in mice. Urology 2000;56:521–6.
620. Shusterman S, Grupp SA, Maris JM. Inhibition of tumor growth in a human neuroblastoma xenograft model with TNP-470. Med Pediatr Oncol 2000;35:673–6.
621. Takei H, Lee ES, Cisneros A, et al. Effects of angiogenesis inhibitor TNP-470 on tamoxifen-stimulated MCF-7 breast tumors in nude mice. Cancer Lett 2000; 155:129–35.
622. Hotz HG, Reber HA, Hotz B, et al. Angiogenesis inhibitor TNP-470 reduces human pancreatic cancer growth. J Gastrointest Surg 2001;5:131–8.
623. Kato H, Ishikura H, Kawarada Y, et al. Anti-angiogenic treatment for peritoneal dissemination of pancreas adenocarcinoma: a study using TNP-470. Jpn J Cancer Res 2001;92:67–73.
624. Katzenstein HM, Salwen HR, Nguyen NN, et al. Antiangiogenic therapy inhibits human neuroblastoma growth. Med Pediatr Oncol 2001;36:190–3.
625. Kawano T, Yanoma S, Nishimura G, et al. The inhibitory effects of TNP470 on tumour growth of head and neck carcinoma cell producing interleukin-8. J Laryngol Otol 2001;115:802–7.
626. Rooks V, Beecken WD, Iordanescu I, et al. Sonographic evaluation of orthotopic bladder tumors in mice treated with TNP-470, an angiogenic inhibitor. Acad Radiol 2001;8:121–7.
627. Nagabuchi E, VanderKolk WE, Une Y, et al. TNP-470 antiangiogenic therapy for advanced murine neuroblastoma. J Pediatr Surg 1997;32:287–3.
628. Marcus D, Kim S, Brem H. The effect of an antiangiogenic compound (AGM-1470) on transgenic murine retinoblastoma [abstract]. Invest Ophtalmol Vis Sci 1993;34:226.
629. Brem H, Gresser I, Grosfeld J, et al. The combination of antiangiogenic agents to inhibit primary tumor growth and metastasis. J Pediatr Surg 1993;28:1253–7.
630. Brem H, Gotto F, Budson A. Minimal drug resistance after prolonged antiangiogenic therapy with AGM-1470. Surg Forum 1994;45:674–7.
631. Murata R, Nishimura Y, Hiraoka M. An antiangiogenic agent (TNP-470) inhibited reoxygenation during fractionated radiotherapy of murine mammary carcinoma. Int J Radiat Oncol Biol Phys 1997;37:1107–13.
632. Brem H, Folkman J. Analysis of experimental antiangiogenic therapy. J Pediatr Surg 1993;28:445–51.
633. Morita T, Shinohara N, Tokue A. Antitumour effect of a synthetic analogue of fumagillin on murine renal carcinoma. Br J Urol 1994;74:416–21.
634. Yamamoto T, Sudo K, Fujita T. Significant inhibition of endothelial cell growth in tumor vasculature by an angiogenesis inhibitor, TNP-470 (AGM-1470). Anticancer Res 1994;14:1–3.
635. Yatsunami J, Tsuruta N, Fukuno Y, et al. Inhibitory effects of roxithromycin on tumor angiogenesis, growth and metastasis of mouse B16 melanoma cells. Clin Exp Metastasis 1999;17:119–24.
636. Ma G, Masuzawa M, Hamada Y, et al. Treatment of murine angiosarcoma with etoposide, TNP-470 and prednisolone. J Dermatol Sci 2000;24:126-33.
637. Yoshizawa J, Mizuno R, Yoshida T, et al. Inhibitory effect of TNP-470 on hepatic metastasis of mouse neuroblastoma. J Surg Res 2000;93:82–7.
638. Yanai S, Okada H, Misaki M, et al. Antitumor activity of a medium-chain triglyceride solution of the angiogenesis inhibitor TNP-470 (AGM-1470) when administered via the hepatic artery to rats bearing Walker 256 carcinosarcoma in the liver. J Pharmacol Exp Ther 1994;271:1267–73.
639. Egawa S, Tsutsumi M, Konishi Y, et al. The role of angiogenesis in the tumor growth of Syrian hamster pancreatic cancer cell line HPD-NR. Gastroenterology 1995;108:1526–33.
640. Futami H, Iseki H, Egawa S,et al. Inhibition of lymphatic metastasis in a syngeneic rat fibrosarcoma model by an angiogenesis inhibitor, AGM-1470. Invasion Metastasis 1996;16:73–82.
641. Morishita T, Mii Y, Miyauchi Y, et al. Efficacy of the angiogenesis inhibitor O-(chloroacetyl-carbamoyl)fumagillol (AGM-1470) on osteosarcoma growth and lung metastasis in rats. Jpn J Clin Oncol 1995;25:25–31.

642. Yanai S, Okada H, Saito K,et al. Antitumor effect of arterial administration of a medium-chain triglyceride solution of an angiogenesis inhibitor, TNP-470, in rabbits bearing VX-2 carcinoma. Pharm Res 1995;12:653–7.
643. Kamei S, Okada H, Inoue Y,et al. Antitumor effects of angiogenesis inhibitor TNP-470 in rabbits bearing VX-2 carcinoma by arterial administration of microspheres and oil solution. J Pharmacol Exp Ther 1993;264:469–74.
644. Osawa S, Terashima Y, Kimura G, et al. Antitumour effects of the angiogenesis inhibitor AGM-1470 on rat urinary bladder tumours induced by N-butyl-N-(4-hydroxybutyl) nitrosamine. BJU Int 1999;83:123–8.
645. Landuyt W, Theys J, Nuyts S, et al. Effect of TNP-470 (AGM-1470) on the growth of rat rhabdomyosarcoma tumors of different sizes. Cancer Invest 2001;19:35–40.
646. Brem H, Ingber D, Blood C. Suppression of tumor metastasis by angiogenesis inhibition. Surg Forum 1991;42:439–41.
647. Schoof DD, Obando JA, Cusack JC, Jr.,et al. The influence of angiogenesis inhibitor AGM-1470 on immune system status and tumor growth in vitro. Int J Cancer 1993;55:630–5.
648. Teicher BA, Holden SA, Ara G,et al. Potentiation of cytotoxic cancer therapies by TNP-470 alone and with other anti-angiogenic agents. Int J Cancer 1994;57:920–5.
649. Kato T, Sato K, Kakinuma H,et al. Enhanced suppression of tumor growth by combination of angiogenesis inhibitor O-(chloroacetyl-carbamoyl)fumagillol (TNP-470) and cytotoxic agents in mice. Cancer Res 1994;54:5143–7.
650. Ahmed MH, Konno H, Nahar L, et al. The angiogenesis inhibitor TNP-470 (AGM-1470) improves long-term survival of rats with liver metastasis. J Surg Res 1996;64:35–41.
651. Konno H. Antitumor effect of the angiogenesis inhibitor TNP-470 on human digestive organ malignancy. Cancer Chemother Pharmacol 1999;43(Suppl):S85–S89.
652. Mysliwski A, Bigda J, Koszalka P, et al. Synergistic effect of the angiogenesis inhibitor TNP-470 and tumor necrosis factor (TNF) on Bomirski Ab melanoma in hamsters. Anticancer Res 2000;20:4643–47.
653. Mysliwski A, Szmit E, Szatkowski D, et al. Suppression of growth of Bomirski Ab melanoma and its metastasis in hamsters by angiogenesis inhibitor TNP-470. Anticancer Res 1998;18:441–3.
654. Xia JL, Yang BH, Tang ZY, et al. Inhibitory effect of the angiogenesis inhibitor TNP-470 on tumor growth and metastasis in nude mice bearing human hepatocellular carcinoma. J Cancer Res Clin Oncol 1997;123:383–7.
655. Tanaka H, Taniguchi H, Mugitani T,et al. Angiogenesis inhibitor TNP-470 prevents implanted liver metastases after partial hepatectomy in an experimental model without impairing wound healing. Br J Surg 1996;83:1444–7.
656. Matsumoto K, Ninomiya Y, Inoue M,et al. Intra-tumor injection of an angiogenesis inhibitor, TNP-470, in rabbits bearing VX2 carcinoma of the tongue. Int J Oral Maxillofac Surg 1999;28:118–24.
657. Fujioka T, Hasegawa M, Ogiu K, et al. Antitumor effects of angiogenesis inhibitor 0-(chloroacetyl-carbamoyl) fumagillol (TNP-470) against murine renal cell carcinoma. J Urology 1996;155:1775–8.
658. Konno H, Tanaka T, Matsuda I, et al. Comparison of the inhibitory effect of the angiogenesis inhibitor, TNP-470, and mitomycin C on the growth and liver metastasis of human colon cancer. Int J Cancer 1995;61:268–71.
659. Tanaka T, Konno H, Matsuda I, et al. Prevention of hepatic metastasis of human colon cancer by angiogenesis inhibitor TNP-470. Cancer Res 1995;55:836–9.
660. Mori S, Ueda T, Kuratsu S,et al. Suppression of pulmonary metastasis by angiogenesis inhibitor TNP-470 in murine osteosarcoma. Int J Cancer 1995;61:148–52.
661. Shishido T, Yasoshima T, Denno R, et al, Hirata K. Inhibition of liver metastasis of human gastric carcinoma by angiogenesis inhibitor TNP-470. Jpn J Cancer Res 1996;87:958–62.
662. Morishita T, Miyauchi Y, Mii Y, et al. Delay in administration of CDDP until completion of AGM-1470 treatment enhances antimetastatic and antitumor effects. Clin Exp Metastasis 1999;17:15–8.
663. Gervaz P, Scholl B, Padrun V, et al. Growth inhibition of liver metastases by the anti-angiogenic drug TNP-470. Liver 2000;20:108–13.
664. Oda H, Ogata Y, Shirouzu K. The effect of angiogenesis inhibitor TNP-470 against postoperative lung metastasis following removal of orthotopic transplanted human colon cancer: an experimental study. Kurume Med J 2001;48:285–93.
665. Saito N, Mitsuhashi M, Hayashi T, et al. Inhibition of hepatic metastasis in mice treated with cell-binding domain of human fibronectin and angiogenesis inhibitor TNP-470. Int J Clin Oncol 2001;6:215–20.
666. Offodile R, Walton T, Lee M, et al. Regression of metastatic breast cancer in a patient treated with the anti-angiogenic drug TNP-470. Tumori 1999;85:51–3.
667. Stadler WM, Kuzel T, Shapiro C, et al. Multi-institutional study of the angiogenesis inhibitor TNP-470 in metastatic renal carcinoma. J Clin Oncol 1999;17:2541–5.
668. Moore JD, Dezube BJ, Gill P, et al. Phase I dose escalation pharmacokinetics of O-chloroacetylcarbamoyl) fumagillol (TNP-470) and its metabolites in AIDS patients with Kaposi's sarcoma. Cancer Chemother Pharmacol 2000;46:173–9.
669. Kudelka AP, Levy T, Verschraegen CF, et al. A phase I study of TNP-470 administered to patients with advanced squamous cell cancer of the cervix. Clin Cancer Res 1997;3:1501–5.
670. Madri JA and Pratt BM. Endothelial-cell matrix interactions: in vitro models of angiogenesis. J Histochem Cytochem 1986;34:85–91.
671. Form DM, Pratt BM, Madri JA. Endothelial cell proliferation during angiogenesis. In vitro modulation by basement membrane components. Lab Invest 1986;55:521–30.
672. Herbst TJ, McCarthy JB, Tsilibary EC, et al. Differential effects of laminin, intact type IV collagen, and specific domains of type IV collagen on endothelial cell adhesion and migration. J Cell Biol 1988;106:1365–73.
673. Tsilibary EC, Reger LA, Vogel AM, et al. Identification of a multifunctional, cell-binding peptide sequence from the α1(NCI) of type IV collagen. J Cell Biol 1990;111:1583–91.
674. Tsilibary EC, Koliakos GG, Charonis AS, et al. Heparin type IV collagen interactions: equilibrium binding and inhibition of type IV collagen self-assembly. J Biol Chem 1988;263:19112–8.