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ANGIOGENESIS

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■ **Abstract** Angiogenesis inhibitors for the treatment of cancer have now been approved by the Food and Drug Administration in the United States, and in 28 other countries including China. Clinical application of this new class of drugs is informed by certain principles from angiogenesis research. Oncogenic mutations initiate tumorigenesis, but angiogenesis is necessary for expansion of tumor mass. Two angiogenesis inhibitors have been developed that have a broad spectrum of anticancer activity, yet virtually no side effects. Endogenous angiogenesis inhibitors act as tumor suppressor proteins. The angiogenic response in vivo is based on the genetic background of the host. Several types of angiogenesis inhibitors reveal a biphasic, U-shaped curve of efficacy. "Antiangiogenic chemotherapy" is a novel approach to the treatment of drug resistance.

INTRODUCTION

The field of angiogenesis research originated in 1971 with the publication of the hypothesis that tumor growth is angiogenesis-dependent (1). This paper emphasized the future possibility that angiogenesis inhibitors could be discovered and employed as therapy against cancer. It included the proposal that tumor dormancy could be based on blocked angiogenesis. Although angiogenesis research is strongly linked to cancer biology, many non-neoplastic diseases are also angiogenesis-dependent. These include macular degeneration, psoriasis, endometriosis, arthritis, and others. The molecular basis of angiogenesis is being studied in many branches of biology, including reproduction, development, and repair. Clinical validation of antiangiogenic therapy has been demonstrated worldwide in the past few years. At this writing, angiogenesis inhibitors have been approved by the Food and Drug Administration in the United States, and in 28 other countries.

The success of antiangiogenic therapy is based in part on the following fundamental concepts advanced over the past three decades:

1. Growth of normal and neoplastic tissue mass is angiogenesis-dependent (1–3).

2. Most adults harbor microscopic, nonangiogenic cancers in situ in more than one organ (4), but only ~1 in 600 of these malignancies switches to the angiogenic phenotype and becomes a detectable tumor (5).
3. Naturally occurring endogenous angiogenesis inhibitors defend against the angiogenic switch in pathological conditions and serve to limit physiological angiogenesis, such as in ovulation and wound healing (6, 7).
4. The microvascular endothelial cell recruited by a tumor is an important second target in cancer therapy and has the advantage of being genetically stable. Therefore, treating both the cancer cell and the endothelial cell in a tumor may be more effective than treating the cancer cell alone.

A current goal in the clinical use of angiogenesis inhibitors is to convert cancer to a chronic manageable disease (8).

A future goal is the employment of angiogenesis-based biomarkers, as well as other types of biomarkers, to guide the treatment of cancer years before it can be located anatomically or can cause symptoms (6).

ONCOGENES, ANGIOGENESIS, AND TUMORIGENESIS

It is now widely accepted that oncogenes can induce tumorigenesis. A hallmark of oncogene function was originally discovered by the use of in vitro bioassays. Cells expressing activated oncogenes underwent increased proliferation and decreased apoptosis (9).

Oncogene Dependence

When these in vitro results were extended to animal models in which an oncogene was conditionally overexpressed (i.e., Myc expression under control of doxycycline), tumors grew rapidly and killed their host. After oncogene inactivation, these tumors rapidly regressed, leaving only residual microscopic tumors, or in some animal models, no evidence of tumor. When the oncogene was reactivated, rapid tumor growth resumed. In these in vivo systems, tumor cell proliferation was also increased, and apoptosis was reduced (10, 11). These experiments demonstrated the oncogene dependence of tumor growth. They have led to a widely held belief that an oncogene-induced shift in the equilibrium between tumor cell proliferation and tumor cell apoptosis, resulting in an excess of proliferation over apoptosis, was necessary and sufficient for the emergence of a large tumor that could kill its host.

Essential Role of Angiogenesis in Oncogene-Dependent Tumor Growth

However, this belief is now being challenged by an increasing body of evidence that excessive tumor cell proliferation alone is not sufficient to produce a lethal tumor. The generation of a lethal tumor mass (whether localized or distributed

in the body) requires tumor cell proliferation plus angiogenesis. (Endothelial cell proliferation is a prerequisite, but not the only component, of angiogenesis). Tumor cell proliferation alone, in the absence of angiogenesis, can give rise to dormant, microscopic tumors of $\sim$1 mm^3 or less, but these in situ cancers are harmless to the host (12–19). Furthermore, transfection of an oncogene into tumor cells significantly increases angiogenic activity by increasing their expression of vascular endothelial growth factor (VEGF) (20) and by decreasing their expression of antiangiogenic proteins such as thrombosponin-1. In fact, when thrombospondin-1 is transfected into human tumor cells, and cells are selected for expression of increasing concentrations of this endogenous angiogenesis inhibitor, tumor size increases inversely to thrombospondin-1 expression, but independently of tumor cell proliferation. Tumor cell proliferation remains constant at all tumor volumes, including microscopic dormant tumors (21).

Taken together, these experiments show that oncogene activation can induce increased proliferation over apoptosis in a tumor as well as increased angiogenic activity. This suggests that complete blockade of the angiogenic component of oncogene expression will leave only a residual microscopic, nonangiogenic tumor that is harmless to the host, despite the high proliferation rate of its tumor cells.

A provocative experiment that supports this concept was reported by Fernandez et al. (21a). Human prostate cancer grows to 700 mm^3 in mice by 38 days. Mitomycin C therapy, which blocks tumor cell proliferation, almost completely prevents this tumor growth. However, transfection of the tumor with the bcl-2 oncogene increases tumor cell proliferation, decreases apoptosis, and significantly increases VEGF expression and angiogenesis. Tumors that express bcl-2 escape mitomycin C therapy and grow to $\sim$1000 mm^3. When the bcl-2-expressing tumors are treated with an angiogenesis inhibitor (TNP-470), which selectively inhibits proliferation of endothelial cells at 3 logs lower concentration than it inhibits proliferation of tumor cells or fibroblasts, the bcl-2 effect is nullified, and tumor growth is restricted to $<$10%–15% of that observed in untreated bcl-2-expressing tumors. This experiment provides compelling evidence that generation of tumor mass is impossible without endothelial proliferation (a key component of angiogenesis). Both tumor cell proliferation and angiogenesis together are necessary and sufficient to generate a detectable tumor mass.

ANGIOGENESIS INHIBITORS

The first angiogenesis inhibitor, interferon-α (administered at low doses), was reported in 1980 (22–24). Subsequently, platelet-factor 4 (25), tetrahydrocortisol (26), and by 1990, a fumagillin analogue (27) were found to have potent antiangiogenic activity. Angiostatin, an internal fragment of plasminogen, first revealed that an antiangiogenic peptide could be enzymatically released from a parent protein that lacked this inhibitory activity (28). Endostatin, an internal fragment of collagen XVIII, provided the first evidence that a basement-membrane collagen contained an angiogenesis inhibitory peptide (29, 30) (see Table 1).

TABLE 1 Endogenous angiogenesis inhibitors in the circulation or in matrix (6, 7)

Inhibitor	Clinical trials
1. Alphastatin	
2. Angiostatin	
3. Arresten	
4. Anti-thrombin III (truncated)	
5. Canstatin	
6. Endostatin	Phase II
7. Fibulin-5	
8. Fragment of histidine-rich glycoprotein	
9. Interferon-β	Phase III
10. Maspin	
11. 2-methoxyestradiol	Phase II
12. PEX	
13. Pigment epithelial-derived factor (PEDF)	
14. Platelet factor 4 (PF4)	
15. Semaphorin 3F	
16. sFlt-1	
17. Tetrahydrocortisol	Phase III
18. Thrombospondin-1 (and -2)	Phase II
19. TIMP-2	
20. Troponin I	
21. Tumstatin	
22. Vasostatin	

Endogenous Angiogenesis Inhibitors

At this writing, 27 endogenous angiogenesis inhibitors have been identified in the circulation, in tissues, or both (6, 7).

GENETIC EVIDENCE THAT ENDOGENOUS INHIBITORS SUPPRESS PATHOLOGIC ANGIOGENESIS Tumstatin is a 232-amino-acid antiangiogenic peptide in the $\alpha 3$ chain of collagen type IV (31). In tumstatin-deficient mice (engineered by genetic deletion of the $\alpha 3$ chain), microvessel density is significantly increased in implanted murine tumors, and tumor growth is increased by 300% (Figure 1*a*). Pharmacologic replacement of tumstatin to physiological plasma levels suppresses tumor growth down to the slower growth rate of the wild-type mouse. A similar increased tumor growth rate (250%–300%) is observed in mice depleted of endostatin or thrombospondin-1. In mice deficient for both tumstatin and thrombospondin-1, tumors grow 400% to 500% faster than in wild-type mice (Figure 1*b*). Tumor

proliferation is not increased significantly in these tumors. Tumor growth in these mice correlates directly with increased angiogenesis in the tumor bed and decreased endogenous angiogenesis inhibitors. Prior to these types of experiments, tumors could not be induced to grow beyond their ceiling rate. Therefore, endogenous angiogenesis inhibitors act as tumor suppressor proteins, analogous to the classic tumor suppressor p53. Tumor cell proliferation is regulated by p53. However, p53 also inhibits angiogenesis by at least four distinct mechanisms: by increasing expression of thrombospondin-1 (32), by repressing both VEGF (33) and basic fibroblast growth factor (bFGF) binding protein (34), and by degrading hypoxia inducible factor-1, (35) which, when elevated, upregulates VEGF expression. In contrast, endogenous angiogenesis inhibitors generally have no effect on tumor cell proliferation.

When endostatin, a 20-kD internal fragment of collagen XVIII (29), is overexpressed in the vascular endothelium of mice (by hybridizing it with the vascular endothelium cadherin promoter), tumors grow 300% more slowly in mice expressing only 1.6-fold more endostatin than wild-type mice (36). These mice mimic humans with Down syndrome, whose blood contains 1.6- to 2.0-fold elevated levels of endostatin as a result of a third copy of collagen XVIII on the trisomic chromosome 21 (37). Individuals with Down syndrome are the most protected against cancer of all humans (38). In these individuals, the incidence of all malignant tumors is <0.1 the expected figure, except for testicular cancer and a megakaryocytic leukemia. This correlation with high circulating levels of endostatin also extends to two other angiogenesis-dependent diseases: retinal neovascularization in diabetes (39) and atherosclerosis (40). Individuals with Down syndrome have the same incidence of diabetes as the rest of us, but virtually no diabetic retinopathy, even if such individuals have had diabetes for up to 40 years.

Endogenous angiogenesis inhibitors can also suppress pathologic neovascularization in non-neoplastic diseases. For example, collagen XVIII and its proteolytically released fragment, endostatin, are differentially depleted in blood vessels affected by atherosclerosis (41). Growth of atherosclerotic plaques is angiogenesis-dependent, and plaque neovascularization is further enhanced in atherosclerosis-prone (ApoE-deficient) mice that are subsequently depleted of collagen XVIII/endostatin by the appropriate crosses with collagen XVIII–deficient mice (42). Administration of endostatin inhibits plaque neovascularization and inhibits plaque growth by 85% (41).

THESE NEW FUNCTIONS FOR ENDOSTATIN SUGGEST A NOVEL PARADIGM FOR EMERGENCE OF ATHEROSCLEROTIC PLAQUES Plaque growth results from a shift in the balance between growth factors that stimulate vascular smooth muscle and neovascularization in a plaque, and endogenous angiogenesis inhibitors that normally prevent plaque neovascularization. The loss of endogenous inhibitors may be as crucial to plaque growth as is the downregulation of endogenous angiogenesis inhibitors to tumor growth.

A recently discovered new function of endostatin in atherosclerosis is that endostatin binds both the matrix proteoglycan biglycan and LDL, and it interferes

with LDL retention to biglycan and to subendothelial matrix (43). A peptide encompassing the α-coil in the endostatin crystal structure mediates the major blocking effect of endostatin on LDL retention. Endostatin also inhibits macrophage uptake of biglycan-associated LDL indirectly by interfering with LDL retention to biglycan, but endostatin has no direct effect on macrophage uptake of naive or modified lipoproteins. Therefore, loss of endostatin in advanced atheromas enhances lipoprotein retention in the subendothelial matrix.

BROAD-SPECTRUM ANGIOGENESIS INHIBITORS: CAPLOSTATIN AND ENDOSTATIN

Avastin (bevacizumab) was the first angiogenesis inhibitor approved by the U.S. Food and Drug Administration (for colon cancer), and the first to demonstrate prolongation of survival in patients with advanced cancer. It is an anti-VEGF antibody, and the story of its discovery and manufacture describes a monumental achievement (44). There are ~200 different types of human cancers, and ~60% of these express VEGF. However, many cancers produce other angiogenic proteins as well (45). Furthermore, some cancers may initially produce only VEGF but over time can express redundant angiogenic proteins owing to new mutations. At least six angiogenic proteins have been reported for some types of breast cancer (45; for review see 46). Therefore, in the future, as more patients remain well on Avastin therapy, and as Avastin receives FDA approval for other tumors, it may benefit from being administered together with other angiogenesis inhibitors or other anticancer agents.

Tarceva is an inhibitor of the epidermal factor receptor tyrosine kinase, but its major anticancer activity is to block production of three angiogenic proteins, VEGF, bFGF, and TGF-α. It has received FDA approval for the treatment of lung cancer. The antiangiogenic spectrum of Avastin is expanded when it is administered with Tarceva in clinical trials. However, we have shown that the antitumor activity of Avastin can be greatly synergized when Avastin is administered with Caplostatin, an angiogenesis inhibitor with a much broader spectrum than Tarceva. In fact, some human tumors in animals can be eradicated when Avastin is administered with Caplostatin, but not when either agent is administered alone (R. Satchi-Fainaro, personal communication).

Caplostatin

Caplostatin is a nontoxic synthetic analogue of fumagillin (TNP-470) conjugated to a water-soluble N-(2-hydroxypropyl)methacrylamide (HPMA) copolymer (47, 48). Fumagillin was found by Don Ingber in the Folkman lab to inhibit endothelial cell proliferation without causing endothelial cell apoptosis, when a tissue culture plate of endothelial cells became contaminated with a fungus, *Aspergillus fumigatus fresenius* (27, 49). Scientists at Takeda Chemical Industries (Osaka, Japan) made a synthetic analogue of fumagillin called TNP-470, which, as mentioned

above, inhibits endothelial proliferation in vitro at a concentration 3 logs lower than the concentration necessary to inhibit fibroblasts and tumor cells.

When administered at a dose of 30 mg/kg subcutaneously every other day, TNP-470 was reported by >100 different laboratories to significantly inhibit every tumor tested from 40% up to 100% (i.e., eradication), including primary and metastatic human tumors in mice, as well as mouse, rat, hamster, and rabbit tumors (47). TNP-470 also showed significant inhibition of tumors in clinical trials, including durable complete regressions (48, 49). The clinical utility of TNP-470, however, was limited by neurotoxicity. This side effect was recently overcome when Ronit Satchi-Fainaro in the Folkman lab conjugated it to HPMA to form Caplostatin (48).

Caplostatin has a similar broad antitumor spectrum and can be administered over a dose range more than tenfold that of the original TNP-470 without any toxicity. In addition to its antiangiogenic activity, Caplostatin is the most potent known inhibitor of vascular permeability (48). Caplostatin prevents vascular leakage induced by VEGF, bradykinin, histamine, and platelet-activating factor, and prevents pulmonary edema induced by interleukin-2. The mechanism of inhibiting vascular hyperpermeability is partly explained by TNP-470's inhibition in endothelial cells of VEGF-induced phosphorylation of the receptor for VEGF (VEGFR-2), calcium influx, and RhoA activation. This antihyperpermeability activity of TNP-470 and its polymer conjugate Caplostatin probably contributes to the antiangiogenic activity of these molecules, but the only known molecular target of TNP-470 is methionine aminopeptidase-2 (MetAP2) (50), a cytoplasmic metalloenzyme responsible for removing the N-terminal methionine from nascent protein. However, it is unclear how inhibition of this ubiquitously expressed protein affects TNP-470's and Caplostatin's extremely robust, yet specific, inhibition of endothelial proliferation without affecting any other cell type, including tumor cells. At this writing, there is no evidence that TNP-470 binds an extracellular receptor. Nevertheless, Caplostatin represents the most broad-spectrum anticancer agent known, and it appears not to be restricted by a requirement for activation of a specific endothelial integrin (e.g., $\alpha_v\beta_3$ or $\alpha_5\beta_1$).

Endostatin

Endostatin is a 20-kD internal fragment of the carboxy terminus of collagen XVIII (29). Michael O'Reilly in the Folkman lab found endostatin in the blood and urine of mice bearing tumors, which suppressed angiogenesis in remote metastases. It is the first endogenous angiogenesis inhibitor to be discovered as an internal fragment of a basement-membrane collagen. The two enzymes that release endostatin from collagen XVIII have been reported (51, 52), and its three-dimensional crystal structure has been resolved to 3 Å (53). Endostatin binds to endothelial heparan sulphate with a differential requirement for specific sulphates (54), and it also binds to the integrin $\alpha_5\beta_1$, which is expressed on proliferating endothelial cells in certain tumor beds (55, 56).

Recombinant endostatin was at first produced in *E. coli.* Preparations of inclusion bodies that were endotoxin-free and of low solubility were capable of regressing a variety of established murine tumors when administered subcutaneously (30). We subsequently learned that the low solubility resulted in sustained release from a subcutaneous depot of endostatin (57). But the tendency of these preparations to aggregate at high concentrations made recombinant endostatin from *E. coli* problematic and difficult to send on dry ice to colleagues. A year later, when soluble recombinant endostatin was produced in yeast, active endostatin was produced by numerous laboratories and a wide range of inhibited tumors was reported (for review see 58). Tumors could be inhibited by up to ~68% by daily (bolus) injections of soluble recombinant endostatin, but tumor regression (i.e., >97% inhibition) required continuous administration by an ALZA mini-osmotic pump implanted in the peritoneal cavity (59). At this writing, >750 reports on endostatin reveal significant inhibition of >20 different types of murine, rat, and human tumors (in mice) by administration of the recombinant endostatin protein.

BIPHASIC DOSE-RESPONSE CURVE An interesting property of endostatin is that its inhibition of endothelial cell migration in vitro, its antitumor activity in vivo, and its suppression of circulating endothelial cells in vivo all follow a biphasic pattern revealed as a U-shaped dose-efficacy curve (60) (Figure 2). For example, antitumor efficacy is optimal between very low and very high doses. This property has important implications for the clinical use of endostatin and for some other angiogenesis inhibitors. A similar biphasic phenomenon was first reported for

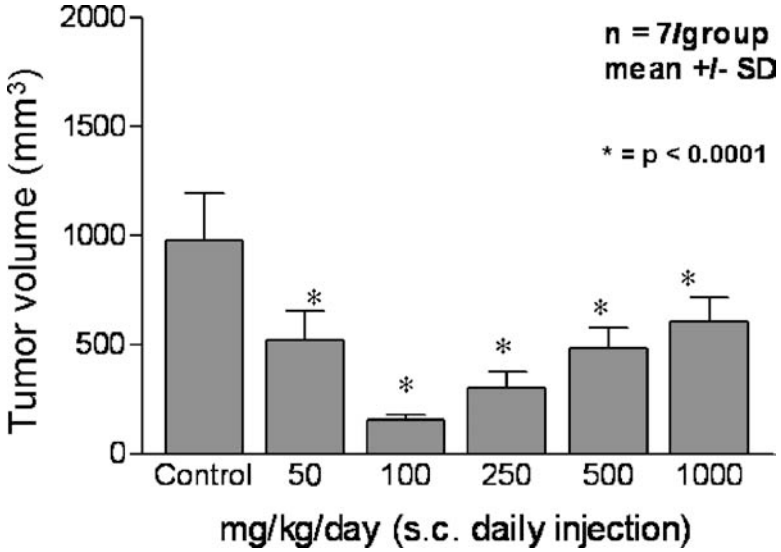

Figure 2 Biphasic U-shaped dose-efficacy curve for human endostatin therapy of human pancreatic cancer (Bx-PC3) in SCID mice, day 20 (60).

the antiangiogenic activity of low-dose interferon-α (61), and subsequently for other angiogenesis inhibitors (62). A biphasic dose-response curve has also been observed for an endostatin peptide of 27 amino acids (63) and for endostatin gene therapy (58, 63). Although experimental endostatin gene therapy successfully inhibits tumor growth in mice (64, 65), some early failures are now understood because they induced excessively high blood levels of endostatin, from three (66) to 800 (67) times the optimum therapeutic concentrations.

MOLECULAR MECHANISMS Endostatin downregulates many signaling pathways that govern proangiogenic activities in human microvascular endothelium and at the same time upregulates many antiangiogenic genes in these cells (68). This was determined by analysis of the genomic response of freshly derived human microvascular endothelial cells to human endostatin using microarrays covering 95% of the human genome, coupled with RT-PCR and phosphorylation analysis (68). For example, endostatin counteracts virtually all of the angiogenic genes upregulated by either VEGF or bFGF, and also downregulates endothelial cell Jun B (fivefold), HIF-1α (3.8-fold), neuropilin (a receptor for VEGF, 3.6-fold), and the epidermal growth factor receptor (EGFR, twofold), among others. Endostatin upregulates endothelial cell sphingomyelinase (a regulator of endothelial cell apoptosis, sixfold), thrombospondin-1 (a potent angiogenesis inhibitor, 2.2-fold), and an HIF-1α inhibitor (HIF-1 AN, 2.45-fold), among others (Figure 3,). Endostatin downregulates the oncogene c-Myc (70). c-Myc plays a key role in the induction of tumor angiogenesis by suppressing thrombospondin-1 expression in tumor cells and in stromal fibroblasts, and it also cooperates with the Ras oncogene (20).

Taken together, these results show that endostatin can molecularly reset *en masse* the angiogenic balance of the endothelial cell genome. The direction of regulation of genes across the genome tends to align with each gene's individual function to promote or suppress angiogenic regulation. Such a widespread alignment of action of the genome to a single agent does not appear to have been observed before. These results are also predictive of the angiogenesis-modifying capacities of genes that have not yet been characterized but may facilitate the search for new molecules that regulate angiogenesis.

ANTIANGIOGENIC CHEMOTHERAPY

Timothy Browder in the Folkman lab was the first to demonstrate this novel concept: By optimizing the dosing schedule of conventional cytotoxic chemotherapy to achieve more sustained apoptosis of endothelial cells in the vascular bed of a tumor, it is possible to achieve more effective control of tumor growth in mice, even if the tumor cells are drug-resistant (71).

Conventional chemotherapy is administered at maximum tolerated doses followed by off-therapy intervals of 2–3 weeks to allow the bone marrow and

gastrointestinal tract to recover. In contrast, antiangiogenic chemotherapy is administered more frequently at lower doses, without long interruptions in therapy, and with little or no toxicity. During antiangiogenic chemotherapy, endothelial cell apoptosis and capillary dropout precede the death of tumor cells that surround each capillary (71). Durable tumor regression and sometimes eradication occur even in drug-resistant tumors. Cyclophosphamide, 5-fluorouracil, 6-mercaptopurine ribose phosphate, and Doxil (the pegylated liposomal formulation of doxorubicin) inhibit angiogenesis when administered on an antiangiogenic dose schedule (70). Two other labs confirmed Browder's findings (72, 73).

Kerbel et al. subsequently showed that continuous administration of cyclophosphamide in the drinking water inhibited tumor growth in mice by 95% and significantly increased circulating levels of thrombospondin-1 (74). Angiogenesis and tumor growth were not inhibited in thrombospondin-1-null mice. This suggests that continuous exposure of vascular endothelium to low concentrations of cyclophosphamide induces increased expression of the angiogenesis inhibitor thrombospondin-1 by endothelium (and perhaps by stromal fibroblasts) in the tumor bed.

Antiangiogenic chemotherapy is also called metronomic chemotherapy (75). The former term emphasizes that chemotherapy dose and schedule are optimized to target endothelial cells instead of tumor cells. The latter term emphasizes *how* chemotherapy dose and schedule are optimized for endothelial cells, i.e., administration at close, regular intervals.

The lesson from these studies is that drug-resistant tumors can be converted to drug-sensitive tumors by optimizing the dose and schedule of conventional cytotoxic chemotherapy for its antiangiogenic activity (76).

GENETIC HETEROGENEITY OF THE ANGIOGENIC RESPONSE

The angiogenic response to a given angiogenic stimulus, such as VEGF or bFGF, can vary with the genetic background of the host. This is a new branch of angiogenesis research that may in the future provide molecular mechanisms to explain a wide spectrum of experimental phenomena and pathologic states. Examples of experimental models that demonstrate genetic regulation of the angiogenic response include the following:

1. Deletion of one allele of Id1 and two alleles of Id3 in mice suppresses the angiogenic response to implanted tumors and significantly inhibits tumor growth (77).
2. Deletion of the tyrosinase gene in black mice (C57Bl/6) yields white mice in which robust angiogenesis is induced in the white iris by a pellet of bFGF implanted in the cornea, in contrast to minimal or no angiogenesis in the wild-type black iris (78).

3. The intensity of corneal neovascularization in response to an implanted intracorneal pellet containing a standardized concentration of either bFGF or VEGF increases in direct proportion to the number of circulating endothelial precursor cells (from bone marrow), and this cell number depends on the mouse strain (79). In mice of 129/Svimj background, circulating endothelial precursor cells are approximately fivefold higher than in C3H/HeJ mice, and corneal neovascularization (the area of angiogenesis) is approximately tenfold higher than in C3H/HeJ mice.
4. Overexpression of thombospondin-1 decreases angiogenesis and inhibits growth of human squamous cell carcinomas in mice (21) and other tumors (80).

Clinical examples of genetic regulation of the host's response to angiogenesis are more difficult to prove than those demonstrated in mice. However, there are some suggestive associations:

1. A novel mutation in endostatin is found in Knobloch syndrome, a form of blindness at birth in which the hyaloid vessels in the fetal vitreous fail to regress after birth (81).
2. An extra copy of collagen XVIII on the trisomic chromosome 21 in individuals with Down syndrome is associated with an elevated level of circulating endostatin and a decreased incidence of cancer (37).
3. A polymorphism in a metalloproteinase (MMP-2 C-735T) is associated with increased angiogenesis in psoriasis (82).

Research on the genetic basis of the host response to angiogenic stimuli is at a very early stage. However, as this field develops, it will almost certainly have important implications for the development of angiogenesis-based biomarkers and the optimization of antiangiogenic therapy, as well as our understanding of vascular and lymphatic malformations (83–85).

CLINICAL APPLICATIONS

Since Avastin (86) received FDA approval for the treatment of colorectal cancer in February 2004, other angiogenesis inhibitors have also been approved in the United States and other countries (Table 2). Avastin is also being studied in numerous clinical trials for patients with other types of cancer, and it is being administered in combination with Tarceva. Antiangiogenic therapy has recently been reported to significantly increase survival in lung cancer and breast cancer, in addition to colorectal cancer (Roy Herbst, in a lecture at the American Society for Clinical Oncology, May 2005).

A lesson from this experience is that when an angiogenesis inhibitor is approved for one type of cancer, it may be prudent to test it in clinical trials for other types of cancer.

TABLE 2 Angiogenesis inhibitors currently approved for clinical use in the United States and in 28 other countries

Date approved	Drug	Place	Disease
December 2003	Thalidomide	Australia	Multiple myeloma
February 2004	Avastin	United States	Colorectal cancer
November 2004	Tarceva	United States	Lung cancer
December 2004	Avastin	Switzerland	Colorectal cancer
December 2004	Macugen	United States	Macular degeneration
January 2005	Avastin	European Union (25 countries)	Colorectal cancer
September 2005	Endostatin	China	Lung cancer

Another lesson from clinical studies is that patients may continue to take angiogenesis inhibitors for prolonged periods of time. For example, thalidomide (87) was approved in Australia for the treatment of advanced multiple myeloma in 2003 and is now used as a first-line therapy. Many patients have been on the drug for 3–5 years without evidence of drug resistance. Thalidomide suppresses production of endothelial cell precursors and downregulates circulating VEGF (88). Endostatin is another example. Four patients at the Dana Farber Cancer Institute, who have carcinoid or islet cell carcinoma with metastases to the liver, have been on daily endostatin for 3.5 years at this writing. They show stable disease or slow tumor regression, without drug resistance and without toxicity.

Another lesson is that certain drugs previously approved by the FDA for another function, and subsequently discovered to be antiangiogenic, are being used successfully to treat refractory recurrent cancers. For example, doxycycline, a chemically modified tetracycline, is a mild antibiotic that has recently been demonstrated to inhibit angiogenesis by increasing thrombospondin-1 (89) and to successfully regress a life-threatening hemangioendothelioma of both lungs (90). Also, low-dose daily interferon-α has been used for the past six years to successfully treat life-threatening hemangiomas and recurrent refractory high-grade giant cell tumor (91, 92).

Antiangiogenic chemotherapy (metronomic chemotherapy) is being used in clinical trials of advanced breast cancer as well as for tumors of the central nervous system (93) and other cancers (for review see 76). UFT (Uracil-tegafur), an orally available prodrug of 5-fluorouracil made by Taiho (Tokyo), is being used in Japan as long-term antiangiogenic (metronomic) chemotherapy.

Recurrent squamous cell carcinoma of the skin is being successfully treated with the application of five topical angiogenesis inhibitors: retinoic acid, Imiquimod, calcipotriene diclofenac, and hyaluronic acid. In a remarkable study reported in June 2005 (94), 61 squamous cell carcinomas (35 invasive and 26 in situ) were treated from 1998 through 2004. A complete response with histopathologic clearance occurred after 12 treatment weeks in 94% of invasive carcinomas and 88% of in situ carcinomas. Among the partial responders (3/26) in the in situ carcinoma

group, all residual tumors cleared with six more weeks of treatment. No recurrence was observed in follow-up to five years. In treated lesions, vascular density was inhibited and micovessels appeared normalized. The doses of these inhibitors were sufficiently low so that there was no skin inflammation or irritation.

Age-related macular degeneration is being treated by an aptamer of VEGF (Macugen, approved by the FDA in 2004). It blocks VEGF when injected into the vitreous and has prevented progression of the disease. Lucentis, an antibody to VEGF, is in phase III clinical trials and has improved sight in some individuals, as well as preventing disease progression in others. It is also injected into the vitreous.

Arthritis is an angiogenesis-dependent disease. Several of the conventional therapies for arthritis have been found to inhibit angiogenesis. These include gold thiomalate, sulfasalazine, methotrexate, and the cyclooxygenase inhibitor celecoxcib (95). In fact, treatment of patients with prednisolone plus salazosulfapyridine significantly raised endostatin in the blood, and decreased VEGF in the blood and in joint fluids (95, 96).

FUTURE DIRECTIONS

The increasing availability of approved angiogenesis inhibitors, their relatively low side-effects profile, and their low incidence of drug resistance has suggested a new direction in cancer therapy. Three angiogenesis-based biomarkers are under development in the Folkman lab: quantification of urinary metalloproteinases (97), analysis of the platelet angiogenic proteome (98), and measurement of blood levels of circulating endothelial cell precursors (CEPs) and mature endothelial cells (CECs) (99). These methods can detect human tumors in mice at sizes of millimeters or less. In the future, it may be possible to detect recurrent cancer by these and other biomarkers and to use them to guide antiangiogenic therapy years before symptoms appear, or before anatomical location of a tumor is possible (6).

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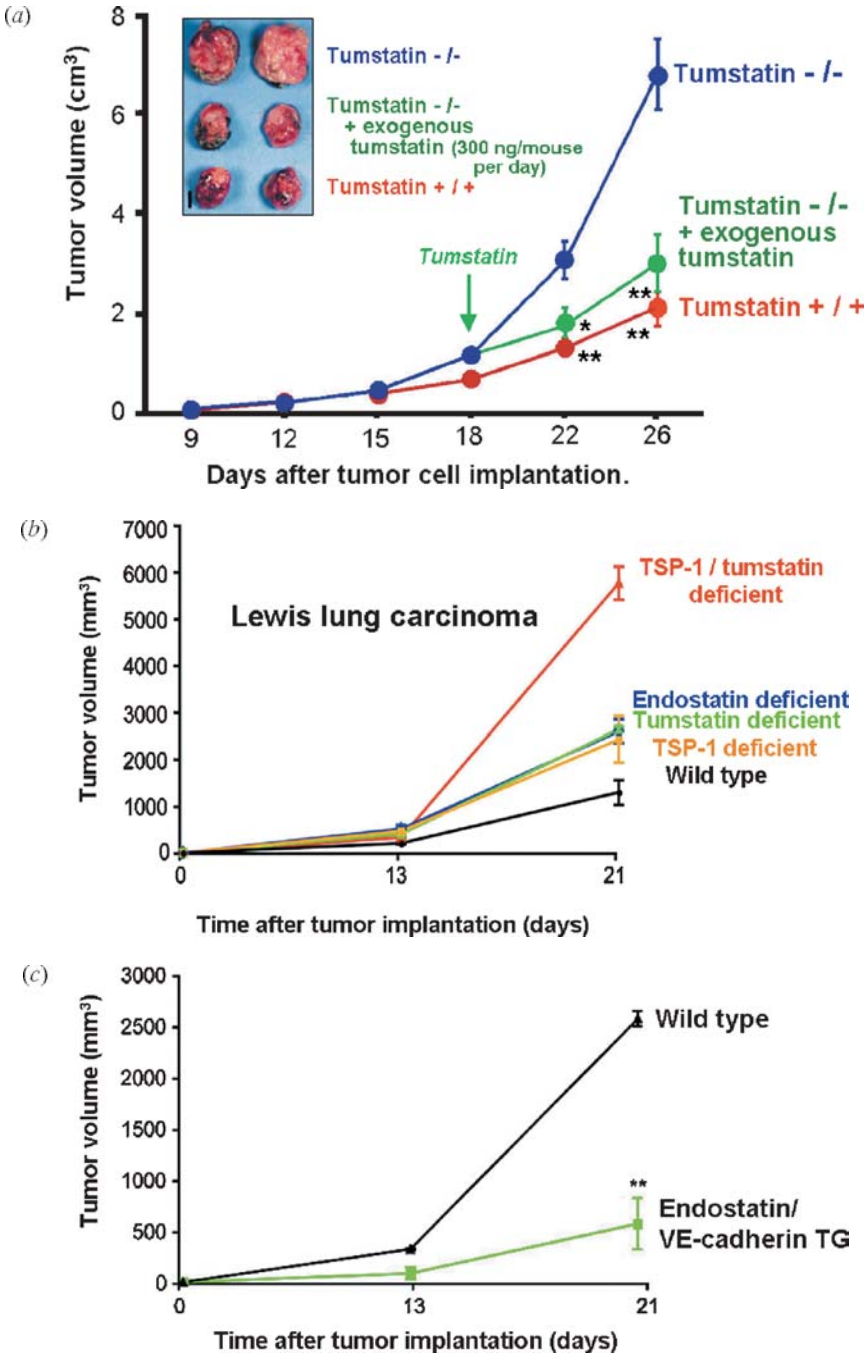

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Figure 1 (*a*) Effect on tumor growth of genetic deletion of the endogenous angiogenesis inhibitor tumstatin (in mice deficient for the α3 chain of collagen IV), and pharmacologic replacement (10). (*b*) In mice deficient for both tumstatin and thrombospondin-1, tumors grow >400% beyond the ceiling rate of growth in wild-type mice (36). (*c*) Overexpression of endostatin by ~1.6-fold in endothelium of tumor-bearing mice suppresses tumor growth by ~300% (36).

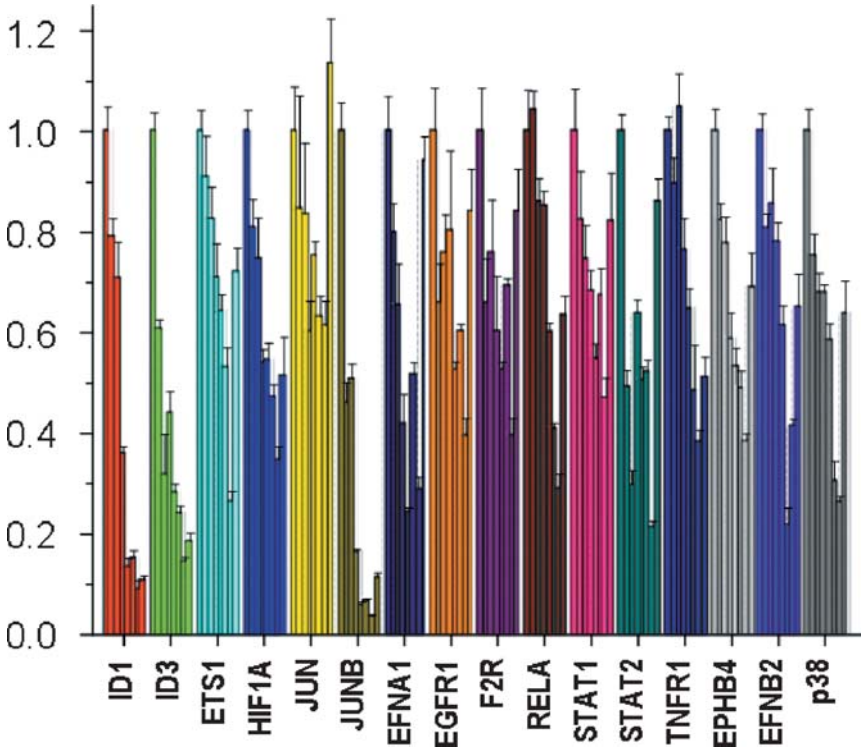

Figure 3 Gene expression in fresh human microvascular endothelial cells after incubation in human endostatin in vitro. Time course of mRNA expression: control, 0.5, 2, 4, 8, 24, and 48 h after 200 ng/ml endostatin (68).

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