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Review Article

Recent advances in antiangiogenesis strategies

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Abstract

Tremendous advancement in basic science in the field of angiogenesis has provided several novel targets and strategies, although there is a large gap between experimental and clinical data. This gap might be minimized through the selection of clinically relevant surrogate biomarkers or biomarkers that correlate with clinical outcome. Additionally, in the design of clinical trials, hard endpoints (such as mortality, quality of life and cost-effectiveness of the antiangiogenesis strategy), as well as surrogate biomarkers and noninvasive imaging of tumors and their microvascular environment, should be used. Adjunctive therapies that maximize the efficacy and minimize the complications associated with cancer and its current standard therapy should also be employed.

Introduction

Tumors cannot grow or metastasize without the development of new blood vessels to supply them with oxygen and nutrients necessary for survival and growth (1). The process of angiogenesis, which is a highly regulated, coordinated series of events, can be divided into an activation phase and a resolution phase, each of which will comprise a variety of processes (2). The activation phase involves increased vascular permeability and extravascular fibrin deposition, vessel wall disassembly, degradation of the basement membrane, cell migration leading to extracellular matrix (ECM) invasion, cell proliferation and tube formation. For the resolution phase of angiogenesis, each of these processes must be halted (cell migration and proliferation), reversed (basement membrane reconstitution) or completed (recruitment of pericytes and smooth muscle cells facilitating vessel maturation). Extracellular proteolysis is a vital biochemical event in all of these processes (3).

Endothelial cells, with their remarkable ability to divide and migrate, are the source of new blood vessels. After an endothelial cell in the wall of an existing capillary becomes activated, it secretes enzymes that degrade the ECM; it then invades the matrix and begins dividing. Eventually, hollow tubes comprised of strings of new endothelial cells create new networks of blood vessels (1, 4, 5).

New capillary growth is tightly controlled by a finely tuned balance between factors that activate endothelial cell growth and those that inhibit it (1, 4). Endothelial cell growth and movement are activated by a number of proteins, including angiogenin, epidermal growth factor (EGF), estrogen, fibroblast growth factors (FGF; acidic and basic), granulocyte colony-stimulating factor (G-CSF), interleukin-8 (IL-8), prostaglandin E1 (PGE1) and E2 (PGE2), tumor necrosis factor-α (TNF-α) and vascular endothelial growth factor (VEGF). Some of the known inhibitors of angiogenesis include angiostatin, endostatin, interferons, IL-1, IL-12, retinoic acid and tissue inhibitor of metalloproteinases (TIMP) (1, 4).

As a tumor grows, it sends out signals to the nearby endothelial cells to activate new blood vessel growth. VEGF and basic FGF are expressed by many tumors and appear to be critical for sustaining tumor growth (1, 3).

The shedding of cells from the primary tumor begins only after the tumor has a full network of blood vessels. In general, tumors with higher densities of blood vessels are more likely to metastasize and are correlated with poorer clinical outcomes. Both angiogenesis and metastasis require matrix metalloproteinases (MMPs), enzymes that break down the ECM, during blood vessel and tumor invasion (1, 2, 4).

Strategies for angiogenesis modulation

Table I describes clinical trials involving angiogenesis inhibitors. Some of these drugs were designed to target specific molecules involved in new blood vessel formation. For the others, the exact mechanism of the drug is not known, but they have been shown to be antiangiogenic in various experimental models of angiogenesis.

Investigators generally use four main strategies to design antiangiogenic agents: 1) using naturally occurring inhibitors of angiogenesis; 2) blocking factors that stimulate angiogenesis; 3) blocking molecules that allow newly formed blood vessels to invade surrounding tissue; and 4) incapacitating newly dividing endothelial cells.

Natural inhibitors of angiogenesis

Naturally occurring negative regulators of angiogenesis balance the positive regulators. The proangiogenic state occurs when there is a shift toward more of the positive regulators and/or less of the negative regulators. Administration of negative regulators might allow for balanced angiogenesis and the treatment of pathological disorders associated with accelerated angiogenesis. Examples of such negative regulators and biological sources are described below.

It has become apparent that the hemostatic system regulates angiogenesis via components of the coagulation and fibrinolytic systems regulating proteolysis in the

Table I: Angiogenesis inhibitors that have reached clinical trials.

Drug	Sponsor	Trial	Mechanism
Compounds that prevent new blood vessels from invading surrounding tissue			
Marimastat	Vernalis	Phase III (discontinued)	Synthetic MMP inhibitor
Tanomastat	Bayer	Phase III	Synthetic MMP inhibitor
Prinomastat	Pfizer	Phase III (discontinued)	Synthetic MMP inhibitor
CGS-27023	Novartis	Phase I	Synthetic MMP inhibitor
COL-3	Collagenex; National Cancer Institute	Phase I	Antibiotic-derived MMP inhibitor
Vitaxin®	MedImmune	Phase II	Antibody to integrin, present on endothelial cell surface
Natural inhibitors of angiogenesis			
Platelet factor-4	Repligen	Phase II (discontinued?)	Inhibitor of endothelial cell growth
Interleukin-12	National Cancer Institute	Phase I/II	Inhibitor of endothelial cell growth
Compounds that block factors that stimulate the formation of blood vessels			
Bevacizumab	Genentech; Roche	L-2004	Monoclonal antibody to VEGF
Semaxanib (SU-5416)	Sugen (Pfizer)	Phase I (discontinued)	Molecule that blocks VEGF receptor signaling
Interferon alfa		Launched	Inhibitor of EGF release
Targeted antivascular therapy			
CM-101	CarboMed	Phase I/II (discontinued?)	Bacterial toxin that binds to new blood vessels and induces inflammatory response
Compounds that interrupt function of dividing endothelial cells			
TNP-40	TAP Pharmaceutical	Phase II (discontinued?)	Synthetic analogue of fungal protein that inhibits endothelial growth
Compounds with unknown mechanism			
Thalidomide	National Cancer Institute	Phase II	
CAI	National Cancer Institute	Phase I/II/III	Nonspecific inhibitor of cell invasion and motility
Squalamine	Genaera	Phase II/III	Extract from dogfish shark liver that inhibits NHE3 sodium-hydrogen exchanger
Suramin	National Cancer Institute	Phase I/II	Nonspecific multisite effects
Oglufanide	Cytran	Phase II (discontinued?)	

milieu and the context of the ECM and the fibrin clot. Cryptic fragments, released by proteolysis from components of the coagulation and fibrinolytic systems, may have a role to play in regulating angiogenesis.

Capillary sprouting results in fibrinolysis at the tips of these sprouts (6). As capillary sprouts mature, the fibrin is replaced by other components of the ECM. Thus, fibrin has a fundamental role to play in angiogenesis by providing a temporary matrix scaffold along which endothelial cells can migrate. Thrombin is the main enzyme involved in generating insoluble fibrin from its soluble precursor fibrinogen, a large glycoprotein found in the blood plasma of all vertebrates. The central enzyme in fibrin proteolysis is plasmin, and its activity and that of thrombin are regulated by a variety of molecules.

The main pathway for initiation of the coagulation cascade is the extrinsic (tissue factor, TF) pathway, whereas the intrinsic (contact factor) pathway serves to augment or amplify the coagulation cascade. Cleavage of high-molecular-weight kininogen (HK) by plasma kallikrein results in the generation of a two-chain form of HK (HKa). The light chain of HKa contains two domains, D5 (which binds to anionic surfaces, including heparin and phospholipids, as well as zinc) and D6.

HKa is reported to bind to endothelial cells via the urokinase receptor (7), and this interaction has been proposed as the basis for the antiendothelial effect of HKa (7, 8). Recent experiments demonstrate that D5, derived from human kininogen, can inhibit angiogenesis. Thus, the protein components involved in the initiation of the intrinsic pathway are also involved in the regulation of angiogenesis.

HKa D5 (also known as kininostatin) has been shown to inhibit endothelial cell proliferation, endothelial cell migration toward vitronectin and angiogenesis in the chicken chorioallantoic membrane assay (7). Structure-function studies have demonstrated that D5 contains a peptide that inhibits only migration, as well as another peptide that inhibits only proliferation (7). Data on a related inhibitory monoclonal antibody have confirmed the role of kininogen in angiogenesis modulation (8).

TF is a membrane-bound protein that initiates coagulation upon blood vessel damage. Upon exposure to blood, TF activates factor VII to factor VIIa (with the aid of calcium and phospholipids), and this complex in turn activates factors IX and X. TF is found on the surfaces of tumor cells, as well as on tumor-associated macrophages and tumor-associated endothelium (9). TF expression may be a hallmark of cancer progression and metastasis mediated by pathways independent of blood coagulation (10). Dramatic reductions in experimental tumor angiogenesis can be induced by inhibiting TF activity via the use of specific antibodies, thus highlighting the importance of TF in tumor angiogenesis (11). Furthermore, the transfection of low VEGF-producing tumor cells with full-length clones of TF restores VEGF production in these cells, whereas transfection of these same cells with a clone of TF lacking the cytoplasmic serine residues does not restore VEGF production, suggesting that the cytoplasmic tail of TF is required for full expression of VEGF (12).

Tissue factor pathway inhibitor (TFPI), a multidomain protein with 3 Kunitz-type proteinase inhibitor domains, may inhibit the proliferation of bFGF-driven endothelial cells (13). Thus, proteins that regulate angiogenesis are present in the entire cascade leading to coagulation and fibrinolysis (3, 9, 14). This may be accomplished in some cases simply by leading to the deposition or degradation of fibrin, which is inherently angiogenic because it sequesters growth factors and guides the migration of endothelial cells. Endothelial cells also appear to be affected by other proteins, either in their native form or after proteolysis leads to the exposure of cryptic domains with pro- or antiangiogenic activity.

Proteinases that function in generating the active form of a given protein can generate many of the cryptic fragments. Many proteins have a more active role than has been thought. For example, plasminogen is activated to plasmin, which can be inactivated by further proteolysis, such as the proteolytic removal of microplasmin from the cell surface. This reaction leads to the rapid inhibition of microplasmin by α_2-antiplasmin and generates angiostatin as a byproduct (15). Maximal angiogenic activity appears to be associated with proteolysis, as evidenced by a healing wound, which is a clear example of the gross coordination of these events. Given the rapid discovery of antiangiogenic fragments of proteins within the coagulation cascade in recent years, more fragments and domains are expected to be discovered in the near future; this will expand our arsenal of antiangiogenic drugs and also contribute to our understanding of the process of wound healing and the roles that coagulation and fibrinolysis play in angiogenesis.

Angiostatin, a polypeptide of approximately 200 amino acids, is produced by the cleavage of plasminogen, a plasma protein vital in dissolving blood clots. Angiostatin binds to subunits of ATP synthase exposed at the surface of the cell embedded in the plasma membrane (15, 16).

Wound healing depends on the processes of blood coagulation and angiogenesis. For example, upon activation by thrombin, platelets (which contain at least a dozen promoters of angiogenesis) may be induced to secrete into the surrounding vasculature. Targeting both the coagulation and angiogenesis pathways may be more effective in treating tumors than targeting either pathway alone. Tumor cells could be used as a model system to study the TF signaling pathway, thus providing new insights into the cellular biology of TF that might be applied to signaling in endothelial cells, smooth muscle cells and fibroblasts. An understanding of the TF signaling pathway might also provide a rational basis for the development of new agents to prevent and/or reduce angiogenesis-related disorders, tumor-associated thrombosis and the positive feedback loop between thrombosis and cancer (17).

The growth and dissemination of cancer cells are stimulated through multiple mechanisms by activating the

blood coagulation system; thus, anticoagulant drugs inhibit the progression of certain cancers. For example, renal cell cancer (RCC) is one of a small number of human tumor types in which the tumor cell contains an intact coagulation pathway leading to thrombin generation and the conversion of fibrinogen to fibrin immediately adjacent to viable tumor cells (18). This is also apparent in melanoma, ovarian cancer and small cell lung cancer (SCLC), but not in breast, colorectal and non-small cell lung cancer (NSCLC) (19). The growth of melanoma and SCLC is inhibited by anticoagulants, but no such effect has been observed in the other tumor types; therefore, based on the relatively unique features of the interaction of the coagulation system with RCC, we hypothesize that RCC will respond to anticoagulation therapy similarly to SCLC and melanoma. An anticoagulant that acts at the TF/VIIa level would most likely have improved efficacy and safety in inhibiting tumor-associated thrombosis, angiogenesis and metastasis.

More than a century ago, Trousseau (20) first described migratory thrombophlebitis complicating gastrointestinal malignancy, thereby recognizing the association between coagulation system activation and systemic thrombosis in human cancers. Greater appreciation in recent years of the interdependency of malignant behavior and the coagulation system has led to an understanding of how an activated coagulation system may enhance cancer cell growth (17, 21). Although this does not establish causality or even a biological association, a recent study revealed that patients with cancer who developed venous thrombosis had significantly shorter cancer-related survival than matched controls who did not develop thrombosis (22). In addition, several studies (including randomized clinical trials) have documented improved cancer-related survival in patients treated with anticoagulants compared to those who did not receive anticoagulants (23-25).

With regard to interactions with the coagulation system, there are two types of tumors: 1) those that activate the coagulation system directly (such as melanoma, RCC, ovarian cancer and SCLC); and 2) those that mediate coagulation activation indirectly via a paracrine mechanism (such as breast and colorectal cancer and NSCLC). Tumors in the first group overexpress procoagulant molecules, such as cancer procoagulant, TF or, in the case of RCC, hepsin, on their cell surfaces. The entire coagulation pathway is assembled on the surface of these tumor cells, leading to fibrin formation that is close to the tumors; this partly explains the clots emanating from the tumor and extending into the renal vein and inferior vena cava seen occasionally in patients with RCC. On the other hand, tumors in the second group tend to activate systemic coagulation by releasing cytokines (*e.g.*, TNF-α, IL-1β), which in turn stimulate the production of procoagulant molecules on the surface of circulating monocytes. Thus, one would predict that tumors in the first group might be more likely to respond to anticoagulation that interferes with TF/VIIa than would tumors in the second group; indeed, prospective trials have shown significant activity for anticoagulants in melanoma and SCLC, but not in colorectal and breast cancer and NSCLC (23-26).

Blocking factors that stimulate angiogenesis

VEGF is an endothelial cell-specific mitogen and an angiogenesis inducer. It is essential for developmental angiogenesis and is also required for bone formation and for female reproductive function. VEGF is implicated in tumors and in intraocular neovascular syndromes. A humanized anti-VEGF monoclonal antibody (rhuMAb VEGF, bevacizumab, Avastin®) was launched in 2004 for use in patients with metastatic colorectal carcinoma in combination with chemotherapy and is currently in phase II and III clinical trials as a treatment for various other solid tumors (27, 28). Phase II studies in patients with colorectal and lung cancer where the antibody was used in conjunction with standard chemotherapy provided evidence of clinical efficacy.

Blocking molecules that allow newly forming blood vessels to invade surrounding tissue

Zinc-dependent endopeptidases that degrade ECM proteins (collagens, laminin and fibronectin) during the process of cancer invasion and metastasis (29), MMPs are produced primarily by reactive stromal and inflammatory cells surrounding tumors rather than by cancer cells. Hydroxamic acid-derived inhibitors of MMPs are currently in clinical trials for the treatment of prostate, lung, gastric, pancreatic and breast cancer. In preclinical trials in mice, these drugs have demonstrated efficacy alone and in combination with chemotherapy, but clinical trials in advanced-stage cancer indicated a lack of efficacy. Further trials in early stages of cancer in conjunction with chemotherapeutic agents are in progress.

We have shown that type IV collagen (COL IV)— specifically, a peptide comprising residues 185-203 of the noncollagenous domain (NCI) of the α3 (IV) chain— has several biological activities, including promotion of tumor cell adhesion and chemotaxis, inhibition of neutrophil activation and inhibition of tumor cell proliferation (30).

Endostatin, a polypeptide globular domain found at the C-terminus of type XVIII collagen (a collagen found in blood vessels) cut off from the parent molecule, has demonstrated potent antiangiogenic and anticancer efficacy in experimental models. It is currently in clinical development (31).

The role of various integrins ($\alpha_v\beta_3$, $\alpha_v\beta_5$ and $\alpha_5\beta_1$) in angiogenesis-mediated disorders has been demonstrated (32-34). The role of $\alpha_5\beta_1$ integrin in angiogenesis was established through the use of monoclonal antibody, cyclic peptide and nonpeptide $\alpha_5\beta_1$ integrin antagonists (34). Additionally, immunoglobulins (IgG) such as PECAM and others have demonstrated crosstalk with key vascular integrins that regulate angiogenesis.

Cancer and angiogenesis

Because diffusion in a mass larger than 2-3 mm^3 is insufficient for oxygen and glucose requirements, tumor cells cannot grow unless they induce angiogenesis. Furthermore, tumor invasiveness and metastasis both require neovascularization. Recent published studies have suggested that acquisition of the angiogenic phenotype is a common pathway for tumor progression and that neovascularization is linked with other molecular steps leading to tumor progression. The angiogenic process is a complex multistep cascade under the control of positive and negative soluble factors, with a paracrine interaction occurring between tumor and endothelial cells.

As mentioned above, angiogenesis involves endothelial cell proliferation, migration and tubule formation with associated changes in the ECM, allowing subsequent new vessel growth toward the tumor. Antiangiogenic therapy could target each of these steps, but these strategies are to be distinguished from direct targeting and destruction of tumor vasculature by cytotoxic agents (1, 4, 5).

Ocular angiogenesis

Inappropriate growth of blood vessels in the iris, cornea, trabecular meshwork and retina is a leading cause of blindness in developed countries. Both MMP and VEGF are involved in the early stages of disease-related angiogenesis. The role of ECM, hypoxia, growth factors and integrins in pigmented epithelial cell biology might contribute to understanding ocular disorders (35-38). Currently, compounds and strategies for inhibiting MMPs and VEGF for preventing neovascularization in patients at risk for blindness are under investigation (35).

Age-related macular degeneration (ARMD) is the leading cause of blindness in people over the age of 55, affecting more than 10 million Americans. Macular degeneration, which affects more Americans than cataracts and glaucoma combined, occurs when the central portion of the retina (the macula) deteriorates. This condition is equally common in men and women and more common in whites than blacks. The cause is unknown, but the condition tends to run in some families. In the most severe form of ARMD (known as “wet” ARMD), irreversible loss of vision results when abnormal angiogenesis occurs under the retina and bleeding from the new blood vessels leaves scars. Laser-based therapy is used to destroy offending blood vessels; however, this treatment is not optimal because the laser can permanently scar the overlying retina, and the offending blood vessels often regrow. Advancing novel angiogenesis inhibitors that effectively and safely inhibit the growth of new vessels in diabetic retinopathy and ARMD —and hence restore the lost vision— is a key objective that remains to be realized.

At present, two direct inhibitors of VEGF are being evaluated for the prevention of retinal neovascularization from ARMD, both requiring administration by intravitreal injection. These compounds are also in or approaching clinical trials for diabetic macular edema (39, 40). One approach involves a humanized anti-VEGF monoclonal antibody fragment that selectively binds all isoforms of VEGF and prevents it from exerting its action. The compound, called rhuFab V2 (ranibizumab, Lucentis®), is produced by Genentech; it was evaluated in a multicenter, randomized, controlled trial in patients with exudative ARMD (39). Another direct VEGF inhibitor is pegaptanib (Macugen®), an anti-VEGF aptamer. This compound, from EyeTech, binds one of the VEGF isoforms and is administered by intravitreal injection (40).

Orally administered agents that may ameliorate diabetic retinal complications are also being evaluated. Protein kinase C (PKC) is an enzyme whose activity is increased early in diabetes. One isoform of this enzyme, PKCβ, appears to be particularly important in the retina. PKCβ plays important roles in diabetes-induced vascular dysfunction, retinal blood flow abnormalities, VEGF expression, VEGF signaling and retinal vascular permeability. It may also play pivotal roles in other diabetes-induced nonocular microvascular complications, such as neuropathy or nephropathy. Thus, oral PKCβ inhibitors might provide an easily administered novel therapy for nonproliferative diabetic retinopathy (NPDR), PDR or macular edema. Two multicenter, randomized, double-masked, placebo-controlled phase II/III clinical trials evaluating once-a-day oral therapy with a PKCβ inhibitor manufactured by Lilly (LY-333531, ruboxistaurin) have just been completed (41).

Multiple myeloma: antiangiogenic therapy

Disease progression in multiple myeloma (MM) is characterized by increased bone marrow neovascularization, and targeting the mechanisms that control angiogenesis could thus represent an innovative therapeutic approach to MM (42). Thalidomide is able to inhibit angiogenesis in murine models, and it has recently been demonstrated to be effective (30-40% response rate) in relapsed/refractory MM, with only mild systemic toxicity. Inhibition of angiogenesis is probably just one of the mechanisms by which thalidomide exerts its action in MM; immunomodulation and inhibition of cytokine production by bone marrow stroma could also be involved. Several ongoing studies are aimed at testing thalidomide-based drug combinations, primarily with dexamethasone but also with conventional chemotherapy. The results obtained so far show a synergistic effect for the drug combinations, with a response rate ranging from 50% to 70% in pretreated patients; there was, however, an increased incidence of venous thrombosis with thalidomide when combined with chemotherapy (43).

Impact of antiangiogenic agents on hemostasis

SU-5416 (semaxanib), an angiogenesis inhibitor, is a potent inhibitor of VEGFR-1 and -2. VEGF may be involved in hemostasis by altering the hemostatic properties of endothelial cells. Kuenen *et al.* (44) examined the effects of SU-5416 on the coagulation cascade and the

vessel wall in patients with advanced cancer. Markers for activation of the protein C pathway, thrombin generation, fibrinolysis and endothelial cell activation were measured in patients with soft tissue sarcoma, RCC or melanoma on days 0, 14 and 28 of treatment with SU-5416. In the fifth week of treatment, 3 of 17 sampled patients developed a thromboembolic event. All patients demonstrated a significant increase in endogenous thrombin potential and of parameters reflecting endothelial cell activation. Endogenous thrombin potential, soluble TF and soluble E-selectin increased to a significantly greater extent in patients experiencing a thromboembolic event.

Endothelial cell dysfunction, which could be associated with an increased incidence of thrombosis, may be induced by antiangiogenic agents (42-45). The effect of antiangiogenic mechanisms on the hemostatic system needs to be studied further.

Role of anticoagulants in angiogenesis

Many patients with cancer reportedly have a hypercoagulable state, which is caused by the impact of cancer cells and chemotherapy on the coagulation cascade (46). Analysis of biomarkers of the coagulation cascade and of vessel wall activation showed significant increases in thrombin generation and endothelial cell perturbation in a treatment cycle-dependent manner when angiogenesis inhibitors and chemotherapeutic agents were combined (43, 44). The incidence of thromboembolic events discouraged further investigation of the combination regimen of SU-5416 plus a chemotherapeutic agent (44). The potential advantage of using an anticoagulant such as heparin or low-molecular-weight heparin (LMWH) was suggested by this study, along with the observation of an increased incidence of deep vein thrombosis in MM patients receiving chemotherapeutic agents and thalidomide. In addition, studies have demonstrated that LMWH or unfractionated heparin (UFH) interferes with various processes involved in tumor growth and metastasis. Clinical trials have indicated a clinically relevant effect for LMWH, as compared to UFH, on the survival of cancer patients with deep vein thrombosis. The effect of LMWH and TFPI released *in vivo* on the regulation of angiogenesis and tumor growth was documented (36, 46). Heparin, steroids and heparin/steroid combinations are effective inhibitors of angiogenesis, as evidenced in a variety of *in vitro* models and *in vivo* models (47, 48). In addition, platelet-tumor cell interactions could play a significant role in metastasis (49).

Standard chemotherapy *versus* angiogenesis inhibitors

Because angiogenesis inhibitors target dividing endothelial cells rather than tumor cells, there are several differences between standard chemotherapy and antiangiogenic therapy. Antiangiogenic drugs are not likely to cause gastrointestinal symptoms, bone marrow suppression or hair loss. Also, since antiangiogenic drugs may not necessarily kill tumors, but rather hold them in check indefinitely, the endpoint of early clinical trials may be different than for standard therapies. It may be appropriate to evaluate increases in survival and/or time to disease progression, rather than looking only for tumor response. A major problem with chemotherapeutic agents is drug resistance. This occurs because most cancer cells are genetically unstable, are more prone to mutations and are therefore likely to produce cells that are drug-resistant. Because antiangiogenic drugs target normal endothelial cells, which are not genetically unstable, drug resistance may not develop. In fact, resistance has not been a major problem in long-term animal studies or in clinical trials. However, while there is some evidence that this lack of resistance may be true for some direct-acting angiogenesis inhibitors, it is becoming apparent that, over time, resistance can develop to many types of angiogenesis inhibitors, including some direct inhibitors, especially when they are used as monotherapy (18). Possible mechanisms for such acquired or induced resistance include the following: 1) redundancy of proangiogenic growth factors when the drug targets a single growth factor or its cognate endothelial cell-associated receptor tyrosine kinase; 2) the antiapoptotic/prosurvival function of growth factors such as VEGF, which, in high local concentrations, can antagonize the proapoptotic effects of various angiogenesis inhibitors; 3) epigenetic, transient upregulation, or induction, of various antiapoptotic effects or molecules in host endothelial cells; and 4) heterogeneous vascular dependence of tumor cell populations. Long-term disease control with antiangiogenic drugs may therefore best be achieved by rational combination therapy. Unlike chemotherapy, the great molecular diversity of antiangiogenic drug targets makes this a particularly attractive therapeutic option (50). Finally, antiangiogenic therapy may prove useful in combination with therapy directly aimed at tumor cells. Because each therapy is aimed at a different cellular target, the hope is that the combination will prove more effective.

Monitoring clinical trials

Traditional/conventional measures of clinical efficacy

Phase I trials are designed to determine the appropriate dose and schedule for further evaluation and describe the pharmacological behavior of the drug and its toxic effects. The goal of phase II trials is to screen agents for evidence of antitumor activity. Antiangiogenesis trials present unique challenges to these traditional paradigms. One of the areas of greatest activity in the design of such trials concerns the means to measure the study's endpoint. In other words, how do you know the drug has antitumor activity?

The tradition of utilizing toxicity as the phase I endpoint is most useful for cytotoxic drug studies because there is a direct relationship between the dose of a drug and both its effects on tumor and its toxicity. This means

that the incidence and severity of a drug's toxicity are directly correlated with dose. Thus, the dose-limiting toxicity is the primary determinant of the dose chosen for further study in subsequent trials because it represents the highest possible dose.

The goal of phase II trials is to screen agents for their potential efficacy. Traditionally, this has been measured by objective tumor regression described using standard criteria (*e.g.*, that of the World Health Organization). Tumor shrinkage has proven useful as a phase II endpoint because it has allowed us to select drugs earlier in the drug development process. It is important to point out that response *per se* is not synonymous with efficacy because the gold standard remains improved cure rates, survival or quality of life.

Novel trial design and evaluation

Antiangiogenic therapeutic trials have successfully challenged traditional paradigms of success. One of the major reasons for this has been the contention that agents that possess high selectivity for a specific target (in this case, the tumor vasculature) will, by definition, possess little crossover toxicity to normal host tissue. This concept has fueled much of the excitement and enthusiasm for such drugs. Indeed, the first generation of antiangiogenesis trials suggested that this prediction may hold true. For example, over a dose range of 100-1000 mg/day, thalidomide possesses almost none of the NCI grade 3 and 4 toxicity that is commonly seen with cytotoxic drugs. Perhaps the most striking example is endostatin. As described above, there were very few grade 2 or higher toxicities with this drug over a log range of doses. Bevacizumab (Avastin®), when administered with 5-fluorouracil (5-FU), was recently approved by the FDA for the treatment of metastatic colon cancer. Data from clinical trials with bevacizumab show a striking absence of the severe toxicities commonly seen in cytotoxic drug clinical trials. One practical implication of this is that selection of the subsequent phase II or III drug dose based on the observed phase I toxicities may be very difficult.

Among the novel features of antiangiogenic drugs is that their lack of toxicity makes them ideal for combination with other therapeutic modalities. Indeed, the combination of bevacizumab with 5-FU provided significantly increased survival in colon cancer patients. Combination with other biological response modifiers is also possible (*e.g.*, interferon), as demonstrated for endostatin in melanoma and for bevacizumab in RCC. Combination with radiation therapy has also been evaluated.

Traditional measures of therapeutic efficacy have come under scrutiny as it becomes increasingly obvious that antiangiogenic drugs may be effective in ways that would not trigger tumor regression. A solid body of experimental evidence suggests that successful inhibition of tumor angiogenesis results in a state of tumor dormancy. This means that a new state of tumor-host equilibrium is induced that prevents further tumor growth and metastasis, although frank tumor regression or shrinkage does not occur. Thus, traditional radiological imaging would not show any tumor change; in the current evaluation systems, this would not be scored as a success, or worse, it would be scored as a therapeutic failure.

There are early hints from angiogenesis clinical trials that a state of dormancy may indeed occur. In addition, new methods of imaging tumors show that blood flow and tumor activity decrease as antiangiogenic therapies take effect. This has led to a re-examination of how success is measured in clinical trials and to devising new measures to assess efficacy. One way is to directly measure the impact of antiangiogenic therapy on tumor blood flow. Imaging methods such as dynamic MRI and positron emission tomography (PET) scanning are particularly useful if serially carried out over the course of therapy.

Success in a trial is also being redefined. The category of "stable disease" as measured radiologically has traditionally been associated with either therapeutic inactivity or failure. Because tumor dormancy may manifest as "stable disease" and because the length of time a tumor is held dormant is an important measure of antiangiogenic efficacy, parameters such as stable disease, minor response and time to tumor progression (TTP) are now part of the evaluation process in therapeutic trials. In addition, surrogate molecular markers of efficacy become even more important in this setting.

What this means for trial design is that the definition of tumor progression has now been tightened to mean a definitive increase in tumor size and no longer includes stable disease. The accuracy of TTP is directly tied to the interval between efficacy assessments. Therefore, the difference between success and failure may rest on when exactly a patient's tumor increases in size. Thus, many antiangiogenesis trials have begun to shorten the interval between tumor assessments to no more than 8 weeks, and sometimes even less.

A substantial number of clinical trials using antiangiogenic therapies are ongoing worldwide, but how to achieve maximum benefit from these therapies and how to monitor patient responses are of paramount concern to investigators. There are currently no available markers of net angiogenic activity of a tumor to aid investigators in the design of antiangiogenic treatment schemes. No marker used alone has been validated as a phase II endpoint (51).

Quantification of various aspects of tumor vasculature might provide an indication of angiogenic activity. Angiogenesis can be assessed directly by counting new blood vessels, or indirectly through the measurement of putative angiogenic factors and their receptors. Selected examples are discussed below.

An often quantified aspect of tumor vasculature is microvessel density (MVD). Studies over the last decade have demonstrated the value of using tumor MVD as a prognostic indicator for a wide range of cancers (52). MVD is the measure of the number of vessels per high-power (microscope) field, and as such, reflects the num-

ber of capillaries and the intercapillary distance. The central concept is that this would decrease as antiangiogenic therapy takes hold, but there are several important caveats to this.

MVD can vary widely. Angiogenesis is a focal event and microvascular “hotspots” occur in tumors (53). Thus, it is not surprising that the minute sampling cores obtained even with the widest biopsy needles in clinical use can miss these hot spots. In addition, there is a significant degree of interobserver variability, further confounding these issues.

The utility of MVD in clinical trials is also variable. A higher MVD predicts for more extensive cancer stage at diagnosis and a worse prognosis in cancers of the lung, breast and colon (54). The lesson from thalidomide in myeloma patients, however, is that the probability of a patient responding to therapy does not correlate with pretreatment MVD, and responders do not show a concomitant decrease in MVD. In contrast, the colon cancer data for bevacizumab suggest that MVD predicted for response. Current evidence therefore indicates that clinical measurement of MVD by itself does not accurately and reliably reflect angiogenic activity or angiogenic dependence of a tumor, and has not yet been shown to be a robust measure to guide or evaluate antiangiogenic treatment (55).Trial design has shifted to the discovery and validation of blood markers that can supplement or supplant MVD.

Survival remains the gold standard for therapeutic clinical trials in cancer. However, this often does not become evident until enough events have occurred, and this eventuality can take years. Indeed, in adjuvant clinical trials where the event rate is low, survival data may not achieve robust significance for close to a decade (56). Because of this long timeline, other means have evolved to evaluate drug efficacy.

Surrogate endpoints are loosely referred to as endpoints that can be used in lieu of traditional endpoints in the evaluation of experimental treatments or other interventions. The need to evaluate treatment benefits as rapidly as possible on easily measured endpoints has always been a goal in clinical research. Whereas dose selection is still primarily guided by pharmacokinetic and safety considerations, several early clinical trials of angiogenesis inhibitors have tried to incorporate biomarkers to help define optimal dosing. One surrogate endpoint is to directly assess effects on the intended drug target to ascertain if this would correlate with the intended clinical response. For example, in trials in which the VEGFR is targeted, the tumor would be sampled to determine if VEGFR is indeed inhibited.

In angiogenesis trials, the most direct measure is to assess the degree to which tumor angiogenesis changes. This is usually reflected in the MVD, as discussed above. This requires that serial biopsies be obtained in the patient undergoing treatment. Although this may be easy in superficial tumor deposits in certain malignancies such as melanoma, sampling deep visceral tumors may be more problematic and riskier for the patient. In trials where serial sampling is performed at the discretion of the investigator, there is often an overrepresentation of superficial tumors. This would not matter if the biology of metastases was site-independent; however, there is molecular evidence that deep visceral metastatic deposits possess a biology distinct from their more superficial counterparts.

Investigators have examined the association between angiogenic factors that are either produced or shed by the tumor and ancillary proteins induced as a result of angiogenesis. Angiogenic factors are an example of the former. The most commonly studied angiogenic factors are basic FGF (bFGF, FGF-2) and VEGF. VEGF mRNA and protein are markedly upregulated in the vast majority of human tumors; in some, VEGF overexpression is associated with a poor prognosis and reduced survival (57, 58). In addition, there is evidence that elevated bFGF levels correlate with more aggressive cancers and decreased overall survival (59). These angiogenic factors are measured in the fluids of the body, including blood, urine, cerebrospinal fluid and pleural effusions. Because these are more readily accessible than tumor biopsies, the evaluation of these surrogate markers in angiogenesis clinical trials is commonly carried out. However, the evaluation of VEGF and FGF levels in this setting is fraught with difficulties. One major issue is that there is wide intra- and interpatient variability in these levels. This is likely a consequence of multiple factors, including tissue and blood sequestration, affinity for heparin-like molecules and other matrix molecules, and the fact that blood may not accurately reflect the tumor microenvironment. Thus, it is not surprising that levels of angiogenic factors have not been found to consistently reflect tumor response. To date, all biomarkers in this field have limitations and none has yet been validated (60).

As discussed earlier, increased procoagulant and prothrombotic factors, as well as decreased natural anticoagulant factors, are common in cancer patients with different tumor types and at different stages (61). Hemostasis activation, increased proinflammatory biomarkers and proangiogenesis biomarkers in cancer patients have been shown to correlate with clinical outcome (61-64).

Normal vasculature is a highly organized structure that possesses a very low mitotic index. In contrast, angiogenic vessels are disorganized, poorly functional and actively growing. This chaotic state results in the shedding of vascular endothelial cells into the circulation; therefore, capturing and quantifying these cells may serve as an index of endothelial activity and, by inference, of the antiangiogenic effect of drugs.

Dynamic contrast enhanced MRI (DCE-MRI) has been increasingly used to evaluate novel antiangiogenic agents, both preclinically and in phase I/II trials. DCE-MRI can be a pharmacodynamic indicator of biological activity of antiangiogenic agents by demonstrating changes in tumor vasculature through parameters reflecting tumor perfusion and permeability. For example, SU-5416 is an intravenous inhibitor of the Flk-1 tyrosine kinase that was

administered to patients with metastatic melanoma involving the liver. In this study, DCE-MRI was able to detect a statistically significant decrease in the ratio of peak contrast uptake in the tumor versus normal liver at 8 weeks when compared to pretreatment. In addition, the changes in tumor permeability and vascularity seen with DCE-MRI were also shown to mirror clinical response (65, 66).

There is a growing need for noninvasive methods to serially assess the status of the coronary, peripheral and tumor vasculature to reflect the prognosis of the disease, as well as the efficacy of treatment (67, 68). These might include the following: MRI and monitoring of angiogenesis, nuclear perfusion imaging of angiogenesis and other modalities.

One of the ways in which trial design has evolved to adapt to the advent of biological therapies, and in particular antiangiogenic therapy, is to move to a rapid titration trial design with the aim of rapidly escalating the dose to the maximum planned dose. The rationale for this is that agents that are not known to exert dose-related toxicities (unlike the dose-related organ dysfunction caused by conventional cytotoxic chemotherapeutic agents) and are specifically targeted to a biological molecule (*i.e.*, endostatin or antibodies) can be given at the maximum planned dose with minimum toxicity. This contention is supported by accumulating data from antibody-based therapies currently under review or approved for use in cancer. Antibody-based therapeutic agents such as Herceptin® (trastuzumab; target: HER2/*neu*), Rituxan® (rituximab; target: CD20), Erbitux® (cetuximab; target: endothelial growth factor receptor [EGFR]), and most recently Avastin® (bevacizumab; target: VEGF) are notable for their lack of acute dose-limiting toxicity. Studies also show a dose-related therapeutic effect, with greater efficacy generally at higher doses. Likewise, a maximum tolerated dose was not established in treatments using the endogenous antiangiogenic molecule endostatin, even when dosing schemes spanned several logs.

There is also growing concern that traditional phase I designs doom the first patients that come onto trial to receive subtherapeutic doses of the agent under study and furthermore lock them in at these dose levels. In addition, the stepwise escalation scheme overstacks patients at the lower dose levels such that the majority of patients on trial are treated at the lower doses.

Conclusions

Antiangiogenic therapy shows great promise as a novel approach for patients with solid malignancies. Most current clinical trials are evaluating antiangiogenic drugs aimed primarily against single angiogenesis stimulators. However, at least three biologically active angiogenesis stimulators (VEGF, FGF and IL-8) are produced by a single solid malignancy, suggesting that tumor angiogenesis results from the activity of multiple angiogenesis stimulators rather than a single one. It has been shown that a combination of antiangiogenic drugs is more effective in inhibiting tumor-induced endothelial cell growth than a single agent, which implies that clinical antiangiogenic strategies for the treatment of solid tumors may be most effective when multiple rather than single antiangiogenic drugs are used in conjunction with standard therapies.

The unique attributes of antiangiogenic agents have challenged the traditional design and evaluation of clinical trials. TTP and stable disease are becoming important parameters of efficacy. Surrogate markers of efficacy become increasingly important in this arena. Tissue measures of MVD remain the most direct measure of antiangiogenic effect. Plasma factors such as VEGF and FGF, as well as newer measures (such as PAI-1 [plasminogen activator inhibitor type 1], coagulation factors and TFs), are currently under study. This area is evolving rapidly as newer and more effective antiangiogenic agents with multiple targets and mechanisms emerge. Additionally, the use of adjunctive therapies that maximize the efficacy of standard cancer therapy and minimize cancer-associated complications should be introduced in upcoming trials.

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