Angiostatin and Anti-angiogenic Therapy in Human Disease

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ABSTRACT

Many diseases have abnormal quality and/or quantity of vascularization as a characteristic feature. Cancer cells elicit the growth of new capillaries during neovascularization in a process termed angiogenesis. In diabetics, pathologic angiogenesis in various tissues is a clinical feature of many common complications. Therefore, the diabetic cancer patient warrants special consideration and extra care in the design of anti-angiogenic treatments without adverse side effects. Some treatment regimens that look promising *in vitro*, in animal models, or in early clinical trials may be contra-indicated for diabetics. This chapter will review the common complications of diabetes, with emphasis on the angiogenic pathology. Recent research related to the mechanism of action and basis for specificity of the anti-angiogenic peptide, angiostatin, will be the focus. The aim is to shed light on areas in which more research is needed with respect to angiostatin and other anti-angiogenic agents and the microenvironmental conditions that affect their activities, in order to develop improved therapeutic strategies for diabetic cancer patients.

I. Introduction

Normal tissue function relies on an adequate supply of nutrients and oxygen from pre-existing blood vessels. It is well established that tumor growth depends on development of a new vascular supply, a process known as angiogenesis (Folkman, 1971). The discovery of factors that mediate this process has significantly increased our understanding of many normal and pathological circumstances. For example, angiogenesis is desirable in wound healing and recovery from cardiac ischemia but undesirable in the pathogenesis of psoriasis (Oates, 2002) or when metastastic tumor tissue develops a blood supply, allowing it to grow and spread (Weidner *et al.*, 1991,1993; Macchiarini *et al.*, 1992). The quest has been to isolate naturally occurring agents or develop new ones that aid in the regulation of this process. Judah Folkman initially developed the hypothesis that naturally occurring agents existed that inhibited angiogenesis. The first two, discovered in his laboratory, were angiostatin (O'Reilly *et al.*, 1994a) and endostatin (O'Reilly *et al.*, 1997).

As tumors grow in size, they become hypoxic and acidotic and elaborate several growth factors to stimulate local blood vessels to sprout branches.

Endothelial cells then begin proliferating towards the tumor and form tubular structures that become blood vessel branches. These new blood vessel branches differ from normal blood vessels. Some agents can target them while not harming intact, mature vasculature elsewhere in the body. For example, newly formed blood vessels are "leaky" or exceptionally permeable (Dvorak *et al.*, 1988). They also are tortuous, with blind ends and incomplete drainage or backflow (Secomb *et al.*, 1993; Kimura *et al.*, 1996) and therefore contain a mixture of arterial and venous blood (Kallinowski *et al.*, 1988; Vaupel, 1997; Vaupel *et al.*, 1989,1998). This makes them poor at removing catabolites, including carbon dioxide (CO_2) (Boyer and Tannock, 1992).

Angiostatin is endogenously produced in the tumor stroma. Therefore, circulating levels can control metastatic cell proliferation until a primary tumor is removed. Before the discovery of angiostatin, it was believed that when cancer recurred after surgical removal of a primary tumor, it was because the surgeon had left some tumor cells behind. It is now generally accepted that micrometastases already have seeded elsewhere in the body but were inhibited from proliferating by endogenous production of angiostatin or other inhibitors from the primary tumor. These micrometastases then came under permissive growth conditions when the primary tumor was removed. The discovery of angiostatin elicited great excitement because it seemed likely that exogenous administration after primary tumor removal could prevent metastatic growth. However, one problem is the short half-life of this peptide, leading to a need for continuous administration. Identifying receptors for these molecules was deemed a high priority, so that alternate molecules could be developed that have the same targets and are more stable, more effective, and easier to manufacture and administer.

Angiostatin is found naturally in significant amounts in the circulation of patients with primary tumors (Canfield *et al.*, 1986; Cao *et al.*, 2000; OSI Pharmaceuticals *et al.*, 2001). When a primary tumor is removed, metastases may experience lower circulating levels of endogenous inhibitors. Local blood vessels respond to the malignant cells' elaboration of vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF). Local blood vessels then respond by sprouting branches to feed the metastases. At this time, small micrometastases may grow beyond the 2-mm size, which is functionally dormant, and become a threat to the patient when rapid growth causes local damage. Angiostatin can maintain metastases in a dormant state in laboratory animals when administered exogenously (O'Reilly *et al.*, 1994a,b,1997). Research on the mechanism of action of angiostatin delineates some parameters affecting its activity that will require evaluation, in order to design treatments that yield the most-effective combinations of agents. More-complicated issues are whether these treatments are applicable in patients having a variety of vascular pathologies. Diabetic patients have other body compartments that feature abnormal vascularization. Understanding how these anti-angiogenic agents work and the conditions in the

various compartments will enable predictions about possible side effects, permitting further drug development. Many agents are in clinical trials (Table I), with alarmingly little known about their mechanisms of action and potential side effects.

II. Stages of Tumor Angiogenesis

Angiogenesis begins when a fibrin clot forms on the adventitial surface of an existing blood vessel (Muthukkaruppan and Auerback, 1970; van Hinsbergh *et al.*, 2001), followed by sprouting of new capillaries. The initial phase begins with increased vascular permeability and local degradation of the vessel wall (i.e., extracellular matrix (ECM) or basement membrane). The endothelial cells enter the tumor stroma, migrate toward a stimulus such as VEGF or FGF, and proliferate behind the leading edge. At this time, the cells may be most vulnerable to agents that interfere with their proliferation, since they lack protection from other cell types (Egginton *et al.*, 2000). The next step in vessel formation is recruitment of pericytes, followed by smooth muscle cells.

III. On/Off Switches for Angiogenesis: a Delicate Balance

Angiostatin is a fragment of a larger protein, plasminogen, with an activity distinct from the parent protein. It may be cleaved from the original precursor protein by enzymes under various conditions; normally, in cases where a pro-angiogenic stimulus first has promoted blood vessel development (such as during wound healing), which must then be inhibited when the desired level of vascularity has been achieved. Angiostatin has a short half-life of about 15 minutes (Gonzalez-Gronow *et al.*, 1990; DeMoraes *et al.*, 2001; Fortier *et al.*, 2001). Consistent with these results, exogenous administration of angiostatin at doses ranging from 15–300 mg/m^2/day showed rapid clearance of this protein and no side effects in a phase I clinical trial performed at Thomas Jefferson University (DeMoraes *et al.*, 2001; Fortier *et al.*, 2001). Based on animal studies, however, if administered continuously in addition to endostatin, metastasis may be discouraged from growth for prolonged lengths of time (Yokoyama *et al.*, 2000). Combination therapies with two new agents are necessarily slow to be tested in humans. Each compound has to be tested individually first in patients and, if they are only effective together, the potential efficacy may not be discernable in early trials. Recent combinations that have been studied in animal models include angiostatin with chemotherapy (te Velde *et al.*, 2002) and angiostatin with radiation (Mauceri *et al.*, 1998). Basic research is needed to understand mechanisms and predict which combinations will be most effective, so that trials can be done in the correct context, the right tumor types, and thus in patients that will benefit the most. Furthermore, deleterious side effects should

TABLE I
Antiangiogenic Agents

Angiogenesis inhibitors	Target	Trial phase	References
ABX EGF, Cl-1033, PKI-166, EGF vaccine, EKB-569, GW2016, ICR-62, EMD 55900, CP358, PD153035, AG1478	EGFR	I	Bier *et al.*, 1995; Schober *et al.*, 1995
IMC-C225 (Erbitux), ZD1839 (Iressa), OSI-774	EGFR	II/III	Robert *et al.*, 2001; Albanell *et al.*, 2002; Baselga *et al.*, 2002; Herbst *et al.*, 2002c; Ranson *et al.*, 2002
Erlotinib (tarceva)	EGFR	III	OSI Pharmaceuticals *et al.*, 2001
Angiostatin	ATP synthase on endothelial cells; intracellular pH regulation; angiomotin; annexin II	I	Moser *et al.*, 1999, 2001; Troyanovsky *et al.*, 2001; Wahl *et al.*, 2001, 2002a; Wahl and Grant, 2002
Arrestin, endostatin	$\alpha_1\beta_1$ on endothelial cells	No, I/II	Colorado *et al.*, 2000; Mundhenke *et al.*, 2001; Eder *et al.*, 2002; Herbst *et al.*, 2002a,b; Sudhakar *et al.*, 2003; Thomas *et al.*, 2003
BAY 12-9566 & w/fluorouracil or doxorubicin	Metalloproteinase inhibitor-2, -3, -9 (multiple target)	I	Heath *et al.*, 2001
Bevacizumab (avastin)	VEGF antagonist	II, III	Chen *et al.*, 2001
Carboxyamidotrizole, and w/paclitaxel	Inhibits endothelial cells calcium influx	I	Kohn *et al.*, 2001
Canstatin, EMD121974, S-24, vitaxin	$\alpha_v\beta_3$ integrin antagonist	I,I,I,I/II	Gutheil *et al.*, 2000; Eder *et al.*, 2002; Herbst *et al.*, 2002b; Thomas *et al.*, 2003
Dimethylxanthenone acetic acid	Unclear, induces TNF-α and nitric oxide I	No	Galbraith *et al.*, 2002
IM862	Activates NK cells	I	Tulpule *et al.*, 2000
Interleukin-12, interleukin-2	Induces CD8+ T-cell receptor & $\alpha\beta$+T cells	I	Brivio *et al.*, 2002
NM-3	VEGF inhibitor	I	Reimer *et al.*, 2002
HuMV833 PTK787, ZK22584	VEGF receptor antagonists	I	Jayson *et al.*, 2002; Turetschek *et al.*, 2002

RhuMab, Angiozyme (ribozyme)	VEGF receptor antagonists	II	Sandberg *et al.*, 2000; Gordon *et al.*, 2001
IMC-1C11	VEGFR-2 antagonists	I	Posey *et al.*, 2003
Neovastat, Marimastat, Prinomastat, BMS-275291, COL-3, MM1270	Matrix metalloproteinase inhibitors	I/II/III	Levitt *et al.*, 2001; Rudek *et al.*, 2001; Batist *et al.*, 2002; Shepherd *et al.*, 2002; Reber *et al.*, 2003
SU101, SU6668, SU11248	PDGFR, VEGFR, bFGF (multiple targets) endothelial cells/pericytes	I	Eckhardt *et al.*, 1999; Whatmore *et al.*, 2002; Bergers *et al.*, 2003
SU5416, with Paclitaxel, w/Gemcitabine & Cisplatin, and w/Irinotecan & Cisplatin and w/radiation	VEGFR-2 antagonists	II,III	Stopeck *et al.*, 2002
Razoxane	Topoisomerase II inhibitor	II	Braybrooke *et al.*, 2000
Squalamine lactate	Sodium/proton antiporter isoform III	I	Akhter *et al.*, 1999; Bhargava *et al.*, 2001
Tecogalan	Inhibits bFGF binding	I	Eckhardt *et al.*, 1996
Temozolomide & PEG interferon α2b	Unknown	I	Agarwala and Kirkwood, 2003
Tetrathiomolybdate	Anticopper agent	I,II	Brewer *et al.*, 2000; Redman *et al.*, 2003
TNP-470	Inhibits endothelial cell proliferation	I	Logothetis *et al.*, 2001
Thalidomide, CC-5013 (immunomodulatory derivative of thalidomide), and with taxotere	Unknown	I	Baidas *et al.*, 2000; Figg *et al.*, 2001; Short *et al.*, 2001; Daliani *et al.*, 2002; Escudier *et al.*, 2002; Gutheil and Finucane, 2002
Tumstatin	$\alpha_V\beta_3$ integrin antagonist	No	Sudhakar *et al.*, 2003
2-methoxyestradiol	Hypoxia inducible factor (HIF1α)	I	Lakhani *et al.*, 2003
VEGF trap	Decoy soluble VEGFR	I	Herbst *et al.*, 2002c

[Abbreviations: EGFR, epidermal growth factor receptor; ATP, adenosine triphosphate; VEGF, vascular endothelial growth factor; TNF, tumor necrosis factor; NK, natural killer; VEGFR, VEGF receptor; PDGFR, platelet-derived growth factor receptor; bFGF, bovine fibroblast growth factor.]

be predictable and avoidable if comparison between compartmental microenvironment and tumor microenvironment are made and the effects of these determinants on the drug activity are understood.

IV. Mechanisms of Action of Angiostatin

One major theme that has emerged from research done thus far on antiangiogenic compounds is that the extent of cell attachment to a matrix and the nature of the matrix are critical determinants of the compound's activity. This is a complex issue because tumor stroma is comprised of a variety of proteins, often in abnormal relative concentrations. Extensive literature indicates that, in addition to tumor cell modulation of stroma composition, stromal components impact tumor cells. This two-directional signaling is part of the malignant phenotype and often is regulated at the post-transcriptional level. This pioneering work, recently reviewed by Roskelley and Bissell (2002), has delineated the importance of tumor stroma in tumor cell behavior. Recent work in the field of angiogenesis has shown that many of the same microenvironmental factors are critical to the endothelial cell response to the tumor microenvironment and can determine the phenotype of these cells and the outcome of exposure to various stimuli (e.g., administration of exogenous angiostatin) (Wahl and Grant, 2002).

Angiostatin is an internal fragment of plasminogen and may contain either the first three (K1–3) or four (K1–4) kringle domains. A similar activity has been reported for kringle 5 (K-5) of plasminogen (Liu *et al.*, 2000; Gonzalez-Gronow *et al.*, 2003). Three receptors on endothelial cells have been reported for angiostatin to date. Adenosine triphosphate (ATP) synthase was the first receptor identified by Moser and colleagues (1999). The presence of this typically mitochondrial enzyme on the endothelial cell surface was somewhat surprising. Binding of angiostatin to surface-associated ATP synthase since has been confirmed by other research groups (Wahl and Grant, 2002; Arakaki *et al.*, 2003). Additional reports have confirmed the unexpected finding that many of the enzymes and components of the mitochondrial electron transport chain and ATP synthesis generating mechanisms are located on the plasma membrane of endothelial cells (Yegutikin *et al.*, 2002; Arakaki *et al.*, 2003). Two other potential target receptors of angiostatin that have been reported are angiomotin (Troyanovsky *et al.*, 2001) and integrin $\alpha_v\beta_3$ (Tarui *et al.*, 2001). The distribution of these receptors on the surface of endothelial cells may be located at sites so proximal to one another that angiostatin may be able to interact with more than one simultaneously. Alternatively, multiple targets could be implicated in binding to the different kringles of angiostatin as a function of receptor and peptide presentation.

Parameters affecting angiostatin's interaction with the ATP synthase receptor have not been completely elucidated but recent work indicates that the

synthase may be situated in a caveolar compartment (Moser *et al.*, 2001) and generates ATP on the cell surface (T.L. Moser, D.J. Cheek, J.A. Roy, T.A. Ashley, M.D. Goodman, A.E. Paradis, B. Li, D.J. Kenan, and S.V. Pizzo, unpublished results). It has been reported that one of angiostatin's receptors is proximal to focal adhesion kinase (FAK) and that binding catalyzes FAK phosphorylation in the absence of integrin clustering that usually is triggered by cell attachment to substrate (Claesson-Welsh *et al.*, 1998). This implies that FAK phosphorylation occurs under aberrant circumstances (i.e., when endothelial cells are not tightly attached to their normal substratum). In the usual scenario, attachment to a substrate such as fibronectin causes integrins to form tetramers. FAK is phosphorylated and organizes, allowing cytoskeletal elements to form structurally organized arrays to carry out cell spreading (Figures 1 and 2). In the tumor microenvironment, several parameters differ. Matrix composition includes collagen, laminin, and many other proteins (Canfield *et al.*, 1986; Baatout and Cheta, 1996; Grant and Kleinman, 1997; Aoudjit and Vuori, 2001). The pH and the oxygen levels are low and the degree of attachment is compromised because cells are rapidly dividing and moving into areas requiring a blood supply. This means that different integrin isoforms may be activated to differing extents

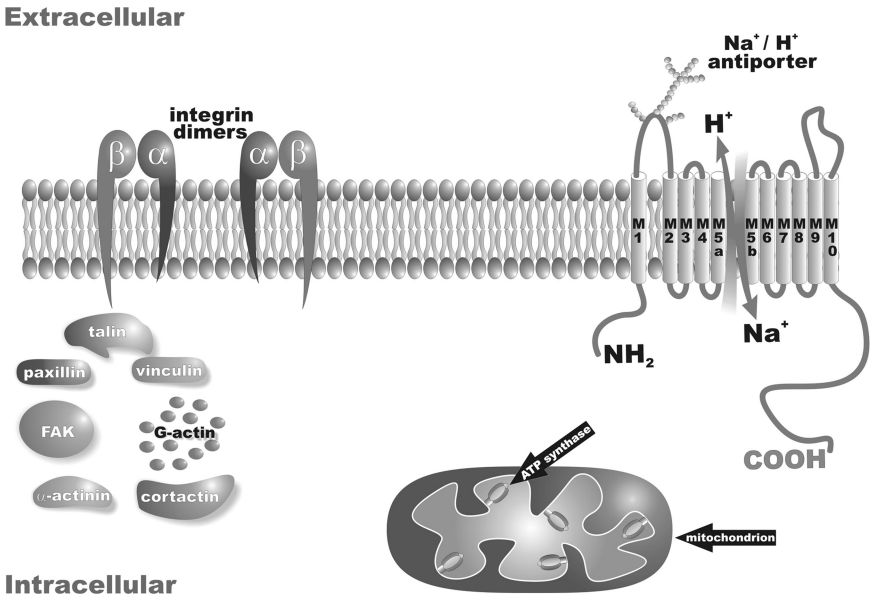

FIG. 1. Endothelial cell membrane in an unattached cell at normal pH (≈ 7.3). The integrin subunit are in $\alpha\beta$ dimers, the sodium proton antiporter (NHE) is minimally active, focal adhesion kinase (FAK) is unphosphorylated, cytoskeletal elements are unassembled, and adenosine triphosphate (ATP) synthase is active primarily within the mitochondria.

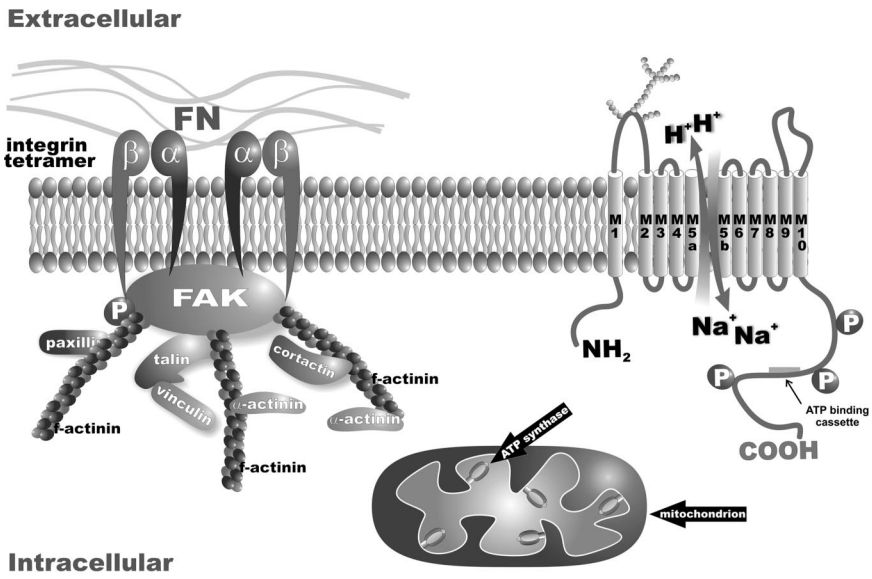

FIG. 2. Endothelial cell attached to fibronectin at normal pH (≈ 7.3). Integrins are clustered into tetramers in response to attachment to fibronectin, FAK is phosphorylated, NHE is more active, cytoskeletal elements are assembled to promote cell attachment and spreading, and ATP synthase remains in mitochondria.

(Schwartz *et al.*, 1991b; Agius *et al.*, 1996; Coopman *et al.*, 1996; Erdreich-Epstein *et al.*, 2000). Thus, the distribution of other molecules on the cell surface and their activities will be affected. When extracellular pH is low, typically the sodium/proton antiporter, isoform 1 (NHE-1), is activated to maintain intracellular pH in the viable range. This occurs as a function of attachment, phosphorylation, ATP binding, and the presence of growth factors in fibroblasts, where it has been studied in the most detail (Schwartz *et al.*, 1991a; McSwine *et al.*, 1994). It has been reported that migrating cells have NHE-1 clustering at the leading edge of lamellopodia in work done using fibroblasts (Grinstein *et al.*, 1993) and melanoma cells (Akasaka *et al.*, 1995) and that they co-localize in partially attached and moving cells with FAK (Schwartz *et al.*, 1991a).

We recently demonstrated that when coupled with extracellular acidification, angiostatin caused a precipitous decline in cytosolic intracellular pH (Wahl *et al.*, 2001,2002a; Wahl and Grant, 2002). Low extracellular pH could affect receptor levels, receptor distribution, angiostatin binding, and angiostatin conformation. ATP synthase distribution on the endothelial cell surface was reported to be more focal at low pH (Wahl and Grant, 2002). Receptor binding interactions with angiostatin as a function of extracellular pH currently are being studied in our laboratories. At normal extracellular pH, both ATP synthase and NHE-1 would

be less active, whether or not cells are attached. However, in the tumor microenvironment (Figures 3 and 4), there is less attachment to a variety of substrate proteins, a lack of integrin clustering, and aberrant FAK phosphorylation. Because of the low extracellular tumor pH, NHE-1 is activated and ATP synthase is activated and organized focally (Wahl and Grant, 2002). When angiostatin enters this scenario, it could bind to ATP synthase, which could, in turn, disrupt FAK, ATP synthesis, and the function of NHE-1. A model for how angiostatin binding affects endothelial cells in a tumor microenvironment — based on recent research, the literature, and conjecture — is shown in Figure 4.

V. Other Anti-angiogenic Agents

Other anti-angiogenic agents have been discovered or developed. In some cases, the receptors have been identified (Table I). However, little is known about how their signals are transduced, the basis for their selectivity, or their degree of

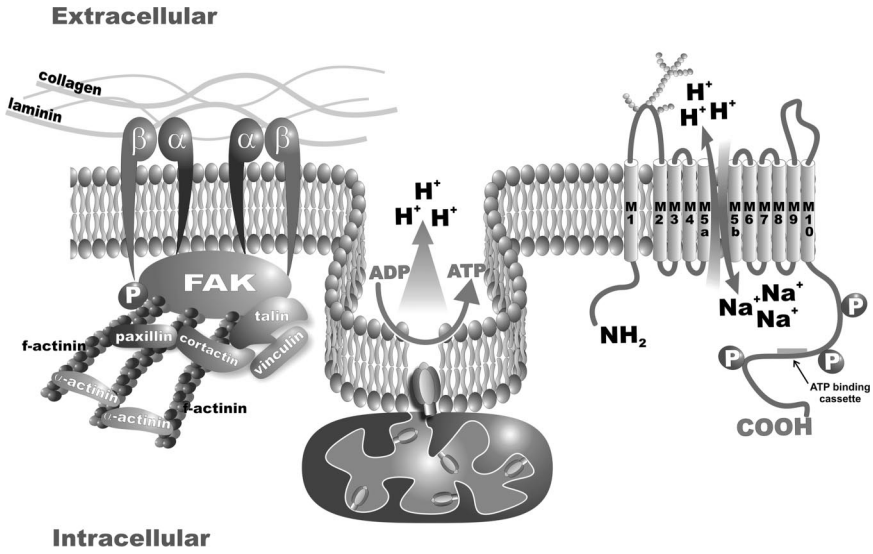

FIG. 3. Endothelial cell in a tumor microenvironment with a low and variable pH. Cells are partially attached to various matrix proteins, including collagen and laminin, different integrin isoforms cluster in tetramers, and ATP synthase appears in caveolae situated near FAK. This ATP synthase now generates ATP on the plasma membrane. FAK is phosphorylated. Extracellular pH is lower (6.7 on average) and migration of endothelial cells is required; therefore, NHE is very active, highly phosphorylated, and has ATP bound on the cytoplasmic tail. NHE molecules congregate at the leading edge of lamellopodia to generate a sodium gradient to bring about cell movement. Signals are transduced to cytoskeleton, which catalyzes a rearrangement of cytoskeletal elements. Cells then proliferate and can differentiate into tubular structures.

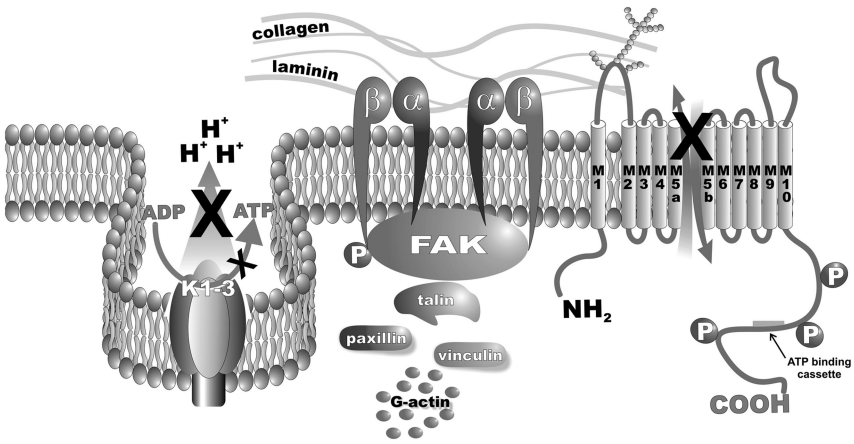

FIG. 4. Proliferating endothelial cells in the tumor microenvironment in the presence of angiostatin. Angiostatin binds to the catalytic subunit of ATP synthase. ATP synthesis ceases and inhibits the synthesis of extracellular ATP. Rapid proliferation loosens attachment of the cells, integrin tetramers dissociate into dimers, and NHE molecules disaggregate. FAK is phosphorylated (as in Figure 2) but under conditions where attachment is not being maintained. NHE is inhibited by lack of attachment, dephosphorylations, and loss of ATP. Intracellular pH decreases below 6.5 and metabolism ceases. The cell has received conflicting signals, which trigger a signal transduction cascade ending in apoptosis.

selectivity. Features of the tumor microenvironment that have been shown to be critical for demonstrating angiostatin activity are delineated below, emphasizing parameters that should be evaluated for other anti-angiogenic agents that may be useful to determine in which clinical scenario(s) each is likely to be most effective.

Some characteristic features of the tumor microenvironment may be shared by other improperly vascularized areas. This means that anti-angiogenic therapies could cause complications for cancer patients with diabetes mellitus. Drugs for treating tumors may have undesirable side effects in other tissues in diabetics to a greater extent than in nondiabetics. Some of these features have been described but have not been characterized fully in terms of application of anti-angiogenic agents. Differences between vasculature arising in different locations, in endothelial cells from the various tumor types, and in different ECM microenvironments are also areas where further basic research is warranted.

Some major parameters that need to be examined for each anti-angiogenic agent under consideration for clinical use are described in Section VI.

VI. Features of the Tumor Microenvironment: Potential Mediators of the Response of Tumor Stromal Endothelial Cells to Anti-angiogenic Drugs

A. VEGF & FGF

VEGF and FGF are two growth factors secreted by metastatic or primary tumor cells to encourage new vessel branch development. These growth factors are used as markers for vessel growth. In the first phase I clinical trial of angiostatin at Thomas Jefferson University, the protocol was designed to administer daily doses of intravenous (IV) angiostatin for 2-week intervals, separated by 1-week interruptions. Both VEGF and FGF rebounded rapidly in the circulation just 1 day after daily dosing stopped after each dosing interval (DeMoraes *et al.*, 2001). This made it evident that daily dosing without interruption would be necessary to maintain the effects of angiostatin. It indicated that agents with a slower clearance time would probably be more practical clinically. Table I lists some anti-angiogenic agents currently in clinical trial. If these are expressed in excess and/or at inappropriate times, hypervascularization could occur.

B. ENDOGENOUS INHIBITORS

Other endogenous inhibitors of angiogenesis have been reported, including angiogenin (Fett *et al.*, 1985), endostatin (O'Reilly *et al.*, 1997), thrombospondin-1 (Nicosia and Tuszynski, 1994), interferons (Dinney *et al.*, 1998), platelet factor-4 (Nicosia *et al.*, 1994), and 16 kDa prolactin (Hanahan and Folkman, 1996). Numerous other inhibitors, both naturally occurring and synthetic, are listed in Table I. Their relative abundance can play a role in the overall picture in various body compartments. Angiostatin has proven to be one of the most potent. *In vitro*, angiostatin is relatively specific for tumor endothelial cells and inhibits their proliferation (Moser *et al.*, 1999), migration (Moser *et al.*, 1999), tube formation (Moser *et al.*, 1999; Wahl *et al.*, 2002a), vessel network formation in the embryonic body model (Eriksson *et al.*, 2003), and formation of sprouts from mouse aortic rings (Hajitou *et al.*, 2002). Exceptions to this specificity include a published study on smooth muscle cells (Walter and Sane, 1999) and one on neutrophils (Benelli *et al.*, 2002). *In vivo*, however, angiostatin inhibits primary tumor metastasis in mice (O'Reilly *et al.*, 1994b), vascular proliferation in the chick chorioallantoic membrane (CAM) assay (Gately *et al.*, 1996), and neovascularization in the corneal pocket assay (White *et al.*, 2003).

Angiostatin is found in normal human plasma at a concentration of $\approx$ 6–12 nM (Soff *et al.*, 1999). The concentration of angiostatin in the urine of cancer

patients is significantly higher than in other patients (Cao *et al.*, 2000). Elevated levels of angiostatin have been observed in the ascites fluid from ovarian cancer patients as well (Yokoyama *et al.*, 2000). More recently, angiostatin was found to be elevated during impaired production of nitric oxide in coronary angiogenesis (Matsunaga *et al.*, 2002) and in the bronchoalveolar lavage fluids from patients with acute respiratory distress syndrome (ARDS) (Lucas *et al.*, 1998). These findings may indicate that angiostatin functions not only as a tumor-specific inhibitor but also as a regulator of angiogenesis in other scenarios.

C. PERICYTES & SMOOTH MUSCLE CELLS

Pericytes and smooth muscle cells are recruited to surround the endothelial cell tubes in developing blood vessels, so reduced numbers of them could affect the levels of various stimulators and inhibitors to which endothelial cells are exposed. Platelet-derived growth factor (PDGF) plays a major role in pericyte recruitment. When vessels initially form, their vulnerability may be maximal in part because these other cell types are not there to provide a mechanical barrier. A recent study indicates that retinal capillary coverage by pericytes was crucial for survival of endothelial cells in retina subjected to hypoxic conditions, particularly under the stress conditions of diabetes (Hammes *et al.*, 2002). Pericytes also secrete similar growth factors and thus modulate the ECM (Allt and Lawrenson, 2001). Smooth muscle cell proliferation also is inhibited by angiostatin (Walter and Sane, 1999).

D. INTRACELLULAR & EXTRACELLULAR pH

Both intracellular and extracellular pH affect angiostatin activity. The average extracellular pH (5.6–7.6) in tumors is lower and more variable than in normal tissue (7.2–7.6), yet tumors have a normal average intracellular pH (Yamagata and Tannock, 1996). It has been reported that angiostatin profoundly affects intracellular pH in endothelial cells (Wahl and Grant, 2002; Wahl *et al.*, 2002a). This effect is manifested only at low extracellular pH. This implicates pH-regulating transporters that are activated at low extracellular pH as a potential class of targets for anti-angiogenic agents in areas of tumors where extracellular pH is low. These potentially include NHE and the H^+-linked monocarboxylate transporter (MCT).

E. ATP SYNTHASE RECEPTOR LEVELS

Modulation of angiostatin's receptor levels may be a determinant of angiostatin activity. Research has shown that ATP synthase distribution can be altered as a function of ECM composition and extracellular pH. When cells are allowed to attach to fibronectin, histochemical assays using a primary antibody

directed against the β subunit of ATP synthase showed no detectable enzyme on the cell surface. However, when cells were plated on Matrigel to simulate the tumor stroma, the enzyme was revealed on the cell surface (Wahl and Grant, 2002). When cells at normal and low extracellular pH were compared, the distribution at low pH was more punctate. This was likely related to the organization of focal adhesion plaques and how they assemble for migration (Wahl and Grant, 2002). Other microenvironmental parameters have not been evaluated in terms of this receptor and these factors have not been evaluated for other anti-angiogenic agents.

F. HYPOXIA

Hypoxia often — but not always — goes hand in hand with acidity, although there is some disparity in spatial distribution (Vaupel *et al.*, 1989,1998; Brizel *et al.*, 1996; Gullege and Dewhirst, 1996; Helmlinger *et al.*, 1997). These two features have not been teased apart experimentally to determine the relative importance of each and the degree of overlap. Most studies thus far are either conducted at low pH or done under hypoxic conditions but not both. Future studies with new anti-angiogenic agents, such as 2-methoxyestradiol (2ME2) or Panzem ® (Entremed, Inc.), a hypoxia-inducible factor (HIF)-1 α inhibitor (Mabjeesh *et al.*, 2003), will help determine the extent to which targeting this receptor will be useful in a variety of tumor types. There is evidence that hypoxia is a feature of diabetic complications such as renal disease (Ries *et al.*, 2003), poor circulation in lower extremities (Fife *et al.*, 2002), and retinopathy (Drasdo *et al.*, 2002).

G. BICARBONATE STATUS

Bicarbonate (HCO_3^-) is a parameter that has not been considered in most *in vitro* work but should receive more attention. In the tumor microenvironment, catabolite removal by inefficient and abnormal blood vessels is poor, resulting in CO_2 buildup (Newell *et al.*, 1993; Helminger *et al.*, 2002). Both these aspects contribute to the low pH environment. In some work at low pH, the pH is lowered by altering sodium bicarbonate concentration (Chu and Dewey, 1988; Chu *et al.*, 1990; Wahl *et al.*, 1996; Owen *et al.*, 1997). In this type of experiment, the buffering capacity of the medium will be low, causing more potential extracellular pH fluctuation. Furthermore, this approach has two experimental variables, pH and bicarbonate, without distinction as to which is causing the effects on cells in the tumor microenvironment. Research studies have been performed where bicarbonate is maintained at 26 mM and the incubator CO_2 is raised to 17% from 5%, to create acidic and tumor-like conditions (Wahl and Grant, 2002; Wahl *et al.*, 2002a,b). This is an appealing model, since physiologic bicarbonate is 26 mM and the tumor is high in CO_2, which is a likely cause of elevated carbonic

anhydrase levels in tumors (Beasley *et al.*, 2001; Giatromanolaki *et al.*, 2001; Koukourakis *et al.*, 2001; Olive *et al.*, 2001; Bui *et al.*, 2003; Swinson *et al.*, 2003). It has been proposed that carbonic anhydrase inhibitors could relax pericytes, cause vasodilation, and enhance blood supply to the retina by increasing intracellular pH and decreasing extracellular pH (Reber *et al.*, 2003). It would be even more physiologic to study tumor cell behavior in a hypoxic chamber inside a 17% CO_2 incubator, to further mimic the part of the tumor microenvironment most likely to promote new blood vessel branch growth. Evaluation of low extracellular pH, low oxygen, and the combination in terms of therapeutic responses to agents and phenotype of cells in such environments should help characterize responses in the tumor microenvironment more completely.

H. LACTIC ACIDOSIS

Another reason, other than high CO_2 and poor perfusion, for the acidic microenvironment in tumors is excessive production of lactic acid (Vaupel *et al.*, 1989; Walenta *et al.*, 1997; Brizel *et al.*, 2001; Wahl *et al.*, 2002b). One indicator of the contribution of lactic acidosis is measuring levels of the transporter used to remove it from the cell. This transporter, MCT, is elevated in human melanoma (Wahl *et al.*, 2002b) and may be elevated in other tumors of neural crest origin, including brain tumors. MCT levels are elevated in the retina of diabetics, indicative of elevated lactic acid in that compartment (Knott *et al.*, 1999). Intracellular and extracellular pH levels, functionality of other transporters used to regulate intracellular pH, and degree to which MCT is elevated may be critical to understanding the degree to which a pH regulation inhibitor can affect a certain cell type and to which angiostatin can affect intracellular pH in endothelial cells in the low pH tumor microenvironment. Intracellular and extracellular pH measurements in normal and pathologic body compartments aside from the tumor microenvironment will help address the relative importance of these parameters. In addition, a relationship between areas of hypoxia and acidity is likely, although the relative importance of each has not been addressed in any one study.

I. GLUCOSE, HYPERGLYCEMIA & ACIDIFICATION

Oral glucose is used clinically to lower tumor pH before thermoradiotherapy (Thistlethwaite *et al.*, 1987; Leeper *et al.*, 1994,1998; Engin *et al.*, 1995). The mechanism is via increased glycolysis in the tumor microenvironment, producing lactic acid (Burd *et al.*, 2001). In diabetes, glucose overload may occur, so the patient's tissues already may be more acidic. This could lead to potentiation of angiostatin's activity in the body, since its activity is greatest when pH is low (Wahl and Grant, 2002; Wahl *et al.*, 2002a). Other enzymes relating to glycolysis may impact this axis, including lactate dehydrogenase (Koslowski *et al.*, 2002),

glut-1 receptor levels (Thews *et al.*, 2003), and hexokinase levels (Wachsberger *et al.*, 2002). In the diabetic, it is believed that complications arise to a greater extent in patients with the most poorly managed glucose levels (DCCT Research Group, 1993), although this is still a matter of considerable debate (Craighead, 1994). Individual variations in glycation (non-enzymatic glycosylation) of proteins are not only affected by degree of management but also by other unknown parameters. Facchiano and coworkers (2002) have reported that FGF (isoform 2) is more glycosylated in diabetic mice and is less chemotactic and pro-angiogenic as a result.

J. MATRIX

Matrix composition could be used to distinguish physiologic compartments, if qualitative and/or quantitative differences can be characterized in nondiabetics and diabetics with and without cancer. With respect to angiostatin, research indicates that the vulnerability of endothelial cells in the tumor microenvironment stems in part from the fact that when they are rapidly proliferating, their attachments are compromised. Furthermore, substrates they come in contact with in the tumor microenvironment differ, leading to different integrin isoform expression, with subsequent differences in signal transduction. This would contribute to the explanation of why mature vessels appear to be unaffected by angiostatin. It also would explain the targeting of endostatin to integrins (Sudhakar *et al.*, 2003), which then would be less apt to be engaged in attachment. Migration of tumor cells is often along the ECM of the basal lamina. When mice are injected with a melanoma cell line selected for metastatic seeding in the lung, the cells migrate specifically and secondary lung tumors are formed. When they are inhibited from binding fibronectin or laminin, over 90% of the cells fail to reach the lungs (Humphries *et al.*, 1986). In order to enter a blood vessel, tumor cells lyse the collagenous matrix by secreting various plasminogen activators. Antibodies directed against plasminogen activators have been shown to inhibit metastasis (Ossowski and Reich, 1983). The urinary-type plasminogen activation complex (u-PA) is one of the major regulators of ECM remodeling. u-PA converts plasminogen to plasmin, which degrades matrix and indirectly activates other metalloproteinases (Vassalli *et al.*, 1991). Enriched levels of u-PA and its receptor (u-PAR) are found on the leading edge of migrating cells. Plasminogen activator inhibitor type 1 (PAI-1), a u-PA antagonist, mediates cell adhesion and spreading by forming a bridge between the cell surface and the matrix directly regulating adhesion (Planus *et al.*, 1997). u-PA expression has been correlated with angiogenesis and poor prognosis (Kaneko *et al.*, 2003).

Matrix metalloproteinases (MMP) are enzymes that digest/degrade matrix proteins, enabling metastasizing cells to migrate. Matrix metalloproteinase inhibitors (MMPI) exist in a balance and can be offset in pathologic conditions

(Spranger *et al.*, 2000). Another factor warranting further study is the composition of matrix in various locations, which will affect the degree of enzyme activity needed to impact upon the matrix. A third consideration is that enzymatic alteration of matrix composition may affect where tumor cells disseminate and may cause digestion of other pathologic matrices in nearby or distant areas. This issue may require consideration in glomerulosclerosis and other complications of diabetes in which basement membrane thickening is a feature. Finally, MMP/MMPI balance may affect wound healing, an important consideration for patients that may need surgery during their course of treatment (Lockhart *et al.*, 2003).

K. TUMOR VESSEL ARCHITECTURE

Tumor blood vessel branches are inefficient and tortuous. In tumor vessels, backflow often occurs, mixing arterial and venous blood, and vessels leak (Secomb *et al.*, 1993; McDonald and Baluk, 2002). Some of these characteristics are potentially exploitable by anti-angiogenic agents and antivascular agents, again, hopefully, with some specificity for the uniquely abnormal features of these vessels. More studies are needed to characterize vasculature in different tumor types, as a function of different body locations of metastasis, and the degree of resemblance to other vasculature in normal and pathologic instances that can coexist in patients.

VII. Models Used to Study Angiogenesis in Various Tissues

A. *IN VITRO* MODELS

Many cell types are used to study angiogenesis from macrovascular vessels, microvascular vessels, various locations in the body, and from various animal species. Although little information is available about the differences between these various cell types, these differences are extremely important if research findings are to be translated into practical, predictive information. Models include the tube differentiation assay (Kubota *et al.*, 1988; Grant *et al.*, 1990,1995; Grant and Kleinman, 1997), the migration/scratch assay, and the rat aortic ring assay (Nicosia and Ottinetti, 1990; Grant and Kleinman, 1997). For angiostatin, data have been confirmed in all three of these models (Wahl *et al.*, 2002a). However, for other agents, one system may demonstrate activity more than another, which will lead to elucidation of what models may be most useful for which agents or diseases. Physiologic models are critical to getting meaningful results.

B. ANIMAL MODELS

Several animal models are available to study angiogenesis, including the corneal pocket (Muthukkaruppan and Auerback, 1970; O'Reilly *et al.*, 1994b; Sood *et al.*, 1999), the chick embryo (Risau and Lemmon, 1988; O'Reilly *et al.*, 1994b; Eriksson *et al.*, 2003), implantation of human cells into a mouse or a rat (xenografts) in various locations (Danielsen and Rofstad, 2000; Shan *et al.*, 2003), and the angioreactor model (Guedez *et al.*, 2003). Which are best to model which tumor types and clinical scenarios? Considerations include the fact that blood vessels in a human xenograft usually are a combination of human and animal vessels. Tumors often are placed in animals in locations other than the ones where they naturally occur. Model evaluation is critical to determining which one may be most physiologically meaningful. A knowledge of the pathogenesis of the particular type of cancer, the degree of vascularity it possesses, and the microenvironment of the location into which it will be grafted are all critical considerations.

C. DESIGN OF CLINICAL TRIALS: TIMING IS EVERYTHING

In the angiostatin phase I clinical trial at Thomas Jefferson University, wound healing was normal in a patient needing acute unexpected surgery while on angiostatin (DeMoraes *et al.*, 2001). Other anti-angiogenic agents may differ, however, so this must be considered, preferably prior to introduction of agents in the clinic. Another consideration in trials is that the typical measure of efficacy — tumor growth delay assessments — may not be perfectly applicable to anti-angiogenic drugs. Some are initially slow to show responses (D'Amato *et al.*, 1994). Others may cause necrosis followed by swelling, which is mostly inflammatory cells and fluid. Thus, even though a large part of the tumor mass is killed, the initial effect is an apparent increase in tumor size (Kaban *et al.*, 1999). Most traditional therapies are directed at achieving maximum results during a brief window of opportunity, while most anti-angiogenic therapies will need to be administered chronically and indefinitely to prevent blood vessel branches from sprouting (Kaban *et al.*, 1999). Therefore, animal model experiments will have to be designed to take these differences into account.

The parameters described above are emerging as important for cancer pathogenesis and treatment outcome, yet most are not considered during experimental work on anti-angiogenic drugs. Conversely, when pro-angiogenic drugs are administered for other conditions, patients with neoplastic disease may have counter-indications. Some of these drugs are listed in Table II.

VIII. Diabetes

Some sequelae of diabetes mellitus include impaired wound healing, characterized by both spontaneous and wound-induced ulcerations, particularly of the

TABLE II
Pro-angiogenic Agents

Drug	Target	Approved or potential use	References
Regranex (becaplurmin)	Unknown	Diabetic foot ulcers (Ortho-McNeil Pharmaceuticals)	Fruhstorfer, 2000
SIKVAV	Unknown	Cardiovascular ischemia prevention	Grant and Zukowska, 2000
Angiopoietin I	Tie-2 on endothelial cells	Cardiovascular ischemia prevention	Cascone *et al.*, 2003; Fiedler *et al.*, 2003
Angiopoietin II	Tie-2 on endothelial cells	Cardiovascular ischemia prevention	Fiedler *et al.*, 2003; Zhang *et al.*, 2003
Neuropeptide Y	NPY2	Wound healing	Grant and Zukowska, 2000; Ekstrand *et al.*, 2003; Lee *et al.*, 2003

lower limbs, and retinopathy. Both these conditions are consequences of insufficient perfusion. If limbs become acidotic and hypoxic, why don't they invoke the growth of new and normal blood vessels? Do they produce compounds that antagonize angiogenesis (e.g., angiostatin, endostatin) in higher amounts than those that promote it (e.g., VEGF, FGF) initially, then not turn off those signals at the right time? If so, exogenous administration of either type of compound could make things worse. With retinopathy, hypervascularization occurs at later stages, where there could be a positive impact from anti-angiogenic therapy. All potential side effects should be evaluated in diabetics, whether or not they have complications, since the eventual complication rate is high. Those that are known to have a vascular component are discussed in the following sections.

A. RETINOPATHY

Retinopathy, followed by macular degeneration, develops in 50–98% of diabetics within 15 years of diagnosis and is the most-devastating complication. It is characterized by basement membrane thickening, pericyte degeneration, microaneurysm formation, and focal capillary closure and acellularity, with subsequent abnormal proliferation of endothelial cells. Although laser treatment can prevent blindness in most cases, vitreous cavity bleeding and retinal detachment are problematic and preventative therapies would be more desirable (Danis *et al.*, 2001).

Among those issues being investigated are the effects of chronic intracellular hyperglycemia and abnormal glycation (Kaban *et al.*, 1999; Noma *et al.*, 2002; Verstappen *et al.*, 2003), disturbances in the polyol pathway (Funatsu *et al.*, 2003), and protein glycation (Funatsu *et al.*, 2002). All sugars, including D-glucose and D-galactose, can initiate protein glycation. They produce stable adducts that accumulate on long-lived biological molecules such as hemoglobin and proteins of the extracellular matrix (e.g., collagen). The rate of this reaction increases with glucose concentration. When basement membranes were glycated heavily *in vitro*, increased proliferation of endothelial cells and decreased proliferation of pericytes was reported (Kalfa *et al.*, 1995; Beltramo *et al.*, 2003). Synergistic effects of diabetes and hypertension have been reported in an animal model and increased number of caveolae in endothelial cells and pericytes, basement membrane thickening, and decreased cell-cell contact (Hillman *et al.*, 2001). As the most metabolically active tissue in the body, the retina has a high demand for oxygen. In diabetes, capillaries supplying the retina may become clogged, resulting in oxygen deprivation (Barinaga, 1995). The retina then produces VEGF but the vessels don't form in a proper or orderly manner. A similar process happens in premature infants. If this abnormal development could be stopped or re-directed, the blindness that often ensues could be preventable. One successful approach to therapy for retinopathy is photocoagulation. After this therapy was administered to diabetic retinas, endogenous angiostatin release was detected, which likely contributes to the success of the treatment (Spranger *et al.*, 2000). Some studies have linked high production of VEGF and low production of endostatin with severity of diabetic retinopathy (Noma *et al.*, 2002), diminished benefits from surgical intervention to treat cataracts (Funatsu *et al.*, 2003), and increased risk of macular edema (Funatsu *et al.*, 2002). Inflammatory mechanisms may influence retinal neovascularisation. Induction of cyclooxygenase-2 inhibitors has been shown to inhibit it by antagonizing prostaglandin E2 (Sennlaub *et al.*, 2003).

B. ULCERATIONS AND DRY GANGRENE

Ulceration and dry gangrene occur in diabetes due to insufficiency of circulation to the lower limbs. What is the mechanism by which this hypovascularity develops? Is there a lack of pro-angiogenic cues or responses to them or counterbalancing anti-angiogenic factors? Recently, Regranex (Ortho MacNeil Pharmaceuticals, Raritan, NJ) was approved for use in combating circulation impairment in the lower extremities. It is important to keep in mind that if a person had an undiagnosed cancer at the time of treatment, his/her malignant condition could be made worse as a result of treatment.

C. OTHER COMPLICATIONS

Other complications in which the role of angiogenesis is unknown include glomerulosclerosis, necrotizing papillitis, focal demyelination, and peripheral neuropathy. Peripheral neuropathy is also a side effect of some chemotherapeutic drugs (Verstappen *et al.*, 2003); thus, alternatives to these should be prescribed for diabetic cancer patients. Neuropathy may be related to the polyol pathway, in which aldose reductase and sorbitol dehydrogenase are the two key enzymes that handle excess glucose by converting it to sorbitol (Oates, 2002). Sorbitol toxicity has been implicated in neuron, kidney, and retinal damage. It may act by creating an osmotic gradient leading to swelling or have direct toxicities (Craighead, 1994). Diabetics have impaired wound healing, increased rejection of transplanted organs, and impaired formation of coronary collaterals, all of which have the lack of angiogenesis in common.

IX. Understanding the Basis for Selectivity Is the Key to Prudent Trial Design

Anti-angiogenic therapies for cancer patients are most desirable if they don't inhibit wound healing. Conversely, development of pro-angiogenic drugs for diabetics that work well in the retina and wounds but don't stimulate tumor growth is needed. Combination therapies that aren't antagonistic can be developed only if there is detailed analysis of the microenvironment and cell types in the different physiologic and pathologic compartments of the patient, so the desirable selectivity can be achieved and undesirable side effects can be avoided. The key is selectivity as a function of cell type, microenvironment, and for the various pathologies in which abnormal vascularity is a feature.

Much remains to be learned about the mechanisms for selectivity of anti-angiogenic agents in the tumor microenvironment and other locations. There is evidence that the tumor microenvironment is acidotic, hypoxic, and pro-angiogenic, so it could follow that pH or oxygen elevation could enhance effects in desired locations for pro-angiogenesis. Could impairment of intracellular pH regulation be causative in the etiology of the reduced perfusion in the limbs of diabetics? If it is compromised, how? There may be abnormalities in pH regulation in the diabetic retina or the ulcerative wound. If so, could counteractive drugs be developed that stimulate ion transport and encourage angiogenesis? Another aspect of drug potency is pH-dependent activity. Many features must be taken into account to understand the interactions that will lead to a positive, negative, or no effect for each agent.

X. Conclusions

In summary, concepts are emerging from research reported on the mechanism of action and basis for specificity of angiostatin. The data indicate that the

many new anti-angiogenic agents that have been developed and tested need to be characterized in terms of microenvironmental influences that affect their activity and other undesirable actions in other types of tissue, in patients with and without secondary pathologies. This chapter delineates some of activity parameters for this class of agents that are needed to enable effective translation to the clinic, with predictive information regarding harmful side effects, so that patients at risk can be handled accordingly.

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