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PROSPECTS

Angiostatin's Molecular Mechanism: Aspects of Specificity and Regulation Elucidated

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Abstract Tumor growth requires the development of new vessels that sprout from pre-existing normal vessels in a process known as ''angiogenesis'' [Folkman (1971) *N Engl J Med* 285:1182–1186]. These new vessels arise from local capillaries, arteries, and veins in response to the release of soluble growth factors from the tumor mass, enabling these tumors to grow beyond the diffusion-limited size of approximately 2 mm diameter. Angiostatin, a naturally occurring inhibitor of angiogenesis, was discovered based on its ability to block tumor growth in vivo by inhibiting the formation of new tumor blood vessels [O'Reilly et al. (1994a) *Cold Spring Harb Symp Quant Biol* 59:471–482]. Angiostatin is a proteolytically derived internal fragment of plasminogen and may contain various members of the five plasminogen ''kringle'' domains, depending on the exact sites of proteolysis. Different forms of angiostatin have measurably different activities, suggesting that much remains to be elucidated about angiostatin biology. A number of groups have sought to identify the native cell surface binding site(s) for angiostatin, resulting in at least five different binding sites proposed for angiostatin on the surface of endothelial cells (EC). This review will consider the data supporting all of the various reported angiostatin binding sites and will focus particular attention on the angiostatin binding protein identified by our group: F_1F_O ATP synthase. There have been several developments in the quest to elucidate the mechanism of action of angiostatin and the regulation of its receptor. The purpose of this review is to describe the highlights of research on the mechanism of action of angiostatin, its' interaction with ATP synthase on the EC surface, modulators of its activity, and issues that should be explored in future research related to angiostatin and other anti-angiogenic agents. J. Cell. Biochem. 96: 242–261, 2005.

Key words: angiogenesis; angiostatin; ATP synthase; endothelial cells

Folkman developed the hypothesis that naturally occurring regulators of angiogenesis exist in vivo and comprised both angiogenic and angiostatic factors [Folkman, 1971]. Folkman's group also identified many of these factors, including the first two described angiostatic proteins: angiostatin [O'Reilly et al., 1994b] and endostatin [O'Reilly et al., 1997]. As tumors grow in size, their oxygen and nutrient exchange requirements exceed the diffusion capacity of the adjacent vasculature, resulting in local hypoxia and acidosis. The hypoxia inducible factor (HIF-1) is in turn activated, resulting in the transcriptional activation of a series of growth factors and enzymes that stimulate local blood vessels to sprout branches towards the hypoxic tumor. These new vessels are architecturally and functionally different from normal blood vessels. They are exceptionally permeable [Dvorak et al., 1988] and tortuous; with blind ends, incomplete drainage, and backflow [Secomb et al., 1993; Kimura et al., 1996]. These vessels, therefore, often mix arterial and venous blood [Kallinowski et al., 1988; Vaupel et al., 1989, 1998; Vaupel, 1997], making them poor at catabolite removal.

Abbreviations used: BAE, bovine arterial endothelial cells; EC, endothelial cells; FGF, fibroblast growth factor; HUVEC, human umbilical vein endothelial cells; MRI, magnetic resonance imaging; MCT, H^+-linked monocarboxylate transporter; MMP, matrix metalloproteinase; NHE, sodium proton antiporter; U-PA, urinary type plasminogen activation complex; VDAC, voltage-dependent anion channel; VEGF, vascular endothelial growth factor.

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Angiostatin and endostatin are endogenously produced in the tumor stroma through the action of proteinases that are induced as part of the angiogenic cascade [Vassalli et al., 1991]. These internal cleavage fragments of plasminogen and collagen XVIII, respectively, therefore exemplify a theme in vascular biology that a given signal initiates a cascade that generates both positive and negative regulators of angiogenesis. Thus, the proteinases that are induced by pro-angiogenic signals lead to the production of signals that ultimately oppose the angiogenic response by cleavage of proteins that play critical regulatory roles in the angiogenesis-related processes of fibrinolysis (plasminogen) and vascular remodeling (collagen XVIII). Herein lies the answer to the teleological question, "Why would a tumor generate a signal that could compromise its blood supply?" The answer is that the angiostatic response is not tumor-specific, but is a consequence of the normal regulatory mechanisms of angiogenesis that couple pro-angiogenic signaling to the induction of an opposing response. It is clear that such an obligatory coupling of pro- and anti- angiogenic signaling is advantageous from an evolutionary viewpoint, since excessive angiogenesis is associated with a variety of disease states [Folkman, 1995; Carmeliet, 2003] and unbridled angiogenesis would compromise normal tissue architecture in growth, morphogenesis, and wound healing. Thus, angiostatin is generated through the actions of matrix metalloproteinases that are activated by pro-angiogenic signaling cascades such as HIF-1 induction and that in turn cleave one of the most abundant protein in blood—plasminogen—to generate a series of internal cleavage products exhibiting activities that oppose the angiogenic response. In a very real sense, production of angiostatin by tumors is a necessary consequence of the activation of pro-angiogenic cascades.

Folkman and his colleagues have put forth the concept of an "angiogenic switch," in which the net drive towards angiogenesis or angiostasis at a given anatomic site is dependent on the sum of the angiogenic signals balanced against the sum of the angiostatic signals. An important extension of the angiogenic switch model is that the switch may be tripped in the proangiogenesis direction at the site of a primary tumor, but in the opposite angiostatic direction at the site of distant metastases. This phenomenon has been described by Folkman and others who, in the surgical treatment of certain cancers, have observed a surge in growth of metastatic tumors following removal of a large primary tumor [O'Reilly et al., 1994b]. Experimental models suggest that the underlying mechanism hinges on differences between the balance of angiogenic and angiostatic signaling at local versus distant sites [Holmgren et al., 1995; Holash et al., 1999; Funatsu et al., 2003]. It is important to note that these models establish that the rapidly growing metastases derive not from seeding of tumor cells at the time of resection of the primary tumor, but rather from pre-existing micrometastases that were somehow held "in check" by the presence of the primary tumor but which are released to grow upon removal of the tumor. Within the primary tumor, a relative abundance of angiogenic signaling molecules trips the angiogenic switch in favor of angiogenesis, thereby supporting growth of the tumor. Angiostatic molecules are also made, but not in sufficient quantities to block angiogenesis at the local site. However, the angiostatic molecules may have a longer half-life in circulation, thereby favoring turning off the angiogenic switch at sites distant from the primary tumor. Upon removal of the primary tumor, these angiostatic factors become much less abundant, enabling the pro-angiogenic factors produced by the metastatic tumors to dominate the angiogenic switch and drive neovascularization of these distant tumors.

The discovery of angiostatin and other angiostatic molecules was met with great excitement because it seemed likely that exogenous administration after primary tumor removal could prevent metastatic growth and progression indefinitely [O'Reilly et al., 1994a, 1997]. Angiostatin alone can maintain metastases in a dormant state in laboratory animals when administered exogenously. Moreover, angiostatin was shown to shrink primary tumors [O'Reilly et al., 1994a,b, 1996]. One barrier to angiostatin therapy is that this polypeptide has a short half-life in the circulation, thus continuous administration is necessary to maintain its effect. The importance of continuous dosing was also observed in the first phase I human clinical trial of angiostatin at Thomas Jefferson University Hospital (Philadelphia, PA). The protocol was to administer daily doses of intravenous angiostatin for 2-week intervals,

separated by 1-week interruptions to evaluate toxicity. Both VEGF and FGF rebounded rapidly in the circulation in less than 1 day each time daily dosing was halted [DeMoraes et al., 2001; Fortier et al., 2001]. These findings strongly suggested that uninterrupted daily dosing would be necessary to maintain the tumor inhibition effects of angiostatin. These results also suggest that agents with a slower clearance time will have greater clinical utility. Other barriers to using angiostatin as a therapeutic agent include difficulties in production and the notorious problems with maintaining activity in storage and transport [Gonzalez-Gronow et al., 2005]. Therefore, identification of angiostatin receptors responsible for its anti-angiogenic effects have been a high priority so that angiostatin-mimetics can be developed. The goal is to develop alternative therapeutic molecules that bind the same receptor(s) with the same activities as angiostatin but that are more stable, easier to produce, and have better pharmacokinetic properties. Research on the mechanism of action of angiostatin delineates some of the parameters affecting its activity that should guide the development of better angiostatic agents for the treatment of cancer.

Before discussing the cellular targets that mediate angiostatin activity, it is important to acknowledge that different forms of angiostatin exist and that different investigators have reached different conclusions using different angiostatins. This complexity is related to the various cleavage sites that exist within plasminogen as well as the various pathways that can produce plasminogen cleavage fragments in different contexts. As shown in Figure 1, plasminogen contains five kringle domains, each of which contains three characteristic disulfide bonds. The kringles are numbered starting from the amino terminus, with kringles 1–3 shown in orange, kringle 4 in red, and kringle 5, closest to the carboxy terminus, in purple. In brief, plasminogen is cleaved in the presence of plasminogen activators to form plasmin [Gately et al., 1996] reduced in the presence of phosphoglycerate kinase [Lay et al., 2000b], cleaved to form angiostatin kringles 1–4.5 by plasmin or other serine proteinases, and then differentially cleaved to either angiostatin kringles 1–3 or 1–4 by the action of various matrix metalloproteinases [Lay et al., 2000a]. Kringle 5 is not generated by this pathway. In an alternative pathway, plasminogen is cleaved by neutrophil elastase to generate angiostatin kringles 1–3, free kringle 4, and so-called "miniplasminogen," consisting of the serine protease domain plus kringle 5. Angiostatin kringle 5 is then released by the action of matrix metalloproteinases. These mechanisms do not preclude a role for miniplasminogen in angiostasis, however no studies in this area have been conducted. Note also that Figure 1 shows two glycosylation sites within the kringle 4 domain. Gonzalez-Gronow et al. [2005] demonstrated that six different glycoforms present in native plasminogen had different binding characteristics to cell surface plasminogen receptor CD26. Moreover, angiostatin fragments prepared from purified plasminogen glycoforms revealed that only the angiostatin 2epsilon glycoform was able to inhibit in vitro endothelial cell (EC) proliferation and tubule formation. These findings highlight the combinatorial complexity and functional consequences of both plasminogen/angiostatin cleavage and post-translational modifications.

ATP Synthase: A Cell Surface Receptor for Angiostatin

In 1995, Moser and Pizzo undertook the identification of EC surface binding sites for angiostatin. A number of cell surface receptors had previously been identified for plasminogen—the parent molecule from which angiostatin is derived—as well as other plasminogen cleavage products such as plasmin [Moser et al., 1999, 2002]. However, none of these other plasminogen products exhibited angiostatic activity, suggesting that angiostatin bound to a different site. Radioiodinated angiostatin consisting of kringles 1–3 (K1–3) and plasminogen were prepared separately and used in cell-binding assays that confirmed angiostatin K1–3 bound to the surface of human umbilical vein EC (HUVEC) at a different site and with different binding kinetics than did plasminogen. For example, Scatchard analysis of angiostatin K1–3 binding revealed an apparent K_d of 245 nM and 38,000 sites per cell, while similar analysis of plasminogen binding revealed an apparent K_d of 158 nM and 870,000 sites per cell. The kinetics of plasminogen binding were consistent with its previously identified binding site, annexin II. Moreover, neither angiostatin nor plasminogen were able to compete with each other for HUVEC binding, further supporting evidence of different binding sites.

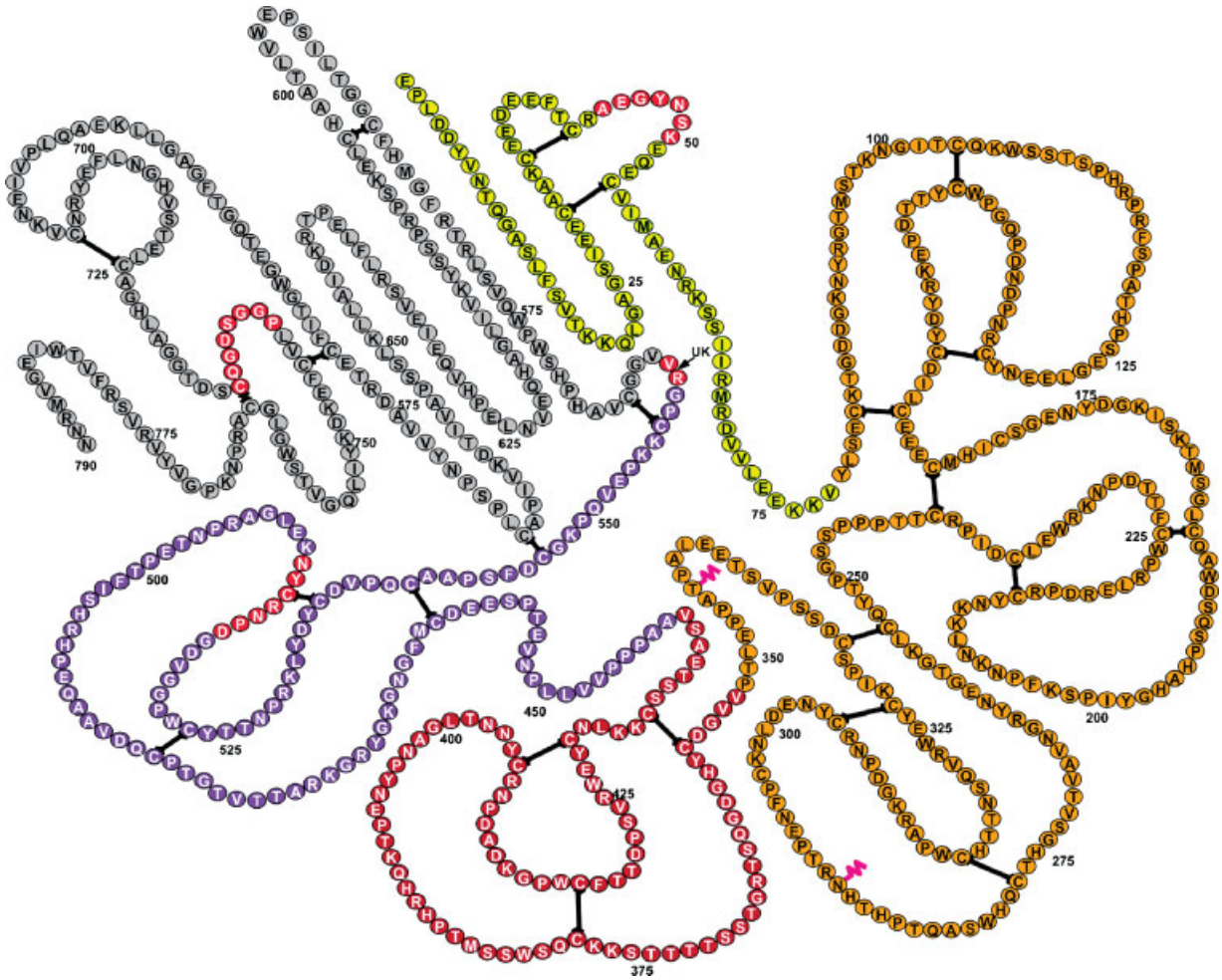

Fig. 1. Modular structure of plasminogen and contained angiostatin fragments. The fulllength plasminogen amino acid sequence is represented as a string of letters, following the 1-letter amino acid code. Disulfide bonds are shown as black bars connecting distant pairs of cysteine residues. Glycosylation sites are represented in the third kringle domain by red wavy lines. The plasmin catalytic domain is represented in gray, kringle 5 in purple, kringle 4 in red, and kringles 1–3 in orange.

To identify the binding site, these investigators prepared an affinity column of angiostatin K1–3 coupled to Sepharose. A separate affinity column of plasminogen-coupled was prepared as a control. Cultured HUVEC were surface labeled with biotin to confirm cell surface location of any identified binding proteins. Plasma membrane extracts of these HUVEC were passed over each column, followed by exhaustive washing to remove nonbound material. Bound proteins were eluted from each column and characterized by SDS–PAGE and Western blot, followed by more extensive proteomic characterization of selected bands.

The plasminogen affinity column yielded a single 44 kDa protein band from HUVEC plasma membranes. This protein was confirmed to be annexin II by immunoassays, thereby validating the sensitivity and specificity of the approach. In contrast, the angiostatin column did not yield a protein band reactive with annexin II antibodies. Instead, a series of protein bands were observed ranging from approximately 20 to 65 kDa, of which the dominant band was approximately 55 kDa. This band was extracted from the gel and subjected to tryptic digestion and mass fingerprinting, which revealed that the 55 kDa band contained the "α"and "β" subunits of F_1F_O ATP synthase.

At the time of this study, the dogma was that F_1F_O ATP synthase was strictly a component of the mitochondrial inner membrane. Thus, the discovery of an EC surface form of F_1F_O ATP

synthase was met with a great deal of skepticism, quite apart from its proposed role in the angiostatin response. A single previous publication had reported detection of F_1F_O ATP synthase on the surface of the tumor cell line A549 [Das et al., 1994]; however, this observation was also met with skepticism and was regarded to be the likely consequence of aberrant trafficking in a genetically unstable tumor cell line or perhaps an artifact of cell culture. Since Das [1998] and Moser and co-authors [Moser et al., 1999, 2001] first presented data that mammalian cells may express certain mitochondrial proteins on the plasma membrane, other investigators have described a wide variety of mitochondrial matrix proteins that have been definitively localized to the plasma membrane surface of certain cells [Soltys and Gupta, 1999]. Moreover, in many cases such matrix proteins have been shown to play functional roles in their plasma membrane locations, including P32 protein, also known as the gC1q receptor for complement protein C1q [Soltys et al., 2000]. Although numerous mechanisms have been proposed to account for the translocation of mitochondrial matrix proteins to extramitochondrial sites [Soltys and Gupta, 1999], no mechanistic details are yet known.

To further characterize the relevance of cell surface F_1F_O ATP synthase, Moser and co-authors [Moser et al., 1999, 2001] tested two hypotheses. First, they asked whether non-inhibitory antibodies against F_1F_O ATP synthase could block angiostatin binding and activity. Second, they asked whether inhibitory antibodies against F_1F_O ATP synthase could mimic the functional effects of angiostatin. To better understand the studies that ultimately confirmed both hypotheses, it is first necessary to consider the structure of the F_1F_O ATP synthase in some detail.

As illustrated in Figure 2, F_1F_O ATP synthase is a mechanochemical enzyme that couples ATP hydrolysis/synthesis (mediated by the F_1 components) to the rotation of a proton translocation assemblage (mediated by the F_O intramembrane components) [Boyer, 1997]. The reaction is bidirectional and can proceed in either the ATP synthesis or the ATP hydrolysis direction, depending on the proton motive force and the availability of ATP and ADP. Given that the polarity of the F_1F_O ATP synthase is subject to a point of reference dependent on mitochondrial versus cell surface localization, in this article we employ the arbitrary polarity designators "above" and "below" the membrane using the orientation illustrated in Figure 2. Note that "above" the membrane may represent either the mitochondrial matrix or the extracellular aspect of the plasma membrane, depending on localization of the enzyme.

Viewed from the top of the complex, counterclockwise rotation of the c-ring—driven by a proton motive force comprising a high concentration of protons "below" the membrane—is associated with transport of protons from beneath the membrane (as represented in Fig. 2) to above the membrane, and with the phosphorylation of ADP to generate ATP. Under conditions in which the proton motive force is diminished and sufficient ATP is present "above" the membrane, the enzyme operates in the opposite direction, with ATP hydrolysis driving the clockwise rotation of the c-ring and consequent pumping of protons from "above" to "below" the membrane.

The F_1F_O ATP synthase complex comprises two subcomplexes. The F_O portion represents the intramembranous components of the proton-pumping rotatory motor, comprised largely of the "a" and "c" subunits. The F1 portion comprises the catalytic portion of the complex, of which the β subunit carries all of the ADP←→ATP catalytic activity. The F_1 and F_O subcomplexes are coupled at two locations—one static and one that can rotate. The dimeric "b" subunits form a stator arm that interfaces between the "a" subunit of F_O and the "δ" subunit of F_1, preventing rotation of the heterohexameric "α-β" ring of F_1. At the second coupling site, the γ subunit of F1 interfaces with the c-ring of F_O so that these two components are rotationally coupled. Thus, as the c-ring rotates due to the proton motive force, the γ subunit rotates within the heterohexameric ring formed by the F1 "α" and "β" subunits. As "γ" rotates, it contacts the c-terminal domains of the "β" subunits, causing conformational changes in the active site that mediates ATP phosphorylation/hydrolysis. Because "γ" has an asymmetric cross-sectional profile, like a cam, it interacts with each of the three "β" subunits in different ways, so that only one "β" subunit is in the active conformation at a time. In the absence of a proton motive force, the complex may run in the opposite direction with hydrolysis of ATP driving clockwise rotation of the "γ" subunit and

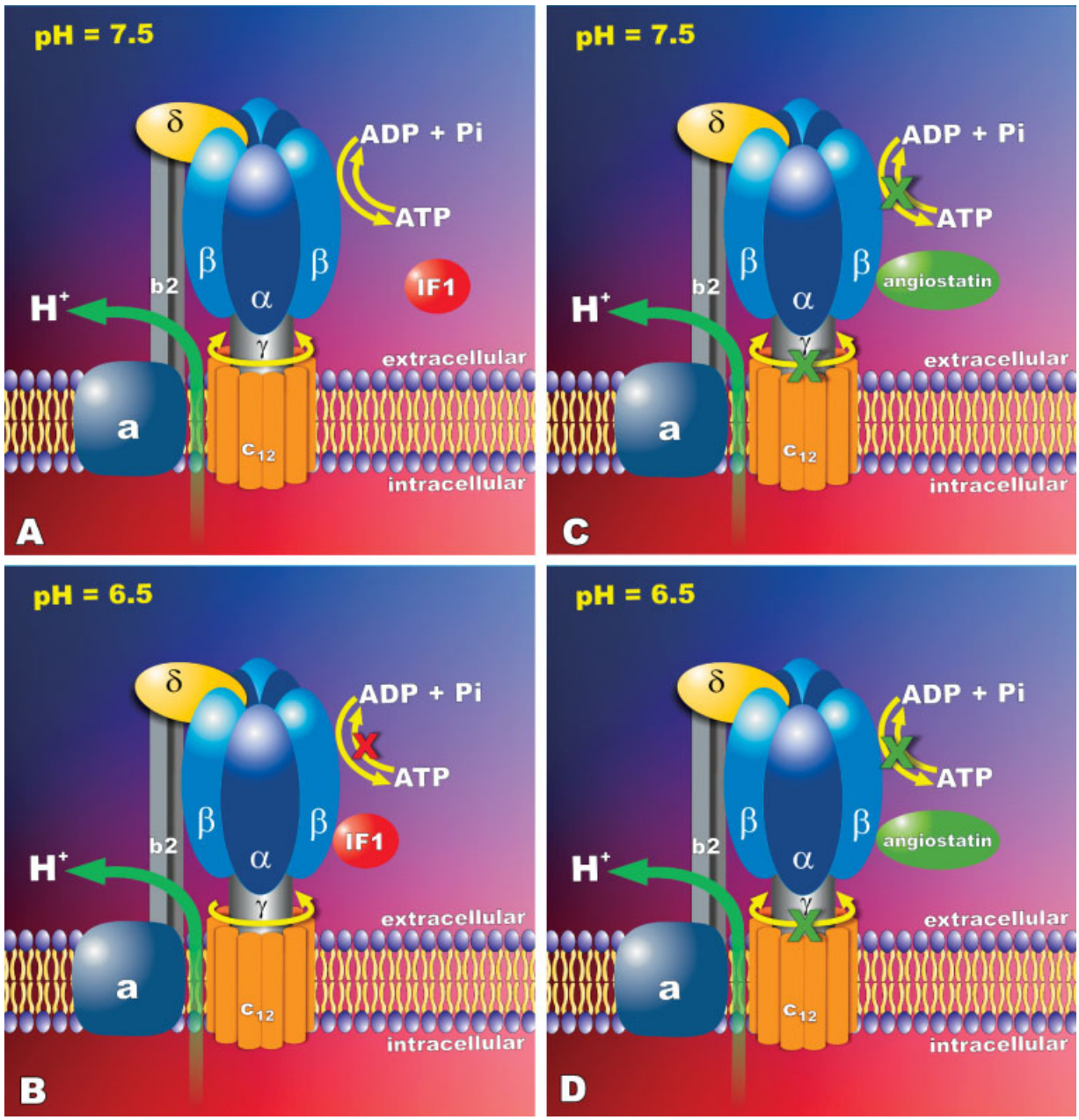

Fig. 2. pH-dependent inhibition of F_1F_O ATP Synthase by IF1 and angiostatin. Cell surface F_1F_O ATP synthase is a multi-component enzyme with mechanochemical catalytic properties. The intramembranous F_O components (including the a and c subunits) constitute the proton transporter machinery. Proton flux is coupled to rotation of the c_{12} ring—in a counter clockwise direction with proton movement in the direction shown. The γ subunit of F1 is coupled to the c_{12} ring, and hence rotates as well. The stator arm (comprising the b_2 and δ subunits) prevents the α-β ring from rotating. Thus, the g subunit rotates inside the α-β ring, inducing conformational changes in the three β subunits that catalyze the phosphorylation of ADP to form ATP. Note that the entire machinery can also work in reverse, indicated by bidirectional arrows. **A**: At pH 7.5, the IF1 factor is dissociated. The complex is free to operate in either direction. **B**: At pH 6.5, the IF1 factor binds and blocks the reverse reaction. Although this mechanism is well characterized in the mitochondrial matrix, its cell surface role remains unclear. **C** and **D**: At either pH 7.5 or 6.5, angiostatin can bind and inhibit F_1F_O ATP synthase activity, blocking all catalytic activity and proton flux. Note that EC at pH 7.5 are able to survive angiostatin treatment, while EC at 6.5 are killed by angiostatin.

"c"-ring, coupled with transport of protons against a gradient.

In the mitochondria, an additional protein, "inhibitor of F_1" (IF1) is available to act as a ratchet, ensuring that under conditions in which the electron motive force is diminished (such as relatively high pH below the membrane), the γ chain cannot rotate in the clockwise

direction and thus cannot needlessly consume ATP resources. It is not known whether IF1 maintains its association with the cell surface form of F_1F_O ATP synthase. Because IF1 dissociates from F_1F_O ATP synthase below pH 6.5, one would expect it to be dissociated, and possibly removed, from the EC surface in the tumor microenvironment. As discussed below, we recently reported that angiostatin and IF1 can compete with each other for ATP synthase binding [Burwick et al., 2005]. These findings suggest but do not prove that angiostatin binds to ATP synthase in proximity to the IF1 binding pocket.

Piceatannol and resveratrol, both polyphenol stilbene phytochemicals that are found in grapes and red wine, are established though non-specific inhibitors of ATP synthase. Both compounds have been found to inhibit numerous cell surface protein kinases and both exhibit cytotoxic effects in a variety of tumor cell lines [Zheng and Ramirez, 1999; Fuggetta et al., 2004; Larrosa et al., 2004; Pozo-Guisado et al., 2005]. These compounds have also been found to inhibit EC tube differentiation in vitro [Belleri et al., 2005], thus indirectly supporting the relevance of cell surface F_1F_O ATP synthase to angiogenesis and tumor biology.

Binding of angiostatin to surface-associated ATP synthase has since been confirmed by other research groups and on some tumor types [Wahl and Grant, 2002; Arakaki et al., 2003]. There have also been additional reports 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 EC [Yegutikin et al., 2002; Arakaki et al., 2003]. Unpublished studies in our laboratories have confirmed that most if not all proteins characteristic of the mitochondrial inner membrane can be found on the surface of EC, suggesting that a still uncharacterized process of bulk transfer of mitochondrial inner membrane domains is responsible for the occurrence of these proteins on the cell surface. A variety of other cell types, including tumor cells, epithelial cells, fibroblasts, and hepatocytes also display a cell surface form of F_1F_O ATP synthase, whereas red blood cells do not (Pizzo et al., unpublished). However, an exhaustive survey of cell types and native tissues has not been conducted. To some degree, these inner mitochondrial membrane domains appear to remain discrete even after fusion with the plasma membrane, as a stable pattern of discrete microfoci is evident in immunofluorescence and co-localization imaging studies [Moser et al., 2001]. It is of interest that only one mitochondrial inner membrane protein has yet been discovered to be absent on the EC plasma membrane. This absent protein is pyruvate dehydrogenase (PDH; data not shown), which is only loosely associated with the mitochondrial inner membrane, and which presumably diffuses away after translocation to the cell surface. It is not yet known whether the electron transport chain and other mitochondrial inner membrane functions remain intact on the cell surface; however, the apparent bulk transfer of the components of these mitochondrial functions suggests that further investigation of EC surface electrophysiology may be worthwhile.

Other Targets of Angiostatin

Like its parent molecule, plasminogen, angiostatin binds multiple cell surface targets that may account for different aspects of angiostatin biology. Moreover, there is evidence that different angiostatin variants—generated by different cleavage termini and with different post-translational modifications—may bind and signal in different manners [Gonzalez-Gronow et al., 1990, 2003, 2005]. Given such combinatorial complexity, a thorough and methodical evaluation of all angiostatin signaling pathways will be required to fully understand its mechanism(s) of action. Such detailed mechanistic analysis remains in its earliest stages. In addition, angiostatin has been shown to bind extracellular targets such as tissue plasminogen activator, which may play a role in regulation of pericellular proteolytic activity that is required for angiogenesis [Stack et al., 1994]. This review will restrict its focus to cell surface targets. Here we briefly discuss progress to date in evaluation of EC surface angiostatin targets other than F_1F_O ATP synthase.

Cell Surface Target: Angiomotin

In 2001, Holmgren's group published the discovery of a novel angiostatin-binding protein they termed "angiomotin," based on its apparent role in EC migration and tube formation [Troyanovsky et al., 2001]. Angiomotin was discovered as an angiostatin binding partner in yeast 2-hybrid screens using as bait a recombinant Gal4-binding domain fused to

kringle domains 1–4 of angiostatin. The bait was screened over a human term placenta cDNA yeast 2-hybrid library, which is rich in vascular endothelium mRNAs. A single interacting peptide was detected from multiple clones, each representing a carboxy-terminal segment of angiomotin.

The yeast 2-hybrid approach, though powerful and robust for many classes of proteins, has at least two significant pitfalls when used to screen membrane proteins. First, post-translation modifications known to be essential for angiostatin signaling [Gonzalez-Gronow et al., 1990] are unlikely to be accurately recapitulated in the yeast nucleus. Second, functional domains that mediate high fidelity protein–protein interactions in the context of the cell surface are known in some cases to bind with reduced or altered specificity as bare domains in a 2-hybrid system. Confirmatory studies demonstrating dose responsive and saturable binding kinetics and antibody competitions of the angiostatin–angiomotin interaction would help to further establish the validity of angiomotin as an angiostatin binding partner. Nevertheless, the discovery of angiomotin as a signaling partner remains a potentially significant discovery that may account for angiostatin's well-known inhibitory effects on EC migration and morphogenesis.

Although definitive validation of angiomotin as an angiostatin target remains incomplete, it is clear that angiomotin is an interesting protein in its own right. Real-time PCR experiments showed that angiomotin expression was detectable in a variety of cell lines, but was most highly expressed in microvascular and umbilical vein EC [Troyanovsky et al., 2001]. The full-length angiomotin gene was cloned and recombinantly expressed, and purified protein was employed to raise polyclonal antiserum. Immunohistochemical staining for angiomotin confirmed its high level of expression in both large vessels and capillaries. Staining also demonstrated a relative absence of expression in most non-EC. Transfection of full-length angiomotin into a variety of cell lines demonstrated upregulation of focal adhesion kinase (FAK) activity as well as colocalization of exogenous angiomotin with FAK. Angiostatin had no effect on cell lines not expressing angiomotin; however, treatment of angiomotin-transfected cells with angiostatin resulted in increased FAK activity and inhibition of both migration and tube formation. EC expressing truncated angiomotin (deletion of three amino acids in the carboxy-terminal PDZ domain) were blocked in both migration and tube formation in vitro [Levchenko et al., 2003]. Moreover, transgenic mice expressing this same mutated form of angiomotin died at embryonic day 9.5 with evidence of non-migration of EC into the neuroectoderm and intersomitic regions. Finally, exogenous expression of human angiomotin in EC resulted in stabilization of established tubes for over 30 days, as well as evidence of enhanced invasion of EC derived from these tubes [Levchenko et al., 2004].

In the context of cell migration and morphogenesis, existing data do not clearly indicate whether the apparent dependence of angiostatin on angiomotin expression is direct or indirect. It should also be noted that angiomotin does not account for other documented effects of angiostatin, including inhibition of proliferation and promotion of EC death [Lucas et al., 1998].

Cell Surface Target: Integrin $\alpha_V\beta_3$

Takada's group investigated bovine arterial endothelial (BAE) cell binding to a variety of proteins, including angiostatin, coated onto plastic microtiter plates [Tarui et al., 2001]. They found that BAE cells bound to angiostatin in a dose- and magnesium-dependent manner, and that binding was saturable at approximately 200 nM angiostatin. This led them to question whether integrins, as major cation-dependent adhesion receptors, might be mediating angiostatin binding by BAE cells. In binding experiments comparing CHO cells transfected with various combinations of recombinant integrins, they found that cells expressing recombinant $\alpha_v\beta_3$, $\alpha_9\beta_1$, and to a lesser extent $\alpha_4\beta_1$ were capable of binding to angiostatin. Moreover, the binding was dose-dependent, saturable, and was blocked by integrin-specific antibodies as well as the RGD peptide. Moreover, integrin-expressing cells bound to various plasminogen fragments including kringles 1–5, 1–4, and 1–3, but not to intact plasminogen. The relative absence of stress fiber formation of integrin-expressing cells on plasminogen-coated plates suggested that angiostatin acts as an antagonist to known angiogenic $\alpha_v\beta_3$ ligands. The investigators suggest that extracellular matrix interactions with $\alpha_v\beta_3$ integrin are essential for maintenance

of mitogen-activated protein kinase by pro-angiogenic factors as well as suppression of apoptosis, and that angiostatin directly antagonizes these proliferative, pro-survival signals. It is known that other antagonists of $\alpha_v\beta_3$ can function as tumor angiogenesis inhibitors [Gutheil et al., 2000].

A potential criticism of the Tarui et al. [2001] study is that the molecular basis of angiostatin–integrin interactions remains completely uncharacterized. We note that there are no RGD motifs found in either human or mouse plasminogen/angiostatin sequences, although other sequences could be involved. Moreover, whole cell binding experiments do not reveal whether the mode of action is direct or indirect, via, for example, a binding site that is distinct from but influenced by an integrin. We further note that Tarui et al. [2001] investigated the heterologous binding of human angiostatin to bovine EC. We are not aware of any homologous human–human angiostatin binding studies as on the date of submission of this manuscript.

Cell Surface Target: Annexin II

Sharma's group investigated whether purified human angiostatin could bind any proteins in bovine aortic EC extracts using a ligand blotting method [Tuszynski et al., 2002]. They observed angiostatin binding to a single 35 kDa band, which was subsequently identified as annexin II. Binding of angiostatin to purified annexin II was efficiently competed by soluble annexin II and partially competed by an anti-annexin II monoclonal antibody. Binding of angiostatin to BAE cells was dose-responsive and saturable, with an apparent K_d of 83 nM, as compared to 101 nM for binding to purified annexin II. Plasminogen binding was similar, with apparent K_d values of 124.5 nM to BAE cells and 164 nM to purified annexin II. Plasminogen could partially compete (approximately 40%) angiostatin binding to BAE cells, while annexin II antibodies competed 68% of the binding. The reverse experiment using angiostatin to compete plasminogen binding blocked approximately 40% of the binding. Finally, these investigators pre-incubated BAE cells with an excess of plasminogen to show that approximately 90% of angiostatin-induced cell death was prevented, further suggesting that at least part a portion of angiostatin biology is mediated by annexin II. A model was proposed of angiostatin signaling through annexin II, resulting in increased intracellular free calcium and therefore decreased EC proliferation and increased apoptosis.

In contrast to the data of Tuszynski et al. [2002], we could find no evidence of angiostatin binding to annexin II on HUVEC [Moser et al., 1999] discussed in "ATP Synthase: A Cell Surface Receptor for Angiostatin"). Possible explanations for this discrepancy include the use of different cell lines (HUVEC vs. BAE), the use of a homologous vs. a heterologous system, inappropriate binding to denatured proteins on Western blots, and differences in the preparation of angiostatin. Given that plasminogen does not exhibit angiostatin-like activity, it is difficult to understand how the annexin II receptor can mediate angiostatin activity unless plasminogen and angiostatin bind the receptor in fundamentally different ways. Clearly more work is needed in this area.

Cell Surface Target: ABSP (Angiostatin Binding Sequence Protein)

Kang, Bang, and Yu [2004] recently published the discovery of a novel angiostatin-binding protein through the use of cDNA phage display interaction screening. A commercially obtained liver cDNA T7 expression library was screened for T7 virions expressing polypeptide fragments that bind angiostatin kringles 1–4. A single partial cDNA expressing a 133 amino acids polypeptide fragment was recovered and subsequently mapped to a larger cDNA encoding an open reading frame of 1998 amino acids. This putative protein has not been characterized and was therefore designated "angiostatin binding sequence protein" (ABSP) by the investigators. The core angiostatin binding sequence (ABS) of 133 residues was recombinantly expressed and found to bind purified angiostatin by co-immunoprecipitation and by surface plasmon resonance, with an apparent K_d of 340 nM. Expression studies demonstrated that ABSP is expressed in a wide range of bulk tissue extracts and further indicated that numerous discrete sizes of the ABSP mRNA were present, suggesting alternative processing. A polyclonal antiserum raised against the core ABS detected 40 and 17 kDa protein bands in a Western blot of HUVEC cell extracts. No protein expression was detected in HepG2 cells. Limitations of the investigation include the fact that no cell-based binding or localization studies were performed and no functional consequences of angiostatin

binding to ABSP were demonstrated. Thus it is impossible to draw any firm conclusions regarding the role of ABSP in angiostatin biology.

Cell Surface Target: c-met

Wajih and Sane [2003] investigated whether angiostatin would influence hepatocyte growth factor (HGF)-induced signaling of EC, based on certain similarities between angiostatin and HGF. Both proteins possess kringle domains and share significant amino acid homology [Nakamura et al., 1989], and both proteins influence EC proliferation and motility, though in opposite directions. Recombinant angiostatin kringles 1–3 were found to inhibit HGF-induced phosphorylation of the HGF receptor, c-met as well as of the downstream signaling mediators Akt and ERK1/2 in HUVEC. Excess HGF could overcome angiostatin inhibition of Akt phosphorylation. Other receptor tyrosine kinase activators such as VEGF, bFGF, and IGF-1 were not blocked by angiostatin in the phosphorylation of Akt or ERK1/2, indicating that the effect was specific to c-met. These effects were specific to angiostatin, as plasminogen had no activity. Finally, HGF inhibited angiostatin binding to HUVEC, and angiostatin inhibited HGF-induced proliferation of HUVEC. Binding of angiostatin to soluble c-met was dose-responsive and saturable, with an apparent K_d of approximately 5 nM and 12,044 sites per cell. These investigators conclude that the ability of angiostatin to block the HGF/c-met interaction may induce anti-angiogenic effects by reducing downstream activation of Akt/phosphatidylinositol kinase, in turn leading to release of suppression of apoptosis and blocking of cell cycle progression into S phase.

Cell Surface Target: NG2 Proteoglycan

Goretzki, Lombardo, and Stallcup [2000] described NG2 proteoglycan binding to angiostatin and plasminogen. NG2 is a developmentally regulated cell surface chondroitin sulfate proteoglycan that is upregulated in the tumor vascular wall. These investigators found that multiple forms of plasminogen, including angiostatin kringles 1–3, bound immobilized NG2 with dose-responsive and saturable kinetics. All forms of protein bound with apparent K_d in the range of 12–75 nM. The anti-angiogenic effect of angiostatin was inhibited by soluble NG2. Soluble NG2 also significantly accelerated the activation of plasminogen by urokinase type plasminogen activator. Although NG2 does not appear to directly mediate signal transduction by angiostatin, it may play an important role in extracellular regulation of angiostatin activity in the tumor vascular bed.

CONCLUSIONS

At first glance, it appears that angiostatin has too many binding partners and cell surface targets. There seem to be no obvious underlying principles that can predict which proteins will bind angiostatin and which will not. In this regard, angiostatins are no different than their parent molecule plasminogen, which itself binds to a plethora of cell surface, soluble, and matrix-associated targets [see Gonzalez-Gronow et al., 1989, 1991, 1994, 1998, 2001, 2004; Stack et al., 1992a,b, 1994] and references therein). As a zymogen that is activated during fibrinolysis, plasminogen undergoes multiple binding events as a normal part of its biology. It's kringle domains clearly alter their binding specificity during proteolytic maturation, as plasminogen does not bind many of the sites that are recognized by its derivative angiostatins and plasmin fragments. Finally, given the combinatorial complexity inherent in the different cleavage forms of angiostatin, it is perhaps not surprising that many investigators have reached different conclusions regarding the key mediators of angiostatin biology. It is possible that some of the described angiostatin binding targets play no true role in mediating its biological effects. On the other hand, as yet there have been no targets described that can mediate all of the described biological effects of angiostatin, including effects on cell survival, proliferation, migration, invasion, morphogenesis, phenotype, and gene expression. In this regard, it is reasonable to propose that angiostatin mediates its effects via multiple binding sites and receptors, including F_1F_O ATP synthase (activity required for EC survival under pH stress inherent in the tumor microenvironment), angiomotin (activity required for EC migration, invasion, and vascular morphogenesis), and integrins (activity required for EC attachment, survival, and migration). Other potential targets such as c-met, and perhaps related receptor tyrosine kinases, may impart activities required for cellular proliferation and survival. The role of still other angiostatin binding targets such as annexin II, NG2, and ABSP are not clearly understood but cannot yet

be excluded as playing important roles in the angiostatin response.

Kringle 5 Also has Anti-Angiogenic Activity

Plasminogen kringle 5 (K5) suppresses growth factor-stimulated angiogenesis via cell cycle G_1 arrest and induction of apoptosis [Ji et al., 1998; Lu et al., 1999; Cao et al., 2000]. K5 confers on plasminogen the capacity to bind to HUVEC with high affinity [Wu et al., 1997]. Interaction of K5 with its receptor, the voltage-dependent anion channel (VDAC1), interferes with both cytosolic intracellular free Ca^{2+} signaling and pH regulation in HUVEC [Gonzalez-Gronow et al., 2003]. Angiogenesis and repair of blood vessels was preceded by increases in cytosolic pH in EC [Komatsu et al., 1999]. Interference of these mechanisms by K5 may be the basis of its anti-angiogenic activity.

Isolated K5 effectively blocks angiogenesis in animal models [Zhang et al., 2001, 2004]. However, unlike angiostatin (K1–3), which is found in the circulation in animal models [O'Reilly et al., 1994b], the in vivo generation of K5 remains to be determined.

The in vivo generation of most angiostatins has been proposed to follow a sequential order of events beginning with conversion of plasminogen to plasmin, followed by reduction of plasmin by disulfide reductases, then serine proteinase-dependent release of kringles 1–4.5, and finally matrix metalloproteinase-dependent trimming of kringles 1–4.5 to either K1–4 or K1–3 [Lay et al., 2000a]. These mechanisms are based on the participation of plasmin reductases [Stathakis et al., 1999], one of which has been identified as the glycolytic enzyme phosphoglycerate kinase (PGK) [Lay et al., 2000a]. PGK is an outer mitochondrial membrane protein that is usually associated with VDAC1 [Adams et al., 1991]. Therefore, binding of plasminogen via K5 to VDAC1 is followed by its conversion to Pm by its physiologic activators, urokinase (u-PA) or tissue-type (t-PA) plasminogen activators. Then plasmin reduction by PGK facilitates angiostatin generation [Lay et al., 2002]. Another plasmin reductase has been identified as the plasminogen/plasmin/t-PA binding protein annexin II [Kwon et al., 2002]. Unlike VDAC1, annexin II binds Pg via the L-lysine binding site in kringle 1 [Hajjar et al., 1994]. Regardless of the plasmin reductase involved in the generation of angiostatin (kringles 1–4), both mechanisms lead to reduction and autoproteolysis of K5 [Kwon et al., 2002; Lay et al., 2002], thereby limiting its efficacy as an anti-angiogenic agent when generated from Pg in vivo. However, for therapy, this limitation can be circumvented by the use of Lysyl 4-aminobenzoic acid derivatives, which mimic K5 [Sheppard et al., 2004]. These molecules show in vitro properties similar to K5 and are able to displace radiolabeled protein from a high affinity binding site on EC [Sheppard et al., 2004].

Other Factors Affecting Regulation of Angiostatin's Activity

Here we review the current state of knowledge with respect to pH, matrix, and receptor distribution and function, then discuss other parameters of the tumor microenvironment that may also modulate the process.

1. At low extracellular pH, angiostatin affects EC intracellular pH. The average tumor extracellular pH (5.6–7.6), measured in vivo using magnetic resonance imaging (MRI), is lower and more variable than in normal tissue (7.2–7.6), yet tumor cells have a normal average intracellular pH [Yamagata and Tannock, 1996]. Angiostatin is more potent at low extracellular pH [Wahl and Grant, 2002], thus has enhanced activity in the tumor microenvironment.

It is important to note that in vivo measurement of intracellular and extracellular pH made with MRI are averaged values from a large number of cells, including stromal, endothelial, and other components and therefore cannot reflect variations between cell types. That is why it is advantageous to also study these parameters in vitro, where conditions can be controlled and manipulated, and a pure population of a particular cell type can be studied.

It has recently been reported that angiostatin has a profound effect in vitro on intracellular pH in EC [Wahl and Grant, 2002; Wahl et al., 2002a]. Thus, angiostatin must have a direct or indirect target that plays a role in pH homeostasis. Moreover, we note that the intracellular pH dysregulation induced by angiostatin is only manifested at low extracellular pH. These observations implicate pH regulating transporters that are active at low extracellular pH. These include the sodium proton exchanger (NHE) [Orlowski and Grinstein, 1997], the H^+-linked monocarboxylate exchanger (MCT)

[Halestrap and Price, 1999]. Recent research indicates that the plasma membrane ATP synthase may be localized in a plasma membrane microdomain where it generates ATP on the cell surface thus it may not directly affect intracellular pH [Moser et al., 2003]. The relative roles of these transporters and the mechanism of the pH effect are under study in our laboratories.

2. Other microenvironmental factors

2a. NHE, pH dependent signal transduction to the cytoskeleton, and angiostatin. It has been reported that angiostatin treatment catalyzes FAK phosphorylation in the absence of integrin clustering [Claesson-Welsh et al., 1998]. Integrin clustering is required for cell attachment to substrate. In the usual scenario, attachment to a substrate such as fibronectin causes integrins to form tetramers, and FAK is phosphorylated, inducing cytoskeletal elements to form structurally organized arrays that promote cell spreading and activating the NHE. In the tumor microenvironment, there are several parameters that are different. The matrix is higher in collagens, laminin, and certain other proteins relative to normal stroma [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 often motile, and the NHE is more active. Various integrin isoforms are expressed differently in tumors relative to normal tissue [Schwartz et al., 1991; Coopman et al., 1996; Erdreich-Epstein et al., 2000], which could have secondary effects on localization of other cell surface molecules and activities. When extracellular pH is low, typically the sodium/proton antiporter, isoform 1 (NHE1) is activated to maintain intracellular pH in the viable range. This occurs as a function of attachment, phosphorylation, ATP binding, and the presence of cytokines [Schwartz et al., 1991; McSwine et al., 1996]. It has also been reported that NHE1 co-localizes with FAK [Schwartz et al., 1991] and clusters at the leading edge of lamellopodia in migrating cells [Akasaka et al., 1995]. It is not known whether the NHE is involved in the pH dysregulation that occurs when angiostatin binds to EC, but there locations relative to FAK make this a possibility.

2b. Receptor distribution is modulated by matrix composition and extracellular pH. Modulation of ATP synthase levels is also a determinant of angiostatin activity. ATP synthase distribution on the EC surface can be altered as a function extracellular matrix composition, metabolic conditions, and extracellular pH. When normal EC are allowed to attach to fibronectin, immunohistochemistry using a primary antibody directed against the β subunit of ATP synthase showed no detectable enzyme on the cell surface [Wahl and Grant, 2002]. However, when cells were plated on Matrigel® to simulate the tumor stroma, the enzyme was expressed 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 could likely related to the organization of focal adhesion plaques and how they assemble for migration [Wahl and Grant, 2002]. Other microenvironmental parameters have not yet been evaluated in terms of this receptor, and these factors have not been evaluated for most other anti-angiogenic agents.

We recently demonstrated that angiostatin causes a precipitous decline in cytosolic intracellular pH, when coupled with extracellular acidification [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 EC surface was reported to be more focally localized at low pH [Wahl and Grant, 2002]. We are currently studying angiostatin binding to ATP synthase as a function of extracellular pH. At a normal extracellular pH of 7.3, ATP synthase, MCT, and NHE1 would all be less active, whether or not cells are attached to substrate. However, in the tumor microenvironment, there is less attachment to variety of substrate proteins, a lack of integrin clustering, and an aberrant phosphorylation of focal adhesion kinase (FAK). Because of the low extracellular tumor pH, the NHE1 and MCT are activated and the 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 the NHE1. The precise location of MCT is not presently known.

2c. Tumor stroma composition. One major theme that has emerged from the research done thus far on anti-angiogenic compounds is that the extent of attachment of cells to a matrix and the nature of the matrix are critical determinants of the compounds' activity. This is a

complex issue because tumor stroma is composed of numerous proteins—often in abnormal relative concentrations. There is considerable literature indicating that tumor cells modulate stromal composition and vice versa. This two-directional signaling is part of the malignant phenotype and plays a central role in tumor cell behavior [recently reviewed in Roskelley and Bissell, 2002]. The emerging view in the angiogenesis field holds that stromal effects also play a critical role in the EC response to the tumor microenvironment. Moreover, stromal effects may influence the outcome of exposure to exogenous stimuli, such as angiostatin [Wahl and Grant, 2002].

With respect to angiostatin, the vulnerability of the EC in the tumor microenvironment comes in part from the fact that when they are rapidly proliferating: their attachments are compromised [Wahl et al., 2002a]. Furthermore, the substrates they come in contact with in the tumor microenvironment, such as collagen and laminin, lead to different integrin isoform expression, with subsequent differences in signal transduction. This may be one of the reasons why mature vessels appear to be unaffected by angiostatin. It would also explain the efficacy of endostatin targeting integrins [Sudhakar et al., 2003], since if they are less engaged in attachment they are more available for interaction. Migration of tumor cells during metastasis is often along the extracellular matrix of the basal lamina. When mice are injected with a melanoma cell line selected for metastatic seeding in the lung, the cells migrate to form secondary lung tumors. When they are inhibited from binding fibronectin or laminin, over 90% of the cells fail to localize in the lungs [Humphries et al., 1986]. In order to enter a blood vessel, tumor cells degrade the collagenous matrix by secreting various proteinases. Antibodies directed against plasminogen activator 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 (MMP) [Vassalli et al., 1991]. Enriched levels of u-PA and its receptor (UPAR) are found on the leading edge of migrating cells. Plasminogen activator inhibitor type I (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]. Expression of u-PA has been correlated with angiogenesis and poor prognosis [Kaneko et al., 2003].

2d. MMP. MMP are enzymes that digest/degrade matrix proteins enabling migration of metastasizing cells. MMP inhibitors 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 also affect the degree of enzyme activity needed to impact upon the matrix. A third consideration is that enzymatic alteration of matrix composition may influence where tumor cells disseminate, and may cause digestion of other pathologic matrices in nearby or distant areas.

3. Sources of acid that can enhance intracellular pH decrease

3a. Carbonic acid/bicarbonate axis. In the tumor microenvironment, there is poor catabolite removal by the inefficient and abnormal blood vessels, leading to accumulation of CO_2 [Newell et al., 1993; Helmlinger et al., 2002]. The discovery of the effect on intracellular pH of angiostatin was manifested in experiments where the extracellular pH was either lowered by lowering the bicarbonate to 7 mM at 5% CO_2, or by raising the CO_2 to 17% and keeping the bicarbonate at 26 mM. However, it is important to consider that buffering capacity will be inefficient at 7 mM bicarbonate, and the pH will thus be fluctuating downward in the days between cell feeding. Another important point is that many experiments performed in commercial media contain 14 mM bicarbonate, which gives a pH of 7.0–7.1. Thus, it is relevant that in studies using such commercial media, angiostatin was reported to decrease EC viability (40%) at "normal" extracellular pH [Moser et al., 1999], whereas experiments using M199 media with 26 mM bicarbonate, which has a pH of 7.3, showed no angiostatin effect on EC viability [Wahl et al., 2001, 2002a]. Another difference between these two studies was that the former was done using recombinant angiostatin obtained from Entremed, Inc. (Rockville, MD), whereas the latter study utilized angiostatin in its native state, purified from plasminogen cleavage reactions. In the most recent collaboration between these two investigators, recombinant angiostatin (from Sigma Chem. Co., St. Louis, MO), native angiostatin from Collaborative (Calbiochem, San Diego, CA),

and native angiostatin prepared in-house all gave identical activities when comparing extracellular pH 7.3–6.7 [Burwick et al., 2005].

Tumor-like conditions can be created experimentally by maintaining bicarbonate at 26 mM and incubator CO_2 at 17%. This creates acidic tumor-like conditions [Wahl and Grant, 2002; Wahl et al., 2002a]. This approach maintains physiologic bicarbonate concentration. Elevated CO_2 is also found in tumors, due to poor catabolite removal (inadequate vasculature), which is likely the basis for elevated carbonic anhydrase IX [Beasley et al., 2001; Giatromanolaki et al., 2001; Koukourakis et al., 2001; Olive et al., 2001; Hui et al., 2002; Bui et al., 2003; Swinson et al., 2003]. Carbonic anhydrase (CA) catalyzes the reversible reaction that hydrates CO_2 and generates a proton and the bicarbonate anion. CA is a transmembrane glycoprotein with an extracellular active enzyme site. It is active under hypoxic conditions [Beasley et al., 2001] and is also induced by acidity [Biskobing and Fan, 2000]. CAII potentiates the activity of the chloride bicarbonate exchanger [Sterling et al., 2001], and has also been implicated in activation of the sodium proton antiporter, NHE1 [Li et al., 2000]. CAII deficiency is associated with a decrease in intracellular pH [Wolfensberger et al., 1999] and, conversely, lowering intracellular pH can upregulate mRNA transcription of the *CAII* gene [Biskobing and Fan, 2000]. CAIX is another mobilizer of HCO_3^- that is overexpressed in hypoxic tissues and probably helps to maintain intracellular pH within the viable range. High expression levels are associated with high microvessel density and poor prognosis in a diverse group of cancers [Beasley et al., 2001; Giatromanolaki et al., 2001; Koukourakis et al., 2001; Olive et al., 2001; Hui et al., 2002; Bui et al., 2003; Swinson et al., 2003]. Conversely, there are reports that suggest that high CAXII expression is associated with good prognosis [Watson et al., 2003], and may be elaborated by stromal cell components of some tumors and hamper angiogenesis. Evaluation of the effects of both low extracellular pH on the angiostatin response has provided insight into its mechanism of action and may also affect the activity of other anti-angiogenic agents.

3b. Lactic acid. In addition to high CO_2 and poor perfusion, a further cause of 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 measurement of the activity of the transporter used to remove it from the cell. Some isoforms of this transporter, the H^+-monocarboxylate exchanger (MCT), are elevated in human melanoma [Wahl et al., 2002b] and may also be elevated in other tumors of neural crest origin. Factors that may influence the degree to which angiostatin can decrease EC intracellular pH in the tumor microenvironment include the degree to which MCT is elevated, functionality of other transporters used to regulate intracellular pH, and the degree of extracellular pH stress within the tumor. Intracellular and extracellular pH measurements in normal and pathologic tissues will help to address the relative importance of these parameters. In addition, there is likely a relationship between hypoxia and acidity, but the relative importance of each has not been addressed within any one study.

3c. Enhanced glycolysis (the Warburg effect). Glut-1 is the glucose uptake receptor active in tumor tissue. Uptake of glucose during hyperglycemia causes a transient lactic acidosis, via stimulation of glycolysis, that can potentiate hyperthermia induced acidification and cell death in human tumors [Thistlethwaite et al., 1987; Leeper et al., 1998; Wachsberger et al., 2002]. Hypoxia is a major contributor to tumor acidity and also activates the hypoxia-inducible factor, HIF-1α. This transcription factor also regulates pro-angiogenic cytokines [Scappaticci, 2002] and promotes expression of genes involved in metastasis [Semenza, 2003].

3d. 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]. No studies to date have systematically determined to relative role of hypoxia and acidosis with respect to tumor pathophysiology or therapeutic response. Most studies are either conducted at low pH, or done under hypoxic conditions, but not both. Studies in the future with Panzem® (Entremed, Inc.), a HIF-1α inhibitor [Mabjeesh et al., 2003], or similar compounds will help to determine the therapeutic benefit of manipulation of the hypoxia response in concert with the angiostatin response.

4. Inhibitor of F1 (IF1)—A potential modulator. Angiostatin has proven to be

rapidly cleared from the circulation in human clinical trials [DeMoraes et al., 2001] as well as in animal studies [O'Reilly et al., 1996]. This has lead to the search for angiostatin-mimetics that would be more practical in the clinic. One candidate molecule was inhibitor of F1 (IF1), which is naturally occurring, and has been shown to block ATP hydrolysis in mitochondria (Fig. 2). Studies using isolated mitochondria and intact EC showed that although IF1 blocked ATP hydrolysis in both cases, which caused conservation of ATP on the cell surface, it did not block ATP synthesis [Burwick et al., 2005]. In contrast, angiostatin blocked both ATP synthesis and hydrolysis. In vitro tube differentiation assays showed that IF1 did not inhibit tube formation, but angiostatin did. The relative concentrations of angiostatin and IF1 may modulate new blood vessel development during angiogenesis [Burwick et al., 2005]. It is noteworthy that IF1 effects are strongly modulated by pH, similar to angiostatin, which should confer specificity for the tumor microenvironment. The binding and activity of angiostatin and IF1 at normal and low pH is depicted in Figure 2A–D. These findings indicate that inhibition of ATP hydrolysis is not the primary anti-angiogenic mechanism of angiostatin, and further suggest that blockade of ATP synthesis is also required. These mechanistic details should be taken into account in the development of new compounds that can replace angiostatin in anti-angiogenic therapy.

5. Acute acidification as potential enhancer in anti-angiogenic therapy. Oral glucose is used clinically to lower tumor pH before thermoradiotherapy [Thistlethwaite et al., 1987; Engin et al., 1995; Leeper et al., 1998]. The mechanism involves increased glycolysis in the tumor microenvironment, producing lactic acid [Burd et al., 2003]. 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]. There are also other enzymes relating to glycolysis that may impact upon this axis, including lactate dehydrogenase [Koslowski et al., 2002].

Unexplored New Uses for Existing Compounds

Many agents evaluated previously for anti-tumor activity could be more toxic to EC because of ease of drug delivery and at lower less toxicity due to the neccessity of chronic metronomic administration to sustain anti-angiogenic effects. Certain candidate compounds that failed as anti-angiogenic agents may have opportunities for revival through the use of improved delivery tools, such as implantable osmotic pumps, that sustain therapeutic levels of drug. Also, since tumor EC are genetically stable, non-malignant cells in an abnormal tumor-induced microenvironment, their signal transduction pathways are less prone to development of resistance to anti-angiogenic compounds. However, it is also possible that tumor cells, being genetically unstable, will activate alternate stimulatory pathways to drive angiogenesis via expression of additional cytokines or stimulatory molecules.

In ongoing research, we are evaluating other pH lowering compounds for anti-angiogenic effects [Contarino et al., 2004] as well as a new generation of camptothecin analogs that are more active at low extracellar pH [Adams et al., 2000a,b]. It's not surprising that many, many novel applications are being suggested, since in normal cells in the tumor microenvironment it should be much easier to follow signal transduction than in cells that harbor numerous mutations and have a high ongoing mutation rate. Thalidomide is another example, rediscovered by Folkman [D'Amato et al., 1994], that has proven to be worth taking a look at in this new context [Figg et al., 2001; Short et al., 2001; Daliani et al., 2002; Escudier et al., 2002; Gutheil and Finucane, 2002].

Conclusions

Although angiostatin exerts greater EC-killing activity at low pH, we have shown that angiostatin is able to bind and inhibit cell surface F_1F_O ATP synthase over a range from pH 6.5 to 7.5 (Fig. 2C,D). It has also been shown that EC can maintain a relatively normal intracellular pH even though the extracellular pH has dropped to tumor-like conditions of pH 6.5 [Wahl and Grant, 2002]. The addition of angiostatin to EC under external pH stress causes a rapid and dramatic decrease in intracellular pH, which is associated with EC death by an unknown mechanism [Wahl and Grant, 2002]. Moreover, angiostatin appears to exert no deleterious effects on EC cultured at normal pH, suggesting that pH stress is the primary mediator of EC cell death by angiostatin. Therefore, it seems likely that angiostatin-mediated inhibition of proton flux via F_1F_O ATP synthase is responsible for the increased sensitivity of EC

to pH stress in the presence of angiostatin. We are currently investigating further mechanistic details of the angiostatin response as it relates to cell surface ATP metabolism and pH homeostasis.

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