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Ectopic Localization of Mitochondrial ATP Synthase: A Target for Anti-Angiogenesis Intervention?

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A receptor for angiostatin was identified on the surface of endothelial cells as F_1–F_0 ATP synthase (Moser *et al*., 1999). *Proc. Natl. Acad. Sci. U.S.A.* **96**, 2811–2816. This ectopic ATP synthase catalyzes ATP synthesis and is inhibited by angiostatin over a wide pH range. Endothelial cells grown at normal pH suffer no ill effects from this angiostatin-mediated inhibition of ATP synthase, whereas endothelial cells grown at low, tumor-like extracellular pH cannot maintain a normal intracellular pH and die. Angiostatin inhibits both ATP synthesis and ATP hydrolysis (Moser *et al*., 2001) and interferes with intracellular pH regulation (Wahl and Grant, 2002; Wahl *et al*., 2002). Although angiostatin administered intravenously is cleared from the circulation in a matter of minutes, angiostatin-mimetics that are more stable have potential for clinical application. An angiostatin-mimetic activity has recently been observed using a polyclonal antibody against the β catalytic subunit of ATP synthase. In order to explore the mechanism of action of angiostatin and its mimetics, further work needs to be done to evaluate clinical applicability, specificity, and contraindications for this class of therapeutics.

KEY WORDS: Angiogenesis; angiostatin; ATP synthase; endothelial cells.

INTRODUCTION

Tumor growth requires the development of new vessels that sprout from preexisting normal vessels in a process known as "angiogenesis" (Folkman, 1971). These new vessels arise from local capillaries, arteries, and veins in response to the release of soluble growth factors from the tumor mass, enabling tumors to grow beyond the diffusion-limited size of approximately 2 mm diameter. Angiostatin, a naturally occurring inhibitor of angiogenesis, was discovered on the basis of its ability to block tumor growth *in vivo* by inhibiting angiogenesis (O'Reilly *et al*., 1994a). Folkman proposed the concept of an "angiogenic switch," in which the net drive towards angiogenesis or angiostasis is dependent upon the sum of theangiogenic vs. angiostatic signals. 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 primary tumor (O'Reilly *et al*., 1994b). This observation was supported in experimental models (Funatsu *et al*., 2003; Holash *et al*., 1999; Holmgren *et al*., 1995). In these models, the rapidly growing metastases derive not from seeding of tumor cells at the time of resection of the primary tumor, but rather from preexisting micrometastases that were held "in check" by the presence of the primary tumor and that are "released" to grow upon removal of the tumor. Because of differences in local concentration of angiogenic and angiostatic factors, the angiogenic switch may be tripped in the angiogenesis direction at the site of a primary tumor, but in the angiostatic direction at metastatic sites. Within

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Abbreviations: HUVEC, human umbilical vein endothelial cells; MRI, magnetic resonance imaging; MCT, H^+-linked monocarboxylate transporter; NHE, sodium proton exchanger.

the primary tumor, the relative abundance of angiogenic signaling molecules trips the angiogenic switch in favor of angiogenesis, 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 longer half-lives 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'S RECEPTOR

In 1995, Moser and Pizzo undertook the identification of endothelial cell 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 and plasminogen were prepared separately and used in cell-binding assays that confirmed that angiostatin bound to the surface of human umbilical vein endothelial cells (HUVEC) at a different site and with different binding kinetics than plasminogen. For example, Scatchard analysis of angiostatin 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 was able to compete with each other for HUVEC binding, further supporting evidence of different binding sites.

To identify the binding site, these investigators prepared an affinity column of angiostatin coupled to Sepharose. A separate affinity column of plasminogen-coupled Sepharose 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 non-bound 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 protein band of with a Mw $\sim$ 44,000 Da on an SDS gel 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 20,000 to 65,000 kDa, of which the dominant band was approximately Mw $\sim$ 55 kDa. This band was extracted from the gel and subjected to tryptic digestion and mass fingerprinting, which revealed this band contained the "α" and "β" subunits of F_1F_0 ATP synthase.

At the time of this study, the dogma was that F_1F_0 ATP synthase was strictly a component of the mitochondrial inner membrane. Thus, the discovery of an endothelial cells surface form of F_1F_0 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 the β subunit of F_1F_0 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 *et al*. (1999, 2001) first presented data that mammalian tumor and endothelial cells may express certain mitochondrial proteins on the plasma membrane, other investigators have described a wide variety of mitochondrial matrix proteins that have been identified on 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_0 ATP synthase, Moser *et al*. (Moser *et al*., 1999, 2001) tested two hypotheses. First, they asked whether non-inhibitory antibodies against F_1F_0 ATP synthase could block angiostatin binding and activity. Second, they asked whether inhibitory antibodies against F_1F_0 ATP synthase could mimic the functional effects of angiostatin.

THE EFFECT OF ANGIOSTATIN ON INTRACELLULAR pH AS A FUNCTION OF EXTRACELLULAR 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 endothelial cells (Wahl *et al*., 2002; Wahl and Grant, 2002). 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 manifest at low extracellular pH. These observations implicate pH regulating transporters that are active at low extracellular pH. The two known transporters of protons are the sodium proton exchanger (NHE) (Orlowski and Grinstein, 1997) and the H^+-linked monocarboxylate exchanger (MCT) (Halestrap and Price, 1999). Studies are underway to determine whether these transporters play a role in the pH effect of angiostatin, and whether the F_1–F_0 ATP synthase is directly or indirectly involved in pumping protons out of the cell. This is a matter of great interest, because the pH gradient in the tumor is inwardly directed, and yet it seems as though the proton trafficking must be in the direction opposite of the gradient in order to maintain a normal intracellular pH in the face of a relatively high concentration of extracellular protons.

INHIBITOR OF F1 (IF1)—A POTENTIAL MODULATOR

Angiostatin was 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. Studies using isolated mitochondria and intact endothelial cells 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 that 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. 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.

NEW APPLICATIONS FOR EXISTING DRUGS

Many existing anti-tumor agents were originally evaluated in escalating dose trials with a goal of maximal tumor kill, often with severe side effects. These drugs can now be reevaluated in terms of their toxicity towards endothelial cells in tumor blood vessels. The ready access to tumor endothelial cells suggests that lower doses, and hence lower toxicity, may be sufficient to achieve an anti-tumor vascular effect.

Certain candidate compounds that failed as anti-angiogenic agents may have opportunities for revival through the use of improved delivery tools, such as implantable timed release osmotic mini-pumps, that can potentially sustain therapeutic levels of drug. Also, since tumor endothelial cells are genetically stable, they are less prone to development of resistance to anti-angiogenic compounds than are tumor cells. 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 analogues that are more active at low extracellular pH (Adams *et al*., 2000a; Adams *et al*., 2000b; Adams *et al*., 2005). 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 (Daliani *et al*., 2002; Escudier *et al*., 2002; Figg *et al*., 2001; Gutheil and Finucane, 2002; Short *et al*., 2001).

CONCLUSIONS

Although angiostatin exerts greater endothelial cells-killing activity at low pH, we have shown that angiostatin is able to bind and inhibit cell surface F_1F_0 ATP synthase over a range from pH 6.5 to 7.5. It has also been shown that endothelial cells can maintain a relatively normal intracellular pH even though the extracellular pH has decreased to tumor-like conditions of pH 6.5–6.7 (Wahl and Grant, 2002). The addition of angiostatin to endothelial cells under external pH stress causes a rapid and dramatic decrease in intracellular pH, which is associated with endothelial cells death by inhibiting metabolic enzymes which all have pH optima above 7.0 (Wahl and Grant, 2002). Moreover, angiostatin appears to exert no deleterious effects on endothelial cells cultured at normal pH, suggesting that pH stress is the primary mediator of endothelial cells cell death by angiostatin. Therefore, it seems likely that angiostatin-mediated inhibition of proton flux via F_1F_0 ATP synthase is responsible for the increased sensitivity of endothelial cells 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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REFERENCES

Adams, D. J., Dewhirst, M. W., Flowers, J. L., Gamcsik, M., Colvin, O. M., Manikuman, G., Wani, M. C., and Wall, M. (2000a). Camptothecin analogues with enhanced antitumor activity at acidic pH. *Cancer Chemother. Pharmacol.* **4**, 263–271.

Adams, D. J., Flowers, J. L., Pommier, Y., Colvin, O., Manikumar, G., Wani, M. C., and Wall, M. (2000b). 7-Substituted camptothecins exhibit enhanced potency and pH modulation in human breast cancer cells. In Proceedings of the *91st Annual Meeting of the American Association for Cancer Research*, San Francisco, CA.

Adams, D. J., Wahl, M. L., Flowers, J. F., Sen, B., Colvin, O. M., Dewhirst, M. W., Manikumar, G., Wani, M. C., and Wall, M. E. (2005). Camptothecins with 7-alkyl substitutions have enhanced activity against human breast cancer: Impact of the tumor pH gradient. *Cancer Chemother. Pharmacol.* **205**, 1–10.

Burwick, N. R., Wahl, M. L., Fang, J., Zhong, Z., Moser, T. L., Li, B., Capaldi, R. A., Kenan, D. J., and Pizzo, S. V. (2005). An inhibitor of the F1 subunit of ATP synthase (IF1) modulates the activity of angiostatin on the endothelial cell surface. *J. Biol. Chem.* **280**, 1740–1745.

Contarino, M. R., Fang, J., Pizzo, S. V., and Wahl, M. L. (2004). The anti-angiogenic potential of pH regulation inhibitors. In *Proceedings of the American Association for Cancer Research*, Orlando, FL, 04-AB-1548-AACR.

Daliani, D. D., Papandreous, C. N., Thall, P. F., Wang, X., Perez, C., Oliva, R., Pagliaro, L., and Amato, R. (2002). A pilot study of thalidomide therapy in refractory solid tumour patients. *Cancer* **95**, 758–765.

D'Amato, R. J., Loughnan, M. S., Flynn, E., and Folkman, J. (1994). Thalidomide is an inhibitor of angiogenesis. *Proc. Natl. Acad. Sci. U.S.A.* **91**, 4082–4085.

Das, A. M. (1998). Regulation of mitochondrial ATP synthase activity in human myocardium. *Clin. Sci.* (*Lond.*) **94**, 499–504.

Das, B., Mondragon, M. O. H., Sadeghian, M., Hatcher, V. B., and Norin, A. J. (1994). A novel ligand in lymphocyte-mediated cytotoxicity: Expression on the β subunit of H^+ transporting ATP synthase on the surface of tumor cell lines. *J. Exp. Med.* **180**, 273–281.

DeMoraes, E. D., Fogler, W. E., Grant, D. S., Wahl, M. L., Leeper, D. B., Zrada, S., Malin, A., Connors, S., Fortier, A. H., Dabrow, M., Sidor, C., and Capizzi, R. L. (2001). Recombinant human angiostatin (rhA): A Phase I clinical trial assessing safety, pharmacokinetics (PK) and pharmacodynamics (PD). *American Society of Clinical Oncology*, phase I trials, Abstract #10.

Escudier, B., Lassau, N., Couanet, D., Angevin, E., Mesrati, F., Leborgne, S., Garofano, A., Leboulaire, C., Dupouy, N., and Laplanche, A. (2002). Phase II trial of thalidomide in renal-cell carcinoma. *Ann. Oncol.* **13**, 1029–1035.

Figg, W. D., Arlen, P., Gulley, J., Fernandez, P., Noone, M., Fedenko, K., Hamilton, M., Parker, C., Kruger, E. A., Pluda, J., and Dahut, W. L. (2001). A randomized phase II trial of docetaxel (taxotere) plus thalidomide in androgen-independent prostate cancer. *Semin. Oncol.* **28**, 62–66.

Folkman, J. (1971). Tumor angiogenesis: Therapeutic implications. *N. Engl. J. Med.* **285**, 1182–1186.

Funatsu, H., Yamashita, H., Noma, H., Mochizuki, H., Mimura, T., Ikeda, T., and Hori, S. (2003). Outcome of vitreous surgery and the balance between vascular endothelial growth factor and endostatin. *Invest. Ophthalmol. Vis. Sci.* **44**, 1042–1047.

Gutheil, J., and Finucane, D. (2002). Thalidomide therapy in refractory solid tumour patients. *Br. J. Haematol.* **110**, 754.

Halestrap, A. P., and Price, N. T. (1999). The proton-linked monocarboxylate transporter (MCT) family: Structure, function, and regulation. *Biochem. J.* **343**, 281–299.

Holash, J., Wiegand, S. J., and Yancopoulos, G. D. (1999). New model of tumor angiogenesis: Dynamic balance between vessel regression and growth mediated by angiopoietins and VEGF. *Oncogene* **18**, 5356–5362.

Holmgren, L., O'Reilly, M. S., and Folkman, J. (1995). Dormancy of micrometastases: Balanced proliferation and apoptosis in the presence of angiogenesis suppression. *Nat. Med.* **1**, 149–153.

Moser, T. L., Kenan, D. J., Ashley, T. A., Roy, J. A., Goodman, M. D., Misro, U. K., Cheek, D. J., and Pizzo, S. (2001). Endothelial cell surface F_1–F_0 ATP synthase is active in ATP synthesis and is inhibited by angiostatin. *Proc. Natl. Acad. Sci. U.S.A.* **98**, 6656–6661.

Moser, T. L., Stack, M. S., Asplin, I., Enghild, J. J., Hojrup, P., Everitt, L., Hubchak, S., Schnaper, H. W., and Pizzo, S. V. (1999). Angiostatin binds ATP synthase on the surface of human endothelial cells. *Proc. Natl. Acad. Sci. U.S.A.* **96**, 2811–2816.

Moser, T. L., Stack, M. S., Wahl, M. L., and Pizzo, S. V. (2002). The mechanism of action of angiostatin: Can you teach an old dog new tricks? *Thromb. Haemost.* **87**, 394–401.

O'Reilly, M. S., Holmgren, L., Chen, C., and Folkman, J. (1996). Angiostatin induces and sustains dormancy of human primary tumors in mice. *Nat. Med.* **2**, 689–692.

O'Reilly, M. S., Holmgren, L., Shing, Y., Chen, C., Rosenthal, R. A., Cao, Y., Moses, M., Lane, W. S., Sage, E. H., and Folkman, J. (1994a). Angiostatin: A circulating endothelial cell inhibitor that suppresses angiogenesis and tumor growth. *Cold Spring Harbor Symp. Quant. Biol.* **59**, 471–482.

O'Reilly, M. S., Holmgren, L., Shing, Y., Chen, C., Rosenthal, R. A., Moses, M., Lane, W. S., Cao, Y., Sage, E. H., and Folkman, J. (1994b). Angiostatin: A novel angiogenesis inhibitor that mediates the suppression of metastases by a Lewis lung carcinoma. *Cell* **79**, 315–328.

Orlowski, J., and Grinstein, S. (1997). Na^+/H^+ exchangers of mammalian cells. *J. Biol. Chem.* **272**, 22373–22376.

Short, S. C., Traish, D., Dowe, A., Hines, F., Gore, M., and Brada, M. (2001). Thalidomide as an anti-angiogenic agent in relapsed gliomas. *J. Neurooncol.* **51**, 41–45.

Soltys, B. J., and Gupta, R. S. (1999). Mitochondrial-matrix proteins at unexpected locations: Are they exported? *Trends Biochem. Sci.* **24**(5), 174–177.

Soltys, B. J., Kang, D., and Gupta, R. S. (2000). Localization of P32 protein (gC1q-R) in mitochondria and at specific extramitochondrial locations in normal tissues. *Histochem. Cell Biol.* **114**, 245–255.

Wahl, M. L., and Grant, D. S. (2002). Effects of microenvironmental extracellular pH and extracellular matrix proteins on angiostatin's activity and on intracellular pH. *J. Gen. Pharm.* (*Vascular*) **35**, 1–10.

Wahl, M. L., Owen, C. S., and Grant, D. S. (2002). Low dose angiostatin induces intracellular acidosis and anoikis cell death in endothelial cells at tumor-like low pH. *Endothelium* **9**, 205–216.

Yamagata, M., and Tannock, I. F. (1996). The chronic administration of drugs that inhibit the regulation of intracellular pH: *In vitro* and anti-tumour effects. *Br. J. Cancer* **73**, 1328–1334.