Introduction
The targeting of tumor vasculature is a promising approach to cancer therapy. Antiangiogenic agents inhibit the growth of new blood vessels, a nutritive network enabling primary tumors to move beyond dormancy and facilitating metastasis of established tumors (1–6). However, tumor expansion and eventual dissemination are complex processes involving a combination of tumor properties besides the ability to stimulate angiogenesis; these include adherence, invasiveness, and microthrombus formation (7). Thus, in contrast to antiangiogenic agents that act exclusively on the endothelial cells lining tumor vasculature, agents with both antiangiogenic and antitumorigenic activities may confer greater therapeutic advantage through additive effects, avoiding reliance on the degree of tumor vascularization and affecting the tumor at multiple stages (8).

Materials and Methods

Materials. Angiostatin kringles 1 to 3, derived from human plasminogen, was obtained from Calbiochem (Darmstadt, Germany) and reconstituted in sterile PBS. Polyclonal antibodies directed against the catalytic β-subunit of ATP synthase were generated and bovine F1 ATP synthase subunit was purified as previously described (11, 12). Cariporide (gift of Dr. Miriam Wahl, Duke University, Durham, NC) was solubilized in sterile water and sterile filtered.

Cell culture. A549, 1-LN, DU145, LNCaP and PC-3 cells were obtained from Duke Cell Culture Facility (Durham, NC). SAOS2 cells were the gift of Dr. Stephanie Gaillard (Duke University, Durham, NC). Human umbilical vein endothelial cells (HUVEC) were isolated from human umbilical veins as described (22). Cells were cultured in DMEM (Life Technologies, Carlsbad, CA) with 1% penicillin-streptomycin and 10% serum replacement medium 3 (Sigma, St. Louis, MO) to minimize the presence of plasminogen. Low-pH (6.7) medium was prepared by reducing bicarbonate to 10 mmol/L at 5%
CO<sub>2</sub> and supplementing with 34 mmol/L NaCl to maintain osmolality or incubation of 22 mmol/L bicarbonate medium under 17% CO<sub>2</sub> conditions. The method of lowering pH used varied by experimental constraints and assay.

Flow cytometry. Cells were cultured in varying pH medium (10, 22, and 44 mmol/L bicarbonate DMEM), under hypoxia (0.5% O<sub>2</sub>, 5% CO<sub>2</sub>, N<sub>2</sub> balanced) versus normoxia (21% O<sub>2</sub>, 5% CO<sub>2</sub>) for 0, 12, 24, 48, and 72 hours. Live cells were blocked, incubated with anti-β-subunit antibody, washed, blocked, incubated with a secondary goat anti-rabbit antibody-FITC (Southern Biotech, Birmingham, AL), and again washed, with all steps at 4°C. Propidium iodide (BD Biosciences, San Jose, CA) was included with all samples to discriminate cells with compromised membranes. The mean fluorescent intensity of FITC in 10,000 cells was quantified by FACSCalibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ) and cells with propidium iodide uptake were excluded to eliminate detection of mitochondrial ATP synthase on CELLquest software (BD Biosciences).

Cell surface ATP generation assay. A549 or 1-LN cells (60,000 per well) in 96-well plates were refreshed with medium and treated with angiostatin, anti-β-subunit antibody, rabbit IgG raised to bovine serum albumin (Organon Teknika, West Chester, PA), picetaninol (a known inhibitor of ATP synthase F<sub>1</sub> used as a positive control, Sigma), or medium alone for 30 minutes at 37°C, 5% CO<sub>2</sub>. Cells were then incubated with 0.05 mmol/L ADP for 20 seconds. Supernatants were removed and assayed for ATP production by CellTiterGlo luminescence assay (Promega, Madison, WI) as described (23). Cell lysates were similarly analyzed to confirm that intracellular pools of ATP did not vary under any conditions. Recordings were made on the Luminoskan Ascent (Thermo Labsystems, Helsinki, Finland). Data are expressed in moles of ATP per cell based on standards determined for each independent experiment.

Cell proliferation assay. The effect of angiostatin on cancer cell lines was assessed with a 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) proliferation assay in serum-free medium. Relative cell numbers in each well of a 96-well microplate after incubation for 20 hours, 37°C, and 5% CO<sub>2</sub> in the presence or absence of angiostatin were determined using the AQueous One Cell Proliferation Assay (Promega) per protocol of the manufacturer. Medium pH was regulated at 5% CO<sub>2</sub> through bicarbonate concentration.

Assessment of cellular cytotoxicity. To quantitate cell death and cell lysis, we measured the activity of lactate dehydrogenase (LDH) released from the cytosol into supernatant with the Cytotoxicity Detection kit (Roche, Indianapolis, IN). A549 and 1-LN cells (5,000 per well) treated with angiostatin, anti-β-subunit antibody, rabbit IgG, cariporide, and Triton X (a detergent used to permeabilize cells as a positive control) were incubated at 37°C and 5% CO<sub>2</sub> for 15 hours at neutral and low-pH conditions, respectively. An index of cytotoxicity was calculated by dividing the average absorbance from treated samples in quadruplicate by the average absorbance from untreated samples in quadruplicate corresponding to the same pH medium.

Assessment of cellular necrosis and apoptosis. The effects of angiostatin, anti-β-subunit antibody, rabbit IgG, and cariporide on A549 cells (5,000 per well) were determined using an ELISA apoptosis and necrosis assay (Roche) that is dependent on detection of extracellular histone-DNA fragments. Apoptosis or necrosis was determined, respectively, the cell lysates or supernatants of quadruplicate samples after 15 hours of incubation at 37°C, in the presence or absence of agents. The apoptotic or necrotic indices were calculated by dividing the average absorbance from treated samples in quadruplicate by the average absorbance from untreated samples in quadruplicate corresponding to the same pH medium. Medium pH was regulated by incubation at 5% CO<sub>2</sub> or 17% CO<sub>2</sub>.

Intracellular pH measurement. pH was measured by fluorescence in cells plated on 35-mm microwell dishes with glass coverslips (MatTek, Ashland, MA). Cells were plated on growth factor–reduced, phenol-red free Matrigel (BD Biosciences). After overnight growth, medium was changed and cells were loaded with the pH-sensitive fluorescent dye cSNARF (Molecular Probes, Eugene, OR) for 15 minutes followed by 20 minutes recovery in fresh medium. Cells were then mounted on a microscope stage at 37°C, 5% CO<sub>2</sub> for 1-hour-long collection of emission spectra from which pH was calculated as described from fields containing between 7 and 15 cells each (21, 24). At the start of spectra collection, medium was removed from the dish and cells were challenged with 1 mL of fresh medium in the presence or absence of pH inhibitors angiostatin, anti-β-subunit, rabbit IgG, or cariporide. Medium pH was regulated by bicarbonate concentration, as described above, with fixed %CO<sub>2</sub>.

Results

ATP synthase, the angiostatin receptor, is expressed on the extracellular surface of tumor cells. We have reported previously that ATP synthase is both present and active on the extracellular surface of endothelial cells and that angiostatin binds to the protein and inhibits its activity. Others have documented extracellular ATP synthase, or its subunits, on various tumor cell lines, but its function remains unknown (16-18, 25). Here, we expand on those results to study the role of ATP synthase on the surface of tumor cells. Using flow cytometry, we first identified the presence of extracellular ATP synthase on seven human tumor cell lines; primary endothelial cells were included for purposes of comparison (Fig. 1A). We observed that ATP synthase is present on all cell lines but that expression is higher in certain cell lines than others. Specifically, the human lung adenocarcinoma line A549 displayed an amount of extracellular ATP synthase equivalent to HUVeCs, whereas the human prostate cancer line 1-LN had a 6-fold lower expression. These two tumor cell lines were subsequently used to contrast effects due to extracellular ATP synthase.

ATP synthase is functional on the cell surface of the tumor cell A549 and its activity is inhibited by angiostatin or anti-ATP synthase antibody. We then studied the activity of tumor cell surface ATP synthase by evaluating enzymatic synthesis of ATP from ADP. Because the antibody directed against the catalytic β-subunit of the F1 complex of ATP synthase binds the A549 cell surface as shown through flow cytometry, we concluded that the F<sub>1</sub> complex is oriented on the outer portion of the membrane. Permeabilized cells were excluded by using propidium iodide. Given this orientation, should the cell membrane–embedded ATP synthase be active, ATP production would occur on the extracellular surface. In an A549 cell surface–based ATP synthesis assay, during a 5-second period, ATP was generated from ADP on a scale of 10<sup>-15</sup> mol/cell (Table 1). This magnitude of ATP synthase activity is comparable with our previous observations on HUVeCs (12, 23). In the absence of ADP, ATP generation was negligible, indicating that the majority of ATP measured could be attributed to the synthase. Prior work showed a role for extracellular ATP synthesis in conditions of acidosis (21). Therefore, we examined the effect of pH<sub>c</sub> on the activity of tumor cell surface ATP synthase. Under conditions of acidosis (pH<sub>c</sub> 6.7), ATP synthesis in A549 cells was nearly double that of cells at pH<sub>c</sub> 7.2. Flow cytometry did not reveal any changes in cell surface ATP synthase expression under a panel of conditions, including acidosis, hypoxia, and a combination of acidosis and hypoxia (data not shown).

Previously, we showed that on the endothelial cell surface, angiostatin inhibits the ability of ATP synthase to synthesize ATP. In A549 cells, angiostatin also inhibited ATP synthesis–driven ATP synthesis (Fig. 1B). Furthermore, in A549 cells, the polyclonal anti-β-subunit antibody showed greater dose-dependent inhibition of ATP synthesis, most markedly under conditions of acidosis, compared with angiostatin and a control rabbit IgG. Both angiostatin and anti-β-subunit antibody were less effective at reducing ATP synthesis on 1-LN cells. To rule out changes in intracellular
ATP contributing to observed effects, intracellular concentrations of ATP were measured. Intracellular ATP was not affected by antibody treatment at either pH, whereas piceatannol, a small molecule inhibitor of ATP synthase, was membrane permeable and inhibited mitochondrial ATP synthesis (Fig. 1C). These results show that, as in endothelial cells, ATP synthase is active on the tumor cell surface and inhibited by angiostatin and anti-β-subunit antibody, confirming specificity of the interaction with ATP synthase. Furthermore, the activity of tumor cell surface ATP synthase is increased by exposure to low pHₐc.

**Angiostatin decreases cell proliferation in tumor cells under conditions of acidosis by inducing cell death.** To observe the consequences in tumor cells of ATP synthase inhibition by angiostatin, we did a proliferation assay on A549 cells treated with angiostatin under conditions of neutral pHₐc (7.2) or acidosis (pHₐc 6.7) because angiostatin decreases endothelial cell proliferation (9, 10, 26). A549 cells exposed to angiostatin and acidosis exhibited a marked decrease in proliferation compared with cells treated with angiostatin at neutral pHₐc (Fig. 1D). To further explore these results, we used an LDH cytotoxicity assay to probe for cell death. Indeed, we observed that under conditions of acidosis, A549 cells exhibited dose-dependent cytotoxicity when treated with angiostatin compared with cells maintained under neutral pHₐc (7.2) conditions (Fig. 2A). To probe the specificity of ATP synthase in these events, we used anti-β-subunit antibody under the same assay conditions and observed enhanced cytotoxicity compared with angiostatin treatment (Fig. 2A). At low pHₐc, maximum cytotoxicity induced by anti-β-subunit antibody was twice that caused by angiostatin. Rabbit IgG, a negative control for the polyclonal antibody, did not elicit any effects on cell viability (data not shown). In comparison, for 1-LN cells treated with angiostatin or anti-β-subunit antibody under acidic conditions, we observed no inhibition of cell proliferation (data not shown) nor did we detect any cytotoxicity (Fig. 2B). To profile A549 cell cytotoxicity induced by angiostatin or the anti-β-subunit antibody, we did time course experiments that showed peak cytotoxicity after 12 hours of incubation with each ATP synthase-inhibiting agent (Fig. 2C and D). Thus, angiostatin is not limited in its antiproliferative effects to endothelial cells; at low pHₐc, angiostatin reduces tumor cell proliferation likely through observed cytotoxicity. Similar

![Image](https://www.aacrjournals.org/877/CancerRes2006;66(2).January15,2006)

**Figure 1.** ATP synthase is present on the tumor cell surface and inhibited by angiostatin or anti-ATP synthase antibody. A, cell surface expression of ATP synthase on tumor cells (dark columns) was compared with that on HUVEC (white columns), a positive control that expresses ATP synthase on the cell surface (11–13). Tumor cells were harvested with EDTA, with all subsequent steps done at 4°C to prevent internalization of surface antigens. Cells were washed, incubated with 2.0 μg of polyclonal rabbit antibody directed against the catalytic β-subunit of human ATP synthase per 10⁶ cells for 1 hour, washed, incubated with goat anti-rabbit IgG-FITC for 1 hour, rinsed, and resuspended with 6.0 μL of propidium iodide per 10⁶ cells. Propidium iodide was used to exclude permeabilized cells and, thus, avoid detection of intracellular ATP synthase. Columns, mean peak intensity calculated for all conditions (n = 5); bars, SD. Data are reported as the increase in the mean intensity over the mean intensity for cells incubated with secondary goat-anti rabbit IgG-FITC alone. B, ATP generation on the surface of A549 cells was inhibited in the presence of angiostatin (1 μmol/L, black columns), anti-β-subunit polyclonal antibody at 40 and 80 μg/mL (gray columns), compared with rabbit IgG as a negative control at 40 and 80 μg/mL (white columns). Inhibitory effects from antibody treatment were greater at low pHₐc (6.7) than at neutral pHₐc (7.2) and this inhibition was reduced in 1-LN (ILN) cells. 1, angiostatin (1 μmol/L); 2, anti-β-subunit antibody (40 μg/mL); 3, anti-β-subunit antibody (80 μg/mL); 4, rabbit IgG (40 μg/mL); 5, rabbit IgG (80 μg/mL). Columns, percent decrease in the mean activity of treated cells compared with the mean of control cells, which were exposed to medium alone at pHₐc 6.7 or 7.2 (n = 5); bars, SD. A Student’s t test was used for statistical analysis by comparing antibody treatment to rabbit IgG treatment at equivalent dosages. *, P < 0.001; **, P = 0.001. C, intracellular ATP generation is not affected by pH. Intracellular ATP pools from (B) did not vary significantly by pH (white columns, pHₐc 7.2: dark columns, pHₐc 6.7) nor by treatment condition with medium alone or anti-β-subunit antibody at 40 μg/mL. In contrast, piceatannol (PC), a membrane-permeable small molecule, was used as a positive control. Intracellular ATP pools from (B) were examined in quadruplicate. Columns, mean; bars, SD. D, angiostatin at various concentrations was added in fresh medium to A549 cells in 96-well plates for 20 hours at either neutral pHₐc 7.2 (□), or low pHₐc 6.7 (△), achieved by reducing bicarbonate concentration to 10 mmol/L. Relative cell numbers were determined using an MTS assay with reagent added directly to cell wells and all samples incubated for 4 hours at 37°C, 5% CO₂. Absorbance was then measured. Points, percentage of inhibition of cellular proliferation calculated from quadruplicate samples by setting proliferation in the absence of angiostatin to 100% for each pHₐc, thus avoiding differences in proliferation due to pHₐc alone; bars, SD.
results obtained with the anti-β-subunit antibody implicate tumor cell surface ATP synthase in the antitumorigenic property of angiostatin.

Characterization of cell death observed in A549 cells treated with angiostatin under conditions of acidosis. To identify the mode by which angiostatin and anti-β-subunit antibody induced death in A549 cells, we used a histone-DNA ELISA to discriminate between apoptotic and necrotic cell death. Surprisingly, angiostatin caused an exclusively necrotic cell death in A549 cells under conditions of acidosis (pHe 6.7; Fig. 3A). This effect was dose dependent and was not observed at neutral pH (pHe 7.2) nor did we detect apoptotic death at either neutral or low-pH conditions. Similarly, treatment of A549 cells with anti-β-subunit antibody combined with acidosis also resulted in necrosis, with a greater index of cell death achieved using anti-β-subunit antibody compared with angiostatin (Fig. 3B). Staurosporine, used as a positive control, induced equivalent apoptotic death across both pH conditions (data not shown). These data suggest that angiostatin or anti-β-subunit antibody in combination with low pH induces necrotic death in tumor cells.

Angiostatin and anti-β-subunit antibody disrupt the regulation of pH in tumor cells under conditions of acidosis. To further explore the mechanism by which angiostatin contributes to tumor cytotoxicity, we investigated whether, under conditions of acidosis, angiostatin could cause deregulation of pH in tumor cells. We used a histone-DNA ELISA to discriminate between apoptotic and necrotic cell death. Surprisingly, angiostatin caused a decrease in pH in endothelial cells at low pH (pHe 6.7). When challenged with low-pH medium (pHe 6.7) containing angiostatin, the pH of A549 cells dropped precipitously (~0.4 pH units (Fig. 3C)), a decrease that was not evident in cells challenged with either low-pH medium alone or angiostatin in neutral pH medium (pHe 7.2; data not shown). A549 cells treated with anti-β-subunit antibody in combination with low-pH medium exhibited the same effect, with an even more rapid decline in pH, whereas rabbit IgG did not elicit any change in pH. Likewise, in a low-pH challenge, anti-β-subunit antibody at a more dilute concentration (40 μg/ml) resulted in a pH decrease of ~0.15 pH units (data not shown). Preincubating angiostatin with purified, soluble bovine ATP synthase F1 protected against the decrease in pH induced by angiostatin under conditions of acidosis (Table 2). Thus, angiostatin or anti-β-subunit antibody in conjunction with low pH causes deregulation of tumor cell pH and intracellular acidification.

Inhibition of the sodium-proton exchanger also decreases pH and causes tumor cell death, but these effects are not enhanced under conditions of acidosis. To test our hypothesis that deregulation of pH induces cytotoxicity, we sought to determine whether the sodium-proton exchange inhibitor cariporide can reduce pH and thereby cause cytotoxicity in tumor cells. Indeed, when challenged under conditions of acidosis (pHe 6.7), the pH of A549 cells simultaneously treated with cariporide decreased 0.5 pH units (Fig. 4A). Cariporide was also cytotoxic to A549 cells.

### Table 1. ATP synthase is active on the tumor cell surface

<table>
<thead>
<tr>
<th>ATP concentration (mol⁻¹·cell⁻¹)</th>
<th>pH&lt;sub&gt;e&lt;/sub&gt; 7.2</th>
<th>pH&lt;sub&gt;e&lt;/sub&gt; 6.7</th>
</tr>
</thead>
<tbody>
<tr>
<td>Medium alone</td>
<td>1.3 ± 0.2</td>
<td>1.5 ± 0.3</td>
</tr>
<tr>
<td>ADP (50 μmol/L)</td>
<td>14.7 ± 0.2</td>
<td>22.9 ± 1.6</td>
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NOTE: Extracellular ATP generation on A549 cells was measured by bioluminescent surface assay following 20-second incubation with medium in the presence or absence of ADP. Low pH<sub>e</sub> (6.7) increases ATP concentration on the surface of A549 by 56% compared with neutral pH<sub>e</sub> (7.2). ATP generation in the absence of ADP was negligible, indicating that extracellular ATP synthesis is primarily due to the activity of cell surface ATP synthase; n = 5 with SD are shown.
Therefore, perturbation of pH \(i\) by inhibiting a membrane-caused both apoptotic and necrotic death, again with increased death observed from ATP synthase inhibition, cariporide treatment the polyclonal anti-

Neutral pH (pHe 7.2) or low pH (pHe 6.7) was achieved by incubation at 5% CO\(_2\) medium was refreshed and cells were loaded with cSNARF, a pH-sensitive fluorescent dye. Samples were then mounted on a microscope stage at 37

A549 cells were plated on 35 mL of Matrigel on glass coverslips at 1.5

angiostatin or anti-ATP synthase antibody cause intracellular acidification and necrosis in tumor cells at low pH. A and B, ELISAs to detect extracellular histone-DNA fragments were done on A549 cells (5,000 per well) in 96-well plates. Angiostatin (A) or anti-\(\mu\)-subunit polyclonal antibody (B) was added in fresh medium and cells were incubated for 12 hours at 37°C. Supernatant was removed to assay for necrosis (\(\times\)), neutral pH; ▲, low pH). Neutral pH (pHe 7.2) or low pH (pHe 6.7) was achieved by incubation at 5% CO\(_2\) or 17% CO\(_2\), respectively. All samples were assayed immediately and compared with pH-matched samples treated with medium alone. Staurosporine (500 nmol/L) was used as a positive control for apoptosis (data not shown). C, angiostatin or anti-ATP synthase antibody decreased pH\(_i\) in tumor cells. A549 cells were plated on 35 mL of Matrigel on glass coverslips at 1.5 \(\times\) 10\(^5\) per microwell dish and allowed to attach overnight. Before pH\(_i\) measurement, medium was refreshed and cells were loaded with cSNARF, a pH-sensitive fluorescent dye. Samples were then mounted on a microscope stage at 37°C, 5% CO\(_2\) for a 1-hour period to collect cSNARF excitation spectra for the calculation pH\(_i\). For each sample, at time 0, medium was removed and fresh medium was equilibrated to pH\(_e\) 6.7 at 5% CO\(_2\) was added in the presence of angiostatin (\(\sim\), 1.05 nmol/L), anti-\(\mu\)-subunit polyclonal antibody (\(\sim\), 0.53 nmol/L), or rabbit IgG (\(\sim\), 0.53 nmol/L) as a negative control for the polyclonal anti-\(\mu\)-subunit antibody.

as assessed in an LDH release assay (Fig. 4B). However, cariporide induced cell death at both neutral and low pH, with a slightly greater toxicity at neutral pH (pHe 7.2). In contrast to the necrotic death observed from ATP synthase inhibition, cariporide treatment caused both apoptotic and necrotic death, again with increased cell death observed at neutral, not acidic, pH (Fig. 4C and D). Therefore, perturbation of pH\(_i\) by inhibiting a membrane-embedded proton transporter other than ATP synthase also causes cytotoxicity. However, the mode of cell death includes apoptosis as well as necrosis. Furthermore, in contrast to the results observed with ATP synthase inhibition, low pH\(_i\) does not enhance the cytotoxicity of cariporide; instead, it seems that at low pH\(_i\), cariporide inhibition has a lesser effect on tumor cell viability than at neutral pH\(_i\).

Discussion

With the anti–vascular endothelial growth factor antibody bevacizumab (Avastin) gaining recognition for clinical success in delaying tumor progression in combination with chemotherapy, the utility of angiogenesis in conjunction with antitumorogenesis for cancer treatment is increasingly apparent (5). In this study, we show that the angiogenesis inhibitor angiostatin is directly cytotoxic to tumor cells. We expand on previous work reporting that angiostatin inhibits endothelial cell surface ATP synthase to reduce proliferation (11, 12). Here, we show that (a) ATP synthase is present on the surface of tumor cells and its expression does not change under conditions of acidosis and/or hypoxia, (b) tumor cell surface ATP synthase is active in the synthesis of ATP, with greater activity under conditions of acidosis (low pH\(_e\)), (c) angiostatin or anti-ATP synthase antibody inhibit cell surface ATP synthase activity, (d) angiostatin or anti-ATP synthase antibody decrease cell proliferation by causing necrotic cell death under conditions of acidosis, and (e) this necrotic cell death is likely a consequence of deregulation of pH\(_i\) subsequent to impaired activity of cell surface ATP synthase. These results are summarized in Fig. 5.

Angiostatin, a proteolytic fragment of plasminogen, potently antagonizes angiogenesis (10, 27, 28). Previously, we showed that angiostatin binds ATP synthase on the endothelial cell surface and inhibits the ATP synthesizing activity of this enzyme with consequent reduction of endothelial cell proliferation (11, 12). ATP synthase is best recognized as a resident of the inner mitochondrial membrane where it functions in the electron transport chain to generate ATP from the trans-membrane proton gradient (29). However, observations of cell surface ATP synthase are growing in number. Besides the endothelial cell, ATP synthase or its subunits have now been localized to the tumor cell surface of HepG2 hepatocellular carcinoma, H1299 non–small cell lung cancer, K562 erythroleukemia, and A549 adenocarcinoma (16–18). Thus, given its role in mediating the actions of angiostatin on the endothelial

Table 2. Soluble ATP synthase subunit Fj protects against intracellular acidification

| Medium alone | 7.337 ± 0.003 |
| Angiostatin (1.05 μmol/L) | 6.925 ± 0.004 |
| Bovine Fj ATP synthase (0.5 μmol/L) and angiostatin (1.05 μmol/L) | 7.162 ± 0.005 |

NOTE: Addition of purified ATP synthase Fj to A549 cells prevents the decrease of pH\(_i\) caused by angiostatin under conditions of acidosis (pHe 6.7). pH\(_i\) was measured in A549 as described. At time 0, fresh medium preincubated for 30 minutes with angiostatin (1.05 μmol/L) and purified bovine Fj ATP synthase (0.5 μmol/L) was introduced; the maximal concentration of Fj ATP synthase possible was used. Intracellular acidification in the presence of soluble Fj ATP synthase was reversed by 0.24 pH units, a 58% change; \(n = 7\) to 15 with SD are shown.
cell, we sought to determine whether ATP synthase is present on a variety of tumor cell lines. Indeed, we found that ATP synthase is present on the tumor cell surface, with certain tumor lines exhibiting higher expression than others. In comparing two prostate cancer lines, PC-3 and its highly metastatic and aggressive derivative 1-LN (30), 1-LN exhibited much lower surface expression of ATP synthase. In light of the present study, it is possible that on 1-LN, a comparative lack of ATP synthase may contribute to the metastatic potential of this cell line; tumor-generated angiostatin (31) would be less effective in containing the growth of a cell line expressing higher surface ATP synthase. In light of the present study, it is possible that on 1-LN, a comparative lack of ATP synthase may contribute to the metastatic potential of this cell line; tumor-generated angiostatin (31) would be less effective in containing the growth of a cell line expressing higher surface ATP synthase.

Having established the presence of tumor cell surface–associated ATP synthase, we next showed the enzymatic activity of ATP synthase by measuring extracellular ATP generation from ADP on intact tumor cells. We were able to rule out the possibility that ATP synthase is merely differentially expressed through flow cytometry experiments on tumor cells exposed to several combinations and durations of pH, and percent O2. In addition to confirming that tumor cell surface ATP synthase is active, we observed that the enzyme had greater activity under conditions of acidosis. Acidosis is a hallmark of the tumor microenvironment, a terrain poorly perfused by chaotic vasculature, and thus frequently characterized by hypoxia and dependency on glycolysis (20, 32). This metabolic reliance on glycolysis can be independent of oxygenation state and typifies many tumors, with consequent generation of lactic acid resulting in low pH, (19). It is possible that tumor cell surface ATP synthase, which couples ATP synthesis to trans-membrane proton transport (29), functions in the regulation of pH, by pumping protons into the extracellular space, with increased activity when the cell is challenged by environmental acidity. It is hypothesized that certain lethal tumor phenotypes, such as invasion and metastasis, arise not only from genetic alterations within the tumor but from tumor microenvironmental stimuli, such as acidosis (32). In tumors, extracellular acidity increases angiogenesis, mutation rate, invasiveness, and resistance to chemotherapy (33–35). Given our observations that angiostatin and anti-ATP synthase antibody inhibit tumor cell surface ATP synthesis, it is possible that angiostatin mediates tumor responses to acidity through tumor cell surface ATP synthase. This would render angiostatin selectively more potent in the acidic tumor microenvironment. Furthermore, we hypothesize that the sensitivity of individual tumors to angiostatin may depend on the degree of microenvironmental acidity within, and the extent of cell surface ATP synthase expressed on, each tumor.

We would expect that if the proton-exporting activity of tumor cell surface ATP synthase indeed functions in pH, homeostasis, then inhibition of the synthase would result in intracellular proton accumulation and pH, decrement. In fact, we did observe that tumor cell pH, dropped dramatically when cells were challenged with low pH, in combination with angiostatin or an antibody directed against the catalytic ß-subunit of the ATP synthase. Preincubating angiostatin with soluble ATP synthase protected against pH, decrease, thereby supporting tumor cell surface ATP synthase interaction with angiostatin as the mechanism behind the observed intracellular acidification. Interestingly, in addition to other pH, regulators, the vacuolar H+ATPases, which hydrolyze ATP and also operate as proton pumps, have recently emerged as regulators of cancer cell pH, at the tumor cell membrane, and show involvement in metastatic potential (36–38). The ATP synthase that we describe is a more complex enzyme, with the bifunctional ß-subunit of the ATP synthase.

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Figure 4. Cariporide-induced cell death is not enhanced at low pH. A, pH was measured in A549 cells as described. Cariporide (50 µM/L) in pH, 6.7 medium was added to cells at time 0. B, cariporide in fresh medium was added to A549 cells (5,000 per well) in 96-well plates, for 12 hours at 37°C before LDH release was assayed. Neutral pH, 7.2 (c) or low pH, 6.7 (o) was achieved by incubation at 5% CO2 or 17% CO2, respectively. C, a histone-DNA ELISA was done on A549 cells (5,000 per well) in 96-well plates. Cariporide was added in fresh medium and cells were incubated for 12 hours at 37°C. Supernatant was removed to assay for necrosis (C) and cells were lysed to assay for apoptosis (D). Neutral pH, 7.2 (c) or low pH, 6.7 (o) was achieved by incubation at 5% CO2 or 17% CO2, respectively. All samples were assayed immediately and compared with pH-matched samples treated with medium alone. Staurosporine (500 nmol/L) was used as a positive control for apoptosis (data not shown).
Intracellular acidification is linked to cell death and has been proposed as the basis for developing new anticancer agents (40, 41). In endothelial cells, angiostatin also causes intracellular acidification at reduced pH, and results in endothelial cell death by a nonapoptotic mechanism (21). We now extend these observations to tumor cells and confirm the role of ATP synthase using an antibody to the enzyme. Our data show that angiostatin or anti-β-subunit antibody in combination with acidosis inhibits tumor cell proliferation and provide evidence that this decrease in proliferation is due to direct cytotoxicity and necrotic cell death. Furthermore, anti-β-subunit antibody shows greater potency than angiostatin, which may result from the interaction of angiostatin with cell surface ligands other than ATP synthase (42) in addition to the greater specificity and binding affinity of antibodies for target ligands. Thus, for the purposes of cancer therapy, the development of antibodies to ATP synthase as angiostatin mimetics may prove advantageous in light of increased specificity, affinity, stability, and serum half-life (4 hours versus 25 days; ref. 28).

Prior work has described angiostatin as inducing apoptosis in endothelial cells. Our results indicate that in tumor cells, angiostatin seems to cause necrosis rather than apoptosis. However, our work is specific to conditions of extracellular acidosis and a survey of various agents interfering with pH, finds that the modality of cell death reported as a consequence of intracellular acidification is varied and inconclusive (43–45). Indeed, it is reported that, in tumors, targeting of the monocarboxylate transporter, another membrane-embedded regulator of pH, causes both apoptosis and necrosis (44). We also observe that targeting of the sodium-proton exchanger with cariporide results in apoptosis and necrosis as well. Interestingly, our data indicate that cariporide inhibition causes greater cell death at neutral pH than at low pH. Acidosis does not render tumor cells more sensitive to inhibition of the sodium-proton exchanger by cariporide, in contrast to the enhancing effect of acidosis on angiostatin-induced tumor cell necrosis. This may suggest that ATP synthase plays a larger role in pH homeostasis at lower pH, rendering cells more susceptible to inhibition by angiostatin under acidic conditions. Such a hypothesis is supported by our observations that the activity of the ATP synthase, as measured by ATP synthesis, is greater at low pH.

Moreover, necrosis is currently thought to be the explosive result of cellular ATP depletion (46), coinciding with our observations linking inhibition of extracellular ATP production to necrosis. Previously, small molecule inhibitors of the ATP synthase, such as oligomycin, were used to preferentially drive cells to necrosis rather than apoptosis by depleting intracellular ATP (47). Such studies may have overlooked the contributions of cell surface ATP synthase to total cellular ATP, as we have shown that these inhibitors also block cell surface ATP synthesis (12). Our data suggest that pH is crucial to the manner in which cells respond to inhibition of cell surface ATP synthase.

Finally, kringle 5, a proteolytic fragment of plasminogen that also possesses antiangiogenic properties, was recently shown to induce apoptosis in stressed tumor cells (48). Whereas kringle 5 binds to different cell surface targets than angiostatin, and transduces its effects through a mechanism independent of ATP synthase (49), these studies are of interest in establishing a known antiangiogenic agent as antitumorigenic. Here, we have provided evidence that the antitumorigenic action of angiostatin should not merely be ascribed to indirect effects from the endothelium, but that direct action targeting the tumor itself must be considered.

In summary, we have shown that angiostatin is directly cytotoxic to tumor cells under conditions of acidosis and that an interaction between angiostatin and tumor cell surface ATP synthase is responsible for inhibition of the synthase, resulting in intracellular acidification, which culminates in cell death. ATP synthase is present and active on the tumor cell surface and its role in pH homeostasis and potentially in cell death pathways necessitates further investigation. We are only beginning to appreciate the influence of the tumor microenvironment on tumor biology. The convergence of these findings may lead ultimately to cancer treatments that address both tumors and the vessels that supply them.

Figure 5. F$_{1}$F$_{0}$ ATP synthase regulates pH$_{i}$ on the tumor cell surface. ATP synthase is composed of a membrane-embedded portion, F$_{0}$, and soluble central stalk, F$_{1}$. F$_{1}$ functions as a proton channel with a rotary motion that drives the F$_{1}$ to synthesize or hydrolyze ATP depending on the direction of rotation (yellow). F$_{1}$ is composed of three α-subunits and three β-subunits alternately arranged to form a cylinder. The α-subunits modulate the activity of the catalytic β-subunits. Angiostatin binds to ATP synthase and inhibits both ATP synthesis and hydrolysis (green; ref. 42).
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49. Gonzalez-Gronow M, Kalfa T, Johnson CE, Dawid S. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
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