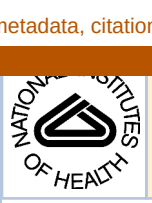

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An inhibitor of the F1 subunit of ATP synthase (IF1) modulates the activity of angiostatin on the endothelial cell surface[1]

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Abstract

Angiostatin binds to endothelial cell (EC)-surface F_1-F_0 ATP synthase, leading to inhibition of EC3 migration and proliferation during tumor angiogenesis. This has led to a search for angiostatin-mimetics specific for this enzyme. A naturally occurring protein that binds to the F1 subunit of ATP synthase and blocks ATP hydrolysis in mitochondria is Inhibitor of F1 (IF1). The present study explores the effect of IF1 on cell surface ATP synthase. IF1 protein bound to purified F_1 ATP synthase and inhibited F_1-dependent ATP hydrolysis consistent with its reported activity in studies of mitochondria. While exogenous IF1 did not inhibit ATP production on the surface of EC, it did conserve ATP on the cell surface, particularly at low extracellular pH. IF1 inhibited ATP hydrolysis but not ATP synthesis, in contrast to angiostatin, which inhibited both. In cell-based assays used to model angiogenesis *in vitro*, IF1 did not inhibit EC differentiation to form tubes and only slightly inhibited cell proliferation compared to angiostatin. From these data, we conclude that inhibition of ATP synthesis is necessary for an anti-angiogenic outcome in cell-based assays. We propose that IF1 is not an angiostatin-mimetic, but it can serve a protective role for EC in the tumor microenvironment. This protection may be overridden in a concentration-dependent manner by angiostatin. In support of this hypothesis, we demonstrate that angiostatin blocks IF1 binding to ATP synthase, and abolishes its ability to conserve ATP. These data suggest that there is a relationship between the binding sites of IF1 and angiostatin on ATP synthase and that IF1 could be employed to modulate angiogenesis.

Keywords

angiostatin; angiogenesis; ATP synthesis; cell surface-associated ATP synthase; endothelial cells; Inhibitory Factor 1; F_1-F_0 ATP synthase

INTRODUCTION

The term angiogenesis refers to the development of new blood vessels from preexisting vessels. This process is essential for maintaining and promoting tumor growth. One of the first anti-angiogenic agents discovered with the aim of treating cancers was angiostatin (1). Our laboratory identified F_1-F_0 ATP synthase as a receptor for angiostatin on the surface of human

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EC (2). This non-mitochondrial ATP synthase catalyzes ATP synthesis and is inhibited by angiostatin at low, tumor-like extracellular pH. The pH dependence explains the selectivity of angiostatin for the tumor microenvironment, where it inhibits EC migration and proliferation (3–5). Angiostatin inhibited both ATP production and ATP hydrolysis in previous studies (6). It was also demonstrated that polyclonal antibodies against the βcatalytic subunit or the α regulatory subunit of ATP synthase inhibited the enzyme bi-directionally and therefore acted as angiostatin-mimetics. However, it was unknown whether a specific inhibitor of ATP hydrolysis could also serve as an angiostatin-mimetic. In order to address this question, we have studied the effects of IF1, a natural inhibitor protein of F_1F_0ATP synthase, on EC surface ATP synthase.

The IF1 protein is a 9.6 kDa basic protein, comprised of 84 amino acids (7), which is known to inhibit the hydrolytic activity of mitochondrial ATP synthase (7,8). IF1 binds to ATP synthase at the F_1 domain, in the COOH-terminal region of the β-subunit (9–11), in an area that is in contact with the central γ - subunit (12). It has been proposed that IF1 disrupts the contact between the ß- and γ - subunits, inhibiting F_1 ATPase function (12). In addition, the binding of IF1 protein to ATP synthase depends on pH (13), with a pH of 6.5 or below favoring a stable complex with the enzyme (14). The ability of IF1 to inhibit ATP hydrolysis is well documented, but its role in the synthesis of ATP has been unclear (15–17). In addition, its ability to inhibit ATP synthesis on the surface of EC had not been explored. We here demonstrate that exogenous IF1 does not inhibit ATP production on the surface of EC; however, the addition of IF1 produced a relative increase in extracellular ATP as a result of inhibition of ATP hydrolysis. We therefore conclude that IF1 serves as a model of uni-directional inhibition of cell-surface ATP synthase, which has an ATP conserving effect. In addition, we demonstrate that IF1 does not have the anti-angiogenic effect of angiostatin, but it may attenuate the anti-angiogenic response to angiostatin.

EXPERIMENTAL PROCEDURES

Expression and purification of IF1

Recombinant human IF1 DNA was obtained as previously described (18). In brief, IF1 DNA (pET15b) containing a 6xHis tag were transformed into BL21(DE3) gold competent cells (Stratagene, La Jolla, CA). IF1 protein expression was induced with isopropyl-β-D-thiogalactopyranoside (IPTG) and batch purified over a Nickel ion-nitrilotriacetic acid (Ni-NTA) column (Qiagen, Valencia, CA) under non-reducing conditions before dialyzing into phosphate-buffered saline (PBS) buffer. A monoclonal antibody against IF1 (anti IF1 IgG_1) was employed to verify the presence of purified protein product by sandwich enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems, Minn. MN) or Western immunoblot as has been previously described (18).

Bovine F_1 ATP Synthase

Fresh bovine heart mitochondria were obtained as previously described (19) and sonicated to yield sub-mitochondrial particles (20). The F_1 portion was separated from membrane-bound F_0 by chloroform extraction. The aqueous layer was centrifuged at 105,000 X g to remove particulate matter before purifying over an S300 gel-filtration column. Human and bovine ATP synthase are highly homologous, differing only by eight amino acid residues in the mature α chains (SWISS-PROT accession nos. P25705 and P19483, respectively) and six residues in the mature β chains (SWISS-PROT accession nos. P06576 and P00829, respectively).

Angiostatin (K1-3)

Human angiostatin consisting of plasminogen kringles 1–3 (angiostatin) was purified as previously described (6,21). The concentration of angiostatin was determined

spectrophotometrically at a λ =280 nm by using an $A^{1\% / 1cm}$ value of 0.8 and a molecular mass of 38 kDa (21).

ELISA Binding Studies

Binding studies were performed with purified bovine F_1 ATP synthase (10 μg/ml) passively adsorbed onto micro-titer 96-well, flat bottomed plates (Dynex Technologies, Chantilly, VA). Briefly, plates were coated with protein in 50 μl of 0.1M Na_2C0_3, pH 9.6, and incubated overnight at 4°C. Nonspecific binding sites were blocked by incubating with PBS, pH 7.0, containing 1% bovine serum albumin (BSA) for 1 h at room temperature. Increasing concentrations of purified recombinant IF1 were added in a 50 μl final volume of PBS, 0.5% BSA and 0.05% Tween 20 for 1h at room temperature. Plates were washed with PBS, 0.05% Tween 20, pH 7.4 and incubated with an anti-IF1 IgG at 1 μg/ml for 1h at room temperature. Plates were washed and incubated with biotin conjugated goat anti-mouse IgG (1:10,000) (Zymed, South San Francisco, CA) for 1 h at room temperature. After washing, plates were incubated with streptavidin horseradish peroxidase (HRP-SA) (Zymed, South San Francisco, CA) at a 1:5000 dilution for 1 h. Plates were washed and 3,3′5,5′-tetramethylbenzidine (TMB) substrate (Sigma, St. Louis, MO) was added to the wells. The reaction was stopped with 50 μl of 1 M H_2S0_4 and color absorbance at λ =450 nm was measured on a SpectraMax® microplate spectrophotometer (Molecular Devices, Sunnyvale, CA).

Microplate F_1 Activity Assay

The forward reaction of ATP synthase results in production of ATP. The complete F_1-F_0 holoenzyme is required to catalyze the reaction. If only the F_1 subunit is present, only the reverse reaction, ATP hydrolysis, can occur. Therefore, to measure IF1 activity, the assay employs only the F_1 subunit. In this assay, the hydrolysis of ATP to ADP is coupled to the oxidation of NADH via pyruvate kinase and lactate dehydrogenase (LDH). The oxidation of NADH to NAD^+ may be read as a decrease in relative fluorescence units (excitation λ =355 nm, emission λ =460 nm). A decrease in fluorescence is a measure of increased F_1 activity. Briefly, purified bovine F_1 ATP synthase (10 μg/ml) was added to 96-well Microfluor® 2 black flat-bottomed plates (Thermo Labsystems, Franklin, MA) at 25 μl volume in PBS, pH 6.5 or pH 7.5. Inhibitors (IF1 or angiostatin), or controls (PBS, 2% sodium azide), were added to wells at increasing concentrations, diluted in PBS pH 6.5 or pH 7.5, for 1 hour. 2X Assay Buffer (4 mM PEP, 0.4 mM NADH, 4 mM ATP, 2% PK/LDH, 50 mM Tris-Acetate, 2 mM $MgCl_2$ in dH_20) was added to each well for 1 h before monitoring NADH fluorescence emission (λ =460nm) on a *fmax*® fluorescent plate reader (Molecular Devices, Sunnyvale, CA).

Cell Proliferation Assay

Human umbilical vein EC (HUVEC) were plated at a density of 5,000 cells/well in EGM medium (Clonetics, East Rutherford, NJ) depleted of FCS overnight to allow the cells to become quiescent. Fresh medium containing 5% FCS, 10 ng/ml basic fibroblast growth factor (FGF), and 3 ng/ml vascular endothelial cell growth factor (VEGF) were added to the wells along with IF1 protein (1 μg/ml or 10 μg/ml), PBS (pH 6.5), or cycloheximide (10 μg/ml). Cell density was measured at 24 h and 48 h using BrdU incorporation Cell Proliferation ELISA® (Roche, Basel, Switzerland) following the manufacturer's instructions.

Endothelial Cell Tube Differentiation

For the experiment in Figure 4, HUVEC were grown at 37ºC in EGM-MV cell medium (−VEGF/FGF) at pH 6.5 or pH 7.5 for 24 h. HUVEC were harvested from flasks using 4 mM EDTA. The cells were then diluted in EGM-MV cell medium (+VEGF/FGF) for a final concentration of 60,000 cells per well. IF1 (1 μM, 2.5 μM), PBS (pH 6.5, 7.5) or cycloheximide (10 μg/ml) were added to cells before incubating on 24-well plates previously coated with

Matrigel® at 37ºC. Tube formation was monitored over a 24 h period and images were taken using a CoolSNAP Digital Color Camera (Image Processing Solutions, North Reading, MA) with Olympus IX70 microscope (Olympus, Orangeburg, NY). For the experiment in HUVEC were grown as above, except during the experimental assay the pH was 6.1. For each error bar, 6–9 fields of tubes from 3 wells in a 96 well plate were quantified using NIH image, and a mean and standard deviation was calculated. Each experiment shown is representative of the two that were performed.

Flow Cytometry

HUVEC (300,000 cells/ml) were re-suspended in ice-cold staining buffer (Hanks' balanced salt solution/1%BSA/0.1% sodium azide) at pH 6.5 or pH 7.5 and incubated on ice for 30 min with anti-IF1 IgG, anti-CD31, or 4 μg exogenous IF1. Cells were washed with ice-cold staining buffer and pelleted in a microcentrifuge at 4ºC. This wash was repeated twice and the cells were re-suspended in ice-cold staining buffer before incubating for 30 min in the dark with goat anti-mouse IgG conjugated to APC. After the final wash, the cells were pelleted and fixed in 10% para-formaldehyde at a density of 10^6 cells/ml. Nonviable cells were identified using DEAD Red stain (Molecular Probes, Eugene, OR) prior to the final wash. The mean relative fluorescence after excitation at a $\lambda = 488$ nm was determined for each sample on a FACScan flow cytometer and analyzed with CELLQUEST software (Becton-Dickinson, Franklin Lakes, NJ).

ATP Generation by Cell-Titer Glo™ luminescence assay

HUVEC which were ~80% confluent in 96-well plates were washed and equilibrated into Custom endothelial basal medium (EBM) from Clonetics (East Rutherford, NJ) containing 0.45 mM NaH_2PO_4 and 0.50 mM Na_2HPO_4. Cells were treated with IF1 (0.5–2.5 μM), angiostatin (5 μM) or piceatannol for 30 min at 37ºC. For competition experiments, angiostatin was allowed to incubate for 30 min at 37ºC before addition of IF1 (or vice versa). All cells were then incubated with 0.05 mM ADP for 20 s. Supernatants were removed and centrifuged before assaying for ATP production by CellTiter-Glo™ luminescence assay. Aliquots (50μl) of cellular supernatants from cell surface ATP assays were analyzed using the CellTiter-Glo™ Luminescent Assay kit (Promega, Madison, WI). In this firefly luciferinluciferase reaction, only ATP is readily detected because the enzymatic reaction of firefly luciferase to oxidize luciferin is specific for ATP relative to all other nucleotides. Samples were injected with the ATP assay mixture and incubated for 10 min to stabilize the luminescence signal. Recordings were then made in a Luminoskan Ascent (Thermo Labsystems, Helsinki, Finland) over a 20 s period. The response in a given sample or standard was quantified as area under the peak of the response and averaged for duplicate determinations. Data are expressed as moles of ATP per cell based on standards determined under the same conditions with each experiment.

RESULTS

IF1 binds to purified bovine F_1 ATP synthase

Bovine F_1 was passively adsorbed onto microtiter wells before addition of increasing amounts of recombinant human IF1 protein. ELISA studies demonstrated that purified IF1 bound to F_1 ATP synthase in a concentration-dependent, saturable manner, and bound at lower concentrations at a pH of 6.5 than at a pH of 7.3 (Fig. 1). Near saturation level was obtained at a pH of 6.5 at 1–2 μM, while at a pH of 7.3, saturation was achieved at 4 μM.

IF1 inhibited F_1 dependent ATP hydrolysis

We employed a micro-plate F_1 activity assay to confirm the ability of our purified IF1 protein to inhibit ATP hydrolysis. The F_1 subunit was sufficient for the reverse ATP hydrolysis

reaction, but not the forward reaction of ATP synthesis, which required the F_1-F_0 holoenzyme. IF1 inhibited the activity of F_1 ATP synthase in a concentration and pH dependent manner (Fig. 2). The inhibitory capacity of IF1 decreased when the pH was raised to 7.5. In contrast, IF1 did not inhibit *E. coli* F_1 activity (data not shown). Sodium azide completely abolished F_1 activity, comparable to IF1 at pH 6.5 and was thus used as a positive control in this and subsequent experiments.

IF1 increases the presence of extracellular ATP on the surface of HUVEC

ATP synthase on the surface of EC is active in producing ATP and is inhibited by angiostatin, as well as by antibodies to the α- and β- subunits of ATP synthase (6), when measured using a CellTiter-Glo^{TM} luminescence assay. We employed this same assay to determine whether IF1 inhibited ATP synthesis on the surface of EC. EC were incubated at pH 6.1 or pH 7.7 before measuring ATP synthase activity on the cell surface. IF1 increased extra-cellular ATP on the surface of HUVEC compared to medium alone (Table 1). This trend is concentration and pH-dependent, with the largest increase seen at pH 6.1, where IF1 (0.5 μM) increases the level of extracellular ATP by approximately 37%. At the same concentration, but at pH 7.7, IF1 increases ATP generation by 13%. Since there is a fixed amount of ADP per well, and ADP alone in medium sets the baseline for ATP production, it is unlikely that there is an active increase in ATP production in the presence of IF1. Rather, the addition of IF1 results in a relative increase in extracellular ATP by inhibiting its hydrolysis. This is in contrast to angiostatin, which inhibited ATP production by greater than 70% in similar studies (see Table 2). Piceatannol, a known inhibitor of mitochondrial F_1F_0 ATP synthase (22,23), inhibited ATP production by 75% at pH 6.1 and 92% at pH 7.7. (see Table 1), which demonstrated that ATP synthase is the major source of ATP in this assay.

Inhibition of HUVEC proliferation in the presence of IF1

IF1 protein was added to quiescent EC at low pH (<7.0) in order to analyze cell proliferation at 24 and 48 h. IF1 (10 μg/ml) inhibited proliferation 20% at 48 h, compared to medium alone and phosphate buffered saline (PBS) vehicle controls (Fig. 3). A known inhibitor, cycloheximide, inhibited cell proliferation 65%. In addition, our laboratory previously demonstrated the ability of angiostatin to inhibit proliferation by 57% in this same assay (6). Therefore, the inhibitory effect of IF1 on EC proliferation is markedly less than that of angiostatin, supporting the argument that bi-directional inhibition of ATP synthase is necessary for a strong anti-angiogenic response.

HUVEC tube differentiation in the presence of IF1

We next studied IF1 in another cell-based assay of neo-angiogenesis, differentiation to form EC tubes, at pH 6.5 and pH 7.5. IF1 demonstrated no inhibition of tube formation at either pH (Fig.4). At pH 6.5, PBS (vehicle) positive controls demonstrated strong tube formation (a) and were comparable to IF1 treated wells (b). At pH 7.5, PBS controls (c) were also identical to IF1 (d). Cycloheximide, a protein synthesis inhibitor, completely inhibited tube formation at both normal and low pH. (e). Consistent with our prior studies, angiostatin also inhibited tube differentiation in similar studies only at low pH (data not shown). These results and the cell proliferation studies indicate that IF1 is a poor angiostatin-mimetic in cell-based assays.

IF1 is endogenously present on the surface of HUVEC

Previously published reports have demonstrated that the α-, β- and γ - subunits of ATP synthase are present on the cell surface (6). Here, we observed that IF1 is also endogenously present on the surface of HUVEC by flow cytometry. IF1 protein was present whether the HUVEC were incubated at pH 7.4 (Fig. 5a) or pH 6.5 (data not shown). The presence of IF1 was compared to CD31, a known marker on the surface of EC. In addition, we also demonstrated that adding

exogenous IF1 protein to EC increased the cell-surface signal (median intensity) by 41% (Fig. 5b). This confirms exogenous IF1 is able to bind the endothelial-cell surface and likely helps saturate IF1 binding to ATP synthase.

IF1 binding to ATP synthase is inhibited by angiostatin

IF1 and angiostatin both bind ATP synthase in a concentration-dependent, saturatable manner. We then performed experiments to determine whether angiostatin inhibited IF1 binding to purified F_1ATP synthase. ATP synthase was pre-incubated with angiostatin before the addition of increasing amounts of IF1. Pre-incubation with 10 μg/ml angiostatin inhibited IF1 binding to ATP synthase by 70% when compared to IF1 binding alone (Fig. 6). The calculated *apparent* K_d was 5 nM.

Similarly, in a cell-surface ATP luminescence assay, pre-incubation with angiostatin abolished the ability of IF1 to conserve ATP at low pH (Table 2). The same effect was seen at pH 7.7 (data not shown). Furthermore, the effect of pre-incubation with IF1 was completely overridden by the addition of angiostatin (see Table 2). Together, these data demonstrated that angiostatin was able to block and compete with IF1 binding to ATP synthase and inhibited its activity. Whether this effect results from direct competition between angiostatin and IF1 cannot be determined from these studies. It is also conceivable that a conformational change in F1 ATP synthase is induced angiostatin and/or IF1.

Angiostatin overrides the effects of IF1 on tube differentiation.

In a series of experiments using the EC tube differentiation assay, we examined the question of whether angiostatin could override the protective effect of IF1 (Figure 7). To optimize the assay, a serum dose response was performed at 6.1 as well as dose responses to IF1 and angiostatin. Tube formation was dependent on serum concentration over the range of 0, 0.1, 1, 5, 10 and 20 percent (v/v). Experiments shown in Figure 7 were performed at the optimal conditions; a pH of 6.1 and 1 mM bicarbonate media containing 0.1% serum. A dose response study revealed decreased tube formation at 0.25 μM and 0.50 μM angiostatin. The data at a concentration of 0.5 μM angiostatin are shown in Figure 7. Decreased tube formation could be observed if angiostatin was added at the time of cell plating, or one or two h subsequent to cell plating. In the experiments with both IF1 and angiostatin, each was added followed by the other 2 h later. A dose response to IF1 revealed a slight protective effect at 1 and 2.5 μM with no further effect at higher doses. These experiments indicated that angiostatin overrides the slight protective effect of IF1 whether the latter is added before or after angiostatin.

DISCUSSION

The role of IF1 inhibitory protein was explored to elucidate its potential for activity on EC surface ATP synthase, its interaction with angiostatin, and it's effect on angiogenesis. In order to determine whether IF1 can be an angiostatin-mimetic, we tested its ability to (1) bind purified F_1 ATP synthase and inhibit its hydrolytic activity and (2) inhibit endothelial cell tube differentiation and cell proliferation. First, we confirmed the ability of IF1 to inhibit F_1 dependent ATP hydrolysis using a microplate F_1 activity assay. However, IF1 did not inhibit ATP production on the surface of EC. From these data, we conclude that IF1 is a unidirectional inhibitor of cell-surface ATP synthase. This is in contrast to angiostatin, which inhibits the reaction catalyzed by ATP synthase in both directions (6). We also demonstrated that IF1 activity is concentration and pH- dependent. Increased inhibitory capacity was observed when the pH is lowered to 6.5, as has been shown with angiostatin (3,4,24). However, in cell-based assays, IF1 did not inhibit EC tube differentiation and only slightly inhibited cell proliferation in contrast with angiostatin (6,25,26).

In order to understand the interactions of IF1 and angiostatin, competition and binding studies were performed with the two proteins and purified F_1 ATP synthase. We have demonstrated that exogenous IF1 was not able to overcome angiostatin-induced inhibition of ATP synthesis on the endothelial cell surface. Furthermore, IF1 binding to purified F_1 ATP synthase was inhibited by pre-incubation with angiostatin. These findings demonstrated that angiostatin was able to inhibit the binding of IF1 to ATP synthase and inhibited its activity on the surface of EC. From a mechanistic standpoint, we propose that the binding of angiostatin induces a conformational change that diminishes the affinity of IF1 towards its binding site.

Finally, we hypothesized that IF1 might be endogenously present on the surface of EC. It is known that the α-, β- and γ-subunits of ATP synthase are present and co-localize extensively on the cell surface (6,27), but the presence of IF1 had not been determined. We have now demonstrated by flow cytometry that endogenous IF1 is present on the surface of EC. Furthermore, exogenously added IF1 is able to increase the signal detected by flow, confirming that EC bind IF1 on the external surface of the plasma membrane.

These data indicate that IF1 is a specific inhibitor of ATP hydrolysis on endothelial cell surface ATP synthase. This inhibition is not sufficient for a sustained anti-angiogenic effect in cell-based assays, suggesting that IF1 is not an angiostatin-mimetic. Rather, we hypothesize that IF1 serves a protective function on EC in the tumor microenvironment by allowing these cells to conserve ATP during periods of low pH. In addition, it now seems likely that angiostatin disrupts this preservation of ATP, tipping the balance towards an anti-angiogenic effect. We therefore also conclude that blockage of ATP hydrolysis is not sufficient to cause inhibition of angiogenesis. Rather, inhibition of ATP synthesis is necessary for an anti-angiogenic outcome. It is likely that the role of IF1 on cell-surface ATP synthase is similar to its role in mitochondria, where IF1 binds ATP synthase in order to conserve ATP. In mitochondria, this binding is favored under anaerobic conditions, when the electrochemical gradient collapses and the pH decreases (28–30).

Although low pH conditions are not present in normal tissues, the tumor microenvironment has an average pH of 6.7 (31,32). This low pH environment favors the binding of IF1 to tumor EC and suggests that IF1 may modulate tumor angiogenesis. The dependence of tumor growth on angiogenesis is already well documented, and it is known that tumor expansion beyond a pre-vascular size (1–3 mm^3) requires the generation of new blood vessels (33). IF1 would give these blood vessels an increased source of ATP at low pH, when mitochondrial ATP synthesis might be shut down. In support of this hypothesis is a study demonstrating that EC maintains ATP levels under hypoxic conditions (34). Although the role of ATP in this environment is still being elucidated, it has been hypothesized that ATP may activate signaling cascades (24) via binding to P2X/P2Y receptors on the cell surface, leading to activation of PI3-kinase (35,36) and stimulation of DNA synthesis and cell replication. Thus, the ability of IF1 to help conserve ATP on the surface of EC may not only promote angiogenesis but also tumor cell population growth.

When mitochondria are in low oxygen conditions, matrix pH decreases, and the proton gradient favorable for ATP synthesis declines. The orientation of the F_1F_o in the EC membrane is such that ATP production occurs on the cell surface (2,6,27). Thus, the ATP generating mechanism in the tumor vascular bed would also face a low ATP, low oxygen situation. The three principle transporters that affect or regulate intracellular pH, are the Na^+/H^+ exchanger (37–39), the Cl^-/HCO_3^- exchanger (40,41) and the H^+-linked monocarboxylate transporter (42–44). All of these take advantage of ion gradients rather than ATP to drive their activity, thus it may be more feasible for the cell in a hypoxic, acidic environment to produce ATP on its surface. This would have the additional benefit of providing ATP on the surface for signaling via the P2X/P2Y receptors (45).

The finding that IF1 is endogenously present on the surface of EC supports the hypothesis that these cells use surface ATP synthase and interactive proteins in a mechanism developed to conserve extracellular ATP. However, it is clear that the protective response afforded by IF1 is not sufficient to overcome the anti-angiogenic effects of angiostatin. In support of this statement, we have shown that the ability of IF1 to conserve ATP on the surface of EC was abolished by angiostatin. This is an example of the balance between pro-angiogenic and anti-angiogenic factors that help determine whether angiogenesis or angiostasis will be favored (46–48). In the present study angiostatin was capable of overriding the protective effect of IF1.

It is now reasonable to propose that angiostatin exerts its anti-angiogenic effect, at least in part, by inhibiting IF1 binding to ATP synthase. This hypothesis would help explain why angiostatin has a stronger anti-angiogenic effect at low pH and little effect at physiologic pH. At physiologic pH, EC would have little use for IF1 as a source of ATP since alternative sources of ATP would be abundant, and therefore the activity of IF1 would be minimal. However, in the low pH, low oxygen, milieu of the tumor microenvironment, EC would have a strong need to conserve ATP through IF1, since oxidative phosphorylation would begin to shut down. In this scenario, the ability of angiostatin to abolish IF1 activity would be devastating to the growing tumor.

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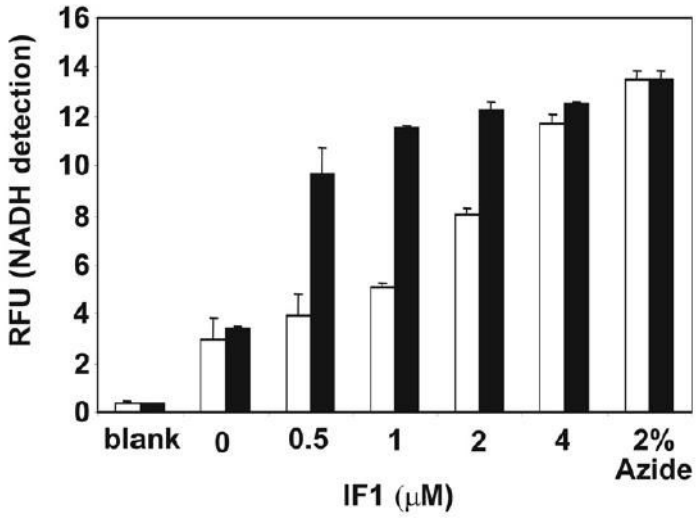

FIG. 1.

Inhibition of purified F_1 ATP synthase activity by IF1. Purified F_1 ATP synthase activity was measured as a change in fluorescence (emission λ =355 nm, excitation λ =460 nm) by coupling the production of ADP to the oxidation of NADH via pyruvate kinase and lactate dehydrogenase. Inhibition of F_1 activity is represented by an increase in relative fluorescence units (RFU). IF1 (0–4 μM) was added to a constant amount of F_1 ATP synthase at either pH 7.5 (open bars) or pH 6.5 (hatched bars). Azide, a known inhibitor of F_1F_0 ATP synthase completely inhibited F_1 activity, similar to IF1 at pH 6.5.

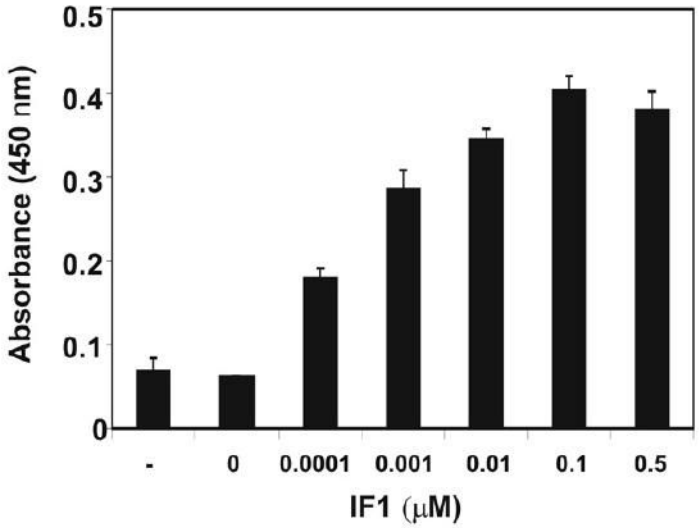

FIG. 2.

Binding of IF1 to purified bovine F_1 ATP synthase. ELISA was employed to demonstrate concentration-dependent binding of IF1 to F_1 ATP synthase. Each well was coated with 10 μg/ml of F_1 ATP synthase before addition of increasing amounts of IF1. Control lane (-) shows binding of secondary antibody only. n=3.

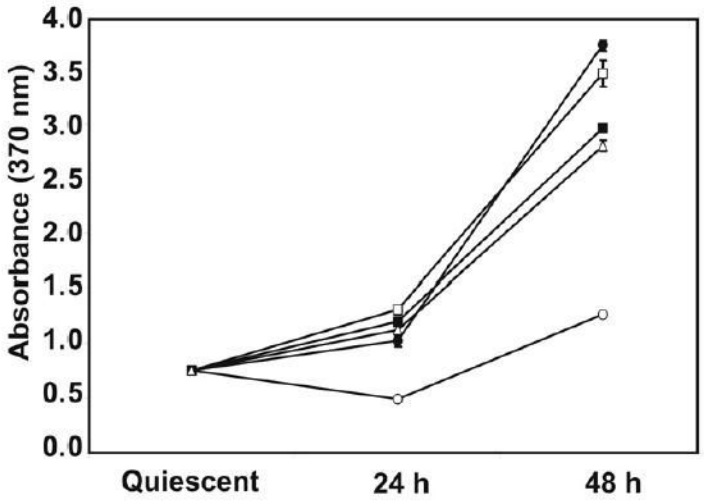

FIG. 3.

Inhibition of HUVEC proliferation at low pH (pH <7.0) in the presence of IF1 as measured by BrdU incorporation. HUVEC proliferation at 48 h was inhibited 20% by IF1 at concentrations of 1 μg/ml (closed squares) and 10 μg/ml (open triangles) compared to media only (open squares) and PBS controls (closed circles). Cycloheximide, an inhibitor of protein synthesis, inhibited cell proliferation by 65%; n=3 (open circles).

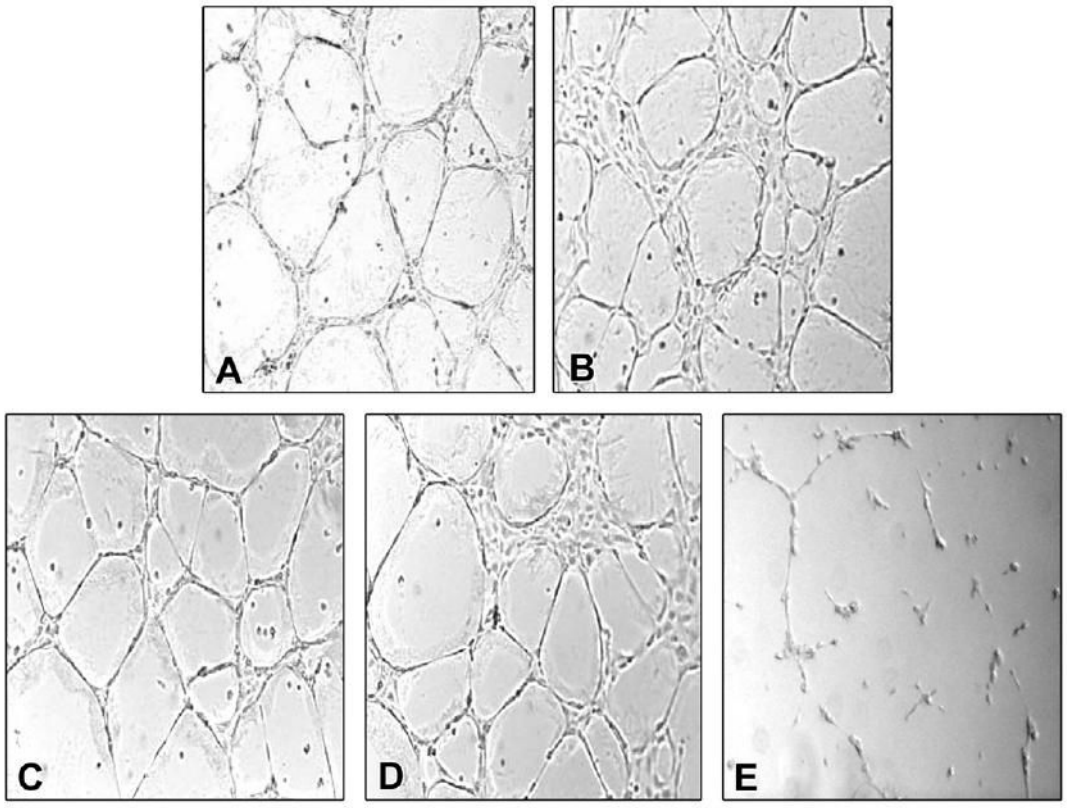

FIG. 4.

HUVEC tube differentiation in the presence of IF1. EC, pre-incubated at pH 6.5 or pH 7.5, were plated on Matrigel-coated wells in the presence of PBS only or 1 μM IF1. At pH 6.5, PBS only positive control (a) was comparable to 1 μM IF1 (b). At pH 7.5, PBS only control (c) was also identical to 1 μM IF1 (d). Cycloheximide, a known inhibitor of protein synthesis, completely inhibited tube formation (e).

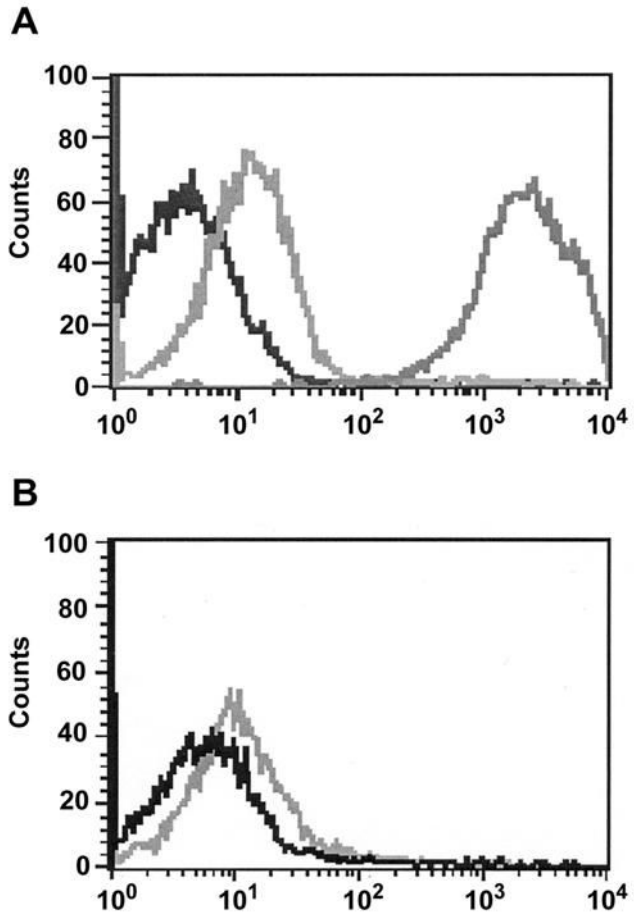

FIG. 5.

Endogenous IF1 on the surface of HUVEC. a) HUVEC were incubated at pH 7.4 overnight before incubating with anti-IF1 and anti-CD31 antibodies. IF1 was shown to be endogenously present on the surface of EC (light gray peak) compared to secondary only control (black peak). CD31, a known marker on the surface of EC was used as a positive control (medium gray peak). b) HUVEC were incubated at pH 7.4 before treatment with exogenous IF1 (IF1 ex) (gray peak) or HBSS+ buffer only to demonstrate endogenous IF1 (IF1 en) (black peak). Exogenous IF1 increased the signal (median intensity) of IF1 on the surface of EC by 41%.

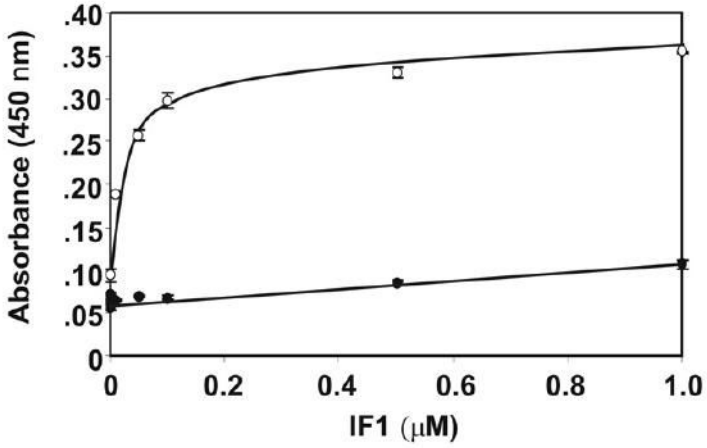

FIG. 6.

IF1 binding to bovine F_1 ATP synthase in the presence of angiostatin by ELISA. Wells were coated with F_1 ATP synthase (10 μg/ml) before incubation with angiostatin 100 μg/ml "+angiostatin" or PBS only "−angiostatin". IF1 was then incubated at increasing concentrations (0–10 μg/ml). Pre-incubation with angiostatin inhibited IF1 (10 μg/ml) binding to ATP synthase approximately 70%. A K_d of 5 nM was calculated from binding data in the binding isotherm using statistics software called Systat for Windows, version 5 (Systat Inc. Evanston, IL).

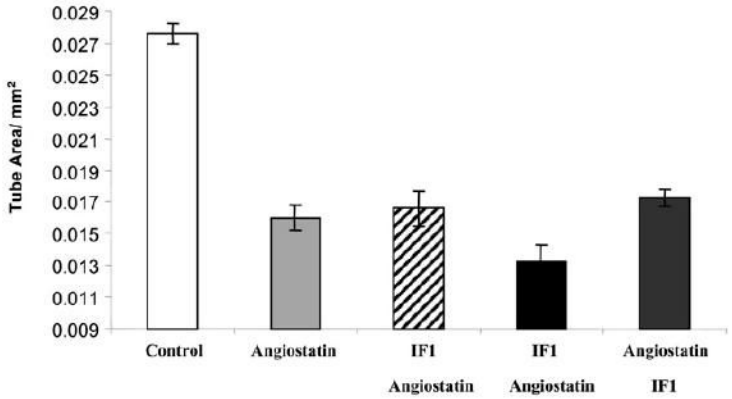

FIG. 7.

The effects of IF1 and angiostatin on EC tube differentiation. Competition experiments with sequential addition of angiostatin and IF1 are shown. The first bar (white) represents the control where cells were plated in the absence of either angiostatin or IF1. The second bar (light gray) represents the effect of 0.50 μM angiostatin. The third bar (hatched) shows the effect of sequential addition of IF1 (1 μM) followed by angiostatin (0.50 μM). The fourth bar (black) represents sequential addition of angiostatin (0.50 μM) and IF1 (5 μM). The fifth bar (dark gray) represents angiostatin (0.50 μM) followed by IF1 (1 μM).

Table 1

ATP generation on the surface of HUVEC as measured by CellTiterGlo™ luminescence assay in the presence of IF1.

	ATP concentration (moles^{-15}/cell) ± SEM	
	pH 6.1	**pH 7.7**
Medium Alone	20.7 ± 2.9	35.8 ± 3.6
IF1 (0.50 μM)	26.5 ± 1.5	36.6 ± 2.0
IF1 (2.5 μM)	28.3 ± 0.4	40.8 ± 1.4
Piceatannol (500 μM)	5.0 ±0.03	2.8 ± 0.08

IF1 increases ATP concentration on the surface of HUVEC as compared to medium alone. The greatest increase is seen at pH 6.1, where IF1 (2.5 μM) increases ATP concentration by 37%. IF1 (2.5 μM) increases ATP concentration 13% at pH 7.7. Piceatannol, a known inhibitor of mitochondrial ATP synthase, inhibited ATP generation by 75% at pH 6.1 and 92% at pH 7.7; n=3.

Table 2

ATP generation on the surface of HUVEC as measured by CellTiterGlo™ luminescence assay in the presence of IF1 and angiostatin.

pH 6.1	**ATP concentration (moles $^{-15}$/cell) ± SEM**
Medium Alone	34.5 ± 2.2
Angiostatin (5 μM)	9.2 ± 1.2
1. Angiostatin (5 μM) **2. IF1** (2.5 μM)	4.7 ± 0.3
1. IF1 (2.5 μM) **2. Angiostatin** (5 μM)	6.9 ± 0.9

ATP production on the surface of EC in the presence of IF1 and angiostatin. Angiostatin alone inhibited ATP production 73%. Subsequent addition of IF1 (2.5 μM) was unable to overcome this inhibition. When IF1 (2.5 μM) was added first, angiostatin-induced inhibition was still the dominant outcome. All treatments were applied to HUVEC in the presence of 50 μM ADP; n=3.