Endostatin has novel ATPase activity, which mediates its anti-angiogenic and anti-tumor activities

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ATP: Adenosine triphosphate, Erk: extracellular regulated protein kinase

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Abstract

Endostatin is an endogenous angiogenesis inhibitor with broad-spectrum anti-tumor activities. Although the molecular mechanisms of endostatin have been extensively explored, the intrinsic biochemical characteristics of endostatin are not completely understood. Here we revealed for the first time that endostatin embedded novel ATPase activity. Moreover, mutagenesis study showed that the ATPase activity of endostatin mutants positively correlated with effects on endothelial cell activities and tumor growth. E-M, an endostatin mutant with higher ATPase activity than that of wild type endostatin, significantly increased endostatin-mediated inhibitory effects on endothelial cell proliferation, migration, tube formation and adhesion. In vivo study showed that E-M displayed enhanced anti-tumor effects compared with wild type. On the other hand, K96A, K96R and E176A, endostatin mutants with lower ATPase activities than that of wild type, showed reduced or comparable effects on targeting both in vitro endothelial cell activities and in vivo tumor angiogenesis and tumor growth. Furthermore, endostatin and its mutants exhibited distinct abilities in regulations of gene expression (Id1, Id3), cell signaling (Erk, p38 and Src phosphorylation), and intracellular ATP levels. Collectively, our study demonstrates that endostatin has novel ATPase activity, which mediates its anti-angiogenic and anti-tumor activities, suggesting that construction of endostatin analogues with high ATPase activity may provide a new direction for the development of more potent anti-angiogenic drugs.
Introduction

Anti-angiogenic agents have been widely considered as the fourth modality in cancer treatment, together with surgery, chemotherapy and radiotherapy. Endostatin, as an endogenous anti-angiogenic inhibitor (1), exhibits potent anti-tumor activities in various mouse models (2) and receives CFDA (China Food and Drug Administration) approval as a new drug for the treatment of non-small cell lung cancer patients in clinic (3). This drug is widely used in China and shows profound clinical efficacy (4-6). Accumulating evidence demonstrates that the N-terminal integrity and correct folding are essential to guarantee the structural stability and biological functions of endostatin (7, 8).

The detailed mechanisms underlying anti-tumor activities of endostatin have been extensively studied. Endostatin specifically diminishes the proliferation, migration and tube formation of endothelial cells in vitro (1, 9). It can initiate endothelial cell apoptosis through induction of VDAC1 phosphorylation, which facilitates the mitochondrial permeability transition pore (mPTP) opening (10). Moreover, it also binds to cell surface receptors such as integrins (11), glypicans (12), laminin (13) and nucleolin (14), and thereby regulates a myriad of signaling cascades. For example, endostatin binding to integrin α5β1 results in the inhibition of FAK/c-Raf/MEK1/2/p38/ERK1 MAPK pathway (15). Besides, the internalization of endostatin by endothelial cells is important, if not essential, for the physiological functions of endostatin (16). Enhancing endostatin uptake by cholesterol-chelating
agents or addition of a macromolecule transduction domain (MTD) significantly increases the therapeutic efficacy on animal models (17, 18). Huber’s group reported that ∼12% of the human genome is modulated by endostatin (19), therefore tips the dynamic angiogenic balance towards the inhibition part.

ATPases play distinct roles in a lot of cellular processes including DNA replication, protein synthesis, protein folding, proteolysis and membrane fusion (20). They have evolved different strategies to recognize ATP and elicit ATPase activities. ATPases have two well-known Walker motifs. The Walker A motif, the best-known motif associated with ATP binding, has a common nucleotide-recognition sequence: GXXXXGK(T/S) (where X is any amino acid residue) (21). The Walker B motif contains a conserved Asp or Glu residue preceded by a consecutive sequence of four hydrophobic residues (hnhhD/E), with this acidic residue coordinating an Mg$^{2+}$ ion (or Mn$^{2+}$ or Ca$^{2+}$) essential for ATP catalysis (21-23). Mutations of key residues in the Walker motifs almost abolish ATPase activities and disrupt normal functions (24-26). For example, a point mutation (K368R) at the Walker A motif of PKCα diminishes the kinase activity and interferes with its downstream signaling (25).

Despite comprehensive investigation, the precise mechanisms of endostatin actions still remain elusive. In the current study, we first discovered novel ATPase activity of endostatin, which has never been reported. Endostatin mutants with decreased ATPase activities (K96A, K96R and E176A) exhibited reduced or comparable levels in the inhibition of endothelial cell activities in vitro and anti-tumor efficacy in vivo compared with wild type endostatin. However, E-M, the endostatin
mutant with higher ATPase activity, showed the more potent anti-tumor and anti-angiogenic effects. Collectively, our study demonstrates that endostatin has novel ATPase activity, which mediates the anti-angiogenic and anti-tumor effects of endostatin.
Materials and Methods

Plasmids, Reagents, Abs

Human endostatin was subcloned into pET30a(+) vector (Addgene) and endostatin mutants (K96A, K96R, E176A, E-M) were constructed using Fast Mutagenesis System (TransGen Biotech) and verified by sequencing (Life Technologies). Recombinant proteins, provided by Protgen Co, Ltd., were expressed in *E. coli* in high densities and cells were harvested and lysed. The insoluble part of the lysates containing the inclusion bodies was collected and purified. The inclusion bodies were subsequently renatured and purified by ion exchange chromatography. The concentrations of recombinant proteins were measured using the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific).

Anti-endostatin antibody was from lab stock. We purchased primary antibodies specifically recognizing GAPDH, Erk, p-Erk, p38, p-p38 (Santa Cruz), pro caspase 3, cleaved caspase 3, Src, p-Src, Id3 (Cell Signaling Technology), CD31 (Abcam) and Id1 (Bioworld Technology). Secondary antibodies were obtained from Santa Cruz.

ATP, ATP-γ-S and ADP-β-S were obtained from Sigma-Aldrich and GTP, CTP, UTP, AMP and ADP were from Sangon Biotech Co., Ltd. (Shanghai, China).

Cell culture

Primary HUVECs were cultured in endothelial cell medium (ECM, ScienCell), and used at passages 2-7. Culture of A549-GFP cells and HMECs was maintained in DMEM and 10% fetal bovine serum (Wisent). All cell lines were purchased from Cell Resource Center, China Infrastructure of Cell Line Resources within 6 months of the
beginning of the project and validated by the supplier, except for A549-GFP cells, which were developed by stable transfection of GFP and were not genetically authenticated.

**ATPase activity assay**

The malachite green assay was used to determine the ATPase activity of endostatin by measuring the release of inorganic phosphate as previously described (27). Briefly, recombinant proteins (typically 5 μg) and ATP or NTP/ADP/AMP/ATP-γ-S/ADP-β-S (final 1 mM) were incubated in imidazole-Cl buffer (10 mM imidazole-Cl, 75 mM KCl, 0.2 mM EDTA, 3 mM MgCl₂, pH 7, final volume of 100 μl) at 37°C for 60 min. Subsequently HCl/Mo was added and 2 min later 0.042% malachite green and H₂SO₄ were added. Absorbance at 650 nm was detected after incubation for 30 min. pH dependence was evaluated using imidazole-Cl buffer at pH 4.5 to 8.5. Bivalent cation dependence was assessed using solutions of MgCl₂, CaCl₂, CuCl₂, MnCl₂, and ZnCl₂. For the determination of enzyme kinetics, ATP concentrations ranging from 6.25 μM to 2 mM were used and Michaelis-Menten calculations were performed with GraphPad Prism 5 software (GraphPad Software, Inc.).

ATP bioluminescent assay was performed with ATP Bioluminescent Assay Kit (Sigma-Aldrich) according to the manufacturer’s protocol.

**ATP binding assay**

Proteins in Tris buffer (10 mM Mg²⁺ and 100 mM NaCl, pH 7.5) were incubated with high-affinity ATP-agarose (Innova) at 4°C for 1 h and then washed three times
with the same Tris buffer. The pelleted resin was mixed with reducing SDS/PAGE loading buffer for immunoblotting (28).

**Tryptophan Emission Fluorescence**

Tryptophan Emission Fluorescence spectra of endostatin and its mutants were measured by a Hitachi F-4500 spectrophotometer equipped with a temperature controlled liquid system as described previously (8). Briefly, the concentrations of proteins in imidazole-Cl buffer were 1 μM, and all measurements were carried out at 37°C, pH 7.4.

**Western blot**

Samples were mixed with reducing SDS/PAGE loading buffer, boiled at 100°C for 15 min, subjected to SDS/PAGE and transferred on to a PVDF membrane (Millipore). The membrane was blocked in TBST (20 mM Tris, 150 mM NaCl and 0.1 % Tween 20) plus 5–10 % dried non-fat skimmed milk for 30 min at room temperature. The membrane was incubated with the indicated primary antibodies in TBST and 1 % dried non-fat skimmed milk for at least 2 h at room temperature or overnight at 4°C, washed three times with TBST for 5 min each time at room temperature, and then incubated with corresponding HRP-conjugated secondary antibodies for 60 min at room temperature. Following five washes with TBST, immunoreactive bands were detected. Images were scanned using Bio-5000 plus (Microtek) and quantified with Image J (NIH).

**Quantitative RT-PCR**

Total RNA was isolated using Direct-zol RNA MiniPrep (Zymo Research), and
cDNA was synthesized using the First Strand cDNA Synthesis Kit (Fermentas). Quantitative RT-PCR (qRT-PCR) was conducted using the Brilliant II SYBR Green qRT-PCR Master Mix Kit (Stratagene).

**Cell viability assay**

2×10³ HUVECs were seeded in sextuplicate in a 96-well-plate and 24 h later, endothelial cells were treated with 20 μg/ml indicated proteins in endothelial cell basal medium containing 1% FBS for 72 h. Cell viability was assessed by 3-(4,5-dimethylthiazol-2-yl)2,5-diphenyl tetrazolium bromide (MTT) as described previously (29). Absorbance at 450 nm was obtained by Varioskan Flash (Thermo Fisher Scientific).

**Transwell migration assay and tube formation assay**

Transwell migration assay and tube formation assay were performed as described previously (29). Images were photographed with an Olympus IX71 optical microscope (Olympus). The number of migrated cells and tube length was quantified with Image-Pro Plus 6.0 software (Media Cybernetics).

**Cell adhesion Assay**

The 48-well plate was coated with matrigel for 1 h, washed for 2 times, and chilled on ice for 1-2 min. Subsequently equivalent cells were added to each well with 20 μg/ml indicated proteins and incubated at 37°C for 30 min. Plates were shaked for 10-15 s and washed for 2-3 times. Adherent cells were photographed with an Olympus IX71 optical microscope and counted.

**Measurement of cellular ATP levels**
HMECs were treated with 20 μg/ml indicated proteins for 24 h. Cellular ATP levels were detected as previously described (30).

**Endostatin internalization assays**

HUVECs were treated with 5 μg/mL endostatin or endostatin mutants at 37°C for 1 h. Next, cells were washed with acidic buffer (pH 3.5) and ice-cold PBS to remove cell surface-binding endostatin. Subsequently, the cells were examined for endostatin internalization by Western blot.

**Animal studies**

A549-GFP tumor cells (10^6) were mixed with Matrigel (Becton Dickinson) and inoculated into the axilla of nude mice subcutaneously. Once the tumor volume approached 0.1 cm³, mice were randomly grouped (n = 6/group) and intravenously treated with PBS, wild type endostatin, K96A, K96R, E176A or E-M (12 mg/kg) every two days. Tumor volumes were measured every other day and calculated as volume = 0.625 × length × (width)^2. After 34 days, mice were sacrificed and tumors were excised and weighed. For immunohistochemistry analysis, tumors were fixed in 4% formaldehyde, and embedded in paraffin (5μm sections). Subsequently, these tissues were stained with Abs and diaminobenzidine, and then counter-stained with hematoxylin. Images were captured by an Olympus IX71 optical microscope (Olympus) and quantification was completed using Image-Pro Plus 6.0 (Media Cybernetics).

All animal studies were approved by the Institutional Animal Care and Use Committee of Tsinghua University (Approval No. 13-LYZ6).
Statistical analysis

All data from individual experiments are represented as means ± standard deviations (SDs) or standard error of the means (SEMs). Comparison was determined using two-tailed Student’s t tests and P values < 0.05 were considered statistically significant.
Results

Endostatin has novel ATPase activity

Walker motifs are found in a broad range of ATPases and thereby provide predictive value for ATPases (31). Through detailed examination of human endostatin sequence, we found that endostatin comprises a Walker A motif variant (GXXGXXXK) (32) and a Walker B motif (Fig. 1A, I and II), suggesting that endostatin may display ATPase activity. Besides, Walker A motif-containing proteins are also frequently found to consist of a RX(2-3)R motif which interacts with the adenine base of ATP with a conserved Arg residue (23). Two RX(2-3)R motifs were identified in the endostatin sequence (Fig. 1A, IIIa and IIIb). Taken together, we proposed that endostatin might have ATPase activity. We then compared the 3D structure of endostatin (33) with that of P97 (34), an AAA-ATPase. As shown in Supplementary Fig. S1, the position of Walker motifs on endostatin 3D structure is similar to that of the characterized ATPase.

To validate whether endostatin indeed exhibits ATPase activity, ATP binding analysis was performed. Endostatin could bind to ATP-agarose, and addition of excessive ATP prevented endostatin from binding (Fig. 1B), excluding the possibility that the interaction between endostatin and ATP-agarose is unspecific. Subsequent malachite green assay revealed that endostatin hydrolyzed ATP in a dose- and time-dependent manner (Figs. 1C and D). Consistently, ATP bioluminescence assay results showed that endostatin indeed directly consumed ATP (Supplementary Figs. S2A and
B), whereas ATP was barely catalyzed by either endostatin storage buffer or angiostatin (K1-3), another endogenous angiogenic factor (Supplementary Figs. S2C and D). From these results, we conclude that endostatin has novel ATPase activity.

Assays to assess the substrate specificity of the endostatin ATPase showed that endostatin could also hydrolyze CTP, UTP and GTP to an equivalent extent (Fig. 2A). In addition, endostatin could hydrolyze ADP, but not AMP (Fig. 2A), showing that endostatin can cleave both β-γ and α-β phosphodiester bonds, similar to apyrase and ecto-ATPase families (35, 36). Concordant with these results, endostatin hydrolyzed ATP-γ-S at a similar rate relative to ADP. However, ADP-β-S could not be hydrolyzed, similar to AMP (Fig. 2A).

We also observed that the endostatin’s ATPase activity varied with pH. The optimal pH of endostatin’s ATPase hydrolysis reaction was 7.0 (Fig. 2B). As a result, all of the subsequent ATPase experiments were performed at pH 7.0. Furthermore, divalent metal ion dependence analysis revealed that endostatin ATPase was dependent on Mg$^{2+}$ (Fig. 2C).

Since the reaction remained linear during the initial 60 min (Fig. 1D), the hydrolysis rate at 60 min was used as the initial rate of ATP hydrolysis for Michaelis-Menten analysis. The results demonstrated a $V_{\text{max}}$ of 2.117 ± 0.071 pmol/min/μg and $K_m$ of 83.36 ±11.21 μM (Fig. 2D). Isolated human Hsp90 exhibits high ATPase activity with a $K_m$ of 80 μM, reflecting similar affinity of endostatin and Hsp90 for ATP (37).

Taken together, our results showed that endostatin has novel ATPase activity,
with high affinity toward ATP.

**Generation of endostatin mutants**

To probe the role of the ATPase activity of endostatin, several mutants harboring mutations within Walker motifs were constructed. The flexible Walker A motif contains an invariant Lys residue. Mutation of this residue frequently leads to decreased nucleotide binding capacity (38), therefore mutants K96A and K96R were generated (Fig. 3A). We also aimed to construct an endostatin mutant with higher ATPase activity. Since myosin has very high ATPase activity (39), the endostatin Walker A motif sequence was thus substituted with the myosin Walker A motif sequence, hereafter referring to this mutant as E-M (Fig. 3A). The Walker B motif (hhhhD/E) also forms contacts with nucleotide, and the aspartate/glutamate residue is proposed to activate water for the hydrolysis reaction (22). Mutation of glutamate interrupts nucleotide hydrolysis, thus endostatin mutant E176A was constructed (Fig. 3A).

Next these mutants were expressed in *E. coli* and purified by ion exchange chromatography (the purity of these proteins was analyzed by SDS-PAGE gel and shown in Supplementary Fig. S3). Since endostatin consists of four tryptophan residues located evenly in space with all of their side chains stretching inside the molecule, therefore tryptophan emission fluorescence can be used to sensitively detect the changes of the tertiary structure of endostatin (40). To rule out the possibility that mutagenesis of Walker motifs may cause pronounced structural changes, tryptophan
emission fluorescence was applied to monitor the tertiary structure of endostatin, K96A, K96R, E176A, and E-M. There was no obvious change in maximal Trp fluorescence emission wavelengths ($\lambda_{\text{max}}$) for all the mutants compared with that of wild type endostatin, implying relatively stable core structures of endostatin mutants (Supplementary Fig. S4). As expected, K96A, K96R and E176A mutants displayed reduced ATPase activities, while E-M showed increased activity relative to that of wild type endostatin (Fig. 3B). We next sought to determine whether the differences of ATPase activities of endostatin mutants were due to distinctions in ATP binding abilities. The ATP binding abilities of K96A, K96R and E176A decreased in comparison to that of wild type endostatin, whereas E-M displayed an enhanced ATP binding ability (Fig. 3C).

The ATPase activity of endostatin mediates its effects on endothelial cell activities

For many ATPases such as AAA+ ATPases, Hsp90 and Dnd, the ATPase activity is vital for their physiological functions (27, 41, 42). To identify whether an intact endostatin ATPase activity is required for its anti-angiogenic effects, we assessed the inhibitory effects of endostatin and its mutants on endothelial cell proliferation, migration, tube formation and adhesion. Wild type endostatin, K96A, K96R, E176A, and E-M attenuated the endothelial cell viability by 39%, 31%, 24%, 22%, and 51%, respectively (Fig. 4A). Strikingly, we noticed that endostatin mutants with lower ATPase activities showed less inhibitory ability relative to wild type endostatin ($P < 0.05$), whereas E-M with the greatest ATPase activity was the most effective in
suppression of cell viability among the proteins tested ($P < 0.001$). Similar effects were observed in the Transwell migration assay (Fig. 4D, quantified in Fig. 4B). Furthermore, the tube formation activity of HUVECs was significantly reduced by endostatin and E-M, while K96A, K96R and E176A treatment had no effects. The tube length was decreased by 21% and 48% on endostatin and E-M treatment, respectively, and this difference was statistically significant ($P < 0.001$) (Fig. 4E, quantified in Fig. 4C). Similar results were obtained in the endothelial cell adhesion assay (Fig. 4F).

The ability of endostatin and its mutants to induce endothelial cell apoptosis was also compared by monitoring both pro and activated (cleaved) caspase 3 levels. All the four endostatin mutants enhanced cell apoptosis to different extents. The cleavage of caspase 3 was significantly increased upon wild type endostatin and K96A treatment and moderately up-regulated by K96R and E176A, while E-M gave rise to drastic cell apoptosis (Fig. 4G).

Altogether, the ATPase activity of endostatin mediates its effects on endothelial cell activities.

**The ATPase activity of endostatin is crucial for its anti-tumor and anti-angiogenic properties**

We next questioned whether the ATPase activity of endostatin has any role in retarding tumor growth and angiogenesis, since it is involved in endostatin-mediated anti-angiogenic properties *in vitro*. To answer this question, an A549-GFP xenograft
tumor model was established and mice received injections of PBS, endostatin, K96A, K96R, E176A or E-M (12 mg/kg) every other day. Primary tumor growth was monitored until 34 days after implantation. As shown in Fig. 5A, tumor volumes are significantly decreased by endostatin and its four mutants, among which E-M leads to the most pronounced tumor growth inhibition ($P < 0.01$ relative to wild type). K96A, K96R and E176A were less effective compared with wild type endostatin ($P < 0.05$). Similar results were obtained in tumor weight measurement (Fig. 5B, tumor photos shown in Supplementary Fig. S5). Notably, no obvious difference of mice weight among different groups was observed, implying that these proteins have relatively low toxicity (data not shown).

Tumor angiogenesis was assessed by immuno-staining of vascular endothelial cell marker CD31. It turned out that tumor angiogenesis was significantly impaired by endostatin, K96R and E-M, whereas no apparent difference was observed in K96A- and E176A-treated tumors compared with control ones (Fig. 5E, quantified in Fig. 5C). Of note, E-M administration led to reduced blood vessels in comparison to endostatin ($P < 0.01$). Apoptotic cells were evaluated using immuno-staining of cleaved caspase 3. Administration of endostatin, K96A, K96R, E176A and E-M all promoted apoptosis. More importantly, the number of cleaved caspase 3 positive cells was significantly up-regulated in E-M-treated tumors (Fig. 5D, representative images shown in Supplementary Fig. S6). Moreover, neither endostatin nor its mutants markedly altered the overall proliferation status of tumors (Supplementary Fig. S7), suggesting that these proteins did not abrogate tumor growth by influencing tumor
proliferation.

Collectively, these findings demonstrate that ATPase activity of endostatin is crucial for its anti-angiogenic and pro-apoptotic effects \textit{in vivo}.

**Endostatin and its mutants exhibit distinct abilities in regulations of gene expression, cell signaling and intracellular ATP levels**

Endostatin is implicated in modulating multiple angiogenesis-related genes (19), thereby we sought to determine whether the ATPase activity is involved in endostatin-mediated gene regulation. The effects of endostatin and its mutants on the mRNA levels of Id1 and Id3 were evaluated, which are important regulators of angiogenesis (43). As expected, endostatin reduced the mRNA levels of Id1 and Id3. Intriguingly, K96A, K96R, and E176A exhibited impaired effects compared with wild type endostatin ($P < 0.05$), while E-M had the most potent inhibitory effects (Fig. 6A). Concordant with qRT-PCR data, Western blot analysis showed that E-M was still the most active in silencing protein expression of Id1 and Id3, and other mutants were not so efficient than wild type (Fig. 6B).

Endostatin directly interacts with the VEGFR2/KDR/Flk1 receptor on the endothelial cells, thus blocking VEGF-induced p38 MAPK and Erk activation (44). To further elucidate the molecular mechanisms why endostatin and its mutants displayed distinguishing anti-tumor and anti-angiogenic activities, we analyzed whether they could block VEGF-induced MAPK/Erk and p38 activation. All the five proteins attenuated MAPK/Erk and p38 phosphorylation to different extents (Fig. 6C).
E-M was more effective than K96A, K96R, and E176A in inhibition of MAPK/Erk and p38 phosphorylation. This may explain why E-M has the most prominent anti-angiogenic capability. Besides, endostatin and E-M significantly suppressed Src phosphorylation (Fig. 6C). Src kinase activity is necessary for turnover of cell-matrix adhesion (45), substantiating our data in cell adhesion assay (Fig. 4F).

ATP is the primary energy currency in the cells and controls energy homeostasis. Intracellular ATP levels are a core determinate of cell fate (46). HMEC cells were treated with 20 μg/ml endostatin, K96A, K96R, E176A, and E-M for 24 h and intracellular ATP concentration was detected as previously described (30). Following endostatin, K96A, K96R and E176A treatment, the ATP content was unaffected but it was markedly reduced in E-M-treated cells (Fig. 6D). This is also an explanation for the most promising efficacy of E-M among these proteins in vitro and in vivo. Further analysis revealed that 20 μg/ml E-M decreased intracellular ATP content by about 50%, whereas elevated concentrations could not further reduce intracellular ATP levels (Fig. 6E). Although low concentrations of endostatin has no effect on the intracellular ATP levels (0 - 20 μg/ml), increment of endostatin amount reduced the intracellular ATP levels and showed a dose-dependent manner (Fig. 6F).

In summary, the ATPase activity of endostatin is involved in Id1, Id3 regulations and MAPK/Erk, p38, Src activation. Meanwhile, these results suggest that E-M exhibits strong anti-tumor and anti-angiogenic effects by intracellular ATP depletion.
Discussion

Endostatin is a potent anti-angiogenic factor. The work presented here is for the first time to demonstrate that endostatin has novel ATPase activity. Through systemic study, we elucidate that the ATPase activity of endostatin is critical for its anti-tumor and anti-angiogenic behaviors both in vitro and in vivo.

Endostatin has novel ATPase activity

The discovery that endostatin exhibits novel ATPase activity is quite surprising, which leads us to consider the functional significance of endostatin’s ATPase activity and whether it is evolutionarily conserved. By comparing the conservation of the particular Walker A and Walker B motifs within endostatin protein sequences from six species, including Homo sapiens, Mus musculus, Rattus norvegicus, Danio rerio, Gallus gallus, Dendrobates azureus, we noticed that only human endostatin consists of the Walker A motif, while the Walker B motif exists in all species. The intriguing fact that the Walker A motif is unique to human endostatin out of six species suggests that this property of human endostatin may make it more potent and its effects more complicated. The explanation for this phenomenon is mysterious at present, and merits further investigation.

Endostatin regulates intracellular ATP levels

Energy homeostasis, the delicate balance between ATP production and consumption, is very critical for many cellular processes (47). Several serious disorders such as cancer and obesity involve the dysregulation of ATP balance in the cellular level and energy homeostasis at the whole body. In this work, we present data
which showed that 20 μg/ml E-M and 500 μg/ml endostatin both led to 50% reduction of intracellular ATP levels. Given the amount of endostatin internalized by cells (Supplementary Fig. S8A) and its catalytic activity, it is unlikely that endostatin can directly catalyze such large amount of ATP in the cells. However, since endostatin is able to affect ~12% of the genome including a bunch of metabolic genes, such as HIF-1α (Supplementary Fig. S8B), aldolase, citrate synthase (19) and hexokinase 2 (10), the latter three of which are direct players in glycolysis and TCA cycle and regulate ATP production, we thus believe that both the ATPase activity and the complex gene regulations of endostatin contribute to the intracellular ATP depletion upon endostatin treatment.

*A novel perspective into endostatin actions: Intimate correlation between enzymatic and biological activities of endostatin ATPase*

Based on previous findings, the molecular mechanisms underlying anti-angiogenic actions of endostatin are mostly attributed to four aspects: interaction with its receptors, internalization by endothelial cells, regulations of multiple signaling pathways, and modifications of gene expression pattern (7). Our study unravels a fifth aspect of endostatin mechanism. We reveal novel insights of endostatin actions by elucidating its biochemical characteristic as an ATPase. Mutagenesis study demonstrates that the ATPase activity of endostatin participates in its inhibitory effects on endothelial cell activities (proliferation, migration, tube formation and adhesion). Furthermore, endostatin low-ATPase-activity mutants show obviously reduced abilities to inhibit tumor growth and angiogenesis compared with
wild type (Fig. 5), while E-M is dramatically more effective than wild type endostatin. Therefore, we conclude that the biological functions of endostatin are intimately correlated with its ATPase activity. This correlation has been well-established in other ATPases. For example, AAA+ ATPases (ATPases associated with diverse cellular activities) rely on ATP binding and hydrolysis to exert their functions (41).

Internalization of endostatin is important for its biological functions (14, 17). Consistently, Jo’s group reported that manipulation of endostatin by addition of a macromolecule transduction domain (MTD) significantly increased endostatin uptake and improved its anti-tumor effects (18). E-M, with the fastest cellular internalization rate (Supplementary Fig. S8A), exhibited the most prominent ATPase activity and anti-tumor activity. It is plausible that enhanced uptake per se contributes to increased anti-tumor effects of E-M compared with that of wild type endostatin.

**Minimum endostatin sequence required for its biological activity**

Several groups have reported the minimum endostatin sequence for its biological activity. Ziche’s group demonstrated that a peptide (sequence 135–184) containing the disulfide bridge Cys135-Cys165 has shown anti-angiogenic activities (48). However, this peptide was treated at the tumor periphery and for only 7 days. In another study (49), a minimum sequence of 27 amino acid synthetic peptide corresponding to the N-terminal zinc binding domain of endostatin fully mimicked its anti-tumor activity. However, the peptide was administrated at a higher frequency (twice a day vs once a day for full-length endostatin). Given the above controversial reports about minimum sequence of endostatin, it seems more likely that different regions or mechanisms...
regulate distinct aspects of endostatin activities.

Our results differ from these groups in that endostatin low-ATPase-activity mutants (K96A, K96R and E176A) retain partial capacity to diminish tumor growth and angiogenesis, indicating that endostatin activities are not exclusively mediated by its ATPase activities. We propose that the endostatin’s ATPase activity, together with its binding to cell surface receptors, internalization (Supplementary Fig. S8A), intracellular signaling pathways and gene expression regulations (Fig. 6 and Supplementary Fig. S8B), all the above-mentioned functions of endostatin cooperate and orchestrate its anti-tumor effects, which leads to a complex but exquisite endostatin network.

Drug design: Endostatin analogs for therapeutics

A good drug should have qualities of high stability and efficacy. Proteins for therapeutic use can be exquisitely optimized through several ways, such as mutagenesis (protein analogues) and chemical modifications (PEGylation or acylation). Although the clinical trials of the *P. pastoris*-expressed endostatin were terminated during phase II in the USA, an N-terminal modified *E. coli*-expressed endostatin was approved by CFDA for the treatment of non-small cell lung cancer patients in 2005 in China (3). The prominent drug design strategy of this drug is to covalently attach a peptide of MGGSHHHHH to the N terminus of endostatin, thus facilitating the N-terminal integrity of endostatin, which is essential for the anti-angiogenic and anti-tumor activities of endostatin (7). In addition, a parallel study conducted by the Folkman laboratory reveals that the N-terminal modified endostatin
is at least twice as potent as *P. pastoris*-expressed endostatin in animal tumor models (50). This provides an excellent example of the analogue approach.

In this study, we found that among the five endostatin proteins we explored, E-M not only is the most effective to attenuate endothelial cell activities and induce apoptosis, but also exhibits the most severe inhibitory effects on tumor growth and angiogenesis. These exciting results lead us to conclude that E-M may be a promising therapeutic agent, and suggest that construction of endostatin analogues with high ATPase activity serve as an efficient approach to optimize endostatin’s therapeutic efficacy.

In conclusion, we demonstrate for the first time that endostatin exhibits novel ATPase activity. The results presented here contribute to a systemic understanding of the link between endostatin intrinsic properties and anti-tumor effects, and provide a brand new direction for the design and development of more potent anti-angiogenic drugs.
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References

Figure legends

Figure 1. Endostatin has ATPase activity.

A, Walker A and B motif analyses of endostatin amino acid sequence. B, endostatin was incubated with ATP-agarose beads in the absence or presence of ATP, and the bound proteins were detected by Western blot. C, ATP hydrolysis by endostatin in a dose-dependent manner. D, ATP hydrolysis by endostatin in a time-dependent manner. Error bars represent SDs.

Figure 2. Biochemical characterization of endostatin’s ATPase activity.

ATPase assays were performed with endostatin to evaluate: A, substrate specificity, B, optimal pH, C, optimal divalent metal ion, and D, kinetic analysis. Malachite green assays were conducted as described in Materials and Methods. Error bars represent SDs.

Figure 3. Generation of endostatin mutants.

A, construction of endostatin mutants with mutations within Walker A or Walker B motifs. B, relative ATPase activity of endostatin mutants compared with that of wild type endostatin. C, equivalent proteins were incubated with ATP-agarose beads and the bound proteins were detected by Western blot. *, P < 0.05, ***, P < 0.001. Error bars represent SDs.
Figure 4. The inhibitory effects of endostatin and its mutants on endothelial cell activities.

HUVECs were treated with 20 μg/ml indicated proteins and examined for A, MTT at 72 hours, B, D, Transwell migration at 6 hours, C, E, tube formation at 6 hours, and F, cell adhesion assay at 1 hour. G, HUVECs were treated with 20 μg/ml indicated proteins for 24 hours, then cells were collected and subjected to Western blot. The pro and cleaved caspase 3 levels were determined by Western blot. *, #, P < 0.05, **, ##, P < 0.01, ###, P < 0.001. Error bars represent SDs.

Figure 5. Anti-tumor and anti-angiogenic efficacies of endostatin and its mutants.

A, tumor volume of A549-GFP xenograft over time. Mice were intravenously administrated with PBS, endostatin, K96A, K96R, E176A or E-M (12mg/kg) every other day. n = 6/group. B, tumor mass was measured. n = 6/group. C, E, quantified results and representative images of tumor sections immunolabeled for CD31. Magnification, 320×. D, tumor sections were immunolabeled for cleaved caspase 3. The number of apoptotic cells was counted and quantified. *, #, P < 0.05, **, ##, P < 0.01, ###, P < 0.001. Error bars represent SEMs.

Figure 6. Endostatin and its mutants showed distinct abilities in regulation of gene expression, cell signaling and intracellular ATP levels

The effects of endostatin, K96A, K96R, E176A, and E-M on A, the mRNA levels, and B, protein expression of Id1 and Id3 at 24 hours. C, HUVECs were treated with
indicated proteins for 1 hour, and stimulated with VEGF<sub>165</sub> for 10 min. The overall
and phosphorylation levels of Erk, p38, and Src were examined by Western blot. D,
HMECs were treated with indicated proteins for 24 hours. Intracellular ATP levels
were detected using ATP bioluminescent assay. E, relative intracellular ATP levels of
HMECs treated with E-M of indicated concentrations. F, relative intracellular ATP
levels of HMECs treated with endostatin of indicated concentrations. *, #, P < 0.05,
**, ##, P < 0.01, ###, P < 0.001. ns, not significant. Error bars represent SDs.
Figure 1

A

1  MHSHRDFQPVLHLVALNSPLSGGMRGIRGADFQCFQQARAVGLAGTFRAF
   \textsuperscript{IIIa}

51  LSSRLQDLYSIVR\textsuperscript{RADRAAVPIVNKLDELFPSWELFSGSEGPLKPGAR}
   \textsuperscript{IIIb}

101  IFSDGKDVLRHPTWPQKSVWHGSVPNGRRLTESYCETWRTEAPSATGQA

151  SSLLGGRLLGQSAASCHHAYIVL\textsuperscript{CLIE\textsuperscript{N}SFMTASK}
   \textsuperscript{II}

B

IB: Endostatin

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<th>ATP (mM)</th>
<th>0</th>
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<tr>
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<td></td>
</tr>
</tbody>
</table>

C

![Bar chart showing phosphate levels at different ATP concentrations]

D

![Bar chart showing phosphate levels over time with varying endostatin concentrations]
Figure 2

A

Phosphate (μM)

ATP  GTP  CTP  UTP  ADP  AMP  ATP-γ-S  ADP-β-S

B

Phosphate (μM)

pH

C

Phosphate (μM)

Mg^{2+}  Ca^{2+}  Cu^{2+}  Mn^{2+}  Zn^{2+}

D

Initial ATP Hydrolysis rate (pmol/min)

ATP (μM)
Figure 3

A

K96A  GSEGPLK → GSEGPLA
K96R  GSEGPLK → GSEGPLR
E176A VLCIE → VLCIA
E-M  GSEGPLK → GESGAGKT

B

Relative ATPase activity

1.5
1.0
0.5
0.0

WT  K96A  K96R  E176A  E-M

C

IB: Endostatin

ATP-Bound

Input

WT  K96A  K96R  E176A  E-M

1  0.78  0.74  0.83  1.21
Figure 5

A

Tumor Volume (mm³)

Days after implantation

PBS  WT  K96A  K96R  E176A  E-M

B

Tumor Weight (g)

PBS  WT  K96A  K96R  E176A  E-M

C

Relative blood vessel density

PBS  WT  K96A  K96R  E176A  E-M

D

Relative number of CC3 positive cells/field

PBS  WT  K96A  K96R  E176A  E-M

E

PBS  WT  K96A  K96R  E176A  E-M
Figure 6

A

B

C

D

E

F

Relative mRNA level of Id1

Relative mRNA level of Id3

GAPDH

Ctrl
WT
K96A
K98R
E176A
E-M

Ctrl
WT
K96A
K98R
E176A
E-M

Ctrl
WT
K96A
K98R
E176A
E-M

Ctrl
WT
K96A
K98R
E176A
E-M

Ctrl
WT
K96A
K98R
E176A
E-M

Ctrl
WT
K96A
K98R
E176A
E-M

###

#

##

#

1 0.66 0.96 0.86 0.98 0

1 0.74 0.85 0.87 0.88 0.09

p-Erk

Erk

p-p38

p-p38/p38

p-Src

Src

p-Src/Src

Ctrl
WT
K96A
K98R
E176A
E-M

1 0.32 0.49 0.59 0.58 0.41

1 0.66 0.52 0.64 0.32 0.18

1 0.56 0.85 1.27 1.59 1.02

ATP conc (nmol/n Blake cells)

Relative cellular ATP level

E-M (µg/ml)

1 0 5 10 20 50 100

WT endostatin (µg/ml)

0 10 20 50 200 500

* ns

** ns

*** ns

Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.
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Endostatin has ATPase activity, which mediates its anti-angiogenic and anti-tumor activities

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