Endostatin Has ATPase Activity, Which Mediates Its Antiangiogenic and Antitumor Activities

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Abstract

Endostatin is an endogenous angiogenesis inhibitor with broad-spectrum antitumor 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 (WT) 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 antitumor effects compared with WT. On the other hand, K96A, K96R, and E176A, endostatin mutants with lower ATPase activities than that of WT, 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 antiangiogenic and antitumor activities, suggesting that construction of endostatin analogues with high ATPase activity may provide a new direction for the development of more potent antiangiogenic drugs.

Introduction

Antiangiogenic agents have been widely considered as the fourth modality in cancer treatment, together with surgery, chemotherapy, and radiotherapy. Endostatin, as an endogenous antiangiogenic inhibitor (1), exhibits potent antitumor 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 (NSCLC) 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 antitumor 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 the 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 the FAK-ERK1/2-P38–ERK1 MAPK pathway (15). Besides, the internalization of endostatin by endothelial cells is important, if not essential, for the physiologic 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). Abdollahi and colleagues (19) reported that approximately 12% of the human genome is modulated by endostatin, therefore tipping the dynamic angiogenic balance toward 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; ref. 21). The Walker B motif contains a conserved Asp or Glu residue preceded by a consecutive sequence of four hydrophobic residues (h-h-h-D/E), with this acidic residue coordinating an Mg2+ ion (or Mn2+ or Ca2+) 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 investigations, the precise mechanisms of endostatin actions still remain elusive. In this 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 antitumor efficacy in vivo compared with wild-type (WT) endostatin. However, E-M, the endostatin mutant with higher ATPase activity, showed the more potent antitumor and antiangiogenic effects. Collectively, our study demonstrates that endostatin has novel ATPase activity, which mediates the antiangiogenic and antitumor effects of endostatin.

**Materials and Methods**

**Plasmids, reagents, and Abs**

Human endostatin was subcloned into pET30a(+) vector (Addgene), and endostatin mutants (K96A, K96R, E176A, and 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 laboratory 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, Id1, Id3 (Cell Signaling Technology), and CD31 (Abcam). Secondary antibodies were obtained from Santa Cruz Biotechnology.

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.

**Cell culture**

Primary HUVECs were cultured in endothelial cell medium (ScienCell), and used at passages 2 to 7. Culture of A549-GFP cells and HMECs was maintained in DMEM and 10% FBS (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 mmol/L) were incubated in imidazole-Cl buffer (10 mmol/L imidazole-Cl, 75 mmol/L KCl, 0.2 mmol/L EDTA, 3 mmol/L MgCl2, pH 7, final volume of 100 μL) at 37°C for 60 minutes. Subsequently, HCl/Mo was added and 2 minutes later 0.042% malachite green and H2SO4 were added. Absorbance at 650 nm was detected after incubation for 30 minutes. 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 μmol/L to 2 mmol/L were used and Michaelis–Menten calculations were performed with GraphPad Prism 5 software (GraphPad Software, Inc.).

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

**ATP-binding assay**

Proteins in Tris buffer (10 mmol/L Mg2+ and 100 mmol/L NaCl, pH 7.5) were incubated with high-affinity ATP-agarose (Innova) at 4°C for 1 hour, 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 μmol/L, and all measurements were carried out at 37°C, pH 7.4.

**Western blot analysis**

Samples were mixed with reducing SDS-PAGE loading buffer, boiled at 100°C for 15 minutes, subjected to SDS-PAGE, and transferred onto a polyvinylidene difluoride membrane (Millipore). The membrane was blocked in TBST (20 mmol/L Tris, 150 mmol/L NaCl, and 0.1% Tween 20) plus 5% to 10% dried non-fat skimmed milk for 30 minutes 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 hours at room temperature or overnight at 4°C. Washed three times with TBST for 5 minutes each time at room temperature, and then incubated with corresponding horseradish peroxidase–conjugated secondary antibodies for 60 minutes at room temperature. Following five washes with TBST, immunoreactive bands were detected. Images were scanned using Bio-5000 plus (MicorTech) 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**

A total of 2 × 10^4 HUVECs were seeded in sextuplicate in a 96-well plate and 24 hours later, endothelial cells were treated with 20 μg/mL indicated proteins in endothelial cell basal medium containing 1% FBS for 72 hours. 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 hour, washed for two times, and chilled on ice for 1 to 2 minutes. Subsequently, equivalent cells were added to each well with 20 μg/mL indicated proteins and incubated at 37°C for 30 minutes. Plates were shaked for 10 to 15 seconds and washed for two to three 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 hours. 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 hour. 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 analysis.

Animal studies

A549-GFP tumor cells (10⁶) were mixed with Matrigel (Becton Dickinson) and inoculated into the axilla of nude mice s.c. Once the tumor volume approached 0.1 cm³, mice were randomly grouped (n = 6/group) and i.v. treated with PBS, WT endostatin, K96A, K96R, E176A, or E-M (12 mg/kg) every 2 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 (5 μm sections). Subsequently, these tissues were stained with Abs and diaminobenzidine, and then counterstained 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 ± SDs or SEMs. Comparisons were determined using two-tailed Student 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 (GXXGXXK; ref. 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 that 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 (Fig. 1C and D). Consistently, ATP bioluminescence assay results showed that endostatin indeed directly consumed ATP (Supplementary Fig. S2A and S2B), whereas ATP was barely catalyzed by either endostatin storage buffer or angiostatin (K1-3), another endogenous angiogenic factor (Supplementary Fig. S2C and S2D). 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 Mg²⁺ (Fig. 2C).

Because the reaction remained linear during the initial 60 minutes (Fig. 1D), the hydrolysis rate at 60 minutes was used as the initial rate of ATP hydrolysis for Michaelis–Menten analysis. The results demonstrated a $V_{max}$ of 2.117 ± 0.071 pmol/(min.μg) and $K_m$ of 83.36 ± 11.21 μmol/L (Fig. 2D). Isolated human Hsp90 exhibits high ATPase activity with a $K_m$ of 80 μmol/L, 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. Because 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). Because 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 ($λ_{max}$) for all the mutants compared with that of WT endostatin, implying relatively stable core structures of endostatin mutants (Supplementary Fig. S4). As expected, K96A, K96R, and E176A mutants
displayed reduced ATPase activities, whereas E-M showed increased activity relative to that of WT 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 with that of WT 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 physiologic functions (27, 41, 42). To identify whether an intact endostatin ATPase activity is required for its antiangiogenic effects, we assessed the inhibitory effects of endostatin and its mutants on endothelial cell proliferation, migration, tube formation, and adhesion. WT endostatin, K96A, K96R, and E176A 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 WT 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, whereas 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 WT endostatin and K96A treatment and moderately upregulated by K96R and E176A, whereas 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 antitumor and antiangiogenic properties

We next questioned whether the ATPase activity of endostatin has any role in retarding tumor growth and angiogenesis, because it is involved in endostatin-mediated antiangiogenic properties in vitro. To answer this question, an A549-GFP xenograft tumor model was established and mice received injections of PBS, WT 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 were 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 WT). K96A, K96R, and E176A were less effective compared with WT 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 immunostaining 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 with endostatin ($P < 0.01$). Apoptotic cells were evaluated using immunostaining 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 upregulated 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 antiangiogenic and proapoptotic effects 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 regulations. 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 WT endostatin ($P < 0.05$), whereas 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 as WT (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 antitumor and antiangiogenic activities, we analyzed whether they could block VEGF-induced MAPK/Erk and p38 phosphorylation. 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 antiangiogenic 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 determinant of cell fate (46). HMEC cells were treated with 20 μg/mL endostatin, K96A, K96R, E176A, and E-M for 24 hours 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 mg/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 antitumor and antiangiogenic effects by intracellular ATP depletion.

**Discussion**

Endostatin is a potent antiangiogenic 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 antitumor and antiangiogenic 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*, and *Gallus gallus*, *Dendrobates azureus*, we noticed that only human endostatin consists of the Walker A motif, whereas 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 that 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, because endostatin is able to affect approximately 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

On the basis of previous findings, the molecular mechanisms underlying antiangiogenic 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 mechanisms. 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 WT (Fig. 5), whereas E-M is dramatically more effective than WT 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, Lim and colleagues (18) reported that manipulation of endostatin by addition of an MTD significantly increased endostatin uptake and improved its antitumor effects. E-M, with the fastest cellular internalization rate (Supplementary Fig. S8A), exhibited the most prominent ATPase activity and antitumor activity. It is plausible that enhanced uptake per se contributes to increased antitumor effects of E-M compared with that of WT endostatin.

Minimum endostatin sequence required for its biological activity

Several groups have reported the minimum endostatin sequence for its biological activity. Morbidelli and colleagues (48) demonstrated that a peptide (sequence 135–184) containing the disulfide bridge Cys135-Cys165 has shown antiangiogenic
activities. 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 antitumor activity. However, the peptide was administered 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 antitumor effects, which leads to a complex but exquisite endostatin network.

Drug design: endostatin analogues 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 United States, an N-terminal modified E. coli–expressed endostatin was approved by the CFDA for the treatment of NSCLC patients in 2005 in China (3). The prominent drug design strategy of this drug is to covalently attach a peptide of MGGSHHHHHH to the N terminus of endostatin, thus facilitating the N-terminal integrity of endostatin, which is essential for the antiangiogenic and antitumor 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 serves 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 antitumor effects, and provide a brand new direction for the design and development of more potent antiangiogenic drugs.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors’ Contributions

Conception and design: S. Wang, X. Lu, Y. Fu, Y. Luo
Development of methodology: S. Wang, X. Lu, P. Liu
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): S. Wang, X. Lu, P. Liu, J. Jia, S. Zhan
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): S. Wang, X. Lu, Y. Fu
Writing, review, and/or revision of the manuscript: S. Wang, X. Lu, Y. Luo
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): Y. Fu, Y. Luo
Study supervision: Y. Luo

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