A 27-Amino-Acid Synthetic Peptide Corresponding to the NH₂-Terminal Zinc-Binding Domain of Endostatin Is Responsible for Its Antitumor Activity

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Abstract

The first recombinant endostatin that elicited strong antitumor activity was expressed in Escherichia coli and administered as a suspension. Under these conditions, the protein retained its full antangiogenic activity. Lack of requirement for a folded structure prompted us to investigate antitumor properties of synthetic peptides corresponding to different regions of endostatin. Here, we show that the entire antitumor, antimigration, and antipermeability activities of endostatin are mimicked by a 27-amino-acid peptide corresponding to the NH₂-terminal domain of endostatin. This peptide contains three histidines that are responsible for zinc binding. Mutations of the zinc-binding histidines abolished its antitumor and antimigration activities, but not antipermeability properties. (Cancer Res 2005; 65(9): 3656-63)

Introduction

Endostatin, a 183-amino-acid proteolytic cleavage fragment corresponding to the COOH terminus of collagen 18, has been the subject of investigation by a number of laboratories because of its antitumor activity with no toxic side effects (1–5). Several antiangiogenic activities have been reported for this protein, such as inhibition of endothelial cell proliferation, migration, and tube formation. Endostatin also suppresses vascular endothelial growth factor (VEGF)–induced vascular permeability (6). However, the mechanism of action of endostatin remains unknown. Endostatin inhibits endothelial cell migration by inhibiting phosphorylation of focal adhesion kinase via binding to α5β1 integrin (7). It also has been shown that cell surface glypicans are low-affinity endostatin receptors (8). Endostatin has been implicated in several signaling pathways, such as down-regulation of c-my c (9), cyclin D1 (10) and RhoA activity (11), blockade of VEGF signaling (12, 13), and inhibition of the wnt-signaling pathway (14). Furthermore, endostatin has been shown to bind and inactivate metalloproteinasces (15–17) and to regulate a spectrum of genes that suppress angiogenesis (18).

The crystal structures of both murine and human endostatin have been elucidated (19, 20) and show a noncovalently held dimer at high concentration required for crystallization (20). The presence of two disulfide bonds results in a highly folded structure.

Endostatin binds one atom of zinc (Zn) per monomer via the three histidines in the NH₂ terminus of the molecule (histidines 1, 3, and 11) and aspartic acid 76. The heparin-binding property of endostatin is mediated by noncontiguous arginines clustered over the three-dimensional globular surface of the molecule (21).

We have previously shown that oligomeric endostatin (NC1 and dimer) is primarily associated with laminin in the basement membrane (22). This association may be important for some of the biological functions displayed by endostatin. On the other hand, the heparin-binding properties of endostatin manifest themselves in its interaction with the cell surface. It is likely that endostatin has several biological functions mediated by different regions of the protein.

Our goal in this report is to identify and characterize the endostatin region responsible for its antitumor activity. Toward achieving this goal, a series of overlapping peptides corresponding to the complete sequence of endostatin were synthesized. We find that the entire antitumor activity of endostatin is located in a 27-amino-acid peptide that binds Zn. We show that Zn binding is required for antitumor and antimigration activities of endostatin but not its antipermeability property.

Materials and Methods

Cell culture and reagents. Human BaPc-3 pancreatic adenocarcinoma and murine Lewis lung carcinoma (LLC) cells (American Type Culture Collection, Rockville, MD) were grown and maintained as described earlier (2, 23). Human microvascular endothelial cells (HMVEC-d, Clonetics, Walkersville, MD) were cultured in microvascular endothelial cell growth medium (EGM-2 MV; Clonetics) and maintained at 5% CO₂ in a 37°C humidified incubator.

Recombinant human endostatin was a generous gift from EntreMed Corporation (Rockville, MD) and recombinant human and murine FeEndostatin were prepared as described earlier (24). Human and murine endostatin peptides were synthesized by SynPep Corporation (Dublin, CA). Peptides were resuspended in PBS or 50 mmol/L Tris, 150 mmol/L NaCl (pH 7.5).

PECAM, purified rat anti-mouse CD31, was obtained from BD PharMingen (San Diego, CA) and human recombinant VEGF was obtained from the NIH (Bethesda, MD).

The thrombospondin-1 (TSP-1) type 1 repeats fragment 3TSR, containing amino acid 361 to 530 of TSP-1 (25), was kindly provided by Dr. Jack Lawler (Beth Israel Deaconess Medical Center, Department of Pathology, Boston, MA). TNP-470 was a generous gift from Takeda Chemical Industries Ltd. (Osaka, Japan).

Animal studies. All animal procedures were done in compliance with Boston Children's Hospital guidelines, and protocols were approved by the Institutional Animal Care and Use Committee. Male (24-27 g) immunocompetent C57Bl/6J mice (The Jackson Laboratory, Bar Harbor, ME) and immunocompromised severe combined immunodeficiency (SCID) mice (Massachusetts General Hospital, Boston, MA) were used. Mice were 7 to 9...
weeks of age. They were acclimated, caged in groups of five in a barrier care facility, and fed animal chow and water ad libitum. Animals were anesthetized via inhalation of isoflurane (Baxter, Deerfield, IL) before all surgical procedures and observed until fully recovered. Animals were euthanized by a lethal dose of CO₂ asphyxiation.

**Tumor models.** BxPC-3 and LLC cells were grown in 900-cm² roller bottles. The cell concentration was adjusted to 50 × 10⁶ cells/mL. Mice were shaved and the dorsal skin was cleaned with ethanol before tumor cell injection. A suspension of 5 × 10⁶ tumor cells in 0.1 mL RPMI 1640 (for BxPC-3) or DMEM (for LLC) was injected s.c. into the dorsa of mice at the proximal midline. BxPC-3 cells were implanted in SCID mice and LLC in C57Bl/6j mice as described earlier (2).

The mice were weighed and tumors were measured every 3 to 5 days in two diameters with a dial caliper. Volumes were determined using the formula \( V = \frac{4}{3} \pi r^3 \) (where \( a \) is the shortest and \( b \) is the longest diameter). Data is represented as volume of treated tumor over control (T/C). At the completion of each experiment, the mice were euthanized with CO₂ asphyxiation. Tumors were fixed in 10% buffered formalin (Fisher Scientific, Fair Lawn, NJ) and paraffin embedded.

For treatment of tumor-bearing mice, tumor volumes were allowed to grow to ~100 mm³, and mice were randomized. Treatment was done by single bolus s.c. injections. Peptides were given twice daily (every 12 hours). Doses indicated for peptides were corrected for the purity of peptides (~70%). For example, mice given injections of 4 mg/kg/d peptide were actually given 2.8 mg/kg/d after correction. BxPC-3 tumors were treated for 28 days and LLC tumors for 14 days. The unpaired Student t test was used for statistical analysis.

**Immunohistochemistry.** Tumors were fixed in 10% buffered formalin overnight at 4°C. The next day, tumors were washed thrice in PBS and paraffin embedded. Sections (5 μm) were permeabilized with 40 μg/mL proteinase K (Roche Diagnostics Corp., Indianapolis, IN) in 0.2 mol/L Tris-HCl buffer (pH 7.6) for 25 minutes at 37°C and washed with PBS. PECAM1 (1:250) was incubated at 4°C overnight. Stainings were amplified using tyramide signal amplification direct and indirect kits (NEN Life Science Products Inc., Boston, MA). Sections were photographed at 200× or 400× magnification using a NIKON TE300 microscope (Melville, NY). Vessel density (average of 10 fields) was determined by IPLab software. The unpaired Student t test was used for statistical analysis.

**Endothelial cell migration assay.** The motility response of HMVEC-d cells was assayed using a modified Boyden chamber. Cells were plated in T75-cm² flasks at 0.5 × 10⁶ cells per flask and allowed to grow for 48 hours (~70% confluent) before the migration assay. To facilitate cell adhesion, the upper of 1:250 was incubated at 4°C overnight. Vessels were captured using a 40× objective, and images were captured with a charge-coupled device camera using SPOT software. Total migration per membrane was quantified from the captured images using Scion Image software (NIH). All experiments were run in triplicate. Migration was normalized to percent migration, with migration to VEGF alone representing 100% migration. The data represents an average of five to six separate experiments. High error bars were observed due to the variability inherent in endothelial cell migration assays. A 3-fold increase in VEGF-induced migration over basal levels was considered the minimum criterion for an experiment to be considered valid. The unpaired Student t test was used for statistical analysis.

**Miles vascular permeability assay.** SCID mice (n = 12) were given s.c. injections of human endostatin (EntreMed; 100 μg/kg/d), murine FcEndostatin (20 mg/kg/d), peptides (either 14 or 2.8 mg/kg/d), and saline (200 μL) for 5 days before performing the Miles assay (26). Briefly, Evan’s blue dye (100 μL of a 1% solution in PBS) was injected i.v. into mice. After 10 minutes, 50 μL of human recombinant VEGF (1 μg/μL) or PBS were injected intradermally into the preshaved back skin. After 20 minutes, the animals were euthanized and an area of skin that included the blue spot resulting from leakage of the dye was removed. Evan’s blue dye was extracted from the skin by incubation with formamide for 5 days at room temperature, and the absorbance of extracted dye was measured at 620 nm with a spectrophotometer. The unpaired Student t test was used for statistical analysis.

**Statistical methods.** Data are expressed as mean ± SD. Statistical significance was assessed using the Student t test. For all statistical comparisons, treated groups were compared with PBS-treated controls or VEGF alone (in migration assays). P < 0.05 was considered statistically significant.

**Results**

**Identification of the endostatin peptide that is responsible for antitumor activity.** The first recombinant endostatin was expressed in *Escherichia coli* and purified using 8 mol/L urea and reducing conditions (1). After dialysis against PBS, the final product, in the form of an insoluble suspension, was injected into immunocompetent C57Bl/6j mice bearing murine LLC, resulting in tumor inhibition (1). Although the above protocol has been repeated by several laboratories, some laboratories failed to reproduce these results, leading to a controversy on the effectiveness of endostatin (27). One possible explanation for the antitumor activity of the *E. coli*–derived insoluble endostatin is that a folded endostatin structure is not required. We hypothesized that endostatin antitumor activity might be mediated by a short synthetic peptide. Toward this goal, we synthesized eight overlapping peptides with 24 to 27 amino acids derived from human endostatin (Table 1). Peptides were approximately one seventh to one eighth the size of full-length endostatin. Three cysteines were replaced by alanines, and cysteine was omitted, to prevent the formation of disulfide bonds. Two additional lysines were added at the COOH terminus of hP8 to increase its solubility.

**Table 1. Overlapping human endostatin peptides**

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Sequence</th>
</tr>
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<tbody>
<tr>
<td>hP1</td>
<td>HSHRDFQPVLIHVILYNASLPLGGMG</td>
</tr>
<tr>
<td>hP2</td>
<td>HSHRDFQPVLIHVILYNASLPLGGMG</td>
</tr>
<tr>
<td>hP3</td>
<td>MIRGBGADFGQQFQARAVGLATFR</td>
</tr>
<tr>
<td>hP4</td>
<td>TFRASLRLQYDLISYRVRBDAAV</td>
</tr>
<tr>
<td>hP5</td>
<td>67AAVIPYKLDELLFPSEALFSGQ</td>
</tr>
<tr>
<td>hP6</td>
<td>90GSEGLPKGARIFSDGKDVLHP</td>
</tr>
<tr>
<td>hP7</td>
<td>111HP7MRQKYSGVHGDPGMLKTSY</td>
</tr>
<tr>
<td>hP8</td>
<td>136EWRTEAPATQAGSSLGLRLIQ</td>
</tr>
</tbody>
</table>

**Note:** Cysteines (33, 165, 173) were substituted by alanines (underlined) or omitted (Cys). The bold letters represent the overlapping amino acids. Two additional lysines were added at the COOH terminus of hP8 to increase its solubility (double underlined).
were ~70% pure. However, no difference in tumor inhibition was observed when peptides of more than 95% purity were used (data not shown).

These peptides were initially tested for antitumor activity using the human pancreatic tumor cells BxPC-3 in SCID mice. Peptides (7 mg/kg/d) were given s.c. twice a day due to the high clearance rate from mouse circulation, whereas full-length endostatin (human FcEndostatin, hFcES; 20 mg/kg/d) was given only once per day. We have previously shown that FcES could successfully inhibit tumor growth (24). The NH2-terminal hP1 peptide of endostatin inhibited BxPC-3 by 39% (P = 0.0077) and full-length endostatin by 44% (P = 0.0057; Fig. 1). Two other peptides, hP2 and hP5, also showed some small antitumor activity, wherein hP2 inhibited BxPC-3 by 19% (P = 0.48) and hP5 by 29% (P = 0.15). The remaining peptides had no effect on inhibiting tumor growth (Fig. 1).

Thus, most of the antitumor activity was associated with the NH2-terminal hP1 peptide compared with full-length endostatin. Tumor inhibition by hP1, hP2, and hP5 was statistically not significant, probably due to the small number of mice per group (n = 3 used). Although peptides were not used at equimolar ratios to full-length endostatin, this data still suggests that the antitumor property of endostatin may be located within its NH2-terminal domain.

The NH2-terminal 27 amino acid peptide of endostatin is responsible for its antitumor property. We next examined the effect of all 8 endostatin peptides on murine Lewis lung carcinoma (LLC) tumor growth. For these experiments, murine analogues of the endostatin peptides were synthesized. The only difference between the human and murine peptides was that murine P1 peptide contained 27 amino acids instead of 25 amino acids for human P1. Unlike the treatment of BxPC-3 tumors, LLC tumors were treated at equimolar concentrations of murine full-length endostatin (20 mg/kg/d) and murine peptides (2.8 mg/kg/d). Eight mice per group were used and control mice were treated with PBS. Peptides were injected (s.c.) twice a day, whereas full-length endostatin and PBS were given once a day. The NH2-terminal mP1 endostatin peptide inhibited LLC by 45% (P = 0.0021), which is similar to the inhibition by full-length endostatin (48%, P = 0.0068; Fig. 2A). No significant tumor inhibition was detected using the remaining peptides (mP2 to mP8) at the same concentration used as the mP1 peptide (Fig. 2A). Thus, this result suggests that the 27-amino-acid mP1 peptide contains all of the antitumor activity associated with endostatin.

We also wanted to determine the effect on angiogenesis after mP1 treatment. Therefore, LLC tumors treated with the endostatin peptides and full-length endostatin at equimolar concentration were analyzed for vessel density (CD31; Fig. 2B and C). Treatment of LLC with mP1 and full-length endostatin reduced vessel density significantly (~50%), whereas the remaining peptides and PBS had no effect. These results suggest that mP1 can inhibit LLC tumor growth by reducing vessel density in similar manner to full-length endostatin.

Histidine residues at position 1 and 3 of endostatin are critical for zinc binding. The crystal structure of endostatin reveals a highly folded molecule (ref. 20; Fig. 2D). However, the NH2-terminal region resembles a random coil structure consistent with our analysis that a synthetic peptide corresponding to this domain can mimic the native molecule (Fig. 2D). There is an atom of Zn associated with each molecule of endostatin (20). Based on our crystal structure analysis, three histidines at positions 1, 3, and 11, plus aspartic acid at position 76, form the four coordinates for this Zn atom (20). Initially, it was found that trypsin digestion of human endostatin resulted in cleavage of four amino acids (HSHR) from the NH2 terminus, which lacked Zn binding (20). Because mP1 contains the three histidine residues mentioned above, it raised the possibility that this peptide was able to bind Zn by having a molecule of water occupying the fourth coordinate (Fig. 3A, left). Previously, our group has shown that site-directed mutagenesis of histidine residues 1 and 3 of endostatin to alanines resulted in the loss of its antitumor property (28). Furthermore, it was shown that deleting the first three amino acids HTH of murine endostatin disrupted Zn binding (28). Therefore, a mutant of peptide mP1 was synthesized and the histidines at positions 1 and 3 were mutated to alanines. This mutant peptide was called mP1-H1/3A. To determine the Zn-binding capacity of mP1 and mP1-H1/3A, flame atomic absorption was done. Each peptide was dissolved in 20 mmol/L Tris (pH 8.0) mixed with excess Zn chloride and extensively dialyzed against the above buffer. These data yielded Zn ratios of 0.1 per molecule of mP1-H1/3A and 0.9 for mP1 (Fig. 3D). Therefore, mutating the histidines at positions 1 and 3 to alanines abolished Zn binding (Fig. 3A, right).

The zinc-binding domain of endostatin is important for its antitumor activity. To determine if Zn binding is also important for the antitumor property of endostatin, mP1 and mP1-H1/3A were tested using the LLC tumor model. Peptides were administered twice a day (s.c.) at a dose of 2.8 mg/kg/d. Peptide mP1 inhibited LLC by 42% (P = 0.031), whereas mP1-H1/3A had no effect (Fig. 3C). To determine if there was a difference in angiogenesis, vessel density (CD31) of LLC tumors was analyzed after mP1 and mP1-H1/3A treatment. There was a considerable decrease in vessel density after mP1 treatment (67% reduction, P < 0.01), whereas mP1-H1/3A was similar to the PBS-treated control (Fig. 3D and E). These data suggest that Zn binding is important for the antitumor property of endostatin.

The NH2-terminal fragment of endostatin inhibits endothelial cell migration. Endostatin has previously been shown to inhibit endothelial cell migration (2, 29). Therefore, we tested the hP1 peptide and the mutant hP1 peptide (hP1-H1/3A) for anti-endothelial cell migration activity. Inhibition of VEGF-induced migration of HMVECs was determined using several doses of
endostatin and endostatin peptides (Fig. 4). Human peptides were used because the cells were of human origin. Two sources of full-length endostatin [EntreMed endostatin (EM-ES) and human FcEndostatin (hFcES)] were used in the migration assay, both of which inhibited migration. The inhibition of full-length endostatin was dose responsive between 100 and 200 ng/mL. Interestingly, there was no additional inhibition with 500 ng/mL. Furthermore, we found that hP1 inhibited endothelial cell migration at lower concentrations than full-length endostatin. Maximal inhibition seems to occur with doses between 25 and 200 ng/mL (Fig. 4).

To determine if the Zn-binding site is important for anti-endothelial cell migration activity, hP1-H1/3A was also tested. No inhibition of endothelial cell migration was observed for hP1-H1/3A at any dose (Fig. 4). As positive controls, two other angiogenesis inhibitors previously used in VEGF-induced endothelial cell migration were also included. As expected, the TSP-1 fragment 3TSR (30) and TNP-470 (31) significantly inhibited endothelial cell migration (Fig. 4).

We also examined two other peptides, hP2 and hP6. As expected, hP2 had no effect on migration at 200 ng/mL (Fig. 4). There was also no inhibition observed at lower concentrations (data not shown). hP6 between 100 and 200 ng/mL had a slight effect on inhibition (only 200 ng/mL is shown). However, lower doses had no effect on inhibiting migration (data not shown).

The endostatin hP1 peptide could inhibit VEGF-induced endothelial cell migration at equimolar concentrations (25 ng/mL) to full-length endostatin (200 ng/mL), whereas hP6 only inhibited at doses of 100 and 200 ng/mL. These results show that the NH₂-terminal P1 peptide of endostatin maintains the ability to inhibit VEGF-induced endothelial cell migration and that the Zn-binding site is critical for this activity.

Figure 2. The NH₂-terminal domain of endostatin is responsible for its antitumor properties. A, treatment of LLC with murine FcEndostatin and murine endostatin peptides. LLC cells were implanted in the s.c. dorsa of C57Bl/6J mice, and systematically treated. Murine FcEndostatin (mES) was given at 20 mg/kg/d s.c., one injection per day. Peptides were given at 2.8 mg/kg/d s.c., two injections per day. Control mice were treated with PBS. T/C is indicated. Group sizes: n = 8. The unpaired Student t test was used for statistical analysis. B, CD31 staining. LLC sections were from PBS-, FcEndostatin- (20 mg/kg/d), and murine endostatin peptide (2.8 mg/kg/d)–treated mice. Peptides were administered s.c. twice a day. LLC tumor sections from day 14 were formalin fixed, paraffin embedded, and then stained with CD31 (PECAM). Sections were photographed at 200× magnification using a NIKON TE300 microscope. CD31 staining is in brown. C, quantification of vessel density based on CD31 staining. Y axis, % CD31/high power field (at 200× magnification). D, crystal structure of endostatin. The orange-shaded area corresponds to P1, which is randomly coiled. Two α helices are designated α1 and α2. Letters A to P, β sheets. Yellow-red strings, disulfide bonds. Black circle, Zn binding to the NH₂ terminus of endostatin.
Antipermeability activities of endostatin peptides. Several pathologic diseases, including tumors, have been associated with increased vascular permeability. Endostatin has been shown to suppress VEGF-induced permeability (6). Therefore, the ability of endostatin peptides to inhibit VEGF-induced permeability was also tested using the Miles assay (26). Previously, endostatin has been shown to inhibit VEGF-induced permeability using the Miles assay.3 Immunocompromised SCID mice were treated for 5 days before performing the Miles assay. Interestingly, both mP1 and mP1-H1/3A inhibited VEGF-induced permeability when tested at both 2.8 mg/kg/d (which is at equimolar ratio to full-length endostatin) and 14 mg/kg/d (Fig. 5). This effect on permeability was similar to that with full-length endostatin. These data indicate that two separate mechanisms or separate regions of the P1 peptide may regulate antitumor and antipermeability activities.

Discussion
We have shown that a synthetic peptide, corresponding to the NH2 terminus of endostatin, is responsible for its antitumor, antitumor, and antipermeability activities. Zinc binding is required for the antitumor and antimigration activities because substitution of the two histidines at amino acid positions 1 and 3 in the peptide completely blocks these properties. According to our crystallography data, histidines 1, 3, 11, and aspartic acid 76 of endostatin are important for Zn binding (20). Mutating these residues to alanine showed reduced antitumor activity of full-length endostatin and the double H1/3A mutant abolished endostatin antitumor activity completely (28). The removal of four amino acids, including the above two histidines from the NH2 terminus of endostatin, resulted in the loss of Zn binding by endostatin (20). In another study, it was shown that deletion of the first three amino acids HTH from the NH2 terminus of murine endostatin prevented Zn incorporation by endostatin (28). However, Zn binding was not required for the antipermeability property of endostatin because the mutant mP1-H1/3A retained its full antipermeability property. It is known that hyperpermeability is regulated by RhoA activity, calcium, tyrosine kinase, and cell junctions, resulting in the disruption of endothelial cell barrier function (32). Endostatin regulates many signaling pathways and it is likely that different mechanisms or separate regions of the P1 peptide regulate endostatin antitumor and antipermeability activities.

3 Shay Soker, personal communication.

Figure 3. The Zn-binding site of endostatin is important for antitumor activity. A, schematic diagram of mP1 and mP1-H1/3A. Zinc binding to mP1 is mediated by three histidines at positions 1, 3, and 11 (left). Histidines at positions 1 and 3 are mutated to alanines in mP1-H1/3A. These mutations prevent Zn binding (right). Sequences of mP1 and mP1-H1/3A are indicated. B, Zn binding to mP1 and mP1-H1/3A. Each peptide was dissolved in 20 mmol/L Tris (pH 8) at a concentration of 0.5 mg/mL and 1 mmol/L Zn chloride was added. Each sample was dialyzed against Tris buffer for 72 hours with three changes in dialysis solution [molecular weight cutoff (MWCO) = 1,000 Da]. Atomic absorption readings of the final Zn concentrations (mg/mL) were determined to be 9.63 and 1.05 for mP1 and mP1-H1/3A, respectively. These data yielded Zn ratios of 0.9 per molecule of mP1 and 0.1 for mP1-H1/3A. Molecular weight of the peptides was taken to be 3,000 Da. C, treatment of LLC with mP1 and mP1-H1/3A. Peptides were given s.c. twice a day. Both mP1 and mP1-H1/3A were given at 2.8 mg/kg/d. D, LLC tumor sections stained with CD31. Sections were photographed at 400× magnification. E, determination of vessel density. The unpaired Student t test was used for statistical analysis.

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The Zn-binding requirement of endostatin for inhibiting tumor formation has been controversial (28, 33, 34). Whereas the earliest report showed that the replacement of histidines 1 and 3 by alanines blocked the inhibitory effect of endostatin in LLC (28), two later publications challenged this finding (33, 34). In one of these reports, a mutant endostatin was prepared by deleting five amino acids in both COOH and NH2 termini (33). This construct elicited antitumor activity, similar to full-length endostatin. However, in the employed renal Re-9 carcinoma tumor model, the administration of endostatin was initiated when the tumor size was 300 mm3 and lasted for only 4 days, when the tumor size reached 500 mm3. The injection sites were at the periphery of the tumor and the injection dosage was \(10^6\) Ag/kg/d. In contrast, in our experiments we initiated treatment when LLC tumors reached a size of \(100\) to \(6,000\) mm3 and continued until tumors were \(6,000\) to 
7,000 mm3. Furthermore, we treated systemically and did not inject into the periphery of the tumor.
Another publication that dealt with the relevance of Zn binding to antitumor activity of endostatin showed that the removal of four amino acids "HSHR" from the NH2 terminus of human endostatin did not affect its antitumor activity (34). Measurements of Zn binding revealed that this mutant bound 2 atoms of Zn per molecule of endostatin, whereas the wild-type bound 10 atoms of Zn per endostatin molecule. However, in our crystal structure studies of endostatin, we have shown that endostatin contains 1 atom of Zn/endostatin molecule and the removal of the four amino acids HSHR from the NH2 terminus results in loss of Zn binding (20).

Endostatin is generated by proteolytic cleavage of collagen 18-1,35,36). The first amino acid at the NH2 terminus of endostatin is a histidine. The presence of histidine is important for conferring Zn binding to endostatin. Consequently, we are led to conclude that the processing of collagen 18 to endostatin may be highly regulated.

Several groups have shown that peptides derived from endostatin have antiangiogenic effects (29, 37-40). An NH2-terminal peptide composed of amino acids 6 to 49 (lacking the Zn-binding histidines) has inhibited endothelial cell proliferation and migration (37, 38). A Matrigel assay using this peptide has resulted in the inhibition of angiogenesis in vivo. However, no antitumor data was presented. In another study, a COOH-terminal peptide (amino acids 135-184) retaining the Cys135-Cys165 disulfide bond, has shown antitumor activity (39). However, the peptide was administered at the tumor periphery and not systemically. Cho et al. have shown that the NH2 terminus, which includes the Zn-binding site, and the COOH terminus of endostatin are not required for antitumor activity (40). However, this peptide and full-length endostatin were not tested at equimolar concentrations. Our results differ from these groups in that the P1 peptide systemically inhibited tumor formation, endothelial cell migration, and permeability at equimolar concentrations to full-length endostatin. Furthermore, at higher concentrations (14 mg/kg/d), mP2 inhibited LLC tumor formation as well as mP1 at 2.8 mg/kg/d (data not shown). However, mP1 at 14 mg/kg/d inhibited LLC tumor formation less than at 2.8 mg/kg/d (data not shown). Thus, a U-shaped curve seems to be associated with antitumor activity of endostatin as a function of the protein concentration. Similar results were observed for full-length endostatin using the pancreatic BxPC-3 and ASPC-1 tumor models.4 Therefore, determination of optimum endostatin concentration may be an important factor. In vitro assays have shown a similar biphasic characteristic by endostatin with the use of matrix metalloproteinases (MMP; ref. 16).

Similar to the mP1 endostatin peptide, antiangiogenic peptides from TSP-1 (ABT-510; ref. 41) and tumstatin (42), which is a fragment of collagen IV α3 chain, have been shown to have antitumor activities. Employing the LLC tumor model, we compared the antitumor activities of the mP1 endostatin peptide with the tumstatin T8 peptide and the ABT-510/TSP-1 peptide using equimolar concentrations. The antitumor activity of mP1 and the tumstatin T8 peptide was similar, whereas mP1 was more effective in inhibiting tumor growth than the ABT-510/TSP-1 peptide (data not shown).

The fact that full-length endostatin is not required for its antitumor activity explains the initial inconsistencies of endostatin activity (27). Endostatin has two disulfide bonds. Aggregation of endostatin from TSP-1 (ABT-510; ref. 41) and tumstatin (42), which is a single protein molecule under reducing conditions, most of the protein in an identical sample does not enter the polyacrylamide gel under nonreducing condition (data not shown). It is probably the degree of nonspecific aggregation that is responsible for the lack of activity in some of the preparations. Endostatin is most likely released from the aggregate in animals over a period of time, resulting in a denatured protein or partial fragments, which are capable of demonstrating antitumor properties due to their NH2-terminal peptide. Presumably, some of the preparations yield larger aggregates, which make such a release inefficient and give rise to a product that is incapable of eliciting an antiangiogenic response in mice.

What is the basis of endostatin’s antitumor activity? Several mechanisms have been proposed. Binding of endostatin to integrin α5β1 has been studied in more detail (7). Based on the findings of these authors, an assembly of several cell surface proteins and components, including α5β1, is responsible for interactions between endostatin and this integrin (11). However, no antitumor data were presented to confirm the above mechanism. More recently, the same authors have shown that an 11-amino-acid peptide derived from endostatin containing arginines and showing heparin binding is responsible for antiangiogenic activity of endostatin (29). We speculate that the phenomena observed by these investigators reflects some of the properties associated with the heparin-binding characteristic of endostatin and not its antitumor activity. Previously, we reported that disruption of heparin binding of endostatin (accomplished by the mutation of two discontinuous arginines on the protein surface) blocked cell motility (43). Furthermore, our endostatin hP3 peptide (see Table 1), which contains the peptide reported by the authors, failed to inhibit tumor growth.

The peptide P1 resembles the catalytic Zn-binding regions of MMPs. Three separate groups have investigated endostatin binding to MMPs. One group has reported that endostatin forms a complex with MMP-2, inhibiting its enzymatic activity (15). A second group has reported the absence of association between MMP-2 and endostatin (44). Finally, a third group obtained data indicating possible interactions between MMP-2 and endostatin (16). Therefore, we investigated the binding of endostatin to MMP-2 and to determine the effect of enzymatic activity using gel zymography. Both endostatin and the peptides could not inhibit the MMP-2 activity when using recombinant MMP-2 and MMP-2 derived from human umbilical vascular endothelial cells (data not shown). However, there still is a possibility of coparticipation of MMPs and endostatin in biological reactions. It is possible that endostatin may bind to the cellular target of MMPs (i.e., extracellular matrix and cell surface proteins) and block their enzymatic activities. Such specific cellular recipients have been poorly characterized at the present time.

The mechanism of endostatin action is unknown. Here, we have shown that a 27-amino-acid peptide corresponding to the NH2 terminal of endostatin, which contains a Zn atom, is responsible for the entire antitumor activity of endostatin. The binding of Zn is crucial for this phenomenon. We believe that the work presented in this publication is probably the most detailed investigation of all the peptides reported for endostatin thus far. This finding may enable us to substitute endostatin with mP1 peptide in clinical trials, hence providing a major saving in time and resources. Moreover, because this peptide retains its antipermeability activity, it can be used to treat other hyperpermeability diseases (i.e., retinal diseases, rheumatoid arthritis, and endometriosis). In addition,

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4 Ilhan Celik and Oliver Kisker, personal communication.
it may be useful in clinical applications by reducing edema or repairing the vascular leak syndrome, as seen in pulmonary edema, ascites, and inflammation. Moreover, these results shed light on our understanding of the molecular mechanism of endostatin.

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A 27-Amino-Acid Synthetic Peptide Corresponding to the NH₂-Terminal Zinc-Binding Domain of Endostatin Is Responsible for Its Antitumor Activity

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