Therapeutic Efficacy of Endostatin Exhibits a Biphasic Dose-Response Curve

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Abstract
We show here that recombinant endostatin protein has a biphasic effect on the inhibition of endothelial cell migration in vitro. In tumor-bearing animals, there is a similar biphasic effect on the inhibition of tumor growth and on circulating endothelial cells after once-daily s.c. injections. This biphasic effect is revealed as a U-shaped curve in which efficacy is optimal between very low and very high doses depending on the tumor type. This result may be applicable to other inhibitors of endothelial growth and to angiogenesis. Furthermore, these results have important implications for clinicians who administer angiogenesis inhibitors for cancer or other angiogenesis-dependent diseases. When these results are taken together with two previous reports of angiogenesis inhibitors with a U-shaped dose-response, they suggest that other regulators of endothelial growth may display a similar pattern. (Cancer Res 2005; 65(23): 11044-50)

Introduction
Tumor growth is angiogenesis-dependent (1). In order to stimulate angiogenesis, tumors up-regulate the production of a variety of angiogenic factors, including the fibroblast growth factors [acidic and basic fibroblast growth factor (bFGF)] and vascular endothelial cell growth factor (VEGF)/vascular permeability factor (1). However, some tumors also generate angiogenesis inhibitors. It is becoming more apparent that the angiogenic phenotype is the result of a net balance between positive and negative regulators of angiogenesis (2–4). A new class of drugs called angiogenesis inhibitors can suppress angiogenesis and therefore inhibit tumor growth, in part, by changing the balance of angiogenic activity in the tumor (5).

One of these inhibitors is endostatin, a 20 kDa COOH-terminal proteolytic fragment (183 amino acid) of collagen XVIII (6). Endostatin is a specific endogenous inhibitor of endothelial cell proliferation, migration (7), and vascular permeability (8). Although it has been shown that endostatin interacts with several receptors and pathways such as α5β1 integrin (9), cell surface glypicans (10), c-myc (11), cyclin-D1 (12), and VEGF signaling pathways (13, 14), additional mechanisms of action are being described (15, 16).

We show here that the in vitro and in vivo activities of endostatin follow a biphasic dose-response curve. The endostatin-induced inhibition of endothelial cell proliferation and migration in vitro increases proportionally with endostatin concentrations; however, further increases in dose result in reduced activity. Endostatin therapy of tumor-bearing mice reveals a similar pattern, i.e., increasing doses correlate with the increasing efficacy of tumor inhibition until an optimal dose is reached beyond which further dose increases result in less activity, a biphasic effect that has a U-shaped configuration.

Materials and Methods
Cell culture and reagents. Human pancreatic cancer cells, BxPC-3 and AsPC-1 (American Type Culture Collection, Rockville, MD), were grown in RPMI 1640 (Life Technologies, Grand Island, NY) supplemented with 10% FCS (Life Technologies) and 1% glutamine-penicillin-streptomycin (Irvine Scientific, Santa Ana, CA). Cells were maintained in T-75 tissue culture flasks (BD Falcon, Becton Dickinson, Franklin Lakes, NJ) or in 900 cm2 roller bottles and grown in 5% CO2/95% air at 37°C in a humidified incubator. Cells were washed with PBS, dispersed in a 0.05% solution of trypsin/EDTA (Life Technologies), and resuspended. After centrifugation (4,000 rpm for 10 minutes at room temperature), the cell pellet was resuspended in RPMI and the concentration was adjusted to 20 × 10⁶ cells/mL.

Tumor cell proliferation assay. Human pancreatic cancer cell lines (BxPC-3 and AsPC-1) were maintained as described above. For the proliferation assay, cells were washed with PBS and dispersed in a 0.05% trypsin/EDTA solution. A cell suspension (10,000 cells/mL) was made with RPMI 1640/10% FCS/1% glutamine-penicillin-streptomycin. Plated into 24-well culture plates (0.5 mL/well) and incubated (37°C, 5% CO2) for 72 hours. After 72 hours, cells were dispersed in trypsin/EDTA, resuspended in Isoton II and counted by Coulter Counter. Proliferation assays of BxPC-3 and AsPC-1 were repeated at least thrice.

Endothelial cell migration assay. Human umbilical vein endothelial cells (HUVEC), passages 4 to 8, were maintained in ECG Medium (PromoCell, Heidelberg, Germany), 2% fetal bovine serum, 50 ng/mL amphotericin B and 50 μg/mL gentamycin, 1 ng/mL bFGF, 0.4% heparin, 0.1 ng/mL EGF, 1 μg/mL hydrocortisone. Cells were trypsinized, centrifuged and diluted in ECG Medium (PromoCell) with 0.05% gelatin. Fifty thousand cells in 250 μL of medium were added per well to 10 mm tissue culture inserts (8 μm pore, Nunc A/S, Roskilde, Denmark) that had been treated with 5 μg/mL of fibronectin. Additionally, 50 μL of the test compound were added to the insert at different concentrations (resulting volume in the upper part of the insert, 300 μL). Cells were preincubated for 20 minutes with or without endostatin at a concentration between 0.03 and 20 μg/mL at 37°C. Medium (300 μL) was added to the bottom wells with or without VEGF (3 ng/mL; R&D Systems, Bad Nauheim, Germany) diluted in ECG Medium with 0.05% gelatin and cells were incubated for 6 hours at 37°C. Cells were washed once with PBS and the cells that had not migrated were removed from the top membrane by scraping with a cotton swab. Cells that had migrated were quantified using a colorimetric assay as follows: cells bound to the bottom of the tissue culture inserts were incubated for 1.5 hours in 400 μL of acid phosphatase substrate [10 mmol/L p-nitrophenol phosphate, 10 mmol/L sodium acetate, and 0.1% Triton X-100].
heart puncture under anesthesia in all animals 24 hours after the last endostatin levels at the end of each experiment, blood was collected by the third to fifth day and the ratio of treated versus control tumor volume was measured. Tumors were massaged every third to fifth day in two treatment groups were significantly different compared with the control group.

**Tumor cell implantation and measurement.** Before tumor cell injection, mice were shaved and the dorsal skin was cleaned with ethanol. Tumors were measured every 3 to 5 days. *, P < 0.001, significantly different compared with the control group.

**Measurement of serum endostatin levels.** Serum endostatin levels were measured by competitive enzyme immunoassay (Accucyte Human Endostatin, Cytimmune Sciences, Rockville, MD) according to the manufacturer's recommendations. The minimum detection limit for this endostatin kit is 1.95 ng/mL.

**Endostatin.** Clinical grade soluble human recombinant endostatin was a generous gift from EntreMed Corporation (Rockville, MD). The recombinant protein was formulated in potassium sucrose octasulfate to a concentration of 130 mg/mL, lyophilized, and stored at 4°C. The lyophilized protein was diluted in double-distilled water in a first step, vortexed for 30 minutes until completely dissolved, followed by further dilutions using PBS to prepare a dilution for the needed dosages. The prepared solutions were cooled immediately (0–4°C) in an ice-bath until application. Tuberculin syringes were filled with endostatin using a 20-gauge 1.5-inch needle (BD Microlance 3, Becton Dickinson, Franklin Lakes, NJ). Injection (s.c.) of endostatin was done using a 30-gauge 1.5-inch needle (BD Microlance 3). During the entire experiment, the room temperature in the animal facility was maintained at ≤ 24°C; the optimum room temperature for mice. At lower temperatures, mice become cold. They then conserve heat by vasoconstriction of skin vessels. This may reduce blood flow to a s.c. tumor and also reduce absorption of drugs injected s.c.7

**Flow cytometry.** Circulating endothelial cells (CEC) in peripheral blood were evaluated using three-color flow cytometry as previously described (17). Briefly, venous blood was obtained from the retro-orbital plexus and red cell lysis was done using ammonium chloride lysis buffer. Cells were then incubated with antibodies against murine CD45 conjugated to FITC (fluorescein) and Flk-1 conjugated to phycoerythrin (both from Becton Dickinson). 7-Aminoactinomycin D was used to assess viability and was purchased from Sigma (St. Louis, MO). Flow cytometry was done using a FACSCalibur flow cytometer (Becton Dickinson Biosciences, San Jose, CA) with analysis gates designed to remove platelets and cellular debris. For each mouse, 50,000 to 100,000 events were typically counted. Mouse blood was added to hemangioendothelioma (EOMA) cells expressing Flk-1 as a positive control.

**Statistics.** All data (tumor volumes) were expressed as the mean ± SD or F. Applying the data was not normally distributed (non-Gaussian

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**Figure 1.** Treatment of human pancreatic carcinoma (BxPC-3) with human endostatin. Mean (± SD) tumor volume after a 20-day treatment with different dosages of rhEndostatin (50, 100, 250, 500, and 1,000 mg/kg/d) in BxPC-3 tumor-bearing mice (group sizes, n = 7). Endostatin was given s.c. once daily. Tumors were measured every 3 to 5 days. *, P < 0.001, tumor volume in all treatment groups were significantly different compared with the control group.

**Figure 2.** Tumor growth under endostatin therapy in BxPC-3 tumor-bearing mice. Mice were treated over a treatment period of 20 days (group size, n = 7). Tumors were measured every 3 to 5 days. PBS-control (●), 1,000 mg/kg/d (▲), 500 mg/kg/d (○), 50 mg/kg/d (△), 250 mg/kg/d (□), and 100 mg/kg/d (▲). T/C, tumor volumes of treated versus control groups; *, P < 0.001, significantly different compared with the control group.

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7 Personal communication with Dipak Panigrahy.
Tumor volumes of the control group was 1,425 mm³ (Fig. 3). At the end of the treatment, the tumor volume in the endostatin at different dosages (100, 250, 500, and 1,000 mg/kg/d; fast-growing pancreatic tumor. These mice were also treated with endostatin with 100 mg/kg/d exhibited little or no growth over the whole course of the treatment compared with the other groups (Fig. 2). In the second experiment, mice were inoculated with AsPC-1, a fast-growing pancreatic tumor. These mice were also treated with endostatin at different dosages (100, 250, 500, and 1,000 mg/kg/d; Fig. 3). At the end of the treatment, the tumor volume in the control group was 1,425 ± 180 mm³. This experiment had to be terminated after 16 days due to the large tumor sizes in the control group. Tumor volumes of 547 ± 83, 201 ± 40, 110 ± 26, and 520 ± 26 mm³ were observed in the endostatin-treated group with 100, 250, 500, and 1,000 mg/kg/d, respectively. All of the tumor volumes in the treated groups were significantly different (P < 0.001) compared with the control group (Fig. 1). The treatment/control ratio decreased progressively during the treatment, and at day 20 was 0.53, 0.16, 0.30, 0.49, and 0.62 for endostatin at dosages of 50, 100, 250, 500, and 1,000 mg/kg/d, respectively. These results represent a 4-fold difference between the 100 and 1,000 mg/kg/d groups. Tumors in mice that were treated with endostatin with 100 mg/kg/d exhibited little or no growth over the whole course of the treatment compared with the other groups (Fig. 2). In order to investigate the in vivo proliferation of both tumor cell lines used, the tumor volumes in BxPC-3- and AsPC-1-bearing mice in the control groups were determined at day 16 after the start of the experiment. This time point was chosen because the tumor volumes in AsPC-1-bearing mice reached 1,425 ± 180 mm³ and the experiment was terminated for this group. Comparison of the tumor volumes at that time point revealed a significantly faster tumor growth for AsPC-1 (1,425 ± 180 mm³) compared with BxPC-3 (701 ± 134 mm³) bearing mice (P < 0.001; see Fig. 6).

In vitro proliferation rate of pancreatic tumor cells (BxPC-3 and AsPC-1). To explain the observed differences in the in vivo tumor growth (see above) and to determine in vitro differences of tumor cell proliferation, proliferation assays using BxPC-3 and AsPC-1 tumor cells were done (see above). The in vitro proliferation rate over 4 days for the two pancreatic tumor cell lines was not significantly different (Fig. 7).
Discussion

Cancer is an angiogenesis-dependent disease. Angiogenesis inhibitors have been shown to inhibit tumor growth in numerous animal models and in patients. In fact, recently, Avastin was the first angiogenesis inhibitor to receive Food and Drug Administration approval to treat advanced colon cancer (23). Throughout the history of chemotherapy, the maximum tolerated dose was always considered the most effective dose. However, because of the relative lack of toxicity of antiangiogenic therapy, the maximum tolerated dose is no longer useful. For antiangiogenic therapy, effective dosing requires a knowledge of whether or not the dose-response curve is linear and also requires a surrogate marker.

In this report, we show that human endostatin inhibits tumor growth in mice not in a linear relationship, but in a biphasic dose-response curve, which is U-shaped. For BxPC-3, optimal inhibition of tumor growth was obtained at a dosage of 100 mg/kg/d by s.c. bolus injection. In contrast, for AsPC-1 (human pancreatic cancer), the optimal inhibition of tumor growth was obtained at 500 mg/kg/d s.c. bolus injection. For both tumors, higher and lower dosages of endostatin showed a less inhibitory effect. For both tumors, the therapeutic efficacy of endostatin was represented by a U-shaped curve, but for the faster-growing tumor (AsPC-1), a proportionally higher dose of endostatin was required.

AsPC-1 tumors exhibited an increase in volume ~2-fold faster compared with BxPC-3 tumors (1,425 ± 180 versus 701 ± 53 mm³ after 16 days; Fig. 6). It is unlikely that this difference in growth rate of tumor mass is due to the proliferation rate of tumor cells because both AsPC-1 and BxPC-3 have previously been shown to have an average proliferating cell nuclear antigen rate of 60% (3). In addition, we showed that the in vitro proliferation rates of BxPC-3 and AsPC-1 were not statistically different (see Fig. 7).

However, the contrast in growth rate may be due to the angiogenic phenotype of each tumor. The microvessel density of AsPC-1 is ~160 vessels/hpf compared with BxPC-3 of 120 vessels/hpf (3). In both tumors, the production of the proangiogenic factors, VEGF and bFGF, did not differ significantly (BxPC-3: VEGF, 6.30 μg/mL; bFGF, 6.2 pg/mL; AsPC-1: VEGF, 5.219 pg/mL; bFGF, 5.6 pg/mL; ref. 24). On the other hand, it has been shown that BxPC-3 tumors generate at least four endogenous angiogenesis inhibitors (angiotatin, ref. 24), cleaved AT III (24), latent AT III (24), and DBP-maf (25) thereby suppressing other tumors in the double-sided tumor model (25). AsPC-1 tumors are not capable of inhibiting the growth of other tumors. At present, only the generation of angiotatin by AsPC-1 tumor cells has been reported, and it is unclear whether this tumor generates any other endogenous angiogenesis inhibitors (26). We therefore hypothesize that BxPC-3 has a greater capacity to produce negative regulators of angiogenesis.

Although tumor inhibition was dose-dependent, exhibiting maximum efficacy at intermediate endostatin doses, we found a linear correlation between increasing doses of endostatin and
endostatin serum levels. It should be noted, however, that the samples were drawn 24 hours after the application of the last dose, which might have resulted in a slight underestimation of the true peak levels of endostatin (Fig. 5).

After these experiments were conducted, it was reported by others that platelets sequester endostatin (27). Therefore, in future clinical studies, it may be prudent to measure the serum and plasma levels of endostatin, especially because endothelial cells are exposed only to plasma levels.

It should be noted that human endostatin was used in these experiments, which may account for the relatively higher doses of endostatin required to inhibit tumor growth compared with our previous reports which employed murine endostatin (6, 19). This finding has potential clinical implications. It is conceivable that serum or plasma endostatin levels in a patient receiving high doses of endostatin (e.g., ~250 mg/m²/d) for a prolonged period of time, could slowly increase over time, eventually reaching less effective endostatin levels. This possible scenario could be misinterpreted as “drug resistance.” In fact, our preliminary observations8 suggest that either lowering the dose or temporarily discontinuing the administration of endostatin would lower endostatin serum levels. This possible scenario could be misinterpreted as “drug resistance.” In fact, our preliminary observations8 suggest that either lowering the dose or temporarily discontinuing the administration of endostatin would lower endostatin serum levels. It should be noted, however, that the samples were drawn 24 hours after the application of the last dose, which might have resulted in a slight underestimation of the true peak levels of endostatin (Fig. 5).

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It is of interest that a genome-wide microarray analysis of endostatin treatment of human microvascular endothelial cells reveals that the expression of several genes which normally up-regulate endothelial proliferation, such as VEGF and hypoxia inducible factor-1α, are not simply down-regulated. Instead the down-regulation follows a U-shaped pattern whereby a low dose or short-time incubation with endostatin is more effective than a very high dose or prolonged incubation (16).

This finding also has implications for gene therapy. Some investigators reported that endostatin gene therapy was poorly effective against tumor growth in mice (28), despite the fact that serum levels reached >20,000 ng/mL. Based on the results of our study, the poor activity of endostatin which Eisterer et al. reported is most likely the result of the exceedingly high levels of circulating endostatin. Eisterer et al. also reported the failure of endostatin gene therapy in mice (29, 30), in which endostatin levels of 300 ng/mL were reported. They were operating at the high end of the U-shaped curve.

In contrast, Shi et al. reported highly effective tumor inhibition by endostatin gene therapy in mice in which the serum concentrations reached only 35 to 40 ng/mL (31). When these results are viewed in light of the effects of endostatin on in vitro migration of endothelial cells, and on tumor growth in vivo, endostatin seems to exert its biphasic effect directly on endothelial cells.

Effect of endostatin on circulating endothelial cells. Several recent studies have established that in both murine models and patients, changes in CECs are observed after treatment with endostatin (17, 21, 22). Consistent with these previous studies, we observed an ~50% reduction in CECs after treatment with endostatin doses which led to maximal antitumor effect (100-500 mg/kg/d) as compared with vehicle controls. Paradoxically, at a higher dose of 1,000 mg/kg/d, there was an increase in CECs compared with controls. There are several possible explanations for this observation. Recent evidence suggests that there are at least two distinct populations of endothelial cells detectable in the circulation: bone marrow–derived endothelial precursors, which are mobilized by VEGF and can contribute to neovascularization (32, 33), and endothelial cells shed from the preexisting vasculature (34, 35). We have recently established that endostatin inhibits the VEGF-induced mobilization of endothelial precursors in non–tumor-bearing mice. Preliminary evidence suggests, however, that antiangiogenic agents may cause an increase in the shedding of endothelial cells from the tumor vasculature (36, 37). Consistent with this hypothesis, a transient increase in phenotypically mature CECs is observed in endostatin-treated patients during the first month of therapy (22). It is possible that at higher doses of endostatin, an increase in endothelial shedding would be observed from established vasculature.

Endostatin is not the first drug to exhibit a U-shaped or “biphasic” concentration-response curve. IFN-α, as reported by Slaton et al. (38), exhibits a U-shaped curve in tumor-bearing animals in which low doses of IFN-α are more effective at inhibiting tumor growth, and reducing bFGF serum levels and microvessel

8 Unpublished observations.
density (38). Furthermore, other inhibitors of endothelial cell migration and/or proliferation have recently been reported to display a biphasic or U-shaped curve. These include rosiglitazone, a PPAR-γ ligand that inhibits angiogenesis and tumor growth (39), thrombospondin (40), angiotatin (41), endostatin (18, 42), a 27-amino acid peptide starting at the NH₂-terminal endostatin (43), and rapamycin (44, 45). Furthermore, in a recently published report of a phase I dose-finding clinical study using recombinant human endostatin (15-600 mg/m²) in patients with solid tumors, biphasic (U-shaped) response curves were also determined by analyzing biomarkers (e.g., changes in microvesSEL density and tumor blood flow) to define an optimal biological dose for endostatin (46). These results are consistent with our findings of a biphasic (U-shaped) dose-response curve in vitro and in vivo for endostatin.

The mechanism(s) for endostatin’s activity, and its U-shaped dose-response curve, remain uncertain. Endostatin has been reported to bind to a number of different cell surface proteins, including integrin α5β1 (9, 15, 47), heparan sulfate proteoglycans, glypicans, and vascular endothelial growth factor receptor-2 (9, 10, 13, 48, 49). For ligands that induce receptor dimerization, a biphasic dose-response curve has also been observed with optimal receptor activation at intermediate doses, and self-antagonism occurring at higher doses at which monomeric ligand-receptor complexes predominate. A similar relationship may exist between endostatin and a key receptor. It is also possible that endostatin may signal through multiple pathways simultaneously, and that optimal output from this antiangiogenic signaling network (16) requires doses which only activate certain pathways.

Recent evidence shows that endostatin is sequestered in platelets (27). Therefore, it may be prudent to measure plasma and serum levels in patients because a fixed dose of endostatin may accumulate in platelets and then spill over into plasma. The clinical implications are that oncologists who employ any drug, which does not have a linear dose-response curve, need to pay careful attention to serum and or plasma levels.

Understanding the mechanism(s) by which endostatin exerts its activity should reveal additional insights into the observed dose-response relationship and provide important guidance for the clinical application of this agent as well as other angiogenesis inhibitors.

Acknowledgments

Received 7/25/2005; accepted 9/21/2005.

Grant support: NIH grant to J.F. (ROI CA66481-09) and by the Breast Cancer Research Foundation (J.F.).

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We thank Catherine Butterfield and Amy Binsner for expert technical assistance, Kristin Gallage for graphic support, William E. Fogler (EntreMed) for the generous support with rhEndostatin, and Dr. Martin Middke for computer support.

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