

# Continuous Administration of Endostatin by Intraperitoneally Implanted Osmotic Pump Improves the Efficacy and Potency of Therapy in a Mouse Xenograft Tumor Model<sup>1</sup>

Oliver Kisker, Christian M. Becker, Daniela Prox, Michael Fannon, Robert D'Amato, Evelyn Flynn, William E. Fogler, B. Kim Lee Sim, Elizabeth N. Allred, Steven R. Pirie-Shepherd,<sup>2</sup> and Judah Folkman<sup>3</sup>

Division of Surgical Research [O. K., C. M. B., D. P., M. F., R. D., E. F., S. R. P.-S., J. F.] and Department of Neurology [E. N. A.], Children's Hospital, Boston, Massachusetts 02115; University Hospital Marburg, Department of General Surgery, Philipps University, Marburg, Germany [O. K.]; University Hospital Benjamin Franklin, Department of Gynecology and Obstetrics, Free University Berlin, Berlin, Germany [C. M. B.]; Departments of Ophthalmology [R. D.] and Surgery and Cell Biology [J. F.], Harvard Medical School Boston, Massachusetts 02115; and EntreMed, Inc., Rockville, Maryland 20850 [W. E. F., B. K. L. S.]

## ABSTRACT

In the first Phase I clinical trials of endostatin as an antiangiogenic therapy for cancer, the protein was administered as an i.v. bolus for ~20–30 min each day. This protocol was based on experimental studies in which animals were treated by s.c. bolus once a day. However, it was not clear in the previous studies whether this schedule could be maximized further. Therefore, we developed experimental models involving continuous administration of endostatin to determine the potency and efficacy of this approach. Endostatin was administered to tumor-bearing mice either s.c. or i.p. in single bolus doses. The efficacy of these regimens was compared with endostatin administered continuously via an i.p. implanted mini-osmotic pump. Our results show that endostatin remains stable and active in mini-osmotic pumps for at least 7 days. We show that endostatin injected i.p. is rapidly cleared within 2 h, whereas endostatin administered continuously via mini-osmotic pump maintains systemic concentrations of 200–300 ng/ml for the duration of administration. Furthermore, continuous i.p. administration of endostatin results in more effective tumor suppression at significantly reduced doses (5-fold), compared with bolus administration. Additional experiments using a human pancreatic cancer model in severe combined immunodeficient mice showed that there was a significant decrease in the microvessel density between the treatment groups and the control group. These data show that continuous administration of human endostatin results in sustained systemic concentrations of the protein leading to: (a) increased efficacy manifested as increased tumor regression; and (b) an 8–10-fold decrease in the dose required to achieve the same antitumor effect as the single daily bolus administration of endostatin. On the basis of this approach, an additional clinical trial has been designed and initiated and is under way in two countries.

## INTRODUCTION

Angiogenesis is essential for the growth and persistence of solid tumors and their metastases. To stimulate angiogenesis, tumors up-regulate the production of a variety of angiogenic factors such as acidic and basic FGFs,<sup>4</sup> and VEGF/vascular permeability factor. However, some tumors are also involved in the generation of angiogenic inhibitors. It is becoming more apparent that the angiogenic phenotype is the result of a net balance between positive and negative regulators of angiogenesis (1, 2).

Compelling evidence that tumor growth is angiogenesis-dependent

Received 3/9/01; accepted 8/27/01.

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<sup>1</sup> This study was supported by NIH Grant P01 CA45548 (to J.F.), by a grant to Children's Hospital from EntreMed, and by a grant from the Deutsche Krebshilfe, Mildred Scheel Stiftung D/97/05974 (to O.K.) and Deutsche Forschungsgemeinschaft Grant Be2199/1-1 (to C.M.B.).

<sup>2</sup> Present address: Attenuon, LLC, 10130, Sorrento Valley Road, San Diego, CA 92121.

<sup>3</sup> To whom requests for reprints should be addressed, at Hunnewell Building (Room 103), Children's Hospital, 300 Longwood Avenue, Boston, MA 02115. Phone: (617) 355-7661; Fax: (617) 355-7662.

<sup>4</sup> The abbreviations used are: FGF, fibroblast growth factor; VEGF, vascular endothelial cell growth factor; SCID, severe combined immunodeficient; hpf, high-power field.

is supported by the discovery that endogenous antiangiogenic factors such as angiostatin (3), endostatin (4), or antiangiogenic antithrombin III (5) can potentially inhibit tumor growth in mice.

Endostatin, a  $M_r$  20,000 COOH-terminal fragment of collagen XVIII was initially isolated from the conditioned medium of heman-gioendothelioma cells (4). Recombinant murine and human endostatin, obtained from *Escherichia coli* and *Pichia pastoris* expression systems, inhibit the proliferation (4) and migration (6–9) of endothelial cells specifically. Both soluble and insoluble recombinant murine endostatin suppress the growth of primary human and murine tumors and metastases in different xenograft mouse tumor models (4, 6, 7, 10, 11). In addition, endostatin has demonstrated antitumor activity against carcinogen-induced primary breast carcinomas in a rat tumor model (12). Additionally, s.c. administration of endostatin in mice and rat tumor models produces tumor regression (11, 12). Thus far, no side effects have been described. Furthermore, antiangiogenic therapy with endostatin did not induce acquired drug resistance (11). Finally, soluble human recombinant endostatin also suppresses tumor growth and metastases in a mouse model (13). This soluble recombinant human endostatin has been in clinical Phase I since October 1999.

However, because endostatin for human use is produced in a large-scale yeast cell culture, any method of administration that reduces the amount of protein per dose would be useful. Thus far, the treatment with murine endostatin requires a s.c. bolus injection of 20 mg/kg applied daily or a single daily i.p. bolus injection of 10 mg/kg (6–12). Using recombinant human endostatin, the dosage had to be increased to 50 mg/kg s.c. twice a day (13), although no attempt was made at titration. We asked if altering the administration route of endostatin may also improve the efficacy of therapy. The reported half-life of human recombinant endostatin in mice after s.c. injection of 50 mg/kg/day is ~10 h (13). This would provide a peak plasma level, but not a continuous level of endostatin. It has been shown that continuous administration of TNP-470, an angiogenic inhibitor of fungal origin (14), decreases the required dose by up to 6-fold and improves the efficiency of therapy in comparison with intermittent bolus s.c. injections (15).

The aim of this present study was to show that the continuous administration of endostatin not only decreases the required dosage, but also improves the efficiency and effectiveness of therapy in various murine and human tumor models. Our results show that endostatin remains stable and active in mini-osmotic pumps for at least 7 days. We show that endostatin injected i.p. is rapidly cleared within 2 h, whereas endostatin administered continuously via mini-osmotic pump maintains systemic concentrations of 200–300 ng/ml for the duration of administration. Furthermore, continuous administration of endostatin results in more effective tumor suppression at significantly reduced doses (5-fold) compared with bolus administration.

These data suggest that continuous administration of endostatin therapy results in sustained systemic concentrations of the protein.

Such sustained systemic concentrations may more effectively inhibit angiogenesis within the vascular bed of a tumor.

## MATERIALS AND METHODS

**Cell Culture.** Human pancreatic cancers cells, BxPC-3, and human fibrosarcoma cancer cells HT1080 (American Type Culture Collection, Rockville, MD) were grown in RPMI 1640 (Life Technologies, Inc., Grand Island, NY) supplemented with 10% FCS (Life Technologies, Inc.) and 1% glutamine-penicillin-streptomycin (GPS; Pharmacia, Sweden). Cells were maintained in T-75 tissue culture flasks (Falcon) and grown in 5% CO<sub>2</sub>/95% air at 37°C in a humidified incubator. For tumor cell injection, the cells were grown in 900-cm<sup>2</sup> roller bottles

**Generation of Recombinant Human Endostatin.** Recombinant human endostatin (currently under evaluation in Phase I clinical trials) was expressed as a soluble protein in *P. pastoris*, in its native form without the addition of extraneous sequences (13). This protein is stable under controlled storage conditions and has been found to retain functional activity under such conditions for >1 year (13).

**Animal Studies.** All animal work was performed in the animal facility at Children's Hospital, Boston, MA, in accordance with federal, local, and institutional guidelines. Male (24–27 g) immunocompromised (SCID) mice (Mass General Hospital, Boston, MA) or C57Bl6/J mice (Jackson Labs, Bar Harbor, ME) 7–9 weeks of age were used. They were acclimated, caged in groups of five in a barrier care facility, and fed with 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 killed by a lethal dose of methoxyflurane (Pittman-Moore, Inc., Mundelein, IL).

**Tumor Models.** Pancreatic cancers cells (BxPC-3) and fibrosarcoma cells (HT 1080) were grown in cell culture as described above. The cell concentration was adjusted to 12.5 × 10<sup>6</sup> cells/ml. Mice were shaved and the dorsal skin was cleaned with ethanol before tumor cell injection. A suspension of 2.5 × 10<sup>6</sup> tumor cells (BxPC-3 or HT1080) in 0.2 ml RPMI 1640 was injected into the s.c. dorsa of mice at the proximal midline.

Animals with Lewis Lung carcinoma (600–800 mm<sup>3</sup> tumors) were killed, and the skin overlying the tumor was cleaned with Betadine and ethanol. In a laminar flow hood, tumor tissue was excised under aseptic conditions. A suspension of tumor cells in 0.9% normal saline was made by passage of viable tumor tissue through a sieve and a series of sequentially smaller hypodermic needles of diameter 22- to 30-gauge. The final concentration was adjusted to 1 × 10<sup>7</sup> cells/ml, and the suspension was placed on ice. The injection of tumor cells (2 × 10<sup>6</sup> cells in 0.2 ml of saline) was performed as described above.

The mice were weighed and tumors were measured by an investigator who did not know the treatment schedule every 3–5 days in two diameters with a dial-caliper. Volumes were determined using the formula  $a^2 \times b \times 0.52$  (where *a* is the shortest and *b* is the longest diameter). At the end of each experiment, the mice were sacrificed with methoxyflurane (Pittman-Moore Inc.) and the tumors weighed and fixed in buffered Formaldehyde-Fresh (Fisher Scientific, Fair Lawn, NJ)

**Treatment of Tumor-bearing Mice.** When the tumor volume was 90–110 mm<sup>3</sup>, mice were randomized. Endostatin treatment with soluble recombinant human endostatin (from *P. pastoris*; a gift from EntreMed, Inc., Rockville, MD) was performed either by single bolus s.c. injections [100 mg/kg/day in 100 mM sodium citrate and 200 mM sodium phosphate (pH 6.2)], single bolus i.p. injections [2, 6, or 20 mg/kg/day in 0.2 ml of 100 mM sodium citrate and 200 mM sodium phosphate (pH 6.2)], or continuous administration of endostatin [2, 6, 12, and 20 mg/kg/day i.p. via mini-osmotic pump (ALZA, Palo Alto, CA)]. These pumps (internal volume, 200 μl) continuously deliver test agents at a rate of 1 μl/h for 200 h (8.3 days). Pumps were replaced every 7 days, thus ensuring continuous delivery of test agents over the course of the experiment. The control group received comparable bolus injections of vehicle (s.c. or i.p.) or i.p. implanted, vehicle-loaded pumps. The s.c. injections were administered at a site distant from the tumor. Continuous administration was performed by loading a mini-osmotic pump with 200 μl of endostatin at different concentrations (2.1, 6.3, 12.5, or 21 mg/ml). The pump was implanted i.p. under sterile conditions after a small midline incision. The incision was closed in two layers with 4.0 Polydioxanone sutures. For each experiment, *n* = 5. Each experiment was repeated at least twice.

**Biological Activity of Endostatin in an *in Vitro* Assay.** To determine the stability of endostatin activity in mini-osmotic pumps, we implanted pumps loaded with endostatin i.p. into SCID mice. Pumps were implanted at days 1, 2, 3, 4, 5, 6, and 7. At day 8 all mice were killed, the pumps were removed, and the remaining endostatin solution was aspirated under sterile conditions. The ability of endostatin to inhibit the migration of endothelial cells was assayed as described below. Human umbilical vein endothelial cells, passage 4, were maintained in Medium 199 (Life Technologies, Inc.), 20% fetal bovine serum, 1% GPS, 10 ng/ml basic FGF, and 100 ng/ml heparin. Cells were trypsinized, centrifuged, and diluted in Medium 199 with 0.05% gelatin. Cells were preincubated for 30 min with or without recombinant human endostatin (EntreMed, Rockville MD) at a concentration of 200 ng/ml at 37°C. Cells (1.5 × 10<sup>5</sup>) in 300 μl were added per well to 10-mm tissue culture inserts (Nunc; 8 μm pore) that had been treated with 10 μg/ml of fibronectin. Medium (300 μl) was added to the bottom wells with or without 10 ng/ml of VEGF (R&D Systems, Minneapolis, MN), and cells were incubated for 9 h 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 quantitated using a colorimetric assay as follows. Cells bound to the bottom of the tissue culture inserts were incubated for 2 h in 400 μl of acid phosphatase substrate [10 mM *p*-nitrophenyl phosphate, 10 mM sodium acetate, 0.1% Triton X-100 (pH 5.8)] at 37°C. Reaction was then quenched with 100 μl of 1 N NaOH, and absorbance of the solution was read at 410 nm. Data (*n* = 3) were compared with VEGF controls using *t* test analysis.

**Determination of the Pharmacokinetics of Recombinant Human Endostatin.** To determine pharmacokinetics, SCID male mice (*n* = 3/treatment group) were dosed either via the i.p. bolus or i.p. continuous route with 20 mg/kg/day of endostatin. Blood samples (*n* = 3) were collected by heart puncture at various intervals. For the pharmacokinetics of single bolus injection, samples were collected at –2, 5, 10, 20, 30, and 60 min, and then at 2, 4, 6, 8, 12, 16, 20, 24, 32, and 36 h post-daily repeated dosing for 7 days. Samples from animals receiving continuous administration via a mini-osmotic pump were collected at –2, 5, 10, 20, 30, and 60 min, and then at 2, 4, 6, 8, 12, 16, 20, 24, 32, and 36 h, and 2, 3, 4, 5, 6, and 7 days after pump implantation. Serum endostatin levels were determined by ELISA (CytImmune Sciences, College Park, MD) according to the manufacturer's instructions. Pharmacokinetic parameters were determined by standard noncompartmental methods using the nonlinear regression program WinNonlin (Scientific Consulting, Inc., Apex, NC). Area under the curve (AUC) and area under the moment curve were calculated by linear trapezoidal method from 0 to 168 h.

**Immunohistochemistry.** Mice were killed at the end of the experiments. Representative tumor tissues (3 from each group) were harvested and fixed in 10% neutral buffered formalin at 4°C for 12 h. All tissues were paraffin-embedded. Sections (5 μm) were first stained with H&E to evaluate tissue viability and quality. The microvessel density was determined by immunocytochemical staining using a Vectastain avidin-biotin detection system (Vector Labs, Burlingame, CA) with anti-CD 31 antibody (monoclonal, dilution: 1:250; PharMingen, San Diego, CA) according to the manufacturer's instructions. Following the method of Weidner *et al.* (16) at low magnification (×40–100) regions of highest vessel density ("hot spot" regions) were scanned and counted at ×200 (0.738-mm<sup>2</sup> field) (2) by an observer who did not know the treatment schedule. At least five fields were counted in a representative tumor section, and the highest count was taken.

**Statistics.** The rate of tumor growth, expressed as the percentage of change from tumor volume at treatment day 0, was plotted against treatment day. Analysis of covariance on the ranks of the percentage of change in volume was used to compare the slopes for the treated and control groups. The Kruskal-Wallis test was used to compare the median microvessel density in treated and control groups. All statistical procedures controlled for the multiple measures from each tumor.

## RESULTS

**Treatment of Tumors with a Single s.c. Bolus Dose of Endostatin.** Human pancreatic cancer cells (BxPC-3) or Lewis Lung carcinoma cells were implanted in mice as described. When the tumors had attained a volume of ~100 mm<sup>3</sup>, therapy was started. Animals received 100 mg/kg of recombinant human endostatin via a s.c. injection.

tion once daily. Control animals received vehicle alone. Therapy was continued for 14 days (Lewis Lung carcinoma), or 23 days (BxPC-3). Tumors were measured every 3–5 days. Endostatin administered by this route and at this dose suppressed the growth of BxPC-3 significantly ( $P \leq 0.001$ ; Fig. 1a) and slowed the growth of Lewis Lung carcinoma significantly ( $P \leq 0.001$ ; Fig. 1b).

**Treatment of Tumors with a Continuous i.p. Dose of Endostatin Administered via Osmotic Pump.** Human pancreatic cancer cells (BxPC-3), fibrosarcoma cells (HT1080) or Lewis Lung carcinoma cells were implanted into mice as described. When the tumors had attained a volume of  $\sim 100 \text{ mm}^3$ , therapy was started. A mini-osmotic pump with an internal volume of  $200 \mu\text{l}$  was filled with a solution of endostatin (12.5 mg/ml). The pump was implanted i.p., resulting in the controlled release of endostatin at a rate of  $1 \mu\text{l/h}$  into the peritoneal cavity. The outlet of the pump faced the animals' diaphragm. The animals received 12–13 mg/kg/day endostatin by this method. Tumor volume was measured every 3–5 days. Endostatin administered by this route and at this dose suppressed the growth of human pancreatic cancer BxPC-3 by 91% (Fig. 2a;  $P \leq 0.001$ ) and human fibrosarcoma HT1080 by 81% (Fig. 2b;  $P \leq 0.001$ ) for 23 days. Lewis lung carcinoma growth was significantly ( $P \leq 0.001$ ) inhibited for 14 days

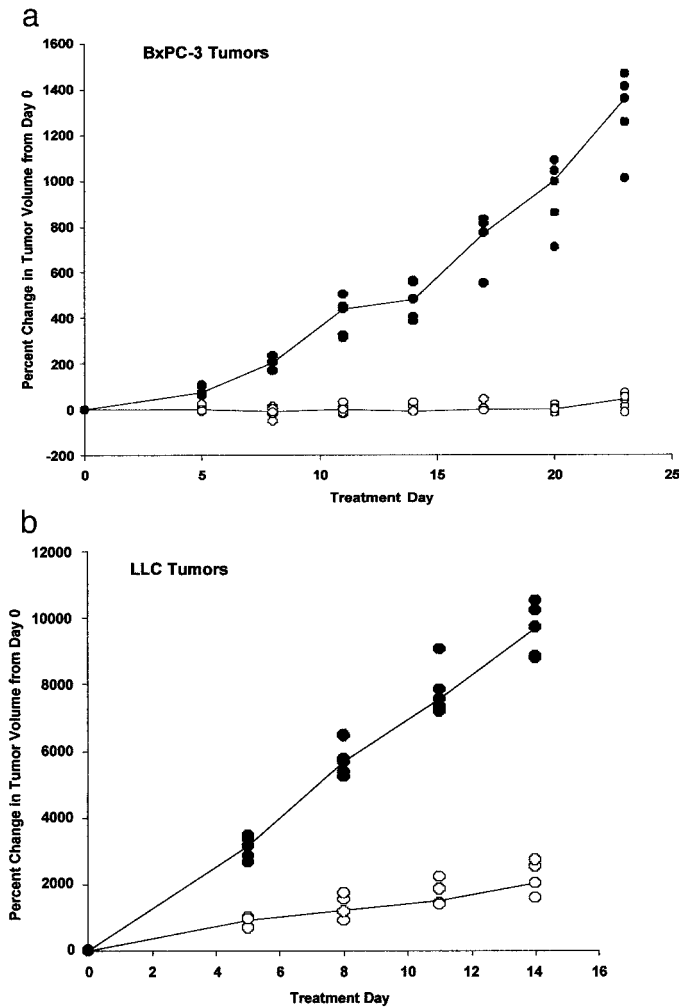


Fig. 1. s.c. administration of human recombinant soluble endostatin. Human pancreatic cancer cells (BxPC-3 or Lewis Lung carcinoma cells) were implanted in the s.c. dorsa of mice, and systemic therapy with human recombinant soluble endostatin (100 mg/kg/day s.c.) was begun when tumors were  $\sim 100 \text{ mm}^3$ . Therapy was continued for 14 days (Lewis Lung carcinoma) or 23 days (BxPC-3). The growth of BxPC-3 was inhibited by 90% (a) and Lewis Lung carcinoma by 76% (b). The slower-growing tumor (BxPc3) had a better response to endostatin treatment than the faster-growing tumor (Lewis Lung). All data are plotted; the curve follows the median. ●, control; ○, 100 mg/kg/day endostatin s.c.

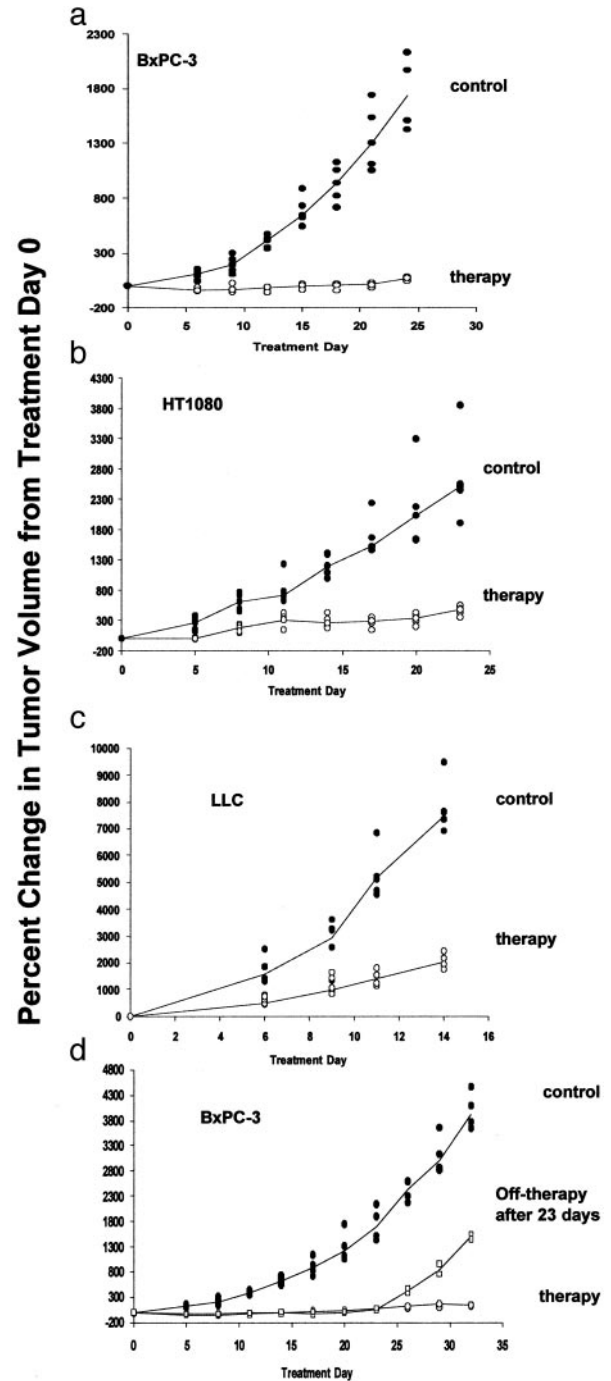


Fig. 2. Continuous administration of endostatin via an i.p.-implanted osmotic pump. Human pancreatic cancer cells (BxPC-3), human fibrosarcoma cells (HT1080), or Lewis Lung carcinoma cells were implanted in the s.c. dorsa of mice. Once the tumors had attained a volume of  $100 \text{ mm}^3$ , osmotic pumps filled with endostatin were implanted in the mice. The pumps release  $24 \mu\text{l/day}$  for 7 days. The animals received 12mg/kg/day. BxPC-3 was inhibited by 91% (a) and HT1080 was inhibited by 81% (b) after 23 days. Lewis Lung carcinoma was inhibited by 72% (c) after 14 days of treatment. In one experiment (d), BxPC-3 cancer cells were implanted in four mice, and the mice were given continuous endostatin therapy via an i.p. implanted osmotic pump. After 23 days, two of the mice were taken off therapy and two mice were maintained on therapy. Tumor volume in the animals taken off therapy began to increase from  $150 \text{ mm}^3$  to  $1500 \text{ mm}^3$  over a period of 10 days. The animals maintained on therapy continued to demonstrate tumor suppression.

by 72% (Fig. 2c). Four animals received continuous administration of endostatin with observable suppression of tumor growth. After 23 days, two of the mice were taken off therapy, and two mice were maintained on therapy. Tumor volume in the animals taken off ther-



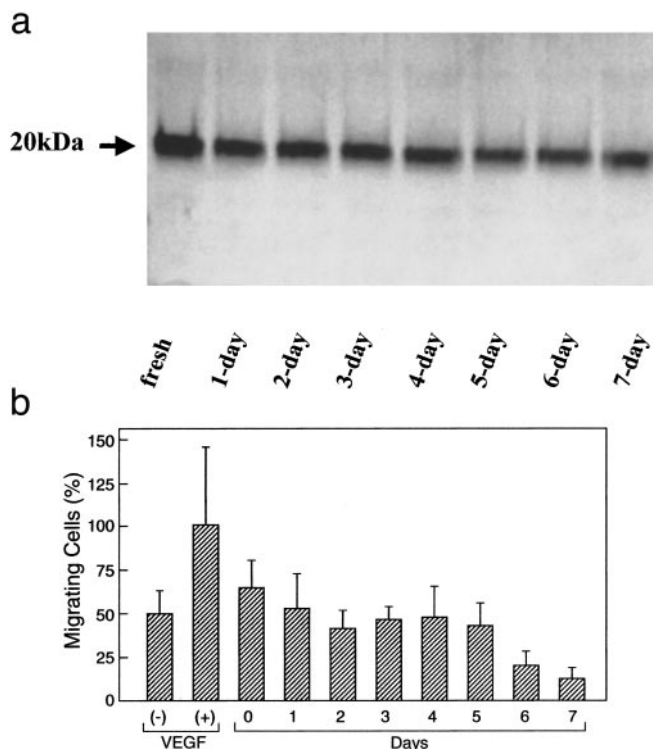


Fig. 3. Stability and biological activity of endostatin incubated in mice for 1–7 days. Pumps were filled with endostatin (20 mg/kg/day), and the pumps were implanted into the i.p. cavity of mice. Pumps were implanted at daily intervals. All pumps were removed after 7 days, resulting in a series of pumps that had been in mice for 1–7 days. The endostatin solution was removed under sterile conditions and stored at 4°C. Endostatin samples were analyzed for proteolytic degradation by SDS-PAGE under reducing conditions. Wells were loaded with equal concentrations of endostatin sample. Notice that no degradation is apparent (a). Analysis of the same samples by nonreducing SDS-PAGE showed no evidence of dimerization via disulfide bridges (data not shown). These samples were also assayed for their ability to inhibit the VEGF-stimulated migration of human umbilical vascular endothelial cells (b). The full biological activity of these samples was still present at day 7, and there appeared to be statistically significant increased activity by days 6 and 7 (compared with VEGF control by *t* test analysis;  $P = 0.058$ ).

apy began to increase from 150 mm<sup>3</sup> to 1500 mm<sup>3</sup> over a period of 10 days (Fig. 2d). This rate of growth was not significantly different from the growth rate of untreated tumors ( $P = 0.62$ ). The animals maintained on therapy continued to demonstrate significant ( $P \leq 0.001$ ) tumor suppression compared with the group taken off therapy. The growth rate of BxPC-3 is slower in comparison to HT1080. Lewis Lung carcinoma was the fastest-growing of the tumors. The slower-growing tumors had a better response to antiangiogenic therapy than the faster-growing tumors. This phenomenon was evident in both treatment regimes (Fig. 1 and 2).

**Determination of Endostatin Stability and Biological Activity in i.p.-implanted Osmotic Pumps.** Mini-osmotic pumps with an internal volume of 200  $\mu$ l were filled with a solution of endostatin (20 mg/ml) and implanted i.p. into mice. Pumps were implanted at 24 h intervals over 7 days. On day 8, all pumps were removed, and the endostatin remaining in the pump was tested for proteolytic degradation and biological activity using an endothelial cell migration assay. No degradation was apparent under reducing SDS-PAGE criteria (Fig. 3a) on day 7. There was also no evidence for dimerization by disulfide bridges as determined by nonreducing PAGE (data not shown). Freshly prepared endostatin will inhibit the migration of endothelial cells by 80–90% at a dose of 200 ng/ml (Fig. 3b). Endostatin removed from mini-osmotic pumps which were implanted i.p. also inhibited the migration of endothelial cells at a dose of 200 ng/ml. Interestingly, endostatin, in a pump implanted in an animal for 7 days, inhibited the migration of endothelial cells more effectively (150–160%) than

endostatin that was in an animal for 1 day (Fig. 3b). Analysis of these data ( $n = 3$ ) by *t* test showed a statistically significant difference compared with VEGF controls ( $P = 0.058$ ). The remaining endostatin was not toxic to endothelial cells *in vitro* as determined by: (a) healthy-looking endothelial cells in the upper well of the tissue culture inserts; (b) no floating endothelial cells in the tissue culture inserts; and (c) no bacterial growth on blood agar plates.

**Pharmacokinetics of Endostatin.** Endostatin was administered as a single bolus injection s.c. or administered via an i.p. implanted mini-osmotic pump. Blood was collected from animals by heart puncture, and the levels of systemic endostatin were measured by ELISA. Pharmacokinetic parameters are tabulated (Table 1).

**Comparison of the Antitumor Effect of Continuously Administered Endostatin versus Single Bolus Injection.** Of the three tumors used in the first part of this study, we noted that the human pancreatic cancer, BxPC-3, maintained the most reproducible rate of tumor growth; therefore, we chose this tumor model for additional studies. The BxPC-3 cells, when implanted on the back of a mouse, will attain a volume of  $\sim 100$  mm<sup>3</sup> after 21 days (Fig. 4a). We have shown (above) that if endostatin is given as a s.c. bolus single injection, a dose of 100 mg/kg/day is required to suppress tumor growth (Fig. 1a). A dose of 20 mg/kg/day, administered as a single s.c. bolus, although not sufficient to stabilize tumor growth, is sufficient to significantly ( $P = 0.007$ ) slow down the rate of tumor growth (Fig. 4a). If the same dose is administered as a single i.p. bolus injection, then the rate of growth is significantly decreased when compared with the growth rate of control tumors (Fig. 4a;  $P \leq 0.001$ ). We have also shown that if the endostatin is administered via mini-osmotic pump, then a reduced dose of 12 mg/kg/day is sufficient to suppress tumor growth (Fig. 2a). To determine whether the increased effectiveness of therapy was attributable to continuous administration or to i.p. administration, we treated animals with increasing doses of endostatin, administered either i.p. as a bolus once daily, or i.p. continuously via a mini-osmotic pump. Animals received equivalent doses of 2, 6, or 20 mg/kg/day.

In all cases, tumor growth was suppressed by either continuous or bolus administration of endostatin (Fig. 4b). The suppression was enhanced with increased dosage by both routes (Fig. 4b). A single i.p. bolus dose administration of endostatin at 2 mg/kg/day resulted in significant inhibition of tumor growth ( $P = 0.001$ ), whereas increasing the dose to 6 or 20 mg/kg/day increased the inhibition in growth rate significantly ( $P \leq 0.001$ ). Continuous administration of endostatin by mini-osmotic pump (Fig. 4b) resulted in a highly significant reduction in tumor growth rate at 2 mg/kg/day. Increasing the dose to 6 mg/kg/day further reduced the growth rate. The lowest growth rate was seen at 20 mg/kg/day endostatin administered via mini-osmotic pump. These data are significantly different ( $P \leq 0.001$ ) from the control tumors. Interestingly, a dose of 2 mg/kg/day continuously was just as effective in suppressing tumor growth as a dose of 20 mg/kg/day i.p. single bolus injection. This equivalence between the two schedules was found to be statistically valid. At the highest dose of endostatin delivered by pump, tumor regression was seen (Fig. 4b).

Table 1 Noncompartmental pharmacokinetic parameters of endostatin  
Parameters were calculated as described in "Materials and Methods."

	$C_{ss}^a$ (ng/ml)	$V_{d_{ss}}$ (ml/kg)	$t_{1/2}$ (h)	Cl (ml/h/kg)	MRT (h)	$AUC_{0-168}/D$ (ng*hr/ml)/(mg/kg)
i.p. bolus	990.44	74964.9	3.33	18601.7	4.03	50.08
i.p. continuous administration	230.23	285674.2	54.52	3631.1	78.67	275.4

<sup>a</sup>  $C_{ss}$ , concentration of drug at steady state;  $V_{d_{ss}}$ , volume of distribution at steady state; Cl, total clearance of drug; MRT, mean residence time;  $AUC_{0-168}/D$ , area under the concentration time curve.

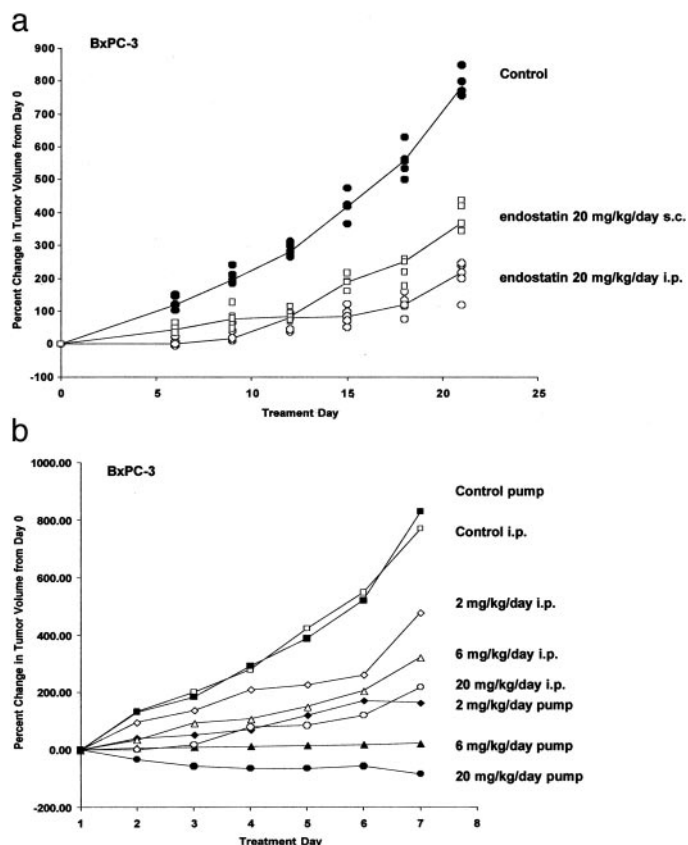


Fig. 4. Treatment of human pancreatic cancer (BxPC-3) with endostatin. Human pancreatic cancer cells (BxPC-3) were implanted in mice as described. Animals received a daily s.c. or i.p. bolus injections of endostatin (20 mg/kg/day). Control animals received vehicle alone. Animals receiving the drug by i.p. administration showed tumor suppression of 66%, whereas animals that received the drug by s.c. administration showed tumor suppression of only 48% (a). In a separate experiment, animals were implanted with BxPC-3 cancer cells as described. One group was treated with a range of endostatin doses given as a bolus i.p. injection, the other group received the same doses via continuous i.p. administration (b).

**Microvessel Density Counts in Tumors Treated with Continuous or Bolus Administration of Endostatin.** Histological sections of s.c. tumors were analyzed for microvessel density (16) using an antibody directed against the endothelial cell marker CD31. There was a significant difference ( $P \leq 0.001$ ) in the microvessel density between the treatment groups and the control group (Fig. 5). The microvessel density per hpf in the control group was  $124.3 \pm 20.3$ . Animals treated with a single bolus injection of 20 mg/kg/day endostatin i.p. had a tumor microvessel density of  $62.1 \pm 10.5$  per hpf ( $P \leq 0.001$ ). Animals treated continuously with human endostatin (20 mg/kg/day) via mini-osmotic pump had a tumor microvessel density of  $20.7 \pm 5.4$  per hpf ( $P \leq 0.001$ ). The difference between the microvessel density in bolus treated and i.p. treated tumors was also significant ( $P \leq 0.001$ ).

## DISCUSSION

We show here that soluble endostatin administered continuously into the peritoneal cavity by a mini-osmotic pump is 50% more effective in tumor suppression than the same dose administered once a day i.p. Furthermore, we show that a 10-fold lower dose, when given continuously, will accomplish the same tumor suppression as a single dose given i.p. daily. Finally, we show that only continuous dosing can achieve tumor regression with human soluble endostatin.

The maintenance of a sustained circulating level of endostatin leads

to an augmentation in both its antitumor and antiangiogenic activity. This conclusion is based on the demonstration that, in comparison to bolus injection, the mean residence time of continuous infusion endostatin is increased from ~4 to 78 h, and dose-dependent steady state levels are achieved.

Our data indicates that endostatin retains biological activity in the osmotic pump for at least 7 days. It is unclear why the biological activity of endostatin is enhanced by incubation at 37°C while it resides in the pump in the mouse. One possibility is that endostatin could undergo some form of aggregation. Importantly, the endostatin does not undergo any obvious proteolytic degradation within an i.p. implanted pump.

Our data also shows that suppression of the angiogenic activity of the tumor, as quantified by microvessel density, correlates with route of administration. A reduction in microvessel density during antiangiogenic therapy has been reported by others, although their protocols did not change the route of administration (3, 4, 5, 10, 17). We emphasize that although intratumoral microvessel density is a good prognostic indicator of risk of future metastasis and mortality, it is less useful to indicate efficacy of antiangiogenic therapy. In a tumor inhibited or regressing under effective antiangiogenic therapy, intratumoral microvessel density can: (a) decrease, if the original tumor was overvascularized; (b) remain unchanged, if capillary drop-out balances tumor cell drop-out; or (c) increase, if the rate of tumor cell death (and autolysis) exceeds the rate of endothelial cell apoptosis. Therefore, a decrease in microvessel density during treatment with an angiogenesis inhibitor suggests that the agent is effective. But the absence of a decrease in microvessel density does not indicate that the agent is ineffective (21).

Previously we have reported that a single s.c. daily dose of murine recombinant endostatin from *E. coli*, caused tumor regression (4). That tumor regression was not achieved in the present study of endostatin at 20 mg/kg/day i.p. was most likely attributable to the differences in the human and murine recombinant proteins. The human protein produced from *P. pastoris* is soluble in contrast with the murine endostatin from *E. coli*, which is insoluble. Additionally, mouse endothelium may be more responsive to murine endostatin than it would be to human endostatin.

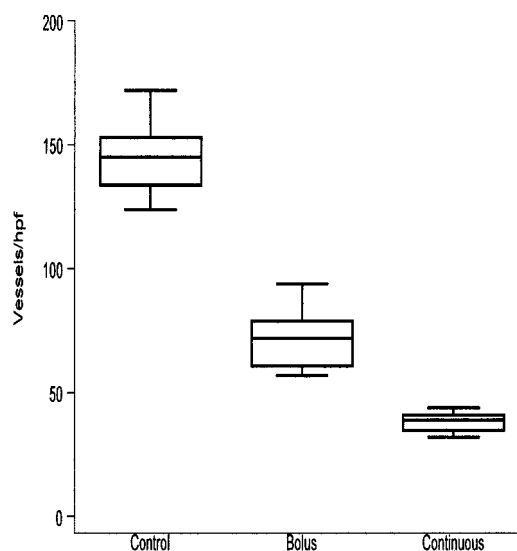


Fig. 5. Histological analysis of excised tumors. Excised tumors were fixed and stained for the presence of the endothelial cell marker CD31. Sections were then quantified for microvessel density. Data were plotted in the box/whisker format. There was a significant difference in the microvessel density/hpf between control groups and groups of mice treated with either bolus injection or continuous administration of endostatin.

Soluble recombinant mouse endostatin (derived from *P. pastoris*) has shown efficacy against a human renal carcinoma in mice when given i.p. as a single bolus dose of 10–20 mg/kg/day. However, only 30% of animals in the treated group demonstrated tumor regression. In the other animals, tumor growth was inhibited but not fully suppressed (6).

Reports of two other angiogenesis inhibitors (15, 18) support our conclusions. Continuous administration of TNP-470 via s.c. implanted osmotic pump was more efficient than single-dose administration in the treatment of primary osteosarcoma and metastatic lung disease in a rat model (15). Furthermore, continuous administration of proteolytically derived human angiostatin by a s.c. implanted osmotic pump was much more effective against colorectal carcinoma in a xenograft model than twice-daily s.c. bolus injection (18). Continuous administration of angiostatin was also effective in inhibiting the outgrowth of liver metastases (18). However, there is one difference between our methodology and the angiostatin study (18). Angiostatin therapy was initiated at the time of implantation of tumors. In contrast, in our study, endostatin therapy was initiated only after tumors had reached 100 mm<sup>3</sup>. The possibility exists that the dose of angiostatin could have been reduced significantly if the pumps had been implanted i.p.

It is of interest that in our study different tumors responded differently to the same dose of endostatin. In general, more-slowly growing tumors are more effectively suppressed by the same dose of endostatin than fast-growing tumors. For example, a continuous administration of 12 mg/kg/day of endostatin inhibited the growth of a slow-growing tumor (BxPC-3) by 90%, whereas the same dose schedule inhibited a rapidly growing Lewis Lung carcinoma by 72%. This phenomenon has also been noted with antiangiogenic treatment of different tumors (19, 20). Therefore, we speculate that it may be possible to measure the angiogenic phenotype of a tumor in “endostatin units” or, indeed, in units of any antiangiogenic drug.

In conclusion, recombinant soluble human endostatin inhibits the growth of tumors in a mouse model. The effective dose can be significantly reduced by altering the route and method of administration. Because the effective dose can be reduced, the tumor suppression can be increased. Thus, it may be more efficacious to treat patients via continuous administration of endostatin therapy. On the basis of our findings, two clinical trials are under way (Boston and Amsterdam) to determine the efficacy of continuous i.v. administration of endostatin by a wearable pump.

## ACKNOWLEDGMENTS

We thank Kristin Gullage for photography. We thank Amy Birsner and Stacy Plum for expert assistance with the animal work. We also thank Jenny Jia Yuan for technical assistance.

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