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Endostatin: An Endogenous Inhibitor of Angiogenesis and Tumor Growth

Michael S. O'Reilly,* Thomas Boehm,* Yuen Shing,* Naomi Fukai,† George Vasios,§ William S. Lane,‡ Evelyn Flynn,* James R. Birkhead,§ Bjorn R. Olsen,† and Judah Folkman*
*Department of Surgery
Children's Hospital, Boston
Departments of Surgery and Cellular Biology
Harvard Medical School
300 Longwood Avenue
Boston, Massachusetts 02115
†Department of Cell Biology
Harvard Medical School
220 Longwood Avenue
Boston, Massachusetts 02115
‡Harvard Microchemistry Facility
16 Divinity Avenue
Harvard University
Cambridge, Massachusetts 02138
§Osteoarthritis Sciences, Inc.
Cambridge, Massachusetts 02139

Summary

We previously identified the angiogenesis inhibitor angiostatin. Using a similar strategy, we have identified endostatin, an angiogenesis inhibitor produced by hemangioendothelioma. Endostatin is a 20 kDa C-terminal fragment of collagen XVIII. Endostatin specifically inhibits endothelial proliferation and potently inhibits angiogenesis and tumor growth. By a novel method of sustained release, E. coli–derived endostatin was administered as a nonrefolded suspension. Primary tumors were regressed to dormant microscopic lesions. Immunohistochemistry revealed blocked angiogenesis accompanied by high proliferation balanced by apoptosis in tumor cells. There was no toxicity. Together with angiostatin data, these findings validate a strategy for identifying endogenous angiogenesis inhibitors, suggest a theme of fragments of proteins as angiogenesis inhibitors, and demonstrate dormancy therapy.

Introduction

Several lines of direct evidence show that angiogenesis is essential for the growth and persistence of solid tumors and their metastases (Folkman, 1989; Hori et al., 1991; Kim et al., 1993; Millauer et al., 1994). To stimulate angiogenesis, tumors upregulate the production of a variety of angiogenic factors, including fibroblast growth factors (aFGF and bFGF) (Kandel et al., 1991) and vascular endothelial cell growth factor/vascular permeability factor (VEGF/VPF). Many malignant tumors, however, also generate inhibitors of angiogenesis (Chen et al., 1995), including angiostatin (O'Reilly et al., 1994; Gately et al., 1996) and thrombospondin (Good et al., 1990). It is becoming clear that the angiogenic phenotype is the result of a net balance between these positive and negative regulators of neovascularization (Rastinejad et al., 1989; Good et al., 1990; O'Reilly et al., 1994; Parangi et al., 1996). Several other endogenous inhibitors of angiogenesis have been identified, although not all are associated with the presence of a tumor. These include platelet factor 4 (Maione et al., 1990; Gupta et al., 1995), interferon-alpha, interferon-inducible protein 10 (Angiolillo et al., 1995; Strieter et al., 1995), which is induced by interleukin-12 and/or interferon-gamma (Voest et al., 1995), gro-beta (Cao et al., 1995), and the 16 kDa N-terminal fragment of prolactin (Clapp et al., 1993). Prior to this report, however, the only known angiogenesis inhibitor that specifically inhibited endothelial cell proliferation was angiostatin (O'Reilly et al., 1994).

We previously reported that angiostatin, a 38 kDa specific inhibitor of endothelial cell proliferation, is an internal fragment of plasminogen containing at least three of the kringles of plasminogen (O'Reilly et al., 1994). In that report, angiostatin was isolated from a subclone of Lewis lung carcinoma in which the primary tumor inhibited the growth of its metastases. Angiostatin, generated by the primary tumor, was shown to potently inhibit angiogenesis. In fact, systemic therapy with angiostatin led to the maintenance of metastases in a microscopic dormant state defined by a balance of apoptosis and proliferation of the tumor cells (O'Reilly et al., 1994; Holmgren et al., 1995). We have subsequently shown that angiostatin therapy can also inhibit the growth of three different types of murine primary tumors, even if therapy is not initiated until tumors are 2% of body weight (O'Reilly et al., 1996). We have not found any tumors that become resistant to angiostatin and have not observed any toxicity due to angiostatin at doses tested, i.e., up to 100 mg/kg. We have not tested angiostatin above this dose. Recombinant fragments of angiostatin have since been made that show inhibitory activity in vitro (Cao et al., 1996). Recently, angiostatin has been shown to be produced by the proteolytic cleavage of plasminogen by a serine protease from several human prostate carcinoma cell lines (Gately et al., 1996).

We now show that a similar rationale as was used for the isolation of angiostatin has led to the isolation of a novel 20 kDa angiogenesis inhibitor from a murine hemangioendothelioma. We have named this inhibitory protein endostatin. Endostatin is a specific inhibitor of endothelial proliferation and is a potent angiogenesis inhibitor. Systemic therapy with endostatin causes a nearly complete suppression of tumor-induced angiogenesis, which results in a strong antitumor activity.

Results

Identification of an Inhibitor of Capillary Endothelial Cell Proliferation from Hemangioendothelioma Cells

A murine hemangioendothelioma cell line, EOMA (Obeso et al., 1990), was evaluated for evidence of the production of inhibitors of endothelial cell proliferation.

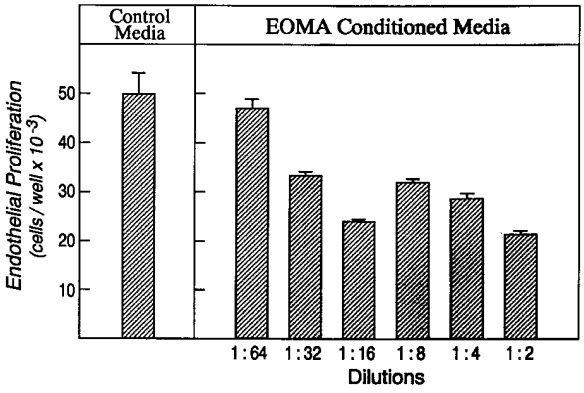

Figure 1. Inhibition of Capillary Endothelial Cell Proliferation by Conditioned Media from EOMA Cells

Conditioned media collected from EOMA cells or base media was applied to bovine capillary endothelial cells with 1 ng/ml bFGF in a 72 hr proliferation assay. Endothelial cell proliferation was inhibited by the EOMA conditioned media. Each bar represents the mean ± SEM.

Many of the known endogenous inhibitors of angiogenesis inhibit the in vitro proliferation of endothelial cells. Conditioned media from EOMA cells was applied to bovine capillary endothelial cells, stimulated with bFGF, in a 72 hr proliferation assay. The conditioned media reversibly inhibited the proliferation of capillary endothelial cells as compared with controls. The pattern of inhibition was consistent with the presence of inhibitory and stimulatory activity of endothelial cell proliferation (Figure 1).

The Inhibitory Activity of Endothelial Cell Proliferation Is Not Due to Angiostatin

To determine whether or not the inhibitor of capillary endothelial cell proliferation produced by the EOMA cells was angiostatin, pooled conditioned media was applied to lysine Sepharose. Lysine Sepharose binds angiostatin and has been used for its purification (O'Reilly et al., 1996). The endothelial cell inhibitory activity was found only in the flow-through fraction and not in the bound fractions (data not shown). The lack of binding of the inhibitory activity to lysine Sepharose suggested that the inhibitor of endothelial cell proliferation was not angiostatin.

Purification of a 20 kDa Protein from the Conditioned Media of EOMA Cells That Specifically Inhibits Endothelial Cell Proliferation

Because several angiogenic inhibitors have an affinity for heparin, we applied the flow-through from the lysine Sepharose column to a heparin Sepharose column. The inhibitory activity bound heparin with relatively high affinity and was eluted with 0.6–0.8 M NaCl in 10 mM Tris (pH 7.4.) Of interest was a stimulatory activity in the conditioned media that eluted from the heparin column with 0.4 M NaCl. To purify further the inhibitory activity, the sample was concentrated and applied to a gel filtration (Bio-Rad Bio-Gel P-100 fine gel or Pharmacia Sephacryl S-200HR gel) column followed by several cycles of reverse-phase HPLC with a C4 column. The inhibitory activity was eluted from the C4 column with 40–45% acetonitrile in 0.1% trifluoroacetic acid. After the final C4 column, the inhibitory activity was associated with a protein of molecular mass 20 kDa (reduced) or 18 kDa (nonreduced), by SDS-PAGE, purified to apparent homogeneity (Figure 2A). In a representative purification, 10 l of conditioned media containing 500 mg of protein was used to purify 2 μg of purified inhibitor. The inhibitory activity was purified by 5000-fold with a 2% recovery of activity.

To characterize further the 20 kDa inhibitor, we tested it on several cell lines of endothelial and nonendothelial origin. Only endothelial cells were significantly inhibited (Figure 3). The dose-dependent inhibition was first observed at doses of 100 ng/ml with maximal inhibition observed at doses of 600 ng/ml or greater (Figure 3A). No significant inhibition was seen for cells of nonendothelial origin (Figure 3B) at doses 1 log-fold higher than those used to inhibit capillary endothelial cells (data not shown). Notably, the proliferation of the EOMA cell line was not inhibited by the purified 20 kDa protein.

Microsequence Analysis of the 20 kDa Protein Reveals Identity to a Fragment of Collagen XVIII

Microsequence analysis of the inhibitor revealed identity to a C-terminal fragment of collagen XVIII. The molecular cloning and sequence of collagen XVIII was first described by Olsen and his coworkers and by Rehn and Pihlajaniemi (Oh et al., 1994; Rehn and Pihlajaniemi, 1994). Collagen XVIII is a novel collagen that consists of an N-terminal region with three splice variants (Muragaki et al., 1995; Rehn and Pihlajaniemi, 1995), a series of collagen-like domains with interruptions, and a 35 kDa C-terminal noncollagenous (NC1) domain. An 18 amino acid N-terminal microsequence analysis of the purified inhibitor of endothelial cell proliferation confirms that it is identical to a C-terminal fragment of this NC1 domain (Figure 2B). We have named this inhibitory fragment endostatin.

Recombinant Endostatin Inhibits Endothelial Cell Proliferation In Vitro

We produced recombinant mouse endostatin in baculovirus and E. coli expression systems. Using sequential heparin Sepharose chromatography, recombinant endostatin was purified to apparent homogeneity from insect cell media. Ni^{2+}-NTA-agarose was used to purify the E. coli–derived endostatin. SDS-PAGE revealed a discrete band of 20 or 22 kDa (reduced) purified to apparent homogeneity for baculovirus and E. coli–derived recombinant endostatins, respectively (data not shown). Both were dialyzed against PBS prior to use. After dialysis, the material from the E. coli system precipitated and the insoluble purified protein was delivered as a suspension for in vivo studies. Recombinant endostatin (baculovirus) specifically inhibited the proliferation of bovine capillary endothelial cells in a dose-dependent fashion (data not shown). The inhibition was similar to that seen for native endostatin (see Figure 3A) with maximal inhibition observed at doses above 600 ng/ml. No

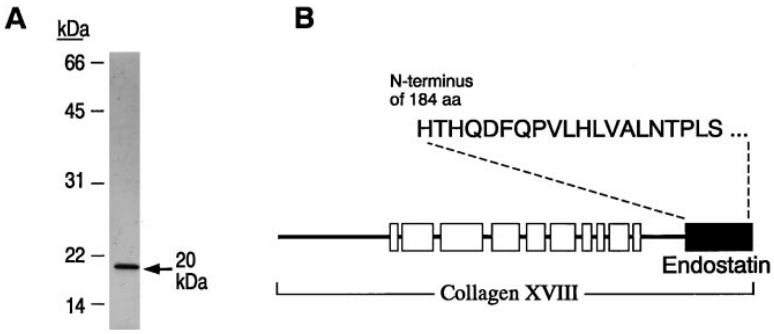

Figure 2. Purification of an Inhibitor of Endothelial Proliferation from EOMA-Conditioned Media

(A) SDS-PAGE. An aliquot (~100 ng) of the purified inhibitor of endothelial cell proliferation was analyzed by electrophoresis in a 10%–18% polyacrylamide gel. Inhibitory activity was associated with a protein of apparent M_r 20,000 (silver stain). Molecular mass markers ($\times 10^{-3}$) are as indicated.

(B) Amino acid sequence. The N-terminal sequence of the purified inhibitor of endothelial cell proliferation (endostatin) is noted in relation to a schematic of collagen XVIII. N-terminal sequence reveals identity of the inhibitor to a 20 kDa C-terminal fragment (solid shading) of collagen XVIII. We have named this inhibitor endostatin. The open boxes represent the collagenous domains of collagen XVIII.

significant inhibition of proliferation of cells of nonendothelial origin or of EOMA cells was observed (Figure 3B) when endostatin was tested at doses up to 1 log-fold higher than those used to inhibit endothelial cells. The precipitated (nonrefolded) material was not testable in vitro because of its insolubility in culture media. However, a small percentage of the material spontaneously solubilized in the PBS during dialysis. In the endothelial cell assays, this material inhibited at concentrations comparable to both the native and baculovirus-derived endostatin. Furthermore, when the recombinant endostatin (E. coli) was refolded, it was soluble and inhibited endothelial proliferation (data not shown). The refolding process, however, resulted in a loss of greater than 99% of the protein. It was therefore not feasible to test this material in the in vivo assays.

Recombinant Endostatin Inhibits Angiogenesis

To test for the ability of recombinant mouse endostatin to inhibit in vivo angiogenesis, we used the chick chorioallantoic membrane (CAM) assay (Folkman, 1985; Nguyen et al., 1994). At doses of 10–20 μg/disc, there was potent inhibition of angiogenesis for the E. coli– and baculovirus-derived endostatins in all of the tested CAMs (n = 5/group). Because of the low yield of E. coli–derived endostatin protein recovered after refolding, we used the nonrefolded purified protein for the in vivo studies. The E. coli–derived endostatin precipitate was observed to dissolve gradually from the discs over five days and produced a sustained antiangiogenic effect. In contrast, the soluble baculovirus-derived endostatin dissolved within 24 hr and gave a maximal antiangiogenic effect within a period of 48 hr. There was no evidence of toxicity in any of the chick embryos tested.

Recombinant Endostatin Inhibits the Growth of Metastases

Because tumor growth is angiogenesis dependent, we treated Lewis lung carcinoma metastases systemically with recombinant mouse endostatin expressed in the baculovirus system. The growth of metastases was almost completely suppressed by the systemic administration of endostatin at a dose of 0.3 mg/kg/day given subcutaneously (7 ± 3 metastases per mouse, n = 4). In contrast, in mice treated with saline after removal of a Lewis lung carcinoma primary tumor, lung metastases grew rapidly (77 ± 7 metastases per mouse, $p < 0.001$). Lung weight, which reflects tumor burden, was 240 ± 25 mg in the endostatin-treated mice versus 760 ± 30 mg in the control mice ($p < 0.001$). Furthermore, there was no weight loss or evidence of toxicity in any of the mice treated with endostatin.

Recombinant Mouse Endostatin Inhibits the Growth of Primary Tumors

The yield of endostatin from the baculovirus system was lower than that of the E. coli system, i.e., 1–2 mg/l versus 30–40 mg/l. We therefore used E. coli–derived endostatin to study the effect of endostatin therapy on primary tumors. We produced recombinant mouse endostatin from E. coli in sufficient quantity to treat several different types of primary tumors growing in syngeneic

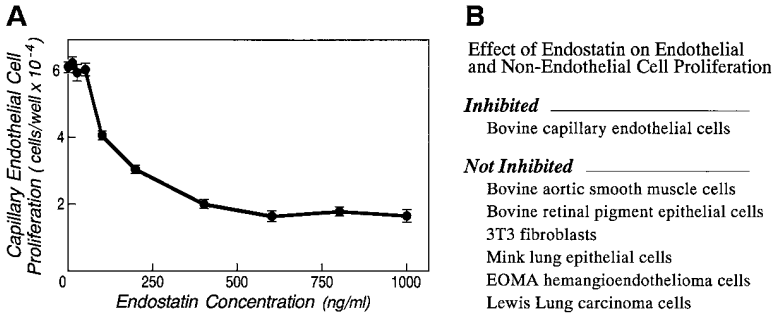

Figure 3. Specific Inhibition of Capillary Endothelial Cell Proliferation by Native and Recombinant Endostatin

(A) Endostatin purified from EOMA-conditioned media was applied to bovine capillary endothelial cells with 1 ng/ml bFGF in a 72 hr proliferation assay. Endostatin inhibited endothelial cell proliferation in a dose-dependent fashion. Each bar represents the mean ± SEM.

(B) Recombinant or native endostatin was applied to a wide variety of nonendothelial types in a 72 hr proliferation assay. No significant inhibition of proliferation was seen at doses up to 1 log-fold higher than those required to inhibit endothelial cell proliferation.

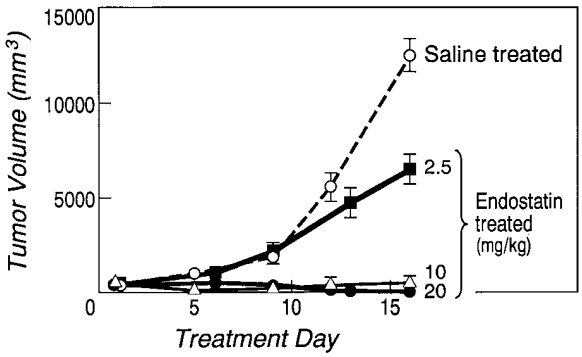

Figure 4. Dose-Dependent Inhibition of Lewis Lung Carcinoma Primary Tumors by Recombinant Endostatin

Mice were implanted with Lewis lung carcinomas and systemic therapy with recombinant mouse endostatin (E. coli) was begun when tumors were approximately 200 mm^3. Mice were treated with doses of 2.5, 10, and 20 mg/kg injected once daily. A dose-dependent inhibition of tumor growth was observed. Representative data for saline treated mice are shown (n = 5). Each point represents mean ± SEM for six mice (20 mg/kg) or four mice (2.5 and 10 mg/kg).

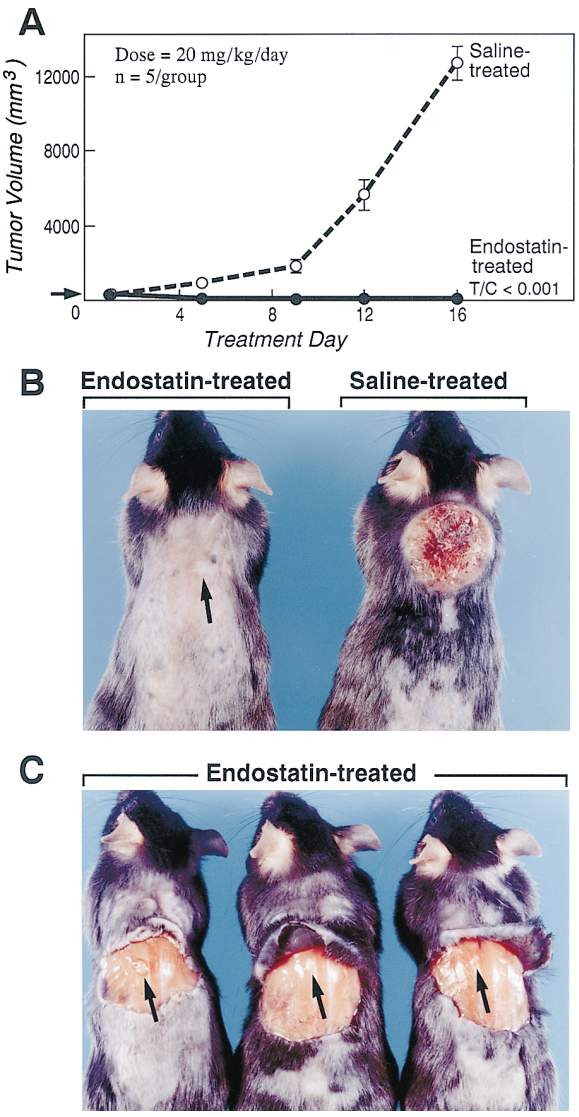

Figure 5. Systemic Therapy with Recombinant Endostatin Regresses Lewis Lung Carcinoma Primary Tumors

The subcutaneous dorsa of mice were implanted with Lewis lung carcinomas.

(A) Results of systemic therapy with recombinant mouse endostatin (20 mg/kg/day) initiated when tumors were ~200 mm^3 (1% of body weight). Tumors in mice treated with endostatin rapidly regressed and were inhibited by >99% relative to saline-treated controls. Each point represents mean ± SEM for five mice. The experiment was repeated with comparable results.

(B) Representative treated and control mice after 11 days of systemic therapy with endostatin. Saline-treated mice (right) had rapidly growing red tumors with ulcerated surfaces. Endostatin- treated mice (left) had small pale residual tumors (arrow).

(C) Residual disease in endostatin treated mice. Three of the five endostatin treated mice were sacrificed after 16 days of therapy. Autopsy revealed small white residual tumors at the site of the original primary implantation (arrows).

mice. Lewis lung carcinoma, T241 fibrosarcoma, EOMA hemangioendothelioma, and B16F10 melanoma were implanted into mice and grown to at least 100–200 mm^3. When purified nonrefolded endostatin was dialyzed against PBS, it precipitated. The precipitated protein was resuspended and the suspension was administered to the mice via subcutaneous injection. The injected precipitate formed a pellet at the injection site. Mice were observed several times daily and the pellet was noted to resorb slowly over a 24–48 hr period. These observations suggest that the injected nonrefolded endostatin protein acts as a subcutaneous depot with release over a 24–48 hr period.

The growth of Lewis lung primary tumors was potently suppressed by systemic endostatin therapy (Figures 4 and 5). Increasing doses of endostatin were associated with improved efficacy (Figure 4). Tumor growth was inhibited by 53% at a dose of 2.5 mg/kg as compared to control mice treated with vehicle alone (Figure 4). At 10 mg/kg, tumor growth was inhibited by 97% (Figure 4). At 20 mg/kg given once daily, in several separate experiments, there was an almost complete regression of established primary tumors (>99% inhibition, $p < 0.001$) (Figures 4 and 5). Immunohistochemical analysis (Figure 6) of the residual small tumors (Figure 5C) showed a potent inhibition of angiogenesis in the endostatin-treated tumors. Furthermore, the proliferative index of tumors in the endostatin- and saline-treated mice was at the same high level in both groups while the apoptotic index increased 7-fold after endostatin therapy (Figure 6). Thus, endostatin therapy results in a similar pattern of tumor dormancy to the one previously described for angiostatin (Holmgren et al., 1995; O'Reilly et al., 1996). Furthermore, there was no evidence of drug-related toxicity in any of the mice.

In a separate series of experiments, syngeneic mice implanted with several other malignant tumors, including B16F10 melanoma, T241 fibrosarcoma, and EOMA hemangioendothelioma were treated with the recombinant endostatin precipitate. All of the tumors tested rapidly regressed and there was no evidence of any toxicity in any of the treated mice (Figure 7). Continued endostatin therapy maintained the tumors in a state of dormancy for as long as it was administered. These data strongly suggest that the antiangiogenic effect of endostatin can be used to target a wide variety of primary malignancies.

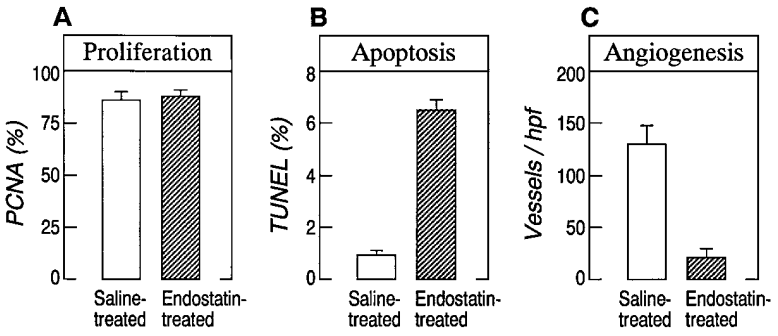

Figure 6. Endostatin Results in an Inhibition of Angiogenesis and an Increase in Apoptosis of Lewis Lung Carcinoma Primary Tumors

Histological sections of tumors from saline- versus endostatin- treated Lewis lung carcinomas were analyzed for proliferation (PCNA), apoptosis (TUNEL), and angiogenesis (vWF). There was no significant difference in the proliferative index of tumor cells (A) in treated versus untreated tumors. In contrast, the apoptotic index of the tumor cells (B) increased 7-fold ($p < 0.001$) in the endostatin-treated mice. Vessel density (C) was determined by counting the number of capillary blood vessels per highpower field (HPF) in sections stained with antibodies against vWF. Angiogenesis was almost completely suppressed in the residual microscopic tumors of the endostatin-treated mice ($p < 0.001$).

After discontinuation of endostatin therapy, a tumor recurred at the primary site within 5–14 days, became vascularized, and eventually killed the mice (data not shown). Notably, we found that E. coli–derived recombinant mouse endostatin with a C-terminal polyhistidine tag, which was expressed, purified and administered in a comparable fashion to the N-terminal-tagged product described above did not inhibit angiogenesis in the CAM assay and had no effect on the growth of Lewis lung carcinomas (data not shown). These data argue strongly that the antitumor and antiangiogenic activity of recombinant endostatin are due to the specific structure of endostatin and not to a contaminant in the sample.

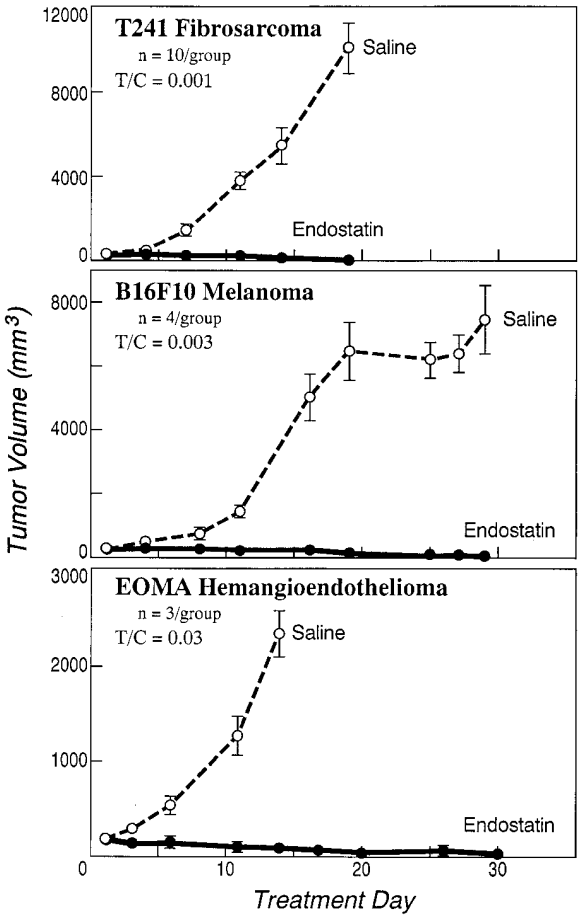

Figure 7. Systemic Therapy with Recombinant Endostatin Regresses a Wide Variety of Primary Malignancies

The subcutaneous dorsa of mice were implanted with Lewis lung carcinomas and systemic therapy with recombinant mouse endostatin (20 mg/kg/day) was begun when tumors were ~200 mm^3 (1% of body weight). Tumors in the mice treated with endostatin rapidly regressed and were inhibited by >99% relative to saline-treated controls ($n = 5$). Each point represents mean ± SEM for ten (T241), four (B16F10), or three (EOMA) mice.

Discussion

These results show that a murine hemangioendothelioma produces a novel and specific 20 kDa inhibitor of endothelial cell proliferation in vitro that is a potent inhibitor of angiogenesis and tumor growth in vivo. The N-terminal sequence of this inhibitor, endostatin, is identical to a C-terminal fragment of collagen XVIII. Systemic administration of recombinant endostatin potently inhibits angiogenesis, maintains metastases at a microscopic size, and regresses primary tumors to less than 1 mm^3, a reduction of over 150-fold. For as long as mice are treated (i.e., in experiments that last 16–25 days), there is no regrowth of tumors, no evidence of drug resistance, and no toxicity.

Collagen XVIII is a recently reported member of a family of collagen-like proteins (Oh et al., 1994; Rehn and Pihlajaniemi, 1994), referred to as the multiplexins, and is localized mainly in a perivascular position around blood vessels (Muragaki et al., 1995). It is tempting to speculate that the presence of a powerful endothelial inhibitor within a protein localized to blood vessels may provide a regulatory mechanism for vessel growth. Mice that are homozygous for collagen XVIII knockout alleles develop normally, however, with no evidence of abnormal vascular morphogenesis (N. F. et al., unpublished data). Thus, collagen XVIII may not represent a rate-limiting negative regulator for vessel growth during embryonic development but may represent a source of regulatory activity released by proteolytic cleavage of the intact molecule under conditions of induced angiogenesis. Therefore, it may become as important to elucidate the role of proteases in the suppression of angiogenesis as it has been to find those proteases that participate in its initiation (Gross et al., 1983).

We found endostatin by the same strategy that we had employed to find angiostatin (O'Reilly et al., 1994), i.e., isolation from a tumor. While it appears to be counterintuitive that tumors should be a source of angiogenesis inhibitors, the results reported here seem to validate

the approach. Furthermore, several different human tumors growing in immunocompromised mice have been shown to be associated with the presence of circulating inhibitors of angiogenesis (Chen et al., 1995).

This leads to the question of why angiogenesis inhibitors should be present in tumors that are angiogenic. One possibility is that an inhibitor could be "left-over" after down-regulation of its production by a tumor cell undergoing the switch to the angiogenic phenotype. This appears to be the case for thrombospondin produced by Li-Fraumeni cells in which the second allele for p53 is mutated or deleted (Dameron et al., 1994). A second possibility is that the proteolytic activity that accompanies tumor growth, and which is an important component of capillary blood vessel growth, may also mobilize circulating angiogenesis inhibitors from precursor proteins that are not inhibitory themselves. Angiostatin, for example, inhibits angiogenesis while plasminogen does not (O'Reilly et al., 1994, 1996). For endostatin, a similar pattern is revealed. Although it is not practical to produce full length collagen XVIII, fragments of its C-terminal domain that are longer than endostatin do not inhibit endothelial cell proliferation (data not shown). Furthermore, the 16 kDa N-terminal fragment of prolactin (Clapp et al., 1993), an internal fragment of platelet factor 4 (Maione et al., 1990; Gupta et al., 1995), synthetic fragments of murine epidermal growth factor (Nelson et al., 1995), fragments of laminin (Grant et al., 1989; Sakamato et al., 1991), and peptides derived from thrombospondin (Good et al., 1990; Tolsma et al., 1993) all inhibit endothelial cell proliferation. The proteolytic cleavage of platelet factor 4, which is itself an endothelial cell inhibitor, with plasmin increases its inhibitory activity by more than 50-fold (Gupta et al., 1995). Also, fragments produced in vitro, such as a 29 kDa plasmin-derived fragment of fibronectin (Homandberg et al., 1985) and a fragment of SPARC (Sage et al., 1995), can inhibit the proliferation of endothelial cells. Thus, a theme appears to be emerging of endogenous inhibitors of angiogenesis arising from larger proteins with distinct and varied functions. If the responsible proteases and the common cleavage sites can be elucidated, a new general mechanism of growth regulation in the vascular system may be revealed, not unlike the cascade of proteolytic events in the coagulation system.

Histology of tumors that regressed with endostatin therapy showed perivascular cuffing of tumor cells surrounding one or more microvessels in which angiogenesis was blocked. Tumor cells displayed high proliferation balanced by high apoptosis, with no net gain in tumor size. These data are consistent with a model of a new type of tumor dormancy that we previously proposed (Holmgren et al., 1995). Furthermore, endostatin inhibited proliferation of endothelial cells in vitro but had no effect on Lewis lung carcinoma cells or other cell types including smooth muscle, epithelium, fibroblasts, and the EOMA cell line from which it was purified.

These results are reflected in the histology of treated tumors, where only angiogenesis appears to be inhibited and not Lewis lung carcinoma proliferation. In fact, the high rate of tumor cell proliferation was not different from tumor cell proliferation in untreated mice. The apoptosis rate of tumor cells in treated mice was 7-fold higher than in untreated mice. This pattern is similar to that observed with angiostatin therapy of primary tumors or their metastases (O'Reilly et al., 1994, 1996; Holmgren et al., 1995), and suggests that suppression of the endothelial cell population may act to withdraw paracrine factors from the tumor cell population.

The fact that a specific inhibitor of endothelial cell proliferation can regress a tumor to a microscopic size and hold it in a dormant state, despite the fact that the tumor cells are refractory to the inhibitor from the outset, indicates that the endothelial population can exert powerful growth regulatory control over the tumor cells. The tumor suppressive effect brought about by endostatin demonstrates, in an even more compelling way than angiostatin (O'Reilly et al., 1996), that dormancy therapy is at least feasible in experimental animals. It remains to be seen whether this approach, based on prolonged and potent antiangiogenic therapy can be translated to cancer patients. The results with endostatin support our previous proposal (Folkman, 1996) that for therapeutic purposes it is fruitful to think about a tumor in terms of two distinct cell populations, a tumor cell population and an endothelial cell population, each of which can stimulate growth of the other. Growth of each cell population may be optimally inhibited by agents that selectively or specifically target that cell type, i.e., cytotoxic chemotherapy and antiangiogenic therapy. Furthermore, combined treatment of both cell populations may be better than treatment of either compartment alone (Teicher et al., 1994).

The systemic administration of precipitated and nonrefolded recombinant endostatin from E. coli permitted sustained release of the purified protein from a subcutaneous depot. It should be noted that the nonrefolded form precipitated in the in vitro endothelial cell assay, and thus could not be tested for antiproliferative activity. When endostatin was refolded by standard methods, it became soluble in tissue culture media and potently inhibited endothelial cell proliferation. There was significant loss of protein, however, during the refolding process. We are unaware of any precedent for the use of an injected depot of nonrefolded recombinant protein as a sustained-release method in animals. Nevertheless, endostatin gradually resorbed in vivo and proved to have potent antiangiogenic activity that resulted in prolonged antitumor activity. Therefore, these data suggest a novel general method for the controlled release of recombinant proteins. Based on this same rationale, we have delivered nonrefolded recombinant angiostatin from E. coli with similar success.

Finally, the discovery of endostatin, its ability to inhibit specifically endothelial proliferation, and its potent antitumor effect provide the most compelling proof of principle to date that tumors are angiogenesis dependent.

Experimental Procedures

Conditioned Media Collection

The murine hemangioendothelioma cell line EOMA was obtained from Cindy Meinenger and Robert Auerbach. The cells were maintained in DMEM supplemented with 10% bovine calf serum (BCS) and 1% glutamine-penicillin-streptomycin (GPS) in a 37°C and 10% CO_2 incubator. To produce conditioned media, 150 ml of DMEM with 3% BCS and 1% GPS was added to near confluent EOMA cells

in 900 cm^2 roller bottles. After 72 hr, at 37°C and 5% CO_2, media was collected, filtered (0.45 μm), and stored at 4°C.

Purification of Inhibitory Activity from Conditioned Media

Lysine Sepharose, heparin Sepharose, Sephacryl S-200 HR gel (Pharmacia, Uppsala, Sweden), Bio-Gel P-100 fine polyacrylamide gel (Bio-Rad Laboratories, Richmond, CA), and a SynChropak RP-4 (100 × 4.6 mm) C4 reverse-phase column (Synchrom, Inc., Lafayette, IN) were prepared according to the manufacturers' recommendations. A heparin Sepharose column (50 × 2.5 cm) was equilibrated with 50 mM NaCl 10 mM Tris-HCl (pH 7.4). Pooled conditioned media was applied and the column was washed with the equilibration buffer. The column was eluted with a continuous gradient of 50 mM–2 M NaCl in 10 mM Tris-HCl (pH 7.4) (200 ml total volume) followed by 100 ml of 2 M NaCl in 10 mM Tris-HCl (pH 7.4). Fractions were collected and an aliquot of each was applied to capillary endothelial cells in a 72 hr proliferation assay. Fractions that inhibited proliferation were dialyzed (Molecular Weight Cut-Off (MWCO) = 6000–8000) against PBS and concentrated using a 4000 MWCO Nanospin concentrator (Gelman Sciences, Ann Arbor, MI).

A Bio-Gel P-100 column or a Sephacryl S-200 HR column (75 × 1.5 cm) was equilibrated with PBS. The sample from heparin Sepharose chromatography was applied and the column was eluted with PBS. Fractions were collected and an aliquot of each was applied to endothelial cells. Fractions that inhibited endothelial proliferation were concentrated and dialyzed as above.

A SynChropak RP-4 (100 × 4.6 mm) column was equilibrated with H_2O/0.1% trifluoroacetic acid (TFA). HPLC-grade reagents (Pierce, Rockford, IL) were used. The sample from gel filtration chromatography was applied, the column was eluted with a gradient of acetonitrile in 0.1% TFA at 0.5 ml/min, and fractions were collected. An aliquot of each was evaporated by vacuum centrifugation, resuspended in PBS, and applied to capillary endothelial cells. Inhibitory activity was further purified to apparent homogeneity by at least two subsequent cycles on the SynChropak C4 column.

Protein Microsequencing

The 20 kDa inhibitor of capillary endothelial cell proliferation was purified to homogeneity from several batches of conditioned media, resolved by SDS-PAGE, electroblotted onto PVDF (Bio-Rad, Richmond, CA), detected by Ponceau S stain, and excised from the membrane. N-terminal sequence was determined by automated Edman degradation on a PE/ABD Model 470A protein sequencer (Foster City, CA) operated with gas-phase delivery of trifluoroacetic acid.

Sequence library searches and alignments were performed against combined GenBank, Brookhaven Protein, SWISS-PROT, and PIR databases. Searches were performed at the National Center for Biotechnology Information through the use of the BLAST network service.

Bovine Capillary Endothelial Cell Proliferation Assay

Bovine capillary endothelial cells were obtained and grown as previously described (Folkman et al., 1979). For the proliferation assay, cells were washed with PBS and dispersed in a 0.05% trypsin solution. A cell suspension (25,000 cells/ml) was made with DMEM + 10% BCS + 1% GPS, plated onto gelatinized 24-well culture plates (0.5 ml/well), and incubated (37°C, 10% CO_2) for 24 hr. The media was replaced with 0.25 ml of DMEM + 5% BCS + 1% GPS and the test sample applied. After 20 min of incubation, media and bFGF were added to obtain a final volume of 0.5 ml of DMEM + 5% BCS + 1% GPS + 1 ng/ml bFGF. After 72 hr, cells were dispersed in trypsin, resuspended in Hematall (Fisher Scientific, Pittsburgh, PA), and counted by Coulter counter.

Nonendothelial Cell Proliferation Assays

Bovine aortic smooth muscle (SMC), bovine retinal pigment epithelial (RPE), mink lung epithelial (MLE), Lewis lung carcinoma (LLC), and EOMA cells and 3T3 fibroblasts were maintained in a 10% CO_2 and 37°C incubator. For the proliferation assays, cells were washed with PBS and were dispersed in a 0.05% trypsin solution. Optimal conditions for the cell proliferation assays were established for each cell type. Fetal calf serum (FCS) was used for the RPE, MLE, and LLC cells, and BCS was used for the other cell types. A cell suspension (20,000 cells/ml for SMC, RPE, MLE; 15,000 cells/ml for 3T3; 10,000 cells/ml for LLC, EOMA) was made with DMEM + 10% bovine serum + 1% GPS, plated onto 24-well culture plates (0.5 ml/well), and incubated (37°C, 10% CO_2) for 24 hr. The media was replaced with 0.5 ml of DMEM + 5% bovine serum + 1% GPS and the test sample applied. After 72 hr, cells were dispersed in trypsin, resuspended in Hematall (Fisher Scientific, Pittsburgh, PA), and counted by Coulter counter.

Chick Chorioallantoic Membrane Assay

Three-day-old fertilized white Leghorn eggs (Spafas, Norwich, CT) were cracked, and embryos with intact yolks were placed in 100 × 20 mm petri dishes (Folkman, 1985). After three days of incubation (37°C and 3% CO_2), a methylcellulose (Fisher Scientific, Fair Lawn, NJ) disc containing endostatin was applied to the CAM of individual embryos. The discs were made by desiccation of endostatin in 10 μl of 0.45% methylcellulose (in H_2O) on teflon rods. After 48 hr of incubation, embryos and CAMs were observed by means of a stereomicroscope. Embryos were observed daily until there was no evidence of inhibitory zones.

Animal Studies

Animal work was carried out in the animal facility of Children's Hospital in accordance with institutional guidelines. Male 6- to 8-week-old C57Bl6/J or 129/J mice (Jackson Labs, Bar Harbor, ME) were used. Mice were acclimated, caged in groups of four or less, and their backs shaved. All mice were fed a diet of animal chow and water ad libitum. Animals were anesthetized in a methoxyflurane (Pittman-Moore Inc., Mundelein, IL) chamber prior to all procedures and were observed until fully recovered. Animals were sacrificed by a lethal dose of methoxyflurane.

Mouse Metastasis Model

Animals with Lewis lung carcinomas of 600–1200 mm^3 tumors were sacrificed, 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 1×10^7 cells/ml and the suspension was placed on ice. After the site was cleaned with ethanol, the subcutaneous dorsa of mice in the proximal midline were injected with 1×10^6 cells in 0.1 ml of saline.

When tumors were 1500 mm^3 in size, approximately 14 days after implant, the mice underwent surgical removal of the tumor. The incision was closed with simple interrupted sutures. From the day of operation, mice received daily intraperitoneal injections of recombinant (baculovirus) mouse endostatin or saline. Mice received 0.3 mg/kg/day of endostatin once daily via subcutaneous injection. When the control mice became sick from metastatic disease, all mice were sacrificed and autopsied. Lung surface metastases were counted by means of a stereomicroscope at 4× magnification.

Treatment of Murine Primary Malignant Tumors

C57Bl6/J mice were implanted with Lewis lung carcinomas, T241 fibrosarcomas, or B16F10 melanomas using the techniques described above. For the EOMA implantations, EOMA cells, grown in culture as above, were washed with PBS, dispersed in a 0.05% solution of trypsin, and resuspended. After centrifugation (4000 rpm for 10 min at 8°C), the cell pellet was resuspended in PBS and the concentration was adjusted to 2.5×10^6 cells/ml. 129/J mice were then injected with 0.1 ml of the suspension as above. Tumors were measured with a dial-caliper, volumes were determined using the formula $width^2 \times length \times 0.52$, and the ratio of treated to control tumor volume (T/C) was determined for the last time point. After tumor volume was at least 100–200 mm^3 (0.5–1% of body weight), which occurred within 3–11 days, mice were randomized into two groups. One group received recombinant mouse endostatin (E. coli) as a suspension in PBS injected subcutaneously at a site distant from the tumor once daily. The other group received comparable injections of the vehicle alone. The experiments were terminated

and mice were sacrificed and autopsied when the control mice began to die.

Expression and Purification of Recombinant Mouse Endostatin from Baculovirus

Recombinant mouse endostatin was expressed using the BacPAK baculovirus expression system (CLONTECH Laboratories) following the manufacturer's protocol. In brief, a cDNA fragment encoding the signal sequence and C-terminal part (endostatin region) of mouse collagen XVIII was inserted into the pBacPAK8 transfer vector. BacPAK6 viral DNA (expression vector) and plasmid DNA of the pBacPAK8-endostatin clone (modified transfer vector) were then cotransfected into insect Sf21 cells. The BacPAK6 was first digested with BSU36 enzyme to make it incompetent for independent replication. Media containing expressed mouse endostatin was collected and applied to a 1.5 × 40 cm heparin Sepharose column that had been equilibrated with 50 mM NaCl 10 mM Tris (pH 7.4). The column was washed with the equilibration buffer and was then eluted sequentially with 0.2 M NaCl, 0.4 M NaCl, 0.6 M NaCl, and 1 M NaCl in 10 mM Tris (pH 7.4). All chromatography was performed at 4°C. The 0.6 M NaCl eluant (which inhibited bovine capillary endothelial cells in a 72 hr proliferation assay) was dialyzed (MWCO = 6000–8000) against PBS and then reapplied to the heparin Sepharose column. The column was eluted with a gradient of 50 mM NaCl–1.2 M NaCl in 10 mM Tris (pH 7.4). An aliquot of each fraction was applied to bovine capillary endothelial cells as above, and fractions that inhibited proliferation were pooled, dialyzed against PBS, and concentrated using a Nanospin Plus (Gelman Sciences) centrifugal concentrator (MWCO = 10,000). SDS-PAGE of the concentrated sample revealed a discrete band of apparent M_r of 20 kDa.

Expression and Purification of Recombinant Mouse Endostatin from E. coli

The C-terminal part of the cDNA of collagen XVIII was used to amplify the cDNA of mouse endostatin that was cloned into the pETKH1 vector (pET11d derivative) (Studier et al., 1990). Induction resulted in the production of a fusion protein carrying the amino acid sequence MARRASVGTD (RRAS = protein kinase A recognition sequence) and six histidine residues at the N-terminus followed by the sequence of mouse endostatin (pTB01#8). The pTB01#8 plasmid was transformed into BL21:DE3 and the fusion protein was purified on Ni^{2+}-NTA-beads as described (QiaExpressionist Handbook, Qiagen). In brief, E. coli were grown until an OD_{600} of 0.8–0.9 and expression of the fusion protein was then induced for 3 hr with 1 mM IPTG. The bacteria were pelleted and resuspended in 8 M urea, 10 mM Tris-HCl (pH 8.0) containing 10 mM imidazole, and incubated for 1 hr at room temperature. The suspension was centrifuged for 15 min at 20,000 g and the supernatant incubated with the Ni^{2+}-NTA-beads for 1 hr at room temperature. The suspension was transferred into a column and washed with 8 M urea, 0.1 M Na-phosphate, 10 mM imidazole, 10 mM Tris-HCl (pH 8.0) followed by the same buffer (pH 6.25). The protein was eluted with the pH 6.25 buffer containing 250 mM imidazole.

Refolding of Recombinant Mouse Endostatin from E. coli

Endostatin produced as above was eluted from a Ni^{2+} column as described and was diluted 10-fold with refolding buffer (0.1 M sodium phosphate, (pH 7.4); 150 mM NaCl, 0.6 M Urea, 2 mM reduced glutathione, 0.02 mM oxidized glutathione, and 0.5 M L-arginine). The final concentration was 0.1 mg/ml and the mixture was gently rotated for 48 hr at 4°C. After extensive dialysis against PBS (MWCO = 6000–8000), the sample was centrifuged for 30 min at 13,000 rpm. The supernatant was concentrated (centricon 10) and used for the BCE proliferation assay. Greater than 99% of the protein was not soluble and was lost during centrifugation.

Preparation of Recombinant Endostatin from E. coli for In Vivo Studies

Endostatin produced as above was eluted from a Ni^{2+} column as described. The fractions containing endostatin were pooled and extensively dialyzed against PBS (MWCO = 6000–8000) at 4°C. During dialysis, the endostatin precipitated. The precipitated endostatin was resuspended in the PBS, the protein concentration was adjusted to 2–4 mg/ml, and the endostatin was stored at −20°C until use. For the mouse studies, endostatin was delivered as a suspension in PBS. The suspension was thawed to 4°C, vortexed, and resuspended through a 26-gauge needle. The subcutaneous dorsa of mice were slowly injected via a 30-gauge needle with the endostatin suspension. The concentration of the endostatin was adjusted with PBS so that each mouse would receive 0.2 ml per 20 g of body weight. For the chick chorioallantoic assay, endostatin was further dialyzed against water and then evaporated by vacuum centrifugation.

Immunohistochemistry

Tumor tissue was fixed for 4 hr in Carnoy's fixative and transferred to 100% ethanol. Tissues were embedded in paraffin according to standard histological procedures. PCNA and von Willebrand factor (vWF) staining were performed as previously described (Holmgren et al., 1995). In brief, sections were pretreated with Proteinase K (vWF) or 10 mM citrate buffer (pH 6.0) (PCNA) and incubated with rabbit antiserum against human vWF (Dako) or with PC10 monoclonal antibody against PCNA (Signet). TdT labeling of fragmented DNA (TUNEL) was performed according to the method of Gavrieli et al. (1992). The proliferative index (PCNA) and the apoptotic index (TUNEL) were estimated by the percentage of cells scored under a light microscope at 200-fold magnification.

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