Intratumoral Administration of Endostatin Plasmid Inhibits Vascular Growth and Perfusion in MCa-4 Murine Mammary Carcinomas

Ivan Ding, Jian Zhong Sun, Bruce Fenton, Wei Min Liu, Paul Kimsely, Paul Okunieff, and Wang Min

Departments of Radiation Oncology [I. D., J. Z. S., B. F., W. M. L., P. O.], Pediatrics [P. K.], and Medicine [W. M.], University of Rochester School of Medicine, Rochester, New York 14642

ABSTRACT

Endostatin, a fragment of the COOH-terminal domain of mouse collagen XVIII is a recently demonstrated endogenous inhibitor of tumor angiogenesis and endothelial cell growth. Antiangiogenic therapy with endostatin in animals requires multiple and prolonged administration of the protein. Gene therapy could provide an alternative approach to continuous local delivery of this antiangiogenic factor in vivo. Established MCa-4 murine mammary carcinomas, grown in immunodeficient mice, were treated with intratumoral injection of endostatin plasmid at 7-day intervals. At the time of sacrifice, 14 days after the first injection, endostatin-treated tumor weights were 51% of controls (P < 0.01). Tumor growth inhibition was accompanied by a marked reduction in total vascular density. Specifically, computerized image analysis showed a 18–21% increase in the median distances between tumor cells and both the nearest anatomical (CD31-stained) vessel [48.1 ± 3.8 versus 38.3 ± 1.6 μm (P < 0.05)] and the nearest tumor-specific (CD105-stained) vessel [48.5 ± 1.5 versus 39.8 ± 1.5 μm (P < 0.01)]. An increased apoptotic index of tumor cells in endostatin-treated tumors [3.2 ± 0.5% versus 1.9 ± 0.3% (P < 0.05)] was observed in conjunction with a significant decrease in tumor perfused vessels (DiOC7 staining), and an increase in tumor cell hypoxia (EF5 staining). Hypoxia resulting from endostatin therapy most likely caused a compensatory increase of in situ vascular endothelial growth factor (VEGF) and VEGF receptor mRNA expression. Increased immunoreactivity of endostatin staining in endostatin-treated tumors was also associated with an increased thrombospondin-1 staining [1.12 ± 0.16 versus 2.44 ± 0.35]. Our data suggest that intratumoral delivery of the endostatin gene efficiently suppresses murine mammary carcinoma growth and support the potential utility of the endostatin gene for cancer therapy.

INTRODUCTION

Angiogenesis, the formation of new blood vessels, is essential for both critical physiological processes (wound healing) and lethal pathological conditions (tumor growth and atherosclerosis; Refs. 1–3). Numerous studies have shown that tumor growth is dependent on angiogenesis (4–7), thus providing a rationale for antiangiogenic therapy. Endostatin is one of the most potent endogenous angiogenesis inhibitors discovered to date. Interestingly, it inhibits pathologic vascular growth while allowing for normal processes. For example, both tumor growth (8) and atherosclerosis (9, 10) are inhibited in experimental animal models, whereas wound healing is unaffected. Moreover, 20,000 endostatin is a proteolytic polypeptide derived from its parent protein (collagen XVIII; Refs. 8, 11, 12). In vitro studies have shown that endostatin specifically inhibits endothelial proliferation without a direct effect on tumor or other nonneoplastic cell growth (12–15). Whether administered as recombinant protein (14, 16), plasmid injection (17–19), or gene transfection (20), endostatin inhibits tumor growth, with reduction of virtually all tumor neovascularization and without detectable systemic toxicity in preclinical models.

To be effective, antiangiogenic therapy with endostatin in tumor-bearing mice requires prolonged administration and high doses of recombinant protein (8, 14). In addition, production of the functional polypeptide has proven difficult, perhaps because of its physical properties and because of variations in the purification procedures utilized by different laboratories (15, 21). However, some preliminary data have shown that local or systemic administration of endostatin is an effective means of application in cancer therapy. A few groups have demonstrated that antiangiogenic gene therapy with viral vectors is a potentially useful approach for inhibiting tumor growth in mouse models (22–23). Although viral vectors have high transfection efficiency and are commonly used in experimental systems, safety issues and the toxicity of these viral vectors likely precludes their use as an i.v. agent. In contrast, we and others have shown that systematically delivered endostatin plasmid possesses low toxicity and high effective antitumor action (17–19). In the current study, we investigated whether intratumoral injection of endostatin plasmid once a week for 2 weeks inhibited mammary tumor growth. We also explored the underlying physiological and molecular mechanisms of endostatin-mediated antitumor effects.

MATERIALS AND METHODS

Endostatin Plasmid. A plasmid containing an expression cassette for mouse endostatin was constructed as described previously (18). The coding sequence of endostatin comprises the COOH-terminal 184 amino acid residues of collagen XVIII. This sequence was directly amplified by PCR from liver cDNA. Plasmids were grown under kanamycin selection in host strain DH5α and purified by alkaline lysis and chromatographic methods using Endofree kit (Qiagen, Valencia, CA). Purified plasmid had the following specifications: <50 Eu/mg endotoxin; <1% protein; and <5% (wt/wt) chromosomal DNA.

Murine Mammary Tumor Models and Treatment. Isotransplants of the murine mammary carcinoma MCa-4 were used. Frozen MCa-4 tumor cells were inoculated i.m. into the hind limbs of 6–8-week-old female BALB/c (nu/nu) mice (National Cancer Institute, NIH, Frederick, MD). Tumors were selected for endostatin plasmid treatment when they reached volumes of between 150 and 250 mm3 (as measured by calipers and the formula: Volume = Diameter3/6). Endotoxin-free endostatin plasmid (45 μg) was injected once a week for 2 weeks into the tumor in the right thigh. All of the tumors (right and left) were averaged. Equal volumes of saline and vector were injected as controls in separate mice. Mice were sacrificed 7 days after the second injection. Tumors were removed, examined, and frozen for later immunohistochemistry or RNA isolation. Guidelines for the humane treatment of animals were followed as approved by the University Committee on Animal Resources.

Measurement of Anatomical (CD31), Perfused (DiOC7), and Angiogenic (CD105) Blood Vessels. Immunohistochemistry methods have previously been described in detail (24). To visualize blood vessels open to flow, an i.v. injected stain, DiOC7 (Molecular Probes, Eugene, OR), was utilized. Injection volumes were administered i.v. at a concentration of 1.0 mg/kg, 1 min prior to freezing. This dose and schedule has been shown to provide optimal visualization of tumor vasculature by preferentially staining cells immediately adjacent to blood vessels. DiOC7-stained vessels emit green fluorescence when excited by blue light. CD31 antibody staining (PharMingen, San Diego, CA) was used for visualizing total structural vessels. In addition, an anti-CD105
antibody (PharMingen) that was recently reported to specifically identify vascular neogenesis was also utilized. CD105 is strongly expressed in activated endothelial cells of various human tumors, including breast cancer, but is either undetectable or only weakly present in mature blood vessels of normal tissues (25–26).

**EFS/Cy3 Hypoxia Marker.** Localized areas of tumor hypoxia were assessed in frozen tissue sections by immunohistochemical identification of sites of 2-nitroimidazole metabolism. A pentafluorinated derivative of etanidazole (EFS) was injected i.v. 1 h before tumor freezing. Protein conjugates of EFS have been previously used to immunize mice from which monoclonal antibodies were developed (27). These antibodies are extremely specific for the EFS drug adducts that form when the drug is incorporated by hypoxic cells, and one of these, ELK3-51, has been well characterized. Regions of high EFS metabolism in tumors (hypoxic regions) were visualized and the area of staining quantified immunochemically using a fluorochrome (Cy3) conjugated to the ELK3-51 antibody and computerized imaging techniques.

**Imaging and Image Analysis.** For each frozen tumor, 4.0-μm sections were cut using a cryostat. The stained sections were imaged using an epifluorescence-equipped microscope, digitized (3-CCD camera), background-corrected, and image-analyzed using Image Pro software (Media Cybernetics) and a 450-MHz Pentium computer. Color images from adjacent microscope fields were automatically acquired and digitally combined to form 4 × 4 montages of the tumor cross-section (using a motorized stage and controller). For each section, two peripheral and two interior image montages were obtained. Each section was then scanned under each of three staining conditions. First, epifluorescence images of the fluorescent green DiOC7 staining were obtained. Each section was then scanned under each of three staining conditions. For each section, two peripheral and two interior image montages were obtained. Each section was then scanned under each of three staining conditions. First, epifluorescence images of the fluorescent green DiOC7 staining were obtained. Each section was then scanned under each of three staining conditions. For each section, two peripheral and two interior image montages were obtained. Each section was then scanned under each of three staining conditions. First, epifluorescence images of the fluorescent green DiOC7 staining were obtained. Each section was then scanned under each of three staining conditions. For each section, two peripheral and two interior image montages were obtained. Each section was then scanned under each of three staining conditions. First, epifluorescence images of the fluorescent green DiOC7 staining were obtained. Each section was then scanned under each of three staining conditions. For each section, two peripheral and two interior image montages were obtained. Each section was then scanned under each of three staining conditions. First, epifluorescence images of the fluorescent green DiOC7 staining were obtained. Each section was then scanned under each of three staining conditions. For each section, two peripheral and two interior image montages were obtained. Each section was then scanned under each of three staining conditions. First, epifluorescence images of the fluorescent green DiOC7 staining were obtained. Each section was then scanned under each of three staining conditions. For each section, two peripheral and two interior image montages were obtained. Each section was then scanned under each of three staining conditions. First, epifluorescence images of the fluorescent green DiOC7 staining were obtained. Each section was then scanned under each of three staining conditions. For each section, two peripheral and two interior image montages were obtained. Each section was then scanned under each of three staining conditions. First, epifluorescence images of the fluorescent green DiOC7 staining were obtained. Each section was then scanned under each of three staining conditions. For each section, two peripheral and two interior image montages were obtained. Each section was then scanned under each of three staining conditions. First, epifluorescence images of the fluorescent green DiOC7 staining were obtained. Each section was then scanned under each of three staining conditions. For each section, two peripheral and two interior image montages were obtained. Each section was then scanned under each of three staining conditions. First, epifluorescence images of the fluorescent green DiOC7 staining were obtained. Each section was then scanned under each of three staining conditions. For each section, two peripheral and two interior image montages were obtained. Each section was then scanned under each of three staining conditions. First, epifluorescence images of the fluorescent green DiOC7 staining were obtained.
endostatin-treated tumors had a significant (28%) reduction in tumors had a 13% increase in median distance to the nearest perfused first endostatin treatment. As shown in Fig. 3, endostatin-treated significant reduction in CD31- and CD105-stained vessels (6). Unpublished studies have shown an contrast, decreased blood vessel density was associated with a significant reduction in total anatomical vessels and angiogenic vessels. Tumor proliferation and necrosis index were not significantly altered. In this experiment, the treated tumors (right leg) had slightly greater growth inhibition than the opposite leg (left) tumors.

To examine the effect of endostatin on vascular growth, anatomical vessels (CD31 staining) and angiogenic vessels (CD105 staining) in the tumors were measured and quantified “Materials and Methods”). As shown in Table 1, endostatin-treated tumors had a significant decrease in total anatomical vessels and angiogenic vessels. Tumor proliferation and necrosis index were not significantly altered. In contrast, decreased blood vessel density was associated with a significant increase in tumor cell apoptosis. The increase in apoptotic index was 1.7-fold [3.2 ± 0.45 versus 1.9 ± 0.3 for saline (P < 0.05); 2.0 ± 0.3 for vector-treated]. An RNase protection assay also showed that endostatin-treated tumors had a significant (28%) reduction in bfl-1 mRNA, as well as 21–26% decrease of bak and bad mRNA gene expression compared with controls (P < 0.05) as shown in Fig. 2. No other differential mRNA expression was observed in the other apoptotic gene (bax), or antiapoptotic genes (bcl-2, bcl-2, and bcl-X) between endostatin-treated and control animals.

The effects of endostatin on tumor functional vessels and oxygenation were evaluated using DiOC6 and EF5 staining 14 days after the first endostatin treatment. As shown in Fig. 3, endostatin-treated tumors had a 13% increase in median distance to the nearest perfused vessel compared with saline controls (P = 0.053). In the same experiment, we also observed that endostatin-treated tumors showed a significant reduction in CD31- and CD105-stained vessels (P < 0.01). Vector-treated tumors also had decreased numbers of perfused vessels compared with saline controls, most likely because of the larger sizes of the tumors (3.4 ± 0.8 g for vector versus 2.5 ± 0.5 g for saline) in this treatment group (Fig. 1B). Unpublished studies have shown an inverse relationship between perfusion and tumor volumes in numerous tumor models.4 The most extensive hypoxia marker uptake, however, was observed in endostatin-treated tumors, despite their smaller tumor size (Fig. 3). The reduction in number of perfused vessels and the increase in tumor hypoxia were associated with an elevation of local tumor VEGF and VEGFR mRNA expression, as detected by in situ hybridization (Fig. 4).

Endostatin-treated tumors also showed increased levels of thrombomodulin-1 protein by immunohistochemistry (Fig. 5). Both thrombomodulin-1 (Fig. 5B) and endostatin protein (Fig. 5A) were detected in cellular (α–c) or stromal (α’–c’) compartment.

DISCUSSION

In the present study, we have shown that intratumoral injection of endostatin plasmid once a week for 2 weeks inhibits murine mammary MCa-4 tumor growth in nude mice. The reduction of tumor growth rate is associated with decreased numbers of tumor anatomical vessels, angiogenic vessels, and perfused vessels. Endostatin-treated tumors also have an increased tumor cell apoptotic index and increased tumor cell hypoxia, which are most likely associated with secondary induction of tumor cell VEGF and VEGFR mRNA expression as well as with elevation of local thrombomodulin-1 protein expression.

Application of antiangiogenic growth factors for gene therapy has been recently used in several tumor models (17, 18). We initially reported the systemic inhibition of tumor growth and metastasis by i.m. administration of the endostatin gene formulated with synthetic polymer in murine Renca and Lewis lung carcinomas, and tumor volume was 40% of control at 13-day posttreatment (18). More recently, we reported that i.v. injection of a mixture of liposome with endostatin plasmid inhibited tumor growth and metastases (19). A similar study has been reported by Chen et al. in human mammary MDA-MB435 xenograft (17). Preclinical gene therapy models, therefore, suggest that this approach to antiangiogenic growth factor therapy will be efficacious. In contrast, achieving tumor regression with recombinant protein has been difficult and requires frequent high-dose injections in animal tumors. Our results and those of others have demonstrated that local or systemic administration of nonviral endostatin plasmid significantly inhibits the growth of several tumor types, including lung, breast, kidney, and sarcoma tumors (17–19). Response, however, is limited to the slowing of tumor growth without complete regression for established tumors. The efficacy may be dependent on tumor type, vector type, and plasmid administration route and dose, as well as the formulation of agents.

Regression of transplanted tumors is a more difficult undertaking than preventing tumor formation when using any cytotoxic or antiangiogenic therapy. Using a transgenic mouse model of a spontaneous pancreatic β-islet cell tumor, Bergers et al. (29) recently reported that recombinant endostatin effectively prevented the promotion from

Table 1  MCa-4 tumor-bearing nude mice treated with endostatin plasmid injections

<table>
<thead>
<tr>
<th>Median distance to tumor vessels</th>
<th>Tumor Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD31 (µm)</td>
<td>CD105 (µm)</td>
</tr>
<tr>
<td><strong>Saline</strong></td>
<td>38.29 ± 1.56</td>
</tr>
<tr>
<td><strong>Vector</strong></td>
<td>41.47 ± 1.13</td>
</tr>
<tr>
<td><strong>Endostatin</strong></td>
<td>48.10 ± 3.76†</td>
</tr>
</tbody>
</table>

*P < 0.05.
**P < 0.01.
†P < 0.05.

4 Unpublished observations.
hyperplastic lesion to small tumor formation but poorly inhibited established tumor growth (progression stage). In our transplantable MCa-4 tumor and others, endostatin succeeded in slowing tumor growth in established tumors as described previously (17–20). The antitumoral action of endostatin should, therefore, be further investigated at different stages of carcinogenesis. Ultimately, we may find that the greatest utility for endostatin is in preventing metastases rather than in inhibiting established tumors (18–19).

Endostatin is believed to specifically inhibit endothelial cell proliferation rather than tumor cell growth (12–15). The underlying molecular mechanisms of antiangiogenesis are presumably related to an increase in endothelial cell apoptosis (13), or an alteration of cell cycle (21). In our animal model, the effects of endostatin-mediated antiangiogenesis are consistent with previous studies. In addition, we quantitatively demonstrated that endostatin-treated tumors showed a clear decrease in perfused vessel density and an increase in tumor cell hypoxia, and that the effects were long lasting, with physiological effects still manifesting 14 days after initial treatment. Direct inhibi-
tion of tumor structural vessels, angiogenic vessels, and perfused vessels can explain the observed endostatin-mediated antitumoral effects. Although we believe that the tumor cell apoptosis was secondary to ischemia, our observation leaves open the possibility that endostatin has direct apoptotic effects on tumor tissue. Likewise, tumor necrosis and apoptosis may have been attributable to ischemia, reperfusion injury, or some as-yet-unexplained indirect endostatin-induced cytotoxicity.

We favor a direct effect of endostatin on tumor vascularity, resulting indirectly in tumor growth reduction. Consistent with this hypothesis, endostatin-treated MCa-4 tumors had a similar proliferative rate to that of controls as measured by mitotic figures (Table 1), which suggests that the tumor cell cycle was not altered by endostatin. Reduced vascularity, however, can deprive tumors of their nutrient supply, and, thus, proliferation can result in environmentally deprived progeny, leading to apoptosis or necrosis after division. Kirsch et al. (30) showed that recombinant angiostatin-treated gliomas caused both neoplastic and endothelial cell apoptosis. They concluded that endothelial cell apoptosis results from vessel thrombosis or regression of pre-existing vessels. In our study, we counted only the tumor cell apoptotic numbers, because very few endothelial cells displayed an apoptotic appearance on the basis of morphology. Thus, we cannot confirm (or refute) the observation of Kirsch et al. (30). Regarding molecules important in promoting or inhibiting apoptosis, there were mixed responses. The antiapoptotic gene bfl-1 decreased, but so did the apoptotic genes bad and bak. Ischemia and reperfusion injury, therefore, appear more important in generating the necrosis and apoptosis than any hypothetical direct endostatin-mediated apoptosis. It is possible, however, that endothelial cells from different tumor types may respond differently to endostatin (20).

Endostatin may exert biological effects directly or indirectly by altering expression of other growth-related molecules. One possible mechanism is a down-regulation of angiogenic molecules by endostatin. Because multiple cell types, such as tumor cells, endothelial cells, activated macrophages, and tumor fibroblasts, in tumors produce angiogenic and antiangiogenic growth factors, and because many angiogenic growth factors can also be mobilized from the extracellular matrix, endostatin-mediated regulation of angiogenic growth factor gene expression is extremely complex and difficult to assess. Kirsch et al. (30) treated three types of malignant gliomas and found a significant reduction of VEGF mRNA by Northern analysis, but an elevation of FGF2 3 weeks after angiostatin treatment. They hypothesized that angiostatin-mediated antiangiogenesis in gliomas may be secondary to down-regulation of certain angiogenic growth factors or to up-regulation of angiogenic factors. We found an up-regulation of VEGF and VEGFR mRNA as well as up-regulation of thrombospondin-1 protein expression in endostatin-treated MCa-4 breast tumors. In contrast to Kirsch et al. (30), our results demonstrated that VEGF and VEGFR were up-regulated rather than down-regulated. These differences could suggest that: (a) the balance of angiogenic and antiangiogenic growth factors as well as their gene regulation may be time-dependent during endostatin treatment. Sampling at different times after endostatin treatment may result in differential gene expression; (b) expression of angiogenic or antiangiogenic growth factors may be tumor size- or tumor histologic type-dependent; (c) endostatin may exert its effects through different mechanisms in different tumors, animal strains, or species; and (d) expression of angiogenic/antiangiogenic factors may be responding to independent stimuli. We believe that tumor hypoxia induced the VEGF and VEGFR mRNA expression, and the up-regulation of thrombospondin-1 may be triggered by endostatin through specific signaling pathways.

In summary, endostatin inhibits tumor growth by reducing structural, angiogenic, and perfused tumor vessels. A lack of adequate blood supply leads to tumor hypoxia and probably accounts for tumor cell apoptosis and the up-regulation of VEGF and VEGFR. Thrombospondin-1 also increased, which suggests that endostatin may regulate this antiangiogenic peptide. However, the molecular mecha-

Fig. 5. Expression of thrombospondin-1 protein in endostatin-treated tumors by immunohistochemistry in MCa-4 tumors. Saline-, vector-, and endostatin-treated tumors were collected 14 days after initial treatment and antiendostatin antibody (A) or antithrombospondin-1 antibody (B) was hybridized with these sections. (a–c), cellular staining pattern; (a’–c’), stromal staining pattern. Endostatin-treated tumors showed the transgene expression and had elevated thrombospondin-1 protein expression.
nisms for endostatin-mediated antiangiogenesis are still unknown at present. Different mechanisms may exist for in vitro and in vivo models. In vitro, several issues need to be addressed: (a) is there a receptor or endostatin-related cell surface molecule on endothelial cells? and (b) if so, what are the signal transduction pathway and target genes? If not, how does it inhibit angiogenesis and is it via stromal effects? In animal models, we also need to consider: (a) does endostatin act on tumor cells through direct or indirect pathways? (b) if by indirect pathways, is it by regulation of other angiogenic or antiangiogenic factors in endothelial, inflammatory, or tumor cells? and (c) can endostatin induce tumor regression rather than just slow growth, and under what circumstances? These mechanistic questions are now under investigation in our laboratory.

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