Endostatin reduces vascularization, blood flow, and growth in a rat gliosarcoma¹

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Endostatin, the 20-kDa C-terminal fragment of collagen XVIII, has previously been shown to inhibit growth and induce regression of different experimental tumors in rodents. In this study, we show that recombinant murine and human endostatin, produced in 293 EBNA cells and yeast, respectively, inhibit ectopic as well as orthotopic growing BT₄C_n gliosarcomas in BD-IX rats. In rats in which s.c. gliomas were grown for a total of 29 days, systemic treatment with recombinant murine endostatin induced about 50% reduction of intratumoral blood flow and tumor size after only 10 days of therapy. In contrast, the blood flow to irrelevant organs was unaffected by endostatin, indicating its specificity of action. Tumors were not observed to increase in size or regrow after cessation of therapy. Furthermore, endostatin-treated rats with i.c. tumors had significantly longer survival time than did untreated controls. In the treated rats, endostatin therapy resulted in a reduced tumor blood vessel volume and an increased tumor cell density with an increased

apoptotic index within a given tumor volume, as verified by flow cytometry and by staining with deoxynucleotidyltransferase-mediated dUTP nick-end labeling. This work verifies the general anti-angiogenic and antitumor effects of endostatin and indicates that the protein may also be considered as a treatment strategy for malignant brain tumors. *Neuro-Oncology* 4, 1–8, 2002 (Posted to *Neuro-Oncology* [serial online], Doc. 01-015, September 27, 2001. URL <neuro-oncology.mc.duke.edu>)

The recruitment of new blood vessels is regarded as essential for continuous tumor growth and metastasis (Folkman, 1990). Recently, several inhibitors of angiogenesis have been identified, and much attention has been paid to endostatin, which is a 20-kDa C-terminal proteolytic fragment of collagen XVIII (O'Reilly et al., 1997). This heparin-binding fragment has been shown to inhibit tumor growth in different experimental model systems (Boehm et al., 1997; Chen et al., 1999; Read et al., 2001; Yamaguchi et al., 1999). The exact mechanism of action is still not understood, but the molecule may induce apoptosis of bovine pulmonary endothelial cells (Dhanabal et al., 1999). However, whether this applies to endothelial cells from the tumor vasculature is unknown.

Murine endostatin, produced as a recombinant protein in *Escherichia coli*, has been shown to inhibit tumor growth when administered daily to mice bearing ectopic Lewis lung carcinoma, T241 fibrosarcoma, or B16F10 melanoma. Importantly, the tumors apparently do not develop resistance to endostatin therapy, but tumor

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³Abbreviations used are as follows: PBS, phosphate-buffered saline; TUNEL, deoxynucleotidyltransferase-mediated dUTP nick-end labeling.

regrowth may be observed upon termination of endostatin treatment (Boehm et al., 1997). However, one study suggests this may be avoided by giving cycled endostatin therapy, which appears to induce a dormant tumor state that persists even after discontinuation of therapy (Boehm et al., 1997).

Most *in vivo* tumor inhibition experiments with endostatin have been performed in mice with s.c. tumors using precipitated endostatin produced in *E. coli* and given s.c. in doses of 3 to 20 mg/kg body weight (O'Reilly et al., 1997). Because the protein also formed disulphide-linked oligomers, it was apparently, to a large extent, not correctly folded (Boehm et al., 1998). It has been shown that recombinant endostatin can be expressed in mouse muscle and secreted into the bloodstream, where it induces a systemic inhibition of angiogenesis (Blezinger et al., 1999). Recently, it was also shown that endostatin can be delivered to brain tumors via genetically engineered cells encapsulated in ultrapure sodium alginate (Read et al., 2001). Also, endostatin given via liposomes or adenoviral vectors may be used for systemic administration to the host (Chen et al., 1999; Feldman et al., 2000). Recombinant murine and human forms of endostatin have also been obtained in a monomeric soluble form from transfected mammalian 293 cells, as well as from yeast (Sim et al., 1999). X-ray crystallography has demonstrated a compact structure and the presence of 2 intramolecular disulphide bonds (Hohnester et al., 1998).

Based on promising results in animals, endostatin is presently undergoing phase I clinical trials. However, based on previous studies, it is not clear how effective the molecule is on tumors grown at different locations. The present work was aimed at studying the effects of endostatin on a rat gliosarcoma model grown intracerebrally as well as s.c. in BD-IX rats. This protocol included the direct assessment of endostatin's effect on both the growth of a vascular network and blood flow in tumors, as well as tumor growth and survival.

Materials And Methods

Generation of Endostatin

Recombinant murine and human endostatins produced in 293 EBNA cells and in yeast (Sim et al., 1999), respectively, were used. Murine endostatin (position 130-315 of the collagen XVIII NC1 domain) was purified and characterized as described previously (Sasaki et al., 1998). Amino acid sequencing showed a single aminoterminal sequence APLAHTHQ-, where APLA is artificially introduced by the insertion of the endostatin cDNA next to a signal peptide sequence in the expression vector. The purified protein showed a single band by polyacrylamide gel electrophoresis (Sasaki et al., 1998) and could be readily crystallized for high-resolution studies (Hohnester et al., 1998), suggesting a native structure.

Recombinant human endostatin was expressed in the methylotropic yeast *Pichia pastoris* (Invitrogen, San Diego, Calif.), in flasks as well as in bioreactors. Shaker flask expression of recombinant endostatin was according to Invitrogen's protocol for MutS shaker flask fer-

mentation. Bioreactor production of endostatin was as described by the manufacturer (Invitrogen). Endostatin was purified with ion exchange Q-Sepharose chromatography followed by direct loading over a heparin-sepharose column (Pharmacia Biotech, Piscataway, N.J.) at a flow rate of 4 ml/min. After loading, the column was washed with 10 mM Tris-HCl buffer, pH 7.4, until the absorbance at 280 nm was below 0.1 optical density. Bound extraneous proteins were eluted with buffer containing 250 mM NaCl. Endostatin was eluted from the column with buffer containing 350 mM NaCl. Endotoxin determination of samples was performed prior to the *in vitro* and *in vivo* studies with the Limulus Amebocyte Lysate Pyrotell reagent (Associates of Cape Cod, Inc., Falmouth, Mass.) following the manufacturer's protocol. Purified recombinant human endostatin protein was assayed and used for the study and conformed to several criteria that included endotoxin levels ≤ 10 ng/mg, presence of expected N-terminal sequence, purity by high-pressure liquid chromatography, and no other contaminating proteins detectable, as visualized by a load of 25 μ g on a sodium dodecyl sulfate-polyacrylamide gel electrophoresis silver-stained gel.

Cell Line

The BT₄C_n tumor was derived from serial *in vivo* passages of the BT₄A tumor, originally induced from transformed fetal rat brain cells after exposure to ethyl-nitrosourea (Mella et al., 1990). The BT₄C_n cells were grown in Dulbecco's modified Eagle's medium with 10% heat-inactivated newborn calf serum and essential amino acids, L-glutamine, penicillin, and streptomycin. The cell line tested negative for viral agents in a rat antibody production test, according to Federation of Laboratory Animal Association recommendations (Rehbinder et al., 1996).

To establish s.c. tumors, 300,000 cells in 0.3 ml PBS³ were injected s.c. in the dorsal flank region of the rat. In the i.c. study, the anesthetized rats were fixed in a stereotactic frame (David Kopf Instruments, Tujunga, Calif.), the skin was incised, and a 1.5-mm burr hole was made to fit the coordinates (3.3 mm posterior to and 2.5 mm to the right of the bregma, depth 2.8 mm). The injection device consisted of a 30-gauge blunt needle connected via a catheter (Small Parts Inc., Miami Lakes, Fla.) to an infusion pump (Harvard Apparatus, Holliston, Mass.). The needle was fixed in the electrode holder of the stereotactic frame and then vertically introduced into the brain. A total of 50,000 cells in 5 μ l PBS/glucose was injected into the brain during a period of 1 min. The needle was kept in place for 2 min and then slowly retracted. Closure was done with bone wax and sutures.

Animals

Inbred BD-IX rats (Harlan Olac Ltd., Bicester, U.K.) of both sexes weighing at least 250 g were caged in pairs or groups of 3 animals in Macrolon III cages at constant temperature and humidity on a 12-h light-and-dark schedule at an air exchange rate of 18 changes/h. Animal care was in accordance with national legislation and institutional guidelines.

The rats were fed a pelleted rat diet (B&K Universal Ltd., Grimston, U.K.) and given tap water ad libitum. The animals tested negative for parasitical, bacterial, and viral agents according to the Federation of Laboratory Animal Association recommendations (Reh binder et al., 1996).

For the surgical procedures, rats were anesthetized with a combination of fentanyl/fluanisone and midazolam, and buprenorphine was used as a postoperative analgetic agent. The rats were given a lethal dose of pentobarbitone i.p. (100 mg/kg).

Treatment Protocol

Treatment of the s.c. tumors was performed with recombinant mouse endostatin. The endostatin was injected daily at a dosage of 0.3 mg/kg s.c. The injections were performed between days 5 and 15 after the initial s.c. inoculation of tumor cells. On day 5, the tumors were palpable, their diameter being approximately 1 mm. In order to avoid a local effect, endostatin was administered s.c. in the flank opposite from the growing tumor. The control animals were treated with a daily s.c. injection of 0.9% NaCl during the same period. Thereafter, the rats were examined regularly for local tumor growth. Tumor volume (TV) was assessed with a caliper by measuring the 2 major diameters by the formula $TV = \pi/(d1 \times d2)^{1/2}$. After 29 days, the rats were killed and the tumors were removed. The tumors were then weighed and prepared for light microscopic, flow cytometric, and immunohistochemical analyses.

In the i.c. study, human endostatin was administered s.c. at a dosage of 0.3 mg/kg or 20 mg/kg daily from day 3 after inoculation, whereas the control animals were given 0.9% NaCl. The last animal in the control group was killed at day 12 because of tumor progression. To maintain a uniform treatment protocol, endostatin treatment was ended at day 12 as well. The animals were given an overdose of pentobarbitone when they showed a weight reduction of 10% and/or clinical signs of raised i.c. pressure (reduced activity, impaired locomotor activity, and lethargy).

Blood Flow

The microsphere embolization method was used to estimate tumor blood flow (Iversen et al., 1992). Briefly, about 500,000 microspheres with a diameter of 15.5 micrometer, labeled with ^{46}Sc (NEN, Boston, Mass.), were injected directly into the left ventricular chamber in deeply anesthetized rats. The rats were given an overdose of pentobarbitone after about 5 min, and the tumors were carefully removed in toto. The extirpated tumors were placed in a gamma counter, and at least 5500 counts were recorded. For comparison, the blood flows to the heart, kidneys, and one skeletal muscle were also measured.

DNA Flow Cytometry

Tumors from each of 3 treated and 3 untreated animals were minced and fixed in ice-cold 96% ethanol and stored at 4°C until use. Before DNA measurements were

taken, the tissues were incubated for 15 min with 0.5% pepsin at 37°C. The isolated nuclei were washed in 0.9% NaCl and treated for 1 min with ribonuclease (1 mg/ml in 0.9% NaCl). Staining of DNA was obtained by adding propidium-iodine (50 µg/ml in 0.9% NaCl) to the nuclei. The cellular DNA content was measured using a FACsort flow cytometer (Becton Dickinson, Palo Alto, Calif.). Normal lymphocytes were used as a standard diploid reference. The DNA histograms were obtained by gating a 2-parameter forward and side-scatter cytogram to a 1-parameter DNA histogram.

TUNEL Assay

Staining for apoptosis was performed using TUNEL assay kit. The staining was performed according to the protocol supplied by the manufacturer (Boehringer Mannheim, Mannheim, Germany), which labels DNA strand breaks generated during apoptosis (Gavrieli et al., 1992). All sections were viewed and evaluated with a confocal laser scanning microscope using transmitted light to visualize tissue texture (see below). In order to visualize the morphology of the sections stained by the TUNEL assay, blood vessels in parallel sections were visualized by immunoperoxidase staining using an anti-von Willebrand factor antibody (Dako, Glostrup, Denmark). The immunostaining was performed using a standard Vectastain ABC Kit (Vector, Burlingame, Calif.).

Immunofluorescence Microscopy

The tumors were embedded in Tissue-Tec (Sakura, Finetek Inc., Calif.) and frozen in isopentane. Frozen sections (100 µm) were placed on poly-L-lysine-coated glass slides. The specimens were then fixed in ice-cold acetone for 3 min and air dried before indirect immunofluorescence was applied.

A polyclonal antibody against von Willebrand factor, recognizing endothelial cells (Dako) was used. The sections were incubated for 24 h at 4°C with the primary antibodies (1:100 dilution anti-von Willebrand factor and 1:50 anti-endostatin). Thereafter, the specimens were washed 3 times for 5 min in PBS and incubated for 1 h with fluorescein isothiocyanate-conjugated swine anti-rabbit IgG (1:20 dilution) and fluorescein isothiocyanate-conjugated rabbit anti-mouse IgG (1:20 dilution) (Dako). To obtain nuclear staining, we washed the specimens in PBS and incubated them for 1 min in 1 mg/ml RNase in PBS, whereupon they were incubated with propidium iodide in PBS (50 mg/ml). To prevent bleaching damage, we used VECTASHIELD mounting medium (Vector Laboratories).

Scanning Confocal Microscopy

The overall architecture of blood vessels within the endostatin-treated tumors was assessed by serial 3-D reconstructions of confocal micrographs of 100-µm-thick frozen sections, immunostained for endothelial cells with an antibody against von Willebrand factor. The measurements were performed on day 29 for the s.c. tumors. We used a confocal laser scanning microscope (Leica TCS

Fig. 1. Effects of endostatin on s.c. tumor growth. The murine endostatin-treated tumors (0.3 mg/kg daily) showed a marked reduction in tumor volume (A) as well as in tumor weight at day 29 (B). Values are means $\pm$ SD, $n = 5$. Asterisk denotes $P < 0.05$.

NT, Leica Lasertechnik GmbH, Heidelberg, Germany) attached to an upright (Leica DM RXA) microscope. For the optical sectioning, a $16\times$ or $63\times$ Leica objective was used, with distilled water as immersion medium. To minimize further bleaching damage, and to obtain comparable results from experiments performed on different days, we analyzed every immunostained section with the same photomultiplier settings and the lowest amount of laser power. The optical sections were recorded with the native Leica software. Typically, 32 optical sections with a resolution of 512×512 per channel were recorded with an appropriate step size. Up to 8 pictures were averaged in order to improve the quality of the slow scan mode. After the measurements, the specimens were checked for bleaching damage. Fluorescein isothiocyanate was excited with the 488-nm line of an Ar-Kr laser. Tetramethyl rhodamine isothiocyanate was excited with the 568-nm line of the Ar-Kr laser, and the fluorescence yield through a long-pass filter (590 nm) was detected. The pinhole settings were optimized to gain maximal confocal quality.

The tagged image files were transferred to a Silicon Graphics O2 workstation and analyzed using Advanced Visual Systems software (Waltham, Mass.). The relative abundance of blood vessels within the tumor was assessed by measuring the amount of cells positive for von Willebrand factor (green fluorescence intensity) in defined areas (0.3 mm^2) on multiple tumor sections. Furthermore, the blood vessel diameters, as well as volumes were determined from the 3-D reconstructions of serial confocal sections. Details on image processing and data visualization in confocal laser scanning microscopy have been published elsewhere (Ehleben et al., 1997; Lorensen et al., 1987; Porwol et al., 1996; Strohmayer et al., 1997).

Statistical Analysis

A nested mixed model (repeated measurements analysis of variance) was used to analyze the tumor volumes. Measurements of tumor diameters from the same animal were

compared at different time intervals. The analysis was implemented using SPSS 10.0 (SPSS Inc., Chicago, Ill.). For detection of differences in the cellular and blood vessel density and the amount of endostatin present within the tissue as well as blood flows, the Mann Whitney U test was used. Significance was assumed for $P < 0.05$.

Results

Reduced Tumor Growth After Endostatin Treatment

When the tumors had grown s.c. for 29 days, the tumors of the rats that received 0.3 mg/kg murine endostatin between days 5 and 15 showed a marked reduction in size (Fig. 1A and 1B). Three days after the onset of the 10-day treatment, an inhibition of growth was observed. The reduced tumor growth among rats given endostatin persisted after the treatment ended on day 15 (Fig. 1A).

Rats bearing i.c. tumors had a 15% longer survival time compared with the control group after daily systemic injections of human endostatin ($P < 0.012$, $n = 5$). There was no evidence that survival time was improved by increasing the dose from 0.3 to 20.0 mg/kg.

Endostatin Induced Reduction of Tumor Blood Flow

For the endostatin-treated group, the blood flow to the s.c. tumors, when measured on day 29, was only about 50% of that observed in the controls ($P < 0.02$, Fig. 2A). No difference in blood flow to the heart, kidneys, or skeletal muscle was noted between treated and untreated rats ($P > 0.05$, Fig. 2B).

Changes in Tumor Morphology After Endostatin Treatment

Light microscopy revealed rather loosely connected cells in untreated tumors with a large number of hyperplastic endothelial cells (Fig. 3A). A marked reduction of

Fig. 2. Effect of endostatin on blood flow to s.c. gliomas. A. Intratumoral blood flow on day 29 in treated rats was about 50% of that in control rats, whereas blood flow to the heart, kidney, and skeletal muscle was unchanged. B. Blood flow to paired organs was very similar, indicating good mixing of indicator microspheres with arterial blood. Values are means $\pm$ SD, $n = 5$. Asterisk denotes $P < 0.05$.

endothelial cell hyperplasia was observed in the treated tumors. This was combined with an increased cellular density within the tumor parenchyma and the appearance of numerous apoptotic tumor cells (Fig. 3B and 3D). Very few apoptotic figures were observed in the control tumors (Fig. 3A and 3C).

Apoptosis

Flow-cytometric DNA measurements from the untreated tumors revealed a diploid as well as a triploid cell population, the diploid cells probably representing normal host cells within the tumor (Fig. 4A). In the treated tumors, the same 2 cell populations were observed, but an abundant number of fragmented cell nuclei were present, in increasing numbers toward the y-axis, indicating nuclear fragmentation (apoptosis) (Fig. 4B). The flow cytometric findings thus confirmed the TUNEL assay, where a consider-

able number of apoptotic cells were identified (Fig. 3D) compared with the control (Fig. 3C).

Immunofluorescence Microscopy

The scanning confocal micrographs revealed that within the endostatin-treated tumors, the blood vessel volume was markedly reduced. Control tumors exhibited an abnormal vascular morphology consisting of numerous tortuous blood vessels (Fig. 5A) that were to some extent normalized after endostatin treatment (Fig. 5B). The blood vessels in the endostatin-treated tumors also appeared to be narrower than in the untreated groups (Fig. 5C).

The relative abundance of blood vessels in the controls compared with the endostatin-treated animals was reduced by about 60% (Fig. 5D).

Discussion

In this study we have treated gliosarcomas, implanted s.c. as well as i.c. in rats, with soluble endostatin. We could show that endostatin markedly decreased tumor growth, led to a reduced vascular volume, decreased blood flow, increased apoptosis within the tumors, and prolonged survival in the treated rats. Direct measurements of tumor vascular volume and blood flow were performed. In contrast to the laser Doppler technique or digital imaging methods, the microsphere method permits determination of perfusion of discrete organs or well-defined parts within an organ (Buckberg et al., 1971). In particular, the present work confirms earlier observations on induction of apoptosis by endostatin. For instance, Dhanabal et al. (1999) have shown that endostatin treatment can lead to a marked reduction in anti-apoptotic intracellular proteins, such as Bcl-2 and Bcl-XL. Whether or not these events represent a direct mechanism of the action of endostatin is at present not clear, especially since it has been demonstrated that endostatin rapidly down regulates many genes in exponentially growing endothelial cells. These include, among others, immediate early response genes, cell cycle-related genes, and genes regulating apoptosis inhibitors (Shichiri and Hirata, 2001). It has also been shown that endostatin can bind to tropomyosin, which may result in a disruption of intracellular microfilaments that in turn may affect several intracellular signaling pathways (MacDonald et al., 2001).

The present work also shows that soluble human endostatin prolonged survival among rats with i.c. tumors. However, the effect of endostatin on the i.c. tumors was not as prominent as that observed for the same tumors grown s.c. This may be explained by the rather rapid i.c. growth of the copiously blood perfused BT₄C_n tumor within the noncompliant skull. This may also explain why endostatin showed no dose-dependent prolongation of survival in these animals. It is possible that the survival end point for the rats with i.c. tumors occurred before a sufficient accumulation of endostatin was achieved within the tumor. In the present work, blood flow to the i.c. tumors was not measured. This is based on the fact that there are large regional blood flow variations between the gray and white matter within the normal brain, complicating any

Fig. 3. Changes in tumor morphology after endostatin treatment. A. Immunoperoxidase staining of s.c. hyperplastic blood vessels in an untreated BT₄C_n tumor. Note the large number of blood vessels (arrows) situated between loosely connected tumor cells. B. After endostatin treatment, a severe reduction in blood vessels is seen (arrows) with an increased intratumoral cellular density. C and D. The TUNEL assay revealed few apoptotic cells in the control tumors (C), whereas numerous apoptotic figures were observed in the treated tumors (D) (arrows). All figures, original magnification $\times 170$; bar = 120 μm .

Fig. 4. Flow cytometry. The DNA histograms from the untreated tumors revealed a diploid as well as a triploid cell population. A. The diploid cells probably represent normal cells present in the tumor. B. In the endostatin-treated tumors, the same 2 cell populations were observed, but in addition, many fragmented cell nuclei were present.

Fig. 5. Immunofluorescence of blood vessels in s.c. gliomas. The overall architecture of blood vessels within the endostatin-treated, 29-day tumors was assessed by serial 3-D reconstructions of confocal micrographs of 100- μm -thick frozen sections, immunostained for endothelial cells with an antibody against von Willebrand factor. The 3-D data showed that, within the treated tumors, the blood vessel volume was considerably reduced. Control tumors exhibited rather chaotic clusters of distorted blood vessels (A), an arrangement that was to some extent normalized after endostatin treatment (B). The blood vessels in the endostatin-treated tumors also appeared to be narrower than those observed in the untreated groups, and the mean vascular diameters were markedly decreased after endostatin treatment (C). The relative abundance of blood vessels was also markedly reduced after endostatin treatment (D). A and B, original magnification $\times 120$.

interpretation of tumor flow data obtained with the microsphere method (Schuier et al., 1987).

As earlier mentioned, in the s.c. tumors, daily doses of murine endostatin as low as 0.3 mg/kg, over a period of 10 days within a 29-day observation time, induced a significant reduction in tumor size, intratumoral blood flow, and vascular volume. Marked endostatin effects on s.c. tumors were detected after only 10 days of daily endostatin treatment (Fig. 1). This may imply that a certain concentration of endostatin is needed within the tumor before reduced tumor growth is evident. As shown in Fig. 3B, the tumor cell density is considerably higher within the treated tumors, which have a limited vascular supply. This can be explained by an increased number of tumor cells within a defined volume as a result of reduced tumor cell invasiveness after endostatin treatment (Kim et al., 2000).

In conclusion, we show significant antitumor effects of endostatin given systemically as monotherapy. The effects were characterized by a reduction in s.c. tumor size, blood vessel volume, tumor blood flow, and prolonged survival. Furthermore, the treatment effects lead to markedly increased apoptosis within the treated tumors. Further studies should be aimed at combining endostatin therapy with both new and conventional treatment methods, including different methods and regimens of drug delivery.

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