Therapy of Hematogenous Melanoma Brain Metastases with Endostatin

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ABSTRACT

Purpose: Cerebral metastases represent the most common type of brain tumors. This study investigated the effects of endogenous endostatin on hematogenous cerebral melanoma metastases.

Experimental Design: Murine K1735 melanoma cells were transfected with the mouse endostatin cDNA. Experimental tumors were induced either by s.c. injection, intracerebral implantation, or via injection into the internal carotid artery to simulate hematogenous metastatic spread. The effects of endostatin expression on tumor incidence, growth pattern, and vascularity were analyzed.

Results: In vitro secretion of endostatin by 2.5 × 10^5 cells within 24 hours was 0.12 ± 0.03 ng, 4.35 ± 0.4, and 1.18 ± 0.7 ng/mL for wild type and two endostatin-transfected K1735 clones termed K1735-endo/2 and K1735-endo/8, respectively. Tumor inhibition in vivo correlated with endogenous endostatin production. Within 25 days, growth of s.c. K1735-endo/2 tumors was <20% compared with wild-type controls. Following intracerebral implantation the average survival time of mice was 27.8 ± 2.6 versus 13.3 ± 3.7 days in the K1735-endo/2 versus the wild-type group, respectively. Intracarotid injection of 1 × 10^5 wild-type cells killed the mice within 24 ± 1.8 days. In contrast, endostatin expression prevented macroscopic metastatic tumor growth in 11 of 12 mice, although viable microscopic tumor pockets were detectable in all animals.

Conclusion: Endostatin inhibits tumor progression of multiple cerebral metastases in vivo. Hematogenous micro-metastases are more efficiently suppressed than tumors resulting from high focal cell numbers which may be due to a higher angiogenic signaling exerted by massive cell deposits.

Endostatin may prevent solid tumor growth more effectively by inhibition of early angiogenesis.

INTRODUCTION

Brain metastases are the most common brain tumors in adults (1, 2). Melanomas represent the third most common primary tumors metastasizing to the brain. Compared with other primary tumors, melanomas have the highest propensity to produce cerebral metastases (3). Thus, up to 46% of clinical and up to 90% of autopsy cases of melanoma were found to have cerebral metastases (3). Once metastasized to the brain, the activity of extracerebral systemic disease progress (4, 5) and the number of cerebral metastases correlates with a poor outcome (6–9). Moreover, up to 45% to 75% of patients with cerebral metastases suffer from multiple lesions (5, 10). Melanoma metastases are particularly unresponsive to conventional therapies and many patients with intracranial metastases will die of cerebral lesions rather than of systemic disease within months (9, 11–13). The relative organ specificity of the metastasizing process, especially with regard to cerebral melanoma metastases, has been attributed to both tumor-specific factors as well as local factors that influence growth patterns and vascularization of tumor seeds in their respective environment (14). Thus, many tumors have already metastasized subclinically at the time of diagnosis and deposited micrometastases, which are clinically silent and undetectable by conventional imaging techniques. Some of these metastases remain dormant until they become vascularized and clinically apparent by yet unknown mechanisms, in particular after removal of the primary tumor (15, 16). This endogenous inhibition of distant metastases by the primary tumor has been attributed to angiogenesis inhibitors like angiotatin and endostatin, which are produced by the primary tumor. Endostatin is a 22-kDa COOH-terminal fragment of collagen XVIII α1 and was originally purified from a murine hemangioendothelioma (17). Angiotatin and endostatin are potent inhibitors of angiogenesis and therefore of tumor growth. Previous studies have shown that focal intracerebral tumors, such as experimental gliomas are amenable to antiangiogenic therapy (18–21).

The purpose of this study was to investigate the growth suppression of diffuse microscopic metastases by endostatin using a model of hematogenous spread of melanoma cells into the brain through an intact blood-brain barrier.

MATERIALS AND METHODS

Cell Lines. The murine K1735 melanoma cell line (kindly provided by Isaiah Fidler, M.D. Anderson Cancer Center, Houston, TX) was cultured from melanomas induced by UV irradiation of croton oil covered skin of C3H/HeN (MTV—)
mice (22). Cell lines and clones were maintained in DMEM (Life Technologies, Grand Island, NY) supplemented with 300 ng/mL t-glutamine, 10 mmol/L HEPES (Biochrom/Seromed KG, Berlin, Germany) and 10% fetal bovine serum. Cultures were grown as monolayers at 37°C in 5% CO2 and 95% air in a humidified atmosphere. Growth curves were obtained after plating of triplicates of 10,000 cells per well (9.6 cm²) of a 6-well plate followed by daily trypan blue counting until several cell layers formed or cells detached spontaneously. Migrational activity was measured in a modified Boyden chamber assay as previously described (23).

Transfection. The expression plasmid (kindly provided by Dr. Indraccolo, Padua, Italy) contained the murine endostatin sequence fused at the NH2 terminus to the human B29 immunoglobulin-related protein leader sequence as well as the neomycin resistance gene (24). Transfection of half-confluent monolayers was done with the FuGene transfection reagent according to the manufacturer’s protocol (Roche, Basel, Switzerland). Control transfections were carried out using either the vector alone or a plasmid containing the endostatin sequence in the antisense direction. Stably transfected clones were selected by geneticin (G418, Roche) treatment. Murine endostatin concentration was measured in the cell culture supernatant of 2.5 × 10⁵ cells conditioned for 24 hours using a specific ELISA (Chemicon International, Temecula, CA). In addition, endostatin-producing cells were identified by immunohistochemical staining using a monoclonal anti-mouse endostatin antibody (R&D Systems, Minneapolis, MN).

Animals. Six to 8-week-old female NMR1nu/nu nude mice were used for all transplantation studies. Mice were maintained under pathogen-free conditions according to international and institutional guidelines.

Subcutaneous Injections. Mice were injected s.c. with 2 × 10⁶ tumor cells suspended in 100 μL PBS into the right flank. On average, K1735 tumors started to grow visibly by day 10 post-implantation. Tumor growth was recorded every other day. The animals were killed when suffering from tumor burden but latest on day 25. Then, the tumors were resected, weighed, embedded in ornithine carbamyl transferase (Tissue-Tek, Miles, Elkhart, IN), quickly frozen in a dry ice/butane bath, and stored at −80°C.

Intracerebral Injection. Mice were anesthetized by i.p. injection of ketamine and xylazine. For intracranial implantation, 5 × 10⁴ cells suspended in a volume of 5 μL PBS were injected into the right frontal hemisphere using a stereotactic fixation device (Stoelting, Wood Dale, IL). To analyze early tumor formation, half of the animals were sacrificed 14 days after implantation, whereas the remaining animals were observed until they became obviously distressed or suffered from neurologic symptoms. The brains were removed, embedded in ornithine carbamyl transferase, quickly frozen in a dry ice/butane bath and stored at −80°C. Intracranial tumor size was determined after cryosectioning.

Injections into the Internal Carotid Artery. Experimental hematogenous brain metastases were produced by selective injection of tumor cells in the right internal carotid artery of the mice, as previously described (25). Mice were anesthetized by i.p. injection of ketamine and xylazine. The right common carotid artery was prepared using a dissecting microscope. The artery was nicked with a pair of microscissors; a glass cannula was inserted into the lumen and threaded forward into the internal carotid artery. After slow injection of 1 × 10⁵ cells in 100 μL PBS, the cannula was removed, the artery ligated with 7-0 silk, and the skin closed with 4-0 silk sutures. The experiment was designed to document tumor spread at two different time points: half of the mice of all groups were sacrificed after 21 days, upon which time first neurologic symptoms in the wild-type K1735 control group occurred. Survival times were recorded in the remaining groups over an observation period of up to 53 days when one animal developed signs of distress.

Histologic Studies. Immunohistochemistry was carried out on acetone-fixed 6-μm frozen sections using the Vectastain Elite avidin-biotin complex kit (Vector Laboratories, Peterborough, United Kingdom). Briefly, the sections were blocked with horse serum for 30 minutes, incubated with primary antibody against von-Willebrand Factor (polyclonal rabbit, Dako, Hamburg, Germany), CD31 (monoclonal mouse, Novocastra, Newcastle upon Tyne, United Kingdom), endostatin (monoclonal mouse, R&D Systems, Wiesbaden, Germany) or Ki67-nuclear antigen (monoclonal mouse, 1:100, Dako) overnight at 4°C and then washed with PBS twice for 10 minutes. Slides were then incubated with the appropriate secondary anti-IgG antibody for 60 minutes and washed. Detection was carried out with avidin-coupled horseradish peroxidase in the presence of chromogen resulting in a brown (3,3′-diaminobenzidine, Dako) or green (Histogreen, Linaris, Dossenheim, Germany) staining of positive cells within 5 to 15 minutes. Slides without the primary antibody served as negative controls. Sections were counterstained with eosin, nuclear fast red, or hematoxylin, depending on the contrasting chromogen and mounted in DePeX (Serva, Heidelberg, Germany).

Microvessel density was measured under light microscopy at 200-fold magnification in a single area of invasive tumor representative of the highest microvessel density (26). Every positive-staining endothelial cell or cell cluster that was separate from other microvessels was counted. The presence of a vascular lumen was not necessary to identify a microvessel (26). Glomeruloid clusters were counted as one microvessel.

For in situ detection of apoptosis, slides were incubated with 20 mg/mL proteinase K for 15 minutes at room temperature and quenched in 2% hydrogen peroxide in PBS for 5 minutes. Apoptosis was detected by the TUNEL method using the Apotag Kit (Oncor, Gaithersburg, MD) followed by counterstaining with nuclear fast red (Vector Laboratories). Sections of postpartum rat mammary gland (Oncor) served as positive control, and slides where terminal transferase was omitted served as negative control. The apoptotic index was defined as the percentage of positively stained cells per 100 nuclei from 10 randomly chosen fields per section, scored under light microscopy at 200× magnification. In addition, standard H&E staining of each tumor specimen was also done.

Statistics. All experiments were done with four to six animals per treatment group. All experiments were repeated at least once. Statistical analysis was carried out using the SPSS.
statistical software suite employing the Mann-Whitney U test and Wilcoxon rank test for in vivo experiments, the Student’s t test for in vitro experiments. \( P < 0.05 \) was considered to indicate statistical significance. Results are indicated as mean ± SD.

RESULTS

Stable Transfectants/Endostatin Production. For generation of stable endostatin expressing K1735 cell lines, 38 independent clones were selected and expanded after FuGene transfection of a plasmid containing the murine endostatin sequence. Secretion of endostatin was determined in triplicate cultures of 2.5 × 10^5 cells of each clone after 24 hours of cultivation. Baseline endostatin production by the parental cell line averaged at 0.12 ± 0.03 ng/24 hours (Fig. 1). This baseline expression was attributed to the expression of the precursor protein of endostatin, procollagen 18, because procollagen 18 as well as endostatin were detectable by immunohistochemistry in wild-type K1735 cells (data not shown). Two clones, K1735-endo/2 and K1735-endo/8, with secreted endostatin levels of 4.35 ± 0.4 and 1.18 ± 0.7 ng in 24 hours (\( P < 0.01 \) and \( P < 0.05 \), respectively, compared with parental wild-type cell line, Fig. 1) were chosen for further characterization. Overexpression of endostatin did not affect tumor cell proliferation or migration as measured in a modified Boyden chamber assay (data not shown).

Tumor Incidence and Survival

Subcutaneous Tumors. Following subcutaneous application of 2 × 10^6 cells of wild-type K1735 melanoma cells into the right flank of nude mice, ensuing melanomas reached an average diameter of 3 cm within 25 days. At this size, the overlying skin began to ulcerate and the animals exhibited signs of distress and were subsequently sacrificed. There was no difference in tumor growth between wild-type K1735 and K1735 transfected with the inversely oriented endostatin cDNA or vector alone (data not shown). Therefore, in all subsequent experiments, only wild-type K1735 cells were used as controls. After s.c. application of K1735-endo/8 and K1735-endo/2 cells, macroscopic disease was observed in 9 of 9 and 8 of 9 animals, respectively, within the observation period of 25 days (Table 1). Average s.c. tumor weight was lower in animals bearing K1735-endo/8 (not significant versus wild type) and K1735-endo/2 tumors (\( P < 0.05 \) endo/2 versus wild type, Fig. 2A) and was inversely correlated with endogenous endostatin expression as measured in vitro (Fig. 1).

Intracerebral Injection. After intracerebral injection of 5 × 10^4 melanoma cells into the right frontal lobe of mice, survival time was 13.3 ± 3.7, 14.1 ± 1.5, and 27.8 ± 2.6 days in mice receiving wild-type K1735, K1735-endo/8 (not significant versus wild type), and K1735-endo/2 cells (\( P < 0.05 \) versus wild type), respectively (Fig. 2B).

To evaluate the incidence of early tumor initiation depending on endogenous endostatin production, six mice per group were sacrificed 14 days post-implantation and focal tumor growth was recorded. Of these animals, all injected with wild-type or K1735-endo/8 melanoma cells developed tumors (Fig. 3A and B), whereas in animals receiving K1735-endo/2 cells smaller tumors were observed in 4 of 6 animals. However, upon histologic analysis using Ki67 and endostatin-specific antibodies, viable tumor cells were detectable in all cases.

Hematogenous Cerebral Metastases. The extent of cerebral metastases induced through injection of wild-type melanoma cells into the right internal carotid artery is shown in Fig. 3C to E. The distribution of wild-type metastases showed some crossover to the left side which is consistent with cross flow between the right and left ICA. Twenty-one days after ICA injection, mice from all groups were analyzed to monitor tumor incidence. Macroscopic intracerebral tumor growth was observed in all wild-type mice and in 4 of 6 mice, which received K1735-endo/8 cells. The average survival time of mice bearing wild-type K1735 metastases was 24 ± 1.8 days. Half of the K1735-endo/8 metastases bearing mice died within 28 ± 2.8 days, whereas the remaining mice survived the observation period of 53 days. Upon histologic examination of the remaining endo/8 mice, three mice showed diffuse microscopic tumor cell nests, whereas three mice did not show any viable tumor cells at all. In contrast, among mice injected with K1735-endo/2 cells, no visible tumor growth was detectable in eight animals after 21 days. The observation period was ended when one animal was 24 days.

Endostatin protein expression of K1735 melanoma

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<th>wt</th>
<th>endo/2</th>
<th>endo/8</th>
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<tr>
<td>ng/24h</td>
<td>0.12 ± 0.03</td>
<td>4.35 ± 0.4</td>
<td>1.18 ± 0.7</td>
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Fig. 1 Endostatin protein expression of wild-type K1735 (wt), K1735-endo/2, and K1735-endo/8 melanoma cells used for animal experiments measured by ELISA (top) and immunohistochemistry (bottom).
in the K1735-endo/2 group developed signs of distress after 53 days. Because survival of animals of this group was already significantly extended compared with the control group all remaining animals were sacrificed at this time and their brains obtained for further analyses. To this end, only one mouse developed macroscopically visible tumor in the K1735-endo/2 group (Fig. 3F), and a singular metastasis was detected upon low power magnification (Fig. 3G−H). However, in all other animals microscopic metastases were detected upon histologic examination using the anti-endostatin antibody (Fig. 3I).

**Histologic Examination.** Melanoma cells were easily recognized on regular H&E-stained slides by their grainy brown cytoplasmic melanin deposits (see Fig. 3D). As depicted in Fig. 3D, the macroscopic morphology of hematogenous metastases showed a tree-like branching pattern. Upon microscopic examination, numerous proliferating melanoma cell cuffs around vessels were observed in wild-type tumors and K1735-endo/8 tumors consistent with a high rate of vascular proliferation and tumor-related angiogenesis (Fig. 3K−M). Using the specific anti-mouse-endostatin antibody on brain sections, tumor micro-pockets and single metastatic K1735-endo/2 and K1735-endo/8 cells were detectable (Fig. 3J and N). In the K1735-endo/2 group, viable melanoma cells were mostly grouped around capillaries (Fig. 3N). However, despite the close relation to the vasculature, no multilayer perivascular cuffing or endothelial cell proliferation was observed.

**Vascular Morphology and Microvessel Density**

**Subcutaneous Tumors.** In subcutaneous tumors, a reduced microvessel count was correlated with the endogenous expression of endostatin as measured *in vitro*. Compared with normal s.c. tissue, microvessel density in wild-type K1735 tumors was higher (Table 2). K1735-endo/2 tumors, however, displayed a lower microvessel density than normal tissue. Apart from vessel paucity, highly endostatin-secreting K1735-endo/2 tumors revealed less irregular vessels, fewer mitotic figures, and hardly any necrotic areas when compared with wild-type and K1735-endo/8 tumors.

**Intracerebral Injection.** Fourteen days after stereotactic implantation of melanoma cells into the brain, wild-type tumors displayed the highest microvessel density followed by K1735-endo/8− and K1735-endo/2− tumors. In normal brain tissue, microvessel density was higher than in any tumor. Microvascular morphology was also affected by endostatin expression. Thus, both wild-type and K1735-endo/8 tumors grew as solid masses harboring irregular and coarse vessels with increased endothelial cell proliferation (Fig. 3K−M). In contrast, K1735-endo/2 tumors harbored vessels displaying a more regular and fine morphology (Fig. 3O−Q). The vessels were more uniform, less branched, and of relatively strong caliber with fewer capillaries (Table 2).

**Cerebral Metastases.** Hematogenous wild-type K1735 metastases were characterized by very coarse and irregular vessels with signs of endothelial cell proliferation (Fig. 3K and L). In comparison with wild-type metastases, K1735-endo/8-derived metastases displayed less irregular vessel morphology with an increased thickness of perivascular tumor cuffs (Fig. 3M).

Whereas among mice receiving K1735-endo/2 cells gross macroscopic disease was found in only one animal (Fig. 3G−H), and histologic analysis revealed the presence of micrometastases and single proliferating cells in all other animals (Fig. 3I, N and S). Microvessel density was not assessable due to limited solid tumor growth. However, K1735-endo/2 cells surrounded capillaries and grew along vascular structures, although strong multilayer perivascular cuffing was not observed (Fig. 3N). Accordingly, no endothelial cell proliferation was noted in K1735-endo/2 mice (Fig. 3P−Q).

**Proliferation and Apoptosis.** A comparison of proliferation and apoptosis between experimental groups was difficult, because endostatin-expressing tumors showed only perivascular cell nests but not solid tumor growth. Proliferative indices measured after staining of Ki67 nuclear antigen

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**Table 1  Tumor incidence**

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<th>Wild type</th>
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<td>S.c.</td>
<td>9/9</td>
<td>8/9</td>
<td>9/9</td>
</tr>
<tr>
<td>Intracerebral</td>
<td>6/6</td>
<td>4/6</td>
<td>6/6</td>
</tr>
<tr>
<td>ICA</td>
<td>12/12</td>
<td>1/12</td>
<td>6/12</td>
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NOTE. Incidence of macroscopic tumor growth in mice after s.c., intracerebral, or hematogenous application (ICA) of K1735 melanoma cells (endo/2: K1735-endo/2, endo/8: K1735-endo/8).

**Fig. 2** S.c. tumor weight (A) was measured 25 days after implantation of K1735 melanoma cells. Survival rate of mice after intracranial implantation of cells into the right frontal lobe is shown in B. *, *P < 0.05. wt, wild type; endo/2, K1735-endo/2; endo/8, K1735-endo/8.
Fig. 3 K1735-endo/8 (e/8) tumor shown 14 days after stereotactic injection into the right frontal lobe (A and B). Depiction of pan-hemispheric metastases 25 days after injection of wild-type (wt) melanoma cells into the right internal carotid artery (C-E). In contrast, the apparent lack of macroscopic K1735-endo/2 (e/2) tumor growth after ICA injection (F). The following slides represent immunohistochemical sections of hematogenous melanoma metastases 14 days after injection into the carotid artery. Using an anti-endostatin antibody (G-I, 3,3’-diaminobenzidine and hematoxylin counterstain), solid tumors and single K1735-endo/2 cells were visualized mostly around capillaries. Non-endostatin producing tumors were detectable by their grainy intracellular melanin deposits as shown in a hematoxylin stain of control tumor in J. Perivascular cuffing was observed in wild type K1735 and K1735-endo/8 melanomas (K and M). Early endothelial cell proliferation in L (endothelial cells stained for CD31 using Histogreen). Double labeling for endostatin (red-brown) and CD31 (green) localizes metastatic K1735-endo/2 cells around capillaries (arrows, N). O-Q, the only additional macroscopic solid K1735-endo/2 melanoma after direct intracerebral injection (O, anti-endostatin antibody; P-Q, anti-CD31 antibody). Tumor spread is within the subarachnoid space along the brainstem. There are only single cells or tumor micropockets within the adjacent brain and the right hemisphere. Ki67 staining of wild-type tumor (R) and K1735-endo/2 (S) tumors after ICA injection. Dispersed single K1735-endo/2 cells without development of solid tumor structures after ICA injection (S and T). Terminal deoxynucleotidyl transferase–mediated nick end-labeling staining of apoptotic nuclei (T, arrows, 3,3’-diaminobenzidine stain) in K1735-endo/2 brain metastases after arterial distribution.
revealed no significant differences between K1735-endo/2 tumors when compared with wild-type controls (4.0 ± 3.2 versus 3.1 ± 1.6, respectively). However, apoptotic indices quantified in situ by labeling fragmented DNA with terminal transferase using the TUNEL method correlated with endogenous endostatin expression as measured in vitro. Apoptotic indices were significantly lower in K1735-endo/2 compared with wild-type K1735 tumors (4.1 ± 0.7 versus 1.4 ± 0.8, respectively; \( P < 0.001 \)). As for wild-type tumors, a high degree of apoptosis was seen at the growing periphery of K1735-endo/2 and K1735-endo/8 tumors. In addition, apoptotic foci were often concentrated within perivascular areas of K1735-endo/2 tumors (Fig. 37).

**DISCUSSION**

We have shown that local endogenous endostatin production of melanoma metastasis reduces the incidence of macroscopic tumor growth without affecting proliferation of micrometastatic cells. This antitumorigenic effect was more pronounced in a hematogenic model of disseminated cerebral metastasis compared with direct s.c. or intracerebral tumors. Moreover, endostatin seems to inhibit metastasis formation in a dose-dependent fashion rendering micrometastases dormant without space-occupying effects.

Overexpression of endostatin did not influence morphology, proliferation, or migration of tumor cells, all of which are variables affecting viability and tumorigenicity before implantation. Previous reports showed that the effect of endostatin is based on the inhibition of the formation of a vascular bed by selective suppression of the endothelial cell compartment within a tumor (27). In our experiments, the tumor cell compartment remained unchanged with respect to proliferation. Thus, tumor cell—specific properties acting on the proliferative capacity seemed to be unaffected by the presence of endostatin. The reduced net growth of tumors seems to result from an increased apoptotic rate in endostatin-expressing tumors. Micrometastatic deposits of melanoma cells expressing endostatin displayed a reduced capacity to recruit a sufficient vascular bed when compared with wild-type melanoma cells. This finding is consistent with previous studies, which have shown that the proliferation of endostatin-producing tumors is counter-balanced by an increase in apoptosis (17, 19, 27, 28). Similar to endostatin, the antiangiogenic endogenous inhibitor angiostatin has been shown to inhibit tumor formation of intracranial tumors in a dose-dependent fashion (19).

There are several experimental approaches to model metastatic growth in vivo. First, the direct implantation of tumor cells into the target organ; second, the systemic hematogenous spread (e.g. via injection into the murine tail vein); third, the selective injection of tumor cells into the vascular bed of a particular organ. Whereas the first method produces reproducible focal tumor growth that can be monitored in any particular tissue environment, it does not mimic the many steps of the metastasizing process (i.e., invasion of the basal membrane of existing vasculature by metastatic cells, survival in the circulation, extravasation at a distant site, and induction of angiogenesis). Whereas the second method mimics most stages of systemic metastasis except for the first step, it produces metastases in various capillary beds. With particular interest in brain metastases, we therefore chose the organ-specific approach by selective injection of tumor cells into the internal carotid artery (25). In contrast to conventional transcranial deposition of tumor cells, this approach most elegantly mimics the process of hematogenous metastasis as it avoids local traumatization and preserves the physiologic environment including the blood-brain barrier (29).

We have shown that micrometastatic outgrowth is more efficiently suppressed by endostatin compared with tumor growth following massive tumor cell deposition as employed in the s.c. and transcranial implantation experiments. One explanation for this observation might be an overriding of endostatin signaling by proangiogenic cues that are relatively stronger in areas of higher cell density as in s.c. and transcranial tumor cell depositions. Along with the local trauma related to the implantation process itself, high cellular density may lead to low oxygen tension rendering these cells hypoxic. Hypoxic cells may be more subjected to selective mechanisms resulting in a more malignant phenotype. In addition, they induce a strong angiogenic response, for example by hypoxia related signaling through hypoxia-inducible factor and consecutively by vascular endothelial growth factor (VEGF; ref. 30). In this regard, tumor deposits with high focal cell number may behave more like prevascular microtumors rather than a micrometastatic single cell dispersion. Endostatin has been shown to inhibit VEGF-induced endothelial cell migration and tumor growth (31, 32). However, the antiendothelial activity of endostatin seems to be dependent on the order in which cells are exposed to endostatin or VEGF (33). Because single metastatic cells are usually not exposed to hypoxia within a given tissue environment, this might explain why the observed inhibitory effect of endostatin is more pronounced in the hematogenous metastases model. In contrast, focal implantation of high tumor cell numbers as in the s.c. and intracranial transplants creates ad hoc a hypoxic microcompartment which may induce an angiogenic response. Thus, tumor initiation and tumor growth resulting from high focal cell concentrations seems to counteract the effect of endogenous endostatin expression.

### Table 2: Microvessel count

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<th>Normal tissue</th>
<th>Wild type</th>
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<th>endo/8</th>
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<tr>
<td>S.c.</td>
<td>11 ± 4 fine, regular</td>
<td>18 ± 7 coarse, irregular</td>
<td>9 ± 9 fine, irregular</td>
<td>12 ± 11 coarse, irregular</td>
</tr>
<tr>
<td>Intracerebral</td>
<td>35 ± 7 very fine, regular</td>
<td>31 ± 7 coarse, irregular</td>
<td>5 ± 9 fine, irregular</td>
<td>29 ± 5 coarse, irregular</td>
</tr>
<tr>
<td>ICA</td>
<td>32 ± 7 very fine, regular</td>
<td>22 ± 10 coarse, irregular</td>
<td>--/-- fine, irregular</td>
<td>--/-- coarse, irregular</td>
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Note. Microvessel count and vascular morphology of tumors grown s.c., intracerebrally after stereotactic injection, or after intracarotid (ICA) injection. Microvessel count of K1735-endo/2 metastases could not be assessed reliably due to their limited size and number.
In line with these observations is the endostatin-mediated inhibition of benzanthracene-induced mammary tumors in rats (34). In this experimental model, tumor formation occurred de novo after exposure to the carcinogen. Endostatin was applied systemically when first palpable tumors occurred. In this model, endostatin was highly efficient in suppressing both growth of existing tumors and development of new tumors at an early stage before vascularization could be established (34).

Previous reports suggest that implantation site might influence tumor initiation and endostatin treatment effects as well. Overexpression of VEGF in experimental gliomas led to an increased angiogenic response if implanted orthotopically rather than s.c. (35). Moreover, endogenous endostatin secretion of rat C6 glioma xenografts was associated with a stronger tumor inhibition in the brain than s.c. (18). With respect to brain tumor development, it is important to note that s.c. tissue is essentially avascular requiring a classic type of angiogenesis for tumor growth. In contrast, the brain is highly vascularized allowing early tumor development to be less dependent on classic angiogenesis. In a similar cerebral melanoma metastasis model, Kusters et al. showed that even VEGF overexpression would not change a co-option type of vascularization, but rather promote tumor progression by dilation of co-opted vessels (36). In this report, only hematogenous brain metastases showed an initial cooption phenotype before new vasculature developed as compared with direct s.c. or intraparenchymal deposition of tumor cells. Endostatin might preferentially inhibit the vascularization by co-option compared with the classic sprouting type of angiogenesis. Although speculative, this explanation might be substantiated by the finding that the only macroscopic K1735-endo/2 metastasis grew within the poorly vascularized subarachnoid space, not in the parenchyma (Fig. 3O–Q). This tumor must have employed sprouting angiogenesis to grow. However, if this hypothesis holds up, one would expect to observe metastasis of the choroid plexus and the meninges more frequently.

To date, several transgenic mouse cancer models including spontaneous primary brain tumors have been developed (37, 38). These models provide an excellent opportunity to analyze the effect of angiogenesis inhibitors at different stages of tumor development. Thus, the Rip1-Tag2 model of pancreatic islet cell carcinomas employs the rat insulin II gene promoter (RIP) to target expression of the SV40 large T tumor antigen (Tag) oncprotein to cells of pancreatic islets (39). In order to investigate the role of angiogenesis on tumor formation RIP1-Tag2 transgenic mice were treated with a set of angiogenesis inhibitors beginning during the period of the angiogenic switch when islet cell carcinomas are formed (40). Treatment with AGM-1470, minocycline, and IFNα/β resulted in a substantial reduction in tumor size and capillary density, as well as increased apoptosis (40). In a second study, the effects of antiangiogenic therapeutic interventions at different stages of pancreatic islet carcinogenesis were compared (41). Specifically, mice were subjected to treatment either when bearing small asymptomatic tumors before the angiogenic switch came into effect or after substantial tumor burden had been established. In this study, endostatin as well as the combination of angiostatin and endostatin strongly inhibited tumor outgrowth by 60% to 63%, thus preventing tumor progression with high efficiency (41). Similarly, small tumors were growth suppressed, whereas treatment of larger tumors led to a prolongation of life span of mice by at least 2 weeks but did not prevent histologic progression to invasive carcinoma (41). Taken together, there is increasing evidence of early, prevascularized antimitastatic effects as a prominent part accounting for endostatin’s antitumor effects (42).

Recently, hypoxia was shown to stimulate VEGF production and concomitantly inhibit endostatin production in cultured endometrial stromal cells (43). Upon extrapolation, these data support the notion that endogenous endostatin expression in micrometastatic tumors is not inhibited and VEGF production not increased due to reduced or absent hypoxia compared with large tumor cell deposits and solid tumors.

An additional mechanism of endostatin action has been proposed in a model of hematogenous hepatic melanoma metastases. Mendoza et al. reported that endostatin inhibits microvascular arrest of circulating tumor cells as shown by endostatin treatment before or after metastatic vascular distribution (44, 45) leading to early metastatic inhibition. This effect was mediated by inhibition of vascular adhesion molecule-mediated adhesion of tumor to endothelial cells. In addition, endostatin reduced vascularity, increased hepatic sinusoidal endothelial cell apoptosis and macroscopic metastatic growth (44, 45). Although an effect on capillary arrest cannot be excluded in our experiments, the unchanged prevalence of micrometastatic K1735-endo/2 deposits at an early (day 21) and a late stage (day 53) of tumor development do not support this notion.

From a clinical perspective, multiple brain metastases continue to represent a major therapeutic challenge especially with respect to microscopic disease. We have excellent means at hand to successfully treat solid single and even multiple lesions (4, 46). However, micrometastases have not yet established a self-sustaining vascular bed and are not visible on advanced diagnostic imaging. At the same time, these micrometastases remain a fierceful threat to the patient. Conceptually, antiangiogenic treatment may help to keep micrometastatic disease dormant. Yet, the clinical applicability of endostatin remains speculative because the results of several phase 1 and phase 2 clinical trials reported thus far suggest no significant therapeutic advantage (47–49). Most of these clinical trials were carried out on end-stage tumor patients enduring tumors that already had an established vascular bed. The data presented here support the notion that the antitumorigenic potential of endostatin may be primarily related to its effects on early angiogenesis before the angiogenic switch. Therefore, a therapeutic strategy based on endostatin should be employed as early as possible before minimal residual postoperative or undiscovered, metastatic disease becomes clinically apparent.

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