Angiostatin Inhibits Bone Metastasis Formation in Nude Mice through a Direct Anti-osteoclastic Activity*

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Bone is a very common metastatic site for breast cancer. In bone metastasis, there is a vicious circle wherein bone-residing metastatic cells stimulate osteoclast-mediated bone resorption, and bone-derived growth factors released from resorbed bone promote tumor growth. The contribution of tumor angiogenesis in the growth of bone metastases is, however, unknown. By using an experimental model of bone metastasis caused by MDA-MB-231/B02 breast cancer cells that quite closely mimics the conditions likely to occur in naturally arising metastatic human breast cancers, we demonstrate here that when MDA-MB-231/B02 cells were engineered to produce at the bone metastatic site an angiogenesis inhibitor, angiostatin, there was a marked inhibition in the extent of skeletal lesions. Inhibition of skeletal lesions came with a pronounced reduction in tumor burden in bone. However, although angiostatin produced by MDA-MB-231/B02 cells was effective at inhibiting in vitro endothelial cell proliferation and in vivo angiogenesis in a Matrigel implant model, we have shown that it inhibited cancer-induced bone destruction through a direct inhibition of osteoclast activity and generation. Overall, these results indicate that, besides its well known anti-angiogenic activity, angiostatin must also be considered as a very effective inhibitor of bone resorption, broadening its potential clinical use in cancer therapy.

Bone is a very common metastatic site for human breast, prostate, lung, kidney, and thyroid cancer (1, 2). Bone metastasis is associated with a high morbidity rate because of intractable bone pain, pathological fractures, hypercalcemia, and nerve compression (1, 2). Current experimental studies support the notion that there is a vicious circle at the bone metastatic site at which bone-residing metastatic cells stimulate osteoclast-mediated bone resorption and bone-derived growth factors released from resorbed bone promote tumor growth (2). These findings (2) have provided the rationale for using bone resorption inhibitors (e.g. bisphosphonates) in the treatment of patients with bone metastases (1, 3). However, these treatments are only palliative and do not provide a life-prolonging benefit to metastatic patients (3). There is, therefore, a need to define new therapeutic targets that could become useful adjunct therapies for the treatment of cancers that metastasize to bone. Tumor-associated neovascularization is necessary for the growth and metastatic spread of solid tumors (4, 5). Angiogenesis inhibitors have been shown to be very effective in the treatment of a wide variety of solid tumors in animals (6). Based on these successful preclinical studies, over 20 antiangiogenic drugs are currently undergoing evaluation in phase I, II, or III clinical trials (6). The contribution of angiogenesis inhibitors in inhibiting the formation of bone metastases caused by solid tumors is, however, unknown. There is some experimental evidence to indicate that human MDA-MB-231 breast cancer cells cause the development of highly vascularized skeletal lesions in animals (7), and, interestingly, the systemic treatment of animals with the anticancer drug TNP-470 (a synthetic analogue of fumagillin with antiangiogenic properties) reduces the formation of bone metastases caused by MDA-MB-231 cells (8). However, the antiangiogenic activity of TNP-470 was not clearly addressed in this study, and the doses used to inhibit bone metastasis formation exhibit some toxicity (8), thereby limiting its clinical utility in the treatment of patients with bone metastases. However, these results (8) do not preclude the use of more specific angiogenesis inhibitors in treating bone metastases.

By using an experimental model of bone metastasis caused by MDA-MB-231/B02 breast cancer cells that quite closely mimics the conditions likely to occur in naturally arising metastatic human breast cancers (9), we demonstrate here that when MDA-MB-231/B02 cells were engineered to produce at the bone metastatic site a specific angiogenesis inhibitor, angiostatin, there was a marked inhibition in the extent of skeletal lesions and a concomitant reduction in the tumor burden in bone. Unexpectedly, beside its ability to inhibit angiogenesis in vitro and in vivo models, we have shown for the first time that angiostatin inhibits cancer-induced bone destruction through a direct inhibition of the osteoclast activity.

**EXPERIMENTAL PROCEDURES**

Construction of Mock- and Angiostatin-inducible Cell Lines Using the Tet-Off-Regulated Expression System—Characteristics of MDA-MB-231/B02 and MDA-MB-231/B02-GFP breast cancer cell lines have been described elsewhere (9, 10). The bi-directional vector pBlU-NAST was constructed by inserting into the pBlU-plasmid (Clontech, Hampshire, United Kingdom) the HindIII/EcoRV fragment encoding for the entire mouse angiostatin sequence (kringles 1–4) and an antigenic epitope tag (hemagglutinin, HA) fused to the COOH terminus of kringles 1–4 (11).

The abbreviations used are: HA, hemagglutinin; TTA, tetR-VP16 protein chimer; TRAP, tartrate-resistant acid phosphatase; VEGF, vascular endothelial growth factor.
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The pTet-Off vector (Clontech) encoding for the tetracycline responsive transactivator (tTA) was co-transfected in the MDA-MB-231/B02-GFP cell line together with a plasmid encoding hygromycin resistance (B02-GFP/TA cells). Stable B02-GFP/TA cells were then Co-transfected with the pBiL/mAST construct and a vector conferring puromycin resistance. A negative control cell line was obtained by introducing into B02-GFP/tTA cells a pBi-L bidirectional Tet-inducible vector encoding for luciferase and β-galactosidase (Clontech). Luciferase induction upon doxycycline withdrawal was used to select inducible clones among stable transfectants. During clonal selection, angiostatin expression was suppressed by the constant addition of doxycycline (100 ng/ml), and the selection of the clones was obtained after growing the cells for 2 weeks in the presence of doxycycline (2 μg/ml). Two angiostatin-inducible transfectant cell lines (B02-GFP/mAST#1 and B02-GFP/mAST#2) were used in the present study. Inducible transfectants were routinely cultured in RPMI 1640 medium (Invitrogen) supplemented with 10% (v/v) fetal bovine serum (Bio-Media, Bous sens, France) and 1% (v/v) penicillin/streptomycin (Invitrogen) at 37 °C in a 5% CO2 humidified incubator. When required, serum-free conditioned media were prepared from transfected or not treated with doxycycline, in the absence of any other antibiotics.

Luciferase Activity Measurement, Western Blotting, and Angiostatin Purification—Luciferase activity was measured on cell lysates following manufacturer’s instructions (Promega, Charbonnière, France) using a luminometer (Berthold, La Garenne Colombes, France). Lysis was carried out with a 2% saline-heparinized solution. Luciferase blot analysis for the immunodetection of angiostatin in conditioned media was performed as previously described (11), with minor modifications. Briefly, conditioned media (2 ml) were incubated overnight at 4 °C with 100 μl of 50% lysine-Sepharose (Amersham Biosciences, Saclay, France). After incubation, Sepharose beads were washed to remove unbound proteins and Sepharose-bound proteins were released by the addition of 30 μl of SDS sample buffer followed by heating at 95 °C for 3 min. Proteins were electrophoresed on a 10% SDS-polyacrylamide gel and transferred to polyvinylidene difluoride membranes. Angiostatin was detected by incubating the membranes with mouse monoclonal anti-HA antibody 12CA5 (Roche Diagnostics, Meylan, France) with a code of practice established by the Experimentation Review Committee (Comité d’Étude, College Park, MD), according to the manufacturer’s instructions (ICN Pharmaceuticals, Orsay, France). 5-μm tumor sections were then subjected to immunohistochemistry using a rat anti-mouse CD31 (PECAM-1) monoclonal antibody that specifically recognizes murine endothelial cells (BD Biosciences). The immunostained blood vessel area/tumor area ratio (expressed as a percentage) was quantified by using a computerized image analysis system (Visiolab 2000, Biocom). Histological analyses were performed on longitudinal medial sections of tibial metaphysis by using a computerized image analysis system (Visiolab 2000, Biocom).

Immunohistochemistry and Assessment of the Microvessel Density—Subcutaneous tumor grafts were fixed and paraffin-embedded according to the manufacturer’s instructions (ICN Pharmaceuticals, Orsay, France). 5-μm tumor sections were then subjected to immunohistochemistry using a rat anti-mouse CD31 (PECAM-1) monoclonal antibody that specifically recognizes murine endothelial cells (BD Biosciences). The immunostained blood vessel area/tumor area ratio (expressed as a percentage) was quantified by using a computerized image analysis system (Visiolab 2000, Biocom) and used to assess the microvessel density. Blood vessels at the bone metastatic sites were quantified by using a method described by Barou et al. (13). Briefly, mice were anesthetized and the left ventricle was catheterized, whereas the right auricle was sectioned to allow perfusion fixation. The vascular network was first rinsed with a 2% saline-heparinized solution, fixed with a 4% paraformaldehyde solution, and finally stained with an India ink solution. Metastatic long bones were then prepared for histological examination, and the area of tumor blood vessels stained with India ink was quantified as described above.

Angiogenesis Assays—In vitro and in vivo Angiogenesis Assays—In vitro endothelial cell proliferation and capillary-like tube formation assays were performed as described previously (14). For the in vivo Matrigel plug assay, experiments were carried out essentially as described by Maeshima et al. (15). Growth factor-depleted Matrigel (BD Biosciences) was mixed with 20 units/ml heparin (Dakota Pharm, Créteil, France), 50 ng/ml vascular endothelial growth factor (R&D Systems, Minneapolis, MN) or an equivalent volume of PBS, and 20 μg/ml of purified angiostatin. The Matrigel mixture was injected subcutaneously into 8-week-old male BALB/C nude mice (Charles River Laboratories). After 6 days, mice were killed and the Matrigel plugs were removed and fixed in 4% paraformaldehyde. The plugs were paraffin-embedded, cut, and stained with hematoxylin and eosin. Sections were examined by light microscopy, and microvessel density of 6–15 high power fields (×400) was counted and averaged using true-color imaging-processing work station Visiolab 2000 (Biocom). All sections were coded and examined in a blind fashion. Each experimental group consisted of 5–6 Matrigel plugs. The microvessel density, i.e. the microvessel area/Matrigel section area ratio, was expressed as a percentage of the untreated bone metastasis formation.

VEGF Immunoassay and Mouse Angiostatin Quantification—The VEGF content in tumor-conditioned media was measured using an enzyme-linked immunosorbent assay kit for the human protein (Cytimmune, College Park, MD), according to the manufacturer’s instructions. Angiostatin content in tumor-conditioned media was quantified by immunoassay, increased cleavage of biotinylated mouse angiostatin was used as a standard. The intensity of each immunoreactive band was then quantified by using the computerized image analysis system ImageQuant (Amersham Biosciences), and data were expressed in ng/ml of cell culture-conditioned medium.

In Vitro Osteoclastogenesis Assay and in Situ Localization of Osteoclasts—The bone metastasis formation experiments were carried out using 8-week-old female OF1 mice (Charles River Laboratories). Spleens were collected and homogenized through a 70-μm mesh (BD Biosciences). The mononuclear cells were isolated using lymphocyte separation media (ICN Pharmaceuticals) and seeded in cell culture
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**RESULTS**

**Structural and Functional Characterization of Angiostatin-inducible MDA-MB-231/B02-GFP Cell Transfectants—Two clones, B02-GFP/mAST#1 and #2, were selected on the basis of their specific and high expression levels of luciferase and angiostatin upon doxycycline withdrawal (Fig. 1a). As a negative control cell line, mock-transfected cells were established by replacing the cDNA for angiostatin to β-galactosidase (B02-GFP/βGal) (Fig. 1a). Therefore, the ability of angiostatin to inhibit angiogenesis in vitro and in vivo was examined. Conditioned media from B02-GFP/mAST clones cultured in the absence of doxycycline induced a 50–60% inhibition of VEGF-mediated endothelial cell proliferation (Fig. 1b) and capillary-like tube formation in vitro (data not shown). To test the ability of angiostatin to inhibit angiogenesis in vivo, angiostatin was first purified by lysine-Sepharose chromatography (Fig. 1c) and then used to study its effect on VEGF-induced angiogenesis with a Matrigel plug assay. As shown in Fig. 1d, plugs containing VEGF were well vascularized, whereas VEGF-induced angiogenesis was almost completely inhibited in the presence of angiostatin. Image analysis quantification showed that angiostatin induced an 80% reduction of the neovascularization (Fig. 1d). Overall, our data indicate that angiostatin produced by the transfectants was biologically active. These results were in complete agreement with previous reports...**
showing that angiostatin inhibits endothelial cell proliferation (16) and in vivo angiogenesis in the Matrigel implant model (17).

**Angiostatin Inhibits the Formation of Osteolytic Bone Metastases Caused by Breast Cancer Cells in Vivo**—We have shown previously that fluorescent MDA-MB-231/B02-GFP#2 cells have the unique property of metastasizing to bone after intravenous tumor cell inoculation into the tail vein of female BALB/C nude mice (9, 10). In addition, radiography and external fluorescence imaging of live metastatic animals allow the measurement of the extent of osteolytic lesions and tumor burden, respectively (9). Using this bone metastasis model, the follow-up of B02-GFP/mAST#2-bearing animals by radiography showed a marked decrease in the extent of osteolytic lesions in untreated animals compared with that observed in doxycycline-fed animals (Fig. 2, a and b and Table I). The extent of tumor burden in B02-GFP/mAST#2-bearing animals was also significantly decreased upon doxycycline withdrawal (Fig. 2, c and d and Table I). Importantly, as shown in Fig. 2, b and d, the derepression of angiostatin did not delay the occurrence of bone metastases but rather inhibited their progression, indicating that angiostatin did not prevent the homing of breast cancer cells to bone. Similar results were obtained when comparing untreated and doxycycline-treated animals bearing B02-GFP/mAST#1 cells (Table I).

Histological examination of metastatic long bones from doxycycline-fed animals revealed that most of the cancellous and cortical bone in proximal tibiae was destroyed and replaced by tumor cells that completely filled the bone marrow cavity (Fig. 2e). In contrast, upon derepression of angiostatin, the cortical bone remained intact and the cancellous bone was only partially destroyed, whereas small to moderate tumor foci were confined to the bone marrow cavity (Fig. 2e). Histomorphometric analyses of metastatic hind limbs confirmed radiographic and fluorescence
analyses and showed that angiostatin not only inhibited the bone loss caused by breast cancer cells (Fig. 2f) but also drastically reduced the skeletal tumor burden (Fig. 2g).

Angiostatin Does Not Inhibit the Growth of Subcutaneous MDA-MB-231/B02 Breast Tumor Xenografts—To determine whether angiostatin has a direct growth-inhibitory effect on MDA-MB-231/B02 breast cancer cells in vivo, B02-GFP/mAST cells were implanted subcutaneously into nude mice. Tumor growth of B02-GFP/mAST#1 and #2 cells in animals was the same, regardless of the presence or absence of the doxycycline repressor (Fig. 3a and results not shown). This lack of effect of angiostatin on tumor growth could not be attributed to a defect of the Tet-Off repressing system, because the luciferase activity in tumor cell extracts from doxycycline-fed animals was drastically repressed when compared with that observed in untreated animals (Fig. 3b). Moreover, upon doxycycline withdrawal, circulating HA-tagged angiostatin was immunodetected at the expected size in the plasma of B02-GFP/mAST#1 tumor-bearing animals (Fig. 3a, inset), indicating that angiostatin degradation did not account for the absence of tumor growth inhibition. To further document this apparent lack of effect of angiostatin on tumor growth in vivo, the immunohistochemical analysis of tumor xenografts was carried out using an anti-CD31 antibody that specifically recognizes murine endothelial cells. There was no difference in the microvascular density of tumors from untreated and doxycycline-fed animals (Fig. 3c). Moreover, despite a substantial inhibition of osteolytic lesions and skeletal tumor burden in untreated animals, the extent of tumor vascularization in bone lesions from untreated and doxycycline-fed animals was the same, as judged by ink staining of tumor blood vessels (Fig. 3d). These results were rather unexpected, because angiostatin purified from

| Cell lines               | Radiographya | Fluorescence imaginga |
|-------------------------|--------------|-----------------------|
|                         | + Dox (%)    | - Dox (%)             | + Dox (%)   | - Dox (%)   |
| B02-GFP#2              | 8.1 ± 0.3 (9) | 2.7 ± 0.2 (12)       | 8.1 ± 0.3 (9) | 2.7 ± 0.2 (12) |
| B02-GFP/βGal           | 8.1 ± 0.3 (9) | 2.7 ± 0.2 (12)       | 8.1 ± 0.3 (9) | 2.7 ± 0.2 (12) |
| B02-GFP/mAST#1         | 8.1 ± 0.3 (9) | 2.7 ± 0.2 (12)       | 8.1 ± 0.3 (9) | 2.7 ± 0.2 (12) |
| B02-GFP/mAST#2         | 8.1 ± 0.3 (9) | 2.7 ± 0.2 (12)       | 8.1 ± 0.3 (9) | 2.7 ± 0.2 (12) |

Data are the mean ± S.E. of six animals per group and are expressed in mm². b, histograms showing the luciferase activity in tumors collected at the day the animals were killed (day 60). As expected, doxycycline (Dox) repressed the luciferase activity. Inset, immunodetection (at day 60) of angiostatin in the plasma of GFP-βGal-bearing animals (left lane), Dox-treated GFP-mAST#1-bearing animals (middle lane), and untreated GFP-mAST#1-bearing animals (right lane). Angiostatin (arrow) was immunodetected only in the absence of Dox. c, angiogenesis in B02-GFP/mAST#1 tumor tissue sections after immunostaining of blood vessels with an anti-CD31 antibody. CD31-positive microvessels are stained in brown. d, assessment of tumor angiogenesis at the bone metastatic site 29 days after intravenous inoculation of B02-GFP/mAST cells into nude mice. Animals were perfused with India ink, and hind limbs were subjected to histological analysis. Blood vessels are stained in gray and tumor cells in blue. Values shown on the upper left of each panel correspond to the extent of the vascularized area (in %) in tumors from animals treated (+) or not treated (−) with Dox. Results are the mean ± S.E. of five to nine animals per group. Bar, 100 µm.

**Figure 3.** Angiostatin does not inhibit subcutaneous breast tumor growth in vivo. a, MDA-B02-GFP/mAST#1 cells were inoculated subcutaneously into the flank of nude mice. The tumor volume from animals treated (black squares) or not treated (white squares) with doxycycline was assessed by bilateral Vernier caliper measurement. Values are the mean ± S.E. of six animals per group and are expressed in mm³. b, histograms showing the luciferase activity in tumors collected at the day the animals were killed (day 60). As expected, doxycycline (Dox) repressed the luciferase activity. Inset, immunodetection (at day 60) of angiostatin in the plasma of GFP-βGal-bearing animals (left lane), Dox-treated GFP-mAST#1-bearing animals (middle lane), and untreated GFP-mAST#1-bearing animals (right lane). Angiostatin (arrow) was immunodetected only in the absence of Dox. c, angiogenesis in B02-GFP/mAST#1 tumor tissue sections after immunostaining of blood vessels with an anti-CD31 antibody. CD31-positive microvessels are stained in brown. d, assessment of tumor angiogenesis at the bone metastatic site 29 days after intravenous inoculation of B02-GFP/mAST cells into nude mice. Animals were perfused with India ink, and hind limbs were subjected to histological analysis. Blood vessels are stained in gray and tumor cells in blue. Values shown on the upper left of each panel correspond to the extent of the vascularized area (in %) in tumors from animals treated (+) or not treated (−) with Dox. Results are the mean ± S.E. of five to nine animals per group. Bar, 100 µm.
B02-GFP/mAST cells was a potent inhibitor of angiogenesis in vivo (Fig. 1, d and e). Therefore, we hypothesized that B02-GFP/mAST#1 and -#2 cells could produce angiogenic factors that counterbalance the anti-angiogenic effect of angiostatin. Indeed, B02-GFP/pGal, B02-GFP/mAST#1 and -#2 cells produced substantial amounts of VEGF (between 6–18 ng/ml), irrespective of the presence or absence of doxycycline. Upon doxycycline withdrawal, the amount of angiostatin produced by B02-GFP/mAST#1 and -#2 cells varied between 178–339 ng/ml. Assuming a molecular mass of 38 kDa for angiostatin and 45 kDa for VEGF, the stoichiometric relationship of angiostatin to VEGF was about 30:1. Using this ratio, angiostatin (1500 ng/ml) did not inhibit angiogenesis induced by VEGF (50 ng/ml) in the Matrigel plug assay (results not shown), whereas it did so at a 10-fold higher concentration (Fig. 1, d and e). Therefore, it is most conceivable that VEGF produced by B02-GFP/mAST#1 and -#2 cells did not modify the proliferation and invasion capabilities of transfectants in vitro as well as their adhesion to bone cortical slices, indicating that angiostatin in vivo was acting on cells other than metastatic cells (results not shown). Because bone-residing metastatic cells stimulate osteoclast-mediated bone resorption (2), we hypothesized that angiostatin could directly inhibit osteoclast functions.

**DISCUSSION**

Tumor-associated neovascularization is necessary for the growth and metastatic spread of solid tumors (4, 5). The contribution of angiogenesis in the development of bone metastases caused by solid tumors is, however, largely unknown. To address this question, we used an experimental model of bone metastasis in which MDA-MB-231/B02 breast cancer cells were engineered to produce at the bone metastatic site a specific angiogenesis inhibitor, angiostatin. To study the function of angiostatin during bone metastasis formation, we used a Tet-Off system in which the derepression of angiostatin was achieved in the absence of doxycycline, thus avoiding any influence of the drug on the angiostatin-induced phenotype. Surprisingly, as opposed to the data presented here, Duivenvoorden et al. (18) have recently reported that the treatment of animals with subcutaneously implanted doxycycline-containing pellets (10 mg/pellet) decreases the formation of skeletal lesions caused by MDA-MB-231 cells. Different circulating doxycycline concentrations cannot account for the difference in results observed in the study by Duivenvoorden (18) and our own study, because the estimated doxycycline plasma concentration in our model was much higher (5 mg/liter) than that reported in their bone metastasis model (0.21 mg/liter). The reasons for this discrepancy are, therefore, currently unclear. However, despite a much higher doxycycline plasma concentration, we clearly observed no inhibitory effect of doxycycline on bone metastasis formation (Table I).
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Using two different angiostatin-inducible B02-GFP/mAST transfectants, we provided clear evidence that angiostatin markedly inhibited the progression of osteolytic lesions. Unexpectedly, although a large number of studies (including our own) already demonstrate that angiostatin exerts anti-angiogenic properties, we demonstrated here that angiostatin inhibited cancer-induced bone destruction through a direct inhibition of osteoclast functions. This contention is supported by the fact that (i) angiostatin substantially decreases the number of osteoclasts at the bone/tumor interface in metastatic hind limbs, and (ii) it inhibits osteoclast generation in vitro. In osteolytic lesions, there is a vicious circle in the bone microenvironment wherein bone-residing metastatic cells stimulate osteolysis-mediated bone resorption, and bone-derived growth factors released from resorbed bone promote tumor growth (2). Angiostatin interrupts this vicious circle. By inhibiting bone resorption, angiostatin indirectly limits the release of bone-derived growth factors which, in turn, reduces skeletal tumor growth. This assumption is supported by the fact that angiostatin reduces skeletal tumor burden (Fig. 2), whereas it does not inhibit the proliferation of B02-GFP/mAST#1 and -#2 cells in vitro (results not shown).

The molecular mechanisms through which angiostatin inhibits osteoclast functions are currently unknown. In endothelial cells, angiostatin binds to integrin αvβ3 (19), angiomotin (20), and cell-surface ATP synthase (21). Osteoclasts also express integrin αvβ3 (22), and integrin αvβ3 antagonists inhibit bone resorption in vitro (23). Therefore, it is possible that angiostatin inhibits bone resorption by binding to integrin αvβ3 expressed by osteoclasts. This very important question deserves further attention.

In conclusion, this study is, to the best of our knowledge, the first one to demonstrate that angiostatin is a strong inhibitor of osteoclast-mediated bone resorption. Besides its well-known anti-angiogenic activity, therefore, angiostatin must be considered to be a very effective inhibitor of osteolytic bone metastases, thus broadening its potential clinical use in cancer therapy.

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