Estradiol and Tamoxifen Regulate Endostatin Generation via Matrix Metalloproteinase Activity in Breast Cancer *In vivo*

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Abstract

Matrix metalloproteinases (MMP) are important regulators of tumor progression and angiogenesis. MMPs generate both proangiogenic and antiangiogenic fragments, such as vascular endothelial growth factor and endostatin. The *in vivo* activation of MMPs and endostatin generation occur mainly in the extracellular environment by interactions of different cell types. Therefore, these processes are necessary to study in the extracellular space *in vivo*. Sex steroids play a dominant role in breast carcinogenesis, by largely unknown mechanisms. In the present study, we used *in vivo* microdialysis to directly quantify MMP-2 and MMP-9 activity and sample endostatin from both stroma (murine) and tumor (human) cells *in vivo* in solid MCF-7 tumors in nude mice. We found that tamoxifen in combination with estradiol increased tumor MMP-2/MMP-9 *in vivo* activity, endostatin levels, and decreased tumor vascularization compared with estradiol treatment only. The stroma-derived endostatin was three to five times higher than cancer cell–generated endostatin. After inhibition of MMP-2/MMP-9, endostatin levels decreased, providing evidence that these proteases are highly involved in the generation of endostatin. Our results support the previously reported concept that MMPs may serve as negative regulators of angiogenesis. The regulation of endostatin generation by modulation of MMP-2/MMP-9 activities suggests a previously unrecognized mechanism of estradiol and tamoxifen, which may have implications for the pathogenesis of breast cancer. (Cancer Res 2006; 66(9): 4789-94)

Introduction

Tumor cell invasion and metastasis is facilitated by proteolytic enzymes that digest the extracellular matrix of the surrounding tissue (1). The matrix metalloproteinases (MMP) constitute a large family of zinc-dependent endopeptidases, which are secreted as latent proenzymes and require proteolytic cleavage to become catalytically active (2). The *in vivo* activation of MMPs occurs mainly in the extracellular environment, either by autoactivation or with the help of a variety of other proteinases (3), and is tightly controlled by endogenous tissue inhibitors, such as tissue inhibitor of metalloproteinases (4–6). The extracellular matrix serves as a reservoir for bioactive molecules, and by reason of their proteolytic capacity, MMPs may regulate the bioavailability and/or activity of several growth factors, cytokines, and adhesion proteins (7, 8). Due to their matrix-degrading abilities and high expression in advanced tumors, MMPs are largely implicated in promoting cancer cell invasion, metastasis, and angiogenesis (2, 9–11). However, recent studies have highlighted the function of MMPs as negative regulators of angiogenesis by their release of antiangiogenic fragments, such as angiostatin, endostatin, and tumstatin (7, 8, 12–15).

Human endostatin is a 20-kDa proteolytic fragment of the COOH-terminal domain NC1 of collagen XVIII and an efficient inhibitor of tumor angiogenesis (16–18). Endostatin causes tumor regression by acting as an inhibitor of endothelial cell proliferation and migration and inducing apoptosis in proliferating endothelial cells (17, 19). Furthermore, endostatin may also down-regulate vascular endothelial growth factor (VEGF)/VEGF receptor (VEGFR) signaling by a direct action on tumor cells or up-regulate other antiangiogenic genes (20, 21). Sex steroids affect the risk of breast cancer occurrence and recurrence, but the mechanisms are still unclear (22, 23). The majority of breast cancers have maintained their estrogen dependency, and antiestrogen treatment is the cornerstone in the treatment of these patients. Sex steroids are potent regulators of angiogenesis in the reproductive tract, but the knowledge of sex steroid–dependent regulation of angiogenesis in breast cancer is limited. We have previously shown that estradiol increased tumor angiogenesis and VEGF secretion in hormone-dependent breast cancer models, whereas tamoxifen, the most widely used nonsteroidal antiestrogen, induced an antiangiogenic response (24–26). If estrogen and tamoxifen affect MMP activities and the generation of endostatin in breast cancer is not known. The *in vivo* activation of MMPs and generation of endostatin occur mainly in the extracellular environment as a result of cell-cell interactions. Hence, it is imperative to investigate these proteins directly in this milieu. Microdialysis is a technique that mimics a blood vessel, and with this technique, it is possible to collect molecules from the interstitial space in a specific tissue or organ *in situ* (27).

In the present study, we investigated the effects of estradiol and tamoxifen on the *in vivo* activity of MMP-2 and MMP-9 and the generation of endostatin. We used microdialysis to sample endostatin and to directly quantify MMP-2/MMP-9 locally in tumor tissue *in vivo*. Our results suggest that there is a hormonal regulation of extracellular endostatin in estrogen receptor–positive breast cancer, and that this generation, at least in part, is dependent on MMP-2/MMP-9 activity.

Materials and Methods

Cells and culture conditions. MCF-7 (HTB-22; human breast adenocarcinoma, estrogen receptor and progesterone receptor positive) cells were obtained from the American Type Culture Collection (Manassas, VA). Cells were cultured in DMEM without phenol red supplemented with 2 mmol/L glutamine, 50 IU/mL penicillin-G, 50 μg/mL streptomycin, and 10% fetal bovine serum at 37°C in a humidified atmosphere of 5% CO_2. Cell culture medium and additives were obtained from Invitrogen (Carlsbad, CA) if not otherwise stated.

Hormone treatment of MCF-7 cells in culture. Confluent cells (10,000 per cm^2) were trypsinized (0.05% trypsin and 0.02% EDTA) and seeded into Petri dishes (Costar, Cambridge, MA). Cells were incubated in the DMEM-based culture medium for 24 hours and then treated with or without 10^{-9} mol/L estrogen (17β-estradiol; Sigma, St. Louis, MO), 10^{-6} mol/L tamoxifen (Sigma), or a combination of estrogen and tamoxifen for 7 days. Hormones were added in serum-free medium consisting of a 1:1 mixture of nutrient mixture F-12 (HAM) and DMEM without phenol red, supplemented with 10 μg/mL transferrin (Sigma), 1 μg/mL insulin (Sigma), and 0.2 mg/mL bovine serum albumin (Sigma). The hormone medium was changed every 24 hours. After hormone treatment, the conditioned medium was collected, and cells were lysed by repeated freeze-thaw cycles after a PBS wash. Total protein content was determined using Bio-Rad Protein Assay with bovine serum albumin as standard (Bio-Rad Laboratories, Stockholm, Sweden). Samples were stored at $-70°C$ until subsequent analyses.

Quantification of MMP-2/MMP-9 activity *in vitro*. The combined activity of MMP-2 and MMP-9 was assayed using a gelatinase activity assay. Conditioned media from hormone-treated MCF-7 cells was mixed 50:50 with 100 μmol/L of a quenched fluorogenic substrate specific for MMP-2 and MMP-9 [2,4-dinitrophenyl (DNP)-Pro-Leu-Gly-Met-Trp-Ser-Arg-OH; Calbiochem, Merck Biosciences Ltd., Nottingham, United Kingdom], in a dark 96-well plate. The mixture was incubated at room temperature for 20 minutes, with gentle agitation. Fluorescence was measured on a Cary Eclipse fluorescence spectrophotometer (Varian, Inc., Palo Alto, CA), with $\lambda_{excitation}$ at 280 nm and $\lambda_{emission}$ at 360 nm. All experiments were done in a low-light or light-free environment. MMP-2/MMP-9 activity was expressed as relative fluorescence units/mg total protein.

Quantification of MMP-2 and endostatin proteins. Microdialysates, plasma samples, and conditioned media from hormone-treated MCF-7 cells were analyzed for MMP-2 and endostatin proteins using commercial quantitative immunoassay kits (human/mouse MMP-2 Quantikine, human endostatin Quantikine, R&D Systems, Minneapolis, MN; mouse endostatin ChemiKine; Chemicon, Hampshire, United Kingdom) without preparation. According to the manufacturers, sensitivities of the assays are 0.16 ng/mL (MMP-2), 0.023 ng/mL (human endostatin), and 9.1 pg/mL (mouse endostatin), and the precision of the ELISA kits were confirmed during the experiments. MMP-2 and endostatin concentrations were correlated to total protein content in cell lysates. All assays were repeated on more than one cell harvest.

MMP-2 and MMP-9 inhibition *in vitro*. MCF-7 cells were treated with 10^{-6} mol/L tamoxifen or a combination of 10^{-9} mol/L estradiol and 10^{-6} mol/L tamoxifen for 7 days. Hormone medium was changed every day. During the last 24 hours of hormone treatment, a synthetic inhibitor of MMP-2 and MMP-9 (MMP-2/MMP-9 inhibitor II; Calbiochem, Merck Biosciences) was also added in two different concentrations (5 or 50 μmol/L). Cells treated with hormones in combination with vehicle (DMSO) served as control cells.

Animals and ovariectomy of mice. Female athymic nude mice (6-8 weeks old) were purchased from Taconic M&B (Ry, Denmark). They were housed in a pathogen-free isolation facility with a light/dark cycle of 12/12 hours and fed with rodent chow and water *ad libitum*. All animal work was approved by the Linköping University animal ethics research board. Mice were anesthetized with i.p. injections of ketamine/xylazine (Apoteket, Linköping, Sweden) and ovariectomized, and 3-mm pellets containing 17β-estradiol, 0.18 mg/60-day release (Innovative Research of America, Sarasota, FL) were implanted s.c. in the animal's back 7 days before tumor induction. The pellets provide a continuous release of estradiol at serum concentrations of 150 to 250 pmol/L, which is in the range of physiologic levels seen in mice during the estrous cycle. One week after surgery, MCF-7 cells (5×10^6 in 200 μL PBS) were injected s.c. on the right hind side flank. Tumor volume was monitored by measuring length, width, and depth of the tumor every 4 days using a caliper. At a tumor size of ~ 300 mm^3, the mice were divided into two subgroups. One group continued with the estradiol treatment only ($n = 12$), whereas tamoxifen (1 mg/every 2 days s.c.) was added to the estradiol treatment in the other group ($n = 10$).

Microdialysis experiments. Tumor-bearing mice were anesthetized with an i.p. injection of ketamine/xylazine and thereafter kept anesthetized by repeated s.c. injections of ketamine/xylazine. A heating pad maintained body temperature. A small skin incision was made and microdialysis probes (CMA/20, 0.5-mm diameter, PES membrane length = 10 mm, 100-kDa cutoff; CMA/Microdialysis, Stockholm, Sweden) were inserted into tumor tissue and sutured to the skin. The probes were connected to a CMA/102 microdialysis pump (CMA/Microdialysis) and perfused at 1 μL/min with saline containing 154 mmol/L NaCl and 40 mg/mL dextran (Pharmalink, Stockholm, Sweden). After a 30-minute equilibration period, the outgoing perfusates (microdialysates) were collected on ice and stored at $-70°C$ for subsequent analysis.

The mean *in vitro* recovery value at room temperature was $6 \pm 0.4\%$ for MMP-2 and $33 \pm 13\%$ for endostatin, at a flow rate of 1 μL/min. After a 30-minute equilibration period, the levels of MMP-2 and endostatin did not change over time when the flow rate was constant. However, the *in vitro* recovery can only be an estimate of the *in vivo* recovery because other factors, such as tissue pressure and temperature, will affect the diffusion of substances (27). Therefore, all microdialysis values are given as original data without recalculations. Although the pore size of the microdialysis membrane would theoretically allow for traversal of MMP-9, which is below the molecular weight cutoff, the recovery of MMP-9 was zero.

At the end of experiments, the mice were euthanized, and the tumors were excised. The removed tumors were weighed, formalin fixed, and subsequently embedded in paraffin for immunohistochemical analysis. Plasma was collected in heparin by cardiac puncture.

Direct quantification of MMP-2/MMP-9 activity *in vivo*. Initial *in vitro* experiments were done to determine the optimal concentration of the substrate for direct quantification of MMP-2/MMP-9 activity using the microdialysis technique. A quenched fluorogenic substrate specific for MMP-2 and MMP-9 (MMP-2/MMP-9 fluorogenic substrate I; DNP-Pro-Leu-Gly-Met-Trp-Ser-Arg-OH; Calbiochem, Merck Biosciences) was used in the microdialysis infusate, and the generated fluorescence was detected using a Cary Eclipse fluorescence spectrophotometer (Varian) with $\lambda_{excitation}$ at 280 nm and $\lambda_{emission}$ at 360 nm, as previously described (28). The *in vitro* experiment was carried out by placing a microdialysis probe (20-kDa molecular mass cutoff, CMA/20, 0.5-mm diameter; PC membrane length = 10 mm; CMA/Microdialysis) in a test tube containing 5 μmol/L of purified active recombinant MMP-2 and MMP-9 (Calbiochem, Merck Biosciences). The microdialysis probes were thereafter perfused with either 10 or 50 μmol/L of the MMP substrate at 2 μL/min. The entire microdialysis system was protected from ambient light. Microdialysis samples were collected at 30-minute intervals and immediately subjected to fluorimetry measurements. Low levels of autofluorescence were detected for both 10 and 50 μmol/L substrate concentrations (1.0 and 3.2 relative fluorescence units, respectively); 50 μmol/L generated the highest fluorescence and was thereby chosen for the *in vivo* experiment. *In vivo*, mice were anesthetized, and microdialysis probes were inserted as described above. After an equilibration period of 30 minutes, microdialysates were collected at 30-minute intervals into chilled amber tubes. Intratumoral MMP-2/9 activity was determined by immediately measuring the relative fluorescence in outgoing microdialysates.

Intratumoral microvessel density. Formalin-fixed, paraffin-embedded tumors were cut in 3-μm sections, deparaffinized, and subjected to anti-von Willebrand's factor (rabbit anti-human von Willebrand; dilution 1:1,000; DakoCytomation, Carpinteria, CA). Sections were counterstained with Mayer's hematoxylin. Negative controls did not show staining. In a blinded manner, 10 high power fields (×200) were examined by section of three different tumors in each group. Vessel quantification of tumor sections was conducted as described previously using a Nikon microscope equipped with a digital camera (29). Percentage of area stained positively for von Willebrand's factor was assessed using Easy Image Measurement software (Bergstrom Instruments, Næstved, Denmark). Tumor sections were also subjected to H&E staining.

Statistical analysis. Statistical analysis was done using SPSS software. One-way ANOVA and Student's *t* test were used to compare means between experimental groups. All statistical tests were two sided. Results are expressed as mean $\pm$ SE. Statistical significance was assumed at Ps < 0.05.

Results

Tamoxifen increased endostatin protein levels in solid MCF-7 tumors growing in nude mice. To investigate whether *in vivo* treatment with estradiol and/or tamoxifen affected the intratumoral generation of endostatin, we did microdialysis to sample the extracellular fluid of solid MCF-7 tumors in nude mice. Mice were maintained with a physiologic level of estradiol during tamoxifen treatment to mimic a clinical situation in at least premenopausal women. Moreover, MCF-7 tumors require estrogen for growth in nude mice; therefore, a nontreated control group is not possible to achieve *in vivo*. All experiments were done on size-matched tumors, and tumor sections did not reveal any necrotic areas upon H&E staining. To measure both stroma derived and endostatin generated from the cancer cells, the outgoing micro-dialysate was analyzed for both murine (stroma) and human (cancer cell) endostatin. Cancer cell–derived (human) endostatin exhibited significantly increased levels in the estradiol + tamoxifen group compared with the estradiol only group ($1,051 \pm 83$ versus 774 ± 47 pg/mL; $P < 0.05$; Fig. 1*A*). There were also significantly higher stroma derived murine endostatin levels in tumors of estradiol + tamoxifen–treated animals than in animals treated with estradiol only ($5,697 \pm 315$ versus $2,468 \pm 390$ pg/mL; $P < 0.001$; Fig. 1*B*). These levels were approximately five times higher compared with cancer cell–derived (human) endostatin. Also in plasma, murine endostatin levels were increased after tamoxifen treatment ($2,688 \pm 390$ pg/mL in the estradiol + tamoxifen group versus $1,699 \pm 80$ pg/mL in the estradiol group; $P < 0.05$). There were no detectable levels of cancer cell–derived (human) endostatin in plasma.

Tamoxifen increased the activity of MMP-2/MMP-9 *in vivo*. The extracellular levels of MMP-2, sampled by microdialysis, were significantly higher in mice that were treated with tamoxifen in combination with estradiol compared with mice exposed to estradiol only (2.7 ± 0.5 versus 1.0 ± 0.2 ng/mL; $P < 0.01$; Fig. 2).

Figure 2. Extracellular levels of MMP-2 in solid MCF-7 tumors in nude mice *in vivo* measured by microdialysis. Mice were treated as described in Fig. 1. Microdialysis was done on size-matched tumors as described in Materials and Methods. Microdialysates were analyzed for MMP-2 using quantitative ELISA. *Columns,* mean; *bars,* SE. **, $P < 0.01$ versus estradiol-treated mice. E_2, estradiol; T, tamoxifen.

To be able to explore the effects of estradiol and tamoxifen on the extracellular activity of MMP-2 and MMP-9 *in vivo*, we perfused the microdialysis probes with a fluorescent MMP-2/MMP-9 substrate. By monitoring the generated fluorescence in the outgoing microdialysate, we could directly quantify intratumoral MMP-2/MMP-9 activity *in vivo* in tumor tissue. The *in vivo* activity of MMP-2 and MMP-9 was significantly increased in tamoxifen + estradiol–treated tumors compared with tumors exposed to estradiol only ($P < 0.001$; Fig. 3).

Tamoxifen increased the generation of endostatin and MMP activity *in vitro*. We furthermore examined whether either estradiol and/or tamoxifen had an influence on the generation of endostatin by the tumor cells themselves, using cell culture. MCF-7 cells in culture were treated with hormones for 7 days. Cell culture media were analyzed for human endostatin using quantitative ELISA. A significant decrease in endostatin secretion was seen after treatment with estradiol alone compared with control ($P < 0.01$; Fig. 4*A*). Tamoxifen exposure induced a significant increase in the
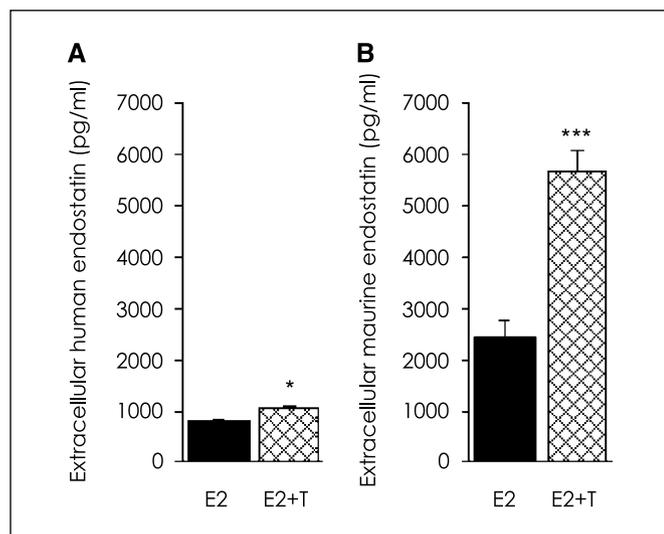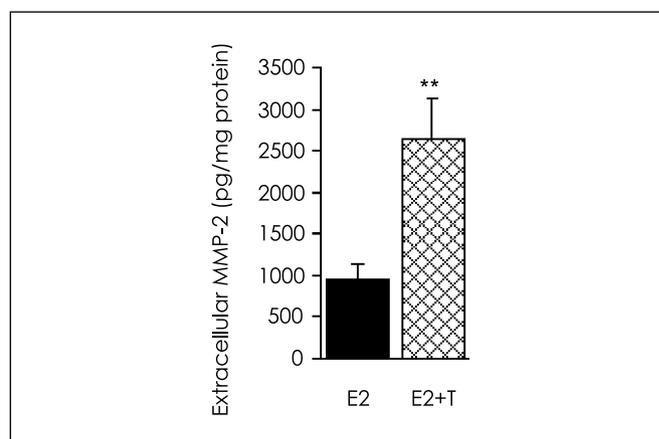

Figure 1. Extracellular levels of endostatin in solid MCF-7 tumors in nude mice *in vivo* measured by microdialysis. Mice were oophorectomized and supplemented with a physiologic level of estradiol (E_2). MCF-7 cells were injected s.c., and tumors were formed on the right hind flank. One group of mice continued with estradiol only, and in the other group tamoxifen treatment was added to the estradiol treatment ($E_2 + T$). Thereafter, microdialysis was done on size-matched tumors as described in Materials and Methods. Microdialysates were analyzed for human (*A*) and murine (*B*) endostatin, using quantitative ELISA. *Columns,* mean; *bars,* SE. *, $P < 0.05$ versus estradiol-treated mice; ***, $P < 0.001$ versus estradiol-treated mice.
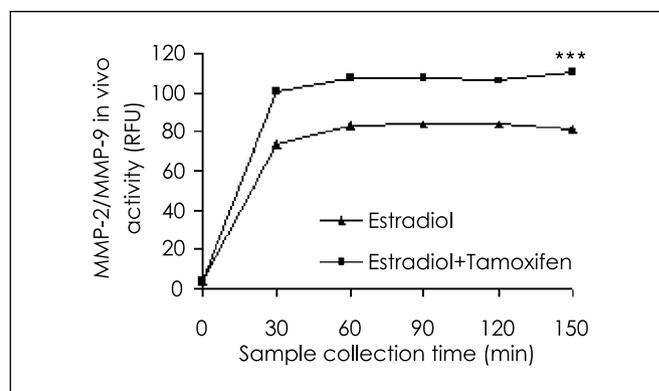

Figure 3. Activity of MMP-2/MMP-9 in solid MCF-7 tumors in nude mice *in vivo* measured by microdialysis. Mice were treated as described in Fig. 1. Microdialysis was done on size-matched tumors as described in Materials and Methods. Microdialysis probes were perfused with a fluorogenic MMP-2/MMP-9 substrate, and the generated relative fluorescence was measured at 30-minute intervals. *Points,* mean ($n = 3$ in each group); *bars,* SE (small error bars are hidden within the dots). ***, $P < 0.001$ versus estradiol only.
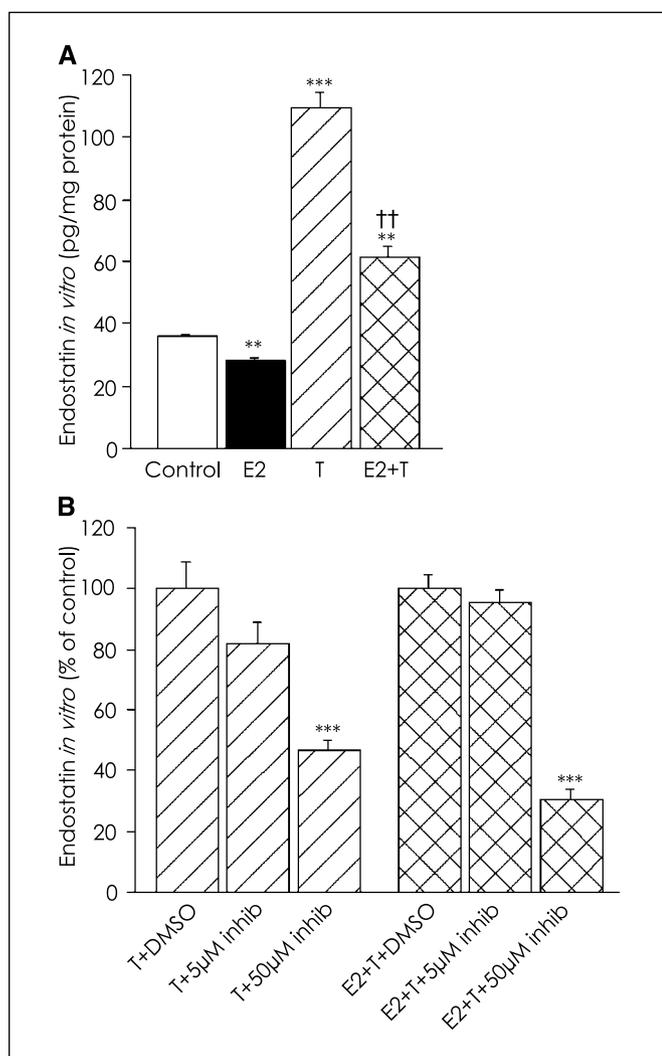
Figure 4. Extracellular levels of endostatin in conditioned medium from hormone-treated MCF-7 cells in culture. *A,* human estrogen receptor–positive breast cancer cells (MCF-7) were cultured without hormones (*C*) or in the presence of estradiol (*E₂*), tamoxifen (*T*), or a combination of estradiol and tamoxifen (*E₂ + T*) for 7 days. Conditioned medium was analyzed for human endostatin using quantitative ELISA. *Columns,* mean; *bars,* SE. **, $P < 0.01$ versus untreated control cells; ***, $P < 0.001$ versus untreated control cells; † †, $P < 0.01$ versus estradiol-treated cells. *B,* endostatin levels following inhibition of MMP-2/MMP-9 activity in hormone-treated MCF-7 cells in culture. Human estrogen receptor–positive breast cancer cells (MCF-7) were cultured in the presence of tamoxifen (*T*), or a combination of estradiol and tamoxifen (*E₂ + T*) for 7 days. A synthetic inhibitor of MMP-2 and MMP-9 was added the last 24 hours of hormone treatment in the concentrations 5 or 50 μmol/L. Cells treated with hormones in combination with vehicle (DMSO) served as control cells. Conditioned medium was analyzed for endostatin using quantitative ELISA. *Columns,* mean; *bars,* SE. ***, $P < 0.001$ versus control cells.

generation of endostatin compared with control (31 ± 2.5 protein versus 16 ± 1.4 ng/mg protein; $P < 0.001$; Fig. 4*A*). The same effect was obtained with a combination of estradiol and tamoxifen ($P < 0.01$, compared with control; Fig. 4*A*). Moreover, the levels of generated endostatin was significantly higher in media from estradiol + tamoxifen–treated cells compared with cells treated with estradiol only (28 ± 1.3 versus 14 ± 1.1 ng/mg protein; $P < 0.01$).

To test if endostatin generation was dependent on MMP activity, we inhibited MMP-2 and MMP-9 and thereafter measured endo-

statin in culture media. A dose-dependent decrease of endostatin was detected after inhibition of MMP-2 and MMP-9; 50 μmol/L of inhibitor significantly decreased the levels of endostatin in the culture media ($P < 0.001$, compared with control; Fig. 4*B*).

MMP activity *in vitro*. To verify our *in vivo* findings of MMP activity, we also analyzed cell culture media from hormone-treated cells for MMP-2/MMP-9 activity using a collagenase activity assay. Tamoxifen treatment induced a significant increase in MMP-2/MMP-9 activity ($P < 0.001$, compared with control; Fig. 5), whereas estradiol significantly decreased the MMP-2/MMP-9 activity ($P < 0.001$, compared with control; Fig. 5).

Tamoxifen decreased tumor vasculature. To examine if the variability of endostatin levels had a biological relevance on tumor vasculature, we quantified vessel area stained with anti-von Willebrand's factor. The vessel area was significantly lower in tumors with high levels of endostatin (estradiol + tamoxifen treated animals) compared with tumors with low endostatin levels (estradiol treatment only; $P < 0.05$; Fig. 6).

Discussion

In this study, we show for the first time that tamoxifen treatment in combination with a physiologic level of estradiol to nude mice bearing breast cancer tumors increased endostatin levels *in vivo* compared with estradiol treatment only. This increase was accompanied by an increase of MMP-2 and MMP-9 activity directly quantified *in situ* using microdialysis. The tamoxifen + estradiol–treated tumors exhibited reduced vessel density quantified by immunohistochemistry. The *in vivo* findings were verified *in vitro* in cell culture, where tamoxifen exposure increased endostatin and MMP-2 and MMP-9 levels, whereas estradiol reduced the levels. Inhibition of MMP-2 and MMP-9 resulted in significantly decreased endostatin levels, indicating that the endostatin generation was dependent, at least in part, on the MMP-2 and MMP-9 activity.

Tumor angiogenesis is regulated by a balance of stimulators and inhibitors (30). One of the most potent endogenous inhibitors of angiogenesis is endostatin. The generation of endostatin takes place in the extracellular space, and conventional techniques detecting cellular expression levels cannot be used for
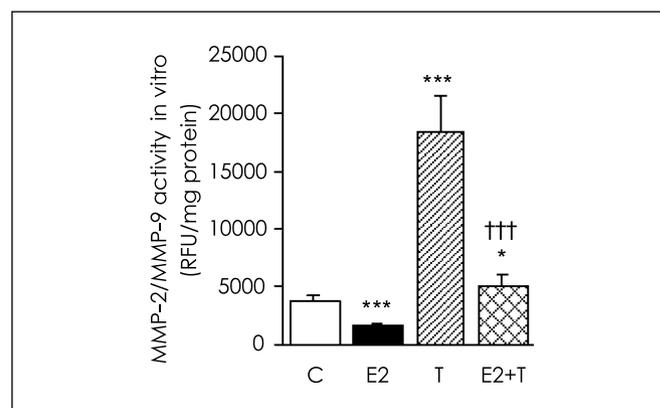

Figure 5. Activity of MMP-2/MMP-9 in conditioned medium from hormone-treated MCF-7 cells in culture. Human estrogen receptor–positive breast cancer cells (MCF-7) were cultured without hormones (*C*) or in the presence of estradiol (*E₂*), tamoxifen (*T*), or a combination of estradiol and tamoxifen (*E₂ + T*) for 7 days. Conditioned medium was analyzed for MMP-2/MMP-9 activity using a collagenase activity assay. *Columns,* mean; *bars,* SE. *, $P < 0.05$ versus control cells; ***, $P < 0.001$ versus control cells; † † †, $P < 0.001$ versus estradiol-treated cells.
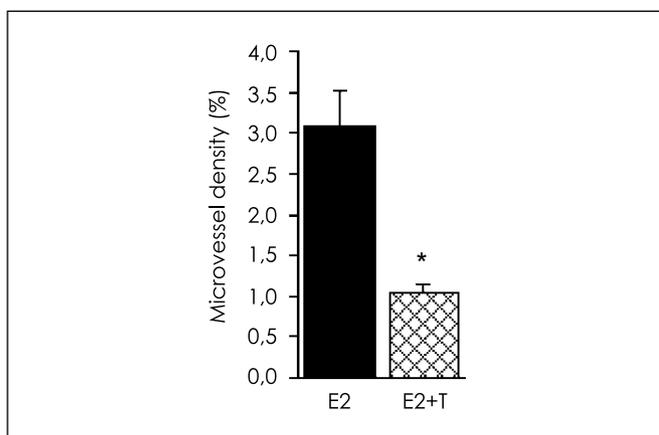
Figure 6. Tumor vessel densities in solid MCF-7 tumors in nude mice. Mice were treated as described in Fig. 1. Tumor sections were stained with anti-von Willebrand's factor, and vessel area was counted on tumor sections. Tumor vessel density quantification was conducted in a blinded manner. Ten randomly selected areas of three different tumors in each group were counted. *, $P < 0.05$ versus estradiol only.

quantifications of this process. Microdialysis provides an excellent tool for sampling of extracellular molecules, and by using this technique, we could quantify tumor tissue levels of endostatin directly *in situ*. The extracellular endostatin sampled in the tumor could theoretically be generated both from the cancer cell and/or from the host tumor stroma microenvironment. To be able to distinguish between endostatin generated from the two compartments, we used a human cancer tumor model grown in nude mice. With this approach, we were able to differentiate stroma endostatin (murine) from cancer cell–generated endostatin (human). Our results show that although the cancer cells indeed contributed to the endostatin production in the tumor, the endostatin generated by the stroma were five times higher. This illustrates the close relationship and the important interactions between cancer cells and the stroma and the need for investigations of tumor biology in the right context. With the microdialysis technique, it is possible to monitor the crucial intercellular crosstalk and signaling taking place in a tumor tissue.

Similar to other endogenous angiogenesis inhibitors, endostatin is naturally present in plasma and various tissues. Plasma levels of endostatin have been shown to be elevated in breast cancer patients or in patients with benign breast disease compared with healthy controls (31). Interestingly, it has been reported that plasma endostatin levels increased after administration of tamoxifen. This is in line with our present results, which showed an increase of the stroma-derived endostatin in plasma after tamoxifen treatment.

Given the tight regulation of MMPs, at the transcriptional and/or posttranslational level, it is imperative to investigate the activities of these proteases at the protein level and in the compartment where they are biologically active, the extracellular space. We were able to directly quantify the intratumoral MMP activity by perfusing microdialysis catheters with a quenched fluorogenic substrate specific for MMP-2 and MMP-9. The net effect of treatment with tamoxifen in combination with a physiologic dose of estradiol was a significant increase in activity of MMP-2/MMP-9. The tumors with high MMP-2/MMP-9 activity and high endostatin levels exhibited decreased vessel density. We confirmed *in vitro* that the generated endostatin was indeed related to MMP-2/MMP-9, as inhibition of these proteases significantly reduced the endostatin levels. In line with our present results, several other studies support the paradigm that MMPs may act in an antitumorigenic fashion by releasing antiangiogenic fragments, thus reducing tumor angiogenesis (11, 13, 15, 32, 33). In a seminal study by Hamano et al., it was shown that tumor growth was accelerated in mice deficient of MMP-9 compared with wild-type mice (15). MMP-9-deficient mice had lower levels of circulating tumstatin, but when levels were restored by supplementing the mice with recombinant tumstatin, tumor growth and angiogenesis decreased (15). This study thereby provides genetic evidence that MMPs may act as negative regulators of angiogenesis (15). Moreover, one study has found that reduced plasma levels of MMP-9 in patients were associated with increased tumor angiogenesis (34). In addition to the effects on endostatin generation by estradiol and tamoxifen described in the present study, we have previously shown that VEGF and sVEGFR1 secretion are affected by these hormones (26, 35). Taken together, these results indicate that sex steroids are potent regulators of angiogenesis in breast cancer by several different mechanisms, and further research in this area is warranted.

In summary, we have shown that tamoxifen in combination with estradiol increased MMP-2/MMP-9 activity, increased endostatin levels, and decreased angiogenesis in solid breast cancer tumors *in vivo* compared with estradiol treatment only. These results were confirmed by *in vitro* cell culture, where inhibition of MMP-2/MMP-9 caused decreased endostatin levels, providing evidence that these two MMPs are, at least in part, responsible for the endostatin generation. We present microdialysis as an innovative technique for quantification of extracellular endostatin as well as a tool for direct measurements of MMP-2/MMP-9 activity in tumor tissue *in situ*.

Our findings provide a novel mechanism by which estradiol may exert a proangiogenic effect and tamoxifen an antiangiogenic effect in breast cancer. These findings may be important both for an estrogen-dependent angiogenic switch in breast cancer progression and for future therapeutic strategies against this disease.

Acknowledgments

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