Angiogenin Regulation by Estradiol in Breast Tissue: Tamoxifen Inhibits Angiogenin Nuclear Translocation and Antiangiogenin Therapy Reduces Breast Cancer Growth In vivo

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Abstract

**Purpose:** Angiogenin, a 14.2-kDa polypeptide member of the RNase A superfamily, has potent angiogenic effects. Nuclear accumulation of angiogenin is essential for its angiogenic activity. Increased angiogenin expression has been associated with the transition of normal breast tissue into invasive breast carcinoma. In this article, we investigated whether estradiol (E2) affected angiogenin in breast tissue.

**Experimental Design:** We used microdialysis for sampling of extracellular angiogenin in vivo. In vitro cultures of whole normal breast tissue, breast cancer cells, and endothelial cells were used.

**Results:** We show that extracellular angiogenin correlated significantly with E2 in normal human breast tissue in vivo and that exposure of normal breast tissue biopsies to E2 stimulated angiogenin secretion. In breast cancer patients, the in vivo angiogenin levels were significantly higher in tumors compared with the adjacent normal breast tissue. In estrogen receptor–positive breast cancer cells, E2 increased and tamoxifen decreased angiogenin secretion. Moreover, E2-induced angiogenin derived from cancer cells significantly increased endothelial cell proliferation. Tamoxifen reversed this increase as well as inhibited nuclear translocation of angiogenin. In vivo, in experimental breast cancer, tamoxifen decreased angiogenin levels and decreased angiogenesis. Additionally, treating tumor-bearing mice with an antiangiogenin antibody resulted in tumor stasis, suggesting a role for angiogenin in estrogen-dependent breast cancer growth.

**Conclusion:** Our results suggest previously unknown mechanisms by which estrogen and antiestrogen regulate angiogenesis in normal human breast tissue and breast cancer. This may be important for estrogen-driven breast cancer progression and a molecular target for therapeutic interventions.

Estrogen is a crucial regulator of normal breast physiology and a major risk factor for breast cancer (1–3). The majority of breast cancers have a maintained estrogen dependency and antiestrogen therapy is a corner stone in medical breast cancer therapy. However, almost 50% of estrogen receptor (ER)–positive breast cancer cases fail to benefit from endocrine therapy. There is a need to elucidate which specific targets are involved in estrogen-driven tumor progression to optimize therapeutic options.

Angiogenesis is a key event in tumor development and progression (4). Sex steroids are crucial for the control of angiogenesis in the female reproductive tract. How steroids regulate angiogenesis in normal breast tissue and breast cancer is yet under investigation.

Angiogenin is a basic heparin-binding protein of 14 kDa and one of the most potent angiogenic factors when examined in various experimental models of angiogenesis (5). Angiogenin binds to endothelial cells (6, 7), undergoes nuclear translocation, a process essential for its angiogenic activity (8–11), and thereby induces a wide variety of responses including cell proliferation, activation of cell-associated proteases, and cell migration and invasion. Moreover, nuclear angiogenin in endothelial cells is necessary for angiogenesis induced by other angiogenic factors including vascular endothelial growth factor (VEGF), acidic and basic fibroblast growth factors, and epidermal growth factor (8). Antagonists to angiogenin have been shown to inhibit the growth and angiogenesis of human tumor cells in athymic mice (12–14). Also, the aminoglycoside antibiotic neomycin and its nontoxic derivative neamine have been shown to abolish nuclear translocation of angiogenin and thereby inhibit its angiogenic activity (15, 16). In breast cancer, increased angiogenin expression has been
Translational Relevance

Estrogen is a crucial regulator of normal breast physiology and a major risk factor for breast cancer. There is a need to elucidate which specific targets are involved in estrogen-driven tumor progression to optimize therapeutical options and develop preventive strategies.

Angiogenesis plays a key role in tumor progression. Angiogenin is one of the most potent proangiogenic factors independent of hypoxia inducible factor and vascular endothelial growth factor.

Here we show, in a translational manner, from single-cell level via experimental breast cancer to normal human breast tissue ex vivo and in vivo to breast cancer patients in vivo, that the expression of angiogenin is estrogen dependent and that antiangiogenin therapy inhibits breast cancer growth. Thus, angiogenin is a potential powerful therapeutic target in breast cancer especially in light of the very high extracellular levels (10,000 pg/mL) of angiogenin detected in breast cancer patients.

associated with the transition from normal to invasive breast carcinoma and with ER expression (17).

There is increasing evidence suggesting that sex steroids may be important in the regulation of angiogenin in breast tissue by affecting the balance between proangiogenic and antiangiogenic proteins (18). We have previously shown that estradiol (E2) acts proangiogenically in normal breast tissue and breast cancer by increasing the extracellular expression of VEGF and its receptor (19–25), increasing interleukin-8, and by decreasing soluble VEGF receptor-1 and endostatin (22, 26–28). Moreover, tamoxifen, the most widely used medical therapy against ER-positive breast cancer, may tip the balance to inhibiting angiogenesis by downregulating extracellular levels of VEGF while at the same time increasing soluble VEGF receptor-1 expression and the generation of endostatin (21, 22, 26).

Recently, the importance of angiogenin as a potent regulator of tumor angiogenin independent of hypoxia inducible factor 1 and VEGF has been highlighted (29). Whether sex steroids regulate angiogenin in breast tissue is unknown. In this article, we show that E2 increases extracellular angiogenin in normal human breast tissue in vivo and in breast cancer cells in culture. We also show that tamoxifen decreases tumor angiogenin both by downregulating extracellular angiogenin in vivo and by inhibiting the nuclear translocation of angiogenin in endothelial cells. Treating tumor-bearing mice with an antibody against angiogenin resulted in tumor stasis, emphasizing the biological significance of angiogenin in estrogen-dependent tumor growth. Moreover, in a novel fashion, extracellular angiogenin was sampled in vivo from breast cancer patients, and this revealed twice as high levels in the cancerous tissue compared with the adjacent normal breast tissue.

Materials and Methods

Subjects

A total of 24 women were investigated with microdialysis for angiogenin sampling in breast tissue in vivo. Fourteen of the women were healthy volunteers: eight were premenopausal (ages 22–25 years) and six were postmenopausal (ages 52–55 years). All had been off sex steroid-containing medication for more than 3 months. All premenopausal women had a history of regular menstrual cycles (cycle length, 28–35 days). Four of the premenopausal women were investigated in the luteal phase of the menstrual cycle whereas four were investigated in the follicular phase. Ten breast cancer patients (ages 51–86 years, postmenopausal without ongoing hormonal treatment) were also investigated preoperatively with microdialysis. The local ethical committee approved the study and all women gave their informed consents.

Microdialysis of healthy volunteers and breast cancer patients

Mepivacaine (5 mg/mL) was administrated intracutaneously before insertion of the microdialysis catheters. In healthy volunteers, the microdialysis catheter was placed in the upper lateral quadrant of the left breast and directed toward the nipple as previously described (24, 30–33). The breast cancer patients were investigated on the day before or the same day as surgery. In these women, one microdialysis catheter was inserted intratumorally in the center of the tumor and one catheter in the adjacent normal breast tissue. The microdialysis catheter (CMA/Microdialysis AB), which consists of a tubular dialysis membrane (10 mm long × 0.52 mm in diameter, 100,000 atomic mass cutoff) glued to the end of a double-lumen tube (100 mm long × 0.8 mm in diameter), was connected to a microinfusion pump (CMA 107, CMA/Microdialysis AB) and perfused with 154 mmol/L NaCl and 40 g/L dextran-70 at a perfusion rate of 0.5 μL/min. The solution entered the catheter through the outer tube and left it through the inner tube, from which it was collected and stored at −70°C for subsequent analysis.

Breast tissue biopsies

Biopsies from human breast tissue were obtained from premenopausal women with no ongoing hormonal treatment and who were undergoing routine reduction mammoplasty as previously described (34). Tissue biopsies containing epithelium, stroma including endothelial cells, and adipose tissue were produced by using an 8-mm biopsy punch (Kai Europe GmbH) and placed in a 12-well plate (Costar). Serum-free medium was used consisting of a 1:1 mixture of nutrient mixture F-12 (Life Technologies) and DMEM without phenol red (Life Technologies) supplemented with transferrin (10 μg/mL; Sigma), insulin (1 μg/mL; Sigma), and bovine serum...
albunin (0.2 mg/mL; Sigma) with or without physiologic levels of estradiol (E2) (17β-E2; 10−9 mol/L; Sigma) or a combination of 10−9 mol/L E2 and 10−8 mol/L progesterone (P4; Sigma) (E2 + P4). The control biopsies were incubated in medium supplemented with the vehicle, equivalent to the hormone-treated groups (0.001% ethanol). The biopsies were treated for 7 days at 37°C in a humidified atmosphere containing 5% CO2 and the medium was changed everyday. The medium was collected and stored at −70°C and the biopsies were formalin fixed and embedded in paraffin for subsequent analyses.

**Cells and culture**

ER- and progesterone receptor (PgR)–positive (MCF-7 and ZR-75-1) and ER- and PgR-negative (MDA-MB-231) breast cancer cell lines were obtained from the American Type Culture Collection. MCF-7 and MDA-MB-231 cells were cultured in DMEM supplemented with 2 mmol/L glutamine, 50 IU/mL penicillin-G, 50 μg/mL streptomycin, and 10% fetal bovine serum, whereas ZR-75-1 cells were cultured in DMEM/F12 without phenol red (1:1) supplemented with 50 IU/mL penicillin-G, 50 μg/mL streptomycin, and 5% fetal bovine serum. Human umbilical vascular endothelial cells (HUVEC) were isolated from fresh umbilical cords obtained from female donors. HUVEC were isolated by collagenase digestion at 37°C for 15 to 20 minutes as previously described (35), and growth medium consisted of Medium-199 without phenol red supplemented with 1% nonessential amino acids, 1.6 mmol/L glutamine, 4 IU/mL penicillin G, 4 μg/mL streptomycin, 20 mmol/L HEPES, 0.02 mg/mL endothelial cell growth factor (Roche Diagnostics), and 16% fetal bovine serum. HUVEC used for the experiments were from passages 2 to 3. All cells were cultured at 37°C in a humidified atmosphere of 5% CO2. Cell culture medium and additives were obtained from Invitrogen if not otherwise stated. In all in vitro experiments, hormones were added in serum-free medium consisting of DMEM/F12 without phenol red (1:1) supplemented with transferrin (10 μg/mL; Sigma), insulin (1 μg/mL; Sigma), and bovine serum albumin (0.2 mg/mL; Sigma). This hormone medium was changed every 24 hours.

**Hormone treatment of cells in culture**

Cells were trypsinized (0.05% trypsin and 0.02% EDTA) and seeded into Petri dishes (Costar) at 20 × 10^3/cm². Cells were then incubated in complete growth medium for 24 hours and were then treated with or without E2 (17β-E2; Sigma), tamoxifen (Tam; Sigma), or fulvestrant (ICI 182,780; Faslodex, Tocris) in serum-free medium for 7 days. For HUVEC, 3 days was chosen for the duration of the treatment due to shorter survival time without the supplements of the complete growth medium. The control group was incubated in medium supplemented with the vehicle (ethanol) at a concentration equivalent to the hormone-treated groups (0.001%). Samples were stored at −70°C until subsequent analyses.

**MCF-7 tumor explants in nude mice**

Female athymic nude mice, BALB/c nu/nu (Taconic M&B), ages 6 to 8 weeks, were housed in a pathogen-free isolation facility with a 12-hight/12-h dark cycle and fed with rodent chow and water ad libitum. The study was approved by the Linköping University animal ethics research board. Mice were anesthetized with i.p. injections of ketamine/xylazine (Apoteket) and ovarioctomized, then 3-mm pellets containing 17β-E2 (0.18 mg/60-day release; Innovative Research of America) were implanted s.c. The pellets provide a continuous release of E2 at serum concentrations of 150 to 250 pmol/L, which is in the range of physiologic levels seen in mice during the estrous cycle (20). One week after surgery, MCF-7 cells (5 × 10^6 in 200 μL of PBS) were injected s.c. on the right hind side flank. The tumors were measured twice weekly and the surface area was calculated (length/2 × width/2) × π. At a tumor size of ~30 mm², the mice were divided into two subgroups such that the mean body weight and tumor size in each group were the same. One group continued with the E2 treatment only, whereas tamoxifen (1 mg every 2 days s.c.) was added to the E2 treatment in the other group. In another set of animals, tumors were established as described above, and at a tumor size of ~15 mm², one group received 60 μg of an antiangiogenin antibody (goat anti-human; R&D Systems) s.c. everyday and the other group was treated with 60 μg of control antibody s.c. everyday (goat IgG, R&D Systems).

**Microdialysis for in vivo sampling of angiogenin**

Tumor-bearing mice were anesthetized i.p. with ketamine/xylazine and thereafter kept anesthetized by repeated s.c. injections of ketamine/xylazine. A heating lamp maintained body temperature. Microdialysis probes (CMA/20; 0.5-mm diameter; 10-mm PES membrane length; 100-kDa cutoff; CMA Microdialysis) were inserted into tumor tissue, sutured to the skin, connected to a CMA/102 microdialysis pump (CMA Microdialysis), and perfused at 0.6 μL/min with saline containing 154 mmol/L NaCl and 40 mg/mL dextran 70 (Pharmalink). After a 30-min equilibration period, the outgoing perfusates (microdialysates) were collected on ice and stored at −70°C for subsequent analysis. At the end of the experiments, the mice were euthanized and tumors were excised. Tumors were measured, weighed, formalin fixed, and subsequently embedded in paraffin for immunohistochemical analysis.

**Quantification of angiogenin by ELISA**

Microdialysates and conditioned media from hormone-treated cells were analyzed using a commercial quantitative immunoassay kit (human angiogenin Quantikine, R&D Systems). The analyzed angiogenin in conditioned media was normalized to the total protein content and expressed as picograms per milligram of protein or to tissue weight and expressed as picograms per milligram of tissue. Total protein content was determined by the Bio-Rad Protein Assay using bovine serum albumin as standard (Bio-Rad Laboratories). E2 and P4 were analyzed using ELISA kits (DRG Instruments GmbH).
Real-time PCR
mRNA was extracted from cells using TurboCapture 8 mRNA kit (Qiagen) according to the manufacturer’s instructions. cDNA was synthesized using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). The relative levels of angiogenin were determined by quantitative reverse transcription-PCR with TaqMan Gene Expression Fast Master Mix in Applied Biosystems 7900HT Fast Real-Time PCR System and normalized to β-actin. Angiogenin RNA expression levels were determined by quantitative reverse transcription-PCR using predesigned primers and probe of the TaqMan Gene Expression Assay for angiogenin (Applied Biosystems) and the Applied Biosystems 7900HT Fast Real-Time PCR System. Angiogenin gene expression was normalized to β-actin (Applied Biosystems) expression. A standard curve for angiogenin was run on each plate, using serial diluted cDNA. All samples were run as triplicates.

HUVEC proliferation assay
HUVEC were seeded (4,000 per well) in gelatin-coated 96-multiwell plates (Costar). After 24 hours of incubation in complete growth medium, the cells were washed and serum-free medium consisting of DMEM/F-12 (1:1) without phenol red, supplemented with 10 μg/mL insulin (Sigma), 1 μg/mL transferrin (Sigma), and 0.2 μg/mL bovine serum albumin (Sigma), was added to minimize the availability of growth factors. Thereafter, the serum-free medium was replaced by conditioned media collected from MCF-7 cells and ZR-75-1 cells (treated for 7 days with or without E2, or a combination of E2 and tamoxifen) in combination with 0.1 μg/mL recombinant human angiogenin (R&D Systems), 50 μg/mL goat anti-human angiogenin neutralizing antibody (R&D Systems), or 50 μg/mL normal goat IgG antibody (R&D Systems), or by serum-free hormone medium with or without 1 nmol/L E2 or a combination of 1 nmol/L E2 and 1 μmol/L tamoxifen (E2 + Tam). The conditioned medium and serum-free medium with supplements were changed every 24 hours. After a total of 48 hours of treatment, HUVEC proliferation was determined using the CellTiter 96 AQ_{"uesous} One Solution Cell Proliferation Assay (Promega). Absorbance was recorded after 2 hours of incubation with CellTiter 96 AQ_{"uesous} One Solution Reagent.

Nuclear translocation of angiogenin in HUVEC
HUVEC were seeded at a density of 5 × 10^4 cells/cm^2 on gelatin-coated coverslips (19-mm diameter; VWR) placed in 12-well plates (Costar). The cells were incubated in complete growth medium for 48 hours, washed thrice with serum-free medium, and incubated with 1 μg/mL recombinant human angiogenin (R&D Systems) at 37°C for 1 hour. Test compounds were added 5 hours before the addition of angiogenin. After incubation with angiogenin, the cells were washed thrice with PBS and fixed with 100% methanol at −20°C for 10 minutes, blocked with PBS/5% bovine serum albumin, and incubated with 20 μg/mL of goat anti-human angiogenin antibody (R&D Systems) for 1 hour. After incubating with primary antibody, the cells were washed thrice and incubated with Alexa 488–labeled rabbit anti-goat IgG (Invitrogen) at 1:500 dilution for 1 hour. The cells were mounted with SlowFade Gold antifade reagent with 4′,6-diamidino-2-phenylindole (Invitrogen) and examined under an Olympus BX41 and IX51 light/fluorescent microscope (×40/0.75); images were captured using an Olympus DP70 charge-coupled device camera (Solna). Negative controls included omission of primary antibody and cells incubated in the absence of angiogenin. The experiment was repeated thrice.

Quantification of tumor microvessel area
Formalin-fixed, paraffin-embedded tumors were cut into 3-μm sections, deparaffinized, and stained with anti-von Willebrand factor (rabbit anti-human von Willebrand; dilution 1:1000; with Envision detection, DakoCytomation). Sections were counterstained with Mayer’s hematoxylin. Negative controls did not show staining. In a blinded manner, hotspot fields (×200) were examined in nine tumors and six normal breast biopsies for each group. The images were digitally analyzed and the percentage of area that stained positively for von Willebrand factor was quantified using ImageJ software version 1.39u (NIH).

Statistical analysis
Statistical analysis was done using GraphPad Prism software. One-way ANOVA with Bonferroni post hoc test and paired or unpaired t test were used where appropriate. All statistical tests were two-sided. Results are expressed as mean ± SEM. Statistical significance was assumed at P < 0.05.

Results
E2 exposure increased extracellular angiogenin levels and angiogenesis in normal breast tissues in healthy volunteers
There were no subsequent complications after the microdialysis experiments. Significant positive correlations between local breast angiogenin levels and local breast tissue E2 and plasma E2 levels were detected. A correlation was observed between breast angiogenin and local E2 (r = 0.83, P = 0.0003; n = 14; Fig. 1A) and between angiogenin and plasma E2 (r = 0.9, P < 0.0001; n = 14; Fig. 1B). There was no correlation between plasma P4 and local extracellular breast angiogenin. To investigate if the positive correlations found in the microdialysis samples were E2 dependent, we set up an experiment using tissue biopsies of normal breasts. We have previously shown that these breast biopsies maintain tissue morphology including endothelial cells and epithelial cells, which proliferate as shown by the proliferation marker Ki67, without developing necrosis during 7 days of culture (34). Exposure of breast biopsies to E2 increased the secretion of angiogenin into the culture media (2,732 ± 398 pg/mg tissue in the control group versus 8,904 ± 1,479 pg/mg tissue in the E2-exposed group (P < 0.01) and control versus
9,660 ± 1,871 pg/mg in the E2 + P4–exposed biopsies (P < 0.01); n = 6 in each group; Fig. 1C]. Next, we investigated if the breast biopsies exhibited increased microvessel area after exposure to the hormones. Fig. 1D shows that both E2 alone– and E2 + P4–exposed biopsies showed increased microvessel area compared with control treated tissue: 1.02 ± 0.13% in the control tissue compared with 5.1 ± 0.9% in the E2 tissues (P < 0.0001) and 5.3 ± 0.7% in the E2 + P4 tissues (P < 0.0001).

**Significantly higher levels of angiogenin in breast cancer versus normal breast tissue in patients**

Ten breast cancer patients were subjected to microdialysis before surgery. One microdialysis catheter was inserted into the center of the tumor and one catheter into the normal adjacent breast tissue. Routine clinicopathologic data such as content of estrogen and P4 receptors (ER and PgR), tumor histology and grade, and stage of the excised tumors were determined. All 10 tumors expressed ER and 5 tumors coexpressed ER and PgR. There were significantly higher levels of angiogenin in cancerous tissue compared with normal breast tissue (9,020 ± 946 pg/mL in cancer tissue compared with 5,074 ± 816 pg/mL in normal breast tissue; n = 10; P < 0.0001; Fig. 2). This difference may represent an absolute difference between the tissues, but other factors such as epithelial density may have also influenced the results.

**Altered extracellular levels of angiogenin in cultured ER-positive breast cancer cells after exposure to E2 and tamoxifen**

To explore if E2 affected the secretion of angiogenin from ER-positive breast cancer cells, the cells were cultured with and without E2. As shown in Fig. 3A, E2 exposure of
MCF-7 cells significantly increased secreted angiogenin in a dose-dependent manner. Addition of 0.1 μmol/L fulvestrant (ICI 182,780 or Faslodex), which is a pure ER antagonist by downregulating the ER, abolished the E2-stimulated angiogenin secretion completely (P < 0.0001; Fig. 3A). As shown in Fig. 3B, E2 also induced a 3-fold induction of the mRNA levels of angiogenin (P < 0.01). Moreover, exposure of the ER-negative MDA-MB-231 cells to E2 did not induce an increased secretion of angiogenin (Fig. 3C).

In our next experiment, we examined how tamoxifen affected angiogenin secretion in the absence or presence of E2 in ER-positive breast cancer cells. As Fig. 3D shows, we found that adding tamoxifen to the E2 treatment of breast cancer cells in culture significantly lowered the angiogenin levels compared to treatment of cells with E2 alone. The effect of tamoxifen increased over time, and after 7 days of culture, a potent inhibition of the estrogen effects on angiogenin secretion was detected (P < 0.0001; Fig. 3D).

Similar results were repeated using another ER-positive breast cancer cell line, ZR-75-1. After 7 days of culture, the levels of angiogenin were 306 ± 14 pg/mg protein in control cells compared with 402 ± 17 pg/mg protein in E2-exposed cells (P < 0.001) and 226 pg/mg protein in the E2 + Tam–exposed group (P < 0.0001, compared with E2 alone; n = 5 in each group).

**Increased proliferation of HUVEC after exposure to conditioned medium from E2-treated cancer cells**

Angiogenin interacts with endothelial cells to induce neovascularization. Therefore, we first set up an experiment...
where HUVEC were treated with E2 and/or tamoxifen directly to confirm the ability of E2 to induce proliferation in our model. Indeed, we found that E2 significantly induced proliferation of HUVEC, whereas tamoxifen completely inhibited this induction [0.3 ± 0.02 in the control cells versus 0.44 ± 0.03 in the E2-exposed cells (P < 0.05) and 0.28 ± 0.02 in the E2 + Tam-treated cells (P < 0.01, versus E2-exposed cells; n = 6 in each group)]. In a tumor, proteins secreted by the tumor cells affect the endothelial cells. We therefore performed a second set of experiments in which we treated HUVEC with cell culture medium collected from hormone-exposed breast cancer cells. Using this approach, we found that HUVEC exposed to conditioned medium from E2-stimulated MCF-7 cells proliferated to a significantly higher extent compared with control cells incubated with medium from non–hormone-treated MCF-7 cells (P < 0.05; Fig. 4A). In addition, this increase in proliferation was completely reversed by the exposure of HUVEC to conditioned medium from E2 + Tam–treated MCF-7 cells (P < 0.01; Fig. 4A). This increase in proliferation by E2 was dependent on the increased angiogenin levels in E2-exposed cancer cells, as we first treated HUVEC with recombinant angiogenin (1 μg/mL) alone or with an angiogenin-neutralizing antibody (50 μg/mL). The proliferation of HUVEC treated with recombinant angiogenin significantly decreased after treatment with the antibody (P < 0.05; Fig. 4B). Conditioned medium from E2-stimulated MCF-7 cells in combination with the antiangiogenin antibody significantly inhibited the increase in proliferation of HUVEC detected after treatment with E2-exposed MCF-7 cell culture medium (P < 0.05; Fig. 4B). This suggests that angiogenin present in E2-treated MCF-7 medium affected the proliferation of HUVEC. The complete block of E2-induced proliferation by antiangiogenin may be explained by several mechanisms: the method is not sensitive enough for fine-tuned differences of HUVEC proliferation; the actions of VEGF and angiogenin are dependent on each other (i.e., by blocking one of them, the action of both is inhibited); the levels of other E2-induced proangiogenic proteins are very low in this cell line.

Again, experiments using the ZR-75-1 cancer cell line confirmed these results. Incubating HUVEC with conditioned medium from E2-treated ZR-75-1 cells in combination with an antiangiogenin antibody inhibited the proliferation increase detected with the E2-treated ZR-75-1 medium [0.47 ± 0.02 in the ZR-75-1 E2 group compared with 0.36 ± 0.03 in the ZR-75-1 E2 + angiogenin antibody group (P < 0.05; n = 6 in each group)].

**Tamoxifen inhibited the nuclear translocation of angiogenin in HUVEC**

The nuclear accumulation of angiogenin in HUVEC is essential for its angiogenic activity (9). We used conditioned medium from E2-treated MCF-7 breast cancer cells to increase the proliferation of HUVEC. HUVEC were isolated from female umbilical cords of female donors. Cells from passages 2 to 3 were seeded into 96-well plates and cultured for 48 h in conditioned medium collected from hormone-exposed MCF-7 cells. Proliferation was assessed by MTS assay. A, proliferation of cultured HUVEC after exposure to conditioned medium from non–hormone-treated MCF-7 cells (MCF-7 control); E2-treated MCF-7 cells (MCF-7 E2); or E2 + Tam–treated MCF-7 cells (MCF-7 E2 + Tam). ***, P < 0.001, compared with control cells; #P, P < 0.001, compared with E2 cells. Columns, mean; bars, SE. B, proliferation of HUVEC after exposure to recombinant human angiogenin with or without an antihuman angiogenin-neutralizing antibody (Ab).
immunofluorescence to monitor the nuclear translocation of angiogenin in HUVEC. Incubation with the antibody without recombinant angiogenin resulted in very weak to absent immunofluorescence (data not shown). As shown in Fig. 5A and B, after 1 hour of incubation in the absence of hormones, the majority of cell-associated angiogenin accumulated in the nucleus. In the presence of 1 μmol/L tamoxifen, the amount of nuclear angiogenin was decreased (Fig. 5C and D). Instead, staining was observed in the cytoplasm. No difference in staining was observed with incubation of cells with E2 only compared with controls cells incubated with recombinant angiogenin only.

Tamoxifen decreased extracellular angiogenin levels and angiogenesis in solid MCF-7 tumors in nude mice

The bioactive site for angiogenin is the extracellular space and no sampling or quantification of angiogenin in this compartment has previously been done. In this study, we used microdialysis to sample extracellular fluid in situ of MCF-7 tumors. These tumors require estrogen for growth in nude mice; therefore, a nontreated control group is not possible to achieve in vivo. Because angiogenin expression is regulated by hypoxia (17, 36), all experiments were done on size-matched tumors, and H&E staining confirmed that tumors did not contain any necrotic areas. As Fig. 6A shows, there were significantly lower levels of extracellular angiogenin in microdialysates from tumors treated with tamoxifen in combination with E2 compared with the levels in tumors treated with E2 only (P < 0.001), confirming our in vitro results in cell culture. These tamoxifen-treated tumors also exhibited decreased vessel area compared with E2-treated tumors (P < 0.001; Fig. 6B). Finally, antiangiogenin treatment resulted in tumor stasis, whereas control treated tumors continued to grow (Fig. 6C). This suggests that angiogenin is involved in estrogen-dependent growth of breast cancer.

Discussion

Here we show for the first time that E2 increases the extracellular levels of angiogenin in normal human breast tissue in vivo. Our in vivo results were confirmed ex vivo by culture of normal human breast biopsies in the presence of E2 alone or together with P4. P4 had neither a counteracting nor an additive effect on the angiogenin levels compared with E2. In breast cancer patients, the extracellular angiogenin levels in cancer tissues were twice as high compared with the normal adjacent breast tissue. We further show that E2 increased and tamoxifen decreased the
secretion of angiogenin from human breast cancer cells in culture. In vivo, in solid human breast cancers in nude mice, tamoxifen therapy decreased the extracellular levels of angiogenin and this was associated with decreased angiogenesis. E2-induced angiogenin derived from cancer cells significantly increased endothelial cell proliferation in vitro. Exposure to tamoxifen of HUVEC in culture significantly decreased the nuclear accumulation of angiogenin. Additionally, treating tumor-bearing mice with an antibody against angiogenin resulted in tumor stasis, suggesting that angiogenin is one mechanism involved in estrogen-dependent breast cancer growth.

Angiogenesis is a prerequisite for the growth and metastasis of solid tumors, and angiogenin has been shown to be one important regulator of this process. Inhibition of angiogenin may prevent and inhibit the growth of human tumors cells inoculated in nude mice (12–14, 37). Moreover, increased tissue levels of angiogenin have been detected in cancer patients with aggressive disease with poor prognosis as recently summarized (38). In breast cancer patients, the results have been heterogeneous, one reason being that different sites, serum or tissue, of angiogenin quantification have been used and no correlation between tissue and plasma levels of angiogenin has been found (39, 40). It has also been suggested that angiogenin circulates inactive in plasma and that only the extravascular-tissue angiogenin promotes angiogenesis (41). Tissue homogenate represents both cell-associated and free extracellular levels in the tissue. Clearly, a technique that quantifies the extracellular angiogenin in vivo directly from the target tissue would more accurately represent the specific bioactive soluble tissue angiogenin. Microdialysis, a technique that mimics a blood vessel within a tissue, allows direct in situ sampling of extracellular proteins as we have described previously (42). In this study, novel data on extracellular angiogenin levels in human breast tissue were achieved by using microdialysis in both normal and cancerous breast tissues. The results clearly show that E2 is a potent regulator of angiogenin in normal breast tissue, as the microdialysis data were confirmed using breast tissue biopsies in culture. In human breast cancers before surgery, the extracellular angiogenin levels were close to 10,000 pg/mL, compared with VEGF levels of ~10 pg/mL as we have previously reported (23). Although some differences in the concentrations of the two proteins may be attributed to different relative recovery [i.e., the ability of the protein to pass over the microdialysis membrane (8% for VEGF and 22% for angiogenin)], it cannot fully explain the magnitude of the difference. Previous data have shown equivalent induction of blood vessel growth in vitro at identical concentrations of VEGF and angiogenin (8). Clinical data have shown a beneficial effect of anti-VEGF (bevacizumab) therapy in breast cancer patients (43). The much higher levels of angiogenin in breast

Fig. 6. Tamoxifen decreases the in vivo levels of angiogenin and angiogenesis and antiangiogenin therapy decreases the growth of MCF-7 tumors in nude mice. A and B, mice were oophorectomized and supplemented with a physiologic level of E2. MCF-7 cells were injected s.c. and tumors were established on the right hind flank. At similar tumor sizes, one group of mice continued with E2 treatment only, whereas in the other group tamoxifen was added to the E2 treatment (E2 + Tam) as described in Materials and Methods. Microdialyses were analyzed for human angiogenin using quantitative ELISA. **, $P < 0.001$. Columns, mean; bars, SE. B, the microvessel area of tumors was quantified as described in Materials and Methods. ***, $P < 0.0001$. Columns, mean; bars, SE. C, size-matched MCF-7 tumors of oophorectomized mice supplemented with E2, as described above, were treated with daily injections of an antiangiogenin antibody (filled squares) or a control IgG (filled circles) and tumor size was followed as described in Materials and Methods. **, $P < 0.001$. 

Estradiol, Tamoxifen, and Angiogenin in the Breast

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cancers suggests that blocking angiogenin would be a powerful molecular approach in inhibiting angiogenesis, and ultimately tumor growth, in breast cancer patients.

In our experimental breast cancer model, we show that tamoxifen reduced the extracellular tumor levels of angiogenin compared with tumors grown in estrogen-treated mice and that tumors with low levels of angiogenin levels exhibited significantly decreased angiogenesis. Our in vivo data confirmed the in vitro cell culture results, using two separate estrogen-dependent breast cancer cell lines, where E2 induced a dose-dependent significant increase of extracellular angiogenin protein levels, which was abolished on addition of the pure ER antagonist fulvestrant. Moreover, E2 induced a 3-fold increase of angiogenin mRNA levels. Adding tamoxifen to a physiologic dose of E2 antagonized the E2-induced increase of angiogenin, which further supports an estrogen-dependent angiogenin regulation in breast cancer cells.

In the process of tumor angiogenesis, cancer cell-derived angiogenin acts on endothelial cells in a paracrine manner. Here we also show that released proteins from E2-exposed cancer cells increased the proliferation of HUVEC. This increase was inhibited by the addition of an antibody against angiogenin, suggesting that the proliferation was, at least in part, mediated by angiogenin. We also show that inhibition of angiogenin by antibody therapy in vivo results in tumor stasis. This strongly suggests that angiogenin is one of the mechanisms behind estrogen-dependent breast cancer growth. Thus, a combination of antiangiogenin therapy with ER targeting compounds may enhance the therapeutic response rate in ER-positive breast cancer.

Nuclear accumulation is essential for the angiogenic activity of angiogenin, and inhibition of this nuclear translocation abolishes the angiogenic activity of the protein (9, 16). The process of nuclear translocation of angiogenin is, to date, largely unknown. The first step of internalizing extracellular angiogenin into the cell requires receptor-mediated endocytosis (9). Further import to the nucleus may involve passive diffusion of angiogenin through the nuclear pore and/or specific proteins involved in nuclear localization signals (44). Here, we show that tamoxifen decreased the important nuclear accumulation of angiogenin in endothelial cells, whereas E2 exerted no additionally effects on the nuclear accumulation of angiogenin compared with control cells treated with recombinant angiogenin only. This may suggest that an already efficient mechanism for the angiogenin translocation to the nucleus is difficult to enhance, but can be inhibited. Hence, tamoxifen exerts two potent direct antiangiogenic mechanisms: decreased secretion of angiogenin from cancer cells and inhibition of the nuclear accumulation of angiogenin in endothelial cells.

In summary, this study shows for the first time that angiogenin is regulated by E2 in normal human breast tissue and that breast cancers in humans express higher extracellular levels of angiogenin compared with normal breast tissue. Tamoxifen counteracted E2-induced angiogenin secretion in breast cancer cells, inhibited the nuclear accumulation of angiogenin in endothelial cells, and reduced endothelial cell proliferation. Taken together, these results suggest previously unknown mechanisms by which E2 may tip the balance to favor angiogenesis and for potent antiangiogenic actions of tamoxifen in breast cancer. This may be important for the understanding of estrogen-dependent breast carcinogenesis as well as for therapeutic interventions of breast cancer.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Angiogenin Regulation by Estradiol in Breast Tissue: Tamoxifen Inhibits Angiogenin Nuclear Translocation and Antiangiogenin Therapy Reduces Breast Cancer Growth In vivo

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