Angiogenin regulation by estradiol in breast tissue: tamoxifen inhibits its nuclear translocation and anti-angiogenin therapy reduces breast cancer growth in vivo

Ulrika W. Nilsson, Annelie Abrahamsson, and Charlotta Dabrosin*

Linköping University, Department of Clinical and Experimental Medicine, Division of Oncology, SE-581 85, Linköping, Sweden

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*Correspondence to: Charlotta Dabrosin MD PhD, Division of Oncology, University Hospital, SE-581 85 Linköping, Sweden
Phone: +46 13 22 85 95; Fax +46 13 22 30 90; E-mail: charlotta.dabrosin@liu.se

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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 in order to optimize therapeutic options and develop preventive strategies.

Angiogenesis plays a key role in tumor progression. Angiogenin is one of the most potent pro-angiogenic factors independent of hypoxia inducible factor and VEGF.

Here we show, in a translational manner, from single cell level, via experimental breast cancer to normal human breast tissue ex and in vivo to breast cancer patients in vivo that the expression of angiogenin is estrogen dependent and that anti-angiogenin 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.
Abstract

Purpose: Angiogenin, a 14.2 kD 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 paper we investigated whether estradiol affected angiogenin in breast tissue. Experimental Design: We used microdialysis for sampling of extracellular angiogenin in vivo. In vitro culture of whole normal breast tissue, breast cancer cells and endothelial cells were used.

Results: We show that extracellular angiogenin correlated significantly with estradiol (E2) in normal human breast tissue in vivo and that E2 exposure to normal breast tissue biopsies stimulated angiogenin secretion. In breast cancer patients, the in vivo angiogenin levels were significantly higher in tumors compared to 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 anti-angiogenin 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 anti-estrogen 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.
Introduction

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 anti-estrogen therapy is a cornerstone 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 in order 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 factor (aFGF and bFGF), and epidermal growth factor (EGF) (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 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 angiogenesis in breast tissue by affecting the balance between pro- and anti-angiogenic proteins (18). We have previously shown that estradiol (E2) acts pro-angiogenic in normal breast tissue and breast cancer by increasing the extracellular expression of VEGF and its receptor (19-25), increasing IL-8, and by decreasing sVEGFR-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 down-regulating extracellular levels of VEGF while at the same time increasing soluble VEGFR-1 expression and the generation of endostatin (21, 22, 26).

Recently, the importance of angiogenin as a potent regulator of tumor angiogenesis independent on the hypoxia inducible factor 1 (HIF-1) and VEGF has been highlighted (29). Whether sex steroids regulate angiogenin in breast tissue is unknown. In this paper we demonstrate that E2 increases extracellular angiogenin in normal human breast tissue in vivo and in breast cancer cells in culture. We also show that tamoxifen decreased tumor angiogenesis both by down-regulating 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 cancers patients, and this revealed twice as high levels in cancerous tissue compared with 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 pre-menopausal (aged 22-25 years) and six were post-menopausal (aged 52-55 years). All had been off sex steroid containing medication for more than three months. All pre-menopausal women had a history of regular menstrual cycles (cycle length 28-35 days). Four of the pre-menopausal women were investigated in the luteal phase of the menstrual cycle whereas four were investigated in the follicular phase. Ten breast cancer patients (aged 51-86, postmenopausal without ongoing hormonal treatment) were also investigated pre-operatively 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 (5mg/ml) was administrated intracutaneously prior insertion of the microdialysis catheters. In healthy volunteers the microdialysis catheter was placed in the upper lateral quadrant of the left breast and directed towards 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 adjacent normal breast tissue. The microdialysis catheters (CMA/Microdialysis AB, Sweden), which consists of a tubular dialysis membrane (10 mm long x 0.52mm in diameter, 100,000 atomic mass cut-off) glued to the end of a double-lumen tube (100 mm long x 0.8mm in diameter) were connected to a micro-infusion pump (CMA 107, CMA/Microdialysis AB, Sweden) and perfused with NaCl 154 mmol/L and dextran-70 40g/l, 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 pre-menopausal women, without ongoing hormonal treatment, 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, Solingen, Germany) and placed in a 12-well plate (Costar, Cambridge, MA). Serum-free medium was used consisting of a 1:1 mixture of nutrient mixture F-12 (GIBCO, Paisley, UK) and Dulbecco's modified Eagle's medium (DMEM) without phenol red (GIBCO, Paisley, UK) supplemented with transferrin (10 μg/ml; Sigma), insulin (1 μg/ml; Sigma), and bovine serum albumin (0.2 mg/ml; Sigma) with or without physiological levels of E2 (17β-E2; E2; Sigma) 10⁻⁹ M or a combination of 10⁻⁹ M E2 and 10⁻⁸ M progesterone (P4) (E2 + P4; Sigma). The control biopsies were incubated in media supplemented with the vehicle, equivalent to the hormone treated groups (ethanol 0.001%). The biopsies were treated for 7 days at 37°C in a humidified atmosphere containing 5% CO₂ and the medium was changed every day. 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 ATCC (Manassas, VA, USA). 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 15-20 minutes as previously described (35), and growth medium consisted of Medium-199 without phenol red supplemented with 1 % non essential 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, Bromma, Sweden), and 16 % FBS. HUVEC used for 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 (Carlsbad, CA, USA) if not otherwise stated. In all in vitro experiments, hormones were added in a serum-free medium consisting of DMEM/F-12 without phenol red (1:1) supplemented with transferrin (10 µg/mL; Sigma, St. Louis, MO, USA), 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, Cambridge, MA, USA), 20×10³ cells/cm². Cells were then incubated in complete growth medium for 24 hours and were then treated with or without E2 (17ß-E2, E2, Sigma), tamoxifen (Tam; Sigma), or fulvestrant (ICI 182,780, Faslodex®, Tocris, Bristol, UK) in serum-free medium for 7 days. For HUVEC, three days was chosen for the duration of 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, in 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 (ages 6–8 wk), from Taconic M&B (Ry, Denmark) were housed in a pathogen free isolation facility with a 12-h light/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, Linköping, Sweden), ovariectomized, and 3-mm pellets containing 17β-E2, 0.18mg/60-day release (Innovative Research of America, Sarasota, Florida, USA) 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 cells in 200 µl 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 x width/2) x π. At a tumor size of approximately 30 mm^2 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, while tamoxifen (1mg 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 approximately 15 mm^2 one group received an anti-angiogenin antibody (goat anti-human R&D Systems, Minneapolis, MN) 60 µg s.c. every day and the other group was treated with control antibody 60 µg s.c. every day (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; PES membrane length 10 mm; 100-kDa cut-off; CMA Microdialysis, Stockholm, Sweden) 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, Stockholm, Sweden). 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 experiments, the mice were euthanized and the 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, Minneapolis, MN, USA). The analyzed angiogenin in conditioned media was normalized to the total protein content and expressed as pg/mg protein or to tissue weight and expressed as pg/mg tissue. Total protein content was determined by the Bio-Rad Protein Assay using bovine serum albumin as standard (Bio-Rad Laboratories, Stockholm, Sweden). E2 and P4 were analyzed using ELISA kits, DRG Instruments GmbH, Germany.

**Real-time PCR**

mRNA was extracted from cells using TurboCapture 8 mRNA kit (Qiagen, Maryland, USA) according to the manufacturer’s instructions. cDNA was synthesized using the High Capacity cDNA reverse Transcription Kit (Applied Biosystems, Foster City, Ca, USA). The relative levels of Angiogenin was determined by qRT-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 qRT-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, Warrington, UK) expression. A standard curve for angiogenin was run on each plate, using serial diluted cDNA. All samples were run as triplicates.
**HUVEC proliferation assay**

HUVECs were seeded (4000 cells/well) in gelatin-coated 96-multiwell plates (Costar). After 24-hour 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 transferrin (Sigma), 1 µg/mL insulin (Sigma), and 0.2 mg/mL BSA (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 medium hormone medium with or without 1 nmol/L E2 or a combination of 1 nmol/L E2 (E2) and 1 µmol/L tamoxifen (E2+Tam). The conditioned medium and serum-free medium with supplements was changed every 24 hours. After a total of 48 hours of treatment, HUVEC proliferation was determined using the CellTiter 96® AQueous One Solution Cell Proliferation Assay (Promega, Madison, WI, USA). Absorbance was recorded after 2 hours of incubation with CellTiter 96® AQueous One Solution Reagent.

**Nuclear translocation of angiogenin in HUVEC**

HUVECs were seeded at a density of 5x10³ cells/cm² on gelatin-coated cover slips (19 mm diameter; VWR, Stockholm, Sweden) 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 prior to 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 % BSA 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 a 1:500 dilution for 1 hour. The cells were mounted in SlowFade® Gold anti-fade reagent with DAPI (Invitrogen), examined with an Olympus BX41 and IX51 light/fluorescent microscope (x40/0.75), and images were captured using an Olympus DP70 CCD camera (Solna, Sweden). 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 in 3-µm sections, deparaffinized, and subjected to anti-von Willebrand’s 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, hot spot fields (x200) were examined of nine tumors and six normal breast biopsies in each group. The images were digitally analyzed and percentage of area stained positively for von Willebrand’s factor was quantified using ImageJ software version 1.39u (NIH, USA).

**Statistical analysis**

Statistical analysis was performed using GraphPad Prism software. One-way ANOVA with the 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 values less than 0.05.
Results

*E2 exposure increased extracellular angiogenin and angiogenesis of normal breast tissue 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. Correlation between breast angiogenin and local E2, r=0.83, p=0.0003, n=14, Fig 1A, and correlation 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). E2 exposure to breast biopsies increased the secretion of angiogenin into the culture media, 2732±398 pg/mg tissue in the control group vs. 8904±1479 pg/mg tissue in the E2 exposed group, p<0.01, and control vs 9660±1871 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. As Fig 1D shows both E2 alone and E2+P4 exposed biopsies showed increased microvessel area compared to 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.

*Significant 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 normal adjacent breast
tissue. Routine clinicopathological 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 ten tumors expressed ER and five tumors co-expressed ER and PgR. There were significantly higher levels of angiogenin in cancerous tissue compared with normal breast tissue, 9020±946 pg/ml in cancer tissue compared with 5074±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 also influence 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 culture with and without E2. As shown in Fig. 3A E2 exposure of MCF-7 cells significantly increased secreted angiogenin in a dose-dependent manner. The addition of 0.1 µM of fulvestrant (ICI 182,780 or Faslodex®), which is a pure ER antagonist by down-regulating the ER, abolished the E2 stimulated angiogenin secretion completely, (P<0.0001; Fig 3A). As Fig. 3B shows, E2 also induced a three fold induction of the mRNA levels of angiogenin (P<0.01). Moreover, E2 exposure to the ER-negative MDA-MB-231 cells did not induce an increased secretion of angiogenin, Fig 3C.

In our next experiment we examined how tamoxifen affected angiogenin secretion in 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 treating cells with E2 alone. The effect of tamoxifen increased over time and after seven 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 seven 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 neo-vascularization. Therefore, we first set up an experiment where human umbilical vein endothelial cells 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 vs 0.44±0.03 in the E2-exposed cells (P<0.05) and 0.28±0.02 in E2+Tam treated cells, (P<0.01 vs E2 exposed cell, 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 media collected from hormone-exposed breast cancer cells. Using this approach, we found that HUVEC exposed to conditioned media from E2-stimulated MCF-7 cells proliferated to a significantly higher extent compared to control cells incubated with media 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 media from E2+Tam-treated MCF-7 cells (P<0.01, compared to E2 only; Fig. 4A). Similar results were found when exposing HUVEC to conditioned media from hormone-stimulated ZR-75-1 cells. Cell culture medium originating from E2-treated ZR-75-1 cells induced proliferation to a significantly larger degree than did control medium, E2 media 0.46±0.02 versus 0.31±0.01 in the control group (P<0.05, n=6 in each group). This increase in proliferation by E2 culture media was inhibited by the exposure of HUVEC to conditioned medium from E2+Tam-exposed ZR-75-1 cells, 0.32±0.01, (P<0.001 versus E2 media, n=6 in each group). To explore
if the increase in proliferation induced by E2 was dependent on the increased angiogenin levels in E2-exposed cancer cells, 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 \( (P<0.05; \text{Fig. 4B}) \). Conditioned medium from E2-stimulated MCF-7 cells in combination with the anti-angiogenin antibody significantly inhibited the increase in proliferation of HUVEC detected after exposure to E2-exposed MCF-7 cell culture medium \( (P<0.05; \text{Fig. 4B}) \). This suggests that angiogenin present in E2-treated MCF-7 medium affected the proliferation of HUVEC. The complete block of E2 induced proliferation by anti-angiogenin may be explained by several mechanisms; the method is not sensitive enough for fine tuned differences of HUVEC proliferation, the action of VEGF and angiogenin is dependent on each other i.e. by blocking one of them the action of both is inhibited, the levels other estradiol-induced pro-angiogenic 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 anti-angiogenin 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 \( (P<0.05, n=6 \text{ in each group}) \).

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

Nuclear accumulation of angiogenin in HUVEC is essential for its angiogenic activity (9). We used immunofluorescence to monitor 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-B, after 1 hour of incubation in the absence of hormones, the majority of cell-associated angiogenin was accumulated in the nucleus. In the presence of 1 µmol/L tamoxifen, the amount of nuclear angiogenin was...
decreased (Fig. 5C-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 levels of angiogenin and decreased angiogenesis in solid MCF-7 tumors in nude mice

The bioactive site for angiogen is the extracellular space and no sampling or quantification of angiogenin in this compartment has previously been performed. 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 non-treated control group is not possible to achieve in vivo. As angiogenin expression is regulated by hypoxia (17, 36), all experiments were performed on size-matched tumors and H&E staining confirmed that tumors not contained 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, anti-angiogenin 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 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 presence of E2 alone or together with P4. P4 had neither a counteracting nor additive effect on the angiogenin levels compared with E2. In breast cancer patients the extracellular angiogenin levels in their cancers were twice as high as compared with 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 of tamoxifen to 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 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 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 as well as free...
extracellular levels in the tissue. Clearly, a technique, which quantifies the extracellular angiogenin \textit{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 \textit{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 was achieved by using microdialysis in both normal and cancerous breast tissue. The results clearly show that E2 is a potent regulator of angiogenin in normal breast tissue as the microdialysis data was 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 approximately 10 pg/ml as we have previously reported (23). Even though some differences of 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 \textit{in vitro} at identical concentrations of VEGF and angiogenin (8). Clinical data have shown a beneficial effect of anti-VEGF (bevacizumab) therapy to breast cancer patients (43). The much higher levels of angiogenin in breast 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 \textit{in vivo} data confirmed the \textit{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 when adding the pure ER.
antagonist fulvestrant. Moreover, E2 induced a three-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 anti-angiogenin therapy with ER targeting compounds may enhance the therapeutic response rate in ER+ 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 the endothelial cells, whereas E2 exerted no additionally effects on the nuclear accumulation of angiogenin compared to 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 whereas it is possible to inhibit. Hence, tamoxifen exerts two potent direct
anti-angiogenic mechanisms; decreased secretion of angiogenin from the 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 than 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 anti-angiogenic 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.
References


Figure legends

**Figure 1. Estradiol (E2) increased extracellular angiogenin and angiogenesis in normal human breast tissue**

A and B; Healthy women were investigated with microdialysis for sampling of extracellular proteins *in vivo* in normal breast tissue. Blood samples were drawn at the time of microdialysis. Eight premenopausal with high E2 levels and six postmenopausal low E2 levels were included.

A. There was a significant positive correlation between extracellular breast angiogenin and extracellular local breast E2, $r=0.83$, $p=0.0003$, $n=14$.

B. There was a significant correlation between extracellular breast angiogenin and plasma E2, $r=0.9$, $p<0.0001$, $n=14$.

C and D; Normal breast tissue biopsies from women undergoing reduction mammoplasties were cultured in the presence of E2 (E2; $10^{-9}$ M), a combination of E2 and P4 (E2+P4; $10^{-9}$ M and $10^{-8}$ M, respectively), or serum-free medium alone supplemented with hormone solvent (C) for seven days.

C. Angiogenin released into the culture media. ** $P<0.001$ compared to control biopsies, Columns, mean; bars, SE

D. Microvessel staining and quantification of normal breast tissue biopsies as described in the materials and methods section. Representative tissue sections from the different treatment groups. *** $P<0.0001$ compared to control biopsies, Columns, mean; bars, SE

**Figure 2. Angiogenin in vivo in breast cancer patients**

Ten postmenopausal breast cancer patients were subjected to microdialysis in the breast cancer and adjacent normal breast tissue before surgery. *** $P<0.0001$, Columns, mean; bars, SE

**Figure 3. Estradiol (E2) increased angiogenin in estrogen-dependent breast cancer cells**
A. Human ER positive MCF-7 breast cancer cells were cultured without hormones (Control) or in the presence of increased concentrations of E2 (E2) and with the addition 0.1 µM of the pure anti-estrogen fulvestrant (Faslodex®) of 7 days. Cell culture supernatants were analyzed for human angiogenin using quantitative ELISA.

B. MCF-7 cells were cultured in 1 nM E2 for 7 days and fold change of mRNA levels of angiogenin were determined by TaqMan qPCR as described in the Materials and Methods section.

C. Human ER negative MDA-MB-231 were cultured in 1 nM E2 for 7 days and cell culture supernatants were analyzed for human angiogenin using quantitative ELISA.

***P<0.0001 compared to control cells, ###P<0.0001 compared to E2 1 nM, †††P<0.0001 compared to E2 10 nM. Columns, mean; bars, SE.

D. MCF-7 cells were cultured for 3, 5, and 7 days in the presence or absence of 1 nM E2, 1 µM tamoxifen (Tam), or a combination of E2 and tamoxifen (E2+Tam). Cell culture supernatants were analyzed for human angiogenin using quantitative ELISA. ***P<0.0001 compared to control cells, **P<0.001 compared to control cells, †††P<0.0001 compared to E2, ††P<0.001 compared to E2. Columns, mean; bars, SE.

**Figure 4. Conditioned medium from estradiol (E2) treated MCF-7 breast cancer cells increased proliferation of HUVEC.**

Human umbilical vein endothelial cells (HUVEC) were isolated from fresh female umbilical cords of female donors. Cells from passages 2-3 were seeded into 96-well plates and cultured for 48 hours in conditioned medium collected from hormone-treated 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 conditioned medium from MCF-7 cells treated with a combination of E2 and tamoxifen...
(MCF-7 E2+Tam). **P<0.001 compared to control cells, ##P<0.001 compared to E2 cells.

*B. Proliferation of HUVEC after exposure to recombinant human angiogenin (Angiogenin)
with or without an anti-human angiogenin neutralizing antibody (Ab) or after exposure to
conditioned medium from E2-treated MCF-7 cells with or without an anti-human angiogenin
neutralizing antibody. *P<0.05 compared to vehicle and MCF-7 control, #P<0.05 compared
to angiogenin alone, †P<0.05 compared to MCF-7 E2 cells. *Columns, mean; bars, SE.

**Figure 5. Tamoxifen inhibited nuclear accumulation of angiogenin in cultured HUVEC**

Human umbilical vein endothelial cells (HUVEC) were isolated from fresh female umbilical
cords of female donors. Cells from passages 2-3 were cultured on cover slips and incubated
with 1 µg/mL recombinant human angiogenin at 37 °C for 1 hour in the absence (A-B) or
presence (C-D) 1 µmol/L tamoxifen. Tamoxifen was added 5 hours prior to the addition of
angiogenin. Angiogenin was visualized by a goat anti-human angiogenin antibody and
ALEXA 488-labeled rabbit anti-goat IgG. Representative image of three independent
experiments is presented.

**Figure 6. Tamoxifen decreased in vivo levels of angiogenin and angiogenesis, and anti-
angiogenin therapy decreased tumor growth of MCF-7 tumors in nude mice**

A and B; Mice were oophorectomized and supplemented with a physiologic level of estradiol
(E2). MCF-7 cells were injected s.c., and tumors were formed 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 the Materials and
Methods section.

A. Microdialysis was performed after 2 weeks of treatment on size-matched tumors as
described in Materials and Methods. Microdialysates were analyzed for human angiogenin
using quantitative ELISA. **P<0.001, Columns, mean; bars, SE.
B. Microvessel area of tumors was quantified as described in Materials and Methods section, 

***$P<0.0001$, Columns, mean; bars, SE.

C. Size-matched MCF-7 tumors of oophorectomized mice supplemented with E2 as described above, were either treated with daily injections of an anti-angiogenin antibody (filled squares) or a control IgG (filled circles) and tumor size was followed as described in the Materials and Methods section, **$P<0.001$. 
Angiogenin regulation by estradiol in breast tissue: tamoxifen inhibits its nuclear translocation and anti-angiogenin therapy reduces breast cancer growth in vivo

Ulrika W Nilsson, Annelie Abrahamsson and Charlotta Dabrosin

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