Tamoxifen Inhibits Angiogenesis in Estrogen Receptor-negative Animal Models[1]

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ABSTRACT

Inhibition of tumor angiogenesis is a therapeutic strategy that can inhibit tumor growth and metastases. The aim of this study was to determine whether the estrogen receptor (ER) ligand drug tamoxifen has antiangiogenic effects. We used three different models of angiogenesis, including measurement of microvessel densities in murine tumors, *ex vivo* aortic ring assays, and corneal pocket assays. ER-negative fibrosarcoma tumors in tamoxifen-treated ovariectomized rats had significantly less vessel formation compared with untreated animals (median microvessel density, 53.6 *versus* 94.3 counts/per ×200 field; $P = 0.002$). Rat aortic rings treated with tamoxifen at several different concentrations demonstrated significantly less vascular sprouting than control rings ($P = 0.0001$). Corneal pocket assays performed in tamoxifen-treated rats compared with control and estrogen-treated rats demonstrated decreased vascular length (0.88 mm *versus* 1.26 mm *versus* 1.47 mm; $P = 0.022$) and vessel area (21% *versus* 34% *versus* 47%; $P = 0.018$). These three animal models all showed significant inhibition of angiogenesis by tamoxifen and suggest a possible contributory mechanism of ER-independent manipulation by tamoxifen in the treatment and prevention of breast cancer. These studies raise the question as to whether or not newer ER ligand drugs might possess even more potent antiangiogenic effects, which in turn could lead to the broadening of the clinical usefulness of these compounds in a number of diseases. More importantly, these studies suggest that the antiangiogenic effects of tamoxifen are due, in part, to ER-independent mechanisms.

INTRODUCTION

Development of a vascular supply is essential for growth of malignant solid tumors and for the survival of metastases (1). This process of vascular development from preexisting capillaries has been termed "angiogenesis." This term defines a series of sequential steps in blood vessel formation, including remodeling and dissolution of the extracellular matrix, endothelial cell proliferation and migration, canalization and anastomosis of vascular loops, and formation of new basement membrane structures around new vessels.

Very little information is available regarding the effects of traditional cancer therapies on pathological and normal angiogenesis. The antiangiogenic effects of tamoxifen, a commonly used hormonal therapy for breast cancer, are not well studied *in vivo* and in tumor models that are not hormonally dependent. Tamoxifen has been shown to inhibit angiogenesis in the CAM[3] assay and in s.c. ER-positive breast cancers (MCF-7) in murine models (2, 3). Tamoxifen has also been shown to inhibit cytokine-induced endothelial cell growth (4). Finally, tamoxifen has been shown to induce hypoxia in ER-positive MCF-7 allografts (5, 6). Our experiments build on previous published work and provide further support that tamoxifen is inhibitory of pathological angiogenesis in several ER-negative models.

Many laboratory assays have been developed to study angiogenesis and its individual processes (7). Some assays, such as examination of tumor MVD, dorsal skin-fold window chambers, and cranial tumor windows, rely on *in vivo* assessment of tumor angiogenesis and can examine the interaction between tumor cells and the microcirculation. Other assays, such as the aortic ring assay, corneal pocket assay, and the CAM assay, do not involve tumor cell implantation.

Our studies used three accepted animal models (MVDs in fibrosarcoma allografts, vascular "sprouting" of aortic rings embedded in Matrigel, and rat corneal pocket model) to study the effects of tamoxifen on angiogenesis. Intratumoral MVD counts, as performed in our study with rat fibrosarcoma, have been shown in many human tumor types to be both predictive for response to therapy and prognostic for patient outcome (8–12). Both the aortic ring assay and the corneal pocket assay have been used for over a decade to screen compounds for their antiangiogenic properties (13–20). Using these three assays, we demonstrate that tamoxifen has significant antiangiogenic properties comparable with the activity of other "antiangiogenic" therapies. In addition, the fibrosarcoma allograft model did not have detectable ER expression, adding to the growing body of evidence that tamoxifen has additional mechanisms of action independent from those regulated through the ER.

MATERIALS AND METHODS

ER Modulators. General laboratory reagents, including tamoxifen and 17β-estradiol, were purchased from Sigma Chemical Co. (St. Louis, MO). All ER ligands were dissolved in 100% ethanol. Sustained release s.c. pellets containing placebo, tamoxifen, or 17β-estradiol were formulated by Innovative Research Inc. (Sarasota, FL).

Fibrosarcoma Model. All rat procedures were carried out under the guidelines and approval of the Duke University Institutional Animal Care and Use Committee Registry. Twenty-six ovariectomized female Fischer 344 rats weighing ∼100 g (Charles River Breeding Laboratories, Raleigh, NC) were randomized to either continuous release placebo or tamoxifen (25 mg) pellets (Innovative Research, Sarasota, FL). After 72 h of treatment, a piece of 1-mm^2 rat fibrosarcoma from a donor rat were implanted s.c. in the left hind limb of each animal. Ten days after xenograft implantation, rats were sacrificed and the tumors were placed in 10% buffered formalin for 24 h.

Immunohistochemistry was carried out using procedures described by Hsu *et al.* (21). Briefly, paraffin-embedded tissues were sectioned (5 μm), and antigen retrieval was performed using heat (to eliminate protein cross-linking that occurs during fixation) in a citrate buffer (which provides a stable pH and osmotic environment for the tissues during heating; Biogenex, San Ramon, CA). Tissues were treated with primary antibody against tissue transglutaminase (1:10, TG100; nonreactive to factor XIIIa),[4] mouse antihuman monoclonal ER (1:100, clone 1D5; DAKO Corp., Carpinteria, CA), and rabbit antihuman polyclonal progesterone receptor as the primary antibodies (1:100; DAKO Corp.). Secondary and tertiary antibodies were provided in a kit (314KLD; Innovex, Richmond, CA), and the location of the reaction was visualized with 3,3′-diaminobenzidine tetrahydrochloride (Sigma Chemical Co.). Slides were counterstained with hematoxylin and mounted with coverslips (22). Negative controls for the immunohistochemistry were treated with normal mouse serum (NMUS) or mouse IgG and were negative for any reactivity. For the purposes of the ER and progesterone receptor assays, nonpregnant rat endometrial tissue was used for a positive control.

MVD counts were done on the transglutaminase-stained sections by two observers (U. L. B. and Z. A. H.) blinded to the treatment assignments. Transglutaminase has been shown to localize to endothelial cells and macrophages and seems to be a specific marker for tumor vasculature (23). Using a ×20 objective and a ×10 eyepiece on a dual-headed microscope, total visualized areas of 725 μm (×200 field) were counted according to the methods of Weidner *et al.* (8). From each tumor, a single section from the central portion of the tumor was examined and three areas of intense vascularity were identified and termed "hot spots." Statistical analysis was performed on the number of vessels for the most intense hot spot (Max MVD) and the median number of vessels for three hot spots within a particular tumor (Median MVD) using a Wilcoxon sum rank test.

Aortic Ring Model. Aortic rings were prepared using a modified protocol first described by Nicosia *et al.* (13, 14).

Table 1 Median size and intratumoral MVD (interquartile range) in murine ER-negative fibrosarcomas treated with tamoxifen

	Control (n = 9)	Tamoxifen treated (n = 14)
Median tumor size (cm)	4.1 (2.1–5.8)	2.9 (2.9–3.5)
	Two-sided P = 0.138	
Median value for mean MVD	94.3 (87–97)	53.6 (48–58)
	Two-sided P = 0.003	
Median value for max. MVD	110.4 (100–119)	67.3 (62–73)
	Two-sided P = 0.0002	

Portions of descending aorta were obtained from female ovariectomized Fischer rats (weight, 75–80 g; Charles River Breeding Laboratories). Rats were anesthetized using 50 mg/kg pentobarbital (Abbott Laboratories) given i.p. Aortic portions were obtained through a dorsal midline incision and were cleaned of surrounding loose connective tissue under a microdissection scope. All aortic samples were maintained in fresh phenol-red free endothelial cell culture medium (EGM-2; Clonetics, San Diego, CA) from the time of removal.

Under sterile conditions, the aortas were cannulated with a 22-gauge needle and flushed with fresh media until no intralumenal debris remained. The aortic portions were cross-sectioned into ∼1-mm sections under the dissecting scope. The 1-mm sections were then placed in separate wells in 12-well cell culture dishes. To be able to stabilize the aortic rings in the center of the dish, enough cold phenol red-free Matrigel (400 μl; Becton-Dickson, Bedford, MA) was added to the center of each well to cover approximately half the height of the ring. Plates were then incubated at 37°C for 30 min. After the incubation and once the rings were fixed to the center of the well, an additional 300 μl of cold phenol-red-free Matrigel was added to each well and plates were incubated for an additional 20 min.

Phenol-red free, endothelial cell media (1 ml, EGM-2; Clonetics) was then added to each well. Half of the wells containing rings from an individual animal were treated with vehicle (pure ethanol, 2 μcl/ml of media), and the other half of the wells were treated with various concentrations (2×10^{-5} M to 2×10^{-7} M) of tamoxifen. All wells had equal concentrations of ethanol added (0.1%).

Images of sprouting within the wells was captured on day 10 using a Scion Image analysis program (Scion, Frederick, MD) and a ×4 objective and a ×10 eyepiece on a Zeiss (Thornwood, NY) light microscope. Wells were graded as: 0, no sprouts; 1, occasional sprouts but not continuous around the aortic ring (sprouts from <50% of the diameter of the ring); 2, continuous sprouts around edge of aorta (sprouts from >50% of the diameter of the ring). All scoring took place by two observers (K. L. B. and Z. A. H.) unaware of the treatment. Once images were captured, aortic rings were removed from culture plates and stored in Histochoice (Amresco, Solon, Ohio) for 24 h. Preserved rings were then embedded in paraffin, sectioned, and immunohistochemically stained for the presence of ER and progesterone receptor, as described for the fibrosarcoma model.

Corneal Pocket Model. Rat corneal pockets were performed using a modified protocol described previously by Pol-

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[4] Unpublished data.

Fig. 1 MVD staining in rat fibrosarcoma xenografts treated with placebo or tamoxifen. Vessel structures in rat fibrosarcoma sections have been stained with transglutaminase, and each section's hot spot had MVD determinations. Control fibrosarcomas (*A*) had significantly higher MVD counts than tamoxifen-treated fibrosarcomas (*B*).
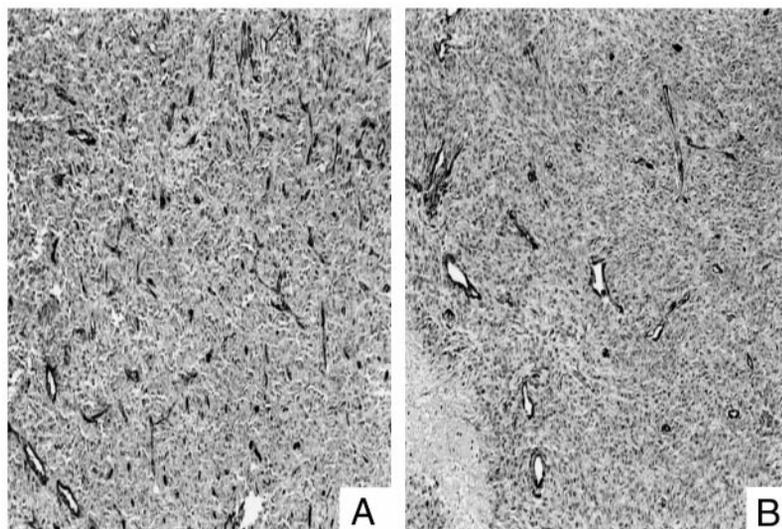
Table 2 Inhibition of microvessel sprouting by tamoxifen in the rat aortic ring model

Treatment	*n*	Sprouting score		
		0	1	2
Placebo-treated rings	42	5	10	27
All tamoxifen-treated rings[a]	48	34	8	0
Tamoxifen (10^{-5} M)	12	11	1	0
Tamoxifen (10^{-6} M)	24	20	4	0
Tamoxifen (10^{-7} M)	12	4	8	0

[a] Difference from placebo-treated rings by Kruskal-Wallis test, $P = 0.0001$.

verini *et al.* (15). Thirty Fischer-344 rats were randomized to receiving either: (*a*) placebo; (*b*) estradiol (1.5 mg); or (*c*) tamoxifen (25 mg) 21-day s.c. continuous release pellets (Innovative Research). Pellets were placed 72 h before corneal surgeries.

The rats were anesthetized using pentobarbital (50 mg/kg) given i.p. and 1 drop of 0.5% proparacaine topically to the cornea. After anesthesia was induced, the study eye was gently proptosed and secured in place by suturing the eyelids. A 1.5-mm incision through the corneal center was made under the dissection microscope. A micropocket within the corneal matrix was created by using a modified spatula with the bottom of the micropocket placed ∼1–1.2 mm from the limbus. A preformed sustained release polymer [poly (2-hydroxyethyl methacrylate); Sigma Chemical Co.] containing basic fibroblastic growth factor (50 ng/3 µl pellet) was placed at the bottom of the micropocket. No suturing or other closure was necessary.

After 7 days of treatment, the animals were deeply anesthetized with i.p. pentobarbital (50 mg/kg). The descending aorta was identified, and a PE 50 catheter was inserted. Each rat was perfused with 50–100 ml of Ringer's lactate, followed by 10 ml of India ink to visualize corneal vessels. The study eye was then enucleated, fixed in formalin overnight, dissected from the eyeball, and then placed on a microscope slide. The corneas were then examined and analyzed at ×10 power using the Scion Image analysis program (Scion) and a Zeiss light microscope. All measurements were made by observers (U. L. B., S. S., W. S.) unaware of the treatment arms. Standardization variables included pellet-limbus distance (line drawn from edge of pellet to most lateral edge of neovascularization) and pellet size. Outcome measurements included circumference of vascularity, maximum vessel length (length of the longest vessel from tip of vessel to limbus), mean vessel length (average of the length of five vessels at set distances from the center of the limbus), and total vessel area (area covered by vessels in three different, preset 0.5 mm² areas/1.5 mm²).

RESULTS

Fibrosarcoma Model. Twenty-three of the 26 rats that had tumors implanted had tumor growth (9 control rats and 14 tamoxifen-treated rats). There was no significant delay in tumor growth seen between the two treatment groups (4.1 cm at 10 days for control tumors *versus* 2.9 cm in tamoxifen-treated tumors; $P = 0.138$; Table 1). Tumors from animals treated with tamoxifen had significantly lower median and maximum MVDs than those tumors in the placebo-treated animals (Fig. 1 and Table 1; two-sided *P*s of 0.002 and 0.005, respectively). In addition, none of the tumor samples tested from either treatment group had immunohistochemically detectable staining for the ER or the progesterone receptor.

Aortic Ring Model. Seventeen rats had their aortas harvested, and a total of 102 rings (6 from each animal) were implanted in Matrigel. The average length (from cut end to cut end) of the rings was 1.2 mm, and the average diameter was 1.8 mm. Twelve rings derived from two rats did not exhibit any sprouting within any of the wells, including the control wells, and were, therefore, excluded from the analysis. Median sprouting scores in the tamoxifen-treated rings were significantly lower than those in the control-treated group (Table 2; $P = 0.0001$). No statistical differences were seen in the sprouting scores of the rings treated with various doses of tamoxifen.

Table 3   Results from use of placebo, tamoxifen, and estradiol in the rat corneal pocket model

Table of means (SEs of the mean) by treatment group.

Variable	Control	Tamoxifen	Estrogen	P (ANOVA F-test)
Standardization variables				
Pellet-limbus distance (pixels)	131 (11.1)	129 (6.95)	124 (7.15)	0.86
Pellet size (pixels)	26310 (1620)	28180 (1444)	24669 (1552)	0.29
Circumference (pixels)	671 (54.2)	630 (32.6)	602 (43.0)	0.56
Result variables				
Mean vessel length (mm)	1.47 (0.21)	0.88 (0.10)	1.26 (0.099)	0.022[a]
Maximum vessel length (mm)	1.85 (0.24)	1.19 (0.13)	1.59 (0.084)	0.027[a]
Vessel area	0.34 (0.074)	0.21 (0.043)	0.47 (0.063)	0.018[b]

[a] These results indicate that the control group and the estrogen group are the same with respect to mean vessel length and maximum vessel length. The overall conclusion of these two tests is that standard dose tamoxifen inhibits the length of the vessels (mean and maximum) as compared with the control or estradiol group.

[b] This result indicates that the estrogen group (higher vascularity) and the tamoxifen group (lower vascularity) are different from the control group with respect to the vessel area.

Corneal Pocket Model.   The results from the corneal pocket model are shown in Table 3. All standardization variables (pellet-limbus distance, bFGF pellet size, and circumference) were equivalent between the treatment groups. Animals treated with tamoxifen had significant inhibition of corneal vessel lengths (mean, $P = 0.022$; maximum, $P = 0.027$) and vessel areas ($P = 0.018$) when compared with control animals (Fig. 2). In addition, corneal vessel areas were significantly different between the three treatment groups ($P = 0.018$). Estradiol-treated corneas had the highest amounts of vessel area (47% of the corneal area covered with vessels), control corneas had intermediate amounts (34% of the corneal area covered with vessels), and tamoxifen-treated corneas had the lowest amounts (21% of the corneal area covered with vessels). There was no difference in vessel lengths between estradiol-treated animals and control animals.

DISCUSSION

Our studies revealed that tamoxifen has significant antiangiogenic properties in several animal models. The therapeutic effects of antiestrogens seen in breast cancer might have contributory mechanisms besides interactions with the ER. The alternative therapeutic mechanisms of tamoxifen include impairment of multidrug resistance proteins (24–26), inhibition of protein kinase C (27), regulation of essential tumor growth factors (28), and, as our study revealed, antiangiogenic effects. These possible antiangiogenic effects are not mechanistically well defined and have been examined through a number of separate, yet hard to compare, techniques.

Our studies tried to minimize interactions between the ER ligand effects of tamoxifen on tumor cells themselves and the possible other ER ligand-independent effects on tumor cells by using estrogen-free (phenol-red free) media, ER-negative tumor lines (fibrosarcoma), and tumor-free *in vivo* angiogenic assays. The *in vivo* fibrosarcoma study revealed that tamoxifen inhibited MVD independent of its direct effect on the ER, as these fibrosarcoma tumors did not have immunohistochemically detectable amounts of ER. Because immunohistochemical staining for the presence of ER has been shown to be superior to ER radiolabeled binding assays in predicting response to tamoxifen therapy, we felt comfortable with the use of immunohistochem-
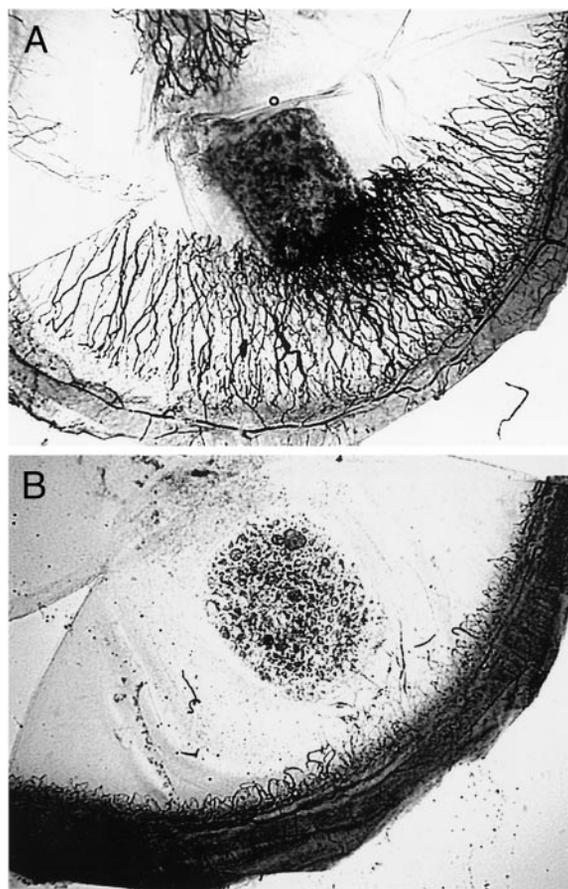

Fig. 2   Rat corneal pocket model using placebo or tamoxifen. Control-treated corneas (*A*) had significantly longer vessel lengths and higher amounts of vascular area than tamoxifen-treated corneas (*B*). Corneas are shown after injection of India ink, fixation, and at a magnification of ×10.

ical staining alone to examine the presence of the ER and progesterone receptor in the fibrosarcoma model (29). The rat aorta assay and the corneal pocket assays demonstrated significant inhibition of angiogenesis independent of the direct toxic effects of tamoxifen on tumors.

The mechanism through which tamoxifen inhibits angiogenesis is still unknown. Postmenopausal women are known to have impaired rates of wound healing, whereas as postmenopausal women receiving estrogen replacement therapy have wound healing rates comparable with those seen in premenopausal women. Moreover, postmenopausal women have decreased levels of TGF-β1 within wounds as compared with premenopausal women, suggesting that estrogens play a role in TGF-β1 modulation and wound healing (30). Tamoxifen has been shown to modulate TGF-β1 in human breast cancer cells *in vivo*, and perhaps this modulation contributes to its antiangiogenic effects (31, 32).

Estrogens also have a role in modulation of another important angiogenic molecule, VEGF. Estrogen-induced rat pituitary tumors express very high levels of VEGF protein and receptor (33). Recent evidence reveals that estrogens and tamoxifen up-regulate VEGF mRNA expression in uterine tissue (34) and cultured breast cancer cells (35). In addition, two ER-binding sites have been identified in the untranslated upstream region of the *VEGF* gene (36). This estrogen-modulated up-regulation of VEGF may play a role in early tumor development. The effects of tamoxifen on VEGF regulation in endothelial cells, smooth muscle cells, and *in vivo* systems is unknown.

Several other hormonal compounds have also been shown to have antiangiogenic activity. The estrogen metabolite 2-methoxyestradiol has previously been shown to inhibit tubulin polymerization by binding at the colchicine-binding site. This estrogen metabolite, with very weak binding affinity to the ER, displays potent antiangiogenic effects in the CAM and corneal pocket model (37, 38). Another hormonal agent, the synthetic progestational agent medroxyprogesterone acetate, has been shown to inhibit neovascularization and tumor growth in the rabbit cornea (16, 39). These compounds provide further evidence for the important role of hormonal compounds in angiogenesis.

The most common and most effective means of treating both early and metastatic ER-positive breast cancer continues to be hormonal manipulation. The role of tamoxifen in the treatment of ER-negative breast tumors remains controversial. Our studies contribute to the growing body of literature demonstrating a multitude of ER-dependent and -independent effects caused by tamoxifen on both tumor and normal host cells. These results also offer a suggestion that either direct endothelial cell damage or inhibited endothelial cell repair might contribute to the increased risk of thromboembolic disease seen in patients treated with tamoxifen.

Furthermore, our studies support previous *in vitro* studies that tamoxifen is a potent inhibitor of angiogenesis at levels comparable with that of other known antiangiogenic agents (16–20). In each of our assays, tamoxifen reduced levels of angiogenesis by more than half of that found in control assays. These effects have implications not only for the treatment and prevention of breast cancer, but for the development of combination antiangiogenic therapies and the discovery of important angiogenic pathways in human breast cancer.

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Tamoxifen Inhibits Angiogenesis in Estrogen Receptor-negative Animal Models

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Clin Cancer Res 2000;6:4359-4364.

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