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Tamoxifen Inhibits Secretion of Vascular Endothelial Growth Factor in Breast Cancer *in Vivo*

Stina Garvin and Charlotta Dabrosin

Division of Gynecologic Oncology, Faculty of Health Sciences, University Hospital, Linköping, Sweden

ABSTRACT

Vascular endothelial growth factor (VEGF) is considered a key mediator of tumor angiogenesis, including neovascularization in human breast cancer. High tissue VEGF levels appear to correlate with poor prognosis and decreased overall survival in node-positive and node-negative breast cancer patients. Hormonal regulation of VEGF expression has been demonstrated, and some reports indicate that tamoxifen, a partial estrogen receptor agonist, increases VEGF mRNA in breast cancer cells. These results appear to contradict the efficacy of tamoxifen as an adjuvant for estrogen-dependent breast cancer, yet clinical data show that tamoxifen prevents metastasis and increases overall survival. In this study, we confirmed previous studies showing that intracellular levels of VEGF *in vitro* increased in response to tamoxifen to levels similar to those observed after estrogen treatment. To further study hormonal effects on the release of VEGF, we used microdialysis to sample the extracellular space, where VEGF is biologically active, in solid tumors *in situ*. We show for the first time that tamoxifen decreased extracellular VEGF *in vivo* in solid MCF-7 tumors in nude mice. These *in vivo* findings were confirmed *in vitro* where extracellular VEGF in the cell culture medium was decreased significantly by tamoxifen treatment. Furthermore, we illustrate that microdialysis is a viable method that may be applied in human breast tissue to detect soluble VEGF *in situ* released by the tumor.

INTRODUCTION

Angiogenesis has been shown to be an essential factor for tumor growth and development of metastasis (1). Vascular endothelial growth factor (VEGF) is a key factor in promotion of tumor angiogenesis (1, 2). In breast cancer tissue, VEGF mRNA expression is increased compared with adjacent normal breast tissue (3). Moreover, high tissue VEGF levels appear to correlate with poor prognosis, and decreased overall survival for node-positive and node-negative breast cancer patients (4, 5). Several VEGF isoforms are produced from a single gene as a result of alternative splicing (6). The isoforms differ in their biological properties and in their abilities to bind heparan sulfate proteoglycans (2). VEGFs are bioactive as freely diffusible proteins in the extracellular space where they become available to endothelial cells, and in one report it has been suggested that the soluble isoforms have greater angiogenic and tumorigenic properties than the heparin-bound isoforms (7).

Estrogen has been shown to modulate angiogenesis in the female reproductive tract under physiologic and pathologic conditions, mainly via effects on endothelial cells (8, 9). Moreover, an estrogen-responsive element in the promoter region of the gene for VEGF has been identified (10). Estrogen exposure is considered a major risk factor for development of breast cancer, and the majority of breast cancers maintain their hormonal dependency (11–13). Therefore, strategies aiming at reducing the influence of estrogen on breast cancer cells have been developed. The most frequently used endocrine therapy for all of the stages of breast cancer is tamoxifen. Tamoxifen is a partial agonist of the estrogen receptor, and it has been shown that breast cancer cells exposed to tamoxifen increase VEGF mRNA expression (14, 15). This result would seem to contradict the efficacy of tamoxifen as an adjuvant for estrogen-dependent breast cancer. However, little is known about the hormonal regulation of VEGF, including the effects of tamoxifen on the levels of VEGF in the extracellular space.

Therefore, we have investigated effects of tamoxifen on VEGF secretion in human estrogen receptor-positive breast cancer cells *in vitro* and in solid tumors *in vivo*. In the present study, we show that the *in vitro* secretion of VEGF was inhibited by tamoxifen, whereas the intracellular levels of VEGF were increased by tamoxifen in the same manner as with estradiol. Moreover, we could verify, using microdialysis, that tamoxifen treatment decreased secretion of VEGF *in vivo* in solid breast cancer tumors in nude mice.

MATERIALS AND METHODS

Cells and Culture Conditions. MCF-7 cells (estrogen receptor-positive and progesterone receptor-positive; American Type Culture Collection, Manassas, VA) were used in all of the experiments. Cells were cultured in DMEM without phenol red supplemented with 2 mM glutamine, 50 IU/ml penicillin-G, 50 μg/ml streptomycin, and 10% fetal bovine serum at 37°C in a humidified atmosphere containing 5% CO_2. Cell culture medium and additives were obtained from Life Technologies, Inc. (Paisley, United Kingdom) if not otherwise stated. Before the experiments, cells were trypsinized (0.05% trypsin and 0.02% EDTA) and seeded into Petri dishes (Costar, Cambridge, MA), 10,000 cells/cm^2. Cells were incubated for 1 day and then treated with or without 10^{-8} M estrogen (17β-estradiol; Apoteket, Umeå, Sweden), 10^{-6} M tamoxifen (Sigma, St. Louis, MO), or a combination of estradiol and tamoxifen. Hormones were added to the MCF-7 cultures in serum-free medium consisting of a 1:1 mixture of nutrient mixture F-12 (HAM) and DMEM without phenol red supplemented with 10 μg/ml transferrin (Sigma), 1 μg/ml insulin (Sigma), and 0.2 mg/ml BSA (Sigma). The medium was changed every day.

Western Blot Analysis. Cells were lysed in 63 mM Tris-HCl (pH 6.8), 10% glycerol, and 2% SDS. Bromphenol blue (0.05%) was added to samples of 12-μg protein each, and the samples were fractionated by 15% SDS-PAGE under nonreducing conditions. The proteins were subsequently transferred to a nitrocellulose membrane, which was incubated in a blocking solution (5% skimmed milk and 0.1% Tween 20 in Tris-buffered saline; *i.e.,* 50 mM Tris-buffered saline supplemented with 0.15 M NaCl) for 90 min at room temperature and subsequently washed in Tris-buffered saline. Thereafter, the membrane was exposed for 16 h at 4°C to a mouse antihuman VEGF antibody (1:500; R&D Systems, Minneapolis, MN), followed by a horseradish peroxidase-conjugated goat antimouse antibody (1:1000; DakoCytomation, Glostrup, Denmark). Bands were visualized using enhanced chemiluminescence (Amersham Biosciences, Uppsala, Sweden). Recombinant human VEGF165 and VEGF121 were used as controls (R&D Systems).

Immunofluorescence Detection of VEGF. Cell cultures were fixed in 4% formaldehyde in PBS for 20 min at 4°C and then processed for immunocytochemistry as described earlier (16). The cells were incubated with a monoclonal mouse antihuman VEGF antibody (dilution 1:100; R&D Systems), followed by a goat antimouse IgG Texas Red conjugate (1:200; Vector Laboratories, Burlingame, CA). Thereafter, the cells were rinsed in PBS and distilled water, and mounted in Vectashield medium (Vector Laboratories). The cultures were examined in a Nikon photomicroscope (Nikon Corporation,

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Requests for reprints: Charlotta Dabrosin, Division of Gynecologic Oncology, University Hospital, SE-581 85 Linköping, Sweden. Phone: 46-13-22-85-95; Fax: 46-13-22-44-60; E-mail: lotda@imk.liu.SE.

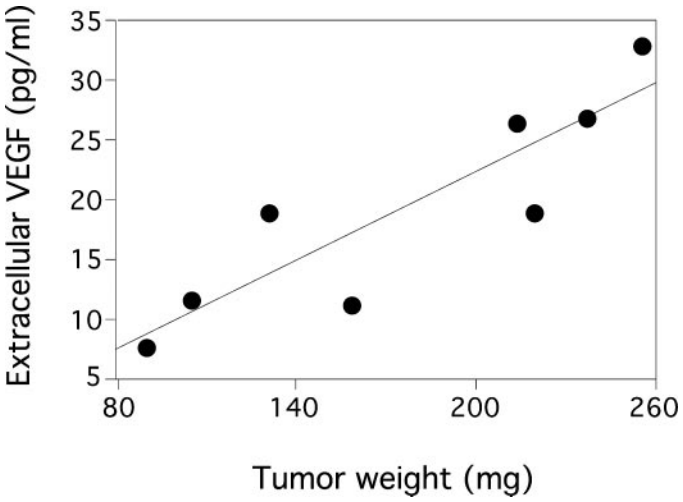

Fig. 1. Correlation between tumor weight and extracellular VEGF *in vivo* measured using microdialysis. Solid MCF-7 tumors in nude mice were subjected to microdialysis for collection of VEGF in the extracellular space. Tumor weight and extracellular VEGF correlated significantly ($r^2 = 0.788$; $P = 0.003$).

Tokyo, Japan) using green exciting light and a 590-nm barrier filter, and photographed with a digital camera. Controls incubated without anti-VEGF antibodies did not stain.

Animals and Ovariectomy of Mice. Female athymic mice (6–8-weeks-old) were purchased from M&B (Ry, Denmark). They 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 Linköping University animal ethics research board approved all of the animal work. Mice were anesthetized with i.p. injections of ketamine/xylazine and ovariectomized; 3-mm pellets containing 17β-estradiol, 0.18 mg/60-day release, or placebo pellets (Innovative Research of America, Sarasota, FL) were implanted s.c. in the back of the animal 7 days before tumor induction. The pellets provide continuous release of estradiol at serum concentrations of 150–250 pM (confirmed by serum analysis), which is in the range of physiologic levels seen in mice during the estrous cycle. One week after surgery, MCF-7 cells (5×10^6 cells in 200 μl PBS) were injected s.c. on the right hind flank. Tumor volume was determined by measuring length, width, and depth of the tumor every 5 days using a caliper. At a tumor size of ~300 mm^3, the mice were divided into two subgroups. One group continued with the estradiol treatment only, whereas tamoxifen (1 mg/every 2 days s.c.) was added to the estradiol treatment for 2 weeks in the other group. Plasma was collected in heparin by cardiac puncture.

Microdialysis Equipment and Experiment. Microdialysis has previously been used extensively for sampling of molecules from the extracellular space from various tissues *in vivo* (17). Tumor-bearing mice were anesthetized i.p. with ketamine/xylazine and were kept anesthetized by repeated s.c. injections. A heating pad maintained the body temperature. A small skin incision was made, and microdialysis probes (CMA/20, 0.5-mm diameter; PES membrane length = 10 mm, 100,000-molecular weight cutoff; CMA/Microdialysis, Stockholm, Sweden) were inserted into tumor tissue and fixed by sutures to the skin. The probes were connected to a CMA/102 microdialysis pump (CMA/Microdialysis) and perfused at 1 μl/min with saline containing 154 mM NaCl and 40 mg/ml dextran (Pharmalink, Stockholm, Sweden). After a 30-min equilibration period, the outgoing perfusate was collected on ice and stored at −70°C for subsequent analysis. We have validated recently the 100,000-molecular weight cutoff membrane for VEGF measurement in murine tumors (18) and also have shown that this technique is suitable for VEGF measurement in human breast tissue (19).

Immunohistochemistry of Tumor Sections. Formalin-fixed, paraffin-embedded tumors were cut in 3-μm sections, deparaffinized, and subjected to antihuman VEGF immunohistochemistry (monoclonal mouse antihuman VEGF; dilution 1:20; R&D Systems, with Envision detection; DakoCytomation) or anti-von Willebrand's factor (rabbit antihuman von Willebrand; dilution 1:1000; DakoCytomation). Sections were counterstained with Mayer's hematoxylin. Negative controls did not show staining. In a blinded manner, 10 high power fields (×200) were examined by section of three different tumors in each group. For VEGF scoring, the whole material was scanned to identify the range of intensity of the staining. Thereafter, the staining on the tumor sections was scored either as weakly or strongly positive. Vessel quantification of tumor sections was conducted as described previously using a Nikon microscope equipped with a digital camera (20). Percentage of area stained positively for von Willebrand's factor was assessed using Easy Image Measurement software (Bergstrom Instruments). Tumor sections were also subjected to H&E staining.

Quantification of VEGF Protein. Microdialysate, plasma samples, and cell culture medium were analyzed for VEGF using a commercial quantitative immunoassay kit for human VEGF (QuantGlo; human VEGF; R&D Systems) without preparation. Cell culture pellets were frozen at −70°C, thawed once, diluted in PBS, and sonicated for 10 s. Protein content was determined using the method described by Lowry *et al.* (21). According to the manufacturer, this kit measures the VEGF165 and VEGF121 isoforms. The sensitivity is <1.76 pg/ml, and intra-assay and interassay precision is 3–8%. The precision of the ELISA kit was confirmed during the experiments. All of the samples were assayed in duplicate.

Quantification of VEGF mRNA. VEGF mRNA was detected using human VEGF Quantikine Colorimetric mRNA Quantitation kit (R&D Systems). Cell lysate samples were made using the provided cell lysate diluent and frozen at −70°C. According to the manual, the whole cell lysate samples were hybridized with the VEGF probe and then transferred to a plate coated with streptavidin. The VEGF mRNA probe and calibrator in this kit cannot distinguish between the different isoforms of VEGF. VEGF mRNA was quantified and correlated to the total amount of protein in the samples.

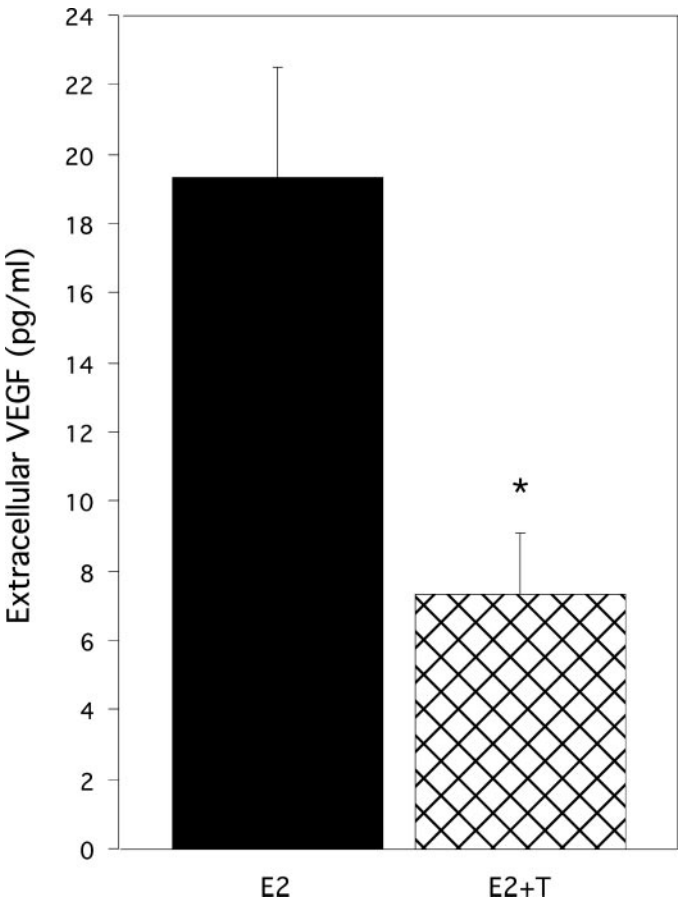

Fig. 2. Extracellular levels of VEGF in solid MCF-7 tumors in nude mice *in vivo* measured with microdialysis. Mice were oophorectomized and supplemented with a physiologic level of estradiol (*E2*). MCF-7 cells were injected s.c., and tumors were formed on the right hind flank. One group of mice continued with E2 only, and in the other group tamoxifen (*T*) treatment was added to the E2 treatment. Thereafter, microdialysis was performed on size-matched tumors as described in "Materials and Methods." The perfusate was analyzed using ELISA ($n = 8$ in the E2 group and $n = 5$ in the E2 + T group; *, $P < 0.05$).

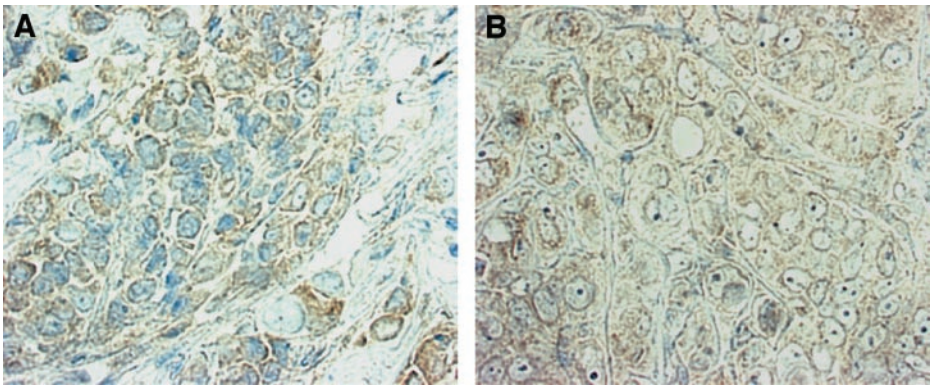

Fig. 3. Immunohistochemistry of VEGF in tumor sections. Mice were treated as described in Fig. 2. There were no differences in distribution or intensity of staining in tumors from the two different treatment groups. Tumor sections from three different tumors in each group and 10 randomly selected areas from each tumor were analyzed. Representative tumors are shown. *A*, tumor section from an estradiol-treated tumor. *B*, tumor section from an estradiol + tamoxifen-treated tumor.

Statistics. The values represent the mean ± SE. Statistical analyses were performed using Student's *t* test, ANOVA with Fisher's post hoc test, or Fisher's exact test where appropriate.

RESULTS

Tamoxifen Decreased Extracellular VEGF in Solid MCF-7 Tumors. MCF-7 tumors require estradiol for growth in nude mice. Therefore, there is no untreated control group or a tamoxifen-alone group in the *in vivo* experimental design. To explore the secretion of VEGF *in vivo*, we performed microdialysis to sample the extracellular fluid of the tumors. Because hypoxia is a potent regulator of VEGF expression, mainly through hypoxia-inducible factor-1, the first experiments were conducted on tumors of different sizes to explore whether tumor size influenced the released extracellular VEGF *in vivo* (22). As shown in Fig. 1, tumor size correlated significantly with extracellular VEGF ($r^2 = 0.788$; $P = 0.003$). Therefore, to avoid this confounding factor, all of the experiments were performed on size-matched tumors (in all of the treatment groups). Tumor weights were 176 ± 22 mg in the estradiol group and 171 ± 21 mg in the estradiol + tamoxifen group. Tumor sections did not reveal any necrotic areas on H&E staining. Microdialysis was performed on estradiol-treated tumors at days 35 and 50 after tumor cell injection, revealing no difference in the secreted VEGF over this timeframe in tumors of similar size. In tumors from mice treated with a combination of estradiol + tamoxifen for 2 weeks, the extracellular levels were 7.3 ± 1.7 pmol/ml; in tumors from mice treated with estradiol only, the levels were 19.4 ± 3.2 pmol/ml ($P < 0.05$; Fig. 2). The difference in the extracellular tumor levels of VEGF was not revealed in plasma of the mice. Plasma levels of VEGF in estradiol-treated animals were 10.5 ± 2 pM compared with 12 ± 0.4 pM in estradiol + tamoxifen-treated mice.

Immunohistochemistry of VEGF in Solid MCF-7 Tumors. Immunohistochemistry of intracellular cytoplasmic VEGF in tumor sections showed no detectable difference between the groups; in tumors from estradiol-treated animals, 22 of 30 sections were scored as strongly positive, whereas 19 of 30 were strongly positive in the estradiol + tamoxifen group ($P = 0.6$). Extracellular VEGF either bound to the cell surface or in the extracellular matrix was not possible to detect. This suggests that the intracellular content of VEGF was similar in the estradiol group and in the group treated with estradiol + tamoxifen. Representative tumor sections are shown in Fig. 3.

Tamoxifen Decreased Tumor Vasculature. To evaluate whether the decreased extracellular levels of VEGF had a biological relevance for tumor vasculature, we quantified vessel area stained with anti-von Willebrand's factor. We found that the vessel area was significantly lower on tumor sections from animals treated with a combination of estradiol + tamoxifen compared with estradiol treatment only (1.2 ± 0.2% of total area *versus* 5 ± 1.1%; $P < 0.05$; Fig. 4*C*). Representative tumor sections are shown in Fig. 4, *A* and *B*.

Tamoxifen Decreased VEGF Secretion into the Cell Culture Media but Increased Intracellular VEGF Protein. To confirm our *in vivo* results, we determined the amount of intracellular and secreted VEGF from MCF-7 cells in culture using a quantitative ELISA in a time course experiment. MCF-7 cells were exposed to the various treatments, and the media were changed every day. Cells and media were harvested on days 1, 3, and 7. Fig. 5*A* shows the secretion, which was increased by estradiol exposure on days 3 and 7, whereas estra-

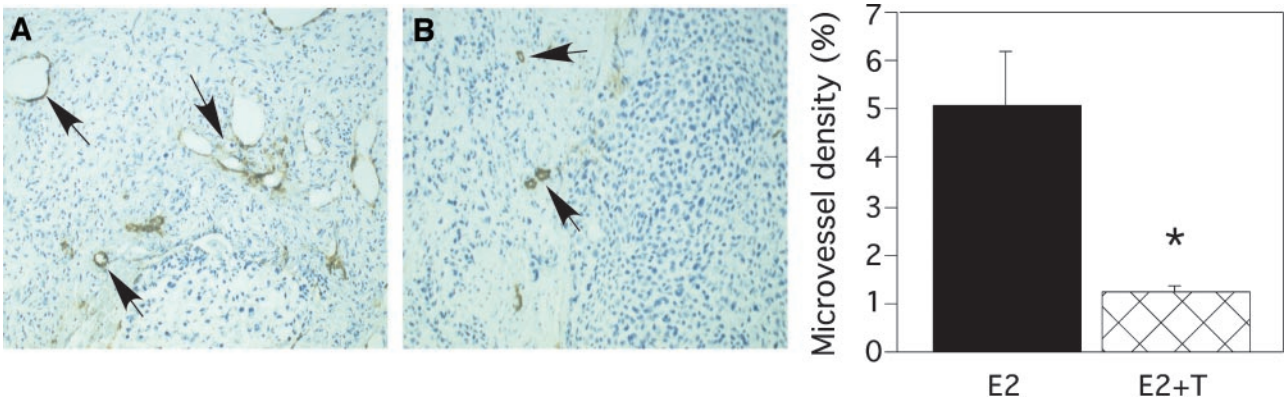

Fig. 4. Tamoxifen (*T*) decreased tumor vasculature. Mice were treated as described in Fig. 2. Tumor sections were stained with anti-von Willebrand's factor, and vessel area was counted on tumor sections. *A*, representative MCF-7 tumor exposed to estradiol (*E2*). *B*, representative MCF-7 tumor treated with a combination of E2 + T. *C*, tumor vessel density quantification was conducted in a blinded manner. Ten randomly selected areas of three different tumors in each group were counted (*, $P < 0.05$).

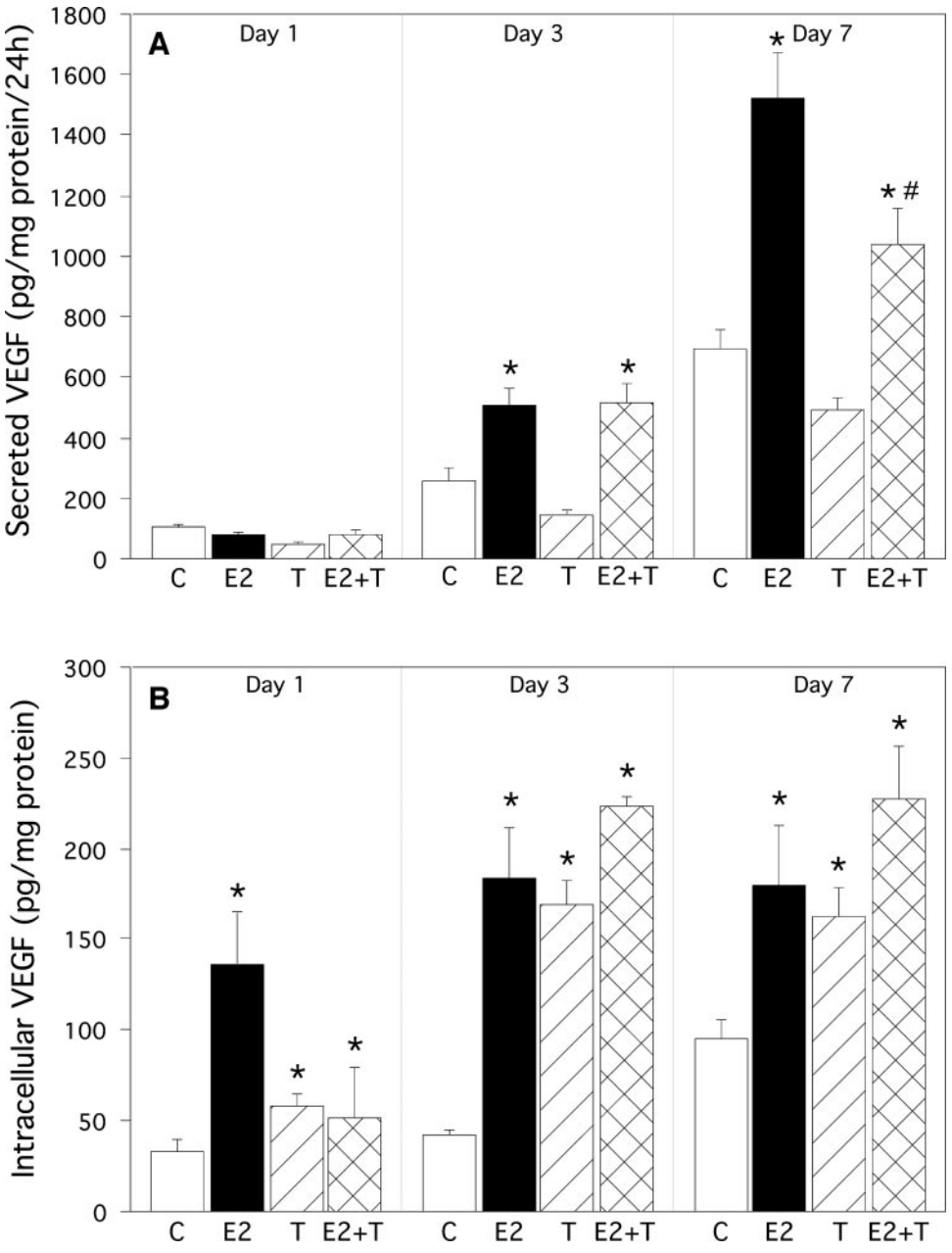

Fig. 5. Secreted and intracellular VEGF after hormone exposure of MCF-7 cells in culture. MCF-7 cells were cultured without hormones (*C*) or in the presence of estradiol (*E2;* 10^{-8} M), tamoxifen (*T;* 10^{-6} M), or a combination of E2 + T. The media were changed every day. VEGF was measured using ELISA after 1, 3, and 7 days in culture (n = 3–8 in each group; *, $P < 0.01$ compared with control cells; #, $P < 0.01$ compared with E2-treated cells).

diol + tamoxifen partially reversed the secretion at the same time points. However, the intracellular levels exhibited a similar increase in all of the hormone treatment groups compared with the control group (Fig. 5*B*).

Western Blot of VEGF. The ELISA kit we used in this study is not validated for measurement of cell culture lysates, and to confirm these results we also performed a Western blot analysis. The Western blot analysis confirmed our ELISA results and showed an increase of VEGF165 in all of the treatment groups compared with control cells (Fig. 6*A*). Three different Western blot analyses were scanned, and densitometry was performed ($P < 0.05$ between hormone-treated cells compared with control cells; Fig. 6*B*).

Estradiol and Tamoxifen Increased VEGF mRNA Levels. To confirm that there was a true increase in synthesis of VEGF and not an increased uptake, we performed mRNA quantification. All of the hormone treatments increased the VEGF mRNA levels ($P < 0.05$; Fig. 7).

Intracellular Localization of VEGF. To further investigate whether hormone treatment also affected the intracellular localization of VEGF, we performed immunofluorescence staining of the cells. As shown in Fig. 8, *B–D*, cells from all of the treatment groups exhibited similar intracellular cytoplasmic localization of VEGF and a more intense staining compared with control cells in Fig. 8*A*.

DISCUSSION

In this study we show for the first time that tamoxifen in combination with estradiol decreases extracellular VEGF, measured with microdialysis, in solid breast cancer tumors *in situ* in nude mice compared with estradiol treatment only. Our *in vivo* findings were verified *in vitro*, where extracellular levels of VEGF in cell culture media decreased significantly after exposure to a combination of estradiol + tamoxifen compared with estradiol exposure only. Immunohistochemistry of the same tumor sections did not reveal any

different staining between the groups. Similarly, *in vitro* tamoxifen increased intracellular VEGF protein and mRNA in a similar fashion as estradiol. It should be noted that the *in vivo* model does not contain a nonestradiol control group, but the estradiol and estradiol + tamoxifen groups exhibited similar results *in vitro* and *in vivo*.

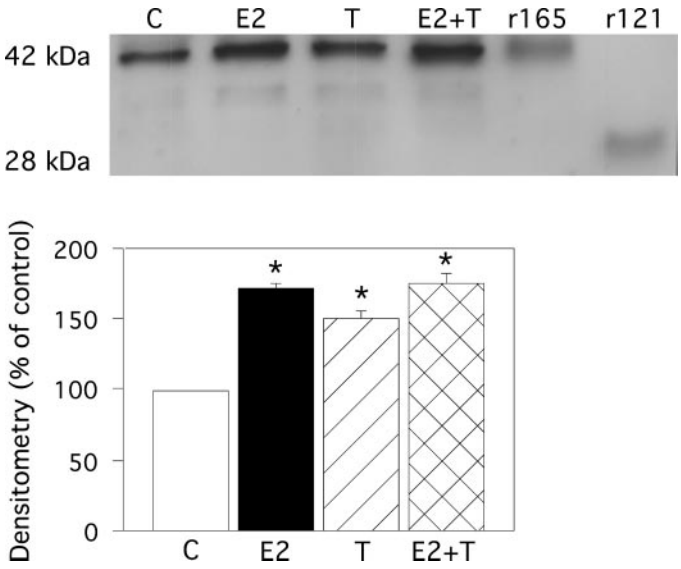

Fig. 6. Western blot analysis of intracellular VEGF in MCF-7 cells. MCF-7 cells were cultured as described in Fig. 5 and in "Materials and Methods." Recombinant VEGF165 and VEGF121 were used as controls. *A*, representative gel of three separate experiments. *B*, densitometry of three different gels (*, $P < 0.05$). An increase in VEGF was observed in all treatment groups in accordance with the VEGF levels quantified with the ELISA kit.

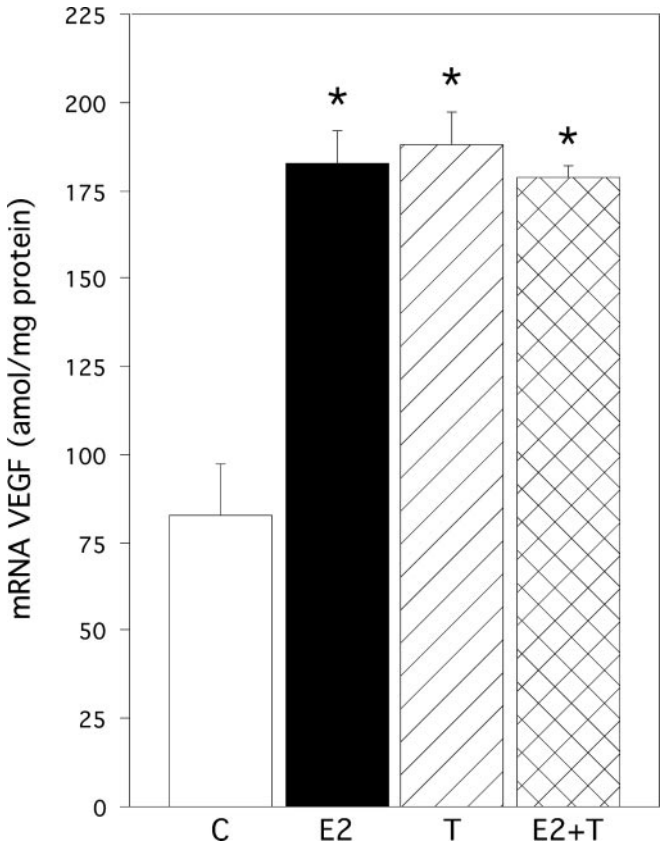

Fig. 7. VEGF mRNA levels. MCF-7 cells were cultured as described in Fig. 5 and in "Materials and Methods" section. Human mRNA VEGF was quantified using a colorimetric mRNA quantitation kit. All treatment groups exhibited significantly higher levels of mRNA compared with the control group (*, $P < 0.01$).

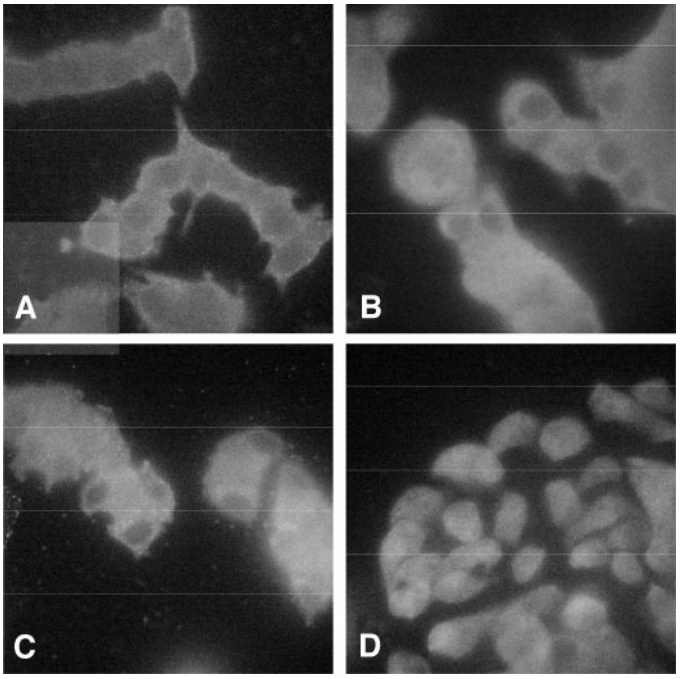

Fig. 8. Subcellular localization of VEGF in MCF-7 cells. MCF-7 cells were cultured as described in Fig. 5 and in "Materials and Methods." *A*, control cells without hormones. *B*, estradiol (*E2*) -exposed cells. *C*, tamoxifen (*T*) -exposed cells. *D*, E2 + T-exposed cells. Cells treated with hormones revealed a more intense staining compared with control cells, but no differences in subcellular localization of VEGF were observed.

Tamoxifen is the most widely used nonsteroidal antiestrogen for the treatment of hormonal-responsive breast cancer. It is well known that tamoxifen has estrogen antagonist/agonist modes of action, and several studies have shown that tamoxifen induces VEGF mRNA levels and intracellular protein (14, 15). This enhanced expression would seem to lead to an increased metastatic potential of cancer cells and is in conflict with clinical data, which show that tamoxifen prevents metastasis and increases overall survival of patients. However, there are, to our knowledge, no studies about effects of tamoxifen on extracellular levels of VEGF, the extracellular space being the biologically active site for VEGF.

The shorter isoforms of VEGF, 121 and 165, are soluble secreted proteins, although a portion of the 165 form remains bound to the cell surface, whereas the larger isoforms bind tightly to heparin and are sequestered in the extracellular matrix (23, 24). VEGFs are bioactive as freely diffusible proteins in the extracellular space, where they act on endothelial cells by stimulating cell proliferation, migration, and tubular organization and increase vascular permeability (24). Previous assessments of VEGF protein have been performed by immunohistochemistry or immunoassay of tissue extracts, and these measurements appear to correlate with microvessel density at least in invasive ductal carcinoma of the breast (25, 26). However, VEGF121, considered to be the most potent stimulator of angiogenesis *in vivo* and the predominate isoform in primary human breast cancer, diffuses freely into the extracellular space from the cells producing it and cannot be detected by immunostaining of tumor sections (7, 27). VEGF measured in blood has been considered as an alternative to these methods, but the interpretation of such studies has been complicated by the fact that most serum VEGF is derived from platelets, which are activated on coagulation (28). Unlike VEGF measured in serum, VEGF measured in plasma has been shown to be significantly higher in breast cancer patients compared with control patients, although the plasma levels do not appear to correlate with intratumoral VEGF assessed by immunohistochemistry (29). Moreover, in the same study tamoxifen treat-

ment was significantly associated with higher circulating and platelet-derived VEGF levels (29). We have shown recently that only ~45% of plasma levels of VEGF in tumor-bearing mice originate from the tumor and do not reflect extracellular VEGF secreted by the tumor (18). In line with these results, the plasma levels in the present study did not reveal the known differences in extracellular VEGF in the tumors detected using microdialysis.

Our results suggest that a regulation of extracellular VEGF may take place at a post-translational level. It is possible that tamoxifen reduces extracellular levels by blocking either the secretion or the release of stored pool of VEGF from the cell. In addition, it has been shown previously that the longer isoforms of VEGF may be converted into soluble, bioactive forms by proteolytic cleavage (24). This cleavage may be especially important in tumors in which the local microenvironment generally expresses a high proteolytic activity (30). It has been shown that increased activities of matrix metalloproteinases are associated with increased VEGF levels and increased tumor angiogenesis (31, 32). A direct measurement of VEGF locally in the tumor, such as that provided by microdialysis, might more accurately reflect the amount of extracellular, and, therefore, theoretically bioactive, protein released by the tumor. We have shown recently that microdialysis is a reliable technique for measurement of VEGF (18). Moreover, we have shown previously that microdialysis is applicable for human breast tissue in providing measurements of VEGF and other high molecular weight proteins and of low molecular weight compounds (19, 33–35).

In a recent study, it was suggested that tamoxifen treatment of nude mice with MCF-7 explants increased VEGF mRNA to levels above those observed with estradiol treatment. The authors interpret that the subsequently increased vascular permeability may lead to a decreased generation of functional microcapillaries (15). These results are in line with our intracellular results; however, the extracellular VEGF in that study was never analyzed, and the hormone treatments differed from ours. In that experiment, supraphysiologic levels of estradiol were used, and the tamoxifen-treated tumors contained large necrotic areas, suggesting that those tumors were highly hypoxic (15). Hypoxia is a strong inducer of tumor angiogenesis and VEGF (22, 36). Thus, hypoxia must be controlled to properly interpret the effects of various interventions, such as hormonal treatments, on VEGF. In our study, all of the tumors were size matched, and microscopic sections of the tumors did not reveal any necrotic areas. The mice in our study received supplements of 17β-estradiol at physiologic levels and were treated with tamoxifen at therapeutic levels because estrogen effects are related to the dose and type of hormone used. Estrogen often exhibits a bell-shaped dose-response curve; therefore, it is important to use physiologic levels of estrogen and naturally occurring 17β-estradiol in pathogenetic studies. Moreover, to mimic the clinical situation, at least for premenopausal women, we chose to give tamoxifen to the mice without discontinuing the supplemented estradiol.

In summary, we show, using microdialysis, that tamoxifen in combination with estradiol decreased extracellular levels of VEGF in solid MCF-7 tumors in nude mice *in vivo* compared with estradiol treatment only. These *in situ* results were verified *in vitro* in cell culture studies of extracellular VEGF release into the cell culture media. However, the intracellular levels of VEGF increased in a similar manner with treatments of either tamoxifen or estradiol. Estrogen and angiogenesis are important factors in breast cancer progression, and metastasis and antiestrogen treatments are cornerstones in breast cancer treatment. Therefore, additional investigations of hormonal regulation of angiogenesis and angiogenic factors in breast cancer are warranted. We believe that our results emphasize the need for studies on the regulation of proteins where they are biologically active—in the case of VEGF, in the extracellular space.

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Stina Garvin and Charlotta Dabrosin

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