Hormonal Regulation of VEGF in Orthotopic MCF7 Human Breast Cancer

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Abstract

Vascular endothelial growth factor (VEGF) mRNA and protein levels were higher in MCF7 breast cancer cells and orthotopic tumors after treatment with tamoxifen, as compared with treatment with estrogen. Accordingly, tumor vascular permeability, evaluated in vivo from contrast-enhanced magnetic resonance imaging, was elevated during treatment with tamoxifen. The results indicate that estrogen regulates angiogenesis in MCF7 tumors by maintaining VEGF at levels sufficient for the generation of functional microcapillaries and consequently facilitates tumor growth. During tamoxifen treatment, estrogen regulation is removed, and VEGF increases to levels that enhance markedly vascular permeability and reduce their perfusion function, leading to inhibition of tumor progression.

Introduction

The progressive growth and metastasis of breast cancer, and other tumors, are angiogenesis-dependent processes (1). The most specific angiogenic agent and endothelial cells mitogen in tumors is VEGF, also called vascular permeability factor because of the permeable capillaries that it induces in the course of the angiogenic agenda (2, 3). Alternative splicing of VEGF pre-mRNA generates several distinct VEGF species, where the most expressed ones are VEGF121, VEGF165, and VEGF189 (4).

Being endocrine-dependent carcinoma, the development of breast cancer necessitates hormones, in particular, estrogen and progesterone (5, 6). Estrogen mediates its activities through nuclear receptors, so-called ERs, which activate transcription and, hence, up-regulate expression of genes that are important for cell growth (7). Multiple lines of evidence indicate that estrogen regulates angiogenesis in the female reproductive system and in breast cancer, although the mechanism of the regulation has not been defined (8). Moreover, the association between VEGF expression and estrogen was studied extensively yielding, however, controversial results, e.g., the expression of VEGF in MCF7 human breast cancer cells was shown to increase on incubation with estrogen, as well as with tamoxifen (9).

The essential functions of angiogenesis and estrogen in the progression of breast cancer, in addition to the widespread pharmacological use of estrogenic and antiestrogenic drugs, bring up the need for acquiring a comprehensive knowledge regarding hormonal regulation of angiogenesis. We have thus investigated VEGF expression in MCF7 breast cancer during hormonal treatments with estrogen and tamoxifen, in vivo, ex vivo, and in vitro. Parametric maps of vascular permeability were obtained from model-based analysis of contrast-enhanced 1H MRI. The results indicate that tamoxifen exerts its antitumor effects through affecting VEGF expression and vascular permeability in an opposite manner to their regulation by estrogen. In addition to characterizing the hormonal regulation of angiogenesis in breast cancer, this study presents a noninvasive methodology to assess VEGF expression via determining its effect on vascular permeability. This methodology can be further extended to monitor the efficacy of therapy, including hormonal treatments.

Materials and Methods

MCF7 Cells and Tumors. MCF7 human breast cancer cells were cultured as described previously (10). For hormonal treatment, cells were cultured in phenol red free DMEM containing 6% dextran charcoal-stripped FCS, in the absence or presence of hormones (estrogen: 3 × 10−8 M or tamoxifen: 2 × 10−6 M, Sigma Chemical Co., Rehovot, Israel). For tumor formation, a pellet of 17β estradiol (0.72 mg/pellet, 60-day release time; Innovative Research of America, Sarasota, FL) was implanted under the back skin of female CD1-NU mice (~6 weeks old) after s.c. inoculation of MCF7 cells (~8 × 106 cells/0.5 ml PBS) into a mammary gland. Tumors were allowed to develop to ~1 cm3 (3–8 weeks). Tamoxifen treatment was applied by replacing the estrogen pellet with a tamoxifen one (5 mg/pellet, 21-day release time; Innovative Research of America). For the MRI measurements, mice were anesthetized by exposure to 1% isoflurane in 3:7 O2/N2O mixture (Medeva Pharmaceuticals, Rochester, NY). Approval for all animal procedures was obtained according to the guidelines of the Committee on Animals of the Weizmann Institute of Science.

Contrast-enhanced 1H MRI and Image Analysis. 1H-MR images were recorded with a 4.7 T Biospec spectrometer (Bruker Analytik, Karlsruhe, Germany). The dynamic T1-weighted spin-echo experiment applied on a central slice of each tumor began with acquisition of a precontrast image, followed by a bolus injection of GdDTPA-dimeglumine (0.4 mmol/kg weight and 100 μl of total volume) and then acquisition of sequential images for ~40 min. Acquisition parameters were: echo time/repetition time = 15/250 ms, two averages, spatial resolution = 0.19 × 0.39 mm2, slice thickness = 1 mm, and time resolution = 1.1 min. The dynamic experiments were applied twice on each animal (n = 3), firstly under estrogen before initiating tamoxifen treatment and secondly after 2 weeks of tamoxifen treatment. The dynamic images were analyzed using model-based algorithms, as was described previously (11, 12). The analysis produced parametric images of rate constant of permeability to GdDTPA (kP, min−1) and a correlation coefficient, which reflects the quality of the fit to the model (R2).

Immunohistochemistry. Mice, treated with estrogen (control, n = 3) or treated for 2 weeks with tamoxifen (n = 3), were euthanized, and the left atrium was perfused with PBS and 2.5% formaldehyde (50 ml each). Tumors were then removed, fixed, and maintained in paraffin blocks. Microsections from each tumor (5 μm) were stained with H&E and immunostained for VEGF. Slides for immunostaining were incubated in peroxide solution (300 μl of H2O2, 100 μl of concentrated HCl, 5 ml of methanol, and 5 ml of PBS; 15 min). Antigen retrieval was achieved in steaming Tris buffer [0.05 M (pH 10); 10 min]. The primary antibody, rabbit antihuman polyclonal against VEGF (BioGenex, San Ramon, CA) diluted 1:10 in fresh blocking solution (20% nonimmune goat serum, 0.5% Triton, PBS; 1 h), was added for overnight incubation at 4°C and visualized by avidin-biotin-peroxidase kit (Zymed Laboratories, South San Francisco, CA), after nuclei counterstaining with hematoxilin. Immunostaining procedure was identical for both estrogen and tamoxifen treatments. The fraction of VEGF staining (brown color) was determined in 10 fields (1002 mm2) within viable tissue of one microsection per

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4 The abbreviations used are: VEGF, vascular endothelial growth factor; ER, estrogen receptor; MRI, magnetic resonance imaging; GdDTPA, gadolinium-diethylenetriamine pentaacetic acid; RT-PCR, reverse transcription-PCR.
tumor, by dividing the area stained brown color by the total field area, using Image-Pro Plus software (Media Cybernetics, Silver Spring, MD).

RT-PCR. RNA was extracted using TRIzol (MRC, Cincinnati, OH). Semi-quantitative RT-PCR reactions were performed with Ready-To-Go RT-PCR beads (Pharmacia Biotech, Piscataway, NJ) with the following conditions: (a) 42°C, 30 min; (b) 94°C, 5 min; (c) 28 cycles of 94°C, 1 min; 56°C, 1 min; 72°C, 1 min; and (d) 72°C, 7 min. VEGF121 and VEGF165 were amplified with specific primers A (sense; 5'-GCTTGCTGCTCTACCTC-3') and B (antisense; 5'-GCTTGCTCATTCTTGTCTC-3''), or A and C (antisense; 5'-CCACA GGGATTTCTTGTCT-3''), respectively. S16 rRNA served as an internal control that is not regulated by hormones and was amplified with primers D (sense; 5'-TCCAA GGGTCCGCTGCAGT-3') and E (antisense; 5'-TCACGATGGGCTTATCGGTA-3'). The conditions for a linear assay were determined in preliminary experiments (data not shown). Each experiment included two negative control samples: (a) lacking RNA; and (b) with inactivated reverse transcription. Products were separated on 2% agarose gels and visualized by ethidium bromide staining after densitometry with Quantity One (Bio-Rad Laboratories, Hercules, CA). cDNA products obtained from the different bands were sequenced to verify their identity.

Statistical Analysis. Data from multiple experiments or groups were expressed as mean ± SE. The Ps were calculated by unpaired two-tailed Student’s t test (unless otherwise stated). A P < 0.05 was regarded as statistically significant.

Results

Hormone-regulated Capillary Permeability in Vivo. Dynamic GdDTPA contrast-enhanced 1H-MRI images, in orthotopic MCF7 tumors, were analyzed at a pixel resolution according to algorithms based on a physiological model (11). The analysis yielded parametric maps of GdDTPA permeability rate constant, \( k^{PS} \), and of a correlation coefficient that reflects the quality of the fit to the model (\( R^2 \); Fig. 1). In all tumors, approximately a third of the pixels (27% compared with the permeability that was measured before treatment) exhibited asymmetric left-skewed distribution around the corresponding mean values (results not shown), and hence, for statistical evaluation the 25th, 50th (median), and 75th percentiles of \( k^{PS} \) were calculated. Tamoxifen treatment caused a significant increase in vascular permeability as compared with the permeability that was measured before treatment with this drug (Fig. 1; Table 1).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Estrogen</th>
<th>Tamoxifen</th>
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<tr>
<td>25th percentile</td>
<td>1.11 ± 0.39</td>
<td>1.62 ± 0.28</td>
</tr>
<tr>
<td>Median</td>
<td>1.14 ± 0.11</td>
<td>3.57 ± 0.28</td>
</tr>
<tr>
<td>75th percentile</td>
<td>2.16 ± 0.24</td>
<td>15.2 ± 0.53</td>
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*Two-week treatment.

Hormone-regulated VEGF Expression ex Vivo. Central slices from tumors of comparable volumes, treated with estrogen (control) or treated for 2 weeks with tamoxifen, were stained with H&E and immunostained for VEGF (Fig. 2). The H&E staining exhibited large central necrotic regions in the tamoxifen-treated tumors, whereas the control tumors were mostly viable and contained a few scattered small necrotic loci (Fig. 2, arrows). Immunostaining with VEGF revealed that within viable regions of the tumors, the expression of this protein in the estrogen-treated tumors was significantly low as compared with tamoxifen-treated tumors (Fig. 2, C–F). Statistical analysis of random fields in viable loci indicated that the percentage of VEGF staining in the control tumors was 0.4 ± 0.23% versus 15.67 ± 3.33% in the tamoxifen-treated tumors.

Hormone-regulated VEGF mRNAs in Vitro. MCF7 human breast cancer cells were found to express four VEGF mRNAs: (a) VEGF121; (b) VEGF165; (c) VEGF145; and (d) VEGF189 (data not shown) with high expression of the first two isoforms. MCF7 cells were deprived of hormones for 3 days and then incubated for 2, 5.5, and 14 h with estrogen or tamoxifen, where control cells were kept untreated (Fig. 3). The levels of VEGF121 and VEGF165 mRNAs in the untreated (control) cells decreased significantly after 5.5 and 14 h of incubation with estrogen. Incubation with estrogen for 2 h did not change significantly the levels of these VEGF mRNAs, as compared with control. Incubation of the cells with tamoxifen for 2, 5.5, and 14 h did not affect significantly the levels of VEGF mRNAs, as compared with control. Incubation of the cells with tamoxifen for 2, 5.5, and 14 h did not affect significantly the levels of VEGF mRNAs, as compared with control.

Discussion

The results of this work demonstrated that in MCF7 human breast cancer, the levels of VEGF mRNAs in vitro, VEGF protein ex vivo,
and of vascular permeability *in vivo* were higher in the presence of tamoxifen as compared with estrogen. High vascular permeability during tamoxifen treatment was shown previously to correlate with increased tumor necrosis and eventually tumor regression, in ectopic MCF7 tumors (13). We would like to emphasize that the in vitro protocol began with depletion from hormones, which is comparable with treatment with tamoxifen, as they both don’t activate the ER. Thus, treatment with this drug exhibited a notable effect on VEGF mRNA levels with respect to estrogen treatment and not in comparison with control (i.e., depletion from hormones; Fig. 3).

To our knowledge, this is the first time that estrogen is shown to regulate angiogenesis at the molecular level in coherence with the physiological level, by suppressing excessive expression of VEGF and, thus, of vascular permeability. Therefore, we suggest the following hypothesis in ER-positive breast cancer: estrogen maintains VEGF expression at moderate levels that enable angiogenesis, the formation of functional microcapillaries, and consequently tumor growth. Accordingly, tamoxifen treatment removes the estrogen regulation leading to increased VEGF expression in parallel to an extreme elevation in vascular permeability. Increased permeability to extreme values interferes with vessel functionality by reducing its perfusion capacity and is therefore destructive to the tumors. The fact that tamoxifen carried out the reverse effect of estrogen also suggests that the estrogenic effect was exerted via the ER and that the therapeutic effect of tamoxifen is in line with its specific antiestrogenic activity, in ER-positive breast cancer (14).

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**Fig. 2.** Expression of VEGF protein in orthotopic MCF7 breast cancer tumors. Microsections of representative MCF7 control tumor (A, C, and E) and tumor treated for 2 weeks with tamoxifen (B, D, and F) were stained with H&E (A and B) and immunostained for VEGF (C–F). Arrows point at necrotic tissues. The tamoxifen-treated tumor is the tumor in Fig. 1B.

**Fig. 3.** VEGF mRNAs in hormone-regulated MCF7 cells. cDNA products of a representative RT-PCR reaction (A) and statistics of repeating RT-PCR experiments (n = 3) applied to amplify VEGF121 (B) or VEGF165 (C) from the total RNA of MCF7 cells incubated with estrogen (E; empty bars) or tamoxifen (T; gray bars). RNA-relative quantities were corrected for loading according to S16 rRNA.
The gradual decrease observed in VEGF mRNA levels during incubation with estrogen (Fig. 3), as well as the significant increase of VEGF protein expression during tamoxifen treatment (Fig. 2), strongly imply that estrogen does not regulate VEGF expression via conventional ER-induced transcription of this growth factor. This hypothesis is supported by recent findings that of the two estrogen-responsive element sequences found in the 3'- and 5'-untranslated regions of the rat VEGF gene, the latter sequence is unusual as it mediates transcriptional activation in one orientation and a significant repression in the opposite orientation (15). Moreover, overexpression of ERα in tumors initiated from Ishikawa human endometrial cancer cells was found to inhibit the expression of VEGF and α,β3 integrin, as well as to suppress the degree of vascularization and, hence, to limit tumor growth (16). These results indicated that high levels of ERα might be beneficial in controlling female cancer because of its inhibitory effect on angiogenic pathways.

Finally, the positive correlation that was established herein between VEGF expression ex vivo and vascular permeability in vivo enables to use the latter noninvasive approach for monitoring angiogenesis during anticancer therapy, particularly targeted anti-VEGF and antivascular therapies. Extension to this methodology is currently being clinically evaluated for the characterization of breast lesion according to estimation of vascular permeability to GdDTPA (17). Assessment of vascular permeability can be also used to determine malignant potential that was shown to correlate with VEGF content (18).

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References

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