Transcriptional Regulation of Vascular Endothelial Growth Factor by Estradiol and Tamoxifen in Breast Cancer Cells: A Complex Interplay between Estrogen Receptors \( \alpha \) and \( \beta \)

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ABSTRACT

Vascular endothelial growth factor (VEGF) is a potent angiogenic and prognostic factor for many tumors, including those of endocrine-responsive tissues such as the breast and uterus. Recent studies indicate that 17\( \beta \)-estradiol (E\(_2\)) modulates VEGF expression in breast and uterine cells, involving transcriptional activation through estrogen receptor (ER) \( \alpha \). However, molecular mechanisms of VEGF regulation mediated by the two ER subtypes and the potential role of ER\( \beta \) in the control of breast cancer angiogenesis have not yet been investigated. In transient transfection assays using the VEGF(\(-2275/+54\)) promoter-luciferase construct, E\(_2\) (1 nM) increased transcription activity in MCF-7 cells (either untransfected or cotransfected with ER\( \alpha \)) and it increased transcription activity in MDA-MB-231 cells cotransfected with ER\( \alpha \) or ER\( \beta \) (1.8- and 2-fold induction, respectively). The positive effect was abolished when MCF-7 cells were treated with pure antiestrogen ICI 182,780 or the agonist/antagonist tamoxifen (1 \( \mu \)M). To identify response elements involved in this transcriptional regulation, MCF-7 or MDA-MB-231 cells were transfected with several deletion constructs of the VEGF promoter. Deletion of 1.2–2.3 kb upstream to the transcription start in the VEGF promoter abrogated E\(_2\)-dependent transcription in these cells. This region contains an imperfect estrogen-responsive element (ERE), ERE1520, and one activator protein 1 site. Transfection of MCF-7 cells (ER\( \alpha \)) with the ERE1520-luciferase construct conferred transcriptional activity with 1 nM E\(_2\) (1.9-fold induction). Also, the imperfect ERE formed a complex with ER\( \alpha \) or ER\( \beta \) proteins in gel shift assay using MCF-7 or MDA-MB-231 nuclear extracts. In contrast to ER\( \alpha \), ER\( \beta \) could transactivate VEGF reporter construct in MDA-MB-231 cells, in the presence of E\(_2\) or tamoxifen, suggesting different transactivation mechanisms between ER\( \alpha \) and ER\( \beta \) in the presence of tamoxifen. Interestingly, E\(_2\) inhibited VEGF transcription in MCF-7 cells transfected with ER\( \beta \) or MDA-MB-231 cells cotransfected with ER\( \alpha \) and ER\( \beta \), suggesting that heterodimerization of ER\( \alpha \)/ER\( \beta \) has the ability to inhibit E\(_2\)-induced VEGF expression in breast cancer cells. These results demonstrate that VEGF is a target gene for ER\( \alpha \) and ER\( \beta \) in breast cancer cells; it remains to be determined whether ER\( \alpha \) and ER\( \beta \) expression in breast biopsies correlates with VEGF expression and vascular density.

INTRODUCTION

Angiogenesis has received much attention over the last few years as a multistep process essential for tumor growth and metastasis, including breast cancer (1, 2). This process requires the combined and coordinated actions of angiogenic factors, extracellular matrix components, and proteases (3). One of the most selective and potent angiogenic factors known is VEGF,\(^3\) also known as vascular permeability factor (4). In vivo studies have shown that suppression of VEGF inhibits tumor growth, and anti-VEGF antibodies or VEGF inhibitors also decrease the metastasis of human tumors implanted in nude mice (5). Also, VEGF transcript levels are elevated in human tumors, including those responsive to steroid hormones such as breast and endometrial tumors (6–10), in association with angiogenesis. VEGF is the major inducer of neovascularization occurring in the normal endometrium, ovary, prostate, and mammary gland (11–15). Recently, up-regulation of VEGF has been demonstrated in vitro with E\(_2\) and prostogens in normal and cancer cells (12, 13, 16–20). Pharmacological studies on MCF-7 cells have shown that E\(_2\) modulates VEGF expression (18), increasing gene transcription and mRNA stability. However, the molecular mechanisms of action on VEGF expression in breast cancer cells via ER have not been elucidated.

The mechanism of the estrogenic effect in breast tumors has been reevaluated based on the recent discovery of a second ER, ER\( \beta \) (21), expressed in various human tissues including mammary gland and breast cancer (22, 23). Based on studies performed in ER\( \alpha \) knockout mice, the presence of ER\( \alpha \), but not ER\( \beta \), seems to be required to mediate E\(_2\)-dependent growth and development of the gland (24, 25). Recent results have highlighted the fact that ER\( \beta \) may be important in human breast tumorigenesis, but the significant involvement of ER\( \beta \) has been controversial because it has been suggested to be of good (26, 27) or bad (28, 29) prognostic value. Recent results have also suggested a protective effect of ER\( \beta \) against the mitogenic activity of E\(_2\) in mammary premalignant lesion (30). Thus far, a possible link between angiogenesis mediated by VEGF and ER status during breast tumorigenesis in vivo and in vitro has not been investigated.

Functional studies have described the regulation of VEGF expression in response to various stimuli: hypoxia (31); growth factors through Sp1/AP2 or AP1 transcription factors (32–34); and E\(_2\) in endometrial cells through an imperfect ERE present in the VEGF promoter (20, 35).

Here we have investigated E\(_2\)-induced VEGF expression according to the ER subtype expressed in two breast cancer cell lines, MCF-7 and MDA-MB-231, using various approaches. Our results suggest that E\(_2\) regulation of VEGF at the transcriptional level in MCF-7 cells is mainly mediated by a specific ERE. Moreover, tamoxifen has agonist or antagonist activity on VEGF transcription, depending on the nature of the ER, ER\( \alpha \) and/or ER\( \beta \), and possibly involves complex interplay with other sequences in the VEGF promoter.

MATERIALS AND METHODS

Hormones and Chemicals. E\(_2\), OH-TAM, and other biochemicals were purchased from Sigma (Saint Quentin, France) unless otherwise stated. ICI

\(^3\) The abbreviations used are: VEGF, vascular endothelial growth factor; E\(_2\), 17\( \beta \)-estradiol; ER, estrogen receptor; hER, human estrogen receptor; ERE, estrogen-responsive element; OH-TAM, 4-hydroxy-tamoxifen; RAL, raloxifene hydrochloride; LUC, luciferase; AP, activator protein; RT-PCR, reverse transcription-PCR; FBS, fetal bovine serum; CMV, cytomegalovirus; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.
182,780 was a gift from Dr. A. E. Wakeling (AstraZeneca, Macclesfield, United Kingdom), and RAL was a gift from Lilly Indianapolis, IN.

**Cell Culture.** MCF-7 and MDA-MB-231 cells (a gift from Dr. D. Chalbos; Montpellier, France) were initially obtained from the American Type Culture Collection. They were routinely maintained in DMEM/Ham’s nutrient mixture F12 (Life Technologies, Inc., Grand Island, NY) and DMEM, respectively, supplemented with 10% FBS, 100 units/ml penicillin, 100 µg/ml streptomycin, and 250 µg/ml amphotericin B (Life Technologies, Inc.) at 37°C and 5% CO2.

**Plasmids and Cloning.** The human VEGF promoter gene (3.5 kb) cloned in pUC8 vector was kindly provided by Dr. J. Abraham (Scios Nova Inc., Sunnyvale, CA; Ref. 35). The VEGF promoter deleted of a 0.7-kb fragment in 3’ was subcloned upstream of LUC cDNA in a pGL3 basic reporter plasmid (pGL3b; Promega) [VEGF(−2275/−549)-LUC; a gift of Dr. J. Plouet; Touliouse, France]. Different constructions containing VEGF promoter fragments (−1176/+54, −88/+54, and −66/+54) cloned in pGL2 basic vector are shown in Fig. 4 and were described previously (33). The 0.7-kb VEGF fragment (+54/+749) 5’-untranslated region containing an AP1 and half ERE sites was prepared from the full-length VEGF promoter using the NheI restriction enzyme and subcloned into the corresponding restriction site of the pGL3b reporter plasmid. The vector (ERE1520)-LUC, containing one copy of the variant ERE of the VEGF promoter (aactagctag at position −1520 from the transcription start), was flank of XhoI and MluI restriction sites and inserted in the corresponding sites of pGL3 promoter vector (Promega). The vector (TRE)6-LUC, containing six copies of TRE elements upstream of the LUC gene, was kindly given by Dr. D. Guédin (Roussel-Uclaf, Romainville, France). All constructs were verified by sequencing. Expression vectors (pSG5) for hERα (HEI) and hERβ were generously provided by Prof. P. Chambon (Strasbourg, France; Ref. 36); data presented in this paper were obtained with the HEI plasmid. A CMV-GAL reporter corresponding to the β-galactosidase gene under the control of the CMV promoter was used as a control of transfection efficiency.

**Transient Transfections and LUC Assays.** Cells seeded onto 12-well plates in DMEM/Ham’s nutrient mixture F12 or DMEM were maintained for 1 week in phenol-red-free medium supplemented with 5% charcoal-stripped FBS. Transient transfection was carried out using the standard protocol for FuGENE reagent (Roche Diagnostics Corp., Indianapolis, IN). Briefly, a 1% FuGENE reagent (Roche Diagnostics Corp., Indianapolis, IN) and 250 µl (final volume). After PCR, the amplified transcripts was qualitatively analyzed by RT-PCR using primers specific to hERα and hERβ transcripts (Fig. 1), as described previously (22); they also express low levels of ERβ. In MDA-MB-231 cells, only the ERβ transcript was detected. However, ERβ protein was clearly detected compared with ERβ, which was not detected in MCF-7 cells using immunocytochemistry (data not shown; Ref. 41).

**RESULTS**

MCF-7 and MDA-MB-231 breast cancer cell lines were chosen to study the molecular mechanisms of VEGF regulation with regard to ER status. Using RT-PCR analysis, MCF-7 cells express high levels of ERα transcripts (Fig. 1), as described previously (22); they also express low levels of ERβ. In MDA-MB-231 cells, only the ERβ transcript was detected. However, ERα protein was clearly detected compared with ERβ, which was not detected in MCF-7 cells using immunocytochemistry (data not shown; Ref. 41).

**Induction of VEGF mRNA and Protein Expression by E2 in MCF-7 Cells.** We first studied the regulation of VEGF mRNA expression in MCF-7 cells stimulated by E2 using semiquantitative RT-PCR (see “Materials and Methods” and Ref. 13; Fig. 2, A and B). As shown in Fig. 2B, the response to E2 (1 nM) occurred in a time-dependent manner; the level of VEGF transcripts significantly increased within 3 h and reached a maximum at 6 h (a 1.6-fold increase; P < 0.05). Dose-dependent experiments indicated that the maximum level was obtained at 1–10 nM (data not shown). VEGF protein significantly increased in the conditioned medium of cells after 12 h of treatment with 1 and 10 nM E2, compared with untreated controls and untreated treated cells were stimulated in serum-free medium with 1 nM E2 at 37°C for 1 h, and nuclear extracts were prepared as described previously (39). Double-stranded oligonucleotides containing the putativeERE located in the fragment of the full-length VEGF promoter (−1570 to −1176) were synthesized (aactagctagctagcagc) and end-labeled by 5’ phosphorylation with T4 polynucleotide kinase and [γ-32P]ATP (NEN Perkin-Elmer). EMSAs were carried out as described previously (40), with minor modifications. Briefly, 32P-labeled ERE (4 × 106 cpm/reaction) was combined with 5–15 µg of nuclear protein extracts and poly-dIdC (Sigma) used as nonspecific carrier DNA in 20 µl of buffer [15 mM Tris-HCl (pH 7.9) containing 75 mM KCl, 0.2 mM EDTA, and 0.4 mM DTT]; the mixture was placed on ice for 15 min. Protein-DNA complexes were separated from the free probe by nondenaturing 5% PAGE at 150 V with running buffer consisting of 25 mM Tris/25 mM borate/1 mM EDTA. Gels were dried, and the radioactivity associated with ERE and ER-ERE complexes was quantified. Binding competition was performed by adding unlabeled probes at 100-fold excess.

**Statistical Analysis.** Student’s t test was used to determine the significance between treatments and untreated controls, and P < 0.05 was considered significant.

**Radioactivity in each band was quantified using Instant-Imager analysis (Packard) and the associated software. Results were expressed as band intensity in each lane relative to that of GAPDH and compared statistically using Student’s t test.**

**RT-PCR of ERs.** PCR amplification was performed using primers chosen at 598–623 and 1392–1416 positions in hERα cDNA (36) and primers chosen at 124–146 and 498–519 positions in hERβ cDNA (21), as described previously (38). GAPDH was used as an invariant housekeeping gene control.

**Preparation of Cell Extract and EMSAs.** MCF-7 and MDA-MB-231 transfected cells were stimulated in serum-free medium with 1 nM E2 at 37°C for 1 h, and nuclear extracts were prepared as described previously (39). Double-stranded oligonucleotides containing the putative ERE located in the fragment of the full-length VEGF promoter (−1570 to −1176) were synthesized (aactagctagctagcagcag) and end-labeled by 5’ phosphorylation with T4 polynucleotide kinase and [γ-32P]ATP (NEN Perkin-Elmer). EMSAs were carried out as described previously (40), with minor modifications. Briefly, 32P-labeled ERE (4 × 106 cpm/reaction) was combined with 5–15 µg of nuclear protein extracts and poly-dIdC (Sigma) used as nonspecific carrier DNA in 20 µl of buffer [15 mM Tris-HCl (pH 7.9) containing 75 mM KCl, 0.2 mM EDTA, and 0.4 mM DTT]; the mixture was placed on ice for 15 min. Protein-DNA complexes were separated from the free probe by nondenaturing 5% PAGE at 150 V with running buffer consisting of 25 mM Tris/25 mM borate/1 mM EDTA. Gels were dried, and the radioactivity associated with ERE and ER-ERE complexes was quantified. Binding competition was performed by adding unlabeled probes at 100-fold excess.

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**Induction of VEGF mRNA and Protein Expression by E2 in MCF-7 Cells.** We first studied the regulation of VEGF mRNA expression in MCF-7 cells stimulated by E2 using semiquantitative RT-PCR (see “Materials and Methods” and Ref. 13; Fig. 2, A and B). As shown in Fig. 2B, the response to E2 (1 nM) occurred in a time-dependent manner; the level of VEGF transcripts significantly increased within 3 h and reached a maximum at 6 h (a 1.6-fold increase; P < 0.05). Dose-dependent experiments indicated that the maximum level was obtained at 1–10 nM (data not shown). VEGF protein significantly increased in the conditioned medium of cells after 12 h of treatment with 1 and 10 nM E2, compared with untreated controls and untreated
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Fig. 2. Induction of VEGF expression by E2 in MCF-7 cells. Cells were cultured as described in “Materials and Methods” and treated with E2 for various periods. A shows a representative agarose gel analyzing RT-PCR products for VEGF (312, 240, and 108 bp, top gel) or GAPDH (bottom gel). L, molecular weight standard. B, the amount of VEGF mRNA was quantified by RT-PCR analysis (19). The values are presented as the mean VEGF:GAPDH mRNA ratio ±SE in a representative experiment (n = 3). C, MCF-7 (open symbols) and MDA-MB-231 (closed symbols) cells were incubated with 1 and 10 nM E2.

The amount of VEGF was determined by ELISA assay in the cell-conditioned medium (triplicate samples). Values are normalized relative to the protein concentrations. A representative result of three experiments is shown. *, statistically significant versus untreated control (P < 0.05 and P < 0.01 in B and C, respectively).

cells (1.7-fold; P < 0.01; Fig. 2C). No further increase of secreted VEGF was seen at longer incubation times and higher E2 concentrations (data not shown). In contrast, E2 has no effect on VEGF released by MDA-MB-231 cells (Fig. 2C), suggesting that endogenous ERβ (see Fig. 1) does not support E2-induced VEGF production.

E2 Modulates VEGF Gene Transcription in MCF-7 Cells through ERα. Previous studies have suggested an effect of E2 on VEGF expression at the transcriptional level in different cells (13, 18, 20), as well as at the posttranscriptional level (18). To determine the molecular mechanisms of transcriptional regulation by E2 in breast cancer cells, MCF-7 cells were transiently transfected with a plasmid containing 2.3 kb of the 5′-flanking region of the VEGF promoter linked to the LUC reporter gene, VEGF(−2275/+54)-LUC or pGL3b, and further treated with E2 for 24 h, as described in “Materials and Methods.” As shown in Fig. 3, this promoter region of the VEGF gene conferred significant hormonal inducibility by 1 nM E2 (1.8- and 2-fold increase, respectively, as compared with untreated cells; P < 0.001). As a positive control, 12-O-tetradecanoylphorbol-13-acetate induced a 3.9- and 15-fold increase of the LUC activity associated with the VEGF gene promoter or the (TRE)6-LUC construct, respectively (see “Materials and Methods”; data not shown).

Parallel experiments were performed in MCF-7 cells transiently transfected with VEGF(−2275/+54)-LUC together with an expression plasmid for hERα or hERβ. The basal transcription level was unchanged, regardless of whether MCF-7 cells were transfected with ERα plasmid or not (data not shown). E2 treatment (1 nM) of MCF-7 cells transfected with ERα resulted in a significant increase in LUC activity (1.72 ± 0.02; P < 0.01) as compared with untreated cells, and similar to that obtained with untransfected cells (Fig. 3). In contrast, no activation was observed in cells transfected with ERβ under similar conditions (Fig. 3).

Transcriptional Up-Regulation of VEGF by E2 Is Dependent on a Distal Region of the Promoter (−2275/−1176) in Breast Cancer Cells. To identify response elements implicated in the transcriptional regulation of VEGF by E2, we next examined transcriptional activities of constructs containing several deletions of the 2.3-kb VEGF promoter (Fig. 4A). In MCF-7 cells containing endogenous ERα (Fig. 4B, pSG5), E2 induced a significant increase of VEGF(−2275/+54)-LUC activity, but it had no effect on cells transfected with deleted vectors (−1176/+54, −88/+54, −66/+54, or +54/+749). Similar results were observed using MCF-7 cells cotransfected with the ERα expression vector (Fig. 4B, hERα). This induction was blocked by cotreatment with ICI 182,780, confirming that ERα is responsible for E2-induced reporter activity (data not shown). In contrast, no effect was observed when MCF-7 cells were transfected with an ERβ expression plasmid and VEGF promoter (2.3-kb or deleted fragments)-LUC constructs (Fig. 4B, hERβ).

We next used MDA-MB-231 cells as a recipient cell to test the effect of E2 on VEGF transcriptional activity in a receptor isof orm-dependent assay (Fig. 5). E2 did not induce VEGF transcription unless cells were cotransfected with ER (Fig. 5A), in agreement with the absence of VEGF protein release in E2-treated cells (Fig. 2C).

In contrast, E2 treatment (1 nM) of cells transfected with either ERα or ERβ expression vector resulted in a significant increase in LUC activity (2× and 1.6×, respectively; P < 0.001), as compared with untreated cells (Fig. 5A). No induction of LUC activity was observed when cells were transfected with p(−1176/+54) or p(+54/+749) (Fig. 5B).
Taken together, these observations indicate that the \((-2275/-1176)\) 5′-flanking region of VEGF promoter contains cis-active elements to mediate E2-induced activation and that the phenomenon is mediated by ERα in MCF-7 cells.

ERE1520 Is Necessary for E2-induced VEGF Transcription in MCF-7 Cells. The presence of an imperfect palindromic ERE has been identified in the VEGF promoter, about 1.5 kb upstream of the transcription start (20). To examine whether this ERE-like sequence functions as an authentic ERE and mediates E2 regulation of the VEGF promoter in breast cancer cells, we next performed gene transfer studies using the ERE1520-LUC plasmid (ERE1520-LUC) in the presence of ER (Fig. 6). As shown in Fig. 6A, incubation of MCF-7 cells with 1 nM E2 resulted in the stimulation of LUC activity (1.9×), similar to that obtained with cells transfected with the 2.3-kb VEGF promoter (see Figs. 3 and 4). No induction was observed when MCF-7 cells were transfected with the ERE1520-LUC and hERβ plasmid (Fig. 6B) or in cells treated with E2 + ICI 182,780 or E2 + OH-TAM or ICI 182,780, OH-TAM, or RAL alone (Fig. 6, A and B). Taken together, these findings suggest that ERE1520 is clearly involved in E2-induced VEGF gene transcriptional regulation in MCF-7 cells that express ERα or overexpress this receptor (trans-
fected cells). Unexpectedly in contrast to the full-length plasmid (see Fig. 5), E2 did not induce LUC activity in MDA-MB-231 cells transfected with ERα and ERE1520-LUC plasmid (Fig. 6c).

VEGF Transcriptional Up-Regulation by E2 and Tamoxifen through ERβ in MDA-MB-231 Cells. Treatments of MDA-MB-231 cells with different hormones allowed further analysis of the regulation of VEGF expression depending on the ER subtype. Whereas E2 did not induce VEGF transcription in MCF-7 cells cotransfected with both ERβ plasmid and the 2.3-kb VEGF promoter, ICI 182,780, OH-TAM, and RAL (1 μM) alone consistently induced LUC activity in these cells (P < 0.01; Fig. 7), suggesting that these agonists/antagonists could modulate VEGF transcription through ERβ.

We next analyzed the regulation of VEGF transcription in MDA-MB-231 cells transfected with ERβ plasmid (Fig. 8). Basal transcription level was unchanged in these cells as compared with recipient cells (data not shown); moreover, transcriptional effect was not observed in untransfected cells (endogenous ERβ). As mentioned previously (Figs. 5a and 6c), E2 (1 nm) resulted in a significant increase in LUC activity in MDA-MB-231 cells transfected with ERβ expression and the 2.3-kb VEGF promoter vector but in no induction when cells were transfected with ERE1520-LUC. Unexpectedly, the transcriptional activation persisted with ERβ-transfected cells cotreated with E2 + OH-TAM or with OH-TAM alone (1.6-fold induction for each treatment; P < 0.05), which was in contrast to the inhibitory effect of OH-TAM on ERα-transfected cells.

The agonistic effect of tamoxifen has been reported previously on ERE-LUC or AP1-LUC constructs depending on ER subtype (42–44). To analyze the mechanism of OH-TAM-positive regulation of VEGF in ERβ-transfected cells, another reporter gene analysis was performed using a construct containing either the ERE1520 or the AP1 sequence located downstream of ERE1520 in the regulatory region (~2275/−1176) (35). As observed for ERE1520 with E2, no increase in LUC activity was observed when cells were treated with OH-TAM or E2 (data not shown), indicating that these elements alone do not represent a potential mechanism for OH-TAM-positive regulation of VEGF via ERβ in MDA-MB-231 cells.

The ERE1520 Sequence Present in VEGF Promoter Binds ERα and ERβ. EMSA with a labeled oligonucleotide containing the putative ERE1520 sequence and MCF-7 or ERα-transfected MDA-MB-231 cell nuclear extracts demonstrated an efficient and similar pattern of protein binding to labeled oligonucleotides (Fig. 9). The binding was competed by adding a 100-fold excess of unlabeled ERE probe, but not with the probe mutated on a 1/2 ERE (ERE1520*), confirming that the identified ERE element binds ER β specifically. Similar experiments conducted in MDA-MB-231 cells (ERβ+) also demonstrated efficient binding of the nucleotide (data not shown).

DISCUSSION

Hormone regulation of growth factors is thought to be largely mediated by autocrine and paracrine factors in breast cancers, but little is known of the possible role of the angiogenic factor VEGF in steroid regulation of breast cancer growth (18, 45). In this study, we show that VEGF is a target gene for ERα and ERβ in human breast cancer cells and that the response depends on the nature of the ER and the ligand. Estrogens induce VEGF expression at the transcriptional level in different breast cancer cell lines, in agreement with previous studies in endometrial cells (12, 13, 20). The similar induction of VEGF levels (mRNA expression and protein secretion) and transcriptional activity observed in E2-treated cells (2-fold induction; P < 0.01) suggest that the up-regulation of VEGF mRNA by E2 is primarily caused by gene transcription under our conditions. However, an effect of E2 on VEGF mRNA stability is not excluded (18). Our results also demonstrate that VEGF induction is mediated by the activation of ERα in the MCF-7 cells: (a) the estrogenic effect is inhibited by treatment with the pure antiestrogen ICI 182,780; and (b) a similar E2 increase in VEGF activation is observed in MCF-7 cells or MDA-MB-231 cells overexpressing ERα in transfection experiments. Interestingly, we show that expressed ERβ can regulate VEGF transcription in MDA-MB-231 cells, with a slightly weaker activity as compared with that of ERα shown in this study (ratio, 0.8) and reported previously in endometrial cell lines (ratio, 0.7; Ref. 20) or with ERE response elements (46, 47). Thus, VEGF is a target gene for ERα and ERβ in breast cancer cells, in addition to pS2, transforming growth factor α, or the cyclin kinase inhibitor p21 (47).

Our data using a series of deletion constructs indicate that the imperfect ERE1520 sequence, which has 70% homology to the consensus ERE sequence, is likely to play an important role in the estrogenic regulation of VEGF production in MCF-7 cells, as deduced from previous findings obtained in endometrial tumor cells (20). In contrast, the 0.7-kb fragment of human VEGF promoter (+54/+749), which possesses one sequence with 80% homology to the consensus ERE and is capable of ER binding in gel shift experiments (48), does not...
confer E2 inducibility in our LUC assay, in agreement with other results obtained in HeLa cells (48). Our study, however, does not exclude the possibility that other ER-binding sites, such as the ERE present in the 3′-flanking region of the VEGF gene, could participate in the E2-induced VEGF expression (48); the possibility of multifactorial control and synergy between E2 and additional regulatory factors also remains to be investigated.

Our in vitro observations further indicate that selective responses to E2 agonists/antagonists depend on the expression and relative levels of each ER subtype. The small but consistent and significant induction of VEGF by OH-TAM in cancer cells expressing ERβ or ERβ/ERα (i.e., MDA-MB-231 or MCF-7 cells transfected with ERβ, respectively), but not in cells expressing ERα, indicates that E2/OH-TAM-liganded ERβ has different transcriptional activities than E2/OH-TAM-liganded ERα at VEGF promoter; this finding may be related to the tamoxifen-associated agonist effects reported previously (19, 49), especially in vascular smooth muscle and uterine cells that express a significant amount of ERβ in the presence of ERα (19, 38). The agonistic effect of tamoxifen has been reported previously on the ERE-LUC construct depending on ER subtype (42) or on the AP1-LUC construct with ERβ (43, 44), suggesting the possibility that the distinct mechanism between ERα and ERβ could involve different activation functions (AF-1 and AF-2). Whereas tamoxifen appears to have no effect on ER1520 (VEGF) or AP1 activity alone in either MCF-7 or in MDA-MB-231 cells transfected with ERα (Ref. 43 and this study), an agonistic effect of tamoxifen on VEGF expression was observed in breast cancer cells expressing ERβ; this finding suggests that E2 inhibition and OH-TAM activation of VEGF expression through ERβ are not ERE mediated but could involve other sequence(s) within the same region of the promoter, as suggested previously (50).

The inhibition of VEGF transcription with E2 in the presence of both ERα and ERβ, in contrast with the induction observed when each receptor is expressed alone, could be explained by the formation of homo- or heterodimers. The heterodimerization between ERα and ERβ was demonstrated previously (51, 52), but the consequence on estrogen signaling has not fully been determined. Our observations suggest that the transactivational mechanisms of ERα/ERα, ERβ/ERβ, or ERα/ERβ are distinct in breast cancer cells, in agreement with a previous report (53). Our observations could also have functional significance. Numerous studies have shown that the ERβ:ERα ratio is decreased in cancerous tissues compared with normal tissue, as observed in breast (26, 30) and ovarian cancers (54), suggesting that ERβ could play a negative role on tumorigenesis. Other studies have suggested that ERβ is a major inhibitor of proliferation and invasion of breast cancer cells (47). Whether modifications of ERα/ERβ in breast cancer could modulate angiogenesis in vivo is still unknown.

Regulation of angiogenesis by steroid hormones is now documented. E2 has been shown to induce VEGF expression in several cell types including endometrial cells, as well as in the uterus (11–13, 16, 20, 49) and in the rodent mammary gland (45, 55). Up-regulation of VEGF has also been demonstrated with progesterone and progestins in some breast cancer cells (17). Taken together, these findings raise the possibility that E2 and progesterone may regulate the growth of breast cancer in part by stimulating VEGF production and thus increasing the density of the microvasculature, as in 7,12-dimethylbenz(a)anthracene-induced rat mammary cancer (45). Tamoxifen itself modulates VEGF expression in several cells (19, 49), including breast cancer cells, suggesting a paracrine effect on endothelial cells; tamoxifen may also have a direct antiproliferative activity on VEGF-stimulated endothelial cell growth (56). In vivo, a significant reduction or an increase in vessel density was observed in responsive or nonresponsive breast tumors, respectively, after tamoxifen administration (57, 58). This is consistent with previous observations reporting that angiogenesis and ER status are to be considered in predicting response to tamoxifen, with patients having the best prognosis characterized by low vascularity and ER positivity, in addition to few lymph nodes and small low-grade tumors (57). Also, the decrease of vessel density with tamoxifen is associated with a decreased VEGF expression in breast tumors (10). However, it is still unknown whether tamoxifen stimulates endothelial cell growth in some breast tumors (tamoxifen-resistant, ERβ+ tumors; Ref. 59).

In summary, this study demonstrates that E2 and its agonists/antagonists induce VEGF expression in different breast cancer cells through transcriptional effects of ERα and ERβ, depending on the nature of ER subtype and the ligand. Our findings raise the possibility that estrogens can directly stimulate the production of this key angiogenic factor in hormone-responsive tumors. It will be of interest to determine whether ERα and ERβ analysis in breast biopsies correlates with VEGF expression and vascular density and adds further prognostic information with regard to tamoxifen response in angiogenesis.

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Fig. 9. Binding of proteins corresponding to ERα and ERβ in EMSA with oligonucleotides containing ERE1520. Gel shift analysis was performed on MCF-7 (left panel) or MDA-MB-231 (right panel) nuclear extracts using the 32P-labeled oligonucleotide corresponding to ERE1520 as the probe (Refs. 20 and 35; see “Materials and Methods”). Binding competition was performed by adding unlabeled probe at 100-fold excess or mutated ERE oligonucleotide EREα. The bottom band corresponds to nonspecific binding because it was not competed by excess unlabeled oligonucleotide.
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