An Angiogenic Switch in Breast Cancer Involves Estrogen and Soluble Vascular Endothelial Growth Factor Receptor 1

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Estrogen is involved in breast tumorigenesis, but the precise mechanisms for its oncogenic and angiogenic actions are poorly understood. Angiogenesis is regulated, in part, by these critical components: vascular endothelial growth factor (VEGF) and its two receptors (VEGFR-1 and VEGFR-2). VEGFR-2 is a positive angiogenic signal transducer, whereas VEGFR-1, especially its soluble form (soluble VEGFR-1), is a negative regulator of VEGF availability. We found that breast epithelial cells express soluble VEGFR-1 and hypothesized that because estrogen can regulate expression of members of the VEGF family, it might stimulate angiogenesis in breast cancer by decreasing expression of soluble VEGFR-1. Soluble VEGFR-1 expression decreased in estrogen receptor (ER)-positive but not in ER-negative breast cancer cell lines treated with estrogen. Pretreatment of the cells with the ER antagonist ICI 182,780 blocked the effect. The estrogen-mediated decrease in soluble VEGFR-1 expression was accompanied by a statistically significant increase in angiogenesis in vivo. Our data suggest that inhibition of soluble VEGFR-1 expression represents a novel mechanism—an estrogen-driven angiogenic switch—possibly responsible for breast carcinoma progression.

Studies of estrogen involvement in breast tumor growth have focused on the molecular mechanisms responsible for increased proliferation. Although these cellular mechanisms undoubtedly contribute to tumor development, estrogen may also play an important role in the regulation of angiogenesis (1,2). However, the precise molecular mechanisms by which estrogen regulates angiogenesis have not been defined.

Vascular endothelial growth factor (VEGF) is a predominant inducer of tumor angiogenesis and an important prognostic factor in breast cancer (3). VEGF binds two high-affinity receptors, VEGFR-1 and VEGFR-2, that have ligand-stimulated tyrosine kinase activity (4). Although VEGFR-2 is recognized as the predominant receptor involved in VEGF-stimulated angiogenesis (4,5), the function of VEGFR-1 is less clear. VEGFR-1 has several unique structural and functional characteristics. The VEGFR-1 gene encodes a full-
length membrane receptor (≈200 kd) and a soluble receptor (≈110 kd), which are generated by alternative splicing of the VEGFR-1 pre-mRNA and contain the extracellular ligand-binding domains but lack the signaling tyrosine kinase domains (4). VEGFR-1 has more than a 40-fold higher affinity than VEGFR-2 for VEGF (4,6). It is believed that VEGFR-1 functions as an inert decoy by binding endogenous VEGF, thereby negatively regulating the availability of VEGF and the activation of angiogenesis through VEGFR-2. Such a decoy function is attributable mainly to soluble VEGFR-1 (4). Several studies have used soluble VEGFR-1 as a VEGF-blocking reagent (i.e., negative regulator of angiogenesis) and as an inhibitor of tumor growth (6–9).

Although expression of VEGFR-1 was previously believed to be restricted to the vascular endothelium, VEGFR-1 has been detected in several types of non-endothelial cells—breast cancer cells in particular (10,11). We used western blot analysis (Fig. 1) and immunofluorescent staining (data not shown) to determine levels of VEGFR-1 protein in normal breast cell lines (Hs578Bst; obtained from American Type Culture Collection [ATCC], Manassas, VA) and breast carcinoma MCF-7 (ATCC) and MDA-MB-231 cells (Dr. P. Steeg, NCI) were grown in RPMI-1640 medium supplemented with 10% charcoal-stripped fetal bovine serum (HyClone, Logan, UT). Proliferating, subconfluent cell cultures were lysed in radioimmunoprecipitation assay buffer. Equivalent amounts of total protein were separated on 4%–12% Bis-Tris NuPAGE gels (Invitrogen, Carlsbad, CA) and analyzed by western blotting with a rabbit polyclonal antibody against human VEGFR-1 (Santa Cruz Biotechnology, Santa Cruz, CA), according to the manufacturer’s recommended protocol. This antibody detects both soluble (≈110 kd) and full-length VEGFR-1 (≈200 kd). Lysate from human umbilical vascular endothelial cells (HUVECs) was used as a positive control for VEGFR-1 protein expression. The membranes were stripped and probed with a monoclonal anti-rabbit (cross-reactive with human) antibody against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Research Diagnostics, Flanders, NJ) to show that equivalent protein levels were loaded.

mRNA were markedly decreased in estrogen-treated MCF-7 cells, whereas levels of full-length VEGFR-1 mRNA were unchanged (Fig. 2, A). We confirmed the estrogen receptor (ER) status of MCF-7, T47D, and MDA-MB-231 cell lines used in these experiments in proliferation assays (data not shown) and then tested whether the observed effect on soluble VEGFR-1 was mediated through the ER pathway by incubating the breast cancer cells with the ER antagonist ICI 182,780 (14) before treating them with estrogen. Estrogen-mediated inhibition of soluble VEGFR-1 mRNA expression was abolished in a dose-dependent manner in cells treated with ICI 182,780 (Fig. 2, B). Estrogen-induced decreases in soluble VEGFR-1 protein levels were also blocked in ER-positive MCF-7 and T47D cells treated with ICI 182,780 (Fig. 2, C). By contrast, estrogen with or without ICI 182,780 had no effect on soluble VEGFR-1 expression in ER-negative MDA-MB-231 breast carcinoma cells (Fig. 2, C). Estrogen-like inhibition of soluble VEGFR-1 mRNA and protein levels was observed in MCF-7 cells treated with the nonsteroidal partial estrogen antagonist tamoxifen (Fig. 2, D). These results demonstrate the involvement of the classical ER pathway in estrogen-mediated soluble VEGFR-1 expression.

We next used the Matrigel plug assay (15) to investigate the estrogen-mediated inhibition of VEGFR-1 expression in vivo and its possible correlation with breast carcinoma–induced angiogenesis. To regulate the in vivo estrogen levels, we removed the ovaries from 6-week-old female BALB/c nude mice and implanted placebo or slow-release estrogen pellets. MCF-7 cells (1 × 10⁸) were mixed with 0.2 mL of Matrigel and injected subcutaneously into the back of each mouse. After 6 days, the Matrigel–cell plugs were removed and analyzed for VEGFR-1 protein expression by immunohistochemistry. Levels of VEGFR-1 protein were lower in Matrigel-embedded MCF-7 cells from mice implanted with estrogen pellets (Fig. 2, E, right panel) than in Matrigel-embedded MCF-7 cells from mice with placebo pellets (Fig. 2, E, left panel). This decrease in VEGFR-1 expression was associated with a pronounced increase in the angiogenic response, reflected by a statistically significant increase in vascular density (mean = 2.08 vessels per microscopic field) measured in the Matrigel plugs from estrogen-treated mice compared with vascular density measured in the Matrigel plugs from placebo-treated mice (0.94 vessels per microscopic field; mean difference = 1.14, 95% confidence interval [CI] = 0.44 to 1.82; P = .002, Welch t test). These data demonstrate that estrogen decreases VEGFR-1 expression in vivo. Consequently, because VEGFR-1 negatively regulates angiogenesis (6–9), this decrease in VEGFR-1 expression may be associated with increased neovascularization within a tumor.

Our results suggest that the inhibition of VEGFR-1 expression could present a novel mechanism by which estrogen exerts pro-angiogenic effects and thus promotes breast cancer development or progression. It is conceivable that normal breast tissue cells constitutively express soluble VEGFR-1 at levels sufficient to absorb endogenous VEGF, thus prevent-
phenol red positive MCF-7 breast cancer cells were cultured in quantitative reverse transcription to assess levels of VEGFR-1 mRNA, comparative semiquantitative reverse transcription–polymerase chain reaction (RT–PCR) was performed in which 1 µg of total RNA was subjected to reverse transcription with oligo(dT) primers in a final volume of 20 µL. The resulting cDNA was diluted to 100 µL. Comparative semiquantitative PCR was performed as follows: glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA was first amplified at a low cycle number (GAPDH primer sequences: sense = 5'-CCACCCATGGCAAATTCCTAGGCA-3'; antisense = 5'-TCTAGACCGGCAGGTCAAGTCCACC-3'); the resulting 600-base-pair products were separated by electrophoresis through a 1.5% agarose gel, visualized after staining with ethidium bromide, and quantified by using an Eagle Eye gel system and its associated software (Stratagene, La Jolla, CA). If needed, the cDNA dilutions were adjusted and used in GAPDH RT–PCR to obtain products with similar signal intensities between all the samples. The adjusted amounts of cDNA were used for PCR with primers specific for soluble VEGFR-1 (5'-CAACAACACACAGGAAAAGG-3' and 5'-GCACTGCACACAAAAAGGC-3') and for full-length membrane-spanning VEGFR-1 (9). The products were separated by 1.5% agarose gel electrophoresis and visualized after staining with ethidium bromide. A representative gel is shown. The intensity of each band was quantified by using Eagle Eye software and expressed as band intensity relative to that of GAPDH. The results of three separate experiments (mean with upper 95% confidence interval) are shown (lower panel). B) MCF-7 cells, prepared as described in (A), were preincubated with the pure estrogen antagonist ICI 182,780 (10⁻⁶ M) for 1 hour before the addition of E₂. VEGFR-1 mRNA expression was assessed by RT–PCR. A representative gel is shown (upper panel). The band intensities were quantified and expressed as band intensity relative to that of GAPDH (lower panel). Bars represent mean (n = 3) with upper 95% confidence interval. C) ER-positive MCF-7 and T47D and ER-negative MDA-MB-231 breast carcinoma cells were preincubated with ICI 182,780 and then incubated with estrogen in the presence or absence of ICI 182,780 for 8 hours. The cells were lysed, and aliquots of total proteins were separated on 4%–12% Bis-Tris NuPAGE gels (Invitrogen, Carlsbad, CA) and analyzed by western blotting with a rabbit polyclonal anti-VEGFR-1 antibody (Santa Cruz Biotechnology, Santa Cruz, CA). Membranes were stained with the protein-binding dye ponceau red (lower panel) for MCF-7 cells or stripped and probed with anti-GAPDH antibody (lower panels for T47D and MDA-MB-231 cells) to ensure equal loading. D) MCF-7 cells were treated with 10⁻⁶ M tamoxifen (TAM) or 10⁻⁹ M E₂ for 8 hours. Cells treated with the diluent, absolute ethanol, were used as a control. Total RNA and protein were isolated and analyzed by RT–PCR (upper panel) and western blotting (lower panel) for VEGFR-1 expression. GAPDH levels are shown as a control for cDNA integrity. E) Ovariectomized nude mice bearing slow-release estrogen or placebo pellets were injected subcutaneously with a mixture of MCF-7 cells and Matrigel. The Matrigel–MCF-7 cell plugs were excised after 6 days and subjected to immunohistochemistry with a rabbit polyclonal anti-VEGFR-1 antibody. The sections were developed using a Dako EnVision kit (DakoCytomation, Glostrup, Denmark). The sections were lightly counterstained with hematoxylin. VEGFR-1 protein expression in Matrigel–MCF-7 cell plug sections from mice implanted with the placebo (left panel) or the estrogen slow-release pellet (right panel) (magnification ×200).

Fig. 2. Effect of estrogen on soluble vascular endothelial growth factor receptor 1 (sVEGFR-1) levels in breast cancer cell lines. A) Estrogen receptor (ER)–positive MCF-7 breast cancer cells were cultured in phenol red–free RPMI-1640 medium supplemented with 10% charcoal-stripped fetal bovine serum to reduce levels of endogenous estrogens. After 96 hours, the cells were cultured in serum-free medium with or without 1 × 10⁻⁹ M estrogen (E₂) for 8 hours. To assess levels of VEGFR-1 mRNA, comparative semiquantitative reverse transcription–polymerase chain reaction (RT–PCR) was performed in which 1 µg of total RNA was subjected to reverse transcription with oligo(dT) primers in a final volume of 20 µL. The resulting cDNA was diluted to 100 µL. Comparative semiquantitative PCR was performed as follows: glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA was first amplified at a low cycle number (GAPDH primer sequences: sense = 5'-CCACCCATGGCAAATTCCTAGGCA-3'; antisense = 5'-TCTAGACCGGCAGGTCAAGTCCACC-3'); the resulting 600-base-pair products were separated by electrophoresis through a 1.5% agarose gel, visualized after staining with ethidium bromide, and quantified by using an Eagle Eye gel system and its associated software (Stratagene, La Jolla, CA). If needed, the cDNA dilutions were adjusted and used in GAPDH RT–PCR to obtain products with similar signal intensities between all the samples. The adjusted amounts of cDNA were used for PCR with primers specific for soluble VEGFR-1 (5'-CAACAACACACAGGAAAAGG-3' and 5'-GCACTGCACACAAAAAGGC-3') and for full-length membrane-spanning VEGFR-1 (9). The products were separated by 1.5% agarose gel electrophoresis and visualized after staining with ethidium bromide. A representative gel is shown. The intensity of each band was quantified by using Eagle Eye software and expressed as band intensity relative to that of GAPDH. The results of three separate experiments (mean with upper 95% confidence interval) are shown (lower panel). B) MCF-7 cells, prepared as described in (A), were preincubated with the pure estrogen antagonist ICI 182,780 (10⁻⁶ M) for 1 hour before the addition of E₂. VEGFR-1 mRNA expression was assessed by RT–PCR. A representative gel is shown (upper panel). The band intensities were quantified and expressed as band intensity relative to that of GAPDH (lower panel). Bars represent mean (n = 3) with upper 95% confidence interval. C) ER-positive MCF-7 and T47D and ER-negative MDA-MB-231 breast carcinoma cells were preincubated with ICI 182,780, and then incubated with estrogen in the presence or absence of ICI 182,780 for 8 hours. The cells were lysed, and aliquots of total proteins were separated on 4%–12% Bis-Tris NuPAGE gels (Invitrogen, Carlsbad, CA) and analyzed by western blotting with a rabbit polyclonal anti-VEGFR-1 antibody (Santa Cruz Biotechnology, Santa Cruz, CA). Membranes were stained with the protein-binding dye ponceau red (lower panel) for MCF-7 cells or stripped and probed with anti-GAPDH antibody (lower panels for T47D and MDA-MB-231 cells) to ensure equal loading. D) MCF-7 cells were treated with 10⁻⁶ M tamoxifen (TAM) or 10⁻⁹ M E₂ for 8 hours. Cells treated with the diluent, absolute ethanol, were used as a control. Total RNA and protein were isolated and analyzed by RT–PCR (upper panel) and western blotting (lower panel) for VEGFR-1 expression. GAPDH levels are shown as a control for cDNA integrity. E) Ovariectomized nude mice bearing slow-release estrogen or placebo pellets were injected subcutaneously with a mixture of MCF-7 cells and Matrigel. The Matrigel–MCF-7 cell plugs were excised after 6 days and subjected to immunohistochemistry with a rabbit polyclonal anti-VEGFR-1 antibody. The sections were developed using a Dako EnVision kit (DakoCytomation, Glostrup, Denmark). The sections were lightly counterstained with hematoxylin. VEGFR-1 protein expression in Matrigel–MCF-7 cell plug sections from mice implanted with the placebo (left panel) or the estrogen slow-release pellet (right panel) (magnification ×200).

ing the induction of inappropriate angiogenesis and cell growth. In agreement with this proposed mechanism, a recent study (16) has shown the presence of endogenous soluble VEGFR-1 at concentrations as high as 440 pg/mL in biologic fluids from normal human subjects. In normal breast tissue, estrogen is unlikely to inhibit VEGFR-1 expression because only a minority of normal breast epithelial cells (7%–17%) express detectable ER levels (17,18). However, in primary breast tumors, the majority of proliferating breast cancer cells are ER-positive (18,19), and estrogen, by reducing the levels of VEGFR-1, may increase the levels of VEGF available to activate angiogenesis. Thus, estrogen triggers an angiogenic switch and further promotes tumor progression. This proposed mechanism is supported by the fact that patients whose tumors had soluble VEGFR-1 levels at least 10-fold higher than their tumor VEGF levels had...
a markedly favorable prognosis compared with patients whose tumors had a much lower VEGFR-1/VEGF ratio (20).

The classical mechanism of estrogen action involves formation of an estrogen–ER complex that binds to the estrogen response element (ERE) in target promoters and modulates (by increasing or decreasing) gene transcription (21). However, analysis of the VEGFR-1 gene 1.5-kilobase regulatory sequence (22) using MatInspector software (23) revealed a lack of formal EREs in the promoter region, suggesting that the effect of estrogen on soluble VEGFR-1 expression is mediated by interactions between the estrogen–ER complex and additional transcription modulators. Such an ER-mediated but ERE-independent pathway has been reported for a number of estrogen-regulated genes (21). Some transcription factors regulating VEGFR-1 expression (i.e., Egr-1, Ets) (24,25) have been previously shown to mediate the cellular effects of estrogen (26,27). Because steroid hormones simultaneously control gene transcription and alternative splicing (28), the observed action of estrogen on VEGFR-1 expression may reflect effects on both transcriptional and post-transcriptional (i.e., alternative RNA processing) regulatory events that are involved in the generation of soluble VEGFR-1.

Regulation of soluble VEGFR-1 by estrogen may represent one of the molecular pathways responsible for the angiogenic switch during breast tumorigenesis. Detailed understanding of the role of estrogen and antiestrogens (i.e., tamoxifen) used in clinical settings to control VEGFR-1 expression may help in the design of new strategies for preventing resistance to endocrine therapy and may also help clarify the emerging role of estrogen in controlling vascularization.

REFERENCES


NOTES

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