Regulation of vascular endothelial growth factor (VEGF) gene transcription by estrogen receptors α and β

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Vascular endothelial growth factor (VEGF) mediates angiogenic activity in a variety of estrogen target tissues. To determine whether estrogen has a direct transcriptional effect on VEGF gene expression, we developed a model system by transiently transfecting human VEGF promoter-luciferase reporter constructs into primary human endometrial cells and into Ishikawa cells, derived from a well-differentiated human endometrial adenocarcinoma. In primary endometrial epithelial cells, treatment with 17β-estradiol (E_2) resulted in a 3.8-fold increase in luciferase activity, whereas a 3.2-fold induction was demonstrated for stromal cells. Our Ishikawa cells had less than 100 functional estrogen receptors (ER)/cell and were therefore cotransfected with expression vectors encoding either the α- or the β-form of the human ER. In cells cotransfected with ERα, E_2 induced 3.2-fold induction in VEGF-promoter luciferase activity. A 2.3-fold increase was observed in cells cotransfected with ERβ. Through specific deletions, the E_2 response was restricted to a single 385-bp *Pvu*II-*Sst*I fragment in the 5′ flanking DNA. Cotransfection of this upstream region with a DNA binding domain ER mutant, or site-directed mutagenesis of a variant ERE within this fragment, resulted in the loss of the E_2 response. Electromobility shift assays demonstrated that this same ERE sequence specifically binds estradiol-ER complexes. These studies demonstrate that E_2-regulated VEGF gene transcription requires a variant ERE located 1.5 kb upstream from the transcriptional start site. Site-directed mutagenesis of this ERE abrogated E_2-induced VEGF gene expression.

The vascular delivery of oxygen and nutrients to tissues is indispensable to their survival. Angiogenesis, the formation of capillary sprouts from preexisting blood vessels, is typically quiescent in the adult, except for pathological situations (e.g., wound healing, diabetic retinopathy, rheumatoid arthritis, cardiac ischemia, tumor growth) and during menstrual cycle-specific physiological processes in the female reproductive system (e.g., ovulation, endometrial growth, implantation, placentation). Whereas oncogene activation and hypoxia trigger angiogenesis in malignant transformation, both physiological and pathological neoangiogenesis are mediated by endogenous paracrine and endocrine factors. The creation of new blood vessels is complex and includes the proteolytic degradation of extracellular matrix, proliferation and migration of endothelial cells, and ultimately the formation of patent capillary tubules supplying the angiogenic stimulus. Many different growth factors and cytokines have been shown to exert chemotactic, mitogenic or inhibitory activities on endothelial cells, smooth muscle cells, and pericytes and can, therefore, be expected to participate in angiogenic processes in either direct or indirect ways.

The current investigation focuses on a single angiogenic protein of hierarchical importance: vascular endothelial growth factor (VEGF)-A. The human VEGF-A gene was localized to chromosome 6p21.3 (1) and is organized in eight exons, separated by seven introns. Alternative exon splicing of the VEGF gene results in the generation of four different mature molecular species, having 121, 165, 189, and 206 aa after signal sequence cleavage (2–4). VEGF is among the most potent and specific angiogenic factors (5) and is active as a glycosylated homodimeric protein (6). Its effects include endothelial cell proliferation, migration, organization into tubules, and enhanced permeability, all of which participate in the angiogenic cascade (7).

VEGF is the principal temporal and spatial mediator of cyclical neovascularization occurring in the ovary and uterus (8–10) and is an important angiogenic factor in other primate estrogen target tissues, including the placenta (11), neonatal (12) and pubertal growth plate cartilage (13), osteoblasts (14), human hair follicles (15), normal breast tissue (16), and some cases of breast carcinoma (17). The classical experiments of Markee, who directly observed endometrial vascularization in heterotopic rhesus endometrium transplanted into the anterior chamber of the eye, emphasize the endocrine regulation of this process (18). The monthly proliferation, and subsequent shedding, of human endometrium provides a coordinated example of programmed angiogenesis. As the functionalis layer of the primate endometrial mucosa grows under the estrogenic stimulation of the proliferative phase, so does the delicate capillary network needed to perfuse and nourish this dynamic tissue. Newly developed *in vitro* models suggest that isolated endometrial endothelial cells themselves express functional estrogen and progestin receptors (19). However, most investigators have failed to identify the presence of classical estrogen or progestin receptors in human endometrial endothelium *in situ* (20, 21).

The current studies address the rapidly remodeled functionalis layer of the endometrium (22, 23). Our experiments test the hypothesis that estrogenic effects on endometrial angiogenesis are mediated indirectly, via production of VEGF that stimulates capillary endothelial mitogenesis and morphogenesis. Analysis of the cloned 3.4-kb human VEGF gene promoter sequence (2) revealed several consensus transcriptional response elements: AP-1, AP-2, GATA-6, IL-6RE, hypoxia-inducible enhancer sequences (2, 24, 25), and several half-palindromic but no classical estrogen response elements (EREs). The estrogen receptor (ER) complex typically binds as a dimer to a consensus, 13-bp, palindromic ERE (26). Whereas very few natural genes contain perfect palindromic EREs, ER complexes also can activate the AP-1 cis complex (27), induce cAMP regulation of

Abbreviations: VEGF, vascular endothelial growth factor; E_2, 17β-estradiol; ER, estrogen receptor; ERE, estrogen response element; PDB, phorbol 12,13 dibutyrate; Act D, actinomycin D; AP-1, activator protein-1; RT-PCR, reverse transcriptase–PCR.

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protein kinase A-mediated genes (28), and bind imperfect EREs (29, 30).

In this paper we demonstrate that 17β-estradiol (E_2) directly regulates VEGF gene transcription in endometrial cells and in Ishikawa adenocarcinoma cells. Estradiol-induced gene transcription is ER dependent and is activated through a variant ERE localized $\approx$1.5 kb upstream from the VEGF transcription start site. Site-directed mutagenesis of this ERE fully abrogates E_2-induced VEGF gene expression, confirming the functional significance of this single motif.

Materials and Methods

Hormones and Chemicals. 17β-Estradiol, phorbol 12,13 dibutyrate (PDB), and other chemicals were obtained from Sigma. ICI 182,780 was from Zeneca (Macclesfield, U.K.).

Reporter Genes and Expression Vectors. A *Kpn*I to *Nhe*I genomic fragment (−2274 to +745) containing the human VEGF gene promoter (2) was cloned upstream of luciferase cDNA in a pGL2-basic vector (Promega). Different reporter constructs were created by digestion with selected restriction enzymes and subcloned into pGL2-basic or pGL2-promoter plasmids (Promega), the latter containing an SV40 promoter, as described previously (25). Mutants were generated with QuickChange site-directed mutagenesis kits (Stratagene), by using oligonucleotides containing the desired mutation. All constructs and mutants were sequenced by the University of California, San Francisco Biomolecular Resource Center to verify that the correct sequences or mutations were present. Expression vectors for ERα, ERβ, and ER mutants have been described previously (27, 31).

Ishikawa Cell Culture and Transfection. Ishikawa cells were grown and serially passaged in DMEM: nutrient mixture F-12 (D-MEM/F12, 1:1 mixture) supplemented with 10% FBS and 50 nM E_2. The cells were grown to 80% confluence, trypsinized, and resuspended in buffer [PBS/0.1% glucose/10 μg/ml Biobrene (Applied Biosystems)] before electroporation (32). Four million cells, 10 μg of VEGF reporter gene, and 3 μg of ER expression vector were added in a single electroporation cuvette. The cells were exposed to a 950-μF, 0.3-kV pulse (Bio-Rad), recovered in E_2-free 10% FBS DMEM/F12 and plated in 12-well dishes. After overnight recovery, the medium was switched to phenol red-free DME H-21 supplemented with 0.1% charcoal-treated calf serum (CS). Hormones, or ethanolic vehicle, were added 12 h later, and the cells were incubated for an additional 20 h. Cell extracts were prepared by washing the cells twice with PBS, lysed with reporter lysis buffer (Promega), and the luciferase activity was measured with a commercially available kit from Promega. Each reporter vector was assayed in at least three different cultures. The results are expressed as fold increase of luciferase activity ($\pm$SD) between untreated cells (controls) and cells treated with 100 nM E_2, 1 μM ICI, or 500 nM PDB.

Preparation and Transfection of Primary Human Endometrial Cell Cultures. Endometrial tissue was collected by Pipelle de Cornier aspiration (Cooper Surgical, Shelton, CT) from nonpregnant volunteers undergoing diagnostic laparoscopy or hysteroscopy. All specimens were obtained from women who provided written informed consent under a protocol approved by the Committee of Human Research at the University of California, San Francisco. All patients had normal ovulatory menstrual cycles and had not received hormonal medication for at least three months at the time of biopsy. Proliferative phase endometrial biopsies were used for the isolation and preparation of endometrial epithelial and stromal cell cultures as described (33). Primary epithelial cells were cultured directly in 24-well dishes. Stromal cells from the same biopsies were passaged twice, to eliminate contaminating immunocytes, and also plated in 24-well plates. Cells were grown to 80% confluence in MEM-α with nonessential amino acids (MEM), 10% FBS, and penicillin/streptomycin (PS). Previous studies, by using cytokeratin and vimentin immunostaining, respectively, verified that the epithelial and stromal cell cultures were more than 95% pure. Moreover, the cultures were free of T cells, granulocytes, monocytes, and other leukocytes as detected by CD3, CD11b, and CD45 immunostaining (33).

Endometrial glandular cells were transiently transfected with 1 μg of VEGF reporter construct containing 2.3 kb of 5′ flanking DNA by using the calcium phosphate (Ca-P) coprecipitation method (34). After overnight incubation, the culture media were replaced with 0.1% CS medium as described above. The cells were then treated with 10 nM E_2 in a final concentration of 0.1% ethanol, and control experiments received an equivalent amount of ethanol as vehicle. The cells were cultured in the presence of hormone for 20 h before lysis and assay of the luciferase activity.

Endometrial stromal cells were transiently transfected with the VEGF reporter plasmid by using Effectene (Qiagen, Valencia, CA), a nonliposomal lipid formulation, according to the manufacturer's protocol. VEGF promoter DNA (1.5 μg) were mixed with Effectene reagent in 24-well plates overnight. After transfection, the cells were recovered in fresh medium for 12 h before the culture media were replaced with MEM containing 0.1% CS. The cells were then cultured in the presence of 10 nM E_2 or ethanol alone for 20 h before assaying the luciferase activity.

Radioligand Binding Assays with [^{3}H]Estradiol. ER concentrations in primary endometrial cells were reported previously (35). ER in Ishikawa cells were measured by using whole cell radioligand binding assays before and after transfection with ERα or ERβ expression vectors. The cells were plated at a density of 50,000 cells/cm^2 and quiesced overnight when they reached 80%–90% confluence. The cells were incubated with 0.1–10 nM [2,4,6,7-^{3}H]17β-estradiol (88 Ci/mmol; New England Nuclear) for 4 h. Nonspecific binding was determined by the addition of a 100-fold excess of unlabeled estradiol. Free label was removed by two washes with ice-cold PBS and bound, intracellular [^{3}H] steroid was extracted with 0.25 M NaOH and quantified in a β-scintillation counter at 35% efficiency. Binding data were analyzed with PRISM version 3.0 (GraphPad Software, San Diego) by the method of Scatchard.

Preparation of Total RNA and Reverse Transcriptase–PCR (RT-PCR) Analysis. Total RNA was extracted from cell cultures by using the TRIzol reagent kit (GIBCO/BRL). RT-PCR was performed by using primers derived from the human VEGF-A sequence. The sense primer began at base 198 (5′ position) and was 28 bases in length. The antisense primer began at base 592 (3′ position) and was 30 bases in length. These amplified a 395-bp PCR product. cDNA was reverse transcribed from total RNA obtained from Ishikawa cells. Ten-fold dilutions of cDNA representing 25 ng to 25 fg of total RNA were amplified. The cDNA was subjected to 30 cycles of PCR amplification consisting of 40 s at 95°C, 20 s at 60°C and 70 s at 75°C. The resulting PCR products were visualized on 4% agarose gels stained with ethidium bromide. Data were analyzed by computer-assisted densitometry (BioImage Intelligent Quantifier, Ann Arbor, MI) and expressed as VEGF:glyceraldehyde-3-phosphate dehydrogenase (GAPDH) ratio as percentage control.

Electrophoretic Mobility-Shift Assay. Double-stranded oligonucleotides containing the putative ERE located in the *Pvu*II-*Sst*I fragment of the VEGF promoter (−1560 to −1175) were synthesized (aatcagactgactggcctcagagccc) and end labeled by 5′

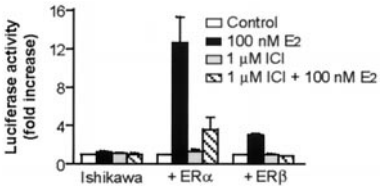

Fig. 1. Ishikawa cells were transfected with ERE-TK luciferase vectors (3 μg per cuvette) with or without expression vectors for ERα or ERβ (1.5 μg per cuvette). The cells were treated for 20 h with 100 nM E_2 and/or 1 μM ICI as indicated in the *Inset*. Error bars show standard deviations among triplicates.

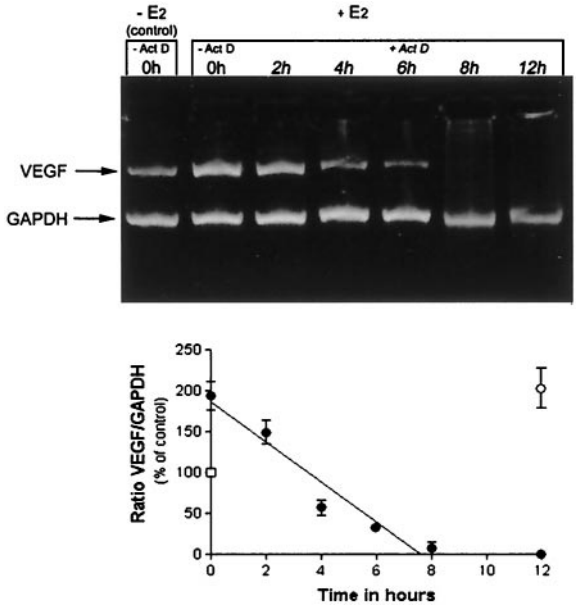

Fig. 2. Ishikawa cells were transiently transfected with ERα. After recovery overnight, the cells were stimulated with ethanolic vehicle [$-E_2$ (control)] or 100 nM E_2 for 15 h without actinomycin D ($-$Act D) before the first time point [0 h; $-E_2$ (□) and $+E_2$ (●)]. Act D (5 μg/ml) was then added to the remaining cell cultures, and total RNA was extracted after an additional 2, 4, 6, 8, and 12 h (●). Control samples (without Act D) were assessed after 12 h (○). RT-PCR was performed as described and visualized on 4% agarose gels stained with ethidium bromide. Data of three independent experiments ($\pm$SD) were analyzed as ratio of VEGF:glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and expressed as percentage of the control. VEGF mRNA half-life was calculated as 4 h.

phosphorylation with T4 polynucleotide kinase and [γ-^{32}P]ATP. DNA-binding reactions were performed in 20 μl buffer containing ^{32}P-labeled ERE and a final concentration of 12 mM Hepes-KOH (pH 7.6)/48 mM KCl/0.8 mM EDTA/4 mM $MgCl_2$/10% glycerol/0.05% Nonidet P-40/2 μg/ml dI-dC). ER expressed in reticulocyte lysates were extracted with TNT Quick Coupled Transcription/Translation Systems (Promega) according to the manufacturer's protocol. The binding reaction was initiated by the addition of expressed ER or 2 μl of Ishikawa cell nuclear extract prepared as previously described (36). Binding competition was performed by adding unlabeled probe at a 100-fold excess. The samples were incubated for 15 min at room temperature, placed on ice, and then separated on 5% nondenaturing polyacrylamide gels at 200 mV with running buffer consisting of 25 mM Tris/25 mM borate/1 mM EDTA.

Results

Ishikawa Cells as a Model for Glandular Endometrial Cells. It is difficult and tedious to obtain primary human endometrial cells. Furthermore, the absolute number of ER and the distribution of ERα and ERβ vary during the menstrual cycle in the human endometrium (35, 37). We therefore chose the Ishikawa cell line, derived from a well-differentiated human endometrial adenocarcinoma, as a model for endometrial epithelial cells. This cell line is reported to express endogenous ER and all four known species of VEGF protein (4).

To quantify the number of ER in our cell line, we performed ligand-binding assays with [^{3}H]estradiol. By Scatchard analysis, our Ishikawa cell line had less than 100 functional ER/cell. We therefore decided to transfect the Ishikawa cells with an expression vector encoding either the α form (ERα) or the β form (ERβ) of the human estrogen receptor. The original human ERα cDNA (HEO) cloned by Green *et al.* (38), was used, which contains a point mutation resulting in the substitution of a valine residue for a glycine residue at position 400 within the ligand-binding domain. This mutation allows normal function of ERα as a transcription factor but reduces the affinity of the receptor for estradiol by about 70%. The transfected receptor absolutely requires added estrogen for activity in cell culture conditions (39). Expression vectors encoding the wild-type ERβ protein were introduced similarly. After transfection with the ER expression vectors, $\approx$20,000 ERα/cell and $\approx$6,000 ERβ/cell could be demonstrated by [^{3}H]estradiol ligand binding assays (data not shown). We recently demonstrated that the ER density of normal endometrial cells is 24,538 $\pm$ 10,169 ER/cell (35). Thus, our transfected Ishikawa cells expressed nearly physiological levels of ER.

To test the functionality of the ER expression vectors, the Ishikawa cells were transfected with an ERE-TK luciferase (Luc) vector alone or together with the ER expression vectors (Fig. 1). When the Ishikawa cells were transfected with ERE-TK-Luc alone, treatment with E_2 resulted in a less than 20% increase in relative luciferase activity (Fig. 1). After cotransfection with ER expression vectors, increases in luciferase activity of 12.6-fold for ERα and 3.0-fold for ERβ, respectively, were noted after E_2 treatment (Fig. 1). ICI 182,780 (1 μM), a pure estrogen antagonist, was used as a control to show that this receptor-dependent increase in luciferase activity was specific to E_2. Pretreatment with ICI antagonized the estradiol effect on luciferase activity by 71% for ERα and by 80% for ERβ respectively (Fig. 1). Treatment with 1 μM ICI 182,780 alone had no significant effect on either of the estrogen receptors (Fig. 1). The transfection efficiency obtained with β-galactosidase reporter vectors was 40% (data not shown).

Inhibition of RNA Polymerase Blocks Accumulation of VEGF mRNA Transcripts. Ishikawa cells were transiently transfected with the expression vector encoding the ERα as described above. After recovery overnight, the cells were treated with ethanolic vehicle (control) or E_2. After 15 h, total RNA was extracted from a set of cell cultures to serve as the first time point, and actinomycin D (Act D, 5 μg/ml) was added when appropriate. The cells were harvested after 2, 4, 6, 8, and 12 h, and RT-PCR was performed and visualized on 4% agarose gels stained with ethidium bromide (Fig. 2).

As shown in Fig. 2, and confirmatory of our previous findings in primary cell cultures (9), a 2-fold increase in steady-state VEGF mRNA was evident after 15 h of E_2. No further increase could be demonstrated after 12 more hours of treatment. Inhibition of cellular transcription by Act D, however, resulted in decay of VEGF mRNA with a calculated half-life of 4 h (Fig. 2). The findings indicate that the E_2-induced increase in gene expression is due to new VEGF mRNA synthesis rather than posttranscriptional regulation.

Estradiol Has a Direct Effect on VEGF Gene Transcription in Primary Cell Cultures. To determine whether the transcription rate of the VEGF gene is altered directly by estrogen, human endometrial

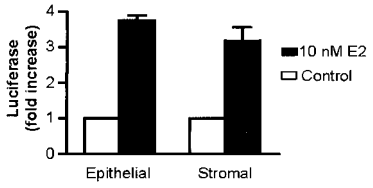

Fig. 3. Human endometrial epithelial and stromal cells were transiently transfected with luciferase fusion vectors containing 2.3 kb of 5′ flanking DNA relative to the VEGF transcriptional start site (VEGF −2274 to +50, *Kpn*I–*Nhe*I). Epithelial cells were transfected by calcium phosphate precipitation. Effectene was used to transfect stromal cells. After transfection, the medium was changed to phenol-red-free DME-H21 with 2.5% dextran charcoal-filtered calf serum. The transfected cells were treated with ethanolic vehicle (control) or with E_2 (10 nM) for 20 h. The cells were lysed, and luciferase activity was measured. Relative luciferase activity is expressed as -fold increase over the controls. Error bars represent standard deviations of three different cultures, each done in triplicate.

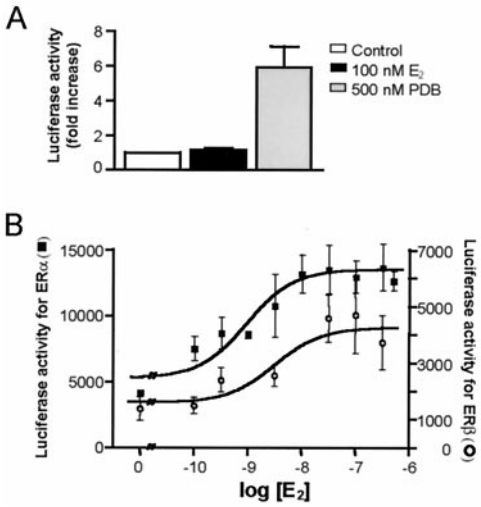

Fig. 4. (*A*) Ishikawa cells were transiently transfected with luciferase fusion vectors containing 2.3 kb of 5′ flanking DNA relative to the VEGF transcriptional start site. After recovery overnight, the cells were treated with ethanolic vehicle (control), E_2 (100 nM), or phorbol-12,13-dibutyrate (PDB, 500 nM) for 20 h. Error bars represent standard deviations from three different cultures. (*B*) Dose–response of E_2 activation of the VEGF gene promoter in the presence of ERα and ERβ. Ishikawa cells were transfected with VEGF luciferase vectors and expression vectors encoding either ERα or ERβ. Transfected Ishikawa cells were treated with increasing E_2 concentrations. The results obtained with ERα are plotted as light units on the left ordinate (■), those obtained with ERβ on the right ordinate (○). Error bars show standard deviations among quadruplicates from a single representative transfection.

epithelial and stromal cells obtained from healthy volunteers were transiently transfected with luciferase fusion vectors containing 2.3 kb of 5′ flanking DNA relative to the VEGF transcriptional start site (VEGF −2274 to +50, *Kpn*I–*Nhe*I). Epithelial cells were transfected by calcium phosphate precipitation, whereas Effectene was used to transfect stromal cells. After transfection, exposure of the cells to estrogens was minimized. Medium free of phenol red, which can act as an estrogen agonist (40), and serum batches that were low in endogenous estrogens and had been further stripped of steroids with dextran-charcoal treatment were used. Under these conditions, the cells were treated with E_2 for 20 h. The cells were then lysed, and luciferase activity was measured and compared with the controls treated with ethanolic vehicle alone. In epithelial cells, treatment with E_2 resulted in a 3.8-fold increase in luciferase activity, whereas a 3.2-fold increase was demonstrated for the stromal cells (Fig. 3). Thus, E_2 has a direct effect on VEGF gene transcription in both endometrial epithelial and stromal cells.

Estradiol Stimulation of the VEGF Gene Transcription Is ER Dependent. Ishikawa cells were transiently transfected with luciferase fusion vectors containing 2.3 kb of 5′ flanking DNA relative to the VEGF transcriptional start site (VEGF −2274 to +50, *Kpn*I–*Nhe*I) by electroporation. After recovery overnight, the medium was changed to phenol-red free DME-H21 with 0.1% dextran charcoal-filtered CS. Ethanolic vehicle (control), 17β-E_2, or 12,13-PDB was then added for 20 h. The cells were lysed, and luciferase activity was measured and compared with the controls. Because four AP-1 sites are located in the VEGF gene promoter (2), 12,13-PDB, a substance known to activate those sites, was used to confirm luciferase activity in the transfected cells. Whereas 12,13-PBD induced a 6-fold increase in the 2.3 Kb VEGF promoter luciferase activity, treatment with E_2 had no effect (Fig. 4*A*).

Knowing that our Ishikawa cell line had less than 100 ER/cell, we cotransfected the VEGF reporter plasmid with expression vectors encoding either ERα or ERβ of the human estrogen receptor. Under these conditions, it was possible to demonstrate 17β-E_2 dose-responsive activation of the VEGF gene promoter (Fig. 4*B*).

VEGF Gene Transcriptional Up-Regulation by 17β-Estradiol Is ERE Dependent. A *Kpn*I to *Nhe*I fragment (−2274 to +745) from the human VEGF gene (2), cloned upstream of luciferase cDNA in the pGL2-basic vector, was transiently transfected into Ishikawa cells in the presence of the ERα expression vector. When compared with controls, treatment with E_2 caused a 3.2-fold increase in transgene activation (Fig. 5). This level of induction is comparable to the documented induction of VEGF mRNA by estradiol in primary human endometrial cells (9). A series of deletion constructs prepared by digestion with restriction enzymes were transiently transfected into Ishikawa cells in the presence of the ERα expression vector. Transfection of a *Kpn*I to *Nhe*I fragment (−2274 to +50) resulted in a 3.8-fold increase of luciferase activity by E_2, whereas transfection of a shorter fragment, *Pst*I to *Nhe*I (−790 to +50), did not respond to E_2 stimulation. This finding indicated that the main functional ERE is located upstream of the *Pst*I (−790) site (Fig. 5). Therefore, other constructs were generated and cloned into the pGL2-promoter vector, which contains an SV40 promoter, and transiently cotransfected with the plasmids encoding ERα. Cotrans-

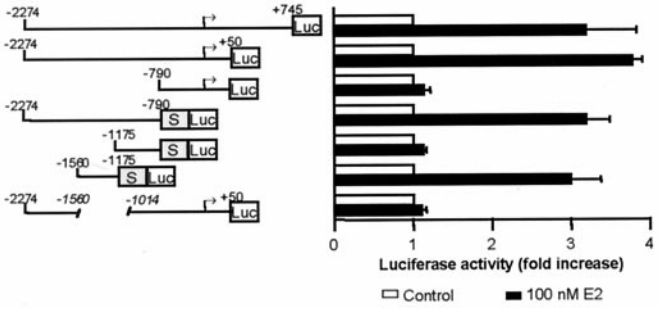

Fig. 5. Specific deletions of the 5′ UTR and 5′ flanking sequences of the human VEGF gene promoter were obtained by digestion with restriction enzymes and transiently transfected into Ishikawa cells with ERα expression vectors. Gene fragments were subcloned into pGL2-basic or pGL2-promoter vectors, where pGL2-promoter contains a minimal SV40 promoter sequence (S). The arrow indicates the transcriptional start site of the VEGF gene. Luciferase activity of each construct was normalized to that of the corresponding empty vector. Error bars represent standard deviations of three different cultures, each done in triplicate.

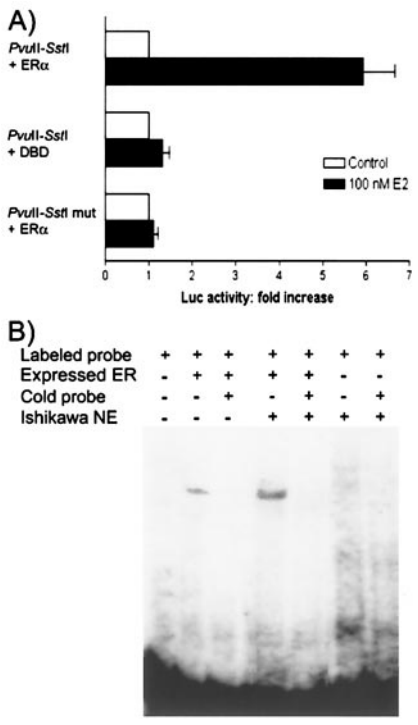

Fig. 6. (*A*) Luciferase fusion vectors containing a *Pvu*II-*Sst*I genomic fragment (−1560 to −1175) of the human VEGF gene promoter were cotransfected with expression vectors encoding either ERα or ERα containing a DNA binding domain mutation (DBD). After mutation of a putative ERE within the targeted upstream region, the luciferase fusion vector was cotransfected with ERα. After recovery overnight, the cells were treated with ethanolic vehicle (control) or 100 nM E_2 for 20 h. Error bars represent standard deviations in three different cultures. (*B*) Electrophoretic mobility-shift assay with oligonucleotides containing the putative ERE. ERs expressed in reticulocyte lysates and/or Ishikawa nuclear extracts (NE) were added to the labeled oligonucleotide probe (aatcagactgactggcctcagagccc). Binding competition was performed by adding cold probe at a 100-fold excess.

fection of the empty pGL2-promoter with ERα stimulated a 1.8-fold increase in luciferase activity after treatment with E_2 (data not shown). As a result of the inherent estrogen response in the pGL2-promoter plasmid, all of the results in Fig. 5 were normalized to the empty vector response. Transfection of the *Kpn*I-*Pst*I (−2274 to −790) upstream region showed a 3-fold increase in luciferase activity after treatment with E_2. Through further digestion, the estradiol effect was restricted to a 385-bp *Pvu*II–*Sst*I fragment localized ≈1.5 kp upstream from the transcription start site (Fig. 5).

Cotransfection of this 5′ *Pvu*II-*Sst*I (−1560 to −1175) fragment with an ER containing a DNA binding domain mutation, or directed mutation of the variant ERE within this upstream region of the VEGF promoter, fully abrogated the E_2-induced effect on luciferase activity (Fig. 6*A*). To verify that the identified element binds ER specifically, we performed electrophoretic mobility-shift assays with oligonucleotides containing the putative ERE. Addition of expressed ER clearly demonstrated DNA binding, whereas addition of Ishikawa cell nuclear extract appears to enhance ER binding but did not bind the label oligonucleotide itself (Fig. 6*B*). The binding was competed by adding unlabeled probe at a 100-fold excess, confirming that the identified element binds ER specifically (Fig. 6*B*). Plasmids containing a tandem repeat of the variant ERE oligonucleotide described in the legend to Fig. 6*B* were constructed in the pGL2-promoter vector. Cotransfection of this construct with ERα and incubation with 100 nM E_2 resulted in a 4.2-fold stimulation of luciferase activity.

Discussion

The focus of the current study is to understand the role of endocrine and paracrine interactions in the vascularization of the human endometrium. Estradiol and VEGF, respectively, are the hypothetical mediators that we chose to investigate. In the human endometrium, VEGF mRNA begins to increase in the midproliferative phase and reaches a maximum in late proliferative and secretory endometrium (9). Beginning in the late proliferative phase, under the influence of ovarian estradiol production, a complex subepithelial capillary plexus develops in the cycling endometrium (41). Endometrial capillary endothelial cells also appear to be the most mitogenically active in tissue explants during this cycle phase when exposed to [^{3}H]thymidine (42). More recent morphometric evaluations of endometrial vessel formation indicate significant variation in endothelial cell proliferation within different regions of the uterus. Samples from hysterectomy specimens show that the endothelial cell proliferation index is significantly elevated in the functionalis compared with the basalis zone (43). Thus, the models used in these experiments were designed to represent cells of the endometrial functionalis layer. Primary human endometrial cell cultures provide an excellent model to study the physiological effects of ovarian steroids on VEGF gene expression, but are difficult to obtain in sufficient amounts for biochemical studies. Ishikawa cells, derived from a well-differentiated human endometrial adenocarcinoma, are reported to express endogenous ER (44) and all four known species of VEGF protein (4). This cell line therefore seemed to be an ideal candidate to investigate the effect of estradiol on endometrial VEGF gene expression. However, we determined that our strain of Ishikawa cells had less than 100 functional ER/cell. Thus, we elected to transfect the cells with expression constructs to generate functional ERα or ERβ in the Ishikawa cells. By Scatchard analyses, 20,000 ERα/cell and 6,000 ERβ/cell could be demonstrated by [^{3}H]estradiol ligand binding assays in the transfected cells, respectively. These concentrations approached the physiological ER density of normal endometrial cells (24,538 ± 10,169 ER/cell) derived from the functionalis zone (35). Furthermore, transfection of either ERα or ERβ expression vectors provided an opportunity to study independently the effect of each receptor. Thus the transfected Ishikawa cell model was designed to recapitulate the ovarian steroid receptor phenotype of functionalis epithelium, which differs from that of the underlying basalis layer (45).

Previous studies indicate that oxygen tension is a key regulator of VEGF gene expression, both *in vitro* and *in vivo* (25, 46). Our current findings indicate that E_2 also increases the steady-state concentrations of VEGF mRNA in human endometrial cells through a predominantly transcriptional mechanism. Inhibition of cellular transcription with actinomycin D after treatment with 100 nM E_2 for 15 h resulted in substantial decay of VEGF mRNA (Fig. 2), suggesting that E_2 induces new VEGF mRNA synthesis rather than mRNA stability. In primary cell cultures transiently transfected with luciferase fusion vectors containing 2.3 kb of 5′ flanking DNA relative to the VEGF transcriptional start site, treatment with 17β-E_2 increased luciferase activity in epithelial and stromal cells. Estradiol-induced gene transcription is ER dependent, as shown in the Ishikawa cells, which have low endogenous levels of ER.

Little is known about the mechanisms of this estrogen effect on VEGF production. Estrogen receptor complexes bind to specific DNA sequences. The classical EREs contain a 13-bp palindromic consensus sequence (GGTCAnnnTGACC) located in the promoter or enhancer regions of estrogen-regulated genes. However, ligand-bound ER also has the capacity to act through different DNA elements (27), and most estrogen-responsive

genes contain ERE sequences that diverge to a varying degree from this consensus sequence (47). Analysis of the cloned 3.4-kb human VEGF gene promoter sequence (2) reveals several half-palindromic but no classical EREs. Because the 5′-flanking region of a gene is the most common location of response elements for steroid receptors, we concentrated our efforts on this part of the VEGF promoter. Cotransfection of a *Pvu*II-*Sst*I genomic fragment, cloned upstream of the luciferase cDNA in a pGL2-promoter vector, with an expression vector encoding for ERα resulted in a 6-fold increase in luciferase activity. However, cotransfection with a DNA binding domain mutant of ERα fully abrogated the E_2-induced effect (Fig. 6*A*), confirming the presence of a functional ERE in the targeted region. Sequence analysis of the relevant 385-bp motif revealed a putative variant ERE (AATCAnnnTGACT), possessing two and one base changes, respectively, in the sequences of each half site relative to the consensus element. However, by electromobility shift assay, we could demonstrate that this sequence binds estradiol-ER complexes and that the binding was competed by adding an excess of cold probe (Fig. 6*B*). Furthermore, site-directed mutagenesis of this ERE eradicates E_2-induced VEGF gene expression, verifying the functional requirement of this single motif.

These studies demonstrate that a single variant ERE is responsible for E_2-induced VEGF gene transcription in endometrial cells. We postulate that this same motif is likely to play an important role in the estrogenic regulation of VEGF production and angiogenesis in other estrogen target tissues (e.g., breast, bone, heart, and skin). Future studies should focus on the relative contributions of ERα and ERβ in the regulation of angiogenesis in these tissues.

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