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Uterine Expression of Vascular Endothelial Growth Factor Is Increased by Estradiol and Tamoxifen[1]

Salman M. Hyder,[2] George M. Stancel, Constance Chiappetta, Lata Murthy, Holly L. Boettger-Tong, and Sari Makela

Departments of Pharmacology [S. M. H., G. M. S., C. C., L. M., H. L. B-T., S. M.], Obstetrics, Gynecology, and Reproductive Sciences [S. M. H.], University of Texas Medical School, Houston, Texas 77225 and Department of Pediatrics, Baylor College of Medicine, Houston, Texas 77030 [L. M.]

ABSTRACT

Vascular endothelial growth factor (VEGF) is an endothelial-specific mitogen with potent angiogenic activity. Because vascular growth accompanies normal endometrial regeneration and may also be involved in uterine tumor growth, we studied VEGF regulation by 17β-estradiol (E_2) and tamoxifen, two agents that can increase uterine cell proliferation and tumor incidence. In immature, ovariectomized rats, E_2 elevates uterine VEGF mRNA transiently, with a peak induction of 15–20-fold within 1 h. A maximum response is produced at a dose of 4 μg/kg E_2, and induction is specific for estrogenic steroids. E_2-dependent VEGF induction is inhibited by actinomycin D but not puromycin, suggesting that the effect is due at least in part to direct estrogen receptor regulation of VEGF transcription. PCR amplification and DNA sequencing indicated that $VEGF_{188}$, $VEGF_{164}$, and $VEGF_{120}$ are all induced by E_2, but the latter two are the predominant forms in the uterus. *In situ* hybridization shows a predominantly stromal expression of VEGF mRNA. The antiestrogens tamoxifen, 4-OH tamoxifen, and nafoxidine produce similar increases in uterine VEGF mRNA levels within 6 h, with 1 mg/kg tamoxifen producing a maximum response of 15–20-fold. The tamoxifen response was also inhibited by actinomycin D but not by puromycin, again suggesting direct transcriptional regulation of VEGF expression by antiestrogens. These findings raise the possibility that estrogen and antiestrogen effects on uterine edema, proliferation, and tumor incidence may involve local increases in tissue VEGF production.

INTRODUCTION

VEGF[3] is a potent mitogen that is specific for endothelial cells and thus associated with angiogenesis (1). This process is an integral part of the neovascularization that occurs in a variety of biological processes, *e.g.*, embryogenesis, corpus luteum formation, wound healing, and the cyclical growth of the endometrium during the estrous cycle of mammals and the menstrual cycle of primates. A second activity of VEGF is the induction of vascular hyperpermeability (1, 2), which generally precedes new vessel growth.

In addition to its role in normal growth and tissue repair VEGF is also thought to be involved in tumor expansion (3). The growth of tumors is limited by nutrient availability from existing vessels and thus invariably involves increased VEGF production, necessary for neovascularization to support the metabolic needs of a growing tumor.

A number of variant VEGF transcripts have been reported, but the significance of these splice variants is not clear (1). In several murine tissues, the two predominant forms of the growth factor contain 164 ($VEGF_{164}$) and 120 ($VEGF_{120}$) amino acids, but the ratio of these splice variants is tissue specific (4). This suggests that the proteins encoded by the different variants have distinct functions, some of which may be unknown at present.

It has recently been shown that VEGF expression is regulated by estrogenic hormones (5, 6). This regulation could thus be involved in a number of physiological and pathological processes, such as endometrial regeneration following menstruation, the growth of hormone-responsive tumors, and possibly even more generalized effects on the endothelium throughout the vascular system. This is an increasingly important area because of the widespread pharmacological use of estrogenic and antiestrogenic drugs. We have thus investigated the regulation of VEGF mRNA expression by both estrogens and antiestrogens in the immature rat uterus, which is widely used as an experimental system to study uterine cell proliferation and function.

Tamoxifen is the antiestrogen that has been extensively used for many years in the treatment of breast cancer, and it is now being considered for prophylactic use in women with risk factors for the disease. However, in contrast to its antiestrogenic activity in breast cancer cells, tamoxifen appears to be an estrogen agonist in the uteri of both humans and animals (7–10), and women receiving the drug have an increased incidence of endometrial cancer (11). At present, it is not known whether tamoxifen alters VEGF expression in either the reproductive tract or the general vasculature.

In this report, we show that both E_2 and tamoxifen increase VEGF mRNA expression in the same cell types and to the same degree in the rodent uterus. This induction is specific for compounds that exhibit estrogenic actions in the uterus, is blocked by actinomycin D, and is unaffected by puromycin, suggesting that regulation is at least in part a direct transcriptional effect. The induction of VEGF is most prominent in the region of the stroma immediately underlying the luminal epithelium.

MATERIALS AND METHODS

Materials. [α-^{32}P]UTP (800 Ci/mmol) was obtained from Amersham Radiochemicals (Arlington Heights, IL) and diluted to 400 Ci/mmol with radioinert UTP from Boehringer Mannheim (Indianapolis, IN) for the RNA polymerase reactions. Guanidine isothiocyanate, cesium chloride, and formamide were obtained from International Biotechnologies, Inc. (New Haven, CT). E_2 was obtained from Steraloids (Wilton, NH), and tamoxifen and nafoxidine were from Sigma Chemical Co. (St. Louis, MO). All other chemicals and reagents were purchased from Sigma and were the highest grade commercially available.

Animals and Hormone Treatment. Immature female Sprague-Dawley rats (21 days old, 40–45 g; Harlan Sprague-Dawley, Indianapolis, IN) were used in most of our studies and were ovariectomized 4–7 days prior to treatment. Some experiments used animals from Charles River (Saint Louis, MO), but the response to E_2 and other compounds was the same, irrespective of the source.

Animals were injected s.c. in the periscapular region with 0.5 ml of 5% ethanol and 95% saline containing estrogens or antiestrogens. Unless otherwise specified in the text or legends, doses of E_2 were 40 μg/kg body weight and doses of tamoxifen and other antiestrogens were 1 mg/kg.

For the metabolic inhibitor studies, animals were treated either with a single dose of puromycin (100 mg/kg) 30 min prior to E_2 or tamoxifen or with two equal doses (4 mg/kg each) of actinomycin D 3 h before and then simulta-

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[2] To whom requests for reprints should be addressed, at Department of Pharmacology, University of Texas Medical School, 6431 Fannin, P.O. Box 20708, Houston, TX 77225.

[3] The abbreviations used are: VEGF, vascular endothelial growth factor; E_2, 17β-estradiol; RT, reverse transcription.

neously with the E_2 or antiestrogen. Both inhibitors were administered by i.p. injection in 0.5 ml vehicle (5% ethanol and 95% saline).

RNA Preparation. Total uterine RNA was prepared by the method of Chirgwin *et al.* (12) as previously described (13). Briefly, uteri were removed from anesthetized animals and immediately homogenized in 5 M guanidinium isothiocyanate using a Polytron homogenizer (Brinkman Instruments, Westbury, NY) set at half-maximal power for 60 s. Uteri from three animals were pooled for the preparation of each RNA sample. RNA was pelleted through 5.7 M CsCl, extracted twice with phenol-chloroform (1:1) and once with chloroform, and precipitated with ethanol. RNA was quantified by absorption at 260 nm.

Northern Blot Analysis. Samples of total RNA (20 μg) were denatured for 30 min in 15 mM methylmercuric hydroxide (Alfa, Salt Lake City, UT) and separated on 1% agarose gels containing 6% (v/v) formaldehyde. The gels were stained with ethidium bromide after electrophoresis, and the rRNA bands were visualized under UV illumination to ensure that samples were undegraded during preparation and processing. To correct for any internal variation in gel loading or transfer, we used the rat *1A* gene, which does not vary following estrogen treatment (14).

The RNA samples were then transferred to Duralon membranes (Stratagene, La Jolla, CA) by electroblotting in 25 mM sodium phosphate (pH 6.5). Membranes were allowed to dry at room temperature and were then prehybridized in 0.8 M NaCl, 2 mM EDTA, 0.5% SDS, 20 mM PIPES, 50% deionized formamide, and 100 μg/ml denatured salmon sperm DNA for 3 h at 62°C.

The ^{32}P-labeled VEGF antisense RNA probe was synthesized from the insert in a pBluescript vector (Stratagene) using Sp6 RNA polymerase (Promega, Madison, WI). The pBluescript contains the $VEGF_{164}$ insert that was cloned following RT-PCR amplification of rat uterine RNA. The probe was sequenced to confirm the presence of the spliced variant. This clone contains the sequences that recognize all the spliced variants of VEGF. The radiolabeled probe was added directly to the prehybridization mixture. Blots were hybridized for 16–24 h at 60°C and then washed twice with 2× SSC [1× SSC is 0.15 M NaCl and 0.015 M Na Citrate (pH 7.0)] containing 0.1% SDS at room temperature for 30 min, washed twice with 0.1× SSC containing 0.1% SDS at 60°C for 30 min each, and then rinsed with 2× SSC. After the rinse, blots were treated with RNase A (1 μg/ml) in 2× SSC for approximately 5 min, washed in 0.1× SSC containing 0.1% SDS for 10 min, and then exposed to X-ray film. Where indicated in the text, films were scanned with a Zeineth Soft Laser scanning densitometer.

PCR and Southern Blot Analysis. The primers and probes for PCR amplification of rat VEGF and the internal probe used for detection of the amplified products have been described previously (5). Primers and probes were synthesized by National Bioscience, Inc. (Plymouth, MN).

RT-PCR was carried out using the Gene Amp PCR kit (Perkin Elmer Cetus, Norwalk, CT) using the conditions recommended by the manufacturer. The PCR parameters (30 cycles) were denaturation at 95°C for 1 min, annealing of primer at 65°C for 1 min, and extension of the product at 72°C for 30 s. The extension step was automatically increased by 2 s with each subsequent cycle.

The PCR products were analyzed on 2% agarose gels containing 1 μg/ml ethidium bromide. Electrophoresis was carried out in 0.5× Tris-borate-EDTA (pH 8.0) at 80 V for 2 h. The gel was photographed under UV light and subsequently transferred to a nylon membrane (Hybond-N; Amersham) by the Southern blot technique. After the transfer, the membrane was baked at 80°C in a vacuum oven for 2 h and stored at room temperature for hybridization.

The internal probe was labeled with [γ-^{32}P]ATP by the 5′ end-labeling method. After hybridization, the membrane was washed in 2× saline-sodium phosphate-EDTA at room temperature for 10 min three times, followed by a 3-min wash in 1× saline-sodium phosphate-EDTA at 70°C. The membrane was exposed to X-ray film at −70°C.

PCR products representing estrogen receptor variants were excised from gels and sequenced as described previously (15). Products were purified by the Wizard PCR purification system (Promega). Purified DNA was used in PCR sequencing with the primers used for amplification and the ABI 373A DNA sequencer (15) with a *Taq* DyeDeoxy Termination Sequencing Kit™ (Perkin Elmer Cetus) as recommended by the manufacturer.

***In situ* Hybridization.** Uteri were removed and fixed overnight in ice-cold 4% paraformaldehyde in PBS (pH 7.2). Samples were then transferred to 70% ethanol and paraffin embedded. Uterine cross-sections (5 μm) were cut and mounted onto slides (Probe-On Plus; Fischer Scientific) and incubated overnight at 42°C.

In situ hybridization was performed using a protocol described previously (16) with minor modifications. The rat VEGF riboprobe described above for Northern blot analysis was used for the generation of sense and antisense probes after appropriate restriction digestions. High specific activity ^{35}S-UTP (>1000 Ci/mol; Amersham) was used to generate VEGF sense and antisense riboprobes following the manufacturer's directions (Promega). Probes were then purified using spin columns (5′-3′ Corp., Boulder, CO), and preparations with >60% incorporation of UTP were used.

Prior to hybridization, sections were deparaffinized in xylene, rehydrated in graded ethanol, and denatured with 0.2 N HCl at 37°C and in 2× SSC at 70°C. Sections were then rinsed with PBS, followed by treatment with RNase-free proteinase K (1 μg/ml) in Tris-EDTA [0.1 M Tris and 0.05 M EDTA (pH 8.0)] for 30 min at 37°C, and again rinsed with PBS. Samples were then postfixed with 4% paraformaldehyde, rinsed in PBS, and equilibrated in 0.1 M triethanolamine. Slides were then acetylated for 10 min at room temperature with 0.25% acetic anhydride in 0.1 M triethanolamine (pH 8.0), rinsed in 2× SSC, dehydrated through graded ethanol, and vacuum desiccated for at least 1 h. Riboprobes were added (to a final concentration of 1×10^7 cpm/ml) to the hybridization solution (50% formamide, 10% dextran sulfate, 0.32 M NaCl, 0.02% Denhardt's solution, 0.001 M EDTA, 2.5 mg/ml tRNA, and 0.05 M DTT in diethylpyrocarbonate-treated water). The mixture was centrifuged at 3000 rpm in a Mistral 2000 centrifuge for 10 min, and 65-μl aliquots of the solution were applied to glass coverslips. Coverslips (22 × 50 mm) were then placed over the sections (four per slide), taking care that no air bubbles formed, and the edges of the coverslips were sealed with rubber cement. Hybridization was then performed overnight at 60°C.

Following hybridization, slides were cooled to room temperature, the rubber cement was removed, and coverslips were soaked off in 4× SSC with orbital shaking for 20 min at room temperature. Slides were rinsed in two changes of 4× SSC (10 min/rinse), and nonspecifically bound probe was digested with 0.02 mg/ml RNase A in 0.5 M NaCl, 0.1 M Tris, and 1 mM EDTA (pH 8.0) for 30 min at 37°C. Slides were then washed sequentially with 2× SSC, 1× SSC, and 0.5× SSC at room temperature (all washes containing 1 mM DTT), followed by a 40-min wash at 50°C in 0.1× SSC. After a final 10-min wash in 0.1× SSC, slides were dehydrated in ethanol (3 min for each wash with 70, 90, 95, and 100% ethanol), drained, and vacuum dried at room temperature for 1 h.

Slides were dipped in photographic emulsion (Kodak NTB-2, diluted 1:1 with water) and exposed for approximately 2 weeks at 4°C prior to development with Kodak D-19. After development, slides were stained with Harris modified hematoxylin, dehydrated through xylenes, and mounted.

RESULTS

Uterine levels of VEGF mRNA are low in control animals and increase dramatically following a single injection of E_2, as illustrated in Fig. 1*A*. The Rat1A transcript was used as an internal loading control, because this mRNA is not regulated by hormone treatment (14). E_2 causes a massive induction of VEGF transcript levels, which is maximal within 1–3 h, remains elevated for up to 6 h, and then gradually declines to near basal levels by 12 h.

As seen in Fig. 1*A*, two VEGF transcripts of approximately 3.7 and 4.5 kb are present in the uterus, as reported previously in other systems (17–20). At present, the relationships of these transcripts to each other and to the multiple forms of VEGF protein and their specific mRNAs are unknown. In our studies, the absolute ratio of the two transcripts was variable between experiments, but E_2 and tamoxifen always produced a similar change in both. For ease of presentation, all quantitative values in the text and figures thus refer to the 3.7-kb transcript, which was the predominant species in most of our experiments, and its intensity is normalized to that of the Rat1A signal in the same samples, as noted in "Materials and Methods." For example, Fig. 1*B* illustrates the estrogenic induction of the 3.7-kb transcript based on densitometric scanning of the autoradiogram shown in Fig. 1*A*.

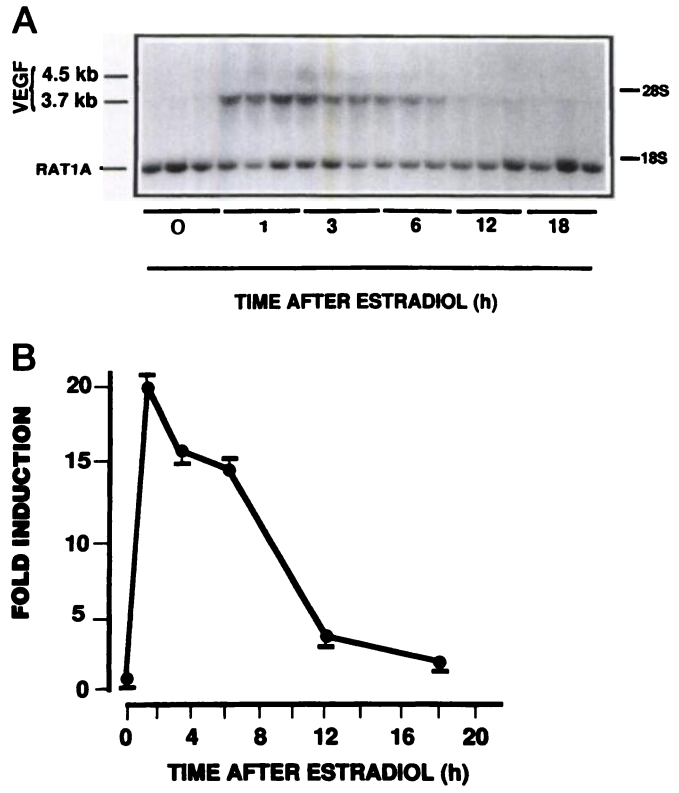


Fig. 1. Time-dependent induction of VEGF in the rat uterus by E_2. *A*, ovariectomized rats were treated with E_2 (40 μg/kg) for the periods indicated. Uteri were then removed, and RNA was prepared as described in "Materials and Methods." Each lane contains 20 μg RNA prepared from pooled tissue of three animals, and each time point was analyzed in triplicate. The Rat1A transcript is an internal standard. *B*, band representing 3.7-kb VEGF message was scanned with a laser densitometer and corrected for internal variation by using the Rat1A signal. The mean values (n = 3) are expressed as fold induction above the vehicle-treated controls. *Bars*, SE.

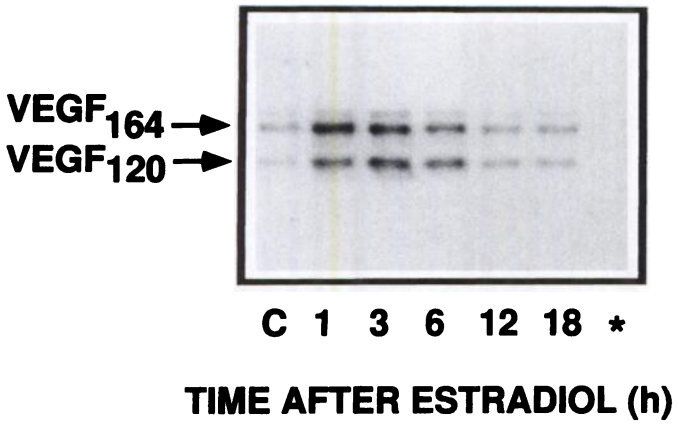


Fig. 2. RT-PCR analysis of VEGF expression in the rat uterus. Uterine RNA from time course samples shown in Fig. 1 were subjected to RT-PCR analysis with primers described in "Materials and Methods." Following separation of the amplified products, the DNA was transferred to a nylon membrane and processed for identification of VEGF-specific bands with an internal primer and the procedure described in "Materials and Methods." *C*, RT-PCR analysis from the untreated animals; *, water control.

Because a number of VEGF splice variants exist in different tissues, we performed PCR analysis to determine which are present in the uteri of control and E_2-treated animals. As shown in Fig. 2, three distinct variants appear to be induced. Direct automated DNA sequencing of these bands identified them as $VEGF_{188}$, $VEGF_{164}$, and $VEGF_{120}$ (data not shown), the latter two being the predominant forms of the growth factor message observed in the rat uterus (Fig. 2).

We next determined the dose-response curve and hormonal specificity for estrogenic induction of VEGF mRNA. For these studies, we gave the animals injections of increasing doses of E_2 (Fig. 3) or nonestrogenic steroids (Fig. 4) and sacrificed them 3 h later. Fig. 3 illustrates that maximum VEGF induction is achieved with a dose of 4.0 μg/kg E_2, and a similar dose-response curve was observed in a second independent experiment. As shown in Fig. 4, androgens, glucocorticoids, and progestins do not produce a significant elevation in uterine VEGF mRNA 3 h after treatment.

The increase in VEGF transcript levels seen above represents *de novo* RNA synthesis and does not require prior synthesis of uterine proteins, as illustrated in Fig. 5. As seen in this figure, induction of VEGF mRNA is blocked by the RNA synthesis inhibitor actinomycin D but not by the protein synthesis inhibitor puromycin. These results suggest that estrogenic induction of the growth factor message in-

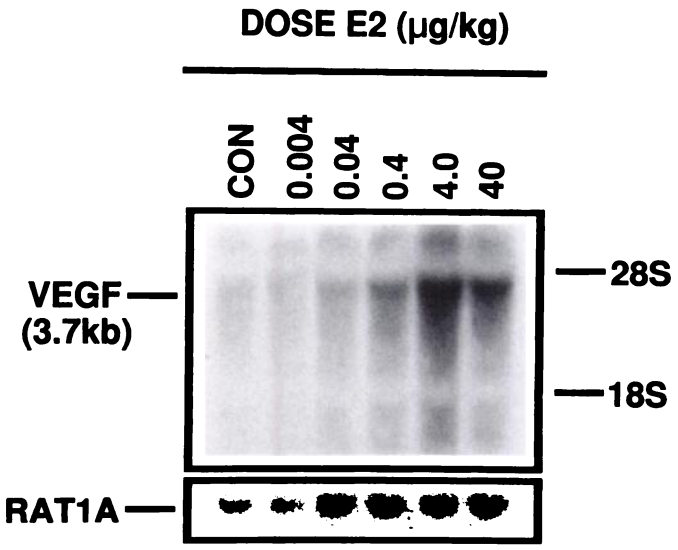


Fig. 3. Dose-dependent induction of VEGF in the rat uterus by E_2. Ovariectomized animals were given the indicated doses of E_2, and 3 h later, RNA was prepared for analysis. Each lane represents pooled RNA from three animals. Northern blot analysis was performed as described in "Materials and Methods." *CON*, control.

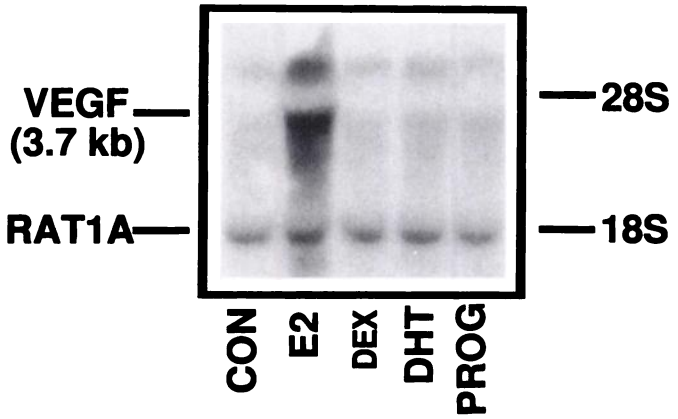


Fig. 4. Hormone specificity of VEGF induction in the rat uterus. Ovariectomized animals were injected s.c. with either vehicle (*CON*), 40 μg/kg E_2, 600 μg/kg dexamethasone (*DEX*), 400 μg/kg 5α-dihydrotestosterone (*DHT*), or 40 mg/kg progesterone (*PROG*). Animals were sacrificed 3 h after steroid treatment, and total RNA was prepared as described in "Materials and Methods." Each lane contains 20 μg RNA pooled from three different animals. Northern blot analysis was performed as described in "Materials and Methods."

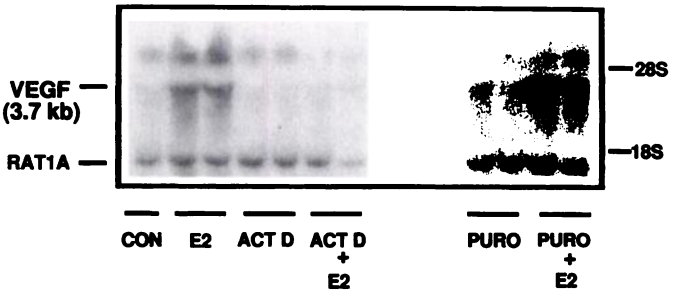
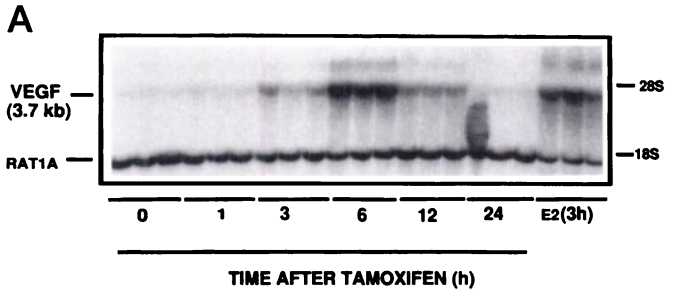


Fig. 5. Effect of actinomycin D (*ACT D*) and puromycin (*PURO*) on VEGF induction. Animals were treated with actinomycin D 3 h before E_2 treatment and then again at the time of E_2 treatment. Animals (three per sample) were sacrificed 3 h after the E_2 injection, and RNA preparation and Northern blot analysis were performed as described in "Materials and Methods." For the puromycin studies, animals received 100 mg/kg of the inhibitor 30 min prior to the E_2 treatment. Three h later, animals were sacrificed, and RNA was prepared. Each lane represents pooled RNA from three different animals. Northern blot analysis was performed as described in "Materials and Methods." *CON*, control.



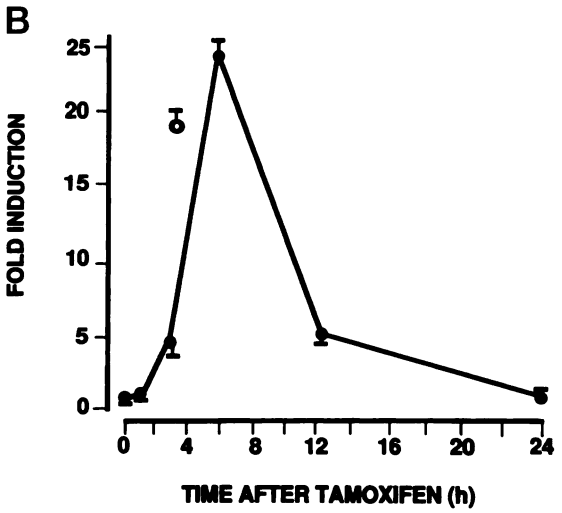


Fig. 6. Time-dependent induction of VEGF in the rat uterus by tamoxifen. *A*, ovariectomized rats were treated with tamoxifen (1 mg/kg) for the times indicated. Uteri were then removed, and RNA was prepared as described in "Materials and Methods." Each lane contains 20 μg RNA from a pool obtained from three animals. The Rat1A probe was used as an internal standard. *B*, the 3.7-kb VEGF message was scanned with a laser densitometer and corrected for internal variation using Rat1A values. The open circle represents E_2-induced VEGF message at 3 h. The mean values (n = 3) are expressed as fold induction above the vehicle treated controls. *Bars*, SE.

volves transcriptional activation, at least in part, but do not rule out additional effects.

The data presented above clearly indicate that E_2 regulates VEGF mRNA synthesis in the uterus, suggesting that this hormonal effect may play a role in the physiological growth of the endometrium during the estrous cycle. We next sought to determine whether the drug tamoxifen, which is an antiestrogen in the mammary gland but produces some estrogenic actions in uterus (21), would also alter VEGF transcript levels.

As seen in Fig. 6, tamoxifen treatment produces a large increase in VEGF mRNA levels. The onset of induction by tamoxifen is slightly slower than that produced by E_2, but the magnitude of the increase is similar after treatment with either agent. Dose-response studies indicated that maximum induction occurs with 1 mg/kg of the antiestrogen (Fig. 7). Tamoxifen causes a significant increase in VEGF mRNA levels in the presence of puromycin, with induction in the presence of the inhibitor being 84% of that caused by the antiestrogen alone (Fig. 8), as judged by densitometry corrected for an internal standard signal, whereas actinomycin D largely abolishes induction (Fig. 9). This suggests that transcriptional activation is likely to be involved with induction by tamoxifen, as well as that previously seen with E_2.

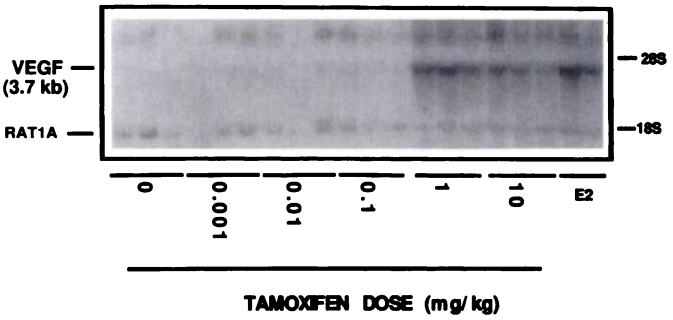


Fig. 7. Dose-dependent induction of VEGF in the rat uterus by tamoxifen. Ovariectomized animals were given the indicated doses of tamoxifen, and 3 h later, RNA was prepared for analysis. Each lane represents pooled RNA from three animals. Northern blot analysis was performed as described in "Materials and Methods."

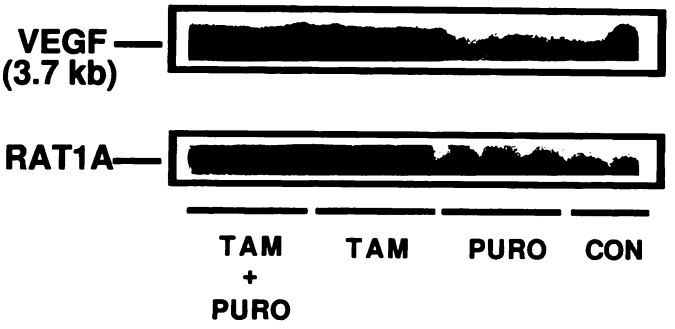


Fig. 8. Puromycin does not inhibit tamoxifen-induced VEGF expression. Animals received 100 mg/kg puromycin (*PURO*) 30 min before the tamoxifen (*TAM*) treatment. Three h later, animals (three per sample) were sacrificed, and RNA was prepared. Each lane represents pooled RNA from three different animals. Northern blot analysis was performed as described in "Materials and Methods." *CON*, control.

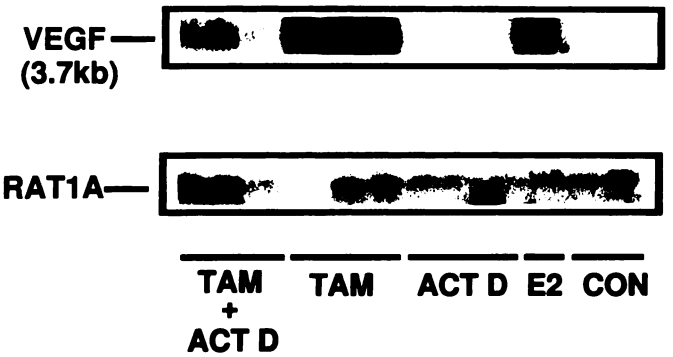


Fig. 9. Actinomycin D inhibits tamoxifen-dependent induction of VEGF. Animals were treated with actinomycin D (*ACT D*, 40 mg/Kg) 3 h before tamoxifen (*TAM*) treatment and then again at the time of tamoxifen (1 mg/kg) treatment. Animals (three per sample) were sacrificed 6 h after the tamoxifen injection, and RNA preparation and Northern blot analysis were performed as described in "Materials and Methods." *CON*, control.

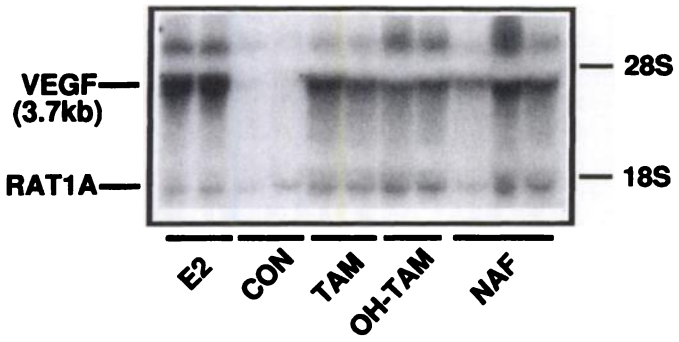


Fig. 10. Induction of VEGF mRNA by various nonsteroidal antiestrogens. Ovariectomized animals were treated with either 40 μg/kg E_2 or 1 mg/kg of the antiestrogens tamoxifen (*TAM*), 4-hydroxytamoxifen (*OH-TAM*), or nafoxidine (*NAF*). Animals (three per group) were sacrificed after 3 h for the E_2 group or after 6 h for all other treatments. RNA was analyzed by the Northern blot procedure as described in "Materials and Methods." *CON*, control.

In addition to antiestrogenic actions mediated by estrogen receptor binding, tamoxifen may produce effects via other actions. We thus determined whether an active metabolite, 4-hydroxytamoxifen, and another triphenylethylene antiestrogen, nafoxidine, also induce VEGF transcript levels in the uterus. As seen in Fig. 10, both compounds clearly increase VEGF mRNA. However, because we did not perform additional studies with these compounds (*e.g.*, time course and dose response), we are uncertain whether they produce the same quantitative changes seen with E_2 and tamoxifen.

The biochemical studies performed to this point establish the induction of VEGF mRNA levels but do not indicate which of the multiple cell types present in the uterus exhibit this response to E_2 or tamoxifen. To answer this question, we performed the *in situ* hybridization studies shown in Fig. 11. As expected, VEGF transcript levels are very low in untreated animals (Fig. 11*B*) and increase dramatically after treatment with either E_2 (Fig. 11*D*) or tamoxifen (Fig. 11*F*). Control studies using a complementary sense probe (Fig. 11, *A*, *C*, and *E*) indicate that the hybridization signal seen in Fig. 11, *D* and *F*, represents specific binding to VEGF transcripts. The increase in specific signal is localized to the region of stromal cells immediately underlying the luminal epithelium, with far less change in the stromal cells adjacent to the myometrium. Epithelial cells, either luminal or glandular, and myometrial cells show little response. The bright-field images of the sections represented in Fig. 11, *B*, *D*, and *F* are represented in Fig. 11, *G–I*.

DISCUSSION

The discovery of VEGF as a potent endothelial-specific mitogen has provided a key marker for studies on the regulation of angiogenesis. In adult mammals, the cyclical regeneration of the endometrium involves extensive angiogenesis and is thus a logical site to examine the regulation of this growth factor. Cullinan-Bove and Koos (5) were

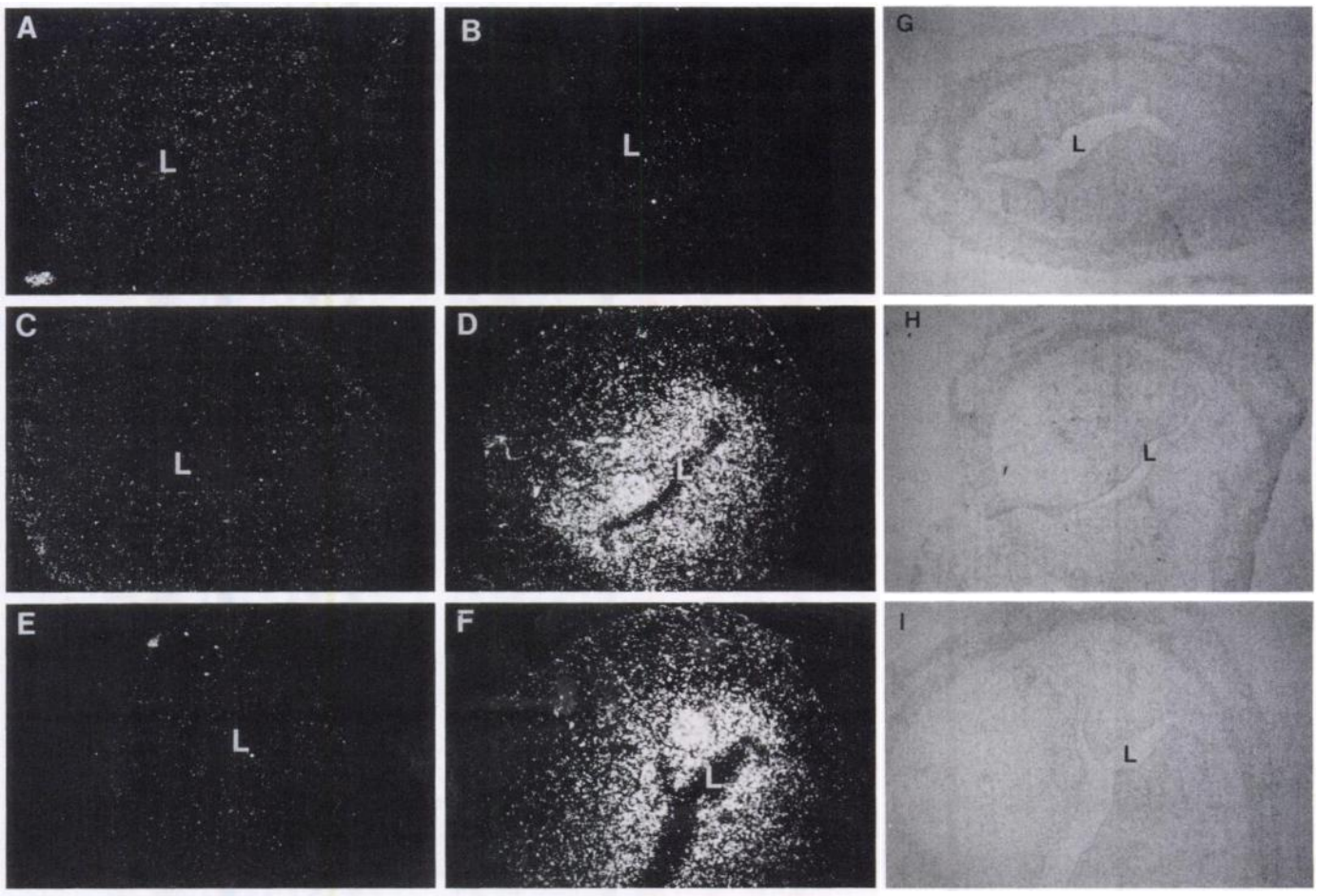


Fig. 11. *In situ* hybridization of VEGF expression in the rat uterus by E_2 and tamoxifen. Sections represented in *A*, *C*, and *E* are treated with the sense probe. *A*, vehicle-treated control; *C*, E_2-treated sample (3 h); *E*, tamoxifen-treated sample (6 h). Sections *B*, *D*, and *F* are treated with antisense VEGF probe. *D* and *F*, corresponding E_2-treated (3 h) and tamoxifen-treated (6 h) samples, respectively; *B*, vehicle-treated control. *L*, lumen of the uterus; the signal obtained is mainly in the stromal compartment beneath the luminal epithelial cells. *G–I*, bright-field images of sections represented in *B* (control), *D* (estrogen-treated samples), and *F* (tamoxifen-treated samples).

the first to show that estrogens regulate the expression of VEGF mRNA in the rodent uterus, and with the minor exceptions noted below, we have confirmed their initial observations in this report. We have now established that antiestrogens such as tamoxifen produce quantitatively similar increases in VEGF transcript levels and have identified the specific uterine cell type in which VEGF induction occurs.

Different forms of VEGF are present in many tissues, and PCR experiments revealed the presence of three VEGF splice variants in the uterus. $VEGF_{164}$ and $VEGF_{120}$ are the major forms and are present the rat uterus in roughly a 1:1 ratio (see Fig. 2), whereas the $VEGF_{188}$ transcript is present is relatively small amounts. Previous studies in humans (22) and rats (5) have also found that $VEGF_{164}$ and $VEGF_{120}$ are the major transcripts and are present in roughly equal amounts. A similar ratio of transcripts has been found in several other rat tissues and cell lines (4). At present, we do not know whether these three forms have different biological functions in the uterus, although we are pursuing this question in other studies. Estrogen treatment appears to increase the level of all three forms (Fig. 3), although our PCR results are qualitative rather than quantitative.

The transient increase in VEGF transcript levels after E_2 treatment is maximal within 1–2 h and is thus the most rapid example of mRNA induction in the uterus of which we are aware. By way of comparison, estrogen induction of other "immediate early response genes" in the uterus (*e.g.*, c-*fos*, c-*jun*, and c-*myc*) is typically maximal 2–3 h after hormone administration (13, 23–29). The rapidity of the uterine VEGF increase may be related to the enhanced vascular permeability seen after hormone treatment. This is one of the most rapid physiological responses to estrogens, and VEGF increases vascular permeability (2) in addition to its angiogenic actions. VEGF may thus mediate two estrogenic actions in the uterus: the water imbibition that occurs hours after hormone treatment and the growth of new blood vessels, which may occur at longer times. It is interesting to note that $VEGF_{164}$ and $VEGF_{120}$, which appear to be the predominant forms induced by E_2 in the uterus, are known to increase both vascular permeability and angiogenesis (1). Similar rapid increases in vascular permeability prior to endothelial cell proliferation are known to occur in other tissues undergoing angiogenesis (2).

Induction of VEGF mRNA by tamoxifen is also rapid, although slower than after E_2 treatment. This may be due to conversion to active metabolites such as 4-hydroxytamoxifen (10) or to other differences in the pharmacokinetics of the two compounds. Time course differences of several hours have also been observed for the induction of other genes in the rodent uterus by estrogens and antiestrogens (21, 30). Similarly, the initial increase in uterine wet weight, which is due to water imbibition, occurs several h later, following antiestrogen treatment relative to estrogen treatment (31). The differences in time courses for VEGF induction thus appear consistent with differences in the time courses of macroscopic organ responses.

Several findings in this report and the previous study by Cullinan-Bove and Koos (5) indicate that estrogenic induction of the VEGF message is due at least in part to direct transcriptional activation. Induction is rapid and is inhibited by RNA synthesis inhibitors but is unaffected by puromycin, indicating that it is not secondary to the induction of other proteins. A similar pattern of sensitivity to inhibitors suggests that tamoxifen also acts by increasing transcription of the *VEGF* gene. However, these results do not exclude other effects of estrogens and antiestrogens, which might increase levels of the growth factor mRNA. In the same vein, a functional estrogen response element has not yet been identified in the *VEGF* gene. Additional studies are thus required to unequivocally determine whether E_2 and tamoxifen increase transcription of this gene via a classic mechanism.

Our results indicate that induction of VEGF transcript levels is specific for compounds that produce estrogenic effects in the uterus (Fig. 4). This is somewhat different from the findings of Cullinan-Bove and Koos (5), who reported that progesterone also increases VEGF mRNA levels, albeit to a lesser degree than E_2. This difference may be due to the fact that their studies used a 6-h treatment with progesterone, and ours used a 3-h treatment. We have not explored this difference further, because our primary interest was to determine whether the estrogenic effect seen at the time of peak induction (*i.e.*, 1–3 h) displayed a hormonal specificity consistent with an estrogen receptor-mediated effect.

Using *in situ* hybridization, we found that there is a striking induction of VEGF mRNA induction is the periluminal region of the stroma. This is accompanied by a decreasing gradient of stromal expression going from the periluminal region toward the stroma. We are not aware of any other genes that show such a striking gradient of expression within a single uterine cell layer following estrogen treatment. Because the estrogen receptor appears to be ubiquitously expressed in cells throughout the entire stroma (32), additional factors that regulate hormonal induction must be present (or absent) in the subset of cells in this tissue layer closest to lumen. Little, if any, expression is seen in either the epithelial or smooth muscle cells of the uterus. From the pharmacological perspective, these studies are also important, because they establish a similar cellular pattern of VEGF expression after treatment with either E_2 or tamoxifen.

This cellular pattern of expression is consistent with the uterine response to estrogenic stimulation and the biological functions of the tissue. The stroma has the highest blood vessel density in the organ and exhibits massive edema after hormone treatment. The subregion of the stroma closest to the uterine lumen is also the main site of microvessel growth in the rodent uterus (6). In primates, a comparable region of stroma (*i.e.*, the functionalis layer of the endometrium) is the primary site of angiogenesis during postmenstruation regeneration of the endometrium (33). The periluminal area of the stroma is also a major site of blood vessel growth that occurs during decidualization following embryo attachment to the uterine wall.

It also seems likely that an increased nutrient supply from the periluminal microvasculature would help support estrogen-induced proliferation, which occurs primarily in the epithelial cell layer of the rat uterus (34). This would in fact represent a very fundamental type of stromal-epithelial interaction that could play a role in hormonal responses.

The finding that VEGF is induced in the uterus by both tamoxifen and E_2 is of potential clinical significance. Although our studies were performed in a rat model, tamoxifen is also known to produce estrogenic responses in the human uterus (35). This raises the possibility that bleeding abnormalities reported in some women receiving tamoxifen treatment (36) may be due in part to increased uterine expression of VEGF. Estrogens (37) and tamoxifen (38) are also known to increase the incidence of endometrial cancer in humans. This raises the question of whether VEGF plays a role in these actions by increasing the vascular supply required to maintain the growth of tumors as their size increases. These questions are increasingly important, because many women are currently receiving tamoxifen for breast cancer and estrogens for postmenopausal estrogen replacement therapy, and because the prophylactic use of antiestrogens for the prevention of breast cancer is currently being discussed (39, 40).

Another interesting question that has not yet been addressed is whether estrogens or antiestrogens have a general effect on VEGF production in the cardiovascular system or in specific organs other than the uterus. Although completely speculative, this is an intriguing possibility, given the established benefits of estrogens on cardiovascular health.

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