Vascular endothelial growth factor is modulated in vascular muscle cells by estradiol, tamoxifen, and hypoxia

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Vascular endothelial growth factor (VEGF) is a polypeptide secreted by a large number of cells that is mitogenic for endothelial cells and induces angiogenesis and vasculogenesis in vivo (see Ref. 15 for review). It is also a potent stimulator of microvascular permeability (12) and a chemotactic factor for endothelial cells and monocytes (9). It acts via its two known receptors, Flt-1 and KDR/Flik-1 (18). Molecular cloning of the complementary DNA (cDNA) for this growth factor revealed that alternative exon splicing of a single VEGF gene results in the generation of several VEGF isoforms of 121, 145, 165, 189, or 206 amino acids (VEGF121, VEGF145, VEGF165, VEGF189, or VEGF206, respectively) (15, 54), with VEGF121 and VEGF165 being the major isoforms. Studies on knockout mice lacking VEGF or its receptor have revealed that VEGF plays a critical role in the development and formation of blood vessel networks (1, 48). Recent studies have shown the presence of VEGF in atheromatous lesions (21) and also suggest that VEGF could participate in the maintenance of the endothelium after injury (25); however, its precise role in the arterial wall is still unknown.

Human and other mammalian cultured arterial smooth muscle cells (SMC) produce VEGF (16), which may constitute a local stimulus for angiogenesis or act as a permeability factor. Factors that upregulate VEGF in various cells include hypoxia (7, 42, 51), multiple growth factors, and cytokines. The expression of VEGF in SMC from aorta or mammary artery appears to be regulated by platelet-derived growth factor-BB or transforming growth factor-β (6), basic fibroblast growth factor (bFGF) (53), and interleukin-1β (27).

Vascular endothelial growth factor is modulated by estradiol (E2) in human endometrial fibroblasts. We report here E2 induction of VEGF expression in human venous muscle cells (smooth muscle cells (SMC) from human saphenous veins; HSVSMC) expressing both ER-α and ER-β estrogen receptors. E2 at 10⁻¹⁰ to 10⁻⁸ M increases VEGF mRNA in HSVSMC in a time-dependent manner (3-fold at 24 h), as analyzed by semiquantitative RT-PCR. This level of induction is comparable with E2 endometrial induction of VEGF mRNA. Tamoxifen and hypoxia also increase HSVSMC VEGF mRNA expression over control values. Immunocytochemistry of saphenous veins and isolated SMC confirms translation of VEGF mRNA into protein. Immunoblot analysis of HSVSMC-conditioned medium detects three bands of 18, 23, and 28 kDa, corresponding to VEGF isoforms of 121, 165, and 189 amino acids. Radioreceptor assay of the conditioned medium produced by E2-stimulated HSVSMC reveals an increased VEGF secretion. Our data indicate that VEGF is E2, tamoxifen, and hypoxia inducible in cultured HSVSMC and E2 inducible in aortic SMC, suggesting E2 modulation of VEGF effects in angiogenesis, vascular permeability, and integrity.

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ESTROGENS ARE KNOWN to have beneficial effects on the cardiovascular system (14) in addition to their important effects on the reproductive system and bone. Estrogens have potent antiatherogenic effects and induce vasorelaxation through mechanisms that may involve direct effects on the artery, mediated by the estrogen receptors ER-α (4, 24, 32, 35, 37) and (possibly the newly discovered) ER-β (20, 26, 30). However, the function of ER-β in the vascular system remains to be elucidated. In contrast to the arterial wall, ER-α is not present in significant amounts in the venous system, especially in saphenous veins (36), and the presence of ER-β has not been established. Recent studies have reported that estradiol (E2) promotes angiogenic activity both in vivo and in vitro (3, 22, 29), but the mechanisms have not been elucidated.

Vascular endothelial growth factor (VEGF) is a permeability factor revealed that alternative exon splicing of a single VEGF gene results in the generation of several VEGF isoforms of 121, 145, 165, 189, or 206 amino acids (VEGF121, VEGF145, VEGF165, VEGF189, or VEGF206, respectively) (15, 54), with VEGF121 and VEGF165 being the major isoforms. Studies on knockout mice lacking VEGF or its receptor have revealed that VEGF plays a critical role in the development and formation of blood vessel networks (1, 48). Recent studies have shown the presence of VEGF in atheromatous lesions (21) and also suggest that VEGF could participate in the maintenance of the endothelium after injury (25); however, its precise role in the arterial wall is still unknown.

Human and other mammalian cultured arterial smooth muscle cells (SMC) produce VEGF (16), which may constitute a local stimulus for angiogenesis or act as a permeability factor. Factors that upregulate VEGF in various cells include hypoxia (7, 42, 51), multiple growth factors, and cytokines. The expression of VEGF in SMC from aorta or mammary artery appears to be regulated by platelet-derived growth factor-BB or transforming growth factor-β (6), basic fibroblast growth factor (bFGF) (53), and interleukin-1β (27). Recently, our laboratory (3) and others (8, 10, 19, 49, 50) have reported the stimulation of VEGF expression by E2 in uterus, both in vivo and in vitro, indicating that the hormone may modulate angiogenesis in these cells via an increase of VEGF expression. An increase of VEGF mRNA in carotid arteries after es-
Estrogen treatment has also been reported in experiments using a rat model of arterial injury (25). However, whether VEGF is regulated by E2 in human vascular SMC is still unknown; in addition, no study has investigated the presence, modulation, and potential signification of VEGF in human veins.

We designed the present study to test whether E2 induces the production of VEGF mRNA and protein in the SMC from human saphenous veins (HSVSMC) and aorta. We report that E2 and the agonist/antagonist tamoxifen induce VEGF mRNA in HSVSMC in a time- and dose-dependent manner and appear to induce VEGF in SMC from human aorta. Our data suggest that E2 may promote formation of new blood vessels or increase vascular permeability by inducing expression of VEGF in vascular SMC.

MATERIALS AND METHODS

Reagents

Reagents for cell culture and DNA amplification were supplied by Gibco BRL (Life Technologies, Cergy-Pontoise, France); 17β-estradiol (17β-E2), tamoxifen, and 4-hydroxytamoxifen (OH-Tam) were supplied by Sigma; TRIzol isolation kit, Moloney murine leukemia virus (MMLV) RT, and Taq polymerase were supplied by Life Technologies; VEGF cDNA and recombinant VEGF165 and VEGF189 were gifts from J. Plouet (Toulouse, France); and the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA was a gift from Y. De Kaiser (Paris, France).

Isolation and Characterization of Cultured Vascular SMC

Fresh human saphenous veins were obtained from patients undergoing coronary artery bypass surgery. Patients were men (n = 14) or women (n = 3) 57–81 yr of age. Some fragments were quickly frozen in isopentane, precooled in liquid N2, and stored in liquid N2 until processing for immunocytochemistry. Other fragments were trimmed of adhering fat and connective tissue. Endothelial cells were discarded by scraping the luminal side of the vein with a scalpel and were suspended in a mixture of medium 199-RPMI 1640 supplemented with 20% human serum. Residual SMC were then put into 0.2% gelatin-coated flasks for culture until confluency. Before steroid stimulation, cells were cultured for 24 h in DMEM containing 5% human or fetal calf serum in the absence of E2 (stripped serum) and phenol red. For stimulation, the medium was replaced with the same phenol red-free DMEM medium in the presence of E2 (10−10 to 10−7 M). Control cells were incubated in phenol red-free medium without hormone. Cells were also stimulated with the estrogen receptor blocker tamoxifen and OH-Tam (10−9 to 10−7 M alone or in combination with E2. E2 immunoassay confirmed the efficiency of the charcoal treatment (E2 < 10 pg/ml).

Hypoxia. The desired O2 mixture was preanalyzed and infused into air-tight incubators with inflow and outflow valves (Haereus). Confluent cells were exposed to a gas mixture of either 94% N2-5% CO2-1% O2 (hypoxia) or 74% N2-5% CO2-21% O2 (normoxia) for 6–24 h at 37°C.

Extraction of RNA from Cells and Northern Blot Analysis

After incubation, confluent cells were scraped into lysing buffer for RNA isolation using a modified guanidium isothiocyanate method (TRIzol), according to the recommendations of the manufacturer. The presence of mRNA encoding VEGF in vascular SMC was determined with the use of Northern blot analysis and RT-PCR using oligonucleotide primers.

mRNA for VEGF was detected by Northern blot, as previously described (3), by use of a human cDNA probe that recognizes all the isoforms. Total RNA (20 μg) was size fractionated in formaldehyde-agarose (1%) gels and transferred to membranes (Hybond, Amersham). Prehybridization and hybridization were carried out in 5× SSC (standard sodium citrate), 5× Denhardt’s, 50% formamide, 0.1% SDS, and 100 ng/ml salmon sperm DNA. Radioactive labeling of the probes was performed by use of the random priming method (9). GAPDH RNA was used to confirm equal RNA loading. Posthybridization washes were carried out for 2 × 30 min with 2× SSC-0.1% SDS at 52°C and for 1 h with 0.1× SSC-0.1% SDS at 60°C, followed by autoradiography and densitometric scanning.

RT and Semiquantitative RT-PCR of VEGF

For the RT stage, single-stranded cDNA was synthesized from 1 μg total RNA in the presence of MMLV RT and the oligo(dT) primers according to the manufacturer’s instructions.

Double-stranded cDNAs were synthesized and amplified using 0.25 U Taq polymerase, 20 mM Tris-HCl (pH 8.0), 50 mM KCl, 0.1 mM of dNTP, 10 pmol of each primer of VEGF, 50 pmol of each primer of GAPDH, 1.5 mM MgCl2, and 4 × 105 counts/min of 33P-end-labeled primer in a 25-ml reaction final volume. The amplification was carried out in a DNA thermal cycler at 95, 60, and 72°C for 30 s, 1 min, and 1 min, respectively, for 28 cycles. To permit semiquantitative analysis, RT-PCR of the housekeeping gene GAPDH was used at 94, 55, and 72°C for 30 s, 1 min, and 1 min, respectively, for 22 cycles. Oligonucleotide primers were chosen from homologous parts of the coding region of the human VEGF and GAPDH genes. The sense primer for human VEGF was 5′-CCATGGACTTCTTGTCTCCCTG-3′, and the antisense primer was 5′-TCCACCCTCTCCTGGTCTAC-3′. The primers for the human GAPDH were 5′-ATCACACATCTTCAGGACG-3′ for the sense primer and 5′-CTGCCCTCCACCCGTCTGTCC-3′ for the antisense primer. PCR was carried out according to DNA amplification reagent kit instructions. The PCR fragments were analyzed by 8% polyacrylamide gel electrophoresis and visualized by ethidium bromide staining. The gel was dried under vacuum, and incorporated radioactivity was counted in an Instant-Imager counter (Packard Instruments).

To determine the relative concentrations of VEGF mRNA in SMC from different treatment groups, a semiquantitative RT-PCR method was established (as previously described for rat SMC; see Ref. 31 and modification of Ref. 44). We first
determined the amount of RNA used to check whether the quantity of PCR products is proportional to the quantity of total RNA used in the RT-PCR analysis. We also checked whether the amount of PCR products increased linearly as a function of the number of cycles. In the exponential range of amplification, the quantity of PCR product derived from a given amount of total RNA (1 μg RNA and 2 μl of the RT solutions) is directly proportional to the number of cycles. Comparison between samples was made by including [33P]dATP in the PCR reaction mixtures and determining the incorporation of label by analysis on Instant-Imager.

Statistics. Data were analyzed from three independent experiments. Two RTs and two PCRs for each RT were carried out for each experiment, performed in triplicate, with reproducible results. The results are expressed as means ± SE. To test for the respective stimulations on VEGF expression, a two-way ANOVA was performed on the data obtained from the different groups. P < 0.05 was considered statistically significant.

Detection of Estrogen Receptors

Total RNA (1 μg) was reverse transcribed with the use of MMLV RT. Five percent of the RT product was used for PCR amplification, with primers chosen at 598–623 and 1392–1416 positions in human ER-α (18) and at 498–519 positions in human ER-β (30) cDNA. GAPDH was used as an internal control. Amplification was performed with the use of 100 μM dNTP, 50 pmol primers, and 0.25 U Taq polymerase in a 20-μl final volume. The parameters for amplification were as follows: 4 min at 94°C for initial denaturation, 40 cycles of 30 s at 94°C, 1 min at 57°C, 1 min at 72°C, and a 10-min final extension at 72°C.

Immunofluorescence and immunocytochemistry of E2 receptor(s) in SMC grown in Labtek chambers (Nunc) were processed as previously described (39) with the use of specific anti-ER-α (Immunotech, Marseilles, France) and anti-ER-β receptor antibodies (generous gift from P. Saunders; see Ref. 47).

Immunocytochemical Detection of VEGF

Immunocytochemical detection of VEGF in frozen-fixed sections from saphenous veins and in SMC was performed with the use of the VEGF antibody, as previously described (3). The immunocytochemical staining included incubation overnight with affinity-purified rabbit polyclonal antibody raised against the first 20 amino acids of human VEGF (1:250 dilution; Santa Cruz Biotechnology), followed by incubation with biotinylated anti-rabbit IgG and streptavidin-biotin peroxidase (Dakopatts, Denmark) or FITC-fluoresced streptavidin (Amersham). The following controls were performed: 1) preabsorption of anti-VEGF antibody with increasing amounts of purified recombinant VEGF (1.5–30 μg/μl diluted antibody) for 12 h at 4°C before immunostaining (3), 2) omission of the first antibody, and 3) incubation of tissue sections with irrelevant rabbit IgG.

Immunoblotting and Radioreceptor Assay of VEGF

Immunoblotting. Conditioned media were collected from E2-treated and untreated cultures, centrifuged, and electrophoresed to nitrocellulose filters. For immunoblotting of VEGF, affinity-purified rabbit anti-VEGF antibodies were used, followed by peroxidase-conjugated anti-rabbit IgG (Dakopatts) and the enhanced chemiluminescence system (Amersham).

Radioreceptor assay. VEGF bioactivity in culture medium was determined by use of a radioreceptor assay using bovine aortic endothelial cells as target cells and iodinated VEGF as a tracer (3, 43).

RESULTS

Expression of Estrogen Receptors in Vascular SMC

Figure 1A shows a phase-contrast microphotograph of HSVSMC in culture. Purity of the vascular SMC preparations was checked by the presence of immunofluorescence staining for desmin and vascular smooth α-actin (Fig. 1B) and by the absence of staining with anti-Von Willebrand factor antibodies (not shown). To investigate the role of E2 on VEGF synthesis in human vascular SMC, we first examined the presence of the estrogen receptors ER-α and/or ER-β in these cells with the use of RT-PCR and immunocytochemistry.

RT-PCR amplification of total RNA isolated from HSVSMC and HASMC, using primers covering exons 4–8 for ER-α and exons 1–5 for ER-β (see MATERIALS AND METHODS), allowed for the detection of specific products (Fig. 2); these PCR products had the expected size of 802 bp (ER-α) (36) and 392 bp (ER-β). ER-β was also shown to be present in HSVSMC isolated from veins of male and female subjects, independent of number of passages (between 3 and 6; not shown).

In addition to the presence of ER-α and ER-β mRNA, the proteins were detected by immunocytochemistry or immunofluorescence performed on HSVSMC (Fig. 1, C and D) with the use of specific antibodies. No immuno-
staining was seen when the primary antibody was replaced by nonimmune rabbit IgG (not shown).

Expression and Induction by E2 of VEGF mRNA in Human Venous SMC

Northern blot analysis. To determine whether cultured HSVSMC express the VEGF gene, Northern blot analysis of total HSVSMC cellular RNA was performed with the use of human cDNA as a probe that recognizes all the VEGF isoforms. As shown in Fig. 3, a major transcript of 3.7 kb and a minor transcript of 4.2 kb were detected. These transcripts very likely correspond to the transcripts for VEGF121 and VEGF165 isoforms. We examined whether VEGF transcript levels were modulated by E2 in HSVSMC. Preliminary experiments indicated that SMC cultured for 24 h in 5% stripped serum express low basal levels of VEGF mRNA (Fig. 3A); this level did not change with time in untreated cells (not shown). The addition of E2 at 10^{-8} M for 24 and 48 h significantly increased VEGF mRNA levels over the control value (Fig. 3A).

RT-PCR analysis. To identify the molecular species of VEGF produced in SMC from saphenous veins, we analyzed RNA from the cells, using RT-PCR and oligonucleotides derived from external exons shared by all differentially spliced VEGF mRNA species. The major amplified species detected were 580- and 450-bp fragments (Fig. 3B). These species were similar to that described in uterine cells (not shown) and probably correspond to the mRNA encoding VEGF165 and VEGF121. A similar pattern of VEGF products was seen in controls and E2-treated cells after 28 cycles of amplification (Fig. 3B).

To compare VEGF transcripts under different hormonal conditions, we developed a semiquantitative RT-PCR assay (see MATERIALS AND METHODS). As shown in Fig. 3C, in the exponential range of amplification, the amount of PCR product derived from a given amount of total RNA in a sample is directly proportional to the number of cycles. Semiquantitative RT-PCR indicated that HSVSMC incubated with E2 produced more VEGF mRNA than did control cells.

Fig. 3. Expression of vascular endothelial growth factor (VEGF) mRNA in HSVSMC. A: Northern blotting of RNA from HSVSMC treated with estradiol (E2; 10^{-8} M) for 24 or 48 h (lanes 2 and 3, respectively) or from untreated cells (lane 1) that received vehicle (DMEM); 20 μg of total RNA were separated on a 1% denaturing agarose-formaldehyde gel, transferred to nitrocellulose filters, and probed with a 32P-labeled human VEGF probe or with a glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probe (as described in MATERIALS AND METHODS). Analysis of the incorporated radioactivity shows an 2.4-fold increase in VEGF mRNA concentration after 48 h of 10^{-8} M E2 compared with that in the absence of treatment. B: an example of RT-PCR analysis of VEGF in HSVSMC. Total RNA was extracted from the cells at the end of each incubation; 1 μg of total RNA was reverse transcribed, and an aliquot of the RT solution was amplified with the use of specific oligonucleotides for VEGF. After electrophoresis, quantification of amplified fragments was determined by counting the incorporated radioactivity with the use of instant-Imager analysis. A typical ethidium bromide gel (top) and an autoradiogram (bottom) of VEGF RT-PCR products from control (lane 1) and E2-treated (lane 2) HSVSMC are shown. The two major products correspond in size to the products expected for the 165-amino acid isoform of VEGF (VEGF165; upper bright band) and the 121-amino acid isoform of VEGF (VEGF121; lower bright band). C: RT-PCR analysis (80°C annealing temperature) of VEGF and GAPDH transcripts in HSVSMC as a function of cycle numbers. One microgram of total RNA was reverse transcribed, and 2 μl of RT were amplified (28 cycles) with the use of specific oligonucleotides for VEGF and GAPDH. After electrophoresis, quantification of amplified fragments was determined by counting the incorporated radioactivity using Instant-Imager analysis. An autoradiogram of a polyacrylamide gel on which the products of RT-PCR assay have been separated (top) and its quantification data with an Instant-Imager (bottom) are shown. GAPDH PCR product is indicated as an internal control. cpm, Counts/min.
Maximal response occurred at $10^{-9}$ M (2.3-fold in 6 h of treatment, $P < 0.01$), and no further increase was seen at higher doses (Fig. 4A). This response to E2 occurred in a time-dependent manner (Fig. 4B); the level of VEGF increased within 2 h and reached a maximum by 24–48 h (a 3-fold increase; Fig. 4B). Taken together, these data demonstrate by two different approaches that E2, at physiological concentrations, induces the expression of VEGF mRNA in HSVSMC.

**Effect of OH-Tam and Tamoxifen on VEGF Expression in HSVSMC**

We next sought to determine whether the drug tamoxifen, which is an antiestrogen in the mammary gland (23) but produces some estrogenic actions in the uterus (19), would also alter VEGF transcript levels in the vascular wall. We used tamoxifen and an active metabolite, OH-Tam. As seen in Fig. 4C, OH-Tam treatment of HSVSMC produced a large increase in VEGF mRNA levels over control values. The magnitude of the increase was similar after treatment with E2 at $10^{-9}$ to $10^{-8}$ M (~3- to 3.5-fold increase after 24 h of incubation), but it was slightly smaller for OH-Tam at $10^{-7}$ M. An increase in VEGF mRNA was also observed in cells treated with tamoxifen, although a higher dose ($10^{-8}$ M) was necessary for the optimal increase (not shown). Thus OH-Tam, tamoxifen, and E2 clearly increased VEGF transcript levels in SMC. In addition, stimulation of cells by E2 and OH-Tam ($10^{-9}$ M; Fig. 4C) or by E2 and tamoxifen ($10^{-8}$ M; not shown) increased VEGF transcripts over the value obtained with OH-Tam or tamoxifen alone, respectively, at the same concentrations.

**Fig. 4. E2 upregulation of VEGF mRNA in SMC.**

A: dose-response stimulation of VEGF transcripts in HSVSMC. Cells were incubated for 6 h with increased concentrations of E2 (0.1–10 nM). RNA was extracted and amplified using RT-PCR (see Fig. 3). Two RT and two PCR per RT were performed in each sample. The gels, representative of 3 samples per treatment cells, were scanned with an Instant-Imager, and the level of RT-PCR products for VEGF in each sample was expressed in relation to the level of GAPDH RT-PCR products. Results are means ± SE of 3 independent experiments. *Significant difference between E2-treated and control cells ($P < 0.05$).

B: time-course stimulation of VEGF expression by E2 in HSVSMC. Cells were treated with E2 ($10^{-8}$ M) for 2–48 h, and total RNA was extracted from the cells at the indicated times. VEGF PCR products were analyzed as described in Fig. 3. C: stimulation of VEGF mRNA levels by E2 and the agonist/antagonist 4-hydroxytamoxifen (OH-Tam). HSVSMC were incubated for 24 h with E2 ($10^{-8}$ M), OH-Tam ($10^{-9}$ to $10^{-5}$ M), both E2 ($10^{-8}$ M) and OH-Tam ($10^{-9}$ M), or vehicle alone; the increase of VEGF mRNA in cells treated with OH-Tam was also observed at 6 h. In another experiment, an increase of VEGF mRNA was observed in cells treated with tamoxifen ($10^{-8}$ M; not shown).

*Significant differences are observed between VEGF mRNA levels in HSVSMC treated by OH-Tam, E2, or E2 + OH-Tam and control cells between OH-Tam-treated and control cells ($P < 0.05$).

D: time-course stimulation of VEGF expression by E2 in SMC from human aorta (HASMC). Cells were incubated with E2 ($10^{-8}$ M) for 2, 6, and 24 h, and total RNA was extracted at the indicated times. This was repeated twice.
Induction by E2 of VEGF mRNA in HASMC

To determine whether the E2-induced increase in VEGF mRNA levels in SMC is specific for saphenous vein, we also performed semiquantitative RT-PCR analysis of VEGF transcripts in HASMC stimulated with E2 for 2–24 h. As shown in Fig. 4D, the level of VEGF mRNA increased in HASMC under 24 h of E2 treatment.

VEGF Protein is Secreted by Isolated HSVSMC

We further examined whether this VEGF transcript was indeed translated into protein. As shown in Fig. 5, normal human saphenous veins were immunoreactive for VEGF. Staining was found in situ in SMC from the media and in the vasa vasorum. Preincubation of the anti-VEGF antibody with recombinant human VEGF significantly reduced the intensity of staining, as previously described in human endometrium (3). HSVSMC were also able to synthesize VEGF, as shown by the presence of immunofluorescence (Fig. 6A). No immunostaining was seen in HSVSMC with the use of nonimmune rabbit IgG instead of the primary antibody (not shown).
To examine whether VEGF was secreted by HSVSMC, the conditioned medium from cultured HSVSMC was concentrated and analyzed by use of both radioreceptor assay and Western blot analyses. Analysis by radioreceptor assay revealed the presence of secreted VEGF; its concentration increased from 0.8 to 1.2 and 2.9 ng/ml for 10^6 cells after the addition of E2 for 48 h and 4 days, respectively. Analysis of the conditioned medium from these cells using Western blotting and anti-VEGF antibodies revealed the presence of bands corresponding to molecular masses of 18, 22–24, and 28 kDa in denaturing conditions (Fig. 6, B and C); these bands correspond to the VEGF_{121}, VEGF_{165}, and VEGF_{189} isoforms, respectively.

Induction by Hypoxia of VEGF mRNA in Human HSVSMC

Hypoxia has been suggested to be a key regulatory factor in the physiopathology of vessels; it has also been previously shown that hypoxia is a strong stimulus of VEGF induction in a variety of cells. To determine whether VEGF is also hypoxia inducible in vascular SMC from saphenous vein, HSVSMC were grown under normoxic (21% O_2) and hypoxic (1% O_2) conditions, and levels of VEGF transcripts were subsequently measured by semiquantitative RT-PCR analysis. As shown in Fig. 7, steady-state levels of VEGF transcripts were only slightly increased within 24 h of growth under hypoxic conditions in the presence of steroids (unstripped serum). No evidence of cell death could be detected in cultures exposed to hypoxia. However, steady-state levels of VEGF transcripts were significantly increased within 6–24 h of growth under low O_2 tension in the absence of steroids (stripped serum) (2.9- and 1.6-fold induction, respectively; Fig. 7).

DISCUSSION

To our knowledge, no previous study has investigated the presence, modulation, and potential significance of VEGF in human veins. The present study demonstrates the expression of VEGF in human saphenous veins in vivo and in HSVSMC. VEGF has been demonstrated to be upregulated by 17β-E2, OH-Tam, and hypoxia in vitro by use of various approaches (immunocytochemistry, Northern blot analysis, and RT-PCR). Additional evidence that the protein is indeed secreted by vascular cells is provided by Western blotting and radioreceptor assay of proteins present in the conditioned medium from cultured HSVSMC. These findings indicate that, in addition to HASMC (Refs. 6, 16, and 27 and this study), venous SMC also express this endothelial growth factor. E2 is thus an additional stimulus for the modulation of vascular VEGF, which may constitute a local source for angiogenesis or may act as a permeability factor for endothelial cells.

Regulation of VEGF Expression in SMC by E2

E2 treatment of human vascular SMC from saphenous veins induces VEGF expression in a time- and dose-dependent manner. The results from Northern blotting in HSVSMC match those of quantitative measurement of the RT-PCR products. RT-PCR analysis detects three mRNA species corresponding to VEGF_{121}, VEGF_{165}, and VEGF_{189}, with VEGF_{121} and VEGF_{165} being predominant. These data extend previous results showing that E2, at physiological doses, increases VEGF mRNA levels in uterine target cells and in macrophages (3, 49). The threefold E2 increase in VEGF mRNA from HSVSMC is obtained at physiological concentrations after 24 h of incubation and is of the same magnitude as that described in uterine stromal cells on E2 stimulation (3, 49). These results were obtained on HSVSMC isolated from saphenous veins from patients undergoing arterial bypass surgery; the patients were old, but the samples themselves were not pathological. Also, stimulation of VEGF expression is observed after E2 treatment of SMC from aorta, and this result confirms previous data on rat carotid artery (25). Thus E2, in addition to hypoxia (7, 42, 51), various cytokines, and growth factors (6, 27, 40, 44, 53), regulates the expression of VEGF in vascular SMC.

Mechanism of E2-Induced VEGF Expression

Our observations suggest that E2 acts directly on SMC, increasing VEGF expression through an E2 receptor(s). This conclusion is supported by the demonstration of the expression of ER-α and ER-β in venous SMC; the expression of ER-α (4, 24, 32, 37, 38) and possibly ER-β (20), which is expressed at high levels in the ovary and prostate (26), was previously shown in the arterial system in males and females. Whether the induction of VEGF in SMC is mediated by ER-α or
ER-β remains unknown. The presence of ER-β in the media (SMC) of veins, both in vivo (unpublished observations) and in vitro, and the absence or expression at a low level of the classical estrogen receptor ER-α (36) in saphenous veins in situ both suggest that the E2-induced VEGF expression in human veins could be mediated by the ER-β receptor. However, the mechanisms by which E2 influences VEGF expression in SMC is not clear. This may be due to a transient increase in VEGF mRNA levels, consecutive to transcriptional induction, as previously suggested in uterine cells (3) and described for E2-regulated genes (5, 13, 33). Other mechanisms of E2-induced VEGF could involve modification of the stability of VEGF mRNA or effects at the posttranscriptional level.

Physiological Expression of VEGF in Veins

Our data indicating that E2 induces VEGF expression in human venous SMC are of interest because the physiological role of E2 in veins is still unclear (36). E2 has been shown to induce the proliferation of venous endothelial cells (29) and to inhibit the proliferation of SMC (11). The expression of VEGF and ER-β in saphenous veins in situ suggests that E2 could also mediate VEGF expression in vivo. The presence of VEGF receptors, Flt-1 and Flk-1/KDR, on venous endothelial cells in vivo and in vitro (unpublished data) also agrees with a paracrine action of VEGF on endothelial cells located in close proximity to SMC in the venous wall. Studies conducted on uterine vessels have confirmed that E2 induces VEGF expression in vivo and in vitro (3, 8, 49) and modifies angiogenesis and capillary permeability. Angiogenesis and hyperpermeability associated with endometrial growth have been shown to be related to E2-induced expression of VEGF in the uterus (3, 10, 49). VEGF has been shown to increase vascular permeability of postcapillary venules by inducing fenestration of the endothelium (46). These effects of VEGF could be mediated, at least partly, by the production of nitric oxide (NO) by endothelial cells (28, 55, 58). The E2-induced VEGF increase in saphenous venous SMC described in this study could be related to an increase in vascular permeability to plasma proteins (12). The expression of VEGF within vasa vasorum of the vein, in addition to that of SMC of the vascular wall, may also be an important phenomenon for maintaining the permeability of nutritive capillaries and preventing the thickening of the wall of vasa vasorum (56).

Whether the physiological role of E2 is similar in veins and arteries remains unknown. Our results in HASMC stimulated by E2 support in vivo studies showing that E2 accelerates functional endothelial recovery after arterial injury (25) via an increase in NO and VEGF expression. In the arterial wall, VEGF produced by SMC has also been suggested to constitute, in synergy with bFGF, a local source of angiogenic factors. Whether VEGF could participate in the protective effect(s) of E2 against the development of cardiovascular disease (14), in addition to vasorelaxation (55), preservation of the endothelium (52), inhibition of SMC proliferation, and antiatherogenic effects (1), is still unknown.

Induction of VEGF by OH-Tam and Tamoxifen

Our data show that OH-Tam and tamoxifen (a compound with mixed agonist/antagonist properties) increase VEGF expression in venous SMC and exert estrogen-like effects in the vascular system. The agonist activity of tamoxifen is useful in the treatment of breast cancer (23), but its effects on the vascular wall are not fully established. Previous studies on the agonist effects of tamoxifen on the arterial wall have described the inhibition of the progression of coronary arterial atherosclerosis in a primate model (57), the inhibition of fatty acid lesions in the normal and apolipoprotein E-deficient mice (45), and the inhibition of the proliferation of SMC in vitro (17). Our data showing the increase in VEGF expression in SMC treated with tamoxifen, OH-Tam, or E2, similar to the increase in VEGF by these compounds in uterus or in breast cancer cells (19), suggest that tamoxifen could be a positive modulator of angiogenesis/vascular permeability mediated by VEGF, at least in veins. Because tamoxifen has recently been shown to counteract the 17β-E2 modulation of other activities in vessels (e.g., NO synthase), further studies will be necessary to analyze the cell specificity and the modulation of gene expression by tamoxifen in the vascular system in the vascular system.

Hypoxia-Induced VEGF in Saphenous Veins

Hypoxia has been shown to increase vasodilation in the saphenous vein (34); however, few studies have investigated its effects on veins compared with arteries. Upregulation of VEGF by hypoxia in human saphenous vein extends previous data obtained both in vitro in arterial cells and in vivo (2, 7). Hypoxia is generally considered to represent a fundamental stimulus for angiogenesis through VEGF production in different tissues and cells (for review, see Ref. 15) and to interfere in pathological conditions such as those found in tumors (42, 51) and diabetic retinopathy (41). This is achieved by the transactivation of the hypoxia-inducible factor I, which binds to the promoter of the VEGF gene. The increase in VEGF observed in saphenous venous SMC under hypoxia in the absence of steroids is higher than that observed under hypoxia in the presence of steroids. These preliminary results are consistent with the clinical observations of venous edema and modifications of permeability observed during the premenstrual period (hormonal deprivation) in women. They suggest that ovarian steroids could protect vascular cells from the effect of hypoxia.

In conclusion, our studies demonstrate the expression of VEGF in human saphenous veins in vivo and in cultured HSvSMC and HASMC and suggest a role(s) of this growth factor in the regulation of endothelial cell function via a paracrine mechanism. Whether VEGF expression varies under different physiological conditions (i.e., pregnancy vs. normal menstrual cycle)
or under pathological conditions (i.e., in varicose veins compared with normal saphenous veins) is unknown. VEGF released by venous SMC and its possible increase by E2, in association with other unknown factors, could contribute to the sex hormone (E2 and progesterone) dependency of varicose vein pathology demonstrated by clinical and epidemiological observations (36). Further studies are required to understand the function of this growth factor in the vascular wall in vivo and the physiopathological relevance of its regulation by E2.

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