Regulation of the vascular endothelial growth factor (VEGF) receptor Flk-1/KDR by estradiol through VEGF in uterus

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Abstract

The induction of vascular endothelial growth factor (VEGF) expression by 17β-estradiol (E_2) in many target cells, including epithelial cells, fibroblasts and smooth muscle cells, suggests a role for this hormone in the modulation of angiogenesis and vascular permeability. We have already described a cyclic increase in Flk-1/KDR-expressing capillaries in the human endometrium during the proliferative and mid-secretory phases, strongly suggestive of an E_2 effect on Flk-1/KDR expression in the endometrial capillaries. However, it is unclear whether these processes are due to a direct effect of E_2 on endothelial cells. Using immunohistochemistry, we report an increase in Flk-1/KDR expression in endometrial capillaries of ovariectomized mice treated with E_2, or both E_2 and progesterone. This process is mediated through estrogen receptor (ER) activation. *In vitro* experiments using quantitative RT-PCR analysis demonstrate that Flk-1/KDR expression was not regulated by E_2 in human endothelial cells from the microcirculation (HMEC-1) or macrocirculation (HUVEC), even in endothelial cells overexpressing ERα or ERβ after ER-mediated adenovirus infection. In contrast, Flk-1/KDR expression was up-regulated by VEGF itself, in a time- and dose-dependent manner, with the maximal response at 10 ng/ml. Thus, we suggest that E_2 up-regulates Flk-1/KDR expression *in vivo* in endothelial cells mainly through the modulation of VEGF by a paracrine mechanism. It is currently unknown whether or not the endothelial origin might account for differences in the E_2-modulation of VEGF receptor expression, particularly in relation to the vascular bed of sex steroid-responsive tissues.
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Introduction

Hormonally controlled angiogenesis is fundamental for endometrial development and the differentiation necessary for implantation, as well as for the uterine changes associated with pregnancy (Giudice 1996, Perrot-Applanat 2000). Physiological angiogenesis is also required to support endometrial regeneration after shedding of the uterine surface in the absence of implantation and to support the proliferation of the human endometrium during the menstrual cycle under the control of estradiol (E_2) and progesterone. Models of endometrial angiogenesis in the proliferative phase describe the growth of vasculature under the influence of estrogen, while the secretory phase involves growth of the coiled arteries mediated by progesterone. Previous studies have demonstrated that E_2 increases the secretion of vascular endothelial growth factor (VEGF) in uterine cells (Cullivan-Bove & Koos 1993, Shifren *et al.* 1996, Bausero *et al.* 1998); this factor has emerged as one of the central regulators of the uterine vasculature (see review by Perrot-Applanat 2000).

In vivo, VEGF induces a potent angiogenic response in a variety of models and acts as a vascular permeability factor based on its ability to induce vascular leakage (see reviews by Ferrara & Davis-Smyth 1997, Ferrara *et al.* 2003). VEGF is also a survival factor for endothelial cells, preventing apoptosis induced by serum starvation. Molecular cloning of the human VEGF gene has revealed that differential exon splicing generates multiple tissue- and function-specific variants containing 121, 165, 189, and 206 amino acids (see review by Ferrara & Davis-Smyth 1997). In most systems, VEGF121 and VEGF165 are the major species expressed, while VEGF189 is only minimally present and VEGF206 is limited to embryonic tissue. In the human endometrium, E_2 up-regulates all VEGF isoforms (Shifren *et al.* 1996, Bausero *et al.* 1998), while progesterone selectively increases the expression of the VEGF189 isoform (Ancelin *et al.* 2002). The role of these isoforms is still debated. Also, little is known about

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the effect of E_2 on the endothelial cells and on VEGF signaling.

The angiogenic effects of VEGF are believed to be mediated by two tyrosine kinase receptors, Flt-1 (Fms-like tyrosine kinase-1 or VEGFR-1) and Flk-1/KDR (fetal liver kinase/kinase-insert domain receptor or VEGFR-2). These receptors initiate different signaling cascades in endothelial cells (Gille *et al.* 2001). Flk-1/KDR is now considered to be the main receptor involved in endothelial cell proliferation, migration and survival (Millauer *et al.* 1993, Ferrara *et al.* 2003). In contrast, Flt-1 has a decoy effect on VEGF signaling, possibly with variations related to the vascular bed type (Ferrara *et al.* 2003). Factors regulating Flk-1/KDR expression *in vivo* in endothelial cells are not clearly defined. In tumors, Flk-1/KDR appears to be regulated by hypoxia, an effect probably mediated by VEGF (Kremer *et al.* 1997). VEGF and its receptor, Flk-1/KDR, which are expressed in most tissues during embryonic development, are down-regulated in the adult in physiological conditions, except in the reproductive tract (Perrot-Applanat 2000, Ferrara *et al.* 2003). In the human endometrium, Flk-1/KDR expression in endometrial blood vessels seems to exhibit menstrual cycle-dependent changes, with higher expression in the proliferative and the early or early-mid secretory phases, suggesting that ovarian hormones influence the expression of this receptor in the uterus (Meduri *et al.* 2000). *In vitro* studies have shown that Flk-1/KDR expression is regulated by several growth factors (Shen *et al.* 1998, Ferrara *et al.* 2003) and by shear stress (Abumiya *et al.* 2002). Treatment of endothelial cells with E_2 directly increases the proliferation and survival of these cells (Morales *et al.* 1995) through inhibition of apoptosis (Spyridopoulos *et al.* 1997). However, although E_2 increases VEGF expression in the endometrium, the relationship between E_2 and Flk-1/KDR expression involved in endothelial cell proliferation is poorly understood.

To better ascertain the role of E_2 in Flk-1/KDR expression, we have examined the role of E_2 and the mechanisms controlling Flk-1/KDR expression in a mouse model of angiogenesis. We have also analyzed the expression of the receptor in endothelial cells treated with E_2 and VEGF.

Materials and Methods

Materials

Reagents for cell culture and PCR were from Gibco (Life Technologies, Cergy-Pontoise, France). Estradiol was purchased from Sigma (Sigma-Aldrich GmbH, Steinheim, Germany). TriZOL isolation kit, Moloney murine leukemia virus (MMLV) reverse transcriptase, and Taq polymerase were from Life Technologies (Cergy-Pontoise, France). Recombinant VEGF was provided by R&D Systems (Minneapolis, MN, USA).

Mice uteri

Mice were housed in the animal care facility at the National Institute of Environmental Health Sciences (NIEHS), Research Triangle Park, NC, USA. They were treated in accordance with NIH guidelines for the humane use of animals in research. The generation and characterization of the estrogen receptor α knockout (αERKO) mice have previously been reported (Couse *et al.* 1995). Adult wild-type (WT) or αERKO mice were ovariectomized and rested for two weeks to clear endogenous hormones before any treatment. Mice (4 animals/group) were implanted with a pellet of E_2 (200 ng), progesterone (35 mg), E_2 and progesterone or placebo over 21 days. Animals were killed by cervical dislocation and the uterus was removed, fixed in 4% paraformaldehyde and embedded in paraffin.

Uteri were also dissected out of C57BL6J/129 Svj mice (8- to 12-weeks-old, random cyclic females), fixed in Bouins and embedded in paraffin as previously described (Kurita *et al.* 2001).

Immunocytochemistry

Immunological detection of VEGF receptor Flk-1/KDR was performed using the polyclonal rabbit antibody CT128 directed against Flk-1/KDR (1:400 dilution; Millauer *et al.* 1993), as previously described (Meduri *et al.* 2000). This antibody has previously been characterized and does not cross-react with other protein kinase receptors. Immunocytochemical staining included overnight incubation at 4 °C with the primary antibody, followed by incubation with biotinylated anti-rabbit IgG and streptavidin-biotin peroxidase (LSAB2 immunostaining kit Dakopatts, Glostrup, Denmark). Controls included omission of the first antibody and incubation of tissue sections with irrelevant rabbit IgG immunoglobulins. Adjacent sections were incubated with a marker of vascular endothelial cells, the polyclonal anti-Von Willebrand factor (vWF) antibody (Dako, Glostrup, Denmark) (Meduri *et al.* 2000).

The number of Flk-1/KDR-stained capillaries in each section was determined after identification of the areas containing the highest number of stained capillaries at low power magnification, as previously described (Meduri *et al.* 2000). Counts of individual immunostained capillaries were performed at higher magnification (×16 objective, 0·322 mm^2 per field), using a stereomicroscope (Leitz, Orthoplan) equipped with a CDD video camera. Five different fields in each section were digitized by image analysis and computerized using the Histolab program (Microvision, Evry, France). Capillary quantification was assessed blindly. The total number of capillaries in each biopsy was previously assessed by vessel counts in serial sections stained by anti-Von Willebrand factor using the same program. Values were expressed as means ± S.E.M.

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Immunohistochemical detection of estrogen receptor (ER) β was performed on mouse uteri using an anti-ERβ sheep polyclonal antibody, as previously described (Kurita *et al.* 2001, Saunders *et al.* 2001).

Endothelial cell isolation and stimulation

Endothelial cells (human umbilical vein endothelial cells; HUVEC) were isolated from fresh human umbilical cords using digestion with Collagenase I according to the method of Jaffe *et al.* (1973). Cells were plated in 0·2% gelatin-coated flasks. They were grown in Medium 199 supplemented with 20% fetal calf serum (FCS) and 2 mM glutamine, 100 μg/ml penicillin/streptomycin, 15 mM HEPES and sodium bicarbonate. Cells were cultured in 5% CO_2 at 37 °C and the media were replaced at 2-day intervals. Immunostaining with Von Willebrand factor antibody (Dako) confirmed their endothelial origin. The presence of Flk-1/KDR was detected by immunostaining of paraformaldehyde-fixed cells with CT128 antibodies, as described above. Second passaged cells were used for experiments. Prior to steroid stimulation, endothelial cells were cultured overnight in a 6-cm Petri dish until confluence in M199 containing 1% FCS in the absence of steroid (charcoal-stripped serum) and phenol red. For stimulation, the medium was replaced with the same phenol red-free medium containing 5% serum in the presence of E_2 (10^{-10} to 10^{-7} M) for 3–72 h. Control cells were incubated in phenol red-free medium without hormone.

Cell culture and infection

Infectious viral particles adr5 (backbone virus), ad-hERα and ad-hERβ were generated by *in vivo* recombination of pACsk12 CMV5-hER plasmid with pJM17 in HEK-293 cells, as previously described (Lazennec *et al.* 2001; Viraquest Inc., North Liberty, IA, USA). Titered virus stock was used to infect HUVEC and HMEC-1 (human microvascular endothelial cells from dermis) using a previously described protocol (Lazennec *et al.* 2001). Briefly, 10^6 HUVEC or HMEC-1 were seeded in a 60-mm diameter petri dish in 10% SVF M199 or MCDB 131 respectively. Cells were infected by an adenovirus at a concentration of 100 or 200 pock formit unit/cell for HUVEC and HMEC respectively at 37 °C in 5% CO_2. Cells were rinsed in PBS and 10% stripped SVF-containing medium was added. Cells were treated with various concentrations of E_2 for 7 or 24 h at 37 °C.

RNA extraction and RT-PCR analysis

Total RNA was isolated from treated or stimulated confluent cultures of human endothelial cells with TriZOL according to the manufacturer's instructions. For the reverse transcription stage, single-stranded cDNA was synthesized from 1 μg total RNA in the MMLV reverse transcriptase and hexamers primers. The presence of mRNA encoding the VEGF receptors and estrogen receptors (ER) in endothelial cells was determined using reverse transcriptase-polymerase chain reaction (RT-PCR) and specific oligonucleotide primers.

Detection of estradiol receptors PCR amplification was performed using 5% RT product and primers chosen at positions 598–623 and 1392–1416 in human ERα cDNA and positions 124–146 and 498–519 in human ERβ cDNA (Bausero *et al.* 2000). The PCR products are 395 and 818 bp for ERβ and ERα respectively. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control (450 bp). Amplification was performed using standard Gibco-BRL PCR buffer with 100 nM dNTP, 50 pmoles primers, 1·5 mM $MgCl_2$ and 1·2 U Taq polymerase, in a 20 μl final volume. The parameters for amplification were: 4 min at 94 °C, 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. Products were separated and visualized in ethidium bromide-stained agarose gels. Quantitative RT-PCR (qPCR) of ERα and ERβ was also performed using Taqman apparatus, according to a method previously described (Bieche *et al.* 2001).

Quantification of Flk-1/KDR expression by real time RT-PCR All PCRs for the detection of Flk-1/KDR were performed using the real-time fluorescence detection method with the LightCycler System and a FirstStart DNA Master SYBR Green I kit (Roche Diagnostic, Meylan, France). The primer sequences for Flk-1/KDR were as follows: forward, 5′ TCTCAATGT GGTCAACCTTCTAG; reverse, 5′ TTTAAACGTCT TAAGGGTGTTAGTG. To avoid amplification of contaminating genomic DNA, two primers were chosen in two different exons. The cycling conditions were as follows: initial denaturation at 95 °C for 10 min, followed by 40 amplification cycles at 95 °C for 15 s, 55 °C for 5 s, and 72 °C for 20 s. A negative control without the cDNA template was performed to assess the overall specificity. Expression levels were normalized to β2-microglobulin, which was unaffected in the different treatment groups. Results are expressed as the mean of 5 independent experiments assayed in duplicate.

Statistical analysis

Student's *t*-test was used to determine the significance between treated and untreated cells, and $P<0·05$ was considered significant.

Results

Estradiol induces Flk-1/KDR expression in vivo

We have previously described the presence and modulation of VEGF and its Flk-1/KDR receptor in the human

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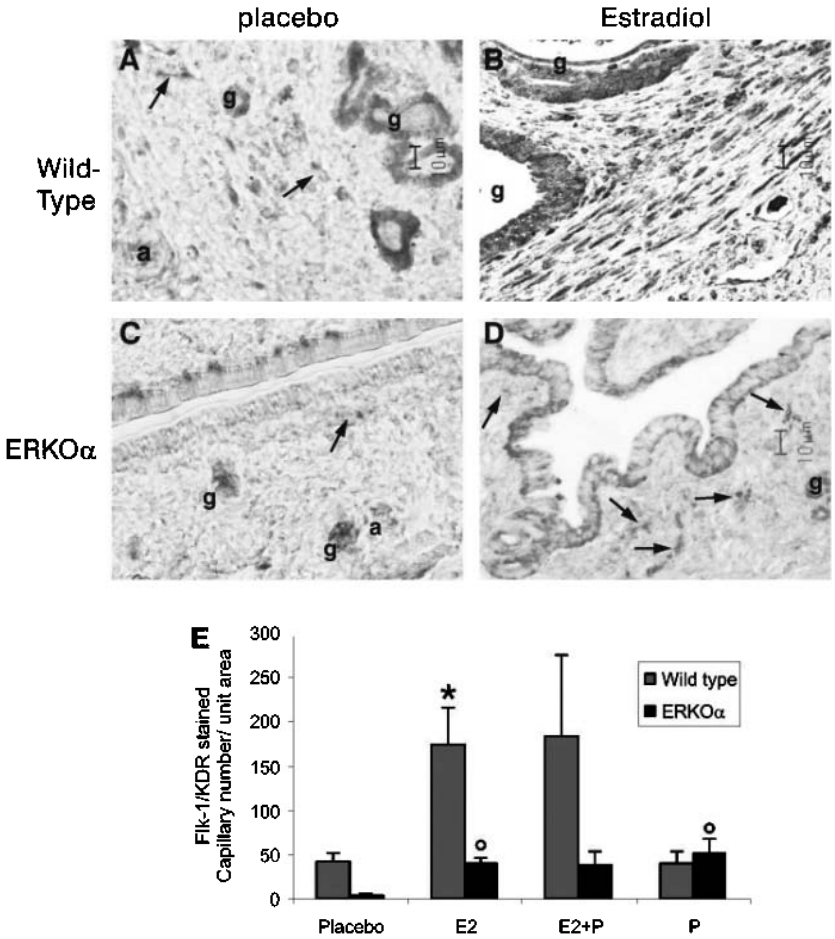

Figure 1 Endothelial Flk-1/KDR expression in mice uterus: immunocytochemical detection. Endometrial sections of ovariectomized (A, B) or αERKO (C, D) mice were immunostained with anti-Flk-1/KDR antibodies. (A) Untreated wild-type mice; (B) E_2-treated wild-type mice; (C) untreated αERKO mice; (D) E_2-treated αERKO mice. Note the immunolabeling in capillaries (arrows) and arterioles (a). The glandular epithelium (g) was also labeled, as previously described in human endometrium (Meduri *et al.* 2000). Note also the tortuous glandular lining in αERKO mice in the presence of estradiol (Fig. 1D). Additionally, numerous elongated cells (20–30%) were stained for Flk-1/KDR in the endometrial stroma of E_2-treated animals as compared with control animals. Original magnification, ×400. (E) Quantitative analysis of the number of capillaries expressing Flk-1/KDR in mouse uteri. Ovariectomized wild-type or αERKO mice were treated or not with E_2 (E2), progesterone (P) or E_2+progesterone (E2+P) over 21 days, as described above. The number of Flk-1/KDR-stained capillaries ± S.E.M. is shown. *P<0·05 placebo versus E_2 in wild-type mice; °P<0·05 placebo versus E_2 or P in αERKO mice. It is interesting to note that progesterone alone also increased Flk-1/KDR in αERKO mice, in a similar manner to E_2.

cyclic endometrium (Meduri *et al.* 2000). The expression of this receptor, deduced from the semi-quantitative analysis of capillaries immunostained for Flk-1/KDR, was maximal in the proliferative and in the mid-secretory periods. This observation suggests a possible effect of E_2 on the expression of Flk-1/KDR in the endometrium.

In order to assess the possible effect of E_2 on Flk-1/KDR expression *in vivo*, we have chosen the mouse uterus model. Ovariectomized mice were treated with E_2 or vehicle, as described in Materials and Methods, and uterine sections were immunostained for Flk-1/KDR (Fig. 1A-D). Quantification of VEGF receptor was examined using a protocol previously described by Meduri *et al.* (2000). Morphometric analysis showed a significant increase in the number of Flk-1/KDR-stained vascular structures expressed per unit area in the uteri of E_2-treated (4·1-fold induction, P<0·05) or E_2+progesterone-treated (4·3-fold induction, P<0·05) mice as compared with

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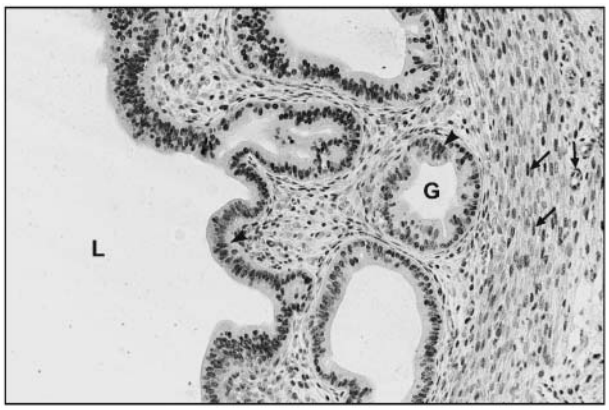

Figure 2 Expression of ERβ in mouse uterus. Uterine sections were immunostained with anti-ERβ antibodies (Saunders *et al.* 2001). ERβ-positive cells are seen in epithelial (arrowheads) and endothelial (open arrow) cells, as well as in some stromal cells (arrows). G, gland; L, lumen. Original magnification, × 20. Insert, high magnification of an immunostained vessel (open arrow).

controls. In contrast, there was no change in the expression of Flk-1/KDR in wild-type progesterone-treated mice (Fig. 1E).

In order to analyze the mechanism of E_2 up-regulation of Flk-1/KDR in mice uteri, similar experiments were performed in E_2-treated mice with a disruption of the ERα gene (αERKO mice) (Fig. 1C,D). As shown in Fig. 1E, the number of uterine Flk-1/KDR expressing vascular structures was significantly lower in E_2- or E_2+progesterone-treated αERKO mice as compared with E_2-treated wild-type mice ($P<0{\cdot}05$), suggesting that ERα could be involved in this regulation process. However, the number of Flk-1/KDR stained vascular structures was also lower in untreated αERKO mice versus untreated wild-type mice ($P<0{\cdot}05$) (Fig. 1E). Also, E_2 or E_2+ progesterone significantly increased Flk-1/KDR expression in αERKO mice ($P<0{\cdot}06$), suggesting that ERβ could also be involved in this regulation. As shown in Fig. 2, endothelial cells from mouse uteri do express ERβ, similar to the situation described in human uteri (Lecce *et al.* 2001). Altogether, our *in vivo* data show that E_2 increases Flk-1/KDR expression via estrogen receptor (α or β) activation. Comparative experiments using immunostaining with anti-vWF (a marker of endothelial cells) indicate that E_2-treatment of mice results in an increase in the total number of blood vessels, but not in an increase in the number of blood vessels expressed per unit area (not shown). Therefore, the increase in Flk-1/KDR-positive blood vessels of E_2-treated mice does not result from an overall increase in blood vessels.

The E_2-induced Flk-1/KDR expression observed *in vivo* could result either from a direct effect of E_2 on endothelial cells, or from an indirect effect through the secretion of VEGF by surrounding cells.

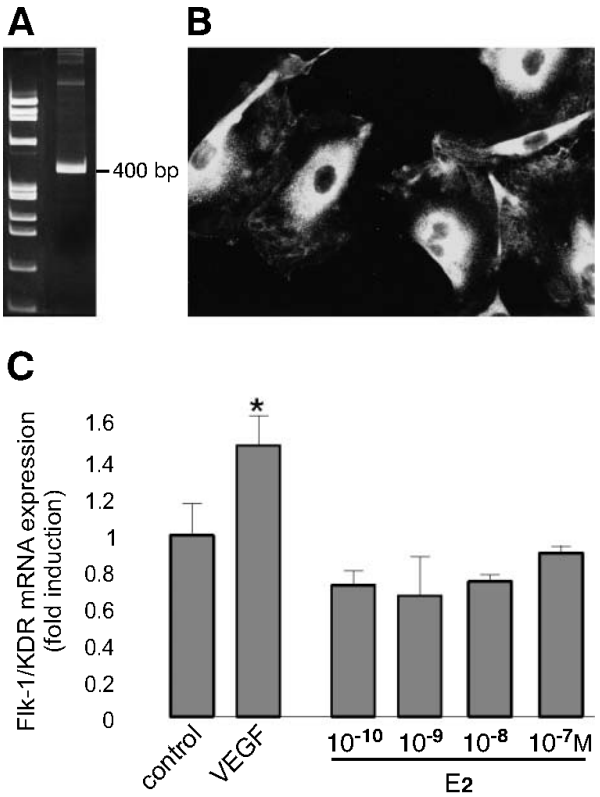

Figure 3 VEGF, but not E_2, up-regulates Flk-1/KDR expression. (A, B) HUVEC (passage 2) express Flk-1/KDR receptors, as shown by RT-PCR analysis (A) and immunocytochemistry with monoclonal anti-Flk-1/KDR antibodies (CT128 clone) and biotinylated fluorescein-streptavidin (B). Note in (B) that Flk-1/KDR is mainly detected at the plasma membrane level and in the cytoplasm. Original magnification, × 40. (C) VEGF up-regulates Flk-1/KDR expression. HUVEC were treated with VEGF (10 ng/ml) or E_2 (10^{-10} to 10^{-7} M) for 7 h. Relative RNA units for Flk-1/KDR were normalized with the β2-microglobulin level, as calculated from standard curves, and compared with untreated cells. The results are expressed as the mean ± S.E.M. of three experiments. *$P<0{\cdot}05$ compared with untreated control cells.

VEGF, but not estradiol, increases Flk-1/KDR expression in endothelial cells (HUVEC)

VEGF levels have previously been shown to increase in human uterine cells in response to E_2 stimulation (Shifren *et al.* 1996, Bausero *et al.* 1998). In order further to analyze the possible effects of E_2 and VEGF on Flk-1/KDR expression *in vitro*, we used endothelial cells prepared from umbilical cord (HUVEC). As shown by immunofluorescence and RT-PCR analysis, these cells expressed Flk-1/KDR receptor (Fig. 3A,B).

The modulation of Flk-1/KDR expression by E_2 and VEGF was further analyzed using quantitative real-time PCR analysis and a LightCycler protocol developed as described in Materials and Methods. This allows the sensitive, specific and quantitative detection of VEGF receptor.

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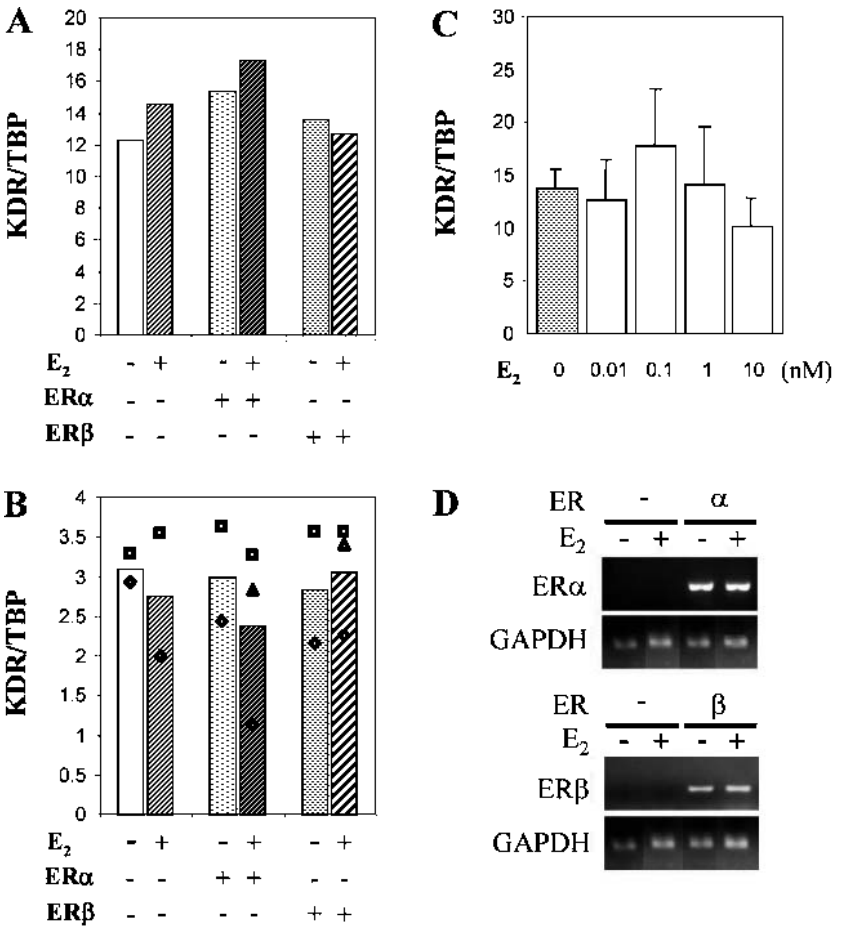

Figure 4 E_2 does not modulate Flk-1/KDR gene expression in endothelial cells that overexpress ERα or ERβ. (A) The HMEC-1 cell line and (B) primary HUVEC were infected overnight with Ad5, Ad-ERα or Ad-ERβ viruses 48 h prior to RNA isolation. Cells were treated with 100% ethanol (−) or 10 nM E_2 (+) for 7 h. (C) HMEC-1 cells infected by Ad-ERβ virus were treated or not with different concentrations of E_2 (nM) for 24 h. Flk-1/KDR mRNA levels were measured by real-time quantitative RT-PCR and were normalized to TATA Box binding protein (TBP) mRNA levels. Results represent one experiment in (A), and the mean of two or three independent experiments in (B) and (C). (D) Adenoviral expression of ERα and ERβ in HUVEC infected with Ad5, Ad-ERα or Ad-ERβ viruses, as checked by RT-PCR. Cells were treated (+) or not (−) with 10 nM E_2 for 7 h. The PCR products have a size of 818 bp, 395 bp and 450 bp for ERα, ERβ and GAPDH respectively. Panel D shows a representative experiment.

Stimulation of HUVEC by VEGF (10 ng/ml) significantly increased the level of Flk-1/KDR mRNA over control values (Fig. 3C). The increase in Flk-1/KDR expression by VEGF was dose- and time-dependent (not shown), with a maximal effect at 10 ng/ml and 7 h (1·5-fold induction, $P<0{\cdot}05$) (Fig. 3C). VEGF increased Flk-1/KDR expression in all HUVEC samples tested ($n=7$).

Treatment of HUVEC endothelial cells with E_2 (10^{-10} to 10^{-7} M) for 7 h (Fig. 3C) or with 10^{-8} M for 0 – 72 h (not shown) did not induce significant Flk-1/KDR expression. Using quantitative RT-PCR analysis (Bieche *et al.* 2001), ERα was absent in all HUVEC samples ($n=7$), while ERβ was present in very low amounts (not shown) with values always set below the lowest range (below the first tertile) found using the same method in a series of 131 primary breast cancer tumors (Bieche *et al.* 2001). The absence of an effect of E_2 on Flk-1/KDR expression in HUVEC could be explained by a loss of ER expression in the primary endothelial cells when isolated from the umbilical cord. Therefore, we have overexpressed ERα or ERβ in macro- (HUVEC) and micro- (HMEC-1) vascular cells, using adenovirus infection as described in Materials and Methods (see Fig. 4D) and have analyzed the modulation of Flk-1/KDR in

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these cells (Fig. 4A-C). Results indicate that E_2 does not affect the Flk-1/KDR expression in endothelial cells, whether E_2 was used at 10^{-8} M (Fig. 4A,B) or at various concentrations (Fig. 4C).

Altogether, these results suggest that the regulation of Flk-1/KDR expression by E_2 in endothelial cells does not involve an autocrine mechanism and mainly occurs through the VEGF pathway.

Discussion

VEGF is a key mediator of angiogenesis. Its expression, abundant in the embryo, is down-regulated in the adult, except during cyclical endometrial growth and corpus luteum formation (Ferrara & Davis-Smyth 1997, Perrot-Applanat 2000). Alterations in the expression of VEGF and its receptors are associated with disruption of ovarian and uterine functions (Ferrara *et al.* 2003). Despite the biological relevance of Flk-1/KDR in angiogenesis, little is known about the molecular mechanisms controlling its expression. In the present work, we describe the up-regulation of Flk-1/KDR by E_2 *in vivo* in the mouse endometrium, while experiments on endothelial cells *in vitro* show the up-regulation of Flk-1/KDR by VEGF in the absence of a direct E_2 effect, suggesting a paracrine mechanism of regulation of Flk-1/KDR *in vivo*.

In vivo *regulation of Flk-1/KDR expression by E_2 in the uterus*

Endometrial cyclical growth depends on capillary proliferation and increased blood flow caused by vasodilatation and changes in vascular permeability (Giudice 1996). These changes are regulated by E_2 and progesterone through activation of their respective nuclear receptors (Perrot-Applanat *et al.* 1994, Lecce *et al.* 2001). E_2 directly modulates VEGF expression in the human endometrium *in vivo* (Shifren *et al.* 1996, Bausero *et al.* 1998). Preliminary observations suggest that E_2 also modulates endometrial Flk-1/KDR (Meduri *et al.* 2000), essential for the development of the uterine vasculature during physiological angiogenesis (Heryanto *et al.* 2003). However, the mechanisms controlling E_2-induced Flk-1/KDR expression are unclear. As functional ERs are essential for the E_2-induced increase in angiogenesis, wild-type and αERKO mice (Couse *et al.* 1995) provide a valuable model to examine the ER-mediated estrogenic effect on uterine Flk-1/KDR expression. We quantified Flk-1/KDR expression in ovariectomized wild-type or αERKO E_2-treated mice using an established protocol (Meduri *et al.* 2000). Our data show that E_2 induces Flk-1/KDR expression through functional ERα activation in the mouse uterus, as previously suggested for the human endometrium (Meduri *et al.* 2000). From our data, ERα does not seem to be the only mediator for Flk-1/KDR expression. Since ERβ is also expressed in uterine endothelial cells, we can anticipate a possible role for ERβ in E_2-induced Flk-1/KDR expression in the absence of ERα. This hypothesis could be strengthened by the study of Kurita *et al.* (2001), who has previously described a role for ERβ in E_2 induction of progesterone receptor in αERKO mice. Further experiments using mice with ERβ invalidation are required to elucidate the respective roles of ERα and ERβ in Flk-1/KDR induction.

We have shown that progesterone modulates VEGF secretion in decidual cells (Ancelin *et al.* 2002). While adjunction of progesterone does not modify the E_2 effect on Flk-1/KDR expression in wild-type and αERKO mice, progesterone alone increases Flk-1/KDR expression only in αERKO but not in wild-type mice (see Fig. 1). We do not know why progesterone has an effect only in the absence of ERα. Previous studies have demonstrated the presence of progesterone receptors in αERKO mice; progesterone is able to induce a decidual reaction and to regulate gene expression in these animals (Curtis *et al.* 1999). Our data on the role of progesterone in Flk-1/KDR expression in the mouse uterus need to be confirmed on a larger series of animals.

E_2 and in vitro *regulation of Flk-1/KDR expression in endothelial cells*

Experimental studies show that E_2 exerts direct effects on endothelial cells, including up-regulation of endothelial nitric oxide synthase activity, modulation of adhesion molecule expression, angiogenic activity and proliferation *in vitro* after 6 days of culture (Morales *et al.* 1995). However, it is unclear whether these effects are mediated through modulation of VEGF receptors. In our study, E_2 did not increase significantly Flk-1/KDR expression in HUVEC samples from early passages (P1 to P2). A small increase in Flk-1/KDR expression with E_2 was observed in one of the 7 samples analyzed (not shown), which could be mediated by ERβ, as suggested by the presence of low levels of ERβ. The presence of ERβ, but not of ERα mRNA, was previously described in HUVEC (Enmark *et al.* 1997, Stefano *et al.* 2000). Our results could be interpreted by the fact that the response could vary among different subjects, or by a loss of ER expression in most samples. Gargett *et al.* (2002) report a moderate increase in Flk-1/KDR expression in E_2-treated myometrial endothelial cells, mediated primarily by ERα. These discrepancies may reflect differences in endothelial cell origin and/or conditions of culture such as the number of passages. We and others have detected only ERβ and not ERα in human and mouse endothelial cells *in vivo* (Critchley *et al.* 2001, Kurita *et al.* 2001, Lecce *et al.* 2001, as shown in this study). To counteract the loss of ER expression in our primary endothelial cells, HUVEC, we have overexpressed ERα or ERβ in macro- (HUVEC) and micro- (HMEC-1) vascular cells. Our results show

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that E_2 does not modulate Flk-1/KDR expression in endothelial cells. In our studies, we have also used transient transfection of ERα and ERβ cDNA along with a Flk-1/KDR promoter-luciferase gene reporter in an umbilical cord cell line (HUV-E-C), which expresses low levels of Flk-1/KDR, as compared with primary HUVEC. E_2 did not activate Flk-1/KDR in these cells but activated an estrogen response element luciferase construct (data not shown). Thus, E_2 does not directly modulate Flk-1/KDR in endothelial cells; a direct effect of E_2 could be limited to the vasculature of sex steroid-responsive tissues, such as the myometrium.

VEGF induces up-regulation of Flk-1/KDR in vitro *and* in vivo

Previous studies have demonstrated that E_2 increases VEGF secretion in human and animal uterine cells (Cullivan-Bove & Koos 1993, Shifren *et al.* 1996, Bausero *et al.* 2000). The present study demonstrates a VEGF-induced up-regulation of Flk-1/KDR mRNA in endothelial cells (HUVEC), in agreement with a previous study on the capillary endothelium of bovine adrenal cortex (Shen *et al.* 1998). By qPCR we can barely detect VEGF mRNA in HUVEC (unpublished results). Altogether, these results suggest that VEGF cannot stimulate Flk-1/KDR via an autocrine mechanism. Up-regulation of Flk-1/KDR protein levels was also reported in other studies using either endothelial cells from human saphenous veins infected with an adenoviral vector encoding VEGF165 (Weisz *et al.* 2001), or mouse cerebral slices incubated with recombinant VEGF165 (Kremer *et al.* 1997). These data suggest that Flk-1/KDR is regulated by VEGF synergistically with other factors, such as transforming growth factor-β, tumor necrosis factor-α and shear stress (Ferrara & Davis-Smyth 1997, Shen *et al.* 1998).

The up-regulation of VEGF expression by E_2 has been established *in vivo* in the injured-carotid model of angiogenesis in which E_2 increases re-endothelialization (Concina *et al.* 2000) while a direct modulation of Flk-1/KDR appears to be very inconsistently observed, suggesting that, in this model also, E_2 regulates angiogenesis mainly through a paracrine VEGF action.

In conclusion, we describe the effects of E_2 on the increase in Flk-1/KDR expression in a sex steroid-responsive tissue - the human and mouse uterus. The increase in Flk-1/KDR expression by E_2 leading to an increase in angiogenesis is secondary to an E_2 up-regulation of VEGF expression, previously observed *in vivo*, associated with VEGF-induced Flk-1/KDR expression in endothelial cells as shown in this study.

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References

Abumiya T, Sasaguri T, Taba Y, Miwa Y & Miyagi M 2002 Shear stress induces expression of vascular endothelial growth factor receptor Flk-1/KDR through the CT-rich Sp1 binding site. *Arteriosclerosis Thrombosis Vascular Biology* **22** 907–913.

Ancelin M, Buteau-Lozano H, Meduri G, Osborne-Pellegrin M, Sordello S, Plouet J & Perrot-Applanat M 2002 A dynamic shift of VEGF isoforms with a transient and selective progesterone-induced expression of VEGF189 regulates angiogenesis and vascular permeability in human uterus. *PNAS* **99** 6023–6028.

Bausero P, Freitas S, Meduri G & Perrot-Applanat M 1998 Paracrine action of VEGF in the human endometrium: regulation by ovarian steroids. *Angiogenesis* **2** 167–182.

Bausero P, Mazucatelli JP, Bloy C & Perrot-Applanat M 2000 Estradiol, tamoxifen and hypoxia modulate vascular endothelial growth factor in human vascular smooth muscle cells. *American Journal of Physiology* 2033–2042.

Bieche I, Parfait B, Nogues C, Andieu C, Vidaud D, Spyratos F, Lidereau R & Vidaud M 2001 The CGA gene as new predictor of the response to endocrine therapy in ER alpha-positive postmenopausal breast cancer patients. *Oncogene* **20** 6955–6959.

Concina P, Sordello S & Barbacanne MA 2000 The mitogenic effect of 17 beta-estradiol on *in vitro* endothelial cell proliferation and on *in vivo* re-endothelialization are both dependent on vascular endothelial growth factor. *Journal of Vascular Research* **37** 202–208.

Couse JF, Curtis SW, Washburn TF, Eddy EM, Schomberg DW & Korach KS 1995 Disruption of the mouse oestrogen receptor gene: resulting phenotypes and experimental findings. *Biochemical Society of Transplantation* **23** 929–935.

Critchley HOD, Brenner RM, Henderson TA, Williams K, Nayak NR, Slayden OD, Millar MR & Saunders PTK 2001 Estrogen receptor beta, but not estrogen receptor alpha, is present in the vascular endothelium of the human and nonhuman primate endometrium. *Journal of Clinical Endocrinology and Metabolism* **86** 1370–1378.

Cullivan-Bove K & Koos RD 1993 Vascular endothelial growth factor/vascular permeability factor expression in the rat uterus: rapid stimulation by estrogen correlates with estrogen-induced increases in uterine capillary permeability and growth. *Endocrinology* **133** 829–837.

Curtis S, Clark J, Myers P & Korach K 1999 Disruption of estrogen signaling does not prevent progesterone action in the estrogen receptor α knockout mouse uterus. *PNAS* **96** 3646–3651.

Enmark E, Pelto-Huikko M, Grandien K, Lagercrantz S, Lagercrantz J, Fried G, Nordenskjöld M & Gustafsson 1997 Human estrogen receptor β gene structure, chromosomal localisation, and expression pattern. *Journal of Clinical Endocrinology* **82** 4258–4265.

Ferrara N & Davis-Smyth T 1997 The biology of vascular endothelial growth factor. *Endocrinology Review* **18** 4–25.

Ferrara N, Gerber HP & LeCouter J 2003 The biology of VEGF and its receptors. *Nature Medicine* **9** 669–676.

Gargett CE, Zaitseva M, Bucak K, Chu S, Fuller PJ & Rogers PA 2002 17-Beta-estradiol up-regulates vascular endothelial growth

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factor receptor-2 expression in human myometrial microvascular endothelial cells: role of estrogen receptor-alpha and -beta. *Journal of Clinical Endocrinology and Metabolism* **87** 4341–4349.

Gille H, Kowalski J, Li B, LeCouter J, Moffat B, Zioncheck TF, Pelletier N & Ferrara N 2001 Analysis of biological effects and signaling properties of Flt-1 (VEGFR-1) and KDR (VEGFR-2). A reassessment using novel receptor-specific vascular endothelial growth factor mutants. *Journal of Biological Chemistry* **276** 3222–3230.

Giudice L 1996 The endometrial cycle. In *Reproductive Endocrinology, Surgery and Technology*, pp 272–300. Eds EY Adashi, JA Rock & Z Rosenwaks. Philadelphia: Lippincolt-Raven Publishers.

Heryanto B, Lipson KE & Rogers PA 2003 Effects of angiogenesis inhibitors on oestrogen-mediated endometrial endothelial cell proliferation in the ovariectomized mouse. *Reproduction* **125** 337–346.

Jaffe EA, Nachman RL, Becker CG & Minick CR 1973 Culture of human endothelial cells derived from umbilical veins. Identification by morphologic and immunologic criteria. *Journal of Clinical Investigation* **52** 2745–2756.

Kremer C, Breier G, Risau W & Plate KH 1997 Up-regulation of Flk-1/vascular endothelial growth factor receptor 2 by its ligand in a cerebral slice culture system. *Cancer Research* **57** 3852–3859.

Kurita T, Lee K, Saunders PT, Cooke PS, Taylor JA, Lubahn DB, Zhao C, Makela S, Gustafsson JA, Dahiya R & Cunha GR 2001 Regulation of progesterone receptors and decidualization in uterine stroma of the estrogen receptor-alpha knockout mouse. *Biology of Reproduction* **64** 272–283.

Lazennec G, Bresson D, Lucas A, Chauveau C & Vignon F 2001 ER beta inhibits proliferation and invasion of breast cancer cells. *Endocrinology* **142** 4120–4130.

Lecce G, Meduri G, Ancelin M, Bergeron C & Perrot-Applanat M 2001 Presence of estrogen receptor beta in the human endometrium through the cycle: expression in glandular, stromal, and vascular cells. *Journal of Clinical Endocrinology and Metabolism* **86** 1379–1386.

Meduri G, Bausero P & Perrot-Applanat M 2000 Expression of vascular endothelial growth factor receptors in the human endometrium: modulation during the menstrual cycle. *Biology of Reproduction* **62** 439–447.

Millauer B, Wizigmann-Voos S, Schnurch H, Martinez R, Moller NP, Risau W & Ullrich A 1993 High affinity VEGF binding and developmental expression suggest Flk-1 as a major regulator of vasculogenesis and angiogenesis. *Cell* **72** 835–846.

Morales DE, McGowan KA, Grant DS, Maheshwari S, Bhartiya D, Cid MC, Kleinman HK & Schnaper HW 1995 Estrogen promotes angiogenic activity in human umbilical vein endothelial cells *in vitro* and in a murine model. *Circulation* **91** 755–763.

Perrot-Applanat M 2000 Hormonal regulation of vascular cell function: angiogenesis. In *Encyclopedic Reference of Vascular Biology and Pathology* pp 157–162. Ed A Bikfalvi. Heidelberg: Springer Verlag.

Perrot-Applanat M, Deng M, Fernandez H, Lelaidier C, Meduri G & Bouchard P 1994 Immunohistochemical localization of estradiol and progesterone receptors in human uterus throughout pregnancy: expression in endometrial blood vessels. *Journal of Clinical Endocrinology and Metabolism* **78** 216–224.

Saunders PTK, Millar MR, Williams K, Macpherson S, Harkiss D, Anderson RA, Orr B, Groome NP, Scobie G & Fraser HM 2001 Differential expression of estrogen receptor ERα and ERβ and androgen receptor in the ovaries of marmosets and humans. *Biology of Reproduction* **63** 1098–1105.

Shen BQ, Lee DY, Gerber HP, Keyt BA, Ferrara N & Zioncheck TF 1998 Homologous up-regulation of KDR/Flk-1 receptor expression by vascular endothelial growth factor *in vitro*. *Journal of Biological Chemistry* **273** 29979–29985.

Shifren JL, Tseng JF & Zaloudek CJ 1996 Ovarian steroid regulation of vascular endothelial growth factor in the human endometrium: implications for angiogenesis during the menstrual cycle and in the pathogenesis of endometriosis. *Journal of Clinical Endocrinology and Metabolism* **81** 3112–3118.

Spyridopoulos I, Sullivan AB, Kearney M, Isner JM & Losordo DW 1997 Estrogen receptor-mediated inhibition of human endothelial cell apoptosis. Estradiol as a survival factor. *Circulation* **95** 1505–1514.

Stefano GB, Prevot V, Beauvillain JC, Cadet P, Fimiani C, Welters I, Fricchione GL, Breton C, Lassalle P, Salzet M & Bilfinger TV 2000 Cell-surface estrogen receptors mediate calcium-dependent nitric oxide release in human endothelia. *Circulation* **101** 1594–1597.

Weisz A, Koren B, Cohen T, Neufeld G, Kleinberger T, Lewis BS & Flugelman Y 2001 Increased vascular endothelial growth factor 165 binding to kinase insert domain-containing receptor after infection of human endothelial cells by recombinant adenovirus encoding the $VEGF_{165}$ gene. *Circulation* **103** 1887–1892.

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