Angionesis and the inception of pregnancy

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7

EFFECTS OF OVARIAN STEROIDS ON HUMAN ENDOMETRIAL ENDOTHELIAL AND STROMAL CELLS. EVIDENCE FOR PARACRINE REGULATION OF ANGIOGENESIS

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Introduction

Angiogenesis is essential for tissue repair and the recovery of endometrial tissue during the menstrual cycle. It also plays a crucial role in the successful implantation of the embryo and its growth. Inadequate endometrial angiogenesis is likely involved in implantation failure and defective placentation, which may have a great impact on a patient's quality of life and pregnancy outcome[1-3]. As such, it is important to gain a better understanding of the process of endometrial angiogenesis.

The process of angiogenesis is under the control of angiogenic growth factors and hormones[4,5]. The ovarian steroids are main regulators of endometrial angiogenesis as already shown in early experiments by Markee and Abel[6,7]. Although many studies have indicated the involvement of these hormones in endometrial angiogenesis, the mechanisms by which 17β-estradiol (E_2) and progesterone act are still not well understood[8-11]. So far it is unclear whether the steroids directly influence the endometrial endothelium or whether they regulate endometrial angiogenesis indirectly via activation of other endometrial cells.

It is generally thought that the regulation of biological responses to estrogens and progesterone is mediated via the interaction of these hormones with their corresponding nuclear receptors; estrogen receptor-alpha (ERα), -beta (ERβ) and the progesterone receptor (PR). Bound to their receptors these hormones form a complex in the nucleus of the cell that binds to estrogen or progesterone response elements (EREs and PREs) in the promoter regions of many genes. This results in an altered expression of these genes. In addition ERs on the surface of endothelial cells participate in the rapid regulation of the vascular tone[12]. The existence of various splicing variants of the PR and ERs, which may have different transactivation properties *in vivo*, further adds to the complex regulation by the ovarian hormones[13-17]. Several studies reported on the expression of these receptors in endometrial cells *in vivo* and *in vitro*, but their conclusions have not been equivocal[16,18-21].

Mediators by which steroid hormones might influence the endometrial angiogenic process are locally produced angiogenic growth factors and cytokines. These factors affect endothelial proliferation, invasion and migration, which all play a role in angiogenesis. VEGF-A, in particular, is a potent mediator in the endometrium, as it is able to influence all these processes[1,19,20,22]. In the human endometrium VEGF-A is produced by epithelial cells, stromal cells and stromal leukocytes[23-25]. Steroid hormones may also affect angiogenesis by modulating factors that are involved in the local proteolytic remodeling of matrix proteins important for the invasion and migration of the endothelial cells, such as matrix metalloproteinases, urokinase (u-PA) and their inhibitors[4,22].

Endothelial cells from various tissues display organ-specific characteristics, which at least in part are retained in culture[26]. Therefore, endometrial angiogenesis is preferably studied with hEMVEC. As few other investigators, we have been able to isolate, culture and characterize hEMVEC[20,22,27,28]. In the present study we have examined the influence of E_2 and progesterone on hEMVEC and human stromal cells (hESC). This was done in order to get a better understanding of the steroidal regulation of endometrial angiogenesis and the mutual role of the hEMVEC and hESC in this process.

Materials and Methods

Materials

Medium 199 (M199) without phenol-red, and supplemented with 20 mM HEPES was obtained from BioWhittaker (Verviers, Belgium); newborn calf serum (NBCS) and human serum as previously described[22,29]. For experiments with steroids, charcoal-treated sera were used. Tissue-culture plastics, endothelial cell growth factor (ECGF), human bFGF, heparin, TNFα, thrombin, human fibrinogen, factor XIII and fibronectin were obtained as previously indicated[22,29]. Human recombinant VEGF-A was purchased from RELIATech (Braunschweig, Germany). E_2 (E-2758) and progesterone (P-8783) were purchased from Sigma (St Louis, USA) and ICI 182.780, a potent and specific anti-estrogen[30], (Faslodex (TM), fulvestrant) was from AstraZeneca (Alderley Park, UK). Stock solutions of steroids and ICI 182.780 (10mmol/L) were prepared in DMSO and stored at -20°C; further dilutions were made in M199 without phenol red with 0.1% pyrogen-free human serum albumin (HSA), and finally in incubation medium immediately before the start of an experiment. Oligonucleotides used for RT-PCR were obtained from Biosource Europe SA (Nivelles, Belgium).

Cell culture

HEMVEC were isolated from endometrial tissue as previously described and maintained in M199 without phenol-red supplemented with 20 mM HEPES (pH 7.3), 20% human serum, 10% heat-inactivated NBCS, 150 mg/mL ECGF, 5 ng/mL VEGF-A, 5 U/mL heparin, 100 IU/mL penicillin and 100 mg/mL streptomycin (= hEMVEC culture medium)[22].

HESC were isolated from the primary heterogeneous cell population, which was obtained after the endometrial tissue was minced, incubated in collagenase and transferred into a culture dish. After 2-4 hours the non-adhered and CD31-negative cells (hESC and epithelial cells), were transferred and cultured in hEMVEC medium with

10% human serum and without VEGF-A in gelatin-coated dishes. Endometrial epithelial cells were quickly lost upon serial passage in culture. HESC were characterized as fibroblasts by immunofluorescence staining with anti-human fibroblast (ITK diagnostics, Uithoorn, The Netherlands). The cells were negative for the endothelial cell markers CD31 and von Willebrand Factor. Very few cells (<1%) at low passage number stained positive for the epithelial cell markers cytokeratine-8 and -18 or smooth-muscle actin (data not shown).

Cells were cultured on fibronectin-coated or gelatin-coated wells at 5% CO_2 / 95% air until confluence and subcultured with a split ratio of 1:3. The medium was renewed at 2-3 day intervals.

Immunohistochemistry

Human endometrial tissue specimens were embedded in paraffin and cut into 4 μm sections. After deparaffinization and blocking with 0.3% H_2O_2-methanol, the sections were washed in PBS. For antigen retrieval they were cooked in citrate buffer (0.01M, pH 6.0) in a microwave for 10 min. Subsequently they were washed and incubated for 15 min in a "block"-buffer (5% bovine serum albumin (BSA) in PBS) to reduce background staining. Then the specific steroid receptor antibody (ERα: DAKO 1D5, ERβ: Serotech MCA1974, PR: ABR PR-AT 4.14) was added (diluted in 1 % BSA/PBS), followed by an overnight incubation at 4ºC. The next day, after three washes in PBS, the sections were incubated with biotine-conjugated horse-anti-mouse Ig (1:300 in PBS-1% BSA) for 1 h at room temperature. After additional washing and amplification with Avidine Biotin Complex, the sections were stained with NOVA-RED for 10 min. They were counterstained with Mayers' haematoxylin.

RNA Isolation and RT-PCR

Total RNA from hEMVEC and hESC (30 cm^2/condition) was isolated as described by Chomczynski and Sacchi [31]. cDNA was synthesized from 1 μg total RNA with 0.5 μg oligo dT primer and 15 U AMV Reverse Transcriptase (Promega, Madison). PCR amplification of 1 μL cDNA was performed on a Robocycler (Stratagene) in 40 μL reaction mixtures containing 4μL 10x PCR buffer, 25 mM of each dNTP, 10 pmol of each primer, and 0.2 μL of *Taq* polymerase (Amersham Pharmacia Biotech Inc, Piscataway). The following cycling conditions were used: 94°C for 4 min; 35 cycles of 94°C for 1 min, 60°C (ERβ) and 67°C (ERα, PR) for 1 min, and 72°C for 1 min; followed by 72°C for 7 min. After 35 cycles the PCR was stopped and amplification products were evaluated by 1% agarose gel electrophoresis.

Oligonucleotide primers

The following primer sequences were used in the RT-PCR to detect receptor mRNA: for the ERα mRNA: sense 5′-TGATGGGGAGGGCAGGGGTGAAGTG-3′ and antisense 5′-TAGGCGGTGGGCGTCCAGCATCTCC-3′[32]. For the ERβ mRNA: *set X*; sense 5′-TTGTGCGGAGACAGAGAAGTGC-3′ and antisense 5′-GGAATTGAGCAGGATCATGGCC-3′[33]. For the ERβ also another set of primers was designed which amplified part of the C-terminal region, *set Y*: sense 5′-CATGATCCTGCTCAATTCCA-3′ and antisense 5′-CTTGTTACTCGCATGCCTGA-3′. For the PR mRNA: sense 5′-GTGGGCGTTCCAAATGAAAGCCAAG-3′ and antisense 5′-AATTCAACACTCAGTGCCCGGGACT-3′[32]. For β-actin mRNA: sense 5′-AAGATGACCCAGATCATGTTTGAG-3′ and antisense 5′-AGGAGGAGCAATGATCTTGATCTT-3′.

Cell proliferation

Incorporation of ^{3}H-thymidine in DNA was determined as a measurement of endothelial and stromal cell proliferation as previously indicated[22].

In vitro angiogenesis assay

Human fibrin and collagen matrices were prepared as previously described[22,29]. Highly confluent hEMVEC were detached and seeded in a split ratio of 2:1 on the surface of the fibrin or collagen matrices and cultured for 24 h in M199 medium without indicator supplemented with 20% human serum, 10% NBCS, and penicillin/streptomycin. Subsequently hEMVEC were stimulated with the mediators indicated for 3-4 days to form capillary-like tubules. The culture medium with additions was renewed every day, because of the high turnover rate of E_2 and progesterone. The formation of tubular structures by hEMVEC in the three-dimensional matrix was quantified by non-phase contrast microscopy as previously given[22,29].

Assays

U-PA and PAI-1 were assayed by EIA as previously indicated[22]. VEGF antigen was determined by VEGF ELISA (R&D system, Minneapolis, USA).

Statistical analysis

The data are expressed as the mean ± SEM/range. Statistical evaluations of the data were performed using the Paired-Sample T-test after the control conditions were set at a 100%. $p < 0.05$ was considered statistically significant.

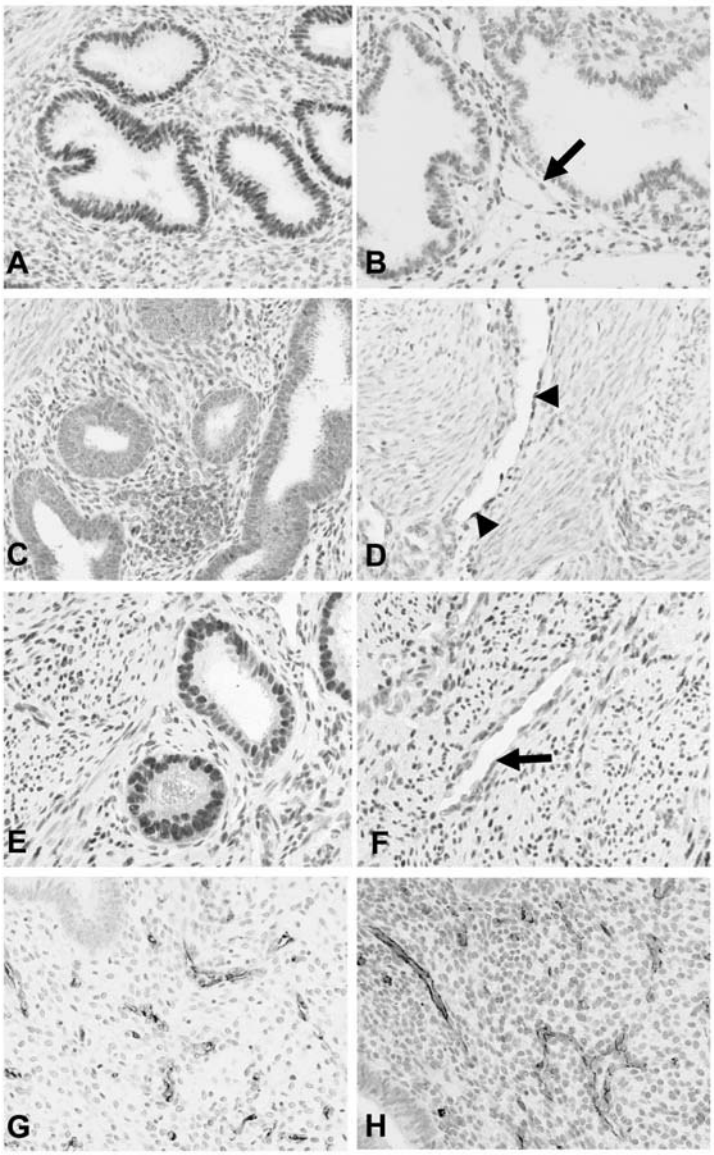

Figure 1. Expression of ERα, ERβ and PR in human endometrial tissue.
Immunohistochemistry was performed with labeled antibodies to ERα, ERβ and PR on paraffin sections of human endometrium, as described in the methods section. Panel A and B; brown staining shows ERα in the epithelium and in the stromal compartment, the endothelium is negative for the ERα. C and D; endometrial stroma, epithelium and endothelium show positive staining for ERβ. E and F; PR staining is seen in the epithelium and in the stroma, the endothelium stains negative for the PR. G and H; von Willebrand and CD31 staining were used to indicate the endothelial cells in the endometrium. Black arrow heads indicate an example of positive endothelial cells, and black arrows indicate negative endothelial cells. *[See appendix: color figures]*

Results

HEMVEC express ERβ, hESC express ERα, ERβ and PR

Immunohistochemical staining and RT-PCR analysis were performed to detect the expression of steroid receptors by hEMVEC and hESC. Endometrial tissue showed specific staining for the ERβ on endothelial cells and in the remaining stromal compartment. The stromal compartment also stained positive for the PR and ERα. The surface and glandular epithelium was highly positive for ERα and PR and also contained ERβ (Fig. 1).

Subsequently, we investigated the presence of ERs and PR in hEMVEC *in vitro*. Because the purification of hEMVEC includes several passages, the cells were evaluated from 5 passages onward. No notable ERα or PR mRNA was detected in hEMVEC from passages 5 to 14 of 4 different donors (Fig. 2A and E). In contrast, ERβ mRNA was clearly expressed in hEMVEC at passages 5 to 14 (cells from 2 different donors) (Fig. 2C); ERβ was detected by both *primer sets X* and *Y* (not shown). The ERβ remained expressed during serial passage of hEMVEC. In one of the 4 isolations a very weak signal for PR was shown in a higher passage (not shown).

In hESC the ERα was expressed in 2 (passages 4 and 6) out of 3 isolations from different donors. The hESC isolation that did not express the ERα was at passage 5 (Fig. 2A). HESC lost their expression of ERα above passage 5-6 (Fig. 2B). Unexpectedly, hESC, from passages 4 to 6 of 3 different donors, did not express ERβ when *primer set X* was used. However, when we used a different primer set, that amplified part of the C-terminal region of ERβ (*primer set Y*), ERβ mRNA was detected in hESC, suggesting an alternatively spliced ERβ (Fig. 2D).

Ovarian steroids and proliferation of hEMVEC and hESC

Increasing amounts of E_2 (10^{-10}-10^{-7}M) and progesterone (10^{-8}-10^{-6}M) were tested for their effect of hEMVEC proliferation under basal conditions (contains 0.75 ng/mL VEGF for hEMVEC maintenance) and in the presence of 6.25 ng/mL VEGF-A. E_2 had no effect on the basal and VEGF-A enhanced proliferation of hEMVEC (Fig. 3A). Progesterone did also not affect the basal and VEGF-A-mediated proliferation of hEMVEC (Fig. 3B).

The effects of E_2 and progesterone were subsequently evaluated in hESC in control medium (without additional growth factors) and in the presence of bFGF, which enhances hESC proliferation. A slight non-significant increase in basal proliferation of hESC was observed after 48 h incubation with high concentrations of E_2 only. This effect was absent when these cells were also stimulated by bFGF (Fig. 3C). Progesterone did not alter the proliferation of hESC either under control conditions, or in the presence of bFGF (Fig. 3D).

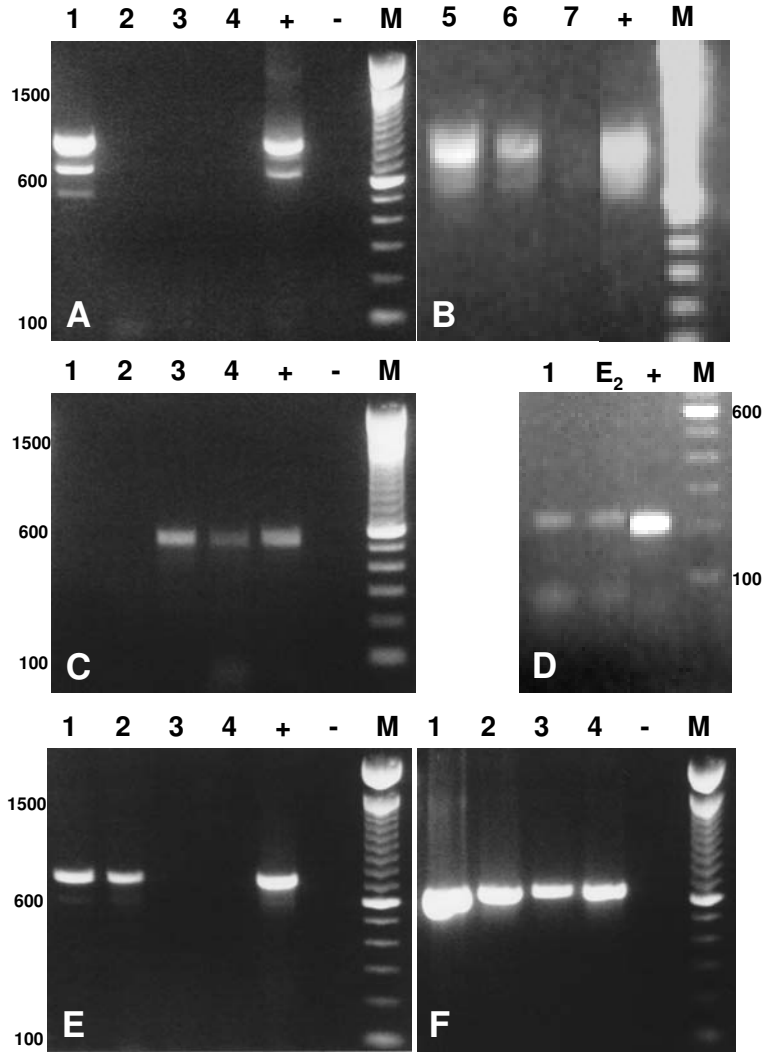

Figure 2. Expression of ERα, ERβ and PR in endometrial cells as determined by RT-PCR.
HEMVEC and hESC were cultured till confluence on fibronectin- or gelatine-coated dishes in (hEMVEC) culture medium. RNA was isolated from these cells and cDNA was synthesized as described in Material and Methods. PCR amplification was performed with primers for ERα (panel A, B), ERβ (panel C, D) and PR (panel E) as described. The expected length of the amplified DNA fragment of the ERα is 832 bp, of the ERβ 541 bp (C) and 208 bp (D) and of the PR 737 bp. As a positive control for the expression of all three receptors, RNA isolated from T47D cells (human breast cancer cell line) was used. To check the quality of cDNA, primers specific to the human β-actin gene (panel F) were used; the expected length of the amplified DNA fragment was 647 bp. As negative control for the PCR reaction 1 μL of H_2O was used. Data were obtained with endometrial cells from different donors. Lane 1: hESC passage 4, lane 2: hESC passage 5, lane 3: hEMVEC passage 5, lane 4: hEMVEC passage 8, lane 5: hESC passage 1, lane 6: hESC passage 2, lane 7: hESC passage 3, +: positive control, -: negative control, M: molecular weight marker, E_2: hESC under E_2 stimulated conditions.

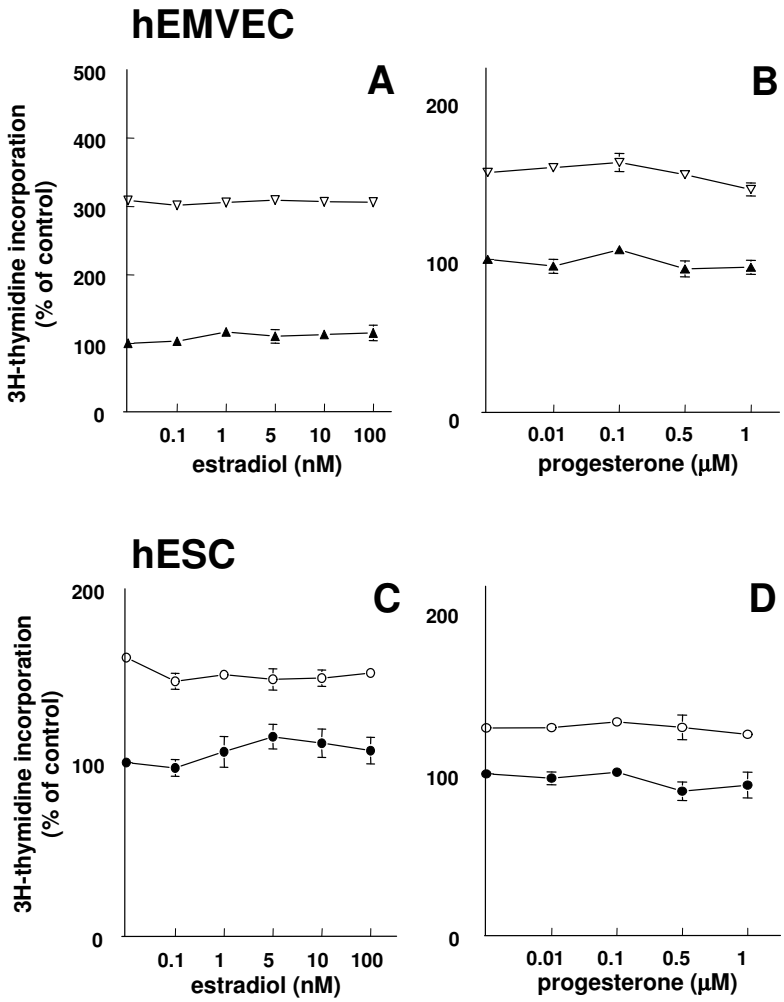

Figure 3. **Effects of estradiol and progesterone on hEMVEC and hESC proliferation.**
A,B: Non-confluent hEMVEC (passages 4 to 9 of 3 different donors) were cultured for 48 h in the presence of increasing amount of daily added E_2 (A) or progesterone (B) in M199 without phenol-red supplemented with 10% charcoal-treated NBCS and 0.75 ng/mL VEGF-A (-▲-) or 6.25 ng.mL VEGF-A (-▽-) . After 42 h, tracer amount of ^{3}H-thymidine was added to the medium and the incubation continued in the same medium for another 6 h and ^{3}H-thymidine incorporation was determined as described[23]. The data are expressed as a percentage of the control and represent mean ± SEM of 9 (E_2), 6 (P), 4 (E_2-or P+VEGF-A-) independent experiments performed in duplicate wells.
C,D: Non-confluent hESC, from passages 2 and 3 of 4 different donors, were cultured for 48 h in the absence or presence of an increasing amount of E_2 added daily (panel C) or progesterone (panel D) either in combination with (-○-) or without (-●-) bFGF (2.5 ng/mL) in M199 without phenol-red supplemented with 10% charcoal-treated NBCS. 3H-thymidine incorporation was assayed as given under A,B. The data are expressed as a percentage of the control and represent mean ± SEM/range of 4-5 (in the absence of bFGF) and 2-3 (in the presence of bFGF) independent experiments performed in duplicate wells.

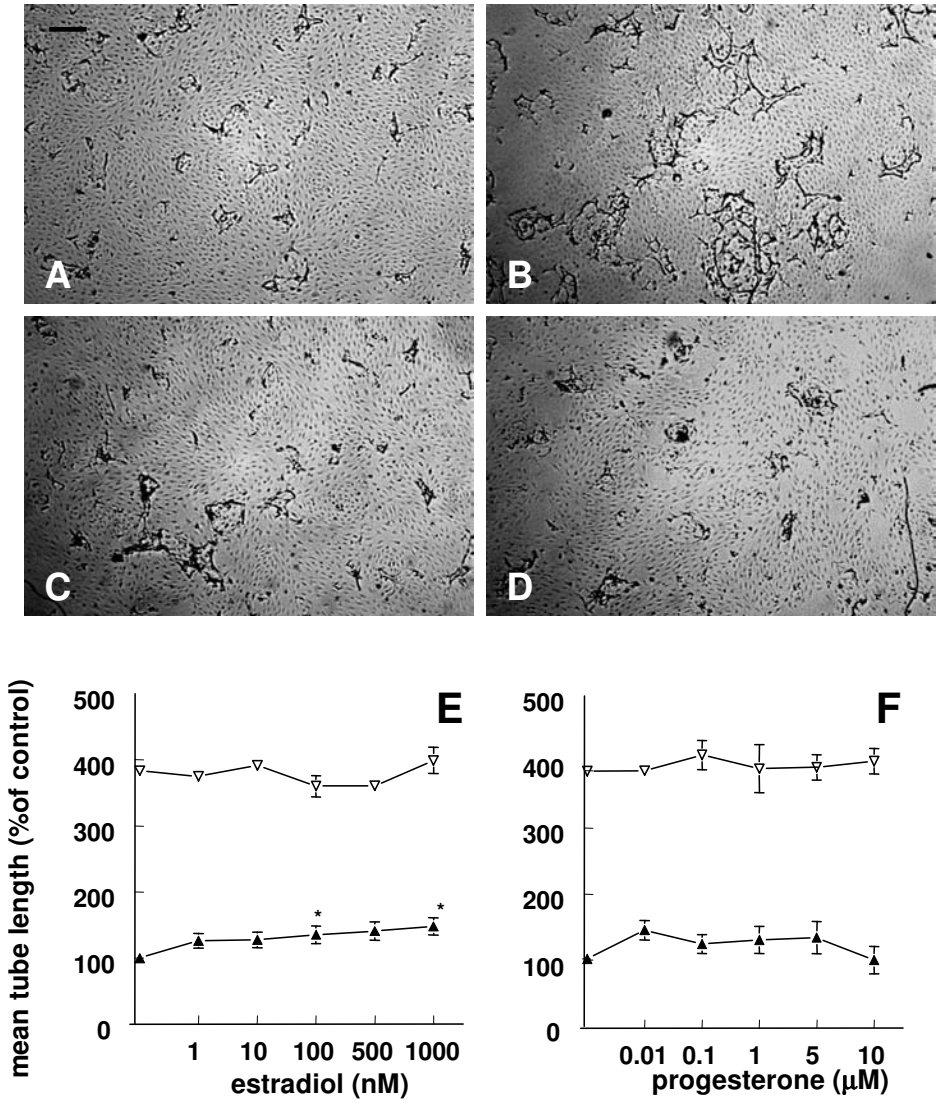

Figure 4. Effect of VEGF-A and ovarian steroids on capillary tube formation by hEMVEC.
A-D: Nonphase contrast views were taken which show growth of capillary-like tube structures in all panels. Panel A; control situation, panel B; stimulation with 10 ng/mL VEGF-A, panel C; stimulated with E_2 (10^{-7}M) and panel D; stimulation with progesterone (10^{-6}M). *Bar* = 300 μM.
E,F: hEMVEC, from passages 5-9 of 4 different donors, were cultured on top of a three-dimensional fibrin matrix in M199 supplemented with 20% human serum and 10% NBCS and stimulated with increasing amounts of E_2 (panel E) or progesterone (panel F) in the presence (-▽-) or absence (-▲-) of VEGF-A (10ng/mL). After 2-5 days of culturing, mean tube length was measured by image analysis as described and expressed as mean tube length as a percentage of the control ± SEM/range of 5 (absence of VEGF-A) or 2 (presence of VEGF-A) independent experiments performed in duplicate wells (* differs significantly, $p<0.05$, from 0 M condition).

Effect of VEGF-A, estradiol and progesterone on capillary-like tube formation

To evaluate whether ovarian hormones directly affected endothelial tube formation, we used an *in vitro* model, in which hEMVEC were cultured on top of a 3D-fibrin matrix[29]. Under basal conditions a limited number of hEMVEC invade the fibrin matrix and form tubular structures (fig. 4A). The presence of VEGF-A markedly enhanced the extent of capillary tube formation by hEMVEC (Fig. 4B, E). This increase amounted 3.8 ± 0.8-fold (11 independent experiments with hEMVEC from 4 different donors). The stimulation of capillary-like tube formation was completely prevented by the addition of VEGF-receptor-2/KDR blocking monoclonal antibodies (not shown).

Subsequently, the influence of increasing amounts of E_2 and/or progesterone on *in vitro* angiogenesis of hEMVEC was studied. A slight increase in tube formation was visible when hEMVEC were stimulated with E_2, which was borderline significant at 10^{-7}M (135±13%, $p=0.034$) and 10^{-6}M (148±13%, $p=0.046$, Fig. 4C,E). The minor stimulation by 10^{-7}M E_2 was completely inhibited by the addition of ICI 182.780 (not shown). Progesterone had no effect on tube formation (Fig. 4D, F). The amounts of u-PA and PAI-1 produced by the tube forming endothelial cultures were not significantly altered (not shown).

Various concentrations of E_2 or progesterone had no effect on VEGF-A-enhanced tube formation (Fig. 4E, F). Similarly, no effect on tube formation was seen, when E_2 (10^{-7}M) and progesterone (10^{-6}M) were simultaneously added, (not shown). When hEMVEC were cultured on collagen type-I matrices, E_2 (10^{-8}M) and/or progesterone (10^{-8}, 10^{-7}M), both with and without VEGF-A, had no significant effect on tube formation (not shown).

Estradiol and progesterone enhance VEGF-A production by hESC

A more distinct effect of E_2 and progesterone on endometrial angiogenesis might occur indirectly via activation of other endometrial cells that are in close contact with hEMVEC, in particular hESC. The presence of functional ER and PR in hESC was verified by measuring the IL-6 production in non-stimulated and IL-1β-stimulated hESC (0.014 ng/ml and 25 ng/mL IL-6, respectively). Both in non- and IL-1β-stimulated hESC, E_2 and progesterone (10^{-11}-10^{-8}M) reduced IL-6 production (Fig. 5).

Because hEMVEC responded well to VEGF-A, we evaluated whether E_2 and/or progesterone induced VEGF-A production by hESC. Increasing amounts of E_2 stimulated the VEGF-A production 10- to 29-fold (Fig. 6). ICI 182,780 blocked this effect by 50%. Increasing amounts of progesterone stimulated the VEGF-A production 9- to 15-fold. Progesterone did not further enhance the E_2-increased VEGF-A production (Fig. 6).

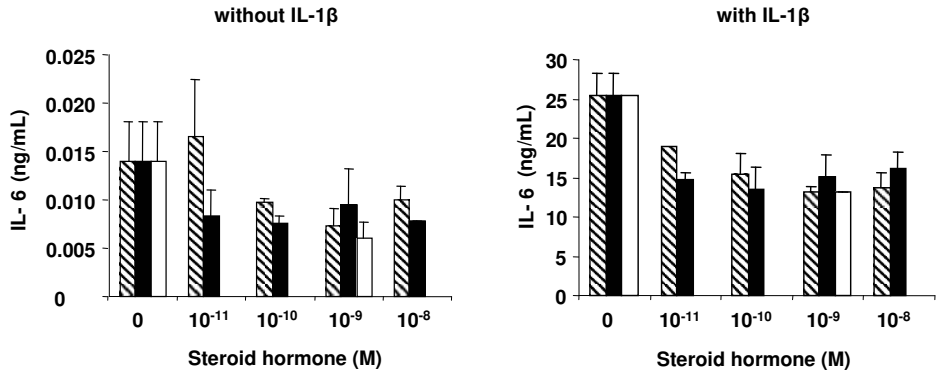

Figure 5. Effect of ovarian steroids on IL-6 production by hESC.
HESC were incubated for 20 h in the presence of the indicated concentrations of steroid hormones in 0.25 ml/cm^2 M199 medium (phenol-free) supplemented with 10% bovine charcoal-treated serum and 10% human charcoal-treated serum. After 1 h 125 pg/ml hr-IL-1β was added to part of the wells (right panel). After the 20 h incubation period IL-6 was determined in the conditioned medium by ELISA. Hatched bars, E_2; closed bars, progesterone; open bars (none and 10^{-9} M), E_2 plus progesterone. Note the difference in scale of the two panels. The data (mean ± range) are given for a representative experiment. Similar data were obtained with hESC cultures derived from another donor.

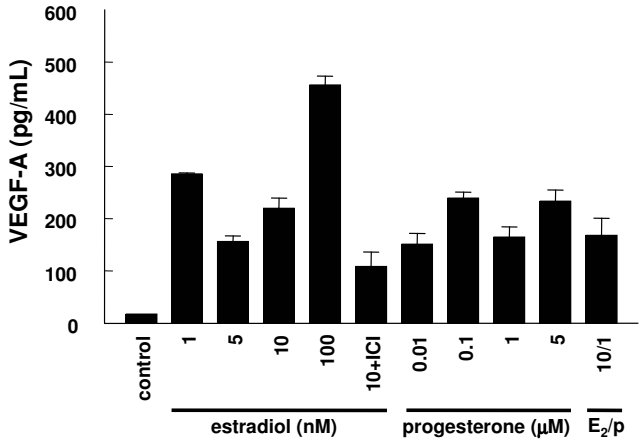

Figure 6. Estradiol and progesterone stimulate VEGF-A expression by hESC.
Confluent hESC on gelatin-coated wells were preincubated with M199 without phenol-red supplemented with 10% charcoal-treated NBCS for 24 h and subsequently stimulated with increasing amounts of E_2 or progesterone, or 10 nM E_2 plus 10 μM ICI 182.780 (ICI was added 1 h before E_2 was spiked) or the combination of 10 nM E_2 and 1 μM progesterone in M199 without phenol-red supplemented with 10% charcoal-treated NBCS. After 24 h E_2 and progesterone were spiked in the medium. After 48 h, supernatants were collected. VEGF-A was determined in 48 h conditioned medium by ELISA. The data are expressed as mean ± range of duplicate wells and are representative of two experiments performed with hESC from different donors.

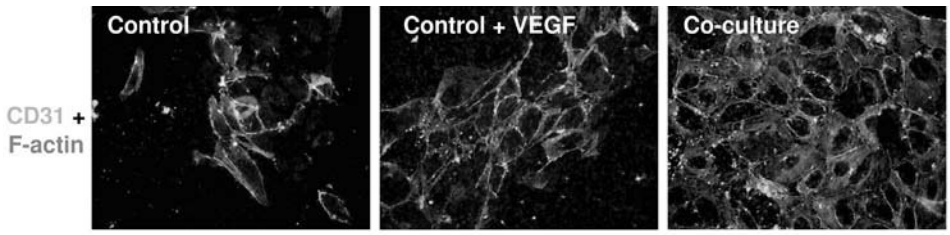

Figure 7. HESC and VEGF contribute to maintenance of hEMVEC monolayers.
Cultures of hEMVEC and hESC were detached and seeded on the surface of a filter (hEMVEC) or dish (hESC) of a transwell™ system (Costar). The next day the hEMVEC-covered filters were transferred into the wells in which hESC had been grown or wells without cells (control). To half of the control conditions VEGF-A (10 ng/mL) was added. HEMVEC were immunostained for CD 31 (green) and F-actin was visualized by rhodamine-falloidin (red). HEMVEC monolayers remained intact in co-culture, but showed holes in control cells. The addition of VEGF-A improved the quality of the monolayers. *[See appendix: color figures]*

Interaction between hESC and hEMVEC

In the absence of VEGF, hEMVEC showed discontinuities in their monolayer due to cell detachment. Addition of VEGF-A prevented hEMVEC cell death, and induced the maintenance of intact monolayers by the hEMVEC. When hEMVEC and hESC were co-cultured separated from each other by a porous filter, the monolayers of these hEMVEC maintained the characteristic regular cobblestone pattern even in the absence of VEGF (Fig. 7).This suggests that hESC provide factors, including VEGF-A, that stimulate the maintenance of hEMVEC.

Discussion

In this study we have shown that hESC expressed ERα, ERβ and PR and responded to the ovarian steroids by an increase in VEGF-A expression. HEMVEC express ERβ and show only a marginal angiogenic response to E_2. HEMVEC cultured in close contact with hESC survived better, probably due to paracrine VEGF production by hESC.

The occurrence of ER and PR in endometrial cells

In endometrial tissue, ERα, ERβ and PR were detected in endometrial epithelial cells, stromal cells and ERβ and PR in (perivascular) smooth muscle cells[18,34-39]. In the epithelial cells, and less clearly in the stromal cells, the receptors reflected a cycle-depended expression pattern[18,24,34,37-39]. Endothelial cells showed specific staining for the ERβ but no staining for ERα or PR, which is in agreement with previous reports[18,37,38]. Only

Lecce *et al*. detected, although only occasionally, both ERβ and ERα in endometrial endothelial cells and an up-regulation of ERβ during the late secretory phase[38].

In cultured hESC we detected ERα, ERβ and PR mRNA expression. The ERβ expression was less prominent than that of ERα, in agreement with previous observations[38,40,41]. Interestingly, the ERβ could only be detected in hESC by the primer set that amplified part of the C-terminal region of ERβ. HESC lost their ERα upon serial passage under these culture conditions. It is likely that the single hESC isolation, that did not express the ERα, had already lost its ability to express the ERα. The expression of PR appeared to be less dependent on the passage number of the hESC.

Cultured hEMVEC expressed ERβ, in accordance with *in vivo* studies[18,21,38]. Iruela-Arispe *et al*. reported the presence of ER on hEMVEC[19,20]. As these authors made no distinction between ERα and β, the ER detected may well have been ERβ. Because different splicing variants of ERα have been described caution should be taken to conclude definitively on the absence of ERα in endothelial cells[15,16]. However, none of the presently known splicing variants could be detected in hEMVEC in our experimental conditions.

The precise physiological function and importance of ERβ in the endometrium, as well as in other organs, is still unclear[42]. It has been shown that in the human uterus ERβ is less abundant compared to ERα. ERβ-knockout mice were fertile but showed small litter size, likely due to impaired ovarian function, and multiple resorbed fetuses[42,43]. Furthermore, it has been suggested that a role of ERβ may be antagonizing and/or modulating ERα-mediated actions. Examples of this are the exaggerated induction of VEGF by E_2 via ERα in ERβ-knockout mice and the absence of the physiological down-regulation of PR in the luminal epithelium by E_2 via ERα in ERβ-knockout mice[42,44]. When both ERα and ERβ are co-expressed, they can form homo-or heterodimers and it seems that ERβ preferentially forms heterodimers when ERα is present[45,46].

Krikun *et al*.[21] found, in addition to ERβ, also PR mRNA in hEMVEC. However, they could not confirm the presence of PR immunohistochemically. We detected PR mRNA in only one hEMVEC isolation, although to a very minor extent. In the present and other immunohistochemical studies PR could not be detected in endometrial endothelial cells[18,37]. The faint PCR signal for PR mRNA may reflect a minor contamination of the culture with hESC that has escaped our inspection, or indicate that the cells, when at a higher passage, undergo some kind of decidualization, a condition in which endothelial cells have been described as expressing PR and reacting to ovarian steroids[47,48]. Iruela-Arispe *et al*. detected PR on hEMVEC, in addition to ER, although the levels of PR were significantly lower than those displayed by stromal cells[19,20,49]. These authors reported that only a subpopulation of endothelial cells in normal endometrium stained positive for PR[19].

The effects of E_2 and progesterone on hEMVEC

E_2 and progesterone did not affect the proliferation of hEMVEC to a biologically significant level in our study. Iruela-Arispe *et al*. reported a stimulatory effect of E_2 and an inhibitory effect of progesterone on comparable cells, but only in the presence of high concentrations of VEGF-A and bFGF[20]. Peek *et al*., who examined decidual endothelial cells, found an increased proliferation upon exposure to E_2; a lower dose of E_2 and a high dose of progesterone inhibited proliferation[48]. A positive proliferative response to E_2 was described for hUVEC, but other investigators were unable to confirm this[50-52]. On the contrary, hESC responded well to estrogens. In agreement with Irwin *et al*., we found that hESC showed a slight, though not significant, proliferative response to E_2[53]. When our study was completed, Kayisli *et al.* reported that both E_2 and progesterone stimulated hEMVEC proliferation, albeit to a limited extent, and tube formation in a collagen matrix[54]. However, their finding that the hEMVEC responded to 10^{-12} M progesterone is difficult to explain in the light of their own finding that these cells did not express PR. Taken together, our data and those by other investigators indicate that the effect of E_2 and progesterone on hEMVEC is absent or very small as compared to the effect of these ovarian hormones on hESC.

The meaning of the observed minor effect of E_2 on *in vitro* angiogenesis by hEMVEC is doubtful. This effect was only very small compared to the effect seen after stimulation with VEGF-A[22]. Estrogens can rapidly up-regulate VEGF expression in endometrial stromal and epithelial cells by the direct transcriptional action of the ER. VEGF has been shown to be responsive to E_2 and progesterone[23,41,55,56]. Functional DNA sequences, called estrogen response elements (ERE), have been identified in the VEGF gene that functions as a classical enhancer for both ERα and ERβ. Although the ERE can bind both ERs, it might exhibit a more selective response to ERα[57,58]. Also progestins have a direct effect on VEGF gene transcription as analysis of the sequence of the VEGF promoter revealed three functional progesterone response elements (PREs) and full VEGF promoter activation required all three. Although PR-mediated transcriptional regulation of the VEGF-promoter appeared to be complex and could not be localized to confined PRE sequences, other response-element motifs are thus likely to play a contributory role[59].

When the hEMVEC were co-cultured with hESC, improved survival of hEMVEC was seen. This is probably due to the local generation of VEGF, which resembles the *in vivo* situation, in which a positive correlation between stromal VEGF immunostaining and endothelial cell density was found[24]. Other (angiogenic) factors, such as PDGF and bFGF, may be involved as well, as hEMVEC survived better in co-culture than after sole addition of VEGF-A. The epithelial cells of the endometrium also express VEGF-A. Albrecht *et al*. found that myometrial endothelial cells in co-culture with endometrial epithelial cells formed more tube-like structures than with stromal cells[56]. However, it remains un-

certain to what extent the VEGF-A produced by these cells is available to the endothelial cells, as a mainly apical secretion has been described[60].

To summarize, this study indicates that hEMVEC proliferation and *in vitro* angiogenesis is not much influenced by the ovarian steroids despite ERβ expression in these cells. Ovarian steroids stimulate hESC to produce VEGF-A, a factor to which hEMVEC highly respond. These findings suggest that E_2 and progesterone are indirect regulators of endometrial angiogenesis.

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