Angionesis and the inception of pregnancy

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ENHANCED ANGIOGENIC CAPACITY AND UROKINASE-TYPE PLASMINOGEN ACTIVATOR EXPRESSION BY ENDOTHELIAL CELLS ISOLATED FROM HUMAN ENDOMETRIUM

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Introduction

Human reproduction depends on the rapid cyclical development of a receptive maternal environment necessary for implantation and placentation. Indispensable for this physiological process is angiogenesis, the forming of new blood vessels. It is required for supporting the proliferation and differentiation of glandular and surface epithelial cells and stromal cells, of which the endometrium is mainly composed[1].

Studies have indicated that there are three successive episodes of physiological angiogenesis in the endometrium during the menstrual cycle[2]. The first episode can be seen as post-menstrual repair and occurs during the early proliferative phase, the second episode takes place during the mid-proliferative phase under the influence of estradiol, and the third occurs during the estradiol- and progesterone-mediated secretory phase, when the coiled arteries grow. Together with the changes in vascular permeability throughout the menstrual cycle a transformation of a thin, dense endometrium into a thick, highly edematous secretory endometrium takes place[3].

Regulation of the outgrowth of new vessels is the result of a delicate balance between stimulators and inhibitors and involves several steps. After stimulation of the endothelial cells by angiogenic factors, the basement membrane is degraded by proteolytic enzymes, in particular matrix-degrading metalloproteinases (MMPs) and enzymes of the plasminogen activator system[4]. The cells will then invade, migrate and proliferate under the influence of angiogenic factors into the underlying interstitial matrix and will form new capillary structures[5,6]. It has been suggested that angiogenesis in the endometrium may occur by a process of elongation and expansion of pre-existing vessels[7], a process that differs from the traditional concept of angiogenesis[5,6].

It is generally assumed that urokinase-type plasminogen activator (u-PA) and its inhibitor, the plasminogen activator inhibitor I (PAI-1), are involved in regulation of the first steps of angiogenesis, *i.e.* local proteolytic remodeling of matrix proteins and migration of endothelial cells[6,8,9]. U-PA converts plasminogen into the broadly acting serine protease plasmin, which, in turn, is able to both degrade matrix proteins and activate several MMPs[10-12].

Studies have shown organ-specific characteristics among microvascular endothelial cells[13]. Physiology and pathology involving the endothelium could therefore be best addressed by studies of endothelial cells of the affected organ. As the endometrium is a tissue unique for its cyclic destruction and rapid regeneration of blood vessels, the angiogenic behavior of its endothelial cells is expected to differ from that of endothelial cells in other tissues (e.g. human microvascular foreskin endothelial cells (hFMVEC)). To date, only a few reports describe the isolation and characterization of endothelial cells of the endometrium[14-16]. These isolated human endometrial microvascular endothelial cells

(hEMVEC) express estradiol and progesterone receptors and display an enhanced expression of the VEGF receptor type 2 (VEGFR-2)[14]. Furthermore, the expression of extracellular matrix proteins elastin, collagens and fibronectin by hEMVEC was not detectable, whereas endothelial cells from the human umbilical vein (HUVEC) do express these proteins[15].

The aim of this study was to examine the growth characteristics of hEMVEC and to study the fibrinolytic capacity of these cells and their ability to form capillary-like tubular structures in a three-dimensional (3-D) fibrin matrix. A comparison was made with hFMVEC and HUVEC.

Materials and methods

Materials

Penicillin/streptomycin, L-glutamine and medium 199 (M199) with or without phenol red, and supplemented with 20 mM HEPES was obtained from BioWhittaker (Ver viers, Belgium); new born calf serum (NBCS) and collagenase type II was obtained from Life Technologies (Grand Island, NY). Tissue culture plastics came from Costar/Corning (Cambridge, MA) and Falcon (Becton Dickinson, Bedford, MA). A crude preparation of endothelial cell growth factor (ECGF) was prepared from bovine hypothalamus as described by Maciag *et al*.[17] Human serum was obtained from a local blood bank and was prepared from fresh blood from 10-20 healthy donors, pooled and stored at 4°C. It was heat-inactivated before use. Heparin and thrombin was obtained from Leo Pharmaceutics Products (Weesp, the Netherlands). Human fibrinogen came from Chromogenics AB (Mölndal, Sweden). Dr. H. Metzner and Dr. G. Seemann (Aventis Behring, Marburg, Germany) generously provided factor XIII (Fibrogammin-P). Fibronectin was a gift from Dr. J. van Mourik (Sanquin, Amsterdam, The Netherlands). In addition, the mouse antihuman CD31 antibodies (clone CLB-HEC/75) and human serum albumin (HSA) was obtained from Sanquin (Amsterdam, The Netherlands). Recombinant human vascular endothelial growth factor (VEGF-A) and human placenta growth factor-2 (PlGF-2) were commercially obtained from RELIATech (Braunschweig, Germany) and tumor necrosis factor-α (TNFα), containing 2.45 x 10^7 U/mg protein and less than 40 ng lipopolysacharide/mg protein, a gift from Dr. J. Travernier (Biogent, Gent, Belgium). Recombinant human basic fibroblast growth factor (bFGF) was purchased from PeproTech (Rocky Hill, NJ). Tosyl-activated Dynabeads M450 and goat antimouse IgG coated Dynabeads were from Dynal AS (Oslo, Norway). Ulex europaeus agglutinin-1 (UEA-1) was from Sigma Chemical Co. (St. Louis, USA), and UEA-1 conjugated with fluorescein isothiocyanate (UEA-1-FITC) was from Vector Laboratories (Burlingame, CA). Monoclonal antibodies (mAbs) against cytokeratin 8 (mAb M20) and cytokeratin 18 (mAb M9) were gifts from Dr. G. van Muijen (Dept. Pa-

thology, Academic Hospital Nijmegen, The Netherlands), and the u-PA receptor-blocking mAb H-2 from Dr. U. Weidle (Boehringer Mannheim, Penzberg, Germany)[18]. Rabbit polyclonal anti-u-PA antibodies were prepared in our laboratory. Mouse mAb against smooth muscle cell actin was purchased from Progen Biotechnik GmbH (Heidelberg, Germany). Rabbit antihuman Von Willebrand Factor (vWF) antibodies, FITC-conjugated swine anti-rabbit Ig, FITC-conjugated rabbit antimouse Ig and horseradish peroxidase (HRP)-conjugated goat antirabbit Ig came from Dako Immunoglobulins (Glostrup, Denmark). The rabbit polyclonal antibodies specific for u-PA were prepared in our laboratory[19].

Complementary DNA (cDNA) probes

The following cDNA fragments were used as probes in the hybridization experiments: a 1.02 kb fragment of the human u-PA cDNA[20], a 1.2 kb PstI fragment of rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA (provided by Dr. R Offringa, Leiden University, Leiden, The Netherlands), a 1.05 kb fragment of the human VEGFR-1 cDNA[21], and a 1.4 kb fragment of the human VEGFR-2 cDNA[22].

Cell culture

HUVEC and hFMVEC were isolated and characterized as previously described[23,24]. The HUVEC and hFMVEC were cultured on fibronectin- or gelatin-coated dishes in M199 supplemented with 20 mM HEPES (pH 7.3), 10% human serum, 10% heat-inactivated NBCS, 150 mg/mL ECGF, 5 U/mL heparin, 100 IU/mL penicillin and 100 mg/mL streptomycin (*i.e.* culture medium). HEMVEC were isolated from endometrial tissue as described below and maintained in the above-described culture medium supplemented with 10% human serum and 5 ng/mL VEGF-A (*i.e.* hEMVEC culture medium). Cells were cultured on fibronectin-coated wells at 5% CO_2/95% air until confluence was reached and were subsequently detached with 0.05% trypsin/0.025% ethylenediamine tetraacetate (EDTA) and transferred into fibronectin-coated or gelatin-coated dishes at a split ratio of 1:3. Fresh medium was given three times a week, twice at 2-day intervals and once after a weekend interval. All of the experiments described below were performed in M199 and 20% human serum with HUVEC between passage 1-3, hFMVEC between passage 9-11, and hEMVEC between passage 3-7, respectively.

Isolation and purification of hEMVEC

Endometrial tissue was obtained from pre-menopausal women who had had their uteri removed for benign pathology. All of the patients gave their informed consent according

to the guidelines of the Medical Ethical Review Boards of the Leiden University Medical Center (Leiden, The Netherlands), Bronovo Hospital (The Hague, The Netherlands), and St. Franciscus Gasthuis (Rotterdam, The Netherlands). After removal of the uterus, the endometrial tissue was scraped off and stored into ice-cold storage buffer (140 mM NaCl, 4 mM KCl, 11 mM D-glucose, 10 mM HEPES and 100 IU/mL penicillin and 0.10 mg/mL streptomycin, pH 7.3) at 4°C overnight. The endometrium was minced and incubated in M199/penicillin/streptomycin containing 0.2% collagenase type II at 37°C for two h. Adding the same amount of culture medium stopped the reaction, and all remaining tissue was dissolved by powerful resuspension, resulting in a homogenous solution. After centrifugation (1200 rpm for 5 min at room temperature) the pellet obtained was resuspended in culture medium, and transferred into a fibronectin-coated culture dish. Two to four h later, the nonadhered cells were removed, and the adherent cells were cultured in hEMVEC culture medium.

The primary heterogeneous cell population was grown until near confluence before selection of the endothelial cells using UEA-1-coated Dynabeads. After detachment using trypsin and centrifugation, the cells were resuspended in M199 containing 0.1% HSA with the UEA-1-coated beads (20 beads / target cell). A 15- to 30-min end-over-end rotation was performed at 4°C before the cells that bound to the beads were selected by the use of a magnet (Dynal). The positively selected cell population was cultured in hEMVEC culture medium until confluence and then further isolated using mouse antihuman CD31 antibodies and goat antimouse IgG-coated Dynabeads. Trypsinized cells were incubated with antihuman CD31 antibodies (2 μg/mL in M199/0.1% HSA) for 30 min and kept on ice while being stirred occasionally. The nonbound antibodies were washed away with M199/0.1% HSA before the addition of the goat antimouse IgG-coated Dynabeads. After 15-30 min of incubation with the beads at 4°C, the cells were separated by the use of a magnet. After this selection the CD31-positive cells were cultured in hEMVEC culture medium in fibronectin-coated culture dishes till confluence.

The isolation procedure with anti-CD31 antibodies and antimouse Dynabeads was repeated until a homogeneous culture of endometrial endothelial cells was obtained (as determined after immunofluorescent characterization; see below).

Characterization of the isolated hEMVEC

Immunofluorescent characterization of the hEMVEC was performed on cell monolayers that had been cultured on special optics 96-wells black plates with an ultra-thin clear bottom (Costar/Corning). After washing with M199 containing penicillin/streptomycin, the cells were fixated by the addition of 80% (vol/vol) acetone or 4% formaldehyde for

10 min. The cells were washed with PBS before they were incubated for 30 min with various primary monoclonal or polyclonal antibodies diluted in PBS and 0.3% HSA (PBS/HSA). The control wells were incubated with PBS/HSA only. After washing with PBS/HSA, the cells were incubated with the appropriate second antibody, either FITC-labeled rabbit antimouse (50 μg/mL in PBS/HSA) or FITC-labeled swine antirabbit IgG (20 μg/mL in PBS/HSA).

Incorporation of [^{3}H]thymidine

Incorporation of [^{3}H]thymidine in DNA was determined as the measurement of endothelial cell proliferation. Confluent cultures of endothelial cells were detached by trypsin/EDTA solution and allowed to adhere and spread at a density of 10^4 cells/cm^2 on gelatin-coated dishes in M199-HEPES medium supplemented with 10% heat-inactivated NBCS and penicillin/streptomycin for 18 h. Then the cells were stimulated with bFGF, VEGF-A, or PlGF-2. After an incubation period of 42 h, a tracer amount of [^{3}H]thymidine (0.5 μCi/2 cm^2 well, added in a 10 μL volume) was added to the wells and the cells were incubated for another 6-h period. Subsequently, the cells were washed with PBS and fixated with 100% methanol, ^{3}H-labeled DNA was precipitated in 5% trichloroacetic-acid, and the cells were dissolved in 0.5 mL (0.3 mol/L) NaOH and counted in a liquid scintillation counter. The stimulation index was calculated as follows:

$$\text{Stimulation index} = \frac{(\text{dpm}_{\text{stimulated condition}}) - (\text{dpm}_{\text{background}})}{(\text{dpm}_{\text{control condition}}) - (\text{dpm}_{\text{background}})}$$

Determination of specific u-PA binding

Determination of specific u-PA binding was determined as previously described by Kroon *et al*.[25] In short, cells were incubated for 10 min on ice with 50 mmol/L glycine/HCl buffer (pH 3.0) to remove receptor-bound endogenous u-PA. Subsequently, the cells were incubated on ice with 8 nmol/L ^{125}I-labeled diisopropylfluorophosphate-treated (DIP)-u-PA in endothelial-cell conditioned medium (M199 supplemented with 1% human serum albumin, conditioned for 24 h) for 3 h. After the incubation period, unbound ligand was removed by extensive washing with ice-cold M199. Cell-bound ligand was solubilized with 0.3 mol/L NaOH, and the radioactivity was determined in a γ-counter (Cobra Auto γ, Packard, Meriden, CT). Specific binding was calculated by the subtraction of nonspecific binding from the total binding.

Northern blotting

Total ribonucleic acid (RNA) from hFMVEC and hEMVEC (30 cm^2/condition) was isolated 8 and 24 h after stimulation using the isothiocyanate/phenol acid extraction method described by Chomczynski *et al*.[26] The RNA was dissolved in formamide, and the concentration was determined spectrophotometrically. Equal amounts (7.5 μg) of RNA were separated on a formaldehyde/agarose gel. Subsequently, the separated RNA was transferred to a Hybond-N membrane through capillary force according to the instructions of the manufacturer (Amersham Pharmacia Biotech, Arlington Heights, IL). Hybridization was performed in 7% (wt/vol) SDS, 1 mmol/L EDTA, and 0.5 mol/L NaH_2PO_4/Na_2HPO_4 buffer (pH 7.2) overnight at 63°C with 25 ng of probe labeled with a random primer (Megaprime kit, Amersham Pharmacia Biotech). Thereafter the Hybond membrane was washed twice for 20 min each time with 2x SSC/1% SDS (wt/vol) and three times with 1x SSC/1% SDS (SSC contains 0.15 mol/L NaCl, 0.015 mol/L sodium citrate). Finally, the filters were exposed to a phosphoimager screen and analyzed using a computer.

Enzyme-linked immunosorbent assays

U-PA, tissue-type PA (t-PA), and PAI-1 antigen determinations were performed using commercially available immunoassay kits: u-PA EIA HS Taurus (Leiden, The Netherlands); Thrombonostika t-PA (Organon-Teknika, Turnhout, Belgium), and IMULYSE PAI-1 (Biopool, Umea, Sweden).

Immunohistochemistry

Human endometrial tissues were embedded in paraffin and cut in sections of 4 μm. The cross-sections were prewashed in PBS containing 1% NaN_3 and 0.3% H_2O_2 solution. Subsequently, they were incubated for 15 min in a block buffer (5% BSA in PBS) to reduce background staining. After three wash steps in PBS, the polyclonal anti-u-PA antibody was added (0.01 μg/mL in PBS supplemented with 0.05 % Tween-20 and 0.1% BSA), followed by an overnight incubation at 4°C. The next day, the sections were washed in PBS, horseradish peroxidase-conjugated goat antirabbit Ig (1:1,000 in PBS supplemented with 1% BSA) was added, and the sections were incubated for 1 h at 37°C. Thereafter the sections were washed, and the peroxidase activity was developed with diaminobenzidine. The sections were counterstained with Mayer's haematoxylin and mounted in malinol.

In vitro angiogenesis model

Human fibrin matrices were prepared by the addition of 0.1 U/mL thrombin to a mixture of 2.5 U/mL factor XIII (final concentrations), 2 mg fibrinogen, 2 mg sodium citrate, 0.8 mg NaCl, and 3 μg plasminogen/mL M199 medium. Three hundred microliters of this mixture were added to the wells of 48-wells (1 cm^2) plates. After clotting at 37°C, the fibrin matrices were soaked with M199 supplemented with 10% human serum and 10% NBCS for 2 h at 37°C to inactivate the thrombin.

Type I collagen was solubilized by stirring adult rat tail tendons for 48 h at 4°C in a sterile 1:1,000 (vol/vol) acetic solution (300 mL for 1 g collagen). The resulting solution was extensively dialyzed against 1:10,000 (vol/vol) acetic acid and stored at 4°C[27]. For the collagen gels, 8 volumes of rat tail collagen type I were mixed with 1 volume of 10x M199 and 1 volume of 2% (wt/vol) Na_2CO_3 (mixture pH 7.4). Three hundred-microliter aliquots were added to each well and allowed to gel at 37°C in the absence of CO_2.

Highly confluent hFMVEC and hEMVEC were detached, seeded in a split ratio of 1.25:1 and 2.5:1, respectively, on the surface of the fibrin or type I collagen matrices, and cultured for 24 h in M199 medium without indicator supplemented with 20% human serum, 10% NBCS, and penicillin/streptomycin. Then the endothelial cells were cultured with the mediators indicated for 3-7 days. The culture medium was collected and replaced every 2 or 3 days. Invading cells and the formation of tubular structures of endothelial cells in the 3-D fibrin or collagen matrix were analyzed by phase contrast microscopy.

Statistics

Data for three experiments per well are expressed as the mean ± SEM, and data for duplicate experiments per well are expressed as the mean, with the range between the error bars. Statistical analyses of the data (paired-samples t tests) were calculated using the statistic program SPSS (version 10.0, SPSS, Inc., Chicago, IL).

Results

Isolation and characterization of human endothelial cells from endometrium tissue

HEMVEC were isolated by repeated selection of UEA-1 and CD31-positive cells by means of lectin- or antibody-coated magnetic beads. After two to four rounds of selection, all growing cells were characterized as endothelial cells on the basis of the expression of the classical endothelial markers CD31 and vWF and the capacity to bind UEA-1 (Fig. 1). In

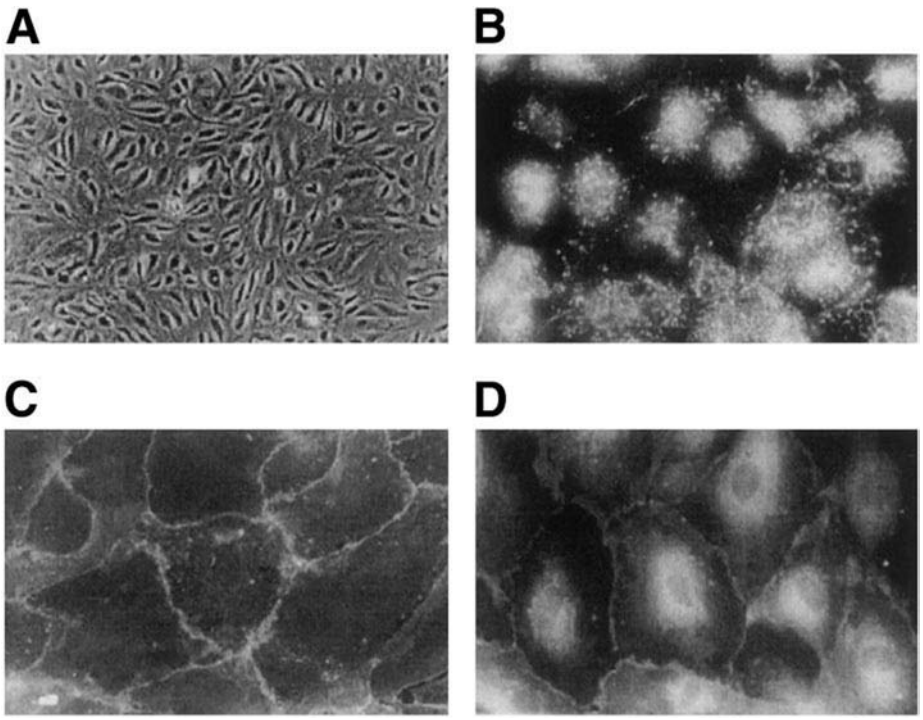

Figure 1. Characterization of hEMVEC.
HEMVEC (passage 5) were cultured to confluence and immunostained as described in *Materials and Methods*. A, Phase-contrast photomicrograph; B-D, indirect immunofluorescent staining of vWF antigen (B) and CD31 antigen (C) or UEA-1-FITC (D) binding.

addition, the cells were negative when stained with antibodies recognizing the epithelial cell markers cytokeratin 8 and 18 and smooth muscle cell actin (data not shown).
In the first instance the isolated hEMVEC were cultured in the presence of high level of serum (20% inactivated human serum and 10% NBCS) and the addition of a crude ECGF preparation on gelatin- or fibronectin-coated culture dishes. This high level of human serum was essential for the maintenance of hEMVEC in culture. Lower amounts of human serum (<20%) resulted in the death of the hEMVEC. Later, after the evaluation of the growth characteristics (see below), the hEMVEC were grown in M199 supplemented with the indicated amount of serum and the combination of 150 μg/mL ECGF and 5 ng/mL VEGF-A. The hEMVEC could be maintained until passage 6-15, varying between the different isolations.

Using the method described, we succeeded in isolating 13 different hEMVEC isolations in 33 attempts. The phase of the menstrual cycle of the women who underwent hysterectomy did not influence the success rate. We succeeded in isolating hEMVEC from proliferative phase tissue as well as from secretory tissue. We

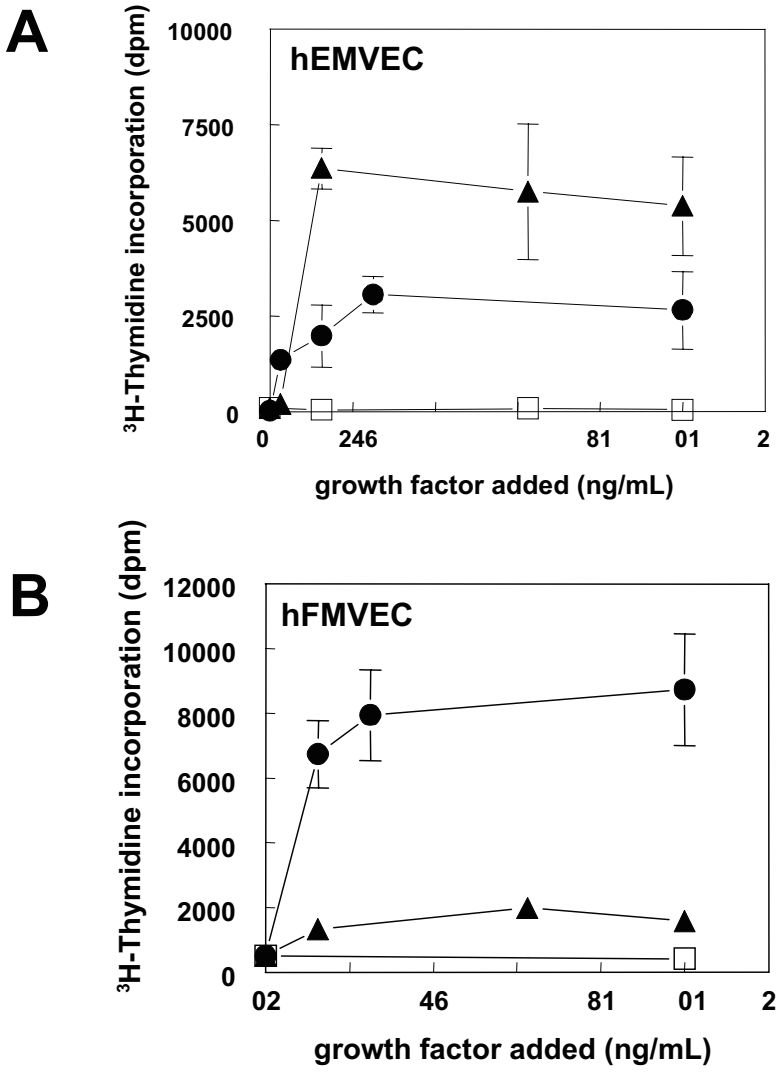

Figure 2. VEGF-A-induced hEMVEC proliferation.
Nonconfluent hEMVEC (A) and hFMVEC (B) were cultured for 48 h in the absence or presence of increasing amount of VEGF-A (▲), bFGF (●), or PlGF-2 (□) in M199 supplemented with 10% NBCS. After 48 h, a tracer amount of [^{3}H]thymidine was added to the medium, the incubation was continued in the same medium for another 6 h, and [^{3}H]thymidine incorporation was determined as described in *Materials and Methods.* The data are expressed as mean ± SEM of triplicate wells and are representative of four experiments performed with different hEMVEC isolations.

never succeeded in isolating hEMVEC from menstruation phase endometrium. Probably the amount of tissue, especially that obtained from the thin (basal) endometrial layer toward the end of the menstruation, was too little to isolate hEMVEC.

Growth characteristics of hEMVEC

The growth characteristics of hEMVEC were compared with those of hFMVEC. HFMVEC (as well as HUVEC, data not shown) were stimulated to proliferate by the addition of bFGF and VEGF-A. These types of human EC react better to bFGF compared with VEGF-A, as determined by the incorporation of 3[H]-thymidine (Fig. 2B). The overall stimulation indexes for HUVEC were 10.9 ± 1.2 and 5.3 ± 0.8 induced by 2.5 ng/mL bFGF and 6.25 ng/mL VEGF-A, respectively ($p=0.00004$; $n=16$), and those for hFMVEC were 12.1 ± 2.0 and. 3.9 ± 0.5 ($p=0.006$; $n=11$). In contrast, VEGF-A was more potent in stimulating hEMVEC to proliferate compared with bFGF (Fig. 2A). The mean stimulation index of hEMVEC was 16.8 ± 4.8 using 2.5 ng/mL bFGF and 30.0 ± 8.3 using 6.25 ng/mL VEGF-A ($p=017$; $n=7$, performed with hEMVEC from four different donors). The stimulation index for hEMVEC was higher than those of HUVEC and hFMVEC due to the lower proliferative capacity of the hEMVEC under control condition (only in the presence of 10% NBCS). Neither hEMVEC nor hFMVEC responded to PlGF-2 (Fig. 2).

The enhanced responsiveness of hEMVEC to VEGF-A was probably due to an enhanced basal expression of the messenger RNA (mRNA) of VEGFR-2 (Fig. 3). Densitometric analysis of the blots revealed a 2.4 fold increase in VEGFR-2 mRNA in hEMVEC compared with hFMVEC. The expression of VEGFR-1 mRNA was very low and comparable in the different endothelial cell types (data not shown).

Production of plasminogen activators and expression of u-PAR by hEMVEC

Compared with other types of humane endothelial cells, unstimulated hEMVEC produced considerably more u-PA. In 24 h, they accumulated 33.8 ± 6.9 ng/10^5 cells ($n=5$) compared with 0.1 ± 0.1 in HUVEC ($n=4$, data not shown). This was not due to a difference in reuptake of u-PA:PAI-1 complexes by the cellular u-PA receptor, because after blockade of the u-PA/u-PAR interaction by monoclonal antibody H-2, the levels of u-PA antigen were 43.2 ± 7.0 (Table 1) and 6.2 ± 2.3 ng/10^5 cells for hEMVEC and HFMVEC (data not shown), respectively. The overall production of u-PA by hEMVEC was enhanced by 1.3-, 1.5- and 1.8-fold after a 24-h incubation with bFGF, VEGF-A or their combination (Table 1). TNFα further enhanced the production of u-PA antigen by hEMVEC. The relatively high u-PA production by hEMVEC compared with hFMVEC was also found at the mRNA level (Fig. 3).

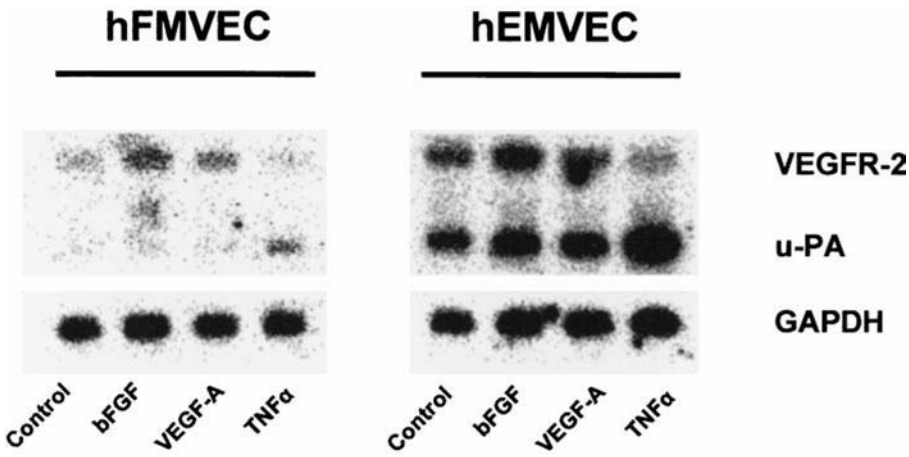

Figure 3. Expression of u-PA, and VEGFR-2 mRNA in hEMVEC.
Confluent (passage 6) hEMVEC and (passage 11) hFMVEC, cultured on fibronectin-coated wells, were stimulated with or without bFGF (10 ng/mL), VEGF-A (100 ng/mL), or TNFα (10 ng/mL). Total RNA was isolated at 24 h and analyzed by Northern blotting for u-PA and VEGFR-2 mRNA. Equal loading was checked by hybridization with a glyceraldehydes-3-phosphate dehydrogenase mRNA. This experiment was performed with two different hEMVEC isolations with similar results.

Table 1. Production of u-PA, t-PA, and PAI-1 by hEMVEC

Addition	u-PA (ng/10^5 cells)		t-PA (ng/10^5 cells)	PAI-1 (ng/10^5 cells)
	- anti-u-PAR mAb	+ anti-u-PAR mAb	- anti-u-PAR mAb	- anti-u-PAR mAb
None	33.8 ± 6.9	43.2 ± 7.0	1.3 ± 0.2	186 ± 42
bFGF	39.1 ± 7.5	54.9 ± 7.7*	1.5 ± 0.3	209 ± 43
VEGF-A	38.8 ± 7.9	64.2 ± 10.1*	2.3 ± 0.4*	218 ± 50
bFGF/VEGF-A	45.8 ± 12.3	78.5 ± 11.2*	2.2 ± 0.3*	196 ± 56
TNFα	91.6 ± 9.8*	nd	1.5 ± 0.3	249 ± 40*
bFGF/TNFα	77.4 ± 8.1*	nd	1.4 ± 0.5	282 ± 58*

HEMVEC were cultured on gelatin-coated wells till confluence in hEMVEC culture medium and preincubated with M199 supplemented with 20% human serum for 24 h. Then the hEMVEC were stimulated with 10 ng/mL bFGF, 100 ng/mL VEGF-A, 10 ng/mL TNFα, or the combination of these mediators in M199 plus 20% human serum. After 24 h, supernatants were collected, and the cells were counted. The u-PA, t-PA, and PAI-1 amounts were determined by ELISA as described in *Materials and Methods*. The data are expressed as the mean ± SEM of 5 experiments/isolations in nanograms per 24 h/10^5 cells. ND, not done.
* $p < 0.05$ compared to control (no addition).

The overall production of t-PA and PAI-1 antigens was 1.3 ± 0.2 and 186 ± 42 ng/10^5 cells, respectively. The production of t-PA antigen was increased by VEGF-A, whereas PAI-1 antigen was enhanced by TNFα (Table 1).

Binding of [^{125}I]DIP-u-PA to cellular u-PA receptor on hEMVEC and hFMVEC revealed comparable binding of u-PA to its receptor and a similar increase induced by VEGF-A, bFGF and simultaneous addition of bFGF/VEGF-A, bFGF/TNFα or VEGF/TNFα (Fig. 4). The average basal u-PA binding to hEMVEC was 4.2 ± 1.4 (n=3) fmol/10^5 cells, whereas that to hFMVEC was 3.3 ± 0.8 fmol/10^5 cells (triplicate wells, n=1, Fig. 4B).

These data indicate that hEMVEC produce very high amounts of u-PA compared with other types of human endothelial cells, whereas their expression and regulation of other fibrinolytic regulators, *i.e.* u-PAR, t-PA and PAI-1, are similar to those of other human endothelial cell types.

Immunolocalization of u-PA in human endometrium tissue samples

To compare the high amount of u-PA accumulation *in vitro* with the *in vivo* situation, sections of endometrium and myometrium were studied. Immunostaining for u-PA was found on the vessels of the endometrium and myometrium (Fig. 5) and in the stroma of the endometrium, whereas surface and glandular epithelial cells were negative for the u-PA antigen (Fig. 5). A negative staining procedure (the same staining procedure but without the primary antibody) showed no staining of the vessels and stroma (data not shown).

In vitro capillary-like tube formation by hEMVEC in 3-D fibrin matrices

Subsequently, we evaluated the ability of hEMVEC to form capillary-like tubular structures in an *in vitro* angiogenesis assay, consisting of human endothelial cells cultured on 3-D fibrin matrices[28]. As we had previously found, hFMVEC form capillary-like tubular structures when stimulated with the combination of VEGF-A (or bFGF) and TNFα after a culture period of 7 days (Fig. 6E)[28,29]. The addition of growth factor alone did not result in the formation of tube-like structures, but induced holes in the monolayer of endothelial cells due to uncontrolled lysis of the fibrin matrix (Fig. 6D)[29]. When hEMVEC were cultured on top of the fibrin matrix in the presence of 20% human serum, they began forming capillary-like tubes within 3 days (Fig. 6A). This spontaneous tube formation was enhanced by the addition of VEGF-A (Fig. 6B). Further enhancement and stabilization of the tube-like structures were seen when the growth factors were added in combination with TNFα (data not shown). Maximal induction of the formation of tube-like structures by hEMVEC was seen on days 2-3, whereas those formed by hFMVEC were maximal on days 7-10. The extent of tube formation was markedly inhibited by polyclo-

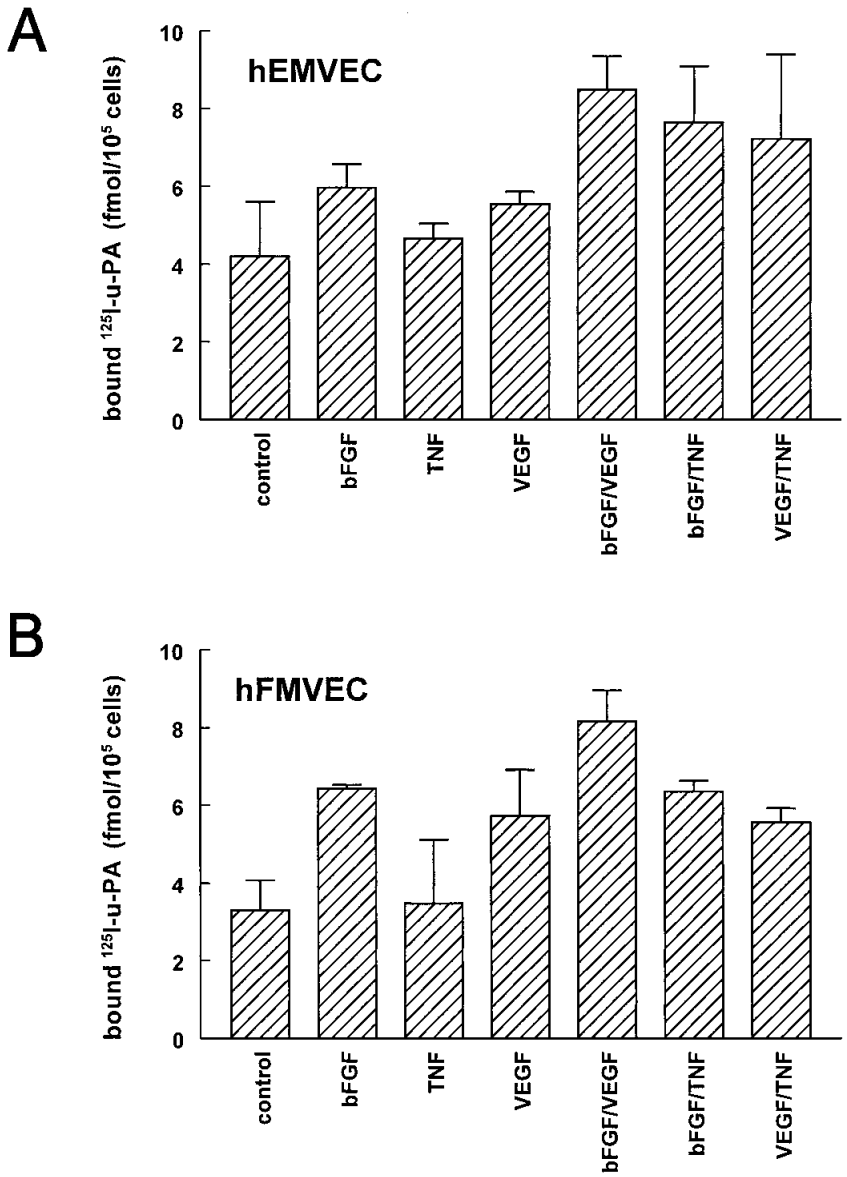

Figure 4. u-PA binding to hEMVEC and hFMVEC.
HEMVEC and hFMVEC were preincubated for 24 h in M199 supplemented with 20% human serum with or without bFGF (10 ng/mL), VEGF-A (100 ng/mL), TNFα (10 ng/mL), or the combination of these mediators. Subsequently, the cells were cooled on ice, and the specific binding of [^{125}I]DIP-u-PA was determined in triplicate wells as described in *Materials and Methods*. A, Data for u-PA binding to hEMVEC expressed as mean ± SEM in femtomoles per 10^5 cells of three independent experiments performed in triplicate wells. B, A representative experiment showing u-PA binding to hFMVEC. The binding is expressed as mean ± SEM in femtomoles per 10^5 cells of triplicate wells.

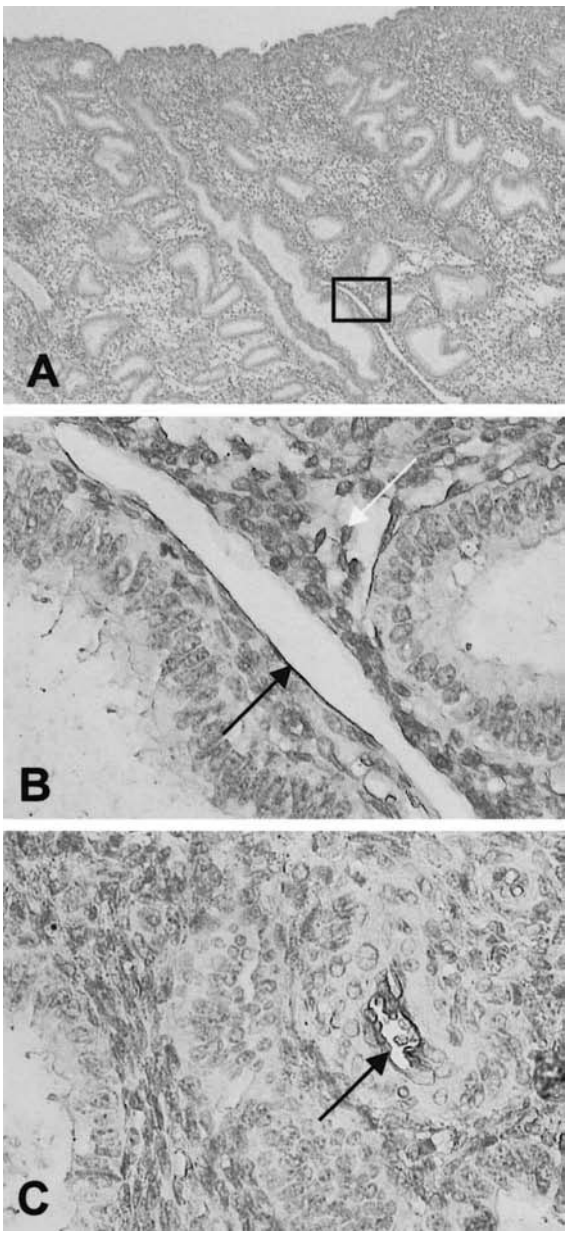

Figure 5. Expression of u-PA in blood vessels in human secretory phase endometrial tissue. Immunohistochemistry was performed with labeled antibody to u-PA on paraffin sections of human endometrium, as described in *Materials and Methods*. A, Brown staining shows accumulation of u-PA in the stromal endometrium; the surface and glandular epithelium are negative for the u-PA antigen (20x magnification). The *rectangle area* is enlarged in B. B and C, *Black arrows* indicate examples of positive endothelial cells, and a *white arrowhead* indicate positive stromal cells (200x magnification). The same results were obtained using proliferative phase endometrium. *[See appendix: color figures]*

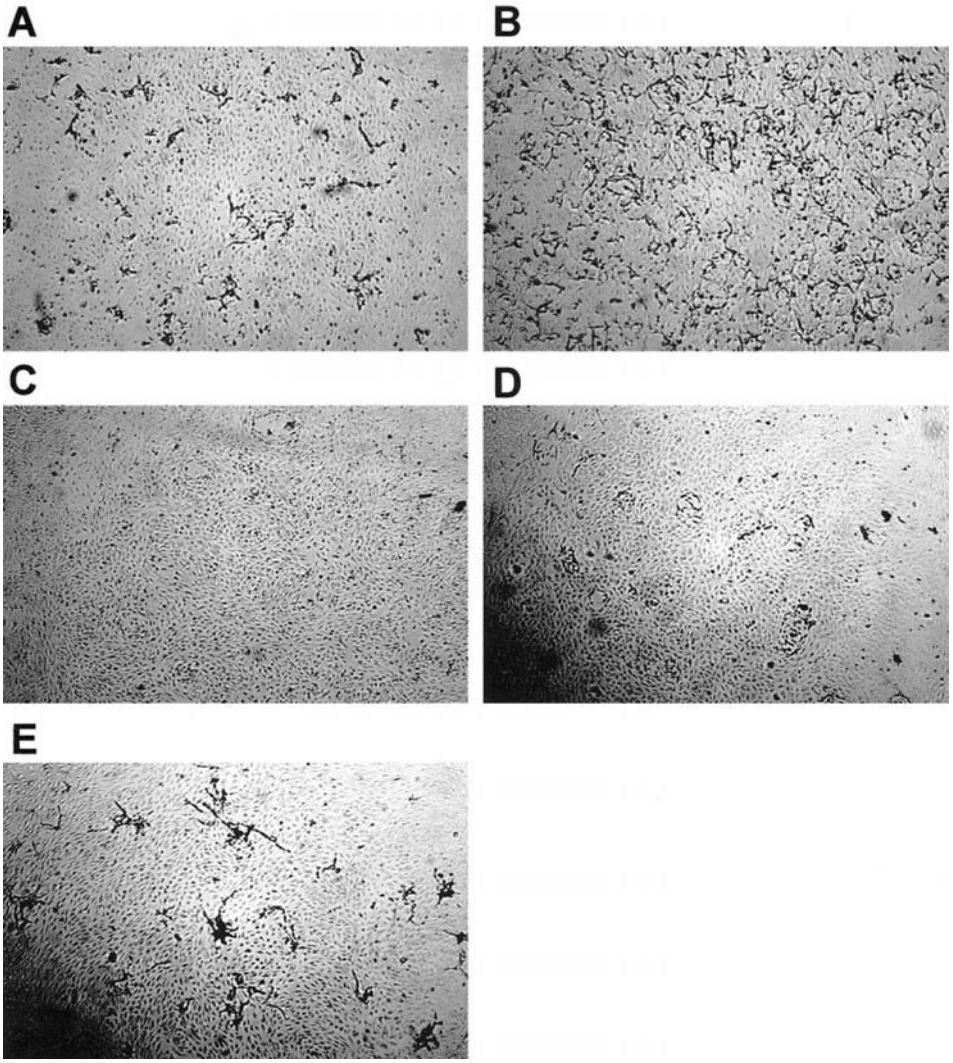

Figure 6. Enhanced capillary-like tube formation by hEMVEC.
HEMVEC (passage 7) were cultured on top of a 3-D fibrin matrix in M199 supplemented with 20% human serum and 10% NBCS and stimulated without (A) or with 10 ng/mL VEGF-A (B). After 2 days of culture, nonphase contrast views were taken, which show in both panels growth of capillary-like tube structures. In contrast, hFMVEC (passage 11) stimulated without (C) or with 25 ng/mL VEGF-A (D) cultured for 6 days show no capillary-like tube structures. E, hFMVEC cultured on fibrin for 6 days in the presence of the combination of 25 ng/mL VEGF-A and 1 ng/mL TNFα. The data are representative of four different hEMVEC isolations.

nal anti-u-PA antibodies under both unstimulated as well as VEGF-A-stimulated (71.2 ± 5.3%; n=4) conditions.

A similar increased angiogenic capacity of the hEMVEC was observed when hEMVEC were cultured on top of rat tail collagen type I matrices. Under our culture conditions, unstimulated hFMVEC did not or hardly invaded the collagen matrix, which was slightly enhanced by addition of the combination of bFGF or VEGF-A and TNFα (data not shown). However, those few hFMVEC that were able to invade the matrix did not form tube-like structures with lumens surrounded by endothelial cells, but formed sprouts at or just beneath the collagen surface[45]. HEMVEC displayed an increased invasion and sprout formation in the collagen matrix that was increased by the addition of VEGF-A alone. However, sprouts of this type of human endothelial cells did not contain clear lumen-like structures (data not shown).

Discussion

Here we describe the isolation and characterization of hEMVEC from various donors. These endothelial cells displayed an enhanced responsiveness to VEGF-A compared with hFMVEC due to an enhanced expression of VEGFR-2. In addition, hEMVEC are more angiogenic when cultured in the presence of 20% human serum on top of a 3-D fibrin matrix or 3-D collagen matrix compared with hFMVEC. The hEMVEC formed tube-like structures within 2-4 days that were enhanced by the addition of VEGF-A alone. This in contrast to hFMVEC, which had to be stimulated with the combination of VEGF-A and TNFα for a period of 7 days to form tubes. The enhanced angiogenic behavior of the hEMVEC was probably due to an increase in expression of u-PA, facilitating an enhanced proteolytic capacity to the hEMVEC.

Overt angiogenesis takes place in the endometrium throughout the reproductive life of the female during each menstrual cycle. Therefore, the endothelial cells of the endometrium must be able to respond quickly to changes in steroids and environmental conditions and to angiogenic mediators to form a new vascular bed to allow proper implantation and placentation. To study the process of angiogenesis in the endometrium we isolated hEMVEC using a standard isolation procedure of endothelial cells consisting of a digestion of the endometrial tissue and selection of endothelial cells using UEA-1 or anti-CD31-coated magnetic beads. HEMVEC were difficult to maintain in culture. Only in the presence of 20% human serum and VEGF-A (and/or ECGF) were the cells were able to proliferate and to be passed up to passages 6-15. The sensitivity of the cells to the culture condition may be a reflection of the *in vivo* situation, where rapid apoptosis of the cells in the endometrium starts in the secretory phase and peaks in the menstrual phase[30].

The family of the VEGF growth factors is thought to play an important role in the process of angiogenesis[31,32]. Both the expression of VEGF[3,33,34] and the specific VEGF receptors VEGFR-1 and VEGFR-2 are found in the endometrium during the three stages of the menstrual cycle[35,36]. In particular, the expression of VEGFR-2 on the vessels was increased during the proliferative phase[35]. Cultured hEMVEC also display enhanced expression of VEGFR-2 compared with hFMVEC or HUVEC, whereas the expression of VEGFR-1 is comparable among these EC types. As it is generally accepted that VEGF-A-induced endothelial proliferation is mediated *via* VEGFR-2[37,38], the enhanced expression of this VEGFR may be an explanation for why hEMVEC are more reactive toward VEGF-A than to bFGF compared with hFMVEC. These data are in accordance with recently published data by Iruela-Arispe *et al.*[14], who also showed that hEMVEC expressed increased levels of VEGFR-2 and an increased proliferation in response to VEGF-A.

Most striking was the finding of the relative high u-PA expression by hEMVEC, whereas the levels of the other compounds of the plasminogen system, t-PA and PAI-1, were not significantly enhanced compared with those in hFMVEC or HUVEC[28,39]. The average u-PA binding to the u-PAR was also similar (4.2 ± 1.4 (n=3) fmol/10^5 cells vs. 6.4 ± 3.2 (n=17)[28], and 2.6 ± 1.8 (n=8)[28] fmol/10^5 cells for hEMVEC, HUVEC, and hFMVEC, respectively). Under basal conditions, hFMVEC do not express such high levels of u-PA (0.2 ± 0.1 ng/10^5 cells; n=9)[28,40]. Only when stimulated with the inflammatory mediator TNFα, but not with VEGF-A or bFGF, do both hFMVEC and HUVEC start to secrete considerable levels of u-PA (up to 1 ng/10^5 cells)[28,39,41]. However, production of u-PA by activated hFMVEC and HUVEC is still a magnitude lower than basal or TNFα-stimulated u-PA production by hEMVEC. In contrast to that by hFMVEC[28], u-PA production by hEMVEC increased after addition of the angiogenic growth factors bFGF, VEGF-A, or the combination of these mediators with TNFα. The high expression of u-PA by the hEMVEC *in vitro* was confirmed by immunohistochemical staining of endometrial tissue obtained from healthy premenopausal women. The endothelial cells of the vessels in the myometrium as well as the vessels of the endometrium *in vivo* showed expression of the u-PA antigen. In normal human tissue the expression of endothelium-associated u-PA is hardly detectable, but there is an increase in endothelial cell expression of u-PA detectable in inflamed tissues, such as during appendicitis[42], and tumor angiogenesis[43], in atherosclerotic vessels[44], and in vessels in atherosclerotic plaques[25].

Simultaneously with the enhanced u-PA expression, hEMVEC displayed an enhanced capacity to form tube-like structures in 3-D fibrin matrices and sprout formation in collagen matrices compared to hFMVEC. The formation of tube-like structures of hFMVEC in fibrin matrices depends on u-PAR-localized u-PA activity[25] and occurs only after stimulation of the cells with the combination of a growth factor (either bFGF or VEGF-A) and the inflammatory mediator TNFα[28], which is a potent stimulator of the production of u-PA

by human EC. It is possible that the spontaneous tube formation by the hEMVEC may be due to a response of the hEMVEC (mediated *via*, for instance, the increased VEGFR-2 expression) to growth factors in human serum in combination with the enhanced basal u-PA expression, as shown by the inhibition of tube formation after the addition of neutralizing anti-u-PA antibodies. The enhanced u-PA/plasmin activity, which is also able to activate several MMPs, such as MMP-1, MMP-3, and MMP-9 *in vitro*[10-12], may provide endothelial cells in the endometrium with enhanced angiogenic capacity, as shown *in vivo*.

In conclusion, we show that human endometrium-derived endothelial cells display an enhanced proteolytic capacity and an enhanced angiogenic capacity. These data provide us with a better understanding of the regulation, production, and physiological responses of the vasculature in the endometrium and may lead to new insight into pathology during pregnancy, which may be related to diseases later in life and therapeutic strategies in the future.

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