Soluble factors from human endometrium promote angiogenesis and regulate the endothelial cell transcriptome

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BACKGROUND: Angiogenesis and vascular remodeling play critical roles in the cyclical growth and regression of endometrium. They also appear to play roles in the pathogenesis of endometriosis. METHODS and RESULTS: Supernatants were collected from cultured endometrium isolated from women with and without endometriosis. These supernatants induced endothelial cell proliferation and angiogenesis in vitro. They contained vascular endothelial growth factor (VEGF)-A, and their proliferative effects on endothelial cells were partially abrogated by a blocking anti-VEGF-A antibody. Gene array analysis showed that culture supernatants from proliferative phase endometrium, and to a lesser extent secretory phase endometrium, induced significant changes in the transcriptome of endothelial cells. We could not detect any association between endometriosis and the ability of endometrial-derived soluble factors to promote angiogenesis or to regulate the endothelial transcriptome. In addition, we could not detect any association between endometriosis and the concentration of VEGF-A in supernatants from cultured endometrium or in menstrual effluent. CONCLUSIONS: We have shown that endometrium cultured in vitro produced soluble factors, including VEGF-A, that promoted angiogenesis. Proliferative phase endometrium promoted significant endothelial cell transcriptome changes that appear overall to be pro-angiogenic. These transcriptome changes provide insight into the dynamic control of vessel structure on which both eutopic endometrium and endometriotic lesions depend.

Key words: endometrious/gene array/VEGF-A/transcriptome

Introduction

Angiogenesis and vascular remodeling play critical roles in the cyclical growth and regression of the endometrium (reviewed by Gargett et al., 2001). In the proliferative phase of the menstrual cycle, angiogenesis occurs in the stratum functionalis as the endometrium approximately quadruples in thickness. In the secretory phase of the cycle, spiral arterioles lengthen and become more coiled and the sub-epithelial capillary plexus matures. In the post-menstrual phase, angiogenesis is involved in the repair of the superficial layer of the remaining stratum basalis. Endometrial angiogenesis is tightly coordinated with recruitment of vascular smooth muscle cells to the nascent vessels (Kohnen et al., 2000). Endometrial angiogenesis may be directly controlled by reproductive steroids (Iruela-Arispe et al., 1999; Hague et al., 2002) and indirectly controlled by angiogenic regulators expressed by the diverse cell lineages present in the endometrium. Potential regulators of endometrial angiogenesis include: nitric oxide (Cameron et al., 1998), vascular endothelial growth factor (VEGF)-A (Charnock-Jones et al., 1993; Donnez et al., 1998), VEGF-B and -C (Mints et al., 2002; Moller et al., 2002), relaxin (Unemori et al., 1999), fibroblast growth factors (FGF) (Ferriani et al., 1993), matrix metalloproteinases (MMPs) (Freitas et al., 1999), thymidine phosphorylase (Fujwaki et al., 1999), angiopoietin-2 (Krikun et al., 2000), erythropoietin (Yasuda et al., 1998), adrenomedullin (Nikitenko et al., 2000) and epidermal growth factor (EGF) (Sandberg et al., 2001). Leukocyte-derived factors may play a particularly important role. Angiopoietin-2, VEGF-C and placental growth factor (PIGF) expressed by endometrial NK leukocytes (Li et al., 2001) may drive spiral arteriole and sub-epithelial capillary plexus changes during the secretory phase of the cycle (Sandberg et al., 2001), while VEGF-A expressed by intra-vascular neutrophils may promote angiogenesis in the stratum functionalis during the proliferative phase of the cycle (Gargett et al., 2001).

Endometriosis, the growth of ectopic endometrial fragments in the peritoneum, is a debilitating disease that causes...
pain with menstruation, pain with intercourse and other symptoms. In addition, endometriosis also causes infertility (Haney, 1990). Despite a significant recent increase of the disease coincident with the introduction of widespread fertility regulation, the cause of the problem remains unknown. Tissues that grow to greater than \( \sim 1 \) mm\(^3\) need to establish a blood supply to prevent necrosis (Folkman, 2002). Therefore, for endometriotic lesions to survive they must recruit a vasculature from the surrounding tissues (reviewed by Smith, 1997). However, the processes that promote this angiogenesis are as yet uncertain. They may involve both endometrial and peritoneal factors—several examples of each have been proposed. Potential pro-endometriotic endometrial factors include increased proliferation capacity of endometrial endothelial cells (EC) (Wingfield et al., 1995) and elevated expression of several molecules including: the pro-angiogenic integrin \( \alpha v \beta 3 \) (Hii et al., 1998), hepatocyte growth factor and its c-Met receptor (Khan et al., 2003), endoglin (Kim et al., 2001), nitric oxide synthases (Wu et al., 2003), urokinase receptor (Sillems et al., 1998) and tissue inhibitor of metalloproteinases-2 (TIMP-2) (Sillems et al., 1998). Potential peritoneal factors include: macrophage migration inhibitory factor (MIF) in peritoneal fluid of endometriotic women (Kats et al., 2002), steroidally-regulated VEGF-A production by peritoneal macrophages (McLaren et al., 1996), elevated tumour necrosis factor (TNF)-\( \alpha \) production from peritoneal macrophages (Noda et al., 2003; Richter et al., 2004) and elevation of soluble TNF receptor (Steff et al., 2004), angiogenin (Steff et al., 2004) and PI GF (Suzumori et al., 2003).

Previously, the programmed variation of the transcriptome of whole endometrium through the menstrual cycle has been characterized (Borthwick et al., 2003; Kao et al., 2003). However, the effects of soluble factors produced by cells within the endometrium on endothelial gene expression, cell biology and angiogenesis have not been fully investigated. In this study, we have assessed the ability of the soluble factors secreted by endometrium collected from non-endometriotic and endometriotic women in the proliferative and secretory phases of the menstrual cycle to promote EC proliferation and angiogenesis and to modulate the EC transcriptome. Given the important role played by VEGF-A in angiogenesis, we have also assessed the concentration of VEGF-A in endometrial culture supernatants and in menstrual effluent from women with and without endometriosis.

**Materials and methods**

**Collection and culture of endometrium**

This study was approved by the local ethical review committee and patient consent was obtained for the collection of all human tissues used in this study. Since anti-inflammatory medications or medications related to reproductive steroids alter the cellular composition of endometrium and are therefore likely to alter the pro-angiogenic properties of endometrium, women currently on these medications as well as women who had received courses of these drugs in the previous 3 months were excluded. Women were also excluded who had irregular menstrual cycles or who were using an intrauterine device. In all women in the endometriotic group of this study the clinical diagnosis of endometriosis was confirmed laparoscopically. Women in the non-endometriotic group had no symptoms of endometriosis and in addition, 6 out of the 10 women in this group had the absence of endometriosis confirmed by laparoscopy. Only three of the women in the study were smokers (two non-endometriotic proliferative phase donors and one endometriotic secretory phase donor). Endometrium was collected by Pipelle biopsy, and the approximate stage of the menstrual cycle confirmed by histology. Adhering blood clot and included myometrial tissue were removed by washing and dissection. The endometrium was then disaggregated by incubation with agitation in phosphate-buffered saline (PBS) containing 100 mg/ml collagenase (Sigma, UK) and 100 mg/ml DNase (Sigma, UK). The disaggregated endometrial cells were then washed twice in PBS and plated at 70% confluence in tissue culture dishes in a fully humidified atmosphere of 5% CO\(_2\) in a proprietary culture medium (LVEC, large vessel endothelial cell medium; TCS, Botolphi, UK) supplemented with a mixture of heparin, hydrocortisone, epidermal growth factor, fibroblast growth factor, 2% fetal calf serum (FCS), gentamycin and amphotericin. This culture medium maintained both EC (for which it was designed) and endometrium, since the appearance of disaggregated endometrium after 4 days in this culture medium was equivalent to that of endometrium cultured in DMEM/Hams F12 (50:50 mix) supplemented with 10% FCS (data not shown). No reproductive steroids were added to the culture medium since reproductive steroids (Morales et al., 1995; Alvarez et al., 1997) and their metabolites (Yue et al., 1997) have been shown to affect EC. Disaggregated monolayer cultures were chosen in preference to explant cultures to maintain optimal oxygenation and nutrition for all cells, and to allow visible assessment of endometrial cell viability. After 24 h in culture the endometrial monolayers were washed twice in medium in order to completely remove dead cells and erythrocytes. After a further 24 h in the LVEC medium, culture supernatants were collected, centrifuged and stored at \(-70^\circ\)C. To assess the cellular composition of the endometrial cultures, proliferative and secretory phase endometrium were disaggregated as described above, cultured on chamber slides for 24 h, fixed at \(-20^\circ\)C with 50:50 acetone:ethanol, immunostained with an anti-pan cytokeratin antibody cocktail (DAKO N1589) and a fluorescein isothiocyanate (FITC)-conjugated secondary antibody and detected using an epifluorescence microscope.

**EC proliferation and angiogenesis assays**

Human umbilical vein EC(s) (HUVEC) were isolated by collagenase digestion as described (Jaffe et al., 1973) and cultured to passage 5 in LVEC medium. Once at passage 5, HUVEC were plated into 24-well plates at 70% confluence and supernatants from endometrial cultures added (20% V/V) for 24 h. Viable HUVEC numbers were then counted using a haemocytometer after trypan blue staining (0.2% Sigma, UK). Four wells were used for each experimental condition. The 20% V/V dilution of the endometrial cell culture supernatants was chosen empirically since it on average induced maximal HUVEC responses without inducing signs of stress in these cells (data not shown). To prevent confounding, medium of identical composition (LVEC medium described above) was used for culture of all endometrial explants and all HUVEC in this study. Since we have shown that quiescing HUVEC by transfer to low serum medium reduces the ability of these cells to mount transcriptional responses to growth factors (Johnson et al., 2003), the HUVEC were not quiesced before incubation with the endometrial culture supernatants. In additional experiments, a blocking anti-VEGF-A monoclonal antibody (clone 26503, R&D Systems, UK) was added
to HUVEC cultured as above, and relative cell numbers estimated using a tetrazolium compound (MTS) assay (CellTiter 96 Aqueous Non-Radioactive Cell Proliferation Assay; Promega) used according to the manufacturer’s instructions.

In vitro angiogenesis assays consisted of co-cultures of HUVEC and human dermal fibroblasts (Bishop et al., 1999). They were purchased pre-plated in 24-well tissue culture plates from TCS Cellworks (ZHA-1000; Botolph, UK) and cultured in the LVEC medium for 11 days with or without the addition of endometrial culture supernatants (20% V/V, replaced every 4 days). After 11 days cultures were fixed in 2% paraformaldehyde for 20 min at 4°C, permeabilized in 0.1% Triton X-100 and 0.1% Tween-20 for 30 min at room temperature, stained with Ulex–FITC (Sigma L9006, UK) at a concentration of 10 μg/ml and Hoescht Bisbenzimide (33342 (Calbiochem 382061, UK) at a concentration of 1 μg/ml. For each experimental condition, the number of branches and total length of capillary-like structures was measured by imaging 21 randomly chosen 1 mm² fields using a confocal microscope (Lieca, Germany). Images were processed to quantify angiogenesis using the TCS Angiogenesis assessment software (TCS, Botolph, UK).

**Gene array analysis**

RNA was prepared from HUVEC using Trizol reagent (Gibco/BRL, UK) followed by clean up through an RNeasy spin column (Qiagen, UK) and ethanol precipitation. RNA integrity was assessed using an Agilent 2100 bioanalyzer (Agilent, UK). β²P-labelled complex cDNA probes were prepared from the RNAs and hybridized to an array of ~1000 cDNAs immobilized onto nylon filters as described (Evans et al., 2003). Hybridization was quantified using a phosphoimager (Molecular Dynamics Inc., CA) and IMAGENE software (BioDiscovery, CA). After global and local normalization (Evans et al., 2003), transcript abundance data were compared using the CyberT algorithm (version 7.03; sliding window = 301, Bayes estimate = 10). This algorithm is an unpaired t-test, modified by the inclusion of a Bayesian prior based on the variance of other transcripts in the data set (Long et al., 2001). For further statistical analysis, the ‘R’ statistical software system and GeneSpring Expression Analysis Software (Silicon Genetics, Redwood City, CA) were used.

**Quantitative polymerase chain reaction analysis**

The ABI PRISM 7700 Sequence Detection System (TaqMan) was used to perform real-time polymerase chain reactions according to the manufacturer’s protocols. ‘Ct’ values for each transcript were compared to those for cyclophilin A, which according to the gene array results remained relatively constant in abundance. Primers and probes for GSTP-1, VE-cadherin, IL1RL-1 and vimentin were proprietary oligonucleotides obtained from Applied Biosystems (ABI, UK) ‘Assays-on-Demand’ and used a 5’ FAM reporter and 3’ non-fluorescent minor groove binder.

**ELISA for VEGF-A**

To quantify VEGF-A in endometrial culture supernatants an ELISA assay was used (R&D systems, UK) according to the manufacturer’s instructions, and the VEGF-A concentration determined by measuring absorbance at 490 nm by comparison with a standard curve. For each measurement six replicates were performed.

**Collection of menstrual effluent**

Menstrual effluent was collected by volunteers by vaginal insertion of a menstrual cup (‘The Keeper’™, Eco Logique Inc., Canada) in the second 24 h of bleeding. The cup was left in situ for 4 h, the fluid sample collected and the volume measured. Menstrual cup samples were washed with an equal volume of PBS and centrifuged at 200 g for 10 min at 4°C. The supernatant was stored at −70°C for ELISA which was performed as described above.

**Statistical analysis**

All experimental data except those generated from gene arrays were analysed using the ‘R’ statistical software package (http://rweb.stat.umn.edu/R/doc/html/index.html). Before comparisons were made, data were tested for normality using Chi squared tests and then for equal variance between groups using Bartlett’s method. Based on these results, we were able to use multi-way analysis of variance (ANOVA). In all cases where ANOVA suggested a statistically significant effect, post hoc Tukey’s Multiple Comparison Tests were used to assess the source of the effect.

**Results**

**Supernatants from cultured endometrium promote endothelial cell proliferation and angiogenesis in vitro**

To assess the effects of soluble factors derived from endometrium on endothelial cell biology, endometrium was collected from five women without endometriosis in each of the mid-proliferative (days 6–10) and mid-secretory (days 16–21) phases of the menstrual cycle using Pipelle biopsy. Endometrial cells were disaggregated, cultured (see Methods) and culture supernatants collected over a 24 h period. These proliferative and secretory phase endometrial culture supernatants were applied to HUVEC (20% V/V). After 24 h exposure to these supernatants or to medium alone (control) or 10 ng/ml VEGF-A, cell numbers were counted. Relative to cultures supplemented with 10 ng/ml VEGF-A (standardized as 100%), cultures supplemented with medium alone contained 40 ± 3.5% cells, while cultures supplemented with the proliferative and secretory phase endometrial supernatants contained 88 ± 19% and 84 ± 10% cell numbers, respectively (expressed as mean ± 2 × standard error). ANOVA (P ≤ 0.05) followed by Tukey’s post hoc tests indicated that the differences in cell number between the control cultures and the cultures supplemented with either proliferative or secretory phase endometrial supernatants were significant. However, the effects of the supernatants from proliferative and secretory phase endometrial cultures were not significantly different from one another.

To assess whether soluble factors derived from endometrium could promote angiogenesis in addition to promoting endothelial proliferation, endometrial culture supernatants were added (20% V/V) to co-cultures of HUVEC and human dermal fibroblasts for an 11 day period. These co-cultures spontaneously formed capillary-like structures (in vitro angiogenesis; Bishop et al., 1999). We found that endometrial culture supernatants promoted the acquisition of capillary length and branching (estimated by counting junction number) more effectively than medium alone. Medium alone (n = 3) induced vessels with a mean length of 4.0 ± 0.1 mm/mm² and a mean branching of 12 ± 1.5/mm², respectively (mean ± SEM). However, proliferative phase supernatants (n = 4) induced vessels with a mean length of 6.5 ± 0.1 mm/mm² and a mean branching of 26.3 ± 0.9/mm² (mean ± SEM) and secretory phase supernatants (n = 2) induced vessels with a
mean length of 5.8 and 6.0 mm/mm² and a mean branching of 19 and 21/mm² (Figure 1). Due to the small number of secretory phase endometrial supernatants, for statistical purposes the results for the two secretory phase and four proliferative phase endometrial supernatant groups were combined, and a Student’s t-test indicated that both length and branching were significantly elevated when compared to the three control cultures ($P \leq 0.05$). The promotion of EC proliferation and in vitro angiogenesis appears to be specific to endometrium, since supernatants from HUVEC themselves, primary vascular smooth muscle cells, Jurkat T lymphocytes and primary human macrophages cultured in identical conditions to the endometrium had no significant effect on HUVEC cell biology or in vitro angiogenesis (data not shown). To assess the possibility that differences in the cellular composition of the endometrial cultures were responsible for these effects, we analysed the cellular composition of disaggregated endometrium derived from three patients in the mid-proliferative and two in the mid-secretory phases of the menstrual cycle after culture for 24 h. These cultures contained variable proportions (7–11%) of cytokeratin$^{+}$ epithelial cells. The cellular proportions were not significantly different (ANOVA; $P > 0.05$) in the cultures derived from either proliferative or secretory phase endometrium.

**Supernatants from cultured endometrium regulate the endothelial cell transcriptome**

The pattern of EC transcript abundance (transcriptome) contributes to the unique physical characteristics and functions of EC, and the response of EC to extracellular signals is in part mediated through transcriptome alteration. To assess the transcriptome changes that were associated with (and may have driven) endometrial supernatant-induced EC proliferation and angiogenesis, we collected RNA from HUVEC that were exposed to proliferative and secretory phase endometrial culture supernatants (20% V/V) for a 24 h period. Radio-labeled complex cDNA probes generated from these RNAs were hybridized to nylon filter gene arrays that

![Image](http://example.com/image1.png)

**Figure 1.** (A) Supernatants from cultured endometrium promoted in vitro angiogenesis. Endothelial cells co-cultured with dermal fibroblasts were detected with Ulex-FITC (Sigma, UK). Representative images of 320 µm x 1 mm fields are shown for: CO (untreated control), VE (10 ng/ml VEGF-A165), PR and SE (supernatant from cultured proliferative- and secretory-phase endometrium, respectively). (B and C) The length of vessels and number of inter-vessel junctions (a measure of branching) per mm² were established for 21 randomly chosen 1 mm² fields for each of the following conditions: three untreated cultures (control), one culture treated with 10 ng/ml VEGF-A, two cultures each treated with secretory phase endometrial supernatants derived from different patients and four cultures each treated with proliferative phase endometrial supernatants derived from different patients. Bars represents group means ± SE. The mean length and number of inter-vessel junctions of HUVEC exposed to the endometrial supernatants ($n = 6$, composed of HUVEC exposed to two secretory phase and four proliferative phase endometrial supernatants) was significantly greater than the mean length and number of inter-vessel junctions of untreated HUVEC (Student’s t-test; $P \leq 0.05$).
contained ~1000 genes related to endothelial cell biology (Evans et al., 2003). Secretory phase endometrial supernatants induced few large-scale changes in HUVEC transcript abundance (Figure 2A). In contrast, proliferative phase endometrial supernatants induced numerous large-scale changes in HUVEC transcript abundance (Figure 2B). Transcript abundance in HUVEC treated with proliferative-phase and secretory-phase endometrial supernatants is compared in Figure 2C. The gene array results for four transcripts differentially regulated by proliferative- and secretory-phase endometrial supernatants were compared with results from TaqMan quantitative PCR and found to be concordant (Figure 2D). For all four transcripts, t-tests on the TaqMan data indicated a significant difference in expression between HUVEC exposed to supernatants from proliferative and secretory phase endometrium ($P \leq 0.01$).

To select those genes most significantly regulated in HUVEC by cultured endometrial supernatants relative to control medium-treated HUVEC, transcripts were selected where the ‘fold change’ was $\geq 2$-fold up or down and Baysian $t$-test $P$-value was $\leq 0.05$. When untreated HUVEC ($n = 3$) were compared to HUVEC cultured in the presence of proliferative-phase endometrial supernatants ($n = 5$) 26 transcripts met these criteria—these are shown in Table I. However, when untreated HUVEC ($n = 3$) were compared to HUVEC cultured in the presence of secretory phase endometrial supernatants ($n = 5$) only two transcripts met these criteria (ADP ribosylation factor-like 2, $\uparrow 2.1$; and CD36, $\downarrow 2.1$). However, several transcripts regulated by proliferative-phase endometrial supernatants were in fact also regulated by secretory-phase endometrial supernatants, but to a lesser degree. For example, 50% of transcripts regulated $^\geq 2$-fold

*Figure 2.* Gene array analysis of the response of HUVEC to endometrial culture supernatants is presented as a set of scatter-plots. White spots denote transcripts regulated up or down, on average, by $\geq 2$-fold. Error bars denote SE. (A) Untreated HUVEC ($n = 3$) compared to HUVEC cultured in the presence of secretory phase endometrial supernatants ($n = 5$). (B) Untreated HUVEC ($n = 3$) compared to HUVEC cultured in the presence of proliferative phase endometrial supernatants ($n = 5$). (C) HUVEC cultured in the presence of proliferative phase endometrial supernatants ($n = 5$) compared with HUVEC cultured in the presence of secretory phase endometrial supernatants ($n = 5$). (D) TaqMan quantitative PCR confirmed the regulated abundance of four transcripts in five replicate experiments. Transcript abundance is presented relative to the housekeeping transcript cyclophilin-A. Black and white dots represent transcript abundance in HUVEC incubated with proliferative and secretory phase endometrium, respectively. Horizontal lines denote means. For all four transcripts, $t$-tests (with Bonferroni correction to allow for four simultaneous comparisons) indicated a significant difference between expression levels in HUVEC exposed to supernatants from proliferative vs secretory phase endometrium ($P \leq 0.01$).
Table I. Transcripts differentially regulated in HUVEC cells by incubation with supernatants from cultured proliferative phase endometrium

<table>
<thead>
<tr>
<th>Name</th>
<th>By P-value</th>
<th>Fold change</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADP ribosylation factor-like 2</td>
<td>0.000168</td>
<td>+2.61*</td>
</tr>
<tr>
<td>Angiopoietin 1</td>
<td>5.60E-06</td>
<td>−2.39*</td>
</tr>
<tr>
<td>c-Jun N-terminal kinase 3</td>
<td>0.000704</td>
<td>−2.02*</td>
</tr>
<tr>
<td>Collagen IV alpha 6</td>
<td>4.13E-05</td>
<td>+3.28*</td>
</tr>
<tr>
<td>CXC-receptor-4</td>
<td>0.001395</td>
<td>+2.18</td>
</tr>
<tr>
<td>Cystatin C</td>
<td>0.000768</td>
<td>+2.10*</td>
</tr>
<tr>
<td>EST DKFZP56600424</td>
<td>0.000291</td>
<td>+2.25</td>
</tr>
<tr>
<td>Endoglin</td>
<td>0.004326</td>
<td>+2.40</td>
</tr>
<tr>
<td>Fibronectin-1</td>
<td>0.000247</td>
<td>+4.49</td>
</tr>
<tr>
<td>Glutathione S transferase p1</td>
<td>0.000212</td>
<td>+3.55*</td>
</tr>
<tr>
<td>Glyceraldehyde-3-phosphate dehydrogenase</td>
<td>5.00E-13</td>
<td>+2.11*</td>
</tr>
<tr>
<td>Insulin-like growth factor binding protein-2</td>
<td>0.000591</td>
<td>+3.33</td>
</tr>
<tr>
<td>Interferon gamma receptor-2</td>
<td>0.000271</td>
<td>+2.14</td>
</tr>
<tr>
<td>Interleukin 1 receptor like-1</td>
<td>7.53E-07</td>
<td>+3.26</td>
</tr>
<tr>
<td>Mitogen-activated protein kinase-12</td>
<td>0.002626</td>
<td>+2.69*</td>
</tr>
<tr>
<td>Matrix metalloproteinase-2</td>
<td>0.000444</td>
<td>+2.15*</td>
</tr>
<tr>
<td>Matrix metalloproteinase-3</td>
<td>0.000164</td>
<td>+2.24</td>
</tr>
<tr>
<td>Myosin light chain regulator-B</td>
<td>0.001236</td>
<td>+2.28</td>
</tr>
<tr>
<td>Plasminogen activator inhibitor-1</td>
<td>8.47E-06</td>
<td>+3.09*</td>
</tr>
<tr>
<td>PECAM-1 (CD31)</td>
<td>2.13E-05</td>
<td>+2.19</td>
</tr>
<tr>
<td>Rho 7</td>
<td>0.003470965</td>
<td>+2.14*</td>
</tr>
<tr>
<td>Ryanodin receptor-1</td>
<td>1.89E-06</td>
<td>−2.29*</td>
</tr>
<tr>
<td>SIVA</td>
<td>0.000277083</td>
<td>+2.40</td>
</tr>
<tr>
<td>Tissue factor inhibitor-2</td>
<td>0.006458425</td>
<td>+3.32*</td>
</tr>
<tr>
<td>VE-Cadherin</td>
<td>6.43E-05</td>
<td>+3.20</td>
</tr>
<tr>
<td>Vimentin</td>
<td>4.93E-05</td>
<td>+3.19</td>
</tr>
</tbody>
</table>

Transcripts that were significantly regulated in HUVEC by incubation with proliferative phase endometrium are listed. In all cases mean transcript abundance regulation (‘fold change’) relative to control medium-treated HUVEC was ≥ 2-fold up (+) or down (−), and Baysian t-test P-value (By P-value) was ≤ 0.05. Asterisks denote those transcripts that were also up- or down-regulated in abundance ≥ 1.6-fold by exposure to supernatants from secretory phase endometrium.

up or down by proliferative-phase endometrial supernatants were also regulated, in the same direction, ≥ 1.6-fold up or down by secretory phase endometrial supernatants (asterisked in Table I).

Supernatants from cultured endometrium contain active VEGF-A

VEGF-A is an important regulator of endothelial cell survival, proliferation and angiogenesis, and appears to play a particular role in regulating angiogenesis in the endometrium. Therefore, we wished to determine whether VEGF-A may mediate, in part, the effects of cultured endometrial supernatants on EC. Using an ELISA, we confirmed that five proliferative and five secretory phase endometrial culture supernatants contained VEGF-A at a mean concentration of 5.2 ± 0.9 ng/ml and 5.0 ± 1.2 ng/ml, respectively (group mean ± SEM). These VEGF-A concentrations were significantly greater (ANOVA; P ≤ 0.01 and Tukey’s test) than those contained in medium alone (0.36 ± 0.2 ng/ml) or in medium conditioned by HUVEC themselves (0.43 ± 0.1 ng/ml). The concentration of VEGF-A secreted by proliferative and secretory phase endometrial cultures was not significantly different (ANOVA; P > 0.05). We then assessed the effects of saturating quantities (80 ng/ml) of an anti-VEGF-A monoclonal blocking antibody (clone 26503) on the ability of endometrial supernatants to promote HUVEC culture expansion. We found that the blocking antibody significantly (but not completely) inhibited the responses of the HUVEC to four out of the six endometrial supernatants tested. While the blocking antibody had no significant effect on HUVEC cultured in their optimal medium, it did significantly reduce the expansion of serum-starved HUVEC, presumably by reducing the VEGF-A-derived survival signals available to these stressed cells (Figure 3).

Endometriosis did not detectably alter the ability of endometrium to secrete soluble factors that influence endothelial cells

Angiogenesis is strongly implicated in the pathogenesis of endometriosis. Therefore, we wished to assess whether soluble factors produced by endometrium from women with endometriosis have a greater effect on EC than soluble factors produced by endometrium from women without the disease. Endometrial biopsies were collected from endometriotic patients and their secretion in culture of factors that promoted angiogenesis and EC transcriptome change assessed as above. Firstly, we assessed the effects on HUVEC proliferation of proliferative (n = 3) and secretory (n = 3) phase endometrial culture supernatants derived from eutopic endometrium of women without the disease. Secondly, we assessed the effects of
cultured endometrial supernatants from women with endometriosis on the HUVEC transcriptome. The effects of proliferative phase endometrial culture supernatants derived from women with and without endometriosis on HUVEC transcript abundance were indistinguishable (Figure 4B). Clustering of the array results separated HUVEC exposed to supernatants from proliferative vs secretory phase endometrial cultures, but could not separate HUVEC exposed to supernatants from endometrial cultures derived from endometriotic vs non-endometriotic women (Figure 4C). In addition, we could detect no differences between the concentrations of VEGF-A in endometrial supernatants derived from women with and without the disease (ANOVA; \( P \geq 0.05 \); Figure 4D). Since retrograde menstruation is thought to be associated with endometriosis, and since VEGF-A within menstrual debris may promote angiogenesis when endometriotic lesions first implant in the peritoneum, we compared the concentration (Figure 4E) and total mass (data not shown) of VEGF-A in menstrual effluent from women with (\( n = 16 \)) and without (\( n = 24 \)) endometriosis using ELISA. No significant differences were detected (ANOVA; \( P > 0.05 \)).

**Discussion**

We have shown that soluble factors derived from cultured endometrium promote HUVEC proliferation, angiogenesis and transcriptome change. One factor responsible for these effects appears to be VEGF-A, since we detected VEGF-A by ELISA in endometrial supernatants, and since a blocking anti-VEGF-A antibody partially inhibited EC proliferation in response to endometrial supernatants. In the small number of patients in our study, we were unable to correlate VEGF-A production with the stage of the menstrual cycle at which the endometrium was collected. This is not surprising, given that steroidal regulation of endometrial VEGF-A levels is limited (Sharkey et al., 2000) and that regulation of VEGF-A production in endometrium may occur on a local (e.g. in intravascular neutrophils; Gargett et al., 2001) rather than tissue-wide scale. Other endometrial factors may also promote EC proliferation, angiogenesis and transcriptome change. Previously published gene array studies of endometrium (Borthwick et al., 2003; Kao et al., 2003) provide clues to these. For example, the pro-angiogenic enzyme MMP-3 is within the most abundant 2% of endometrial transcripts (Borthwick et al., 2003). SPARC is equally abundant in endometrium, and proteolysis of SPARC by MMP-3 produces potent pro-angiogenic peptides (Sage et al., 2003). We could detect no difference between the pro-angiogenic abilities of proliferative and secretory phase endometrial supernatants, although this may have been due to the low sample number (secretory \( n = 2 \) and proliferative \( n = 4 \)) examined. This concurs with the previous observation that endometrial EC proliferation indices *in vivo* do not correlate with the menstrual cycle (Rogers et al., 1998). However, when we used gene arrays as a more sensitive indicator of EC responses to endometrial supernatants, we found that soluble factors secreted by proliferative phase endometrial cultures were more effective than those secreted by secretory phase endometrial cultures in promoting EC transcriptome change. This suggests that, even when disaggregated and removed from their specific endocrine environments, cultured cells isolated from proliferative and secretory phase endometrium retain their subtly different pro-angiogenic properties. A possible explanation is suggested by a previous study (Borthwick et al., 2003), which found that the potent pro-angiogenic factors Intestinal Trefoil Factor (Rodrigues et al., 2003) and MMP-11 (Nishizuka et al., 2001) were many times more abundant in proliferative than in secretory phase endometrium. Although it is possible that cultures derived from proliferative and secretory phase endometrium contain different ratios of epithelial to stromal cells, and therefore secrete different amounts of factors such as VEGF-A (Gargett et al., 1999), we suggest that this is unlikely to completely explain our results. Immunohistochemistry could not detect significant differences in the proportions of epithelial and stromal cells between cultures derived from mid-proliferative and mid-secretory phase endometrium. In addition, the concentrations of VEGF-A did not differ significantly when supernatants from cultures derived from mid-proliferative and mid-secretory phase endometrium were compared.

The transcriptome changes induced in EC by endometrium-derived factors may partly underlie the angiogenesis
that is also induced by these factors, since many of the regulated EC transcripts encode promoters of angiogenesis (Table I). These include transcripts previously found to be up-regulated during in vitro angiogenesis (Kahn et al., 2000) such as CXC receptor-4, collagen 1α6 and tissue factor-inhibitor-2. Given that VEGF-A was secreted by the cultured endometrium, it is interesting that transcripts previously found to be up-regulated in EC by VEGF-A (Weston et al., 2002; Yang et al., 2002) were also up-regulated by endometrial supernatants, such as CXC receptor-4, MMP-2 tissue factor-inhibitor-2 and VE-cadherin. Several other transcripts regulated by the endometrial supernatants have clear associations with EC survival and proliferation. For example, angiopoietin-1 was down-regulated. Angiopoietin-1 usually functions to stabilize vessels, but is functionally inhibited by angiopoietin-2 during angiogenesis. Endoglin (up-regulated) is a component of the transforming growth factor beta receptor complex that is essential for vessel development (Zhang et al., 2002). PECAM-1 and VE-cadherin (both up-regulated) are EC adhesion molecules that promote EC survival through protein kinase B (AKT) signaling. Other regulated signaling molecules include: ADP ribosylation factor-like-2, interleukin-1 receptor-like-1, interferon γ receptor-2, MAP kinase-12, Rho-7 and SIVA. Several transcripts associated with the extracellular matrix were regulated in EC by endometrial supernatants. These may contribute to cyclical remodeling of both vessels and other elements of endometrium. For example, Plasminogen activator-inhibitor-1 and fibronectin-1 were up-regulated. MMP-2 and MMP-3 were also up-regulated and have previously been associated with cyclical endometrial remodelling (Ueda et al., 2002) and endometriosis (Cox et al., 2001).

Previous studies (summarized in the Introduction) have suggested that both endometrial and peritoneal factors promote the angiogenesis that underlies endometriosis. Our study investigated the role played by endometrial factors. We found that endometriosis had no effect on the capacity of endometrial culture supernatants to induce angiogenesis or gene expression changes in EC. In addition, we found no relationship between endometriosis and VEGF-A production by cultured endometrium or VEGF-A concentration or mass in menstrual effluent. This suggests that differences between the soluble pro-angiogenic factors secreted by the endometrium of endometriotic and non-endometriotic women may not be a major determinant of this disease. It is possible that the small size of our study (dictated by our stringent patient selection criteria and the relative rarity of unmedicated endometriotic patients) has caused us to miss a subtle role played by soluble endometrial factors. However, it is also possible that confounding and the lack of stringent patient selection may have caused some previous investigators to overestimate the role played by endometrial factors in this disease. The importance of stringent patient selection criteria and measures to reduce confounding has been highlighted in a recent study published in this journal of soluble ICAM-1 in endometriotic patients (Steff et al., 2004). Models of endometriosis in which human endometrium is transplanted into immunocompromized mice (Hull et al., 2003) will provide powerful tools to dissect the relative roles of endometrial and peritoneal factors.

In conclusion, we have shown that endometrium cultured in vitro produces soluble factors, including VEGF-A, that promote angiogenesis. Endometrium, especially from the proliferative phase of the menstrual cycle, also promotes significant pro-angiogenic changes to the EC transcriptome. However, we were unable to detect any association between endometriosis and the effects of soluble endometrial factors on EC biology or gene expression. We believe that the endometrial factor-induced EC transcriptome changes identified here provide insight into the dynamic control of vessel structure on which both the eutopic endometrium and endometriotic lesions depend. They also provide potential therapeutic targets for the modulation of endometrial angiogenesis.

Acknowledgements

We wish to acknowledge Kate Day and Isabelle Perault for technical assistance and Andrew Sharkey and Stephen Charnock-Jones for advice on endometrial culture and gene array analysis, as well as Mr Andrew Prentice and the staff of the Rosie Hospital, Cambridge, for assistance with tissue collection. This study was funded by a Wellbeing project grant. C.P. was funded by a Wellcome Trust fellowship.

References


Endometrial factors regulate endothelial biology


Submitted on November 5, 2003; resubmitted on May 18, 2004; accepted on June 16, 2004