The effect of etonogestrel on VEGF, oestrogen and progesterone receptor immunoreactivity and endothelial cell number in human endometrium

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Contraceptive use often leads to disrupted endometrial bleeding patterns in women. In this study, two different contraceptive regimes (Mircette, a monophasic oral contraceptive and Implanon, a long-acting gestagen) were used and their effects on the immunoreactivity of vascular endothelial growth factor (VEGF), oestrogen receptor (ER), progesterone receptor (PR) and endothelial cell number were determined. During the untreated normal cycle, there was a significant increase ($P = 0.005$) in glandular VEGF immunoreactivity and a significant decrease ($P < 0.05$) in PR immunoreactivity in the mid- and late secretory phases compared with the proliferative phase. There was a significant positive correlation ($\gamma = 0.38, P = 0.046$) between stromal VEGF immunoreactivity and endothelial cell number. This correlation was also apparent during treatment with Implanon, but not with Mircette. Disrupted bleeding patterns were associated with Implanon and, to a lesser extent, with Mircette. Both contraceptives significantly reduced glandular VEGF immunoreactivity. Implanon significantly increased ($P = 0.016$) glandular PR staining, but Mircette significantly reduced ($P = 0.027$) stromal PR staining when compared with secretory before-treatment biopsies. There were no changes in endothelial cell number or glandular or stromal ER during the normal cycle, or with use of either contraceptive. There was no association between the parameters measured with bleeding patterns and histological category.

Key words: angiogenesis/bleeding/contraceptive/endometrium/endothelium

Introduction

Discontinuation of oral contraception is often a result of unwanted side-effects, such as breakthrough bleeding or spotting, or androgenic effects such as weight gain or acne (Kaunitz, 1993). Desogestrel, a pro-drug whose active metabolite is etonogestrel, was developed to provide a more selective progestational activity and reduced androgenic activity that would improve cycle control and minimize metabolic changes and adverse effects (Wilde and Balfour, 1995). Desogestrel and the newer progestagens also result in less pronounced antagonism of the beneficial effects of ethinyl oestradiol on lipid metabolism and the cardiovascular system (Kuhl, 1996).

Steroid hormones produced by the ovary regulate the menstrual cycle including the changes in the endometrial vasculature. Differing levels, types and distribution of their specific receptors, i.e. oestrogen receptors (ER) $\alpha$ and $\beta$, and progesterone receptors (PR) A and B, presumably mediate their actions together with locally produced polypeptide factors, e.g. epidermal growth factor (EGF; Gordon et al., 1995). Vascular endothelial growth factor (VEGF) is a potent angiogenic growth factor (Ferrara et al., 1992). It is produced in both glands and stromal cells of the endometrium, as well as smooth muscle cells of the myometrium (Charnock-Jones et al., 1993). VEGF mRNA is up-regulated in the immature rat uterus in response to oestradiol, oestriol and progesterone (Cullinan-Bove and Koos, 1993; Hyder et al., 1996). VEGF is, therefore, a possible candidate as a mediator of steroid hormone action on the endometrial blood vessels. Alterations in the normal distribution of VEGF due to the administration of synthetic steroids may be responsible for any resulting aberrant bleeding.

The aims of the present study were to compare parameters which may be important in endometrial bleeding: VEGF, oestrogen receptors (ER), and progesterone receptors (PR) as reflected by immunohistochemistry and endothelial cell number in the endometrium; (i) throughout the normal menstrual cycle; and (ii) after using two different contraceptives, Implanon and Mircette. The effects of these parameters on the observed bleeding patterns and histology were analysed.

Materials and methods

Subjects

Normally menstruating women from the USA were recruited into one of two separate study groups using different methods of contraception. The first 14 subjects used a progestin-only contraceptive, Implanon, a non-biodegradable implant with a duration of action of 3 years containing and releasing etonogestrel. The next 20 subjects used Micrette, a 21 day monophasic combined oral contraceptive containing 20 ?g ethinyl oestradiol and 150 ?g desogestrel, with 10 ?g ethinyl oestradiol/day administered for 5 days immediately before the next pill cycle.

Endometrial biopsies were taken on an out-patient basis by Pipelle before treatment and ~12 months after commencement. Specimens were formalin fixed and embedded in paraffin (56–57°C melting point, Surgipath Medical Industries, Richmond, IL, USA). Sections (5 ?m) were mounted on 2% aminopropyltriethoxyxilane (Sigma, St
Louis, MO, USA) coated slides. One section was stained with haematoxylin and eosin for classification by a gynaecological histopathologist. These categories were normal proliferative, early secretory (i.e. days dated 15–19), mid-secretory (days 20–22) and late secretory (days 22–28; Hendrickson and Kempson, 1980), atrophic, or oral contraceptive effect (i.e. consistent with exogenous progestin administration). Additional sections were used for immunohistochemistry as described below.

**Immunohistochemistry**

**Vascular endothelial growth factor**

Sections were stained for VEGF with an antibody directed against amino acids 1–20 of mature human VEGF and which therefore recognizes the 121, 145, 165, 189 and 206 amino acid splice variants. Sections were dewaxed, rehydrated and washed in phosphate-buffered saline (PBS). Endogenous peroxidase was quenched with 3% hydrogen peroxide in PBS (10 min), before application of primary antibody: goat anti-rabbit immunoglobulin G (IgG; Zymed Laboratories, San Francisco, CA, USA), diluted to 0.5 μg/ml in 1% bovine serum albumin (BSA)–PBS, incubated for 1 h at 37°C. This was followed by a biotinylated secondary antibody: streptavidin–peroxidase (Zymed) diluted 1/1000 in PBS (10 min). Colour was developed with aminoethylcarbazole (AEC) substrate chromogen mix (Zymed) for 5–10 min. Sections were counterstained with diluted Mayer’s haematoxylin (1/20) for 1 min, then mounted in aqueous mounting medium (Clearmount Mounting Solution; Zymed). For negative controls 0.5 μg/ml normal rabbit IgG was substituted for the primary antibody.

**Oestrogen and progesterone receptors**

Separate sections were stained for ERα and PR using mouse monoclonal antibodies: ER 1D5 (Dako, Glostrup, Denmark) and PR-AT 4.14 (ABR; Golden, Colorado, USA). Antigen retrieval was carried out by microwaving in 0.01 mol/l sodium citrate buffer (pH 6.0) for 10 min at 500 W. Antibodies were diluted 1/50 in protein blocking solution (1.5% normal horse serum, 2% BSA in PBS–TWEEN) and incubated overnight at 4°C after a 30 min pre-block. The secondary antibody was biotinylated horse anti-mouse (Vector Laboratories, Burlingame, CA, USA), diluted 1/200 for 45 min. This was followed by the Vectastain Elite ABC complex (1/200, 30 min), then metal-enhanced diaminobenzidine (Pierce Europe BV, Oud Beijerland, The Netherlands) in peroxidase buffer, 10 min and haematoxylin for 30 s. Sections were dehydrated and mounted in DePXY (Merck, Poole, UK).

The glandular epithelium and stromal cell compartments from each stained section were scored subjectively on a scale of 0–4 by two or three independent observers. For VEGF, the scores were based on the staining intensity of the majority of cells. For ER and PR however, modified H-scores were assigned (Ravn et al., 1993). First an estimate was made for the fraction (%) of stained cells in each compartment: 0 = 0–9%, 1 = 10–39%, 2 = 40–69%, 3 = 70–89%, and 4 = 90–100%. Second, the staining intensity (I) was scored: 0 = no staining, 1 = weak but definite staining, 2 = moderate staining, 3 = pronounced staining and 4 = intense staining. The H-score was then calculated by the formula (%)×I / 4. The averaged subjective scores were analysed by the non-parametric Wilcoxon test for paired samples (before- versus after-treatment).

**Endothelial cell number**

Separate sections were stained to determine the number of endothelial cell (EC) nuclei per mm². The method used was

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**Table I. Median subjective semi-quantitative scores and their ranges (in brackets), and n for each group during the normal menstrual cycle (i.e. from the before-treatment biopsies), and after treatment with Implanon or Micelle; for vascular endothelial growth factor (VEGF), oestrogen receptor (ER) and progesterone receptor (PR) immunostaining for the endometrial glandular, and stromal cell compartments; and endothelial cell densities. Biopsies during the normal cycle were divided into histological categories according to the criteria of Hendrickson and Kempson (1980)**

<table>
<thead>
<tr>
<th>Phase</th>
<th>VEGF immunostaining score</th>
<th>ER immunostaining score</th>
<th>Endothelial cell number</th>
</tr>
</thead>
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<td></td>
<td>Glands (n)</td>
<td>Stroma (n)</td>
<td>Glands (n)</td>
</tr>
<tr>
<td>Proliferative</td>
<td>1.00 (0.75–2.0)</td>
<td>1.00 (0.17–2.5)</td>
<td>1.75 (1.0–2.25)</td>
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<tr>
<td>Early secretory</td>
<td>1.63 (0.25–2.25)</td>
<td>1.38 (0.5–1.5)</td>
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<tr>
<td>Mid-secretory</td>
<td>*2.59 (1.3–3.5)</td>
<td>0.88 (0.17–2.0)</td>
<td>1.50 (1.5–1.8)</td>
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<tr>
<td>Late secretory</td>
<td>*2.30 (1.17–3.0)</td>
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<td>1.23 (0.75–2.0)</td>
</tr>
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<td>Contraceptive treatment</td>
<td>Implanon</td>
<td>0.625 (0.3–2.5)</td>
<td>1.67 (0.75–3.0)</td>
</tr>
<tr>
<td></td>
<td>Micelle</td>
<td>0.83 (0.2–5)</td>
<td>1.00 (0.25–2.30)</td>
</tr>
</tbody>
</table>

*P < 0.05 compared with proliferative phase (Dunn’s multiple comparisons test).

**Table I. continued**

<table>
<thead>
<tr>
<th>Phase</th>
<th>PR immunostaining score</th>
<th>Endothelial cells</th>
</tr>
</thead>
<tbody>
<tr>
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<td>Glands (n)</td>
<td>Stroma (n)</td>
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<tr>
<td>Proliferative</td>
<td>1.90 (1.5–2.8)</td>
<td>2.10 (1.0–2.3)</td>
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<tr>
<td>Early secretory</td>
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<tr>
<td>Mid-secretory</td>
<td>*0.29 (0.0–0.78)</td>
<td>2.29 (2.28–2.63)</td>
</tr>
<tr>
<td>Late secretory</td>
<td>**0.28 (0.0–2.3)</td>
<td>2.30 (1.69–3.0)</td>
</tr>
<tr>
<td>Contraceptive treatment</td>
<td>Implanon</td>
<td>2.0 (1.0–3.5)</td>
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<tr>
<td></td>
<td>Micelle</td>
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**Oestrogen and progesterone receptors**

Separate sections were stained for ERα and PR using mouse monochlonal antibodies: ER 1D5 (Dako, Glostrup, Denmark) and PR-AT 4.14 (ABR; Golden, Colorado, USA). Antigen retrieval was carried out by microwaving in 0.01 mol/l sodium citrate buffer (pH 6.0) for 10 min at 500 W. Antibodies were diluted 1/50 in protein blocking solution (1.5% normal horse serum, 2% BSA in PBS–TWEEN) and incubated overnight at 4°C after a 30 min pre-block. The secondary antibody was biotinylated horse anti-mouse (Vector Laboratories, Burlingame, CA, USA), diluted 1/200 for 45 min. This was followed by the Vectastain Elite ABC complex (1/200, 30 min), then metal-enhanced diaminobenzidine (Pierce Europe BV, Oud Beijerland, The Netherlands) in peroxidase buffer, 10 min and haematoxylin for 30 s. Sections were dehydrated and mounted in DePXY (Merck, Poole, UK).

The glandular epithelium and stromal cell compartments from each stained section were scored subjectively on a scale of 0–4 by two or three independent observers. For VEGF, the scores were based on the staining intensity of the majority of cells. For ER and PR however, modified H-scores were assigned (Ravn et al., 1993). First an estimate was made for the fraction (%) of stained cells in each compartment: 0 = 0–9%, 1 = 10–39%, 2 = 40–69%, 3 = 70–89%, and 4 = 90–100%. Second, the staining intensity (I) was scored: 0 = no staining, 1 = weak but definite staining, 2 = moderate staining, 3 = pronounced staining and 4 = intense staining. The H-score was then calculated by the formula (%)×I / 4. The averaged subjective scores were analysed by the non-parametric Wilcoxon test for paired samples (before- versus after-treatment).

**Endothelial cell number**

Separate sections were stained to determine the number of endothelial cell (EC) nuclei per mm². The method used was
Figure 1. Human endometrium immunostained for vascular endothelial growth factor (VEGF, red), with light haematoxylin counterstaining (pale blue) A–D are from the same subject (01003), in the Implanon study; and E–H are from subject 0108 in the Mircelle study. A, B, E, and F are from before-treatment (normal cycle) biopsies; C, D, G, and H are from after-treatment biopsies. A, C, E, G bar = 200 µm; B, D, F, H bar = 50 µm. Note the reduction in glandular VEGF staining after contraceptive treatment.

essentially as described previously (Goodger Macpherson and Rogers, 1994) and used an antibody which stains all endometrial vessels. Sections were treated as for VEGF above, except the primary antibody was mouse anti-human CD34 (QBEND 10; Serotec, Oxford, UK) diluted 1/25 in 1% BSA–PBS incubated for 45 min at 37°C, and the secondary antibody was biotinylated rabbit anti mouse IgG (Zymed). All cell nuclei were stained with undiluted haematoxylin. From these sections, an estimate of the endothelial cell number was obtained, i.e. the number of EC nuclei per mm². Each section was viewed under a microscope at ×400, connected via a video camera to a personal computer and sampled with software using a uniform systematic random sampling method with a meander algorithm after outlining the section (Grid stereological software; Interactivision, Silkeborg, Denmark). In this way, 50 or 100 fields of known area per section were sampled and EC nuclei counted by one observer.

Bleeding records
Daily records of vaginal bleeding were kept by each participant for at least 13 cycles, with the following entries: no bleeding or spotting;
spotting, requiring no more than one sanitary napkin or tampon per day; or bleeding, requiring more than 1 sanitary napkin or tampon per day. Records were analysed using a 90 day reference period immediately prior to the day of the after-treatment biopsy (biopsy day = day 91). Definitions used were as follows (Rogers et al., 1993): (i) a bleeding/spotting episode 1 or more consecutive days during which blood loss (bleeding or spotting) was recorded, each episode being bounded by two or more bleeding/spotting free days; a single bleeding/spotting free day within a bleeding/spotting episode being counted as part of the episode surrounding it; and (ii) a bleeding/spotting free interval 2 or more consecutive days during which blood loss had not occurred; each interval being bounded by bleeding/spotting days.

Bleeding during this 90 day reference period was categorized as: (a) amenorrhoea – no bleeding/spotting; (b) prolonged – one or more bleeding/spotting episodes lasting 10 days or more; (c) frequent – more than four bleeding/spotting episodes; (d) infrequent – less than two bleeding/spotting episodes; (e) irregular – range of length of bleeding/spotting free intervals (i.e. greatest interval minus smallest interval) >17 days; (f) regular – two to four bleeding/spotting episodes, no bleeding/spotting episode lasting 10 days or more, with a range of length of bleeding/spotting free intervals of 17 days or less.

The bleeding patterns in the women receiving the oral contraceptive were described as withdrawal bleeding, absence of withdrawal, early withdrawal, breakthrough bleeding or continued withdrawal. Statistical analysis was performed using non-parametric tests to look for possible relationships between the VEGF, ER, and PR glandular and stromal staining and endothelial cell number, and their influence on bleeding category and histological classification of the after-treatment biopsy.

**Results**

**Normal menstrual cycle**

**VEGF immunoreactivity**

All before-treatment biopsies (i.e. from normal cycles) from both studies stained for VEGF (n = 31 overall) were divided into four histological groups based on the stage of the menstrual cycle. The groups were normal proliferative (n = 8), early secretory (n = 8), mid-secretory (n = 4) and late secretory (n = 11). See Table I for median scores and ranges for glands and stroma. Analysis of subjective VEGF immunostaining scores using the Kruskal–Wallis test showed that there was a significant increase in glandular VEGF immunostaining across the cycle (P = 0.005); and Dunn’s multiple comparisons test showed that the staining during the mid- and late secretory phases were significantly greater than that of the proliferative phase (P < 0.05). There was no significant change in stromal VEGF staining.

**ER and PR immunoreactivity**

PR immunoreactivity significantly decreased in the glands across the normal cycle (P = 0.002; n = 26 overall; proliferative phase n = 8; early secretory n = 6; mid-secretory n = 4; late secretory n = 8). Dunn’s multiple comparisons test showed that the staining during the mid- and late secretory phases was significantly weaker than that of the proliferative phase (P < 0.05, P < 0.01 respectively). There were no significant changes in endometrial ER across the normal cycle in glands or stroma, or in the stromal PR staining. See Table I for median scores and their ranges.

**Endothelial cell number**

There were no significant changes in endometrial endothelial cell number across the normal cycle (n = 28 overall; proliferative phase n = 8; early secretory n = 7; mid-secretory n = 4; late secretory n = 9). See Table I for median scores and ranges.

**Correlations between immunohistochemical parameters**

In order to look for possible relationships between the various immunohistochemical parameters measured, Spearman rank correlation tests were used. There were significant positive correlations between glandular and stromal VEGF staining (r = 0.65, P < 0.0001), glandular ER and glandular PR (r = 0.57, P = 0.003) and stromal VEGF and endothelial cell number (r = 0.38, P = 0.046); and a negative correlation between glandular VEGF and glandular PR staining (r = –0.49, P = 0.01).

**Implanon study**

Patients (n = 14) participated, with a median age of 26.0 years, range 18–40 years. Detailed results showing averaged subjective scores for VEGF, ER and PR staining in the glands and stroma, together with their endothelial cell numbers, histological classification and bleeding summaries are shown in Table I.

**Routine histology**

All before-treatment biopsies were taken during the secretory phase of the cycle as judged by routine histology. Use of Implanon altered the histological appearance of the endometrium, resulting in reduced endometrial volume. A total of 11 after-treatment biopsies were judged to be atrophic, one weakly proliferative, one proliferative and one menstrual.

**Immunohistochemistry**

Seven of the after-treatment biopsies were judged to be inadequate, so these patients were excluded from analysis where appropriate. No staining was seen in negative controls. VEGF staining was always cytoplasmic. See Table I for median scores and their ranges for the immunohistochemical parameters in after-Implanon treatment biopsies. There was a significant reduction in VEGF staining in the glands with treatment (n = 6 before- versus after-treatment biopsy pairs, P = 0.031). There was no change in stromal VEGF staining with treatment. See Figure 1A–D for photomicrographs of examples of VEGF staining of endometrium from the same patient before and after Implanon treatment. Specific ER and PR staining was always nuclear and an example of this is shown in Figure 2A–D. The glands showed significantly increased PR staining with treatment (n = 7 pairs, P = 0.016) and a trend (although not significant) towards increased glandular ER staining (n = 6 pairs, P = 0.063). There was no change in stromal ER and PR staining. Endothelial cell number did not change with treatment.

**Correlations between immunohistochemical parameters**

There was a significant positive correlation (Spearman rank correlation) between endothelial cell number and stromal VEGF (r = 0.857, P = 0.024) in the after-treatment biopsies. There were no other correlations between the immunohistochemical parameters measured in the after-Implanon treatment biopsies.
**Menstrual bleeding pattern versus immunohistochemical parameters**

Implanon disrupted the normal menstrual bleeding pattern in all subjects and resulted in amenorrhoea in only four out of 14. Kruskal–Wallis analysis of variance showed that there were no significant differences between the observed bleeding categories during the 90 day reference period and any of the immunohistochemical parameters measured in the after-Implanon treatment biopsies.

**Mircette study**

Twenty patients participated, with a median age of 29.5 years (range 23–47). Detailed results showing averaged subjective scores for VEGF staining in the glands and stroma, together with their endothelial cell numbers, histological classification and bleeding summaries are shown in Table I.

**Routine histology**

Eight before-treatment biopsies were taken during the proliferative phase of the cycle, 11 during the secretory phase, and one was judged to be inactive. Like Implanon, Mircette use altered the histological appearance of the endometrium. Of the after-treatment biopsies, three were judged to be proliferative, two secretory, seven atrophic, seven to show an exogenous progestogen effect, and one an exogenous progestogen effect with shedding.

**Immunohistochemistry**

One of the after-treatment biopsies was judged to be inadequate for immunohistochemical staining due to the small volume of tissue, so this patient was excluded from analysis where appropriate. No staining was seen in negative controls. See Table I for median scores and their ranges for the immunohistochemical parameters in the after-Mircette treatment biopsies. Statistical analysis showed that overall there was a significant reduction in VEGF staining in the glands with treatment ($n = 19$ before- compared with after-treatment biopsy pairs, $P = 0.007$). However, this reduction with treatment is not apparent when comparing only paired proliferative before-treatment biopsies against after-treatment samples ($n = 7, P = 0.813$), but is quite marked when comparing only paired secretory before-treatment biopsies against after-treatment samples ($n = 11, P = 0.001$). There was no significant difference in VEGF staining when comparing proliferative before-treatment biopsies ($n = 8$) with secretory before-treatment biopsies ($n = 11, P = 0.129$). Nor was there any significant change in stromal VEGF staining with treatment. See Figure 1E–H for photomicrographs of VEGF staining of endometrium from the same patient before and after Mircette treatment.

Overall, there were no changes in ER or PR staining with treatment in either the glands or stroma. There were no significant changes in glandular ER or PR when comparing only paired proliferative before-treatment biopsies against after-treatment samples, or paired secretory before-treatment biopsies against after-treatment samples. There were also no significant changes in stromal ER or PR when comparing only paired proliferative before-treatment biopsies against after-treatment samples. However, when comparing only paired secretory before-treatment biopsies against after-treatment samples, there was significantly reduced stromal PR staining with treatment ($P = 0.027$) and a trend towards increased stromal ER staining with treatment ($P = 0.065$). Endothelial cell number did not change significantly with treatment.

**Correlations between immunohistochemical parameters**

There was a significant positive correlation (Spearman rank correlation) between glandular EGF and stromal VEGF ($\rho = 0.61, P = 0.006$) after treatment. There were no other significant correlations between the immunohistochemical parameters measured in the after-Mircette treatment biopsies.

**Histological category versus immunohistochemical parameters**

Kruskal–Wallis tests showed that the histological category of the after-treatment biopsy did not influence any of the immunohistochemical parameters.

**Menstrual bleeding pattern versus immunohistochemical parameters**

Mircette resulted in a regular bleeding pattern in the majority of subjects ($11/19$, 58%). Of the remaining subjects, seven experienced bleeding in addition to withdrawal bleeding and one woman failed to have regular withdrawal bleeding. Bleeding pattern data were analysed against each of the immunohistochemical parameters by the unpaired non-parametric test (Mann–Whitney) using two groups only: regular bleeders versus the rest; due to the very low subject numbers in each of several different bleeding categories. Results showed that there were no significant changes to the immunohistochemical parameters with bleeding pattern, however there was a trend towards increased stromal ER staining with non-regular bleeding ($P = 0.051$).

**Discussion**

During the normal menstrual cycle VEGF immunoreactivity of the endometrial glands changed significantly, with a significant increase during the mid- and late secretory phases compared to the proliferative phase. Stromal VEGF immunoreactivity did not change through the cycle. These findings contrast with those of other authors (Li et al., 1994), who reported strong VEGF glandular staining that did not change during the cycle, with the strongest stromal staining being found in discrete cells during the mid-proliferative phase. However the pattern of VEGF staining reported here agrees with previous work (Shifren et al., 1996; Torry et al., 1996), though these studies had fewer subject numbers; and none of the previous studies made any attempt at quantification. The findings of all of these groups differ from that of Lau et al. who failed to show significant differences in staining for VEGF in either glandular or stromal staining (Lau et al., 1999). This difference may reflect the specificity of the different antibodies used by the groups and this group’s findings do not concur with earlier in-situ hybridization studies (Charnock-Jones et al., 1993). A study with small numbers of cynomolgus monkeys during the normal cycle also showed a significant increase in glandular VEGF staining during the secretory phase compared with the proliferative phase (Greb et al., 1995). It is also of interest that in the present study a simple comparison within the
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Figure 2. Human endometrium immunostained for oestrogen receptor (A, B) and progesterone receptor (C, D). A and C show tissue obtained before treatment, while B and D show tissue after Implanon treatment. Bar = 50 µm.

before-treatment (normal cycle) group from the Mircette study of glandular VEGF staining during the proliferative phase \((n = 8)\) versus the secretory phase \((n = 11)\) showed no significant difference.

The present study showed that glandular PR staining was significantly reduced during the mid- and late secretory phases of the cycle compared to the proliferative phase. These results agree with those of others (e.g. Snijders et al., 1992; Critchley et al., 1993) and is consistent with down-regulation by progesterone (Chauchereau et al., 1991; Snijders et al., 1992). The continued presence of PR in the stroma through the cycle also agrees with previous work (Critchley et al., 1993). The lack of statistically significantly reduced glandular or stromal ER staining as the cycle progressed in the current study is probably due to insufficient subject numbers. Trends towards reduced ER staining in the secretory phase were, however, apparent.

The correlation between VEGF immunoreactivity in the glands and stroma suggests a similarity in regulation, although the variation in the glands is more pronounced. The relationship between glandular ER and glandular PR is reflected by similar regulation of each receptor by oestrogen and progesterone, i.e. oestrogen alone up-regulates ER and PR, whereas progesterone down-regulates ER and PR (McDonnell et al., 1995). The negative correlation between glandular VEGF and glandular PR is of interest, since this is consistent with the hypothesis that endometrial glandular VEGF is regulated by progesterone \textit{in vivo} in the normal human adult. The finding that progesterone increases expression of the most abundant forms of VEGF in the uterus of immature rats is also consistent with this hypothesis (Cullinan-Bove and Koos, 1993). The antibody used in this study does not distinguish between the different spliced forms of VEGF and it is suggested that the smaller 121 form of VEGF is the main variant expressed by stromal cells (Huang et al., 1998). However, 165 is the main variant of glandular expression and like the 121 variant is regulated by ovarian steroids.

The correlation between stromal VEGF staining and endothelial cell number has not been previously reported. More work, with larger subject numbers, is required to investigate this interesting relationship further. As an angiogenic factor, it seems probable that VEGF could influence endometrial vascular density. In tumours, microvessel density has been used as a measure of tumour angiogenesis, with increased intratumoral microvessel density correlating with increased metastasis and/or decreased patient survival (Vartanian and Weidner, 1994). However, in the present and previous studies, neither endometrial endothelial cell number nor microvascular density (Rogers et al., 1993; Goodger Macpherson and Rogers, 1994) changed throughout the normal cycle and indeed changes in endothelial cell number do not necessarily indicate changes
in angiogenic activity (Goodger Macpherson and Rogers, 1995). The lack of change in intensity of stromal VEGF staining and endothelial cell number during the cycle suggest that these parameters may be regulated by non-steroidal mechanisms. However, this is at odds with the suggestion that glandular and stromal VEGF are regulated by similar mechanisms and that glandular VEGF is regulated by progesterone.

The contraceptives Implanon and Mircette, which contain the pro-form of the same progestagen in combination with ethinyl oestradiol, both significantly reduced endometrial glandular immunoreactive VEGF. After 12 months of use there was no difference in endothelial cell number compared to before-treatment, and there was no correlation between glandular VEGF staining and endothelial cell number for the after-treatment biopsies from either the Implanon or Mircette studies. In the light of the observation that stromal VEGF correlates with endothelial cell number during the normal cycle, however, the reduction in glandular VEGF with contraceptive treatment may be biologically unimportant. Indeed there was a similar positive correlation between stromal VEGF and endothelial cell number with Implanon treatment, but not Mircette treatment. The significantly increased glandular PR staining with Implanon use shows that etonogestrel alone fails to down-regulate the progesterone receptor in the same way that progesterone does. It is likely that the balance of agonist/antagonist actions and the progesterone receptor type contributes to the end result of progestin action. On the other hand, Mircette resulted in significantly reduced stromal PR compared with normal secretory phase values in response to progesterone. For both contraceptives, however, neither bleeding patterns during the 90 day reference period just prior to the after-treatment biopsy, nor histological category of the after-treatment biopsy showed any significant differences with VEGF, ER or PR staining, or endothelial cell number.

The lack of change in endothelial cell number with the two contraceptives used in this study contrasts with the action of Norplant (an implant which releases 80 µg/day levonorgestrel); high doses of norethisterone and medroxyprogesterone acetate (MPA). Norplant results in significantly increased microvascular and endothelial cell number compared with that seen during the normal cycle (despite significantly reduced endothelial cell proliferation), probably due either to increased regression of tissue surrounding the endometrial vessels, or a reduced rate of endothelial cell death (Rogers et al., 1993; Goodger Macpherson et al., 1994; Hickey et al., 1999). The increased vascular density did not seem to be directly related to increased endometrial bleeding. On the other hand, treatment with higher doses of norethisterone or MPA resulted in a significantly reduced endometrial vascular density (Song et al., 1999). The present study confirms the apparent dissociation between endometrial endothelial cell number and endometrial bleeding. It has also been proposed that there may be considerable endometrial microvascular heterogeneity, particularly in response to Norplant treatment resulting in focal bleeding sites (Rogers, 1996). The same may be true with other steroid treatments.

In conclusion, the present study shows that immunoreactive VEGF increases significantly in the glands of the endometrium during the normal menstrual cycle, while immunoreactive PR decreases. This study also shows that Implanon and Mircette significantly reduce glandular VEGF staining. This is not associated with changes in endothelial cell number, bleeding pattern, or histological category of the endometrium. Stromal staining for VEGF correlated positively with endothelial cell number although neither parameter changed significantly throughout the menstrual cycle, or influenced the endometrial bleeding pattern.

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References
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