Human Reproduction, Vol. 15, (Suppl. 3), pp. 85–95, 2000

The effect of progestins on vascular endothelial growth factor, oestrogen receptor and progesterone receptor immunoreactivity and endothelial cell density in human endometrium

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One common side-effect of contraceptive use is that it often leads to disrupted endometrial bleeding patterns. This may be due to changes in endothelial density and vessel integrity. To investigate whether the level of endometrial immunoreactive vascular endothelial growth factor (VEGF), oestrogen receptor or progesterone receptor (PR) have any role in this, women were treated with either Mircette®, a monophasic oral contraceptive, or Implanon®, a long-acting gestagen, and immunohistochemistry performed. In addition a small number of endometria were studied from women treated with levonorgestrel released from an intrauterine coil. During the untreated normal cycle, there was a significant increase in glandular VEGF immunoreactivity and a significant decrease in PR immunoreactivity in the mid- and late secretory phases compared to the proliferative phase. There was a significant positive correlation between stromal VEGF immunoreactivity and endothelial cell density. 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 but the intrauterine treatment with levonorgestrel resulted in strong glandular epithelial staining and intense staining of decidualized stromal cells. Implanon significantly increased glandular PR staining, but Mircette significantly reduced stromal PR staining when compared to secretory phase before-treatment biopsies. There were no changes in endothelial cell density or glandular or stromal ER during the normal cycle, or with use of either contraceptive. There was no association of the parameters measured with bleeding patterns or histological category.

Key words: angiogenesis/bleeding/contraceptive/endothelium

Introduction

One form of contraception which has many attractions is that of a long-acting gestogen which is either administered systemically or locally by an intrauterine system (IUS). Such a regimen does not require daily treatment and has long-term contraceptive efficacy. However, a serious disadvantage of such methods is frequent or irregular bleeding episodes. These complications are the most frequent cause of discontinuation of such contraceptive methods.

Hormonal control of gene expression is of fundamental importance in many tissues. The uterine

endometrium is a prime example of such a tissue and is highly responsive to the ovarian sex steroids. These responses include rapid tissue growth and remodelling, blood vessel development and protein secretion. Each of these processes necessitates complex cell–cell interactions. Advances made in the understanding of reproductive function have largely centred on the control of production and action of steroid hormones. Indeed most gynaecological medical intervention is based on steroidal/antisteroidal regulation of the uterus. However, while it is clearly recognized that steroids control endometrial function, paracrine and autocrine factors are now being seen as key mediators of reproductive function, albeit interacting with steroids. For example, in the ovariectomized mouse the uterus undergoes marked growth in response to single doses of oestradiol. This effect can be blocked by anti-transforming growth factor-α (TGFα) antibody suggesting that the mitogenic effects of oestrogen in this tissue are mediated by TGFα. Within the endometrium the cytokine/growth factor signals that pass between the stroma and epithelium and the epithelium and embryo are at present unclear. In addition other cell types in the endometrium such as leukocytes and endothelial cells also respond to ovarian steroids, although this may well be an indirect action.

One growth factor which could mediate this interaction is vascular endothelial growth factor (VEGF). This is a potent angiogenic growth factor (Ferrara *et al.*, 1992) and is produced in both glands and stromal cells of the endometrium and also myometrial smooth muscle cells (Charnock-Jones *et al.*, 1993; Smith, 1998). 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). Alterations in the normal distribution of VEGF due to the administration of synthetic steroids may be responsible for any resulting aberrant bleeding. This may be due to an increase in vascular permeability, changes in the thrombotic balance (VEGF induces endothelial uPA and PAI-1) or increased capillary number.

The aims of the present study were to compare parameters which may be important in endometrial bleeding: VEGF, oestrogen recpetor (ER), and progesterone receptor (PR) as reflected by immunohistochemistry and endothelial cell density in the endometrium (i) throughout the normal menstrual cycle and (i) after using two different contraceptives: Implanon and Mircette. The relationship between these parameters and the observed bleeding patterns and histology was analysed. In addition a study was performed in which endometrial VEGF immunoreactivity and macrophage distribution was investigated in women before and while using a levonorgestrel releasing intrauterine coil.

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 (Organon, Cambridge, UK), a non-biodegradable implant with a duration of action of 3 years containing and releasing etonogestrel. The next 20 subjects used Mircette (Organon), a 21 day monophasic combined oral contraceptive containing 20 μg ethinyl oestradiol and 150 μg desogestrel, with 10 μg ethinyl oestradiol/day administered for 5 days immediately before the next pill cycle.

Endometrial biopsies were taken on an outpatient basis by Pipelle suction curette 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 μm) were mounted on 2% aminopropyltriethoxysilane (Sigma, Poole, UK)-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 23–28 (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

VEGF

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 (without prior antigen unmasking): rabbit anti-human VEGF (A-20; Santa Cruz Biotechnology, Santa Cruz, 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: goat anti-rabbit IgG (Zymed Laboratories, South San Francisco CA, USA), 1/100 in PBS (10 min); then horseradish peroxidase–streptavadin conjugate (Zymed) 1/400 in PBS (10 min). Colour was developed with aminoethylcarbazole (AEC) substrate chromogen mix (Zymed), 5–10 min. Sections were counterstained with diluted Mayer's haemotoxylin (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.

Levonorgestrel study

Women (n = 12), seeking medical attention for the typical symptoms of oestrogen deficiency such as hot flushes, excessive sweating or vaginal dryness were recruited to this study. The intrauterine device used released with 5 (n = 6) or 10 (n = 6) μg/24 h of levonorgestrel (Leiras Oy, Turku, Finland) and all women also received transdermal 17β-oestradiol (50 μg/25 h Estraderm; Novartis, UK). Endometrial biopsies were collected by Pipelle prior to treatment and also after both 6 and 12 months treatment.

The immunostaining of the endometria from these women was performed in a similar manner except that the final chromogenic substrate was diaminobenzidine (DAB) which resulted in brown staining. Macrophages (and some other cells of myeloid origin) were identified by specific staining for CD68 (Dako M0876, High Wycombe, Bucks, UK) and the double-stained macrophages and VEGF positive cells detected fluorescently with fluoroscein isothiocyanate and TRITC (tetramethylrhodamine isothiocyanate) labelled anti-mouse and rabbit antibodies respectively.

ER and PR

Separate sections were stained for oestrogen receptor alpha (ERα) and PR using mouse monoclonal antibodies: ER 1D5 (Dako) and PR-AT 4.14 (ABR, Golden Co., CO, 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, Chester, UK) in peroxidase buffer, 10 min, and haematoxylin for 30 s. Sections were dehydrated and mounted in DePX (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 density

Separate sections were stained to determine the number of endothelial cell (EC) nuclei per mm^2. The method used was essentially as described previously [Goodger (Macpherson) and Rogers, 1994] and used an antibody which stained all endometrial vessels. Sections were treated as for VEGF above, except that the primary antibody was

mouse anti-human CD34 (QBEND 10, Serotec, Oxford, UK) diluted 1/25 in 1% BSA 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 density was obtained, i.e. the number of EC nuclei per mm^2. 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 one 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: one 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. (ii) A bleeding/spotting-free interval: two 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 follows. Amenorrhoea: no bleeding/spotting; prolonged: one or more bleeding/spotting episodes lasting ≥10 days; frequent: more than four bleeding/spotting episodes; infrequent: less than two bleeding/spotting episodes; irregular: range of length of bleeding/spotting free intervals (i.e. greatest interval minus smallest interval) >17 days; regular: two to four bleeding/spotting episodes, no bleeding/spotting episode lasting ≥10 days, with a range of length of bleeding/spotting-free intervals of ≤17 days.

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 density, and their influence on bleeding category and histological classification of the after-treatment biopsy. For some analyses the samples were paired (i.e. before- and after-treatment samples from the same woman).

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 was 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; overall n = 26; 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 density

There were no significant changes in endometrial endothelial cell density across the normal cycle

Table I. Table showing 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 Mircette, 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

	VEGF immunostaining score				ER immunostaining score				PR immunostaining score				Endothelial cells	
	Glands	*n*	Stroma	*n*	Glands	*n*	Stroma		Glands	*n*	Stroma		/mm^2	*n*
Normal cycle phase														
Proliferative	1.00 (0.75–2.0)	8	1.00 (0.17–2.5)	8	1.75 (1.0–2.25)	8	1.88 (1.4–3.0)		1.90 (1.5–2.8)	8	2.10 (1.0–2.3)		345 (227–554)	8
Early secretory	1.63 (0.25–2.25)	8	1.38 (0.5–1.5)	8	1.50 (1.0–2.25)	6	1.33 (1.15–1.75)		1.10 (0.70–2.0)	6	2.30 (1.3–3.0)		270 (233–421)	7
Mid-secretory	2.59 (1.3–3.5)*	8	0.88 (0.17–2.0)	8	1.50 (1.5–1.8)	4	1.80 (1.5–1.9)		0.29 (0.0–0.78)*	4	2.29 (2.28–2.63)		307 (201–527)	4
Late secretory	2.30 (1.17–3.0)*	11	1.17 (0.5–3.0)	11	1.23 (0.75–2.0)	8	1.63 (1.0–2.1)		0.28 (0.0–2.3)**	8	2.30 (1.69–3.0)		269 (217–464)	9
Contraceptive treatment														
Implanon	0.625 (0.3–2.5)	6	1.67 (0.75–3.00)	7	2.25 (1.3–2.5)	6	1.65 (1.3–3.0)		2.0 (1.0–3.5)	7	2.5 (1.5–3.5)		266 (187–414)	7
Mircette	0.83 (0–2.5)	19	1.00 (0.25–2.30)	19	1.5 (0.8–2.0)	20	2.0 (1.0–2.5)		1.5 (0.3–2.8)	20	1.9 (1.0–3.0)		308 (184–496)	19

Biopsies during the normal cycle were divided into histological categories according to the criteria of Hendrickson and Kempson (1980).
*$P < 0.05$ compared to proliferative phase (Dunn's multiple comparisons test).
**$P < 0.01$ compared to proliferative phase (Dunn's multiple comparisons test).

(P = 0.806; overall n = 28; 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 density (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

Fourteen patients 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 densities are shown in Table I.

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 density did not change with treatment.

Correlations between immunohistochemical parameters

There was a significant positive correlation (Spearman rank correlation) between endothelial density 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 4/14. 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 years. Detailed results showing averaged subjective scores for VEGF staining in the glands and stroma, together with their endothelial cell densities, 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

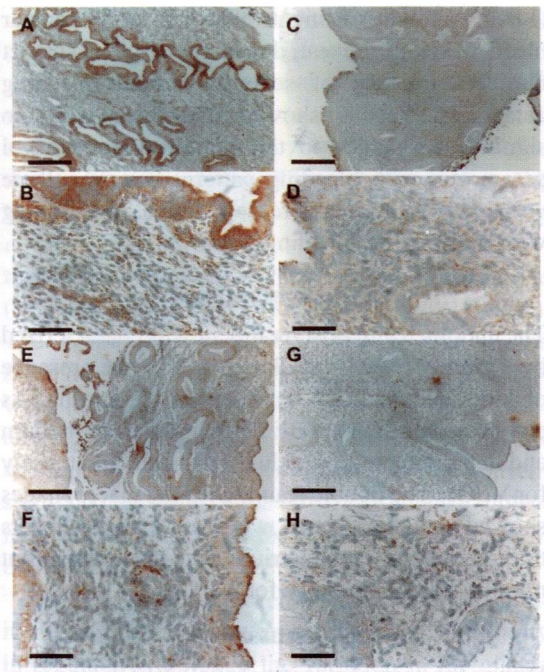

Figure 1. Photomicrographs of human endometrium immunostained for vascular endothelial growth factor (VEGF) (red), with light haematoxylin counterstaining (pale blue); **A–D** are from the same subject, in the Implanon study; **E–H** are from a single subject in the Mircette study. (**A**, **B**, **E**, **F**) Before-treatment (normal cycle) biopsies; (**C**, **D**, **G**, **H**) 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.

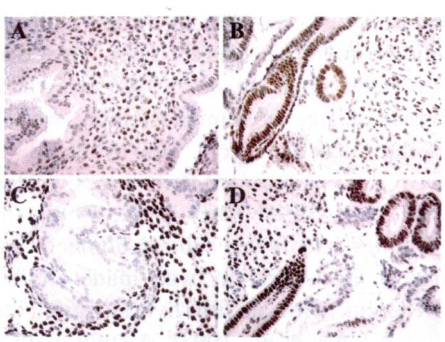

Figure 2. Photomicrographs of human endometrium immunostained (brown) for oestrogen receptor (**A**, **B**) and progesterone receptor (**C**, **D**). (**A**, **C**) Tissue obtained before treatment; (**B** and **D**) tissue after Implanon treatment (Original magnification ×400).

staining was seen in negative controls. See Table I for median scores and their ranges for the immunohistochemical parameters in after-Mircette

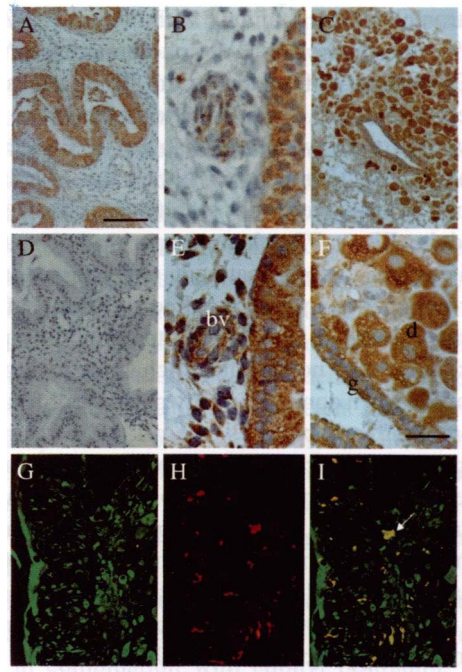

Figure 3. Photomicrographs of human endometrium stained for vascular endothelial growth factor (VEGF) (brown in **A–F**, green fluorescence in **G** and **I**). (**A**, **B**, **D**, **E**) Normal secretory phase endometrium; (**D**) control stained with non-immune IgG. (**B**, **E**) Serial sections of a blood vessel (bv) stained without and with antigen retrieval by heat treatment respectively. Remaining sections are from biopsies following treatment with an intrauterine levonorgestrel-releasing coil. g = glandular epithelium; d = decidualized stromal cell; arrow identifies a VEGF positive macrophage. (**G–I**) Sections are stained for VEGF (green) and CD 68, (macrophages, red) with the double-stained cells appearing yellow in panel **I**. Scale bar in **A** = 200 μm and applies to **A**, **C**, **D**, **G**, **H**, **I**; scale bar in **F** = 50 μm and applies to **B**, **E** and **F**.

treatment biopsies. Statistical analysis showed that overall there was a significant reduction in VEGF staining in the glands with treatment (n = 19 before versus 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) against secretory before-treatment biopsies (n = 11) P = 0.129. There was no significant change

in stromal VEGF staining with treatment (P = 0.639). 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 density did not change significantly with treatment.

Correlations between immunohistochemical parameters

There was a significant positive correlation (Spearman Rank Correlation) between glandular VEGF and stromal VEGF (r = 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 (11/19, 58%) of subjects. Of the remaining subjects, four experienced irregular cycles, and one each was categorized as having either frequent, frequent and irregular, infrequent, or prolonged 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).

Levonorgestrel study

The endometrium post treatment was characterized by a greatly reduced thickness and by the presence of large decidual-like cells within the stroma. As can be seen in Figure 3F the glandular epithelium and in particular the decidual cells stained intensely for VEGF. The double staining for the macrophages showed that these cells were present in clusters throughout the endometrium and that many (but not all) stained strongly for VEGF.

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 Li *et al.* (Li *et al.*, 1994), who reported strong VEGF glandular staining that did not change during the cycle, and the strongest stromal staining being found in discrete cells during the mid-proliferative phase. They also contrast with a study (Lau *et al.*, 1998) in which no significant change in immunostaining was found across the menstrual cycle. However, the pattern of VEGF staining reported here agrees with others (Shifren *et al.*, 1996; Torry *et al.*, 1996), though these studies had fewer subject numbers and no attempt at quantification was made. It is also in agreement with 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 to the proliferative phase (Greb *et al.*, 1995). It is also of interest that in the present study a simple comparison within the before-treatment (normal cycle) group from the Mircette study of glandular VEGF staining during the prolif-

erative 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 (Snijders *et al.*, 1992; Critchley *et al.*, 1993) and are consistent with down-regulation of PR in the glands by progesterone (Chauchereau *et al.*, 1991; Snijders *et al.*, 1992). The continued presence of PR in the stroma throughout 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 apparent, however.

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. 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 *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). These data are also consistent with our observation that local treatment with a gestogen increases VEGF level.

The correlation between stromal VEGF staining and endothelial cell density has not been previously reported. This has been investigated by others (Gargett *et al.*, 1999) who found no correlation. This suggests that 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 density nor microvascular density [Goodger (Macpherson) and Rogers, 1994; Rogers *et al.*, 1993] changed throughout the normal cycle and indeed changes in endothelial cell density 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 density during the cycle suggest that these parameters may be regulated by non-steroidal mechanisms. This is, however, 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 contains the pro-form of the same progestagen in combination with ethinyl oestradiol, both significantly reduced endometrial glandular immunoreactive VEGF. Endothelial cell density after 12 months of use showed no difference compared to before treatment, and there was no correlation between glandular VEGF staining and endothelial cell density 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 density 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 EC density 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 PR in the same way that progesterone does. It is likely that the balance of agonist/antagonist actions and the PR type contributes to the end result of progestin action. Mircette, on the other hand, resulted in significantly reduced stromal PR compared to normal secretory phase levels 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 density.

The lack of change in endothelial cell density with the two contraceptives used in this study contrasts with the action of three other contraceptives, for example, Norplant (an implant which releases 80 μg/day levonorgestrel), high doses of norethisterone and medroxypogesterone acetate (MPA). Norplant results in significantly increased microvascular and endothelial cell density compared to 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]. 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 decreased endometrial vascular density (Song *et al.*, 1995). None of these studies, however, investigated VEGF immunoreactivity. Another study investigating endometrial microvascular density in various conditions of atrophy found no differences compared to endometrium removed from normally cycling women (Hickey *et al.*, 1996). The present study confirms the apparent dissociation between endometrial endothelial cell density 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; Rogers *et al.*, 1998). The same may be true with other steroid treatments.

In this study we also carried out VEGF immunostaining of endometrium from patients who had been treated with levonorgestrel from an IUD. These samples showed very strong staining in the decidualized stromal cells as well as the uterine epithelial cells. The latter stained strongly despite the pronounced thinning of the epithelial layer. We also observed strong staining in $CD68^+$ cells within this tissue. The distribution of the macrophages was extremely heterogeneous and due to the paucity of tissue in many specimens accurate quantitation of their number was not attempted. Macrophages are a well-known source of VEGF and therefore this observation in itself is unremarkable; what is of more interest is the high average frequency of macrophages in the levonorgestrel-treated tissue. Further studies in which specific staining for macrophages is carried out using endometrial tissue exposed to other contraceptive steroids would determine whether this is a common occurrence following such treatment

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 density, bleeding pattern, or histological category of the endometrium. Stromal staining for VEGF correlated positively with endothelial cell density although neither parameter changed significantly throughout the menstrual cycle, or influenced the endometrial bleeding pattern. However, the precise role of progesterone in controlling glandular VEGF is complicated by the fact that long-term local treatment causes both the epithelial cells to remain immunopositive for VEGF and decidualized stromal cells to stain very intensely. It is likely that understanding the down-regulation (or otherwise) of the PR under these conditions may offer an explanation for this.

Acknowledgements

This work was supported in part by NV Organon, The Netherlands. We thank Dr S.D.Holman from the Cambridge University Department of Anatomy for use of his equipment for endothelial cell quantification, and Mr M.A.Baas and Miss D.van Ingen-Schenau from NV Organon for ER and PR staining.

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