The effects of levonorgestrel implants on vascular endothelial growth factor expression in the endometrium

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Vascular endothelial growth factor (VEGF) expression and the microvascular density of the endometrium were studied in Norplant users and normal controls, using immunohistochemistry on formalin-fixed paraffin-embedded endometrial sections. The VEGF staining index was quantified using computerized image analysis. The VEGF staining index between stages of the menstrual cycle and between normal and Norplant endometria were compared. Norplant VEGF staining index was analysed for correlation with microvascular density, duration of Norplant use, the number of bleeding/spotting days in the reference period up to 90 days prior to biopsy, and the length of time since the last bleeding/spotting episode. The results showed that immunoreactive VEGF was detected predominantly in endometrial glands but weakly expressed in the stroma throughout the menstrual cycle, and also in Norplant users. Large variation in the VEGF staining index between individuals was observed and no significant difference in the VEGF staining index was detected between stages of the menstrual cycle for the glands and stroma. The glandular and stromal VEGF staining indices were significantly higher in Norplant than in normal endometrium (P < 1 x 10^-4). No correlation was found between the Norplant VEGF staining index and endometrial microvascular density, duration of Norplant use, the number of bleeding/spotting days in the reference period, and the length of time since the last bleeding/spotting episode. The VEGF staining index was higher in glands than stroma for both normal and Norplant endometrium. The results suggest a differential control of endometrial glandular versus stromal VEGF expression, and possible positive effects of levonorgestrel on VEGF expression.

Key words: breakthrough bleeding/blood vessel/endometrium/Norplant/VEGF

Introduction

Norplant (Leiras, Turku, Finland) is a levonorgestrel-containing subdermal contraceptive implant which provides effective contraception for 5 years. Side-effects associated with the use of Norplant include menstrual disorders such as prolonged bleeding, irregular bleeding (i.e. breakthrough bleeding) and amenorrhoea. The mechanism(s) controlling these menstrual disorders is not known.

The endometrium of most Norplant users is regressed. The average thickness of Norplant endometrium is ~10% of the thickness of endometrium from the normal menstrual cycle during the luteal phase (Rogers, 1996). However, the microvascular density is significantly higher in the Norplant endometrium than the normal endometrium (Rogers et al., 1993). The cause of the elevation of microvascular density in Norplant endometrium is not known.

Vascular endothelial growth factor (VEGF) is a member of the VEGF growth factor family. Members of the family include VEGF, VEGF-B (Olofsson et al., 1996), VEGF-C (Lee et al., 1996), VEGF-D (Yamada et al., 1997), VEGF-2 (Hu et al., 1997) and placental growth factor (Maglione et al., 1991). VEGF is a vascular permeability and an angiogenic factor. VEGF has been shown to stimulate endothelial cell growth in vitro and angiogenesis in vivo (Leung et al., 1989; Kim et al., 1993). Homozygous and heterozygous VEGF-deficient mouse embryos have impaired and abnormal blood vessel formation with embryonic death occurring at mid-gestation (Carmeliet et al., 1996; Ferrara et al., 1996). Two VEGF receptors, flt-1 (De Vries et al., 1992), flk-1/KDR (Terman et al., 1992) have been identified. Five VEGF isoforms termed according to the number of amino acid residues in the molecule, i.e. 121, 165 and 189 (Torry et al., 1996) 145 (Charnock-Jones et al., 1993), and 206 (Houck et al., 1991), have been identified. Four isoforms (VEGF_121, VEGF_145, VEGF_165, and VEGF_189) have been found in the human endometrium, of which VEGF_121 and VEGF_165 are predominant (Charnock-Jones et al., 1993; Torry et al., 1996).

VEGF has been immunolocalized in the human endometrium (Li et al., 1994; Shifren et al., 1996; Smith, 1996; Torry et al., 1996). However, there is an inconsistency in the reported patterns of VEGF expression in human endometrium during the normal menstrual cycle. Glandular VEGF immunoreactivity has been reported to be lower (Shifren et al., 1996; Torry et al., 1996) during the proliferative phase than the secretory
phase, or not different across the menstrual cycle (Li et al., 1994; Smith, 1996). Stromal VEGF immunoreactivity was generally reported to be low across the menstrual cycle (Shifren et al., 1996; Smith, 1996; Torry et al., 1996), although Li et al. (1994) showed strong stromal VEGF immunoreactivity during the mid-proliferative phase. Despite these reports on VEGF expression, there are no quantitative comparisons of VEGF staining between the various stages of the menstrual cycle.

The aims of the present study were to use immunohistochemistry: (i) to study VEGF expression by quantifying VEGF staining in endometrium during the normal menstrual cycle and in Norplant users; and (ii) to investigate whether VEGF expression in the Norplant endometrium was correlated with microvascular density, duration of Norplant use, the number of bleeding/spotting days in the period up to 90 days prior to biopsy, and the length of time since the last episode of bleeding/spotting.

Materials and methods

Subjects

All normal endometrial biopsies and the majority of Norplant biopsies were archival tissues which were collected for our previous studies (Rogers et al., 1993; Goodger et al., 1994; Lau et al., 1996). This consisted of 49 samples from across the normal menstrual cycle and 72 Norplant samples. All subjects were recruited on the basis of fully informed consent. Ethical approval was obtained from Monash University Standing Committee on Ethics in Research on Humans (for samples from the normal menstrual cycle) and the Medical Faculty of the University of Indonesia Ethics Commission in Research on Humans (for Norplant samples). Biopsies from the normal menstrual cycle were dated by an experienced histopathologist and categorized into nine stages of the normal menstrual cycle (Rogers et al., 1993) as follows: menstrual (n = 6), early proliferative (n = 7), early-to-mid proliferative (n = 5), mid-proliferative (n = 3), mid-to-late proliferative (n = 7), late-proliferative (n = 4), early secretory (n = 7), mid-secretory (n = 4) and late secretory (n = 6). The Norplant biopsies were taken from women, 19–415 days after Norplant insertion. The number of spotting/bleeding days, as defined by the World Health Organization (Rogers et al., 1993) in the 90 day reference period prior to biopsy was recorded from a menstrual diary. In subjects where the time between Norplant insertion and biopsy was <90 days, the whole period of Norplant use was considered to be the reference period. There were varying degrees of breakthrough bleeding between subjects, ranging from 0 to 79 days in the reference period. All biopsies were routinely fixed in 10% buffered-formalin in phosphate-buffered saline (PBS, pH 7.2) for 4–6 h. Following formalin fixation, biopsies were stored in PBS prior to routine histological processing and paraffin wax embedding. Norplant biopsies were shipped to Monash University, Australia, while stored in PBS, for processing.

VEGF and CD34 immunostaining

Serial sections (6 μm) were dewaxed, washed in 100, 100 and 75% ethanol and then rehydrated for standard immunohistochemical staining for VEGF and the endothelial cell marker CD34. A horse-radish peroxidase-conjugated streptavidin system was used. Endogenous peroxidase activity was blocked by incubating the sections with 3% H2O2 in 50% methanol for 10 min at room temperature. Non-specific binding was overcome by pre-absorbing the sections with 10% normal goat serum (for VEGF; Zymed Laboratories, San Francisco, CA, USA) or 10% normal rabbit serum (for CD34; Hunter Antiserum, Sydney, Australia) for 10 min at room temperature.

For VEGF staining, antigen retrieval was effected by boiling sections in sodium citrate buffer (10 mM, pH 6) for 10 min and then cooled in the hot buffer for 10 min prior to H2O2 treatment. A rabbit anti-human VEGF polyclonal antibody (Sc-152; Santa Cruz Biotechnology Inc, Santa Cruz, CA, USA) was used. This antibody was raised using a peptide corresponding to amino acids 1–20 of the N-terminal of VEGF. According to the manufacturer, this antibody reacts with VEGF 121, VEGF 165 and VEGF 189. VEGF antibody was applied to sections at 2 μg/ml and incubated at 37°C for 2 h. Biotinylated goat anti-rabbit immunoglobulin (Ig)G (Zymed, used undiluted) was then applied and incubated at room temperature for 30 min.

For CD34 staining, the procedures are the same as those described previously (Rogers et al., 1993). A mouse monoclonal anti-human CD34 antibody (QBEND10; Serotec, Oxford, UK) was used at 0.25 μg/ml in PBS (10 mM, pH 7.4) containing 1% BSA. Incubation was carried out at 37°C for 45 min. Biotinylated rabbit anti-mouse polyclonal antibody (Zymed) was then applied at 6 μg/ml and incubated for 15 min at room temperature.

For both VEGF and CD34, horse-radish peroxidase-conjugated streptavidin (Zymed) was applied after the second antibody stage at 1:400 dilution and incubated at room temperature for 15 min. H2O2 and the aminothio carbazole chromogen (Zymed) mixture was applied to the sections and incubated at room temperature for 10 min. At the end of the incubation period sections were rinsed with distilled water and mounted with an aqueous mount (Clearmount; Zymed).

Positive and negative controls using human placenta (for VEGF) or a human endometrium (for VEGF and CD34) were included in every run. The negative control substituted the primary antibody with a non-immune rabbit IgG (for VEGF, Santa Cruz Biotechnology Inc) or mouse IgG1 (for CD34; Chemicon, Temecula, CA, USA) at the same protein concentrations. VEGF immunostaining pilot studies using endometrial tissues from the normal menstrual cycle and Norplant users indicated that preincubating the VEGF antibody with 10-fold and 20-fold molar concentrations of VEGF peptide (Santa Cruz Biotechnology Inc) reduced the staining index by 83 and 89% respectively.

Image analysis

Images of VEGF staining were randomly captured using a digital video camera (Fujix: Fuji, Tokyo, Japan) mounted onto a microscope and connected to an Analytical Imaging Station (AIS: Image Research Inc, Ontario, Canada). One to four images per section (equivalent to 0.57–2.27 mm2 of tissue area) were captured. This area represents the whole endometrium in a number of normal and Norplant biopsies. VEGF staining intensity for the glands and stroma and the proportion of stained area were measured using the AIS software. An average of 7 (range 1–26) measurements of glandular staining and an average of 3 (range 1–18) measurements of stromal staining were taken per section. These measurements covered all glands and stromal areas in a number of images captured from both normal and Norplant endometrium. A staining intensity index for glandular and stromal compartments was calculated by multiplying the background-adjusted staining intensity by the proportion of stained area. For the measurement of glandular staining, the lumen was excluded from the area measured. Scattered VEGF-positive individual cells were excluded in the measurement of stromal VEGF staining. All sections were also semi-quantitatively scored by eye and analysed to validate the image analysis system.
Figure 1. Vascular endothelial growth factor (VEGF) staining in glands and stroma of endometrium from the normal menstrual cycle during: (a) early proliferative; (b) mid-proliferative; (c) late proliferative; (d) early secretory; (e) mid secretory; (f) late secretory, and from: (g) a Norplant user, and (h) negative control normal late secretory endometrium. Bar = 100 μm.
The scoring was carried out blind and all samples were scored in one session to reduce variability.

For CD34 analysis, 1–3 images representing 0.15–0.45 mm² tissue area were captured per sample. This area is the entire tissue area for a number of samples. The number of blood vessels on the image was counted manually, the scanned area was measured and the microvascular density was calculated.

**Statistical analysis**

Initial analyses indicated that VEGF indices for all except VEGF staining index for the stroma of normal endometrium assumes a normal distribution. For consistency, non-parametric tests were performed. The Kruskal–Wallis Test was used to compare VEGF staining indices between various stages of the cycle. The Wilcoxon Rank-Sum Test for two groups was used to compare normal VEGF staining indices between various stages of the cycle. The Wilcoxon Rank-Sum Test for two groups, pool of all stages of the menstrual cycle, by pooling all proliferative phases only, pool of secretory phases only were compared. The inter-assay coefficients of variation for glandular and stromal staining indices were 22.2 ± 9.15% and 15.0 ± 7.14% (n = 3) respectively.

Similar results were obtained from analyses of the semi-quantitative scores of VEGF staining as those obtained by image analyses. No difference in VEGF staining scores was detected between the stages of the menstrual cycle for the glands or the stroma. VEGF staining score was significantly higher in the glands than the stroma (P < 0.0002).

**Results**

VEGF was detected predominantly in endometrial glands throughout the normal menstrual cycle (Figure 1). Weak VEGF staining was also detected in the stroma throughout the menstrual cycle. Relatively large variations in VEGF staining between individuals at the same stage of the cycle were observed. No statistically significant difference in VEGF staining index between the various stages of the cycle was detected for the glands and the stroma (Kruskal–Wallis Test) (Figure 2). The VEGF staining index was significantly higher in glandular than stromal compartments (Wilcoxon Rank-Sum Test for two groups, pool of all samples P < 5 × 10⁻⁶; Figure 3, pool of proliferative phases only P < 0.005, pool of secretory phases only P < 0.0002). The inter-assay coefficients of variation for glandular and stromal staining indices were 22.2 ± 9.15% and 15.0 ± 7.14% (n = 3) respectively.

Similar results were obtained from analyses of the semi-quantitative scores of VEGF staining as those obtained by image analyses. No difference in VEGF staining scores was detected between the stages of the menstrual cycle for the glands or the stroma. VEGF staining score was significantly higher in the glands than the stroma (P < 0.0002).

VEGF was also detected in individual cells scattered in the stroma of 32 samples. The number of positive individual cells varied greatly between samples of the same stage of the cycle. The number of positive individual cells did not appear to be associated with the stage of the menstrual cycle. Blood vessels were largely negative for VEGF staining. Nine samples across the menstrual cycle showed varying degrees of VEGF staining in some blood vessels but the staining did not appear to be cycle specific.

VEGF was also detected in Norplant endometrium (Figure 1). As for normal endometrium, VEGF staining index was significantly higher in the glandular than the stromal compartments in Norplant endometrium (Wilcoxon Rank-Sum Test for two groups, P < 1 × 10⁻⁶). Both glandular and stromal VEGF staining indices were significantly higher in Norplant than in normal endometrium (Figure 3: Wilcoxon Rank Sum Test for two groups, P < 1 × 10⁻⁶). Both glandular and stromal VEGF staining indices were significantly higher in Norplant than in normal endometrium (Figure 3: Wilcoxon Rank Sum Test for two groups, P < 1 × 10⁻⁶ for both comparisons). VEGF was also detected in scattered individual cells in eight, and in blood vessels in two, Norplant endometrial samples.

Regression analyses showed no correlation between duration of Norplant use and VEGF glandular and stromal staining indices (R² = 0.012, P > 0.38 for glands; and R² = 0.005, P > 0.55 for stroma). There was a similar lack of correlation between VEGF staining indices and the number of bleeding/spotting days in the reference period prior to biopsy (R² =...
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VEGF mRNA levels were reported to be lower during the secretory phase than the proliferative phase (Charnock-Jones et al., 1993). In contrast, another report from the same group indicated a lack of difference in VEGF mRNA levels across the menstrual cycle, although VEGF mRNA levels appeared to be higher during the menstrual phase of the cycle but no significant difference was observed (Smith, 1996). By using ribonuclease protection assay, Torry et al. (1996) and Shifren et al. (1996) showed a 3–6-fold increase in VEGF mRNA during the secretory phase compared with the proliferative phase. However, ribonuclease protection assay cannot differentiate between glandular and stromal VEGF mRNA.

Taking together all the available data, it appears that VEGF protein expression does not vary across the normal menstrual cycle despite an increase in VEGF mRNA during the secretory phase. Similar discordance between VEGF and VEGF mRNA levels has been reported in the normal human myometrium (Harrison-Woolrych et al., 1995).

VEGF immunoreactivity has previously been reported in the Norplant endometrium (Smith, 1996). In this study, we extended the finding that VEGF staining was significantly higher in Norplant endometrium than in normal endometrium. The cause of a higher VEGF staining in the Norplant endometrium is not clear. A number of factors have been shown to influence VEGF and/or VEGF mRNA expression, including oestrogen (Charnock-Jones et al., 1993), hypoxia (Namiki et al., 1995), tumour necrosis factor-α (TNF-α, Yoshida et al., 1997), gonadotrophins (Christensen and Stouffer, 1997), interleukin (IL)-1 (Ferrer et al., 1997) and IL-5 (Horiiuchi and Weller, 1997). However, oestrogen seems unlikely to be the cause of this increase in VEGF immunoreactivity, as only 20% of Norplant users have been shown to have ovulatory-like cycles (Faundes et al., 1991). Even in these women, the plasma oestrogen concentrations are much lower than those observed during the normal menstrual cycle (Rogers et al., 1996).

In addition, immunoreactive oestrogen receptor levels in the Norplant endometrium were minimal (Critchley et al., 1993). The possible role of other factors in Norplant endometrial VEGF regulation has not been investigated. The higher VEGF staining in Norplant than in normal endometrium suggests a positive effect of levonorgestrel on VEGF expression. Levonorgestrel is known to act through binding to progesterone receptors (Lemus et al., 1992). Progesterone receptor protein and mRNA have been found in glands and stroma of Norplant endometrium (Critchley et al., 1993; Lau et al., 1996).

Despite an apparent increase in VEGF immunoreactivity, and our previous study showing higher microvascular density in Norplant endometrium compared with normal endometrium, no correlation between blood vessel density and VEGF staining indices in Norplant endometrium was observed. It is not clear if this lack of correlation was due to the fact that most Norplant endometrial samples were collected at least 3 months after the commencement of Norplant use. A stable vascular density may have been achieved in these tissues at the time of endometrial biopsy despite an apparent high level of VEGF in these tissues. Alternatively, endometrial microvascular density is highly variable and it is possible that while both VEGF and microvascular density are significantly elevated compared

Discussion

This study has shown that immunoreactive VEGF is present in glands and the stroma of the normal endometrium throughout the menstrual cycle. Glandular staining is much stronger than stromal staining. No significant difference in glandular and stromal VEGF staining indices between various stages of the menstrual cycle was detected.

In the literature, there is a lack of consensus with regard to the levels of immunoreactive VEGF in the human endometrium. This study, and those by Li et al. (1994) and Smith (1996), showed that there is a lack of difference in glandular VEGF immunoreactivity across the menstrual cycle. However, Shifren et al. (1996) and Torry et al. (1996) showed an increase in glandular VEGF immunoreactivity during the secretory phase. With regard to stromal VEGF staining, this study and those by Shifren et al. (1996), Torry et al. (1996) showed low levels of stromal VEGF immunoreactivity across the menstrual cycle, whereas Li et al. (1994) and Smith (1996) showed strong stromal VEGF immunoreactivity during the proliferative phase. The underlying reasons for the observed differences between various studies are not known. In three of the studies (Shifren et al., 1996; Smith, 1996, Torry et al., 1996), endometrial samples were broadly categorized into ‘proliferative’ and ‘secretory’ stages. In contrast, in this study and in the study of Li et al. (1994), endometrial samples were further categorized into various stages of proliferation and secretion. It is not known whether the differences in the classification of endometrial samples had contributed to the reported differences. In this study, if VEGF staining indices from all proliferative stages and all secretory stages were pooled into ‘proliferative’ and ‘secretory’ stages respectively, a significant difference was achieved for stromal VEGF staining index with higher VEGF staining index during the proliferative stage being detected. No difference for glandular VEGF staining indices was observed.

In this study, we have found a large variation in VEGF staining indices between individuals within each stage of the menstrual cycle, although it is not known whether this inherent variability has contributed to the lack of statistical difference in VEGF staining in the glands and the stroma across the menstrual cycle. Variability in endometrial VEGF staining has also been reported previously (Li et al., 1994).

As with VEGF immunoreactivity, inconsistent patterns of VEGF mRNA expression have also been reported. Glandular VEGF mRNA levels were reported to be higher while stromal VEGF mRNA levels were reported to be lower during the secretory phase than the proliferative phase (Charnock-Jones et al., 1993). In contrast, another report from the same group indicated a lack of difference in VEGF mRNA levels across the menstrual cycle, although VEGF mRNA levels appeared to be higher during the menstrual phase of the cycle but no significant difference was observed (Smith, 1996). By using ribonuclease protection assay, Torry et al. (1996) and Shifren et al. (1996) showed a 3–6-fold increase in VEGF mRNA during the secretory phase compared with the proliferative phase. However, ribonuclease protection assay cannot differentiate between glandular and stromal VEGF mRNA.

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0.038, \( P > 0.12 \) for glands; and \( R^2 = 0.0001, P > 0.92 \) for stroma) and the number of days from the last bleeding/spotting day to the day of biopsy \( (R^2 = 0.059, P > 0.057 \) for glands; and \( R^2 = 0.005, P > 0.55 \) for stroma).

The average blood vessel density in the Norplant endometrium was 269.3 \( \pm 20.9/\text{mm}^2 \). This microvascular density was consistent with those reported in our previous study (Rogers et al., 1993). Regression analyses showed no correlation between blood vessel density and glandular VEGF staining index \( (R^2 = 0.017, P > 0.36) \) or stromal staining index \( (R^2 = 0.036, P > 0.14) \).
with endometrium from the normal menstrual cycle, the inherent variability in VEGF staining and microvascular density between endometria may obscure a more precise correlation between these two events.

The present study also found no correlation between VEGF staining in the Norplant endometrium with either the number of bleeding/spotting days, or the duration since the last bleeding/spotting episode, i.e. there is no difference in VEGF staining between endometria with amenorrhea and those with recent breakthrough bleeding. In fact, there was a slight tendency for a decrease in VEGF staining with recent breakthrough bleeding, although the decrease was not statistically significant ($R^2 = 0.058, P = 0.0575$). VEGF has been implicated in blood vessel repair since expression of the VEGF receptor, flt-1 was found to be elevated in healing skin wound (Peters et al., 1993). After an episode of breakthrough bleeding, damaged blood vessels have to be repaired and regenerate. It might therefore be expected that active angiogenesis is occurring in these tissues. A similar argument may apply to menstruation after which the endometrium regrows very rapidly. However, no increase in VEGF staining was observed in Norplant endometrium where frequent and/or recent breakthrough bleeding had occurred, or during the menstrual phase of the normal menstrual cycle. These results thus do not provide evidence to support the role of VEGF in vascular repair during wound healing in the human endometrium. The mechanism controlling endometrial wound healing may be different to that in the skin, as the endometrium does not form scars after menstrual shedding or breakthrough bleeding. The role of VEGF in the Norplant endometrium is thus not clear, although it has been suggested that VEGF may be involved in the maintenance of endometrial vasculature by inhibiting endothelial cell apoptosis (Smith, 1996).

This study also found higher VEGF staining in glands than stroma for both normal and Norplant endometrium. These results suggest a differential control of glandular and stromal VEGF expression. Similar differential glandular and stromal VEGF expression has been reported in the normal human endometrium (Li et al., 1994; Charnock-Jones et al., 1996; Shifren et al., 1996). The mechanisms controlling this differential expression and the precise function of glandular versus stromal VEGF await to be determined.

Finally, VEGF immunoreactivity was also detected in scattered individual cells in the stroma of the normal and Norplant endometrium. The origin of these cells was not determined in the present study, although it is likely that they are macrophages. Smith (1996) showed that VEGF-positive individual cells in the stroma of normal and Norplant endometrium are also CD68 positive. This showed that tissue macrophages also produce VEGF. However, the importance of macrophage-derived VEGF in endometrium angiogenesis is not known.

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