Influence of different hormonal regimens on endometrial microvascular density and VEGF expression in women suffering from breakthrough bleeding

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BACKGROUND: The aim of this study was to quantify blood vessel density (BVD) and immunoreactive vascular endothelial growth factor (VEGF) levels in endometrial biopsies taken from women suffering breakthrough bleeding (BTB) under different exogenous hormonal regimes. METHODS: Endometrial biopsies from women in Melbourne with BTB were divided into four groups: combined–continuous hormone therapy (HT) (estrogen and progesterin taken daily), cyclical HT (daily estrogen with progesterin for 14 days each cycle), progestin-only, or no HT. Subjects from Barcelona were using the Mirena intrauterine levonorgestrel-releasing system for contraceptive purposes, with menstrual diaries for classification into four groups (amenorrhea, infrequent, regular and prolonged). Control biopsies from Melbourne were included in the study. Endometrial samples were immunostained for VEGF and blood vessel localization using an antibody to CD34. RESULTS: Results showed that BVD was significantly reduced in the progestin-only treated group compared with the other three treatment groups (P = 0.028). In addition, all four Mirena BTB groups had significantly reduced BVD compared with controls. Considerable heterogeneity was observed in VEGF immunostaining within and between individual samples with no major differences between HT or Mirena. CONCLUSION: These results provide strong evidence that unopposed progestins reduce endometrial BVD and that there is no link between VEGF immunostaining and BVD or BTB.

Key words: breakthrough bleeding/endometrium/microvascular density/progestin/VEGF

Introduction

Endometrial breakthrough bleeding (BTB) is defined as any irregular or unpredictable bleeding that is not part of the normal menstrual process. Unlike menstrual bleeding, which occurs primarily from the spiral arterioles in response to falling levels of estrogen and progesterone (Markee, 1940), BTB occurs from the endometrial capillaries and smaller vessels and is a common side effect of progestin-only contraceptives (D’Arcangues, 2000). BTB can also occur under a wide range of other hormonal conditions and commonly used hormone therapy (HT) regimens (Hickey et al., 2003). Understanding of the cellular mechanisms that lead to BTB remain elusive because of both the wide range of hormonal conditions under which it can occur and the high degree of variability between different women in terms of their endometrial response to exogenous hormones and their susceptibility to BTB. Thus, while administration of exogenous hormones—and in particular progestin-only regimens—will increase the incidence of BTB, the link between hormones and BTB is not direct. Numerous studies over the past 10–15 years have been undertaken with the aim of developing a better understanding of the local endometrial mechanisms responsible for BTB. Unfortunately, the majority of these studies have failed to identify mechanisms that might be responsible for BTB or, where possible mechanisms have been proposed, widespread consensus as to their importance does not yet exist within the field.

It has been proposed that, in women with BTB, there is increased endometrial vascular fragility, leading to increased susceptibility to damage and rupture (Hickey et al., 2000). This concept is supported by the finding that perivascular support cells (pericytes and vascular smooth muscle cells) are reduced around microvessels in the endometrium of women using progestin-only contraception who suffer from BTB compared with those without BTB (Rogers et al., 2000). In addition to providing structural stability, perivascular cells, and in particular pericytes, play a key role in blood vessel maturation during angiogenesis (Hanahan, 1997). It is thought that a newly formed capillary remains responsive to vascular endothelial growth factor (VEGF) until it is sheathed by pericytes, and will thus grow or regress depending on the presence or absence of
Angiogenesis, or the formation of new vessels from pre-existing vasculature, can occur by several different mechanisms that can be measured in a variety of direct or indirect ways. One of the commonest ways of indirectly assessing angiogenic activity is to measure and compare the net gain or loss of microvessel numbers between two tissues of interest. Though not a true measure of vessel density, the technique most commonly used for this purpose is to count the number of vessel cross-sections or profiles occurring in a defined area of a histological section. A number of studies using this approach have reported what the authors define as endometrial blood vessel density (BVD) in women with and without BTB. Of these studies, one of the earliest reported an ∼50% increase in BVD in the endometrium of women using the subdermal levonorgestral (LNG) releasing implant Norplant®, although there was no correlation between BVD and severity of BTB (Rogers et al., 1993). The finding of increased endometrial BVD in Norplant® users has subsequently been confirmed (Hickey et al., 1999; Hickey and Fraser, 2002), suggesting a proangiogenic effect of LNG from Norplant® on the endometrium. In contrast, other studies have reported an ∼40% reduction in endometrial BVD in women using the progestins, norethisterone (NET) or medroxyprogesterone acetate (MPA), at higher doses than occurs with Norplant® (Song et al., 1995), suggesting that at higher doses progestins have an antiangiogenic effect on the endometrium. More recently it has been reported that the intrauterine LNG-releasing device, Mirena®, which is used both as a contraceptive and as a medical treatment for menorrhagia (Stewart et al., 2002), causes an increase in endometrial BVD following both short-term (McGavigan et al., 2003) and long-term use (Hague et al., 2002). Given that endometrial levels of LNG are significantly higher with intrauterine delivery than sub-dermal implant (Hague et al., 2002), these last two studies do not support a relationship between increasing progestin dose and reducing endometrial BVD. Further studies are required to clarify this issue.

One possible mediator of an endometrial angiogenic response to progestins is VEGF. Although the precise mechanisms by which VEGF regulates endometrial angiogenesis are poorly understood (Gargett et al., 1999), with recent evidence for a previously unsuspected level of complexity (Gargett et al., 2001; Heryanto et al., 2004), it is possible that bulk shifts in endometrial expression of VEGF could influence BVD. Some evidence to support this hypothesis exists, with reports of elevated VEGF in the endometrium of Norplant® users (Lau et al., 1999), and a significant correlation between endometrial stromal cell VEGF expression and endothelial cell density in controls and in women using the etonogestrel-releasing subdermal implant Implanon® (Charnock-Jones et al., 2000). However, other studies have failed to find any relationship between VEGF expression and changes in endometrial BVD (Laag-Fernandez et al., 2003).

The aim of the current study was to quantify BVD and immunoreactive VEGF levels in endometrial biopsies taken from women suffering from BTB under a range of exogenous hormonal regimes.

**Materials and methods**

**Subjects and sample collection**

A total of 82 endometrial biopsies were collected from women in Melbourne complaining of BTB who were undergoing diagnostic curette or other gynaecological procedure. The only inclusion criteria for the study were clinical diagnosis by the gynaecologist of BTB. A further 24 control endometrial tissue specimens, collected either from hysterectomy operations performed on ovulating women suffering from menorrhagia (n = 7) or from curettes taken for diagnostic purposes (n = 17) were accessed from the Centre for Women’s Health Research tissue bank. These control subjects had not received exogenous hormones in the 3 months prior to hysterectomy, had no clinically reported BTB, and their endometrial tissue was assessed as normal by routine histopathological examination. Ethical approval for the collection of endometrial tissue was obtained from Southern Health Human Research and Ethics Committee C, and informed consent was obtained from all subjects.

A total of 49 subjects in Barcelona were recruited from women using the Mirena® intrauterine LNG-releasing system for contraceptive purposes only, with no prior reported bleeding disturbances. Menstrual diaries were used to record BTB for a minimum of 5 months prior to endometrial biopsy by Pipelle suction curette. Bleeding categories were based on World Health Organization (WHO) definitions for BTB (see Rogers et al., 1993). Women participating in the study gave signed consent and ethical approval was obtained from Institut Universitari Dexeus Universitari Ethics Committee. Clinical groupings and age ranges for all subjects are given in Table I.

All endometrial biopsies underwent identical routine fixation and processing (immediate fixation in 10% buffered formalin, followed by dehydration and paraffin wax embedding). Once embedded in wax, biopsies collected in Barcelona were shipped to Melbourne for subsequent immunohistochemical analysis.

**Immunohistochemistry**

Tissue samples were formalin-fixed and paraffin-embedded prior to 5 μm serial sectioning. VEGF and blood vessel localization using an antibody to CD34 (an endothelial cell marker), were detected in adjacent sections. For both protocols, dewaxed sections were rehydrated in phosphate-buffered saline (PBS). Endogenous peroxidase was blocked with 3% hydrogen peroxide in 50% methanol for 10 min and

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**Table 1.** Clinical data, hormone therapy and breakthrough bleeding (BTB) groupings, and endometrial blood vessel density (BVD) data for each subject group

<table>
<thead>
<tr>
<th>Subject group</th>
<th>n</th>
<th>Age ± SEM</th>
<th>Adequate biopsy (n)</th>
<th>BVD/mm² ± SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>cc-HT</td>
<td>19</td>
<td>61.0 ± 2.0</td>
<td>9</td>
<td>218.3 ± 27.9</td>
</tr>
<tr>
<td>Cyclical HT</td>
<td>22</td>
<td>43.3 ± 2.9</td>
<td>12</td>
<td>183.4 ± 16.9</td>
</tr>
<tr>
<td>Progestin-only</td>
<td>16</td>
<td>52.5 ± 2.0</td>
<td>10</td>
<td>132.1 ± 16.8</td>
</tr>
<tr>
<td>No HT</td>
<td>19</td>
<td>52.7 ± 1.9</td>
<td>12</td>
<td>211.2 ± 21.1</td>
</tr>
<tr>
<td>Control proliferative</td>
<td>12</td>
<td>35.3 ± 2.8</td>
<td>12</td>
<td>227.7 ± 31.7</td>
</tr>
<tr>
<td>Control secretory</td>
<td>12</td>
<td>34.5 ± 2.3</td>
<td>12</td>
<td>188.4 ± 16.0</td>
</tr>
<tr>
<td>Mirena® amenorrhea</td>
<td>15</td>
<td>36.2 ± 1.4</td>
<td>13</td>
<td>98.5 ± 10.1</td>
</tr>
<tr>
<td>Mirena® infrequent</td>
<td>5</td>
<td>38.0 ± 3.4</td>
<td>5</td>
<td>79.5 ± 23.9</td>
</tr>
<tr>
<td>Mirena® irregular</td>
<td>3</td>
<td>39.3 ± 3.0</td>
<td>2</td>
<td>81.0</td>
</tr>
<tr>
<td>Mirena® regular</td>
<td>12</td>
<td>38.4 ± 1.9</td>
<td>10</td>
<td>102.0 ± 20.9</td>
</tr>
<tr>
<td>Mirena® prolonged</td>
<td>9</td>
<td>41.3 ± 1.5</td>
<td>7</td>
<td>92.4 ± 14.7</td>
</tr>
<tr>
<td>Mirena® unclassified</td>
<td>5</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

cc-HT = combined-continuous hormone therapy; HT = hormone therapy.
then washed with PBS. A serum-free protein-blocking reagent (Dako, High Wycombe, UK) was added for 10 min at room temperature.

VEGF antigen was detected using a rabbit polyclonal antibody (#sc-152, Santa Cruz Biotechnology Inc., Santa Cruz, California, USA) at 1 mg/l incubated overnight at 4°C. Negative control was rabbit IgG at 1 mg/l. CD34 antigen was detected using a mouse monoclonal antibody supplied as a supernatant with the concentration given as a range between 10–50 mg/l (Serotec, Oxford, UK) diluted 1/50 and incubated for 1 h at 37°C. Primary antibody step was followed by biotinylated secondary antibody and streptavidin-peroxidase steps at room temperature for 15 min each, using reagents of the LSAB+ Kit (Dako, Carpentaria, California, USA). AEC substrate (Zymed, San Francisco, CA, USA) was applied for 5 min at room temperature as a chromogen.

**Vessel counting (microvascular density)**

Blood vessel profiles (stained with the endothelial-cell marker, CD34) were counted with the operator blinded as to which clinical grouping the tissue belonged to. Sections were viewed at magnification ×200 and vessels counted in up to six random fields of each section—or the whole section if the sample was too small to provide six fields. The microvascular density (number of vessel profiles/mm²) was calculated by averaging all the fields counted.

**VEGF staining intensity**

VEGF was semi-quantitatively scored for staining intensity from 0 (no immunostaining) to 3 (strong immunostaining) in each of four tissue compartments—surface epithelium, glands, stroma and decidual cells (where present).

**Statistical analysis**

Values are presented as a mean ± SEM. All statistical tests were performed using SPSS for Windows, Version 11.0.0 (SPSS Inc., Illiniois, USA). After testing that data conformed to the assumptions of ANOVA (analysis of variance), microvascular density was analysed using one-way ANOVAs followed by Tukey post hoc tests. The semi-quantification of VEGF immunostaining was analysed using Kruskal–Wallis non-parametric tests. Individual means were analysed using Mann–Whitney U-tests. A P value < 0.05 was considered significant.

**Results**

**Subject information**

Of the 82 women with BTB recruited in Melbourne, 76 could be classified into one of four broadly defined hormone therapy groups. These groups were: (i) combined–continuous hormone therapy (cc-HT; estrogen and progestin taken on a daily basis); (ii), cyclical HT (estrogen taken daily with a progestin taken for 14 days each cycle); (iii) progestin-only; and (iv) no HT.

For the women receiving progestin-only HT, the majority were taking either Primolut (norethisterone, 5 mg/day taken orally) or Provera (depot-medroxyprogesterone acetate, 150 mg injected intramuscularly every 3 months). Within the cyclical and cc-HT groups, women received a wide range of HT products with no more than two women in either group receiving exactly the same formulation. Sufficient endometrial biopsy tissue for immunohistochemical analysis was obtained from 43 of the 76 subjects (see Table I). The 24 controls were classified by histopathology as 12 from the proliferative phase of the menstrual cycle, and 12 from the secretory phase. Of the 49 Mirena® subjects recruited in Barcelona, seven were rejected because of inadequate biopsy material for immunohistochemical analysis and five because of incomplete menstrual bleeding records. The remaining 37 subjects had been using Mirena® for an average of 12.3 months (range 5–48 months) and were divided into five groups based on BTB history as follows: amenorrhoea (n = 13); infrequent (n = 5); irregular (n = 2); regular (n = 10); prolonged (n = 7).

**Blood vessel density results**

A summary of BVD results is given in Table I and Fig. 1. Among the 43 subjects from Melbourne with BTB and from whom adequate endometrial biopsies for immunohistochemical analysis were obtained, BVD was significantly reduced by ANOVA in the progestin-only treated group compared with the other three treatment groups (P = 0.028). Post hoc testing confirmed that BVD in the progestin-only group was significantly reduced compared with both the cc-HT group (P = 0.040) and the group that did not receive HT (P = 0.044). There was no difference in BVD between the proliferative and secretory control groups by t-test (P = 0.281). When the combined control (208.1 ± 17.7 vessels/mm²) group was included in the ANOVA with the four Melbourne BTB groups, the result just failed to reach significance (P = 0.063).

For analysis of the Mirena® subjects, the irregular BTB group, which only contained two subjects, was excluded. There was no difference by ANOVA in BVD between the four groups of subjects using Mirena® (P = 0.855). When the proliferative and secretory control groups were included in the ANOVA, the results were highly significant (P < 0.001). Post hoc testing demonstrated that all four Mirena® BTB groups had significantly reduced BVD compared with both proliferative and secretory controls (Fig. 2A and B).

**VEGF immunostaining results**

Considerable heterogeneity was observed in VEGF immunostaining within and between individual samples (Fig. 2C and D), with some examples of glands scoring 3 and 0 within the same specimen.

When heterogeneity occurred within a sample, average scores were estimated.

The stroma sometimes had very strong patches of stain that generally appeared to be associated with haemorrhage rather than stromal cell staining.

The staining sometimes appeared cytoplasmic with clear nuclei, and sometimes appeared diffuse throughout the whole cell including the nucleus.

Results for VEGF immunostaining are shown in graphical form in Fig. 1. There were no differences by ANOVA in glandular or stromal VEGF immunostaining between any of the four HRT groups and the proliferative or secretory controls (P = 0.351 for glands and P = 0.340 for stroma). In contrast, significant differences were detected in luminal epithelial VEGF immunostaining (P = 0.025 by ANOVA), with post hoc testing showing that control proliferative phase luminal epithelial VEGF immunostaining was significantly reduced compared with all other groups (cc-HT: P = 0.013; cyclical HT: P = 0.006; no HT: P = 0.045; progestin-only: P = 0.031; secretory
phase: $P = 0.038$). Within the Mirena® subject groups, no statistically significant differences were detected in any tissue compartment for VEGF immunostaining.

**Discussion**

Results from the current study demonstrate that among 37 women who used the levonorgestrel-releasing intrauterine system (LNG-IUS) Mirena® for an average of 12.3 months, endometrial blood vessel density (BVD measured as vessel profiles per unit area) was very significantly reduced compared with controls. We have also shown that among a group of women receiving varied treatments for BTB, those receiving progestin-only HT had significantly reduced endometrial BVD. We have been unable to show any relationship between endometrial BVD and immunohistochemical expression of VEGF, or BTB. The observed reduction in BVD may be due to either a decrease in vessel length per unit volume of tissue or...
and 6 months following insertion of Mirena® (Roopa et al., 2003). Once again, further independent studies will be required to resolve this issue.

Even without the disagreement between our study and previous studies on the effects of Mirena® on endometrial BVD, the relationship between progestin exposure and endometrial BVD remains contradictory. In the first study to use immunohistochemical techniques to measure endometrial BVD, it was shown that exposure to the LNG-releasing subdermal implant Norplant® significantly increased endometrial BVD (Rogers et al., 1993). This finding has subsequently been confirmed by Hickey et al. (1999). However, it has also been reported that in women taking norethisterone 5–30 mg/day, or medroxyprogesterone acetate 15–80 mg/day, endometrial BVD was significantly reduced (Song et al., 1995). One conclusion from this study was that higher dose progestins may have an inhibitory effect on endometrial BVD, unlike the low dose delivered by Norplant®, which appears to stimulate an increase in BVD. Our data showing decreased endometrial BVD for women taking progestin-only HT agree with the findings of Song et al. (1995). Based on our results, the addition of estrogen under a range of conditions appears to prevent the progestin-induced reduction in endometrial BVD. Others have reported results that do not contradict this finding (Wahab et al., 2000; Jondet and Dehennin, 2003).

Given the complexity of the relationship between progestins and BTB, a better understanding of how progestins regulate endometrial BVD will only come from more detailed mechanistic studies using human cells and animal models. Understanding of how progesterone and progestins interact with endothelial cells and blood vessels is limited. It appears that most endothelial cells express functional progesterone receptors, although at very low levels (Vázquez et al., 1999). Stimulation of endothelial cells in vitro by progestrone results in a receptor mediated anti-proliferative effect, and aortic strips show decreased re-endothelialization in the presence of progesterone (Vázquez et al., 1999). However, using a mouse sponge angiogenesis assay, Hague et al. (2002) demonstrated that four different progestins had in vivo angiogenic effects, although results varied depending on the progestin and dose. In another mouse study, subdermally administered progestins increased endometrial BVD, again with some differences between progestins (Girling et al., 2004). As with the contradictory effects of progestins on endometrial BVD, it seems paradoxical that the direct effect of progesterone and/or progestins on endothelial cells is anti-proliferative while, in in vivo animal models, it appears to be pro-angiogenic. Additional complications arise from the differences seen between different progestins and the different dose-related effects.

As stated in the Introduction, counting vessel profiles per unit area (N_A) in a tissue section is not a true measure of blood

Figure 2. Representative photomicrographs showing CD34 immunostaining of: (A) control proliferative endometrium; (B) endometrium taken from a woman using Mirena®; (C) strong vascular endothelial growth factor (VEGF) immunostaining in endometrium from a woman receiving cyclical HRT; and (D) weak VEGF immunostaining in control proliferative endometrium. Note reduced blood vessel density (BVD) in endometrium from Mirena® user. Differences in VEGF immunostaining intensity did not show a consistent pattern between groups. Scale bars = 100 μm.
vessel density. Despite this, this methodology has been widely used in the literature in many different fields, with the most prominent being cancer biology where increased BVD has been shown to be prognostic for poorer outcomes in many different tumour types. This observation supports the argument that measuring BVD as $N_A$ in tissue sections is biologically relevant, possibly because vessel spacing per se, rather than total vessel content, is more important in terms of tissue perfusion and fluid exchange. More appropriate stereological measures of blood vessels in a tissue include length ($L_V$), surface area ($S_V$) or volume ($V_V$) of vessels per unit volume of tissue. When multiplied by total reference volume, these measures give absolute values for the blood vessel content of a tissue. However, it is often not possible to calculate total reference volume, particularly when working with human tissue biopsies as in the current study. Other issues that need to be considered when choosing stereological approaches for this type of study include limits on how thin it is possible to cut tissue sections from routine wax embedded hospital pathology blocks, and whether the blood vessel orientation within the tissues is random (isotropic) or not (anisotropic). Ultimately, the quantitative measures used are always only partial structural descriptors of a functionally complex biological system. Thus BVD from tissue sections, however measured, gives no indication of either total blood flow or which individual vessels were perfused in the living tissue.

One feature of this study is the highly heterogeneous nature of the HT protocols given to the different clinical groups. However, the subjects represent a typical cross-section of women who present to their gynaecologist for endometrial BTB problems, and are therefore an important and clinically relevant group. The variability in HT reflects in part the empirical clinical approach that is often taken to BTB problems, where the prescribed HT protocol is altered until a satisfactory outcome is achieved. Similar variability in HT regimens has been reported in other studies (Hickey et al., 2003). A weakness of the current study is the BTB data for the HT subjects, which were obtained retrospectively. It is possible that better records may have identified some relationship between BTB and the other variables measured in this study, although the lack of any such relationship in the Mirena® groups where accurate prospective BTB records were obtained argues against this.

From a clinical perspective, management of progestin-induced BTB remains problematic. The results of this and previous studies highlight the continuing lack of understanding of the underlying mechanisms that contribute to BTB. Until these gaps in our knowledge are filled, the development of effective clinical strategies to deal with BTB remains unlikely.

In conclusion, the results from this study provide strong evidence that higher dose progestins reduce endometrial BVD and that there is no apparent link between luminal epithelial, glandular or stromal immunoreactive VEGF and BVD or BTB.

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