Perivascular smooth muscle α-actin is reduced in the endometrium of women with progestin-only contraceptive breakthrough bleeding

Peter A.W.Rogers¹,³, Debbie Plunkett¹ and Biran Affandi²

¹Monash University Department of Obstetrics and Gynaecology, Monash Medical Centre, 246 Clayton Road, Clayton, Victoria, 3168, Australia and ²Department of Obstetrics and Gynaecology, Faculty of Medicine, University of Indonesia, Jakarta, 10430, Indonesia.

³To whom correspondence should be addressed at: Monash University Department of Obstetrics and Gynaecology, Monash Medical Centre, 246 Clayton Road, Clayton, Victoria, 3168, Australia. E-mail: peter.rogers@med.monash.edu.au

It has been shown that the endometrium of women using progestin-only contraceptives has increased vascular fragility, although the structural basis for this weakness is unknown, as is its role in breakthrough bleeding (BTB). Perivascular cells such as pericytes and vascular smooth muscle cells surround capillaries during the maturation process following angiogenesis, and act to strengthen and stabilize the vessels. The aim of the present study was to quantify endometrial perivascular smooth muscle α-actin (αSMA) expression in women using Norplant® with and without BTB problems, and compare it to controls. Using immunohistochemical techniques, vessels were classified as level 0, 1 or 2 depending on whether perivascular αSMA was absent, discontinuous or continuous. In 15 controls the subepithelial plexus had significantly more level 0 vessels than either the functionalis or basalis (61 ± 4 versus 31 ± 6 and 37 ± 4%, P = 0.0006 and P = 0.0007 respectively). In contrast the functionalis and basalis had significantly more level 2 vessels than the subepithelial plexus (20 ± 3 and 23 ± 2 compared to 4 ± 1%, P = 0.0005 and P = 0.0007 respectively). The major finding of the study was that in Norplant users, where the relatively atrophic endometrium cannot be divided into different regions, women with BTB problems (n = 20) had significantly more level 0 vessels than those with reduced bleeding (n = 17) (60 ± 4 versus 46 ± 4%, P = 0.0302). Norplant users with BTB problems also had a non-significant reduction in level 2 vessels compared to women without bleeding problems (4 ± 2 versus 11 ± 4%, P = 0.0667). These results demonstrate that perivascular αSMA is reduced around the endometrial vessels of Norplant users with BTB compared to those with no bleeding problems, and strongly support the concept that reduced vascular structural integrity plays a key role in endometrial BTB.

Key words: breakthrough bleeding/endometrium/Norplant®/smooth muscle α-actin/vascular fragility

Introduction

Abnormal endometrial bleeding, and specifically prolonged and irregular breakthrough bleeding (BTB), is a major unwanted side-effect of progestin-only contraception. Since progestin-only contraceptives are used by more than 12 million women worldwide, and up to 50% of those who start using progestin-only contraceptives may discontinue in the first year because of menstrual bleeding problems, progestin-only contraceptive BTB has become a major social and medical problem. Because significant differences in BTB patterns occur between women on identical contraceptive regimens, it is generally believed that local mechanisms acting within the endometrium are the major cause of abnormal BTB. However, despite significant research effort in this field over the past several years (Findlay and Affandi, 1996), little progress has been made into identifying local...
mechanisms that have been shown to play a role in BTB.

One hypothesis that has been put forward as a possible cause of endometrial BTB is increased microvascular fragility (Fraser and Peek, 1992). In theory there are a number of physical factors that could influence the fragility of the endometrial blood vessels. These include the state of the endothelial cells, including their thickness and cytoskeletal arrangement, the strength of the intercellular junctions between the endothelial cells, and the type and number of adhesion molecules anchoring the endothelial cells to the underlying basement membrane. Other factors that may be involved include the structure and composition of the basement membrane, as well as the absence or presence of the perivascular cells such as pericytes and smooth muscle cells. In a previous study we have investigated the presence or absence and basic composition of the microvascular basement membrane in the endometrium of women using Norplant® (Palmer et al., 1996). In that study no differences were found in endometrial microvascular basement membrane structure that might explain increased capillary fragility in Norplant users.

More recently there has been increasing interest in the role of pericytes and smooth muscle cells in blood vessel maturation following angiogenesis (Goede et al., 1998). This interest has been fuelled by the discovery of angiopoietin-1 and -2, which play a major role in post-angiogenic vessel maturation through interaction with the tyrosine kinase receptor Tie-2 (Suri et al., 1996). Among other putative roles, the angiopoietins play a key role in the recruitment and loss of pericytes and smooth muscle cells from the developing and regressing vasculature.

The aims of this study were: (i) to compare the expression of perivascular smooth muscle α-actin (αSMA) at three different endometrial sites in tissues taken from women during the normal menstrual cycle; (ii) to compare the expression of perivascular αSMA around endometrial blood vessels from women using Norplant contraception who were either suffering from prolonged BTB or who had minimal or no BTB; (iii) to compare endometrial perivascular αSMA expression between the normal menstrual cycle and women taking Norplant contraception. We postulated that increased BTB in some Norplant users occurs due to increased endometrial vascular fragility as a consequence of reduced perivascular αSMA expression.

**Materials and methods**

**Endometrial tissues**

Full-thickness endometrial tissue samples (n = 15) were obtained from reproductive age (i.e. not menopausal and with normal menstrual cycle lengths) women in Melbourne undergoing hysterectomy for a variety of reasons including fibroids, menorrhagia, adenomyosis, prolapse and tubal metaplasia. Tissue samples were only selected from areas of the uterus that appeared normal by gross pathological examination, and this normal appearance was also confirmed by routine histology. Endometrial biopsies were also collected by Pipelle suction curette (Prodimed 60530; Neuilly-en-Thelle, France) or Karman cannula (Rocket, London, UK) (Hadisaputra et al., 1996) from Indonesian women (n = 37) attending Raden Saleh Clinic in Jakarta with between 3 and 12 months exposure to Norplant contraception. Each subject maintained a daily menstrual record card recording either no-bleeding or spotting/bleeding. From the menstrual diary, biopsies were categorised as being from bleeders (n = 20) or non-bleeders (n = 17) based on an assessment of the 90 days prior to the time of biopsy. Bleeders were defined as subjects with spotting or menstrual bleeding for ≥25 out of the 90 days and non-bleeders as having spotting and bleeding for ≤10 days.

All biopsies were routinely fixed in 10% buffered formalin for 4 h, processed and embedded in paraffin. Sections, 5 μm thick, were cut and stained with haematoxylin and eosin. Control cycle endometrial tissues were classified by an experienced histopathologist using the criteria established by Noyes et al. (1950) into five menstrual cycle stages (menstrual, early proliferative, late proliferative, early secretory, late secretory).

Ethical approval for this study was obtained from Monash Medical Centre Human Ethics Committee, The Human Ethics Committee of the Faculty of
Medicine at the University of Indonesia, and The World Health Organization.

**αSMA–CD34 double immunostaining**

Paraffin sections, 5 μm thick, were cut from each biopsy and mounted on aminopropyltriethoxysilane (APES; Sigma, St Louis, MO, USA)-coated slides. The sections were air-dried overnight at 37°C. The immunostaining for αSMA–CD34 used a double-staining protocol. The primary antibodies used were monoclonals, mouse anti-human αSMA (clone 1a4; mIgG2a; Dako, CA, USA) and mouse anti-human CD34 (clone qBEND10; mIgGl; Serotec, Oxford, UK). αSMA reacts with smooth muscle cells of vessels, pericytes, myoepithelial cells, and some stromal cells in the intestine, testis, breast and ovary. It is used as a marker for smooth muscle cell localization and differentiation. CD34 recognizes a 110 kDa glycoprotein expressed on haematopoietic cells. Predominant staining is on the endothelial cell membrane; however, some cross-reaction with vascular associated adventitia and basement membranes occurs.

Tissue sections were dewaxed to dH₂O. Slides were washed in phosphate-buffered saline, pH 7.2 (PBS) between all steps unless otherwise stated. Endogenous peroxidase was blocked with 3% H₂O₂ in PBS for 10 min at room temperature (RT). Slides were incubated with protein blocking agent (PBA; Lipshaw Immunon, Pittsburgh, PA, USA) for 10 min, directly followed by αSMA antibody [0.094 μg/ml in 1% bovine serum albumin (BSA) in PBS] for 45 min, 37°C. The Dako LSAB + Peroxidase kit was used for the biotinylated secondary antibody and streptavidin–peroxidase complex. These were applied sequentially for 15 min at RT. Aminoethylcarbazole (AEC) red chromogen (Zymed, San Francisco, CA, USA) was applied for 10 min at RT, then washed with dH₂O. Incubation with double stain enhancer (Zymed) for 30 min was performed, followed by washing in dH₂O, then PBS. The sections were incubated with the second primary antibody, CD34 (0.05 μg/ml in 1% BSA/PBS) for 45 min at 37°C. The DAKO LSAB + Alkaline Phosphatase kit (DAKO) was used for the biotinylated secondary antibody and streptavidin–alkaline phosphatase steps. These were applied sequentially for 15 min at RT. Vector Blue (Vector, Burlingame, CA, USA) chromogen was applied for 10 min at RT in the dark. Sections were rinsed in dH₂O, mounted in Clearmount (Zymed) and air-dried overnight. Negative controls were performed, substituting the primary antibodies with isotype-matched controls at the same concentration as the primary antibodies.

**Evaluation and scoring of immunostaining**

The endometrium was divided into three zones: basalis, functionalis and subepithelial plexus. These were defined morphologically with the assistance of a haematoxylin and eosin-stained section. Norplant samples have no defined basalis and functionalis, therefore, vessels were counted randomly in each biopsy. Forty vessels were counted in each zone of the endometrium and in each Norplant biopsy. Sections were scored under oil immersion (×1000) and each vessel was rated as 0, 1 or 2 according to the αSMA staining around it. A score of 0 represents no αSMA, 1 represents αSMA around part of the circumference of the vessel (presumably a pericyte), and 2 indicates that the entire circumference of the vessel has αSMA staining (presumably smooth muscle cells around an arteriole).

**Statistics**

Statistical analysis was undertaken using the Mann–Whitney U-test (SPSS for Windows, Release 6.1). Comparisons between functionalis and basalis from the same sample were treated as related pairs and analysed by the Wilcoxon matched-pairs signed-ranks test (SPSS for Windows, release 6.1). $P < 0.05$ was taken as the value for statistical significance.

**Results**

CD34–αSMA double immunostaining gave clear identification of the endometrial vessels in all specimens, with endothelial cells stained blue and pericytes/smooth muscle cells stained red (Figure 1). All tissues examined had examples of level 0, level 1 and level 2 perivascular αSMA immunostaining. Under superficial examination there was no difference in general structure between vessels in the three different zones of control endometrium or tissues from women using Norplant.
Despite some variability, quantification of level 0, 1 and 2 vessels showed no obvious pattern of differences within each of the three different zones of normal endometrium across the five different stages of the menstrual cycle (Table I). For statistical analysis, menstrual, early proliferative and late proliferative data were combined and tested against early and late secretory combined. This statistical analysis confirmed that there was no difference between the proliferative and secretory stages of the cycle in either the subepithelial plexus, the functionalis or the basalis.

When functionalis was tested against basalis using Wilcoxon matched-pairs signed-ranks test, there was no difference in the numbers of level 0, 1 or 2 vessels between these two endometrial compartments. As a consequence, data from functionalis and basalis were combined for further analysis.

The data show that during the normal menstrual cycle the subepithelial plexus had significantly more level 0 vessels than either the functionalis or basalis (61 ± 4% versus 31 ± 6% and 37 ± 4%, \( P = 0.0006 \) for subepithelial plexus versus functionalis and basalis combined). In contrast the functionalis and basalis had significantly more level 2 vessels than the subepithelial plexus (20 ± 3% and 23 ± 2% compared to 4 ± 1%, \( P = 0.0005 \) for functionalis and basalis combined versus subepithelial plexus) (Figure 2). The functionalis and basalis combined also had more level 1 vessels than the subepithelial plexus (\( P = 0.0155 \))

Table I. Summary data of the results (mean ± SEM), expressed as a percentage of blood vessels in each region of the endometrium, or group of Norplant users, that were scored as either level 0, level 1 or level 2

<table>
<thead>
<tr>
<th></th>
<th>Subepithelial plexus</th>
<th>Functionalis</th>
<th>Basalis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( n )</td>
<td>Level 0</td>
<td>Level 1</td>
</tr>
<tr>
<td><strong>Menstrual</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Early P</td>
<td>2</td>
<td>38 ± 66</td>
<td>65 ± 8</td>
</tr>
<tr>
<td>Late P</td>
<td>4</td>
<td>62 ± 96</td>
<td>32 ± 8</td>
</tr>
<tr>
<td>Early S</td>
<td>2</td>
<td>55 ± 42</td>
<td>42 ± 3</td>
</tr>
<tr>
<td>Late S</td>
<td>5</td>
<td>70 ± 3</td>
<td>25 ± 2</td>
</tr>
<tr>
<td><strong>Combined average</strong></td>
<td>13</td>
<td>61 ± 4</td>
<td>35 ± 4</td>
</tr>
</tbody>
</table>

Graphs of combined data are shown in Figure 2.

\( P = \) proliferative; \( S = \) secretory.

Figure 1. Examples of level 0, level 1 and level 2 smooth muscle \( \alpha \)-actin (\( \alpha \)-SMA) immunostaining (red/brown colour) around endometrial blood vessels (endothelial cells immunostained blue with an antibody to CD34). (a) Level 0 vessels have no \( \alpha \)-SMA, (b) level 1 vessels have broken staining around their circumference and (c) level 2 vessels have continuous \( \alpha \)-SMA around them. Scale bar = 5\( \mu \)m.
The most significant finding of this study was that endometrium taken from Norplant users with prolonged BTB had significantly more level 0 vessels than endometrium from women with reduced bleeding (46 ± 4% versus 60 ± 4%, \( P = 0.0302 \)) (Table I and Figure 2). There was also a non-significant reduction in level 2 vessels in the endometrium of bleeders versus non-bleeders (11 ± 4% versus 4 ± 2%, \( P = 0.0667 \)).

There was no statistical difference in numbers of level 0, 1 or 2 vessels between the endometrium from Norplant users with BTB problems and control subepithelial plexus. By contrast, the endometrium from Norplant users without bleeding problems had significantly fewer level 0 vessels than control subepithelial capillary plexus (46 ± 4% versus 61 ± 4%, \( P = 0.0210 \)).

Endometrium from Norplant users with BTB problems was different from control functionalis/basalis by having significantly more level 0 vessels (\( P = 0.0002 \)), and significantly fewer level 1 (\( P = 0.0474 \)) and level 2 (\( P = 0.0000 \)) vessels. By contrast, the endometrium from Norplant users without bleeding problems had similar numbers of level 0 and 1 vessels as control functionalis/basalis, although level 2 vessel numbers were significantly reduced (\( P = 0.0015 \)).

**Discussion**

The major finding of this study is that perivascular \( \alpha \)SMA is significantly reduced around the endometrial microvasculature of Norplant users with increased BTB, compared to those with minimal BTB. Perivascular \( \alpha \)SMA is found in pericytes and smooth muscle cells, and both of these cell types add to the structural integrity and strength of blood vessels. Thus, the results from this study provide the first evidence for a correlation between structural changes that increase vascular fragility, and increased endometrial BTB.

A second important finding of this study is the difference in normal endometrial perivascular \( \alpha \)SMA between the subepithelial capillary plexus and the functionalis or basalis. Sixty-one per cent of the vessels in the subepithelial capillary plexus have no \( \alpha \)SMA, compared with only 31% in the functionalis and 37% in the basalis. Conversely, only 4% of vessels in the subepithelial capillary plexus have continuous \( \alpha \)SMA around them (defined as level 2 vessels in this study, and presumably representing arterioles), compared with 20% in the functionalis and 23% in the basalis. These results highlight the importance of taking into account regional differences in structure and function when studying the endometrial vasculature.

The results of the current study agree to some extent with those of Hourihan *et al.* (1986), who found a reduction in arterioles at the endometrial/myometrial junction in patients using the progestins norethisterone and levonorgestrel. In the present study we found the number of arterioles reduced from 20% in functionalis and 23% in basalis, to
4% in Norplant users with BTB and 11% in those with minimal BTB. In the present study we did not differentiate between spiral and non-spiral arterioles. However, based on observations from routine histological sections, spiral arterioles are virtually absent from Norplant-exposed endometrium, and so the few arterioles that were seen in Norplant-exposed endometrium in this study were not spiral. Hourihan et al. (1986) also found increased veins and dilated veins just below the surface epithelium. In the present study we were not specifically looking for veins or dilated veins, although it is possible that some of the vessels without αSMA were veins and not capillaries.

When comparing the perivascular αSMA patterns in Norplant users with increased versus minimal BTB, it is interesting to note that those with increased BTB have an appearance similar to normal subepithelial capillary plexus, while those without BTB are more similar to functionalis or basalis. This dichotomy in vascular structure between bleeders and non-bleeders, with parallels to subepithelial capillary plexus on one hand and functionalis or basalis on the other, may provide a clue to the mechanisms that underlie progestin-induced BTB. One of the consequences of Norplant use is the regression of the endometrium to a state in which it is not possible to differentiate between functionalis or basalis (Rogers, 1996). However, this regressed endometrium can still have different histological appearances (Rogers et al., 1993), demonstrating that individual response to Norplant is varied. Based on the results from the present study, it is clear that the vascular response to progestins also varies from subject to subject. Hence in some women it is possible that the regressed endometrium behaves as a basalis, with a relatively stable vascular bed, while in other women the vasculature remains more like the subepithelial capillary plexus. It is this latter group that subsequently have BTB problems.

The present study provides an important new lead in the search for a better understanding of factors responsible for progestin-induced endometrial BTB. Studies on Tie-1 and -2, and the angiopoietins (Suri et al., 1996; Vikkula et al., 1996; Hanahan, 1997), have started to elucidate their role in vascular maturation and regression, and, in particular, in the recruitment of stabilizing pericytes and smooth muscle cells to the post-angiogenic vasculature. Tie-1 and -2 are endothelial cell-specific transmembrane receptor tyrosine kinases. The ligand for Tie-1 is unknown, whereas angiopoietin-1 has agonist and angiopoietin-2 antagonist/partial agonist effects on Tie-2. Angiopoietin-1 acts to stabilize new vessels by recruiting perivascular cells, whereas angiopoietin-2 can antagonise or reverse this process. It is interesting to note that in adult vasculature angiopoietin-2 is predominantly found in ovary, uterus and placenta, tissues that undergo regular vascular remodelling and angiogenesis (Maisonpierre et al., 1997). There is also evidence for a more direct role for the Tie–angiopoietin system in angiogenesis, with reports that angiopoietin-1 induces endothelial cell sprouting (Koblizek et al., 1998), that its over-expression increases vascularization in mice (Suri et al., 1998), and that Tie-1 and -2 regulate intussusceptive microvascular growth (Patan, 1998). The Tie-angiopoietin system also interacts with vascular endothelial growth factor (VEGF) to modulate postnatal neovascularisation (Asahara et al., 1998).

A number of pieces of evidence now point to links between the mechanisms that regulate endometrial angiogenesis and vessel maturation, and progestin-induced BTB. In the present study we have demonstrated that αSMA levels are reduced around the endometrial vessels of women with progestin-induced BTB. We have also shown that vascular density (Rogers et al., 1993) and immunoreactive VEGF are increased in the endometrium of women using Norplant (Lau et al., 1999). Endometrial vascular smooth muscle cell proliferation increases around spiral arterioles under the influence of progesterone in the secretory phase of the normal menstrual cycle (Abberton et al., 1999). More recently, we have shown that immunoreactive levels of Tie-1 and -2 show temporal and spatial changes across the menstrual cycle in normal endometrium, suggesting at least an indirect role for oestrogen and/or progesterone in regulating their expression (K.M. Abberton et al., unpublished data). Clearly, the link between the Tie receptors, the angiopoietins and sex steroid regulation of endometrial vascular maturation needs to be explored further.

It is also possible that the increased percentage of vessels with no perivascular αSMA in women with BTB is a secondary phenomenon occurring as a
consequence of increased vascular growth, regression and remodelling. It is generally believed that pericytes and smooth muscle cells provide a stabilizing effect on blood vessels, insulating them to some degree from the need for angiogenic ‘survival factors’ (Hanahan, 1997). Thus, in a vascular bed undergoing growth, regression or remodelling, the number of pericytes and smooth muscle cells is significantly reduced. In the case of women with BTB, this could mean that factors that promote either vascular growth or regression are increased, that the vascular bed is remodelling, and that pericyte and smooth muscle cell coverage is therefore reduced.

In conclusion, we have shown a significant difference in endometrial perivascular αSMA coverage between Norplant users with and without increased BTB. This is the first study to demonstrate differences in endometrial vascular structure that could contribute to differences in vascular fragility, and thus help to explain some of the underlying mechanisms that contribute to progestin-induced BTB.

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