Changes in vascular basement membrane in the endometrium of Norplant users

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Progestogen-only contraception is almost invariably associated with changes in menstrual bleeding patterns. Changes in the endometrial vasculature, and in particular an increase in vascular fragility, may contribute to this bleeding. In this study, endometrial vascular density and endothelial cell basement membrane components were examined using immunohistochemistry before and after insertion of Norplant®. Endometrial vascular density was increased from a mean (± SEM) of 189.6 ± 7.0 vessels/mm² during the control cycle to 253.9 ± 80.7 vessels/mm² at 2–13 weeks of Norplant exposure, and to 212.7 ± 12.9 vessels/mm² at 14–42 weeks. During the control cycle, a mean of 161.4 ± 4.5 vessels/mm² stained for collagen IV (85% of all vessels), while at 2–13 weeks, 144.5 ± 13.0 vessels/mm² stained for collagen IV (57% of all vessels) (t ratio = 2.08, P = 0.0057). By 14–42 weeks, 71% of vessels (151.0 ± 9.8 vessels/mm²) were surrounded by collagen IV. This was not significantly different from control values (t ratio = 2.03). Endometrial vascular laminin was also reduced following Norplant insertion, from a mean of 176.0 ± 4.2 vessels/mm² in the control cycle (93% of vessels), to 156.3 ± 6.7 vessels/mm² at 2–13 weeks of exposure (57% of vessels) (t ratio = 2.08, P = 0.01). By 14–42 weeks of exposure to Norplant, 162.5 ± 9 vessels/mm² (76%) stained for laminin. This was not significantly different from control values (t ratio = 2.04). Endometrial vascular heparan sulphate proteoglycan (HSPG) was reduced from 58.6 ± 3.0 vessels/mm² during the control cycle (31% of vessels) to 43.6 ± 5.6 vessels/mm² (only 17% of vessels) at 2–13 weeks (t ratio = 2.08, P = 0.025). At 14–42 weeks, only 19% of vessels stained for HSPG (41.3 ± 5.8 vessels/mm²; t ratio = 2.04, P = 0.009).

Key words: basement membrane/collagen IV/endometrium/endothelial/laminin/Norplant/proteoglycan

Introduction

Long-acting progestogen-only contraceptives are used by up to fifteen million women worldwide. These methods offer highly effective fertility regulation, without adversely affecting blood pressure or increasing the risk of venous thrombosis (McCann and Potter, 1994). Long-acting, zero-order release systems, such as the subdermal implant system, Norplant, offer reliable contraception with a low total steroid dose.

The main disadvantage of progestogen-only contraception is the almost inevitable disruption of menstrual bleeding patterns, particularly during the early months of use (Odlind and Fraser, 1990). Changes in bleeding pattern are the most common reason for discontinuation of all steroidal contraceptives, and account for approximately half of all terminations of progestogen-only methods during the first year of use (Belsey, 1988).

The mechanisms underlying these changes in menstrual bleeding patterns are incompletely understood, but appear to relate to changes in the endometrial microvasculature (Fraser et al., 1996; Hickey et al., 1996). There is little relationship between systemic levels of ovarian steroid hormones and menstrual bleeding patterns in progestogen users (Faundes et al., 1991), and local endometrial mechanisms are likely to be of importance. Prolonged exposure to low-dose progestogens is known to affect endometrial vascular development and morphology (Hourihan et al., 1986; Johanisson, 1990), but studies so far have been unable to link changes in the endometrial vasculature with bleeding patterns in women exposed to low-dose progestogens (Rogers et al., 1993).

Vascular fragility has been proposed as the mechanism by which endometrial vessels break down in breakthrough bleeding, leading to prolonged and frequent episodes of light bleeding and spotting (Odlind and Fraser, 1990), and this has been demonstrated in Norplant users from as early as 3 weeks of use (Hickey et al., 1996).

The cause of this increased vascular fragility is unknown. Small endometrial vessels consist only of endothelial cells and their surrounding basement membrane. Three important constituents of the basement membrane are collagen IV, laminin and heparan sulphate proteoglycan (HSPG). Endothelial cell basement membrane is likely to contribute to vascular strength (Blackwell and Fraser, 1988). Alterations in the structure or integrity of endothelial cell basement membrane might lead to increased vascular fragility and breakthrough bleeding.

The aim of this study was to characterize the endometrial endothelial cell basement membrane in a group of normal-cycling women in the secretory phase of the menstrual cycle, to compare these findings with basement membrane at 1 and 9 months of exposure to Norplant, and to relate these changes to breakthrough bleeding.

Materials and methods

Thirty-four women aged 18 to 40 years, requiring long-term contraception with regular menstrual cycles and using barrier contraception...
were recruited between May 1994 and September 1995. Volunteers were informed of the likely occurrence of bleeding irregularities. Their informed consent was obtained before any investigations were commenced. The institutional ethics committees of The Population Council (New York, USA) and Family Planning NSW, Australia gave approval to this study.

**Serum oestradiol and progesterone**

Before insertion of Norplant, six serum samples were obtained for measurement of oestradiol and progesterone concentrations (once in the first and second weeks of the cycle and twice in the third and fourth weeks). Following Norplant insertion, blood samples were taken on two occasions during the 2 weeks preceding the biopsy. Blood samples were allowed to clot, and centrifuged within 2 h. Serum was stored at −20°C until analysed. Oestradiol and progesterone assays were performed using a chemiluminescent immunoassay (Immulite®, Diagnostic Products Corporation, CA, USA). This assay has a reporting range of 73–734 pmol/l for oestradiol and 0.6–127 nmol/l for progesterone. The lower limit for detection is 44 pmol/l and 0.28 nmol/l respectively, and the inter-assay variability in our laboratory is 10% for both steroid hormones. A progesterone concentration of >10 nmol/l in at least one sample was considered to be suggestive of luteal activity. This assay does not interact with levonorgestrel.

**Bleeding patterns**

Subjects prospectively recorded ‘bleeding’ or ‘spotting’ on a menstrual chart. Bleeding was defined as ‘any bloody vaginal discharge that requires the use of such protection as pads and tampons’, and spotting as ‘any bloody vaginal discharge that is not large enough to require sanitary protection’ (Belsey, 1988). The number of bleeding and spotting days in the 30 days prior to the biopsy were recorded, and whether the subject was bleeding on the day that the biopsy was taken.

**Endometrial biopsies**

One endometrial biopsy was taken during the secretory phase of the pretreatment (control) cycle, and two after insertion of Norplant. The biopsies were taken between 3 weeks and 9 months after insertion of Norplant, with the second biopsy scheduled ~3 months after the first. During the control cycle, endometrial tissue was obtained with a Pipelle suction curette (Prodimed, Cornier, Neully-en-Theille, France). Following insertion of Norplant, a Rocket vacuum aspiration curette was used (Promedicca, N. Ryde, NSW, Australia).

Biopsies were immediately placed in 10% buffered formalin at 4°C for 4–6 h, and then rinsed and stored in phosphate-buffered saline (PBS) at 4°C until processing. Endometrial tissue was processed by paraffin embedding, and 5 µm sections were cut onto sialinized slides for either immunohistochemistry or haematoxylin and eosin staining. One experienced gynaecological pathologist (P.R.) performed the histopathological evaluation. Control biopsies were classified by paraffin-embedded and stained sections contained both glands and stroma, and at least 10 fields of view were counted in blind fashion with a grid eyepiece pre-calibrated using a calibrated slide micrometer. Two individuals counted the slides, using a consistent technique. The results were averaged and calculated as features mainly consistent with the proliferative phase of the normal menstrual cycle: secretion = features mainly consistent with the secretory phase of the normal cycle; shedding = major evidence of shedding including tissue breakdown and fibrin thrombi but with no evidence of a previous secretory phase; atrophic = atrophic endometrium with very little dense stroma; reduced glands with small, cuboidal epithelial cells; progestogenic = evidence of exogenous progestogenic effects, small glands with cuboidal or low columnar epithelium and pseudodecidualized stroma (Rogers et al., 1993).

**Immunohistochemistry**

The Dako Envision™ polymer two-step detection system (K1393, Dako Corporation, Carpenteria, CA, USA) was used for immunohistochemical staining of endometrial vascular endothelium and basement membrane. The visualization system uses a prediluted peroxidase-labelled polymer conjugated with secondary antibodies to rabbit and mouse immunoglobulins.

Following dewaxing and rehydration, the tissue sections were quenched for endogenous peroxidase activity (0.03% hydrogen peroxide), for 5 min at room temperature. Following a buffer rinse, an innocuous protein solution (5% normal goat serum) was applied for 5 min at room temperature to block non-specific staining of the charged sites in collagen and connective tissue. Slides were rinsed in buffer solution between primary antibody and polymer and between polymer and chromagen. The primary antibodies used were mouse anti-human CD34 antigen (Q Bend 10; Novacastra plc, Newcastle, UK) and collagen IV (1:50 dilution), for 30 min at room temperature (Clone CIV 22; Dako Corporation), rabbit anti-human laminin (1:30 000 dilution; Sigma Immunochemicals, St Louis, MO, USA) overnight at 28°C, and mouse anti-human HSPG, for 2 h at 37°C (Clone MAB 458; Chemicon International Inc., Temecula, CA, USA). The labelled polymer was applied for 30 min at room temperature.

Preliminary studies with anti-basement membrane antibodies indicated that sensitivity was optimized by using a two-step epitope retrieval method. This involved initial pretreatment with a proteinase K solution diluted to 1:10 of normal working strength (10 µl in 5 ml) at 20°C for 5 min, followed by a heat-reduction step using Target Retrieval Solution (S 1700; Dako Corp.). The slides were immersed in Target Retrieval Solution and rapidly brought to the boil in a microwave set at maximum power (Model NE-6670; National Co., Tokyo, Japan). The slides were then left to cool for 5 min, placed in the microwave oven on the lowest setting for 10 min continuously, and then allowed to cool.

A negative control of non-immune mouse serum substituted for the primary antibody and a positive control of normal endometrium were used in each staining run. The substrate chromagen used was DAB (brown), for 5 min at room temperature (Universal Envision™ System, Peroxidase DAB version K1392 (Dako Corp.) for basement membrane components and AEC-Red (Universal Envision™ System, Peroxidase AEC version K1393; Dako Corp.) applied for 10 min at room temperature for endometrial endothelium.

All sections were counterstained with Mayer’s haematoxylin (Merck, Darmstadt, Germany), mounted with an aqueous mounting medium (Faramount; Dako Corp.) and examined by light microscopy with a grid eyepiece (Olympus BH2; Olympus Optical Company, Tokyo, Japan).

All red-coloured structures were considered to indicate endometrial vessels, even if a lumen could not be identified, provided that they were not present on the matched negative control slides. Strong and continuous staining around the vessel periphery identified basement membrane. Sections were viewed under the microscope and the presence or absence of each basement membrane component was noted. Assessment was performed in a blinded fashion by a single observer.

**Vessel counting**

Samples were considered suitable for counting if the haematoxylin and eosin sections contained both glands and stroma, and at least 10 random unit areas could be counted at a magnification of ×400. All the slides were counted in blind fashion with a grid eyepiece pre-calibrated with a slide micrometer. Two individuals counted the slides, using a consistent technique. The results were averaged and calculated as vessels per mm².

**Statistical methods**

Statistical analysis was performed using the SAS programme JMP, on a Macintosh 6200/75 computer. Paired t-tests were used to compare

717
endometrial basement membrane components before and after insertion of Norplant. Analysis of variance was used to assess whether there was a significant difference in means between two or more groups. When more than two groups were included in the analysis of variance, the Tukey–Kramer HSD post-hoc test was used to indicate which groups showed significant differences in means from which other groups. Frequency data (contingency tables) were analysed using a chi-square test. Statistical significance was assumed with P values <0.05. Values are given as mean ± SEM unless otherwise stated.

Results

The average age of Norplant acceptors was 28 (range 19–40) years. Sixteen women were nulliparous and nine were nulligravid. All women reported regular menstrual cycles of between 21 to 35 days with no intermenstrual bleeding. Bimanual pelvic examination was normal. No pregnancies occurred during the study period.

Seven women (20%) requested removal of their contraceptive implants during the follow-up period. The average time to Norplant removal in these women was 7.3 ± 1.9 months. The most frequent reasons for Norplant removal were prolonged and frequent episodes of bleeding. These bleeding patterns were confirmed on inspection of menstrual bleeding diaries.

Oestradiol and progesterone concentrations

There was no significant change in mean oestradiol concentrations following insertion of Norplant. Before insertion, the mean oestradiol concentration was 280 ± 20.7 pmol/l and after insertion 198 ± 9.7 pmol/l. All subjects but three showed progesterone values suggestive of luteal activity (>10 nmol/l) in the control cycle. Following insertion of Norplant the mean progesterone concentration was 2.1 ± 0.09 nmol/l, and only two subjects showed progesterone concentrations of >10 nmol/l, suggestive of luteal activity.

Menstrual bleeding patterns

An increase in the mean number of bleeding and spotting days was observed after insertion of Norplant. Before insertion, the mean oestradiol concentration was 280 ± 20.7 pmol/l and after insertion 198 ± 9.7 pmol/l. All subjects but three showed progesterone values suggestive of luteal activity (>10 nmol/l) in the control cycle. Following insertion of Norplant the mean progesterone concentration was 2.1 ± 0.09 nmol/l, and only two subjects showed progesterone concentrations of >10 nmol/l, suggestive of luteal activity.

Endometrial histology

Thirty-four control biopsies and 66 Norplant biopsies were taken (Table I). Two subjects declined a second biopsy because of discomfort during the first procedure. Following insertion of Norplant, a wide range of endometrial histological appearances were seen (Table II). Over the one-year follow-up period, there was no significant change in endometrial histological appearance with time of exposure to Norplant (χ² = 0.01).

Immunohistochemistry

Endometrial vascular density

The staining of the positive control sections was specific, with no background staining, and all relevant negative controls showed no staining. Endometrial biopsies were assessed in the control (pre-treatment) cycle, at 2–13 weeks of Norplant exposure, and again at 14–42 weeks of exposure.

A marked increase in endometrial vascular density was seen following exposure to Norplant compared with control samples taken during the control cycle in the same subjects. Mean endometrial vascular density in the control cycle was 189.6 ± 7.0 vessels/mm², which was significantly less than that seen at either 2–13 weeks of exposure to Norplant (253.9 ± 80.7 vessels/mm² (t ratio = 2.08, P = 0.01)) or at 14–42 weeks exposure (212.7 ± 12.9 vessels/mm² (t ratio = 2.03, P = 0.02)).

The increase in vascular density was observed from as early as 3 weeks after insertion of Norplant. Endometrial vascular density in Norplant users was not apparently related to systemic oestradiol (P = 0.10) or progesterone (P = 0.42) concentrations.

No relationship was observed between vascular density and menstrual bleeding patterns.

Endothelial basement membrane

Endometrial sections were assessed for the presence or absence of the basement membrane components laminin, collagen IV and HSPG. The observations between the two individuals were largely consistent, with a mean variation of 12%.

Collagen IV

The mean number of endometrial vessels showing positive immunostaining for collagen IV was significantly less than control values at 2–13 weeks of exposure (t ratio = 2.08, P = 0.0057). During the control cycle, the mean number of vessels staining for collagen IV was 161.4 ± 4.5 vessels/mm²; this represented 85% of the total vessels. After 2–13 weeks of

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Basement membrane changes in Norplant users

Norplant exposure this had decreased to 144.5 ± 13.0 vessels/mm² (57% of the total vessels), while by 14–42 weeks treatment there was no significant difference between mean control endothelial collagen IV and that in Norplant users, the mean number of vessels staining for collagen IV being 151.0 ± 9.8 vessels/mm² (t ratio = 2.03, P = 0.21; 71% of the total vessels).

No statistically significant relationship was observed between endometrial endothelial cell collagen IV immunostaining and circulating concentrations of either oestradiol or progesterone, or endometrial histological appearance. No relationship between collagen IV and recent or current menstrual bleeding was observed.

**Laminin**

The mean number of endometrial vessels per mm² showing positive immunostaining for laminin was significantly less than control values at 2–13 weeks of exposure (t ratio = 2.08, P = 0.01). During the control cycle, 176.0 ± 4.2 vessels/mm² stained for laminin; this represented 93% of the total endometrial vessels. By 2–13 weeks of exposure this had fallen to 156.3 ± 6.7 vessels/mm² (62% of the total vessels), and by 14–42 weeks of exposure to Norplant there was no significant differences from control values, with 76% of vessels (162.5 ± 9 vessels/mm²; t ratio = 2.04) staining for laminin.

A strongly positive association was observed between collagen IV staining in the endometrial vessels and laminin staining (F ratio = 8.74, P = 0.0045).

No statistically significant relationship was observed between endometrial endothelial cell laminin immunostaining and circulating concentrations of oestradiol or progesterone, or endometrial histological appearance. Recent or current bleeding and spotting was not related to endometrial endothelial laminin.

**Heparan sulphate proteoglycan (HSPG)**

The mean number of endometrial vessels per mm² showing positive immunostaining for HSPG during the control cycle was 58.6 ± 3.0 vessels/mm² (31% of the total endometrial vessels). This was significantly reduced at 2–13 weeks of exposure to 17% of the endometrial vessels (43.6 ± 5.6 vessels/mm²; t ratio = 2.08, P = 0.025) and at 14–42 weeks of Norplant exposure to 19% of the endometrial vessels (41.3 ± 5.8 vessels/mm²; t ratio = 2.04, P = 0.009). No significant relationship was observed between the number of vessels staining for HSPG and those staining for collagen IV (F ratio = 0.58) and for laminin (F ratio = 1.64). In Figure 1, normal endometrial vascular immunostaining for HSPG can be seen in the late proliferative phase around glands and microvessels. In Figure 2, taken 4 months after insertion of Norplant, endometrial microvascular HSPG staining is absent while epithelial HSPG immunostaining is present, apparently not affected by Norplant exposure.

Recent or current bleeding and spotting were not related to endometrial endothelial HSPG.

The results of this study confirm that the basement membrane components collagen IV, laminin and heparan sulphate proteoglycan, are reduced in the endometrial endothelium following Norplant exposure.

Endometrial endothelial basement membrane is a specialized form of extracellular matrix, which contributes to vessel growth, differentiation and cell permeability. Vascular basement membrane is a complex structure composed of several macromolecules, which interact to form the heterogeneous structure which can be defined as basement membrane. The basement membrane also provides a sheet-like structure to which endothe-

**Discussion**

This is the first report of endometrial endothelial cell basement membrane changes during the early weeks of progestogen exposure, and the first to demonstrate changes in vascular basement membrane associated with contraceptive steroid use.

During the normal menstrual cycle, these basement membrane components have been detected in the endometrial microvascular structure and epithelium by strong, continuous immunostaining during the proliferative and secretory phases of the cycle (Aplin et al., 1988; Bulletti et al., 1988). Using immunohistochemistry, Kelly et al. (1995) found collagen IV and laminin to stain all vessels throughout the normal menstrual cycle, while HSPG was detected in only 30–50% of vessels and showed a reduction in staining intensity during the menstrual phase. Ultrastructural studies have shown that microvascular basement membrane undergoes a pattern of elaboration and degradation during the menstrual cycle, with fragmentation preceding bleeding (Roberts et al., 1992). Previous observations in Norplant users at 3–12 months of exposure have failed to demonstrate any differences in immunostaining of these three basement membrane components (Palmer et al., 1996), but these authors (and Kelly et al., 1995) note a large degree of variability from one biopsy to the next in the same subject. This degree of variability was not observed in our study, but heterogeneity in basement membrane components within the endometrium cannot be discounted.

In this study, no association was found between bleeding episodes and basement membrane components in Norplant users. This may suggest that breakthrough bleeding is occurring by a different mechanism from normal menstruation, or that changes in vascular basement membrane represent only one factor in the pathogenesis of breakthrough bleeding.

This decrease in the number of endometrial blood vessels surrounded by basement membrane could have three possible explanations: (i) a decrease or disruption in basement membrane synthesis in the process of endometrial angiogenesis; (ii) an increase in the activity of proteolytic substances in the endometrial extracellular matrix; or (iii) misinterpretation of the immunohistochemical appearance of endothelial cells, for example, some of these ‘blood vessels’ could be lymphatics.

During angiogenesis, the basement membrane is broken down and reconstructed in a regulated sequence, with laminin appearing first. Basement membrane production around stromal cells is gradual and progressive during the secretory phase of the normal menstrual cycle. Exogenous progestogens may disrupt this control (Bulletti et al., 1988). A reduction in basement membrane immunostaining may represent vessel degradation in the process of angiogenesis following levonorgestrel exposure.
An increase in overall endometrial microvascular density has been observed in Norplant users (Rogers et al., 1993), but indices of endothelial cell proliferation were not increased (Goodger et al., 1994).

Norplant may increase the breakdown of endometrial basement membrane. The expression and activity of matrix metalloproteinases (MMP) such as stromelysin-1 (Schatz et al., 1994), matrilysin (Rodgers et al., 1993) and the type IV collagenases (Marbaix et al., 1992) are known to be influenced by ovarian steroids. These MMP appear to be inhibited by progesterone and activated by progesterone withdrawal. Alterations of endometrial progesterone receptor status by levonorgestrel (Critchley et al., 1993) may disrupt the regulation of MMP. In Norplant users, these authors found that progesterone receptor immunoreactivity was increased, but it is unclear whether this represents an increase in functional receptors. However, in women using the levonorgestrel-releasing intrauterine system, Critchley et al. (1998) found an initial reduction in stromal and glandular oestrogen and progesterone receptors, with an increase in subtype A progesterone receptors at 6–12 months of exposure. The authors conclude that this receptor is likely to be the subtype that mediates long-term action of levonorgestrel on the endometrium in these subjects.

Changes in vascular basement membrane are known to contribute to vascular fragility in other organ systems. In diabetes mellitus, there is a characteristic thickening of the basement membrane, but with a loss of functional capacity and ‘leakiness’ leading to retinal vascular fragility (Martin et al., 1988).
tumours and inflammatory conditions characterized by vascular fragility the basement membrane is absent or disrupted (Barsky et al., 1983).

A significant decrease in all three basement membrane components was observed during the first months of exposure to Norplant, and all components except for HSPG appeared to be present at normal concentrations after 12 weeks. The lack of association between endometrial vascular HSPG immunostaining with collagen IV and laminin, and the persistence of reduced HSPG levels, suggests that basement membrane degradation and repair might be disrupted following Norplant exposure. In diabetic retinopathy, a reduction in basement membrane HSPG is thought to lead to a fall in negative charge and subsequent increase in permeability (True, 1991). These defects in assembly and distribution of components may alter the barrier function of the basement membrane.

In this study, endothelial cells were identified using CD34 immunostaining of endometrial endothelial cells using sequential sections. This antibody may occasionally stain the endothelium of lymphatics, and endometrial lymphatics do not express basement membrane (Barsky et al., 1983). Further studies using double staining for endothelial cells and for basement membrane components may help to define more precisely the relationship between endometrial microvessels and the vascular basement membrane. Immunohistochemistry only reliably detects differences in the presence or absence of basement membrane components. In addition, the antibody used to detect laminin does not differentiate between laminin chains. Other techniques such as electron microscopy could yield information on detailed structural and functional differences in basement membrane construction, which might influence the membrane’s strength and integrity and thus contribute to breakthrough bleeding. Similarly, alterations in other basement membrane components such as fibronectin, elastin, dermalan, alkaline phosphatase and mucopolysaccharides require investigation.

In summary, this study has demonstrated a fall in endometrial endothelial cell basement membrane components in the early weeks and months of Norplant use, that have largely returned to normal levels by 3 months of exposure. These changes occur at a time when bleeding problems are common, and may contribute to the subsequent increase in permeability (True, 1991). These defects in assembly and distribution of components may alter the barrier function of the basement membrane.

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