Endometrial breakdown in women using Norplant is associated with migratory cells expressing matrix metalloproteinase-9 (gelatinase B)*

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Norplant, subdermally implanted slow-release levonorgestrel, is an effective and widely used contraceptive agent but has a high rate of discontinuation due to unacceptable abnormal uterine bleeding. Matrix metalloproteinases (MMPs) are expressed in normal cycling endometrium and are postulated to be responsible for the tissue breakdown at menstruation. We have compared the immunolocalization of MMP-9 and migratory cells in endometrium from Indonesian women using Norplant with normal controls. Positive MMP-9 immunostaining was observed intracellularly within stromal and intravascular leukocytes and extra-cellularly in areas of tissue lysis adjacent to these migratory cells. The MMP-9 positive cells were identified as neutrophils, eosinophils, CD3+ T-cells and macrophages. Quantitative assessment revealed that the number of MMP-9 positive cells, neutrophils and eosinophils were significantly increased in those endometrial biopsies from Norplant users displaying a shedding morphology and in normal controls at menstruation. There was no correlation between the number of MMP-9 positive cells and the number of bleeding days reported. Endometrial immunostaining for tissue inhibitor of metalloproteinases was similar in Norplant users and normal controls. These results suggest that MMP-9, an enzyme capable of degrading basement membrane components, may be involved in endometrial breakdown in women using Norplant.

Key words: endometrial bleeding/immunochemistry/leukocyte/ matrix metalloproteinase-9/Norplant

Introduction

Norplant, subdermally implanted slow-release levonorgestrel, is a widely used, effective and reversible progestin-only contraceptive agent. It comprises six silastic rods releasing between 0.05 and 0.08 mg/day in the first 12 months of use, resulting in suppression of ovulation in most women. However, as with other forms of progestin-only contraception, Norplant is associated with a high rate (20–30%) of discontinuation due to unacceptable abnormal uterine bleeding (i.e. bleeding occurring outside the parameters of normal menstruation) which occurs most commonly in the first year after implant insertion (Odlin and Fraser, 1990).

Matrix metalloproteinases (MMPs), a family of zinc-dependent ectopeptidases, act to degrade specific components of the extracellular matrix (for review see Birkedal-Hansen et al., 1993). The enzymes are secreted as latent proenzymes requiring proteolytic cleavage for activation. Regulation of MMPs is complex and occurs at multiple levels, including gene transcription, a cascade of activation in which proteases, including some MMPs, are able to activate MMPs and inhibition by tissue inhibitors of metalloproteinases (TIMPs) by formation of 1:1 complexes. MMP-9 (gelatinase B) is a 92-kDa metalloproteinase which demonstrates substrate specificity for collagen IV (a major component of basement membranes), collagen V, elastin and gelatin.

Studies from our laboratory and others have demonstrated that MMPs are produced in the endometrium and that their expression is closely associated with the process of normal menstruation, the endometrial breakdown being accompanied by uterine bleeding following a normal ovarian cycle (for review see Salamonsen and Woolley, 1996). In menstrual phase endometrium, MMP-9 is present within and around migratory cells, specifically neutrophils, eosinophils and macrophages, especially in areas of tissue lysis (Jezioska et al., 1996). Further, in an in-vitro model, inhibition of MMPs prevented the matrix breakdown of endometrial explants which would have otherwise occurred following progesterone withdrawal (Marbaix et al., 1996). It has thus been postulated that MMPs are responsible for the tissue degradation at menstruation (Salamonsen and Woolley, 1996).

The pathological mechanisms underlying abnormal uterine bleeding associated with progestin-only contraceptives remain ill-defined (for review see Fraser et al., 1996). Morphological and functional endometrial changes have been observed suggesting that focal capillary breakdown is occurring, while increased numbers of endometrial leukocytes have been reported (Ludwig, 1982; Clark et al., 1996; Song et al., 1996). MMPs could increase vessel fragility via actions on the integrity of the basement membrane and contribute to the degradation of the endometrial stroma; the end result being tissue breakdown and bleeding. We postulated that MMPs are associated with the endometrial breakdown and bleeding in women using Norplant and, in particular, that MMP-9 could...
be provided by migratory cells. Thus, the aim of this study was to examine for the presence of MMP-9 and migratory cells in endometrial biopsy samples of women using Norplant.

Materials and methods

Subjects and tissue collection

Indonesian women who presented to the Klinik Raden Saleh in Jakarta, Indonesia for the insertion of Norplant were recruited after informed consent was obtained. After implant insertion, subjects recorded a daily menstrual diary and returned to the clinic 3–12 months later for endometrial biopsy, which was performed by either microhysteroscopy or Pipelle suction curette.

Endometrium was also obtained in Melbourne, Australia from women who were undergoing curettage following laparoscopic sterilization or assessment of tubal patency to allow comparison with normal control tissues. Patients with uterine abnormalities such as leiomyomas, endometrial polyps, endometriosis, or those who had received steroid therapy in the past year were excluded.

All endometrial biopsies were fixed in either 10% buffered formalin or Carnoy’s fixative and embedded in paraffin. Tissue sections were cut at 5 µm, deparaffinized, rehydrated and either stained with haematoxylin and eosin for histological dating according to Noyes et al.’s (1950) criteria or subjected to immunohistochemical staining.

The project was approved by the Human Research and Ethics Committee at the Monash Medical Centre (Monash University Standing Committee on Ethics in Research on Humans), by the Medical Faculty of the University of Indonesia Ethical Commission on Research on Humans and by the World Health Organization.

Menstrual diary records

Subjects recorded a daily menstrual diary from the day of insertion of Norplant to the day of endometrial biopsy. The total duration of implant use was recorded for each subject. Menstrual bleeding charts were analysed using two methods. The total number of bleeding days (any bleeding or spotting) in the 90-day reference period prior to endometrial biopsy and the number of bleeding-free days between the last day of bleeding and the endometrial biopsy were calculated (Marsh et al., 1995).

Immunohistochemical staining

MMP-9 was demonstrated on formalin-fixed tissue using the alkaline phosphatase anti-alkaline phosphatase (APAAP) technique and a mouse monoclonal antibody to human MMP-9 (Insight Biotechnology Ltd., Wembley, Middlesex, UK.). The primary antibody [used at a concentration of 2 µg/ml in Tris-buffered saline (TBS) containing 10% goat serum] was applied and the sections incubated overnight at 4°C. MMP-9 was visualized using goat anti-mouse immunoglobulin G (IgG) (Dako, Glostrup, Denmark) followed by mouse APAAP complex (Dako), repeated twice and with New Fuchsin (Dako) as the chromagen. Endogenous alkaline phosphatase was blocked with 1 mM levamisole.

Resting and activated eosinophils were identified on formalin-fixed tissue using a mouse monoclonal antibody against eosinophil cationic protein, clone EGI (Pharmacia Ltd, Milton Keynes, Bucks, UK) and the APAAP detection system as described previously (Jeziorska et al., 1995). Neutrophil polymorphs were demonstrated on formalin-fixed tissue with a monoclonal mouse anti-human neutrophil elastase (NE; clone NP 57, Dako) using the Dako strept-ABC kit. Following inhibition of endogenous peroxidase with 0.3% H2O2 in methanol, the tissue sections were incubated with 10% normal horse serum in TBS to block non-specific binding. The primary antibody (1.3 µg/ml diluted in 10% normal horse serum/TBS) was then applied and incubated for 2 h at room temperature. After washing, slides were incubated with biotinylated horse anti-mouse IgG for 30 min followed by the detection system used according to the manufacturer’s instructions with diaminobenzidine/H2O2 as the chromagen.

Immunohistochemistry for TIMP-1 and -2 was performed on Carnoy’s fixed tissues (n=10) as described by Zhang and Salamonsen (1997), using sheep anti-human TIMP-1 (a gift from Dr. Hideaki Nagase, Kansas City, KS, USA) and rabbit anti-human TIMP-2 (Triple Point, Forest Grove, OR, USA) as the primary antibodies and the Dako strept-ABC kit and the StrAviGen (Biogenex Laboratories, San Ramon, CA, USA) supersensitive immunostaining system respectively.

Negative and positive controls appropriate for each antibody were included in each series of sections examined. The negative control for MMP-9, EGI and NE was an irrelevant α-lactalbumin monoclonal IgG antibody at the same concentration as the primary antibody. Positive controls included proliferative phase human endometrium (MMP-9), human endometrium (EG1), human tonsil (NE) and human fetal kidney (TIMP-1 and -2). All tissue sections were counterstained with Harris’ haematoxylin. Photography was performed using an Olympus BH2 photomicroscope.

Dual immunofluorescence

On selected specimens, dual immunofluorescent staining was used to identify the cellular source of the MMP-9 using the amplification technique described by Hunyady et al. (1996) which allows double immunostaining using antibodies from the same host species. MMP-9 antiserum, used at a concentration of 0.2 µg/ml, was visualized using the Renaissance TSA Indirect Amplification kit (NEN Life Sciences, Boston, MA, USA) with fluorescein isothiocyanate (FITC) conjugated streptavidin as the detection system. A subsequent conventional fluorescent staining with the second primary antibody and visualization using a sheep anti-mouse or donkey anti-rabbit secondary antibody conjugated to Texas Red (Amersham Life Science, Little Chalfont, Buckinghamshire, UK) was then performed. The second primary antibodies used were: (i) EGI; (ii) NE; (iii) mouse monoclonal anti-CDS68 (clone KP1) to detect macrophages (Dako); and (iv) rabbit polyclonal antibody to CD3 to detect T-lymphocytes (Dako). No detectable signal was observed with conventional immunofluorescent staining using the same MMP-9 antibody concentration as used in the amplification technique. Other controls included omission of the primary antibody with resultant minimal background staining. The tissue sections were mounted with immunomount (Dako) and photographed using an Olympus photomicroscope with filter sets for FITC and Texas Red. Attempts to apply this technique to endometrial granulat lymphocytes (EGL) with mouse monoclonal anti-CD43 (DFT-1; Novocastra, Newcastle-upon-Tyne, UK) (Clark et al., 1996) were unsuccessful, so that the possibility that MMP-9 was also present in EGL could not be excluded.

Assessment of immunostaining

Quantitative analysis of the number of positive cells was undertaken using an Olympus BX-50 microscope and a 40× objective. The image was captured using a Pulinex TMC-6 video camera coupled to a Pentium PC computer using a Screen Machine II FAST multimedia video adaptor (FAST Multimedia AG, Munich, Germany). A software package (Olympus DK CASTGRID V1.10, Olympus, Denmark) was used to generate a counting frame (14 565 µm2) directly on to the video screen. Fields to be counted were selected using a systematic uniform sampling scheme generated by the CASTGRID V1.10 computer program with the aid of a motorized stage (Multicontrol 2000, ITK, Ahornweg, Germany). The number of positive cells...
(excluding intravascular cells) in at least 30 random fields was counted for each section. Stromal cell density was also assessed by counting the number of stromal cells in eight of the random fields above which contained only stroma (i.e. excluding glands and large blood vessels). The number of positive cells was expressed as per 1000 stromal cells. Cell counting was performed by the same observer, with no knowledge of the clinical characteristics of the patient donor.

Distribution of the MMP-9, NE and EGI positive cells within the various endometrial compartments (including luminal and glandular epithelium; perivascular, periglandular or subluminal epithelial stroma; intravascular or tissue breakdown sites) was assessed by two independent observers using an Olympus BH2 microscope and graded from 0 (no cells) to 3 (many cells). TIMP-1 and -2 immunostaining intensity in each endometrial compartment was assessed using the method described by Zhang and Salamonsen (1997), on a scale from 0 (no staining) to 4 (maximal staining intensity).

**Statistical analysis**

Patient characteristics including age, body mass index (BMI), duration of the Norplant implant, number of bleed-free days and the number of bleeding/spotting days in the 90-day period prior to endometrial biopsy were analysed using analysis of variance (ANOVA) or analysis of covariance (ANCOVA). Differences in the number of stromal cells or positively stained cells at different times in the menstrual cycle and between different histological groups of Norplant users were assessed using ANOVA following confirmation of the normal distribution of the data. Differences were taken as significant when *P* < 0.05. Correlation between the number of MMP-9 positive cells and the number of bleeding days or bleed-free days prior to endometrial biopsy was assessed using linear regression.

**Results**

**Histological grouping**

As the morphological response of the endometrium to progestins is very variable, the patients were subdivided into three groups on the basis of the histological appearance of the endometrial biopsies as described previously (Marsh et al., 1995). These included: (i) an atrophic group in which the endometrium displayed a basalis-type appearance with atrophy of the endometrium, stromal fibrosis and inactive glands; (ii) a progesterone-modified group in which small inactive glands were associated with a pseudo-decidualized stromal appearance; and (iii) a shedding group in which there was evidence of endometrial degeneration and shedding. Representative examples of these three histological subtypes are shown in Figure 1 (a–c). Microscopic assessment of the tissues suggested that the stromal cell density varied between histological subgroups and this impression was confirmed on quantitative analysis (Table I) in which the atrophic subgroup had a significantly greater stromal cell density than the progesterone-modified subgroup. On the basis of this finding, the immunohistochemical staining results were expressed as positive cells per 1000 stromal cells.

**Clinical characteristics**

The clinical characteristics of patients using Norplant subdivided according to histological group are shown in Table I. There was no significant difference between patient groups with respect to age and duration of the Norplant implant. The body mass index of the atrophic group was significantly lower than that of the progesterone-modified group. There was no significant difference between the three groups in relation to the number of bleeding-free days or number of bleeding/spotting days in the 90-day period prior to the endometrial biopsy (Table I and Figure 2).

**Immunohistochemical staining of endometrial biopsies from Norplant users**

Positive immunostaining for MMP-9, neutrophil polymorphs and eosinophils was observed in all three histological groups (Figure 1, a–f); however, there was marked variability between individual samples in all groups. MMP-9 immunostaining was present intracellularly in stromal and intravascular leukocytes and extracellularly adjacent to leukocytes in areas of tissue lysis. There was no glandular epithelial, endothelial or stromal cell expression of MMP-9.

Endometrial biopsy samples available for TIMP immunohistochemistry displayed a progesterone-modified morphology; there were no samples with an atrophic or shedding histological appearance. TIMP-1 and -2 positive staining was observed in endometrial epithelial, endothelial and stromal cells but not in leukocytes (Figure 1, g–i). There was no variation in staining intensity when compared with the normal menstrual cycle (Zhang and Salamonsen, 1997).

**Identification of the MMP-9 immunopositive cells**

Dual immunofluorescent techniques were used to identify the nature of the MMP-9 immunopositive cells. Neutrophil polymorphs, eosinophils, macrophages and CD3+ T-cells were detected with Texas Red fluorescent immunostaining and each of these markers colocalized with MMP-9 positive cells detected with FITC immunofluorescence (Figure 1, j–n and j’–n’). However, the dual immunofluorescent staining also revealed that neither all the leukocytes, nor all those of any one specific cell type were positive for MMP-9 within a single biopsy sample, i.e. there were subgroups within each cell type with phenotypes of both MMP-9 positive and negative.

**Quantitative assessment of immunohistochemical staining**

Quantitative assessment was performed to establish the number of immunopositive MMP-9 cells, eosinophils and neutrophil polymorphs in both the Norplant biopsy samples and in control endometrial biopsies taken from women with normal menstrual cycles during the late secretory or menstrual phase (days 26–28 and days 1–3 respectively of the idealized 28-day menstrual cycle). These two phases of the normal menstrual cycle were chosen as controls as previous studies have described semiquantitatively maximal numbers of MMP-9 positive cells, eosinophils and neutrophil polymorphs at these times (Poropatich et al., 1987; Jeziorska et al., 1995, 1996; and A.J.Vincent, unpublished observations).

The shedding subgroup had a significantly greater number of MMP-9 positive cells than the atrophic group (Figure 3). When compared with the number of MMP-9 immunopositive cells in the endometrial biopsies of women with normal menstrual cycles sampled during the late secretory phase or menstrual phase, there was no difference between the shedding...
Figure 1.
Norplant group and the days 1–3 menstrual phase group (Figure 3). The menstrual phase biopsies also had a significantly greater number of immunopositive cells than the Norplant atrophic group.

A similar pattern was also observed for neutrophil polymorphs and eosinophils (Figure 3). Significantly greater numbers of neutrophils were observed in the shedding Norplant group compared with those of the atrophic or progesterone-modified Norplant groups and the late secretory phase group. The number of eosinophils was significantly greater in the shedding Norplant group than in the atrophic Norplant group or late secretory phase control group. There was no difference between the number of immunopositive cells in the shedding Norplant and the menstrual phase group for either neutrophil polymorphs or eosinophils. These results were also consistent with the dual immunofluorescence data, which demonstrated that only some of the migratory cells of any one type in the endometrial biopsies from Norplant users were MMP-9 positive, i.e., the number of MMP-9 positive cells assessed by stereological review was less than the total number of migratory cells observed using the same method.

There was no significant relation between the number of MMP-9 positive cells (data not shown) and the histological group and the number of bleeding days or bleed-free days prior to endometrial biopsy recorded in the menstrual diaries (Table I).

### Table I. Patient characteristics of Norplant users divided according to histological subgroup

<table>
<thead>
<tr>
<th>Histological subgroup</th>
<th>Atrophic</th>
<th>Progesterone-modified</th>
<th>Shedding</th>
</tr>
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<tbody>
<tr>
<td>Age (years)</td>
<td>31 ± 2.4</td>
<td>33 ± 1.2 (range 22–39)</td>
<td>30 ± 1.6 (range 24–36)</td>
</tr>
<tr>
<td>Body mass index</td>
<td>19.3 ± 0.5 (n = 6)</td>
<td>24.0 ± 1.2 (range 25–38)</td>
<td>20.8 ± 1.6 (range 24–36)</td>
</tr>
<tr>
<td>Duration of Norplant (days)</td>
<td>194 ± 32 (range 119–319)</td>
<td>224 ± 21 (range 141–350)</td>
<td>224 ± 34 (range 140–329)</td>
</tr>
<tr>
<td>Number of bleeding/spotting days in the 90-day period prior to endometrial biopsy</td>
<td>40 ± 7c (range 15–71)</td>
<td>24 ± 2 (range 14–32)</td>
<td>37 ± 8 (range 10–57)</td>
</tr>
<tr>
<td>Number of bleed-free days prior to endometrial biopsy</td>
<td>6.4 ± 2.5c</td>
<td>15.9 ± 3.2</td>
<td>5.8 ± 3.3</td>
</tr>
<tr>
<td>Stromal cell density (number of cells × 1000/mm²)</td>
<td>10.71 ± 1.57b</td>
<td>7.85 ± 0.57</td>
<td>9.65 ± 0.66</td>
</tr>
</tbody>
</table>

Results expressed as mean ± SEM.

*Formalin-fixed tissues only.

Statistically significant difference P < 0.05; atrophic subgroup versus progesterone-modified subgroup.

No significant difference between groups using analysis of covariance with body mass index as covariate.

### Distribution of immunopositive cells within the endometrium of Norplant users

MMP-9 positive cells were distributed predominantly within the stroma in areas of tissue breakdown in all histological subtypes (Figure 4) with few present among the epithelial cells. Within the intact epithelium, more positive cells were seen close to the luminal epithelium and intravascularly than elsewhere. A similar pattern was observed for eosinophils and neutrophils (Figure 4).

### Discussion

Uterine bleeding observed at normal menstruation is postulated to involve MMP-mediated degradation of the endometrial extracellular matrix and basement membranes (Salamonsen and Woolley, 1996). However, the pathophysiological mechanisms responsible for abnormal uterine bleeding associated with contraceptive steroid agents, including Norplant, remain ill-defined. Hysteroscopic studies suggest that such bleeding occurs focally, from superficial thin-walled venules and neovascularized areas (Hickey et al., 1996). Increased vascular fragility of endometrial vessels in Norplant users has also been noted on hysteroscopic examination (Hickey et al., 1996). No alteration in immunohistochemically defined endometrial basement membrane components in Norplant users was observed at the light microscopic level (Palmer et al., 1996);...
Figure 2. Number of bleeding days in the 90 days prior to endometrial biopsy, which were reported by women using Norplant grouped according to histological morphology. Each point represents one individual. There were no significant differences between the three groups.

Figure 3. Number of matrix metalloproteinase (MMP)-9, neutrophil elastase or eosinophil cationic protein EG1 immunopositive cells in endometrial biopsies from Norplant users classified according to histological group or day of the idealized 28-day menstrual cycle. Numbers in parentheses denote number of endometrial samples. Results expressed as mean positive cells/1000 stromal cells ± SEM. **P < 0.01; *P < 0.05.

Figure 4. Relative distribution of matrix metalloproteinase (MMP)-9, neutrophil elastase or eosinophil cationic protein EG1 immunopositive cells in endometrial biopsies from Norplant users classified according to either atrophic □, progesterone-modified □, or shedding □ histological group. The cells in different endometrial compartments, including epithelium, glandular lumen (GLumen), intravascular (intravasc), subluminal epithelial stroma (stroma-subLE), stroma adjacent to glands (stroma adj to gl), perivascular stroma (stroma perivasc), stroma at other sites not immediately adjacent to epithelium or blood vessels (stroma other) and areas of tissue breakdown (breakdown sites), were graded from 0 (no cells) to 3 (many positive cells).

However, this study did not specifically include Norplant biopsies with areas of endometrial degradation and did not exclude subtle changes which might only be obvious at the electron microscopic level (Roberts et al., 1992) and yet might contribute to vascular fragility. Alteration in the balance between MMPs and their inhibitors could contribute to vascular fragility.

This study demonstrates that MMP-9, an enzyme capable of degrading basement membrane components, is present within the endometrium in migratory cells, particularly those...
at sites of endometrial breakdown, both during normal menstruation and in women with abnormal bleeding associated with the use of Norplant; its extracellular location at bleeding sites has also been shown. The identity of these MMP-9 positive migratory cells includes eosinophils, neutrophil polymorphs, macrophages and CD3+ T-cells. These findings suggest that MMP-9 may be important in the endometrial breakdown which may contribute to the abnormal uterine bleeding observed in Norplant users.

Differences in objectively measured blood loss in Chinese (higher menstrual blood loss) and non-Chinese women have been reported (Gao et al., 1987). Thus, there may be potential problems when comparing normal controls from Melbourne, Australia (predominately Caucasian ethnicity) and Indonesian women using Norplant. However, no difference in the immunostaining pattern for endothelin and neutral endopeptidase was observed between endometrial biopsies obtained from control women in Melbourne and Indonesian normal controls (Marsh et al., 1995).

The endometrial response to Norplant and other progestin contraceptives is variable, depending on dose, type, method of administration and duration of exposure, with morphological changes involving surface epithelium, glands, vascular structures, leukocytic infiltration and stroma (Ludwig, 1982; Johannisson, 1990; Clark et al., 1996; Rogers, 1996; Song et al., 1996) and functional differences such as the expression of insulin-like growth factor-binding protein-1 (Pekonen et al., 1992), prolactin (Critchley et al., 1998b) and sex steroid receptors (Critchley et al., 1998a). Three different histological morphologies were observed in this study: an atrophic type, a progestosterone-modified endometrial change displaying pseudo-decidualization and inactive glands, and a shedding type displaying endometrial degeneration. No significant correlation between the number of bleeding days and the three histological types was observed; however, this may relate to the small sample size of the shedding and atrophic histological groups. Previous studies have also failed to demonstrate a correlation between endometrial histological changes and bleeding patterns (Johannisson, 1990; Rogers, 1996), although Ludwig (1982) reported decreased frequency of leukocyte infiltration in patients with amenorrhea or atrophic endometria. This failure to demonstrate any correlation between bleeding patterns and histology reflects the complexity of the pathological mechanisms involved and suggests that it is probably not one single factor that is important in the development of bleeding but a complex interplay between many elements.

We observed that stromal cell density appeared to vary in the different endometrial histological groups, a finding confirmed by stereological assessment. The stromal cell compartment of the endometrium is dynamic, altering in response to the variable hormonal milieu during the menstrual cycle and responsible for producing a variety of regulatory molecules, including cytokines (Clark, 1992; Tabibzadeh and Sun, 1992), chemokines (Hornung et al., 1997; Jones et al., 1997), MMPs (Rogers et al., 1993; Hampton and Salamonsen, 1994; Jeziorska et al., 1996) and TIMPs (Zhang and Salamonsen, 1997). Thus, variation in the stromal cell endometrial compartment with concomitant variation in the regulatory environment may have profound effects on endometrial function, including the propensity to undergo degeneration and bleeding. The highest stromal cell density was observed in the atrophic histological group, a morphological appearance associated with amenorrhea (Fraser et al., 1996), which may reflect a net inhibitory effect upon breakdown and bleeding in the endometrium. This may relate to differences in TIMP immunostaining which were not apparent in this study as the TIMP immunostaining was performed only in tissues displaying the progesterone-modified morphology.

Increased numbers of eosinophils and neutrophil polymorphs were observed in areas of tissue breakdown in Norplant-treated endometria as well as in endometria from women at the time of menstruation. These findings are consistent with previous reports of increased numbers of leukocytes, comprising macrophages, neutrophils and endometrial granulated lymphocytes, which were observed during the mid–late secretory phase and immediate premenstrual phase in the normal menstrual cycle (Bulmer et al., 1988; Starkey et al., 1991; Jeziorska et al., 1995). Elevated numbers of macrophages, T-lymphocytes and endometrial granulated lymphocytes have also been described in progestin-exposed endometria including Norplant (Ludwig, 1982; Booker et al., 1994; Clark et al., 1996; Song et al., 1996), but this is the first study to report also increased neutrophils and eosinophils in Norplant users. The number of increased macrophages in the endometria of Norplant users correlates with the number of bleeding days reported by patients (Clark et al., 1996). Increased numbers of neutrophil polymorphs are observed in areas of endometrial breakdown in patients treated with high-dose oral progestins (Song et al., 1996). Ludwig (Ludwig, 1982) reported increased frequency of leukocyte infiltration in patients with recent episodes of bleeding. Each type of leukocyte produces a plethora of regulatory molecules, including various cytokines and proteins, and is thus capable of influencing endometrial structure and function, including endometrial degeneration and the propensity to bleed.

Variation in chemokine expression in the human endometrium throughout the menstrual cycle coincides with the pattern of leukocyte accumulation (Jones et al., 1997). Endometrial stromal cells are a source of chemokines thereby capable of influencing leukocyte infiltration and activation and, potentially, MMP-9 action. The chemokine RANTES (regulated upon activation, normal T-cell expressed and secreted) mRNA and protein have been identified in human endometrium stroma (Hornung et al., 1997); increased expression of RANTES mRNA in cultured endometrial stromal cells was observed in the presence of the cytokines, tumour necrosis factor-α (TNF-α) and interferon-γ. Monocyte chemotactic protein, interleukin-8 (Jones et al., 1997), granulocyte-macrophage colony-stimulating factor (GM-CSF) (Giacomini et al., 1995) and eotaxin (J.Zhang and L.A.Salamonsen, unpublished observations), which are factors chemotactic for macrophages, neutrophils, and eosinophils, are also immunolocalized to the endometrium displaying temporal and spatial variation and may play a role, along with as yet unidentified chemokines, in leukocyte recruitment. Various cytokines are produced by endometrial stromal and epithelial cells, including TNF-α (Hunt et al.,
cytokine generation and secretion (Goetzl et al., 1995). These cytokines have been implicated in the regulation of leukocyte function in other tissues (for review see Weller, 1991; Hallett and Lloyds, 1995; Rutherford et al., 1996) and may also play a role regulating leukocyte function in the endometrium.

Production of MMPs by leukocytes displays a cell-specific pattern, is dependent upon the state of cellular differentiation and is regulated by cytokines and adhesion molecules (for review see Goetzl et al., 1996). MMPs are believed to play a role in leukocyte cellular migration and in regulation of cytokine generation and secretion (Goetzl et al., 1996). MMP-9 is secreted by neutrophils, macrophages, eosinophils and T-cells. In menstrual endometrium, MMP-9 is immunolocalized to macrophages, eosinophils, and neutrophil polymorphs (Jeziorska et al., 1996). In the current study, MMP-9 appears to be localized only to migratory cells (including eosinophils, macrophages, neutrophil polymorphs and CD3+ T-cells) within the endometrium of Norplant users. Importantly, not all cells of any one type are MMP-9 positive, which may reflect differences in cellular differentiation or pattern of leukocyte activation and degranulation. For example, the EG1 antibody used in this study to detect eosinophils detects both resting and activated eosinophils; the MMP-9 positive phenotype may thus be dependent upon the differentiation/activation state of the eosinophil. Although we observed no correlation between the number of MMP-9 positive cells and bleeding patterns reported by women, this may reflect the relatively small sample size of this study, sampling error in regard to biopsy of non-bleeding versus bleeding endometrial sites or indicate that factors in addition to the number of MMP-9 positive cells may be important, such as the MMP-9:TIMP ratio.

Leukocytes, in addition to providing a source of MMPs, including MMP-9 and MMP-8 (specific to neutrophils), also produce factors important in the regulation of MMPs. These regulatory molecules may act at different levels in the MMP pathway, including gene transcription, secretion of prohormones, activation and inhibition. Interaction between the products of different leukocytes may also act to regulate MMPs. For example, neutrophil elastase, an enzyme produced exclusively by neutrophils, inactivates TIMP-1 in the proMMP-9/TIMP-1 complex allowing activation of MMP-9 by MMP-3 (Itoh and Nagase, 1995), while the secretion of proMMP-9 from T-cells is selectively modulated by chemokines and proinflammatory cytokines including TNF-α and IL-1 (Johnatty et al., 1997).

Although MMP-9 is secreted by leukocytes within the endometrium, other MMPs and TIMPs are produced by endometrial stromal and epithelial cells. Products of these cells may act via autocrine and paracrine mechanisms to modulate MMP production and activity. For example, the pro-inflammatory cytokines TNF-α (Hunt et al., 1992) and IL-1 (Tabibzadeh and Sun, 1992) are produced by endometrial stromal and epithelial cells during the mid-late secretory phase of the menstrual cycle and, in vitro, stimulate production of proMMP-1 and -3 from cultured endometrial stromal cells in a dose-dependent manner (Rawdanowicz et al., 1994). Coculture experiments have shown that TGF-β that is produced by endometrial stromal cells in response to progesterone supresses epithelial production of proMMP-7 (Osteen et al., 1994). MMP-3, indirectly via activation of proMMP-7, and MMP-7 are capable of activating proMMP-9. It is reasonable to assume that similar regulatory systems exist in progestin-treated endometrium. The action of TIMPs to inhibit MMPs and their localization to endometrial stroma both in normal cycling (Zhang and Salamonsen, 1997) and Norplant-treated endometrium may contribute to the focal nature of bleeding.

The mechanism by which MMP-9 activity is regulated in Norplant-treated endometrium, which is an environment of continuous exposure to progesterin, is unclear. MMP activity is generally decreased by progesterone but this can be overridden by cytokines such as IL-1 and TNF-α (Singer et al., 1997; Zhang et al., 1998). Alteration in stromal cell production of various cytokines also occurs in response to progesterone (Osteen et al., 1994). Current data suggest that human endometrial leukocytes do not possess progesterone or oestrogen receptors (Tabibzadeh and Satyaswaroop, 1989; King et al., 1996), therefore any effect of these hormones on leukocyte recruitment or function is likely to be indirect, possibly by altering the expression of chemokines and cytokines by stromal cells. Changes in the stromal cell expression of progesterone receptor isoforms in response to prolonged continuous exposure to a progesterin and/or the effect of levonorgestrel as opposed to progesterone may result in autocrine and paracrine alteration in the pattern of cytokine and chemokine secretion by endometrial stromal and epithelial cells, with effects on leukocyte recruitment and activation and MMP production and activity. The net result would be alteration in the MMP:TIMP balance, promoting MMP-9 mediated degradation of the basement membrane and contributing to endometrial degeneration and bleeding.

The pathophysiological mechanisms resulting in abnormal uterine bleeding in women using Norplant are clearly multifactorial and complex. The data presented here suggest that MMP-9 may play a role in endometrial degeneration in Norplant users. Further studies investigating the role of other MMPs in this and in other situations of pathological bleeding are currently in progress.

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