Decreased tissue inhibitor of metalloproteinase in the endometrium of women using depot medroxyprogesterone acetate: a role for altered endometrial matrix metalloproteinase/tissue inhibitor of metalloproteinase balance in the pathogenesis of abnormal uterine bleeding?

A.J.Vincent¹, J.Zhang¹, A.Östör², P.A.W.Rogers³, B.Affandi⁴, G.Kovacs⁵ and L.A.Salamonsen¹,⁶

¹Prince Henry’s Institute of Medical Research, P.O.Box 5152, Clayton, Victoria, 3168, ²Melbourne University Departments of Pathology, Obstetrics and Gynaecology, Parkville, Victoria, ³Monash University Department of Obstetrics and Gynaecology, Clayton Victoria, 3168, Australia, ⁴Human Reproduction Study Group, Department of Obstetrics and Gynaecology, University of Indonesia, Klinik Raden Salah, Jalan Raden Salah 49, Jakarta, 10330, Indonesia and ⁵Monash University Department of Obstetrics and Gynaecology, Box Hill Hospital, Box Hill, Victoria, Australia

²To whom correspondence should be addressed. E-mail: lois.salamonsen@med.monash.edu.au

BACKGROUND: Abnormal uterine bleeding is commonly associated with progestin-only contraceptives, including depot medroxyprogesterone acetate (DMPA), and remains the main reason why these agents are discontinued. Matrix metalloproteinases (MMP), enzymes which degrade specific extracellular matrix components, and leukocytes are implicated in menstruation. Alteration in endometrial MMP-9 and leukocytes has been described in users of other progestin-only contraceptives, suggesting a potential role in the pathogenesis of abnormal uterine bleeding.

METHODS: This study describes the immunohistochemical localization of MMP-9, the tissue inhibitors of metalloproteinases (TIMP)-1, TIMP-2 and TIMP-3, and leukocytes [CD3⁺ T lymphocytes, CD68⁺ macrophages and CD56⁺ uterine natural killer cells (uNK cells)] in the endometrium of women using DMPA. Comparison is made with perimenstrual endometria from normal cycling women. RESULTS: Similar to the perimenstrual period, an influx of MMP-9 positive cells (identified as neutrophils and CD3⁺ T cells on the basis of dual immunofluorescence), macrophages and uNK cells was observed in the endometrium of DMPA users. However, significantly more endometrial T lymphocytes were observed in DMPA users. Immunoreactive TIMP, present in all endometrial compartments, demonstrated a significantly decreased immunostaining intensity score in endometrial epithelium (TIMP-1 and TIMP-2), stroma (TIMP-1, TIMP-2 and TIMP-3), endothelium (TIMP-1 and TIMP-2) and vascular smooth muscle (TIMP-1) of DMPA users compared with controls. No correlation was observed between the parameters studied and bleeding patterns reported by subjects. CONCLUSIONS: These findings provide additional evidence for the importance of the MMP/TIMP balance in the loss/maintenance of endometrial integrity and in the complex pathological mechanisms involved in the troubling side-effect of menstrual bleeding disturbance.

Key words: depot medroxyprogesterone acetate/leukocytes/matrix metalloproteinase-9/tissue inhibitor of metalloproteinase/uterine bleeding

Introduction

Disturbance of menstrual bleeding patterns is the main reason why progestin-only contraceptive agents are discontinued (Odlind and Fraser, 1990). Injectable depot medroxyprogesterone acetate (DMPA), despite being a safe and effective long-acting progestin-only contraceptive, is associated with the greatest perturbation of bleeding patterns of all contraceptive methods (Fraser, 1999). However, the pathogenesis of abnormal uterine bleeding associated with progestin-only contraceptives remains ill-defined. The endometrial response to exogenous progestins is variable depending on dose, type, method of administration and duration of exposure with changes in endometrial morphology, steroid receptor profile, vascular morphology and function, haemostasis and repair mechanisms described (Fraser et al., 1996). Bleeding is thought to arise from capillaries and venules with hysteroscopic evidence of neovascularization and increased vessel fragility observed in vivo in women using the progestin-only contraceptive, Norplant (subdermally implanted levonorgestrel) (Hickey et al., 1996).

Matrix metalloproteinases (MMP) are a family of zinc-
dependent proteases which degrade extracellular matrix (ECM) components (Birkedal-Hansen et al., 1993). Regulation of MMP is complex and occurs at multiple levels including gene transcription, a cascade of activation in which proteases, including some MMP, are able to activate the latent zymogen and inhibition by tissue inhibitors of metalloproteinases (TIMP) with formation of 1:1 complexes with the active enzymes. MMP-9 (gelatinase B) is a 92 kDa metalloproteinase with substrate specificity for collagen IV (a major component of basement membranes), collagen V, elastin and gelatin and is unusual in that both the active and latent enzyme are inhibited by TIMP-1 (Itoh and Nagase, 1995). MMP display spatial and temporal variation within the endometrium during the menstrual cycle, with an increase observed perimenstrually. They are postulated to be involved in the endometrial breakdown observed at menstruation (Marbaix et al., 1995, 1996; Salamonsen and Woolley, 1996). Recent studies have also shown altered MMP (Vincent et al., 1999, 2000; Galant et al., 2000; Marbaix et al., 2000) and TIMP (Galant et al., 2000; Marbaix et al., 2000) expression and activation in endometrial biopsies from women using Norplant.

Leukocytes are an integral component of the endometrium and display variation in type, number, activation status and site across the menstrual cycle and are postulated to play a key role in menstruation (Salamonsen and Lathbury, 2000). Previous immunohistochemical studies have reported both increased (Song et al., 1996; Critchley et al., 1998b; Vincent et al., 1999) and decreased (Clark et al., 1996) numbers of leukocytes in the endometrium of women treated with progesterin-only contraceptives. In addition to providing a source of MMP-9 (Martelli et al., 1993; Shi et al., 1995; Jeziorska et al., 1996, Vincent et al., 1999), endometrial leukocytes produce a range of bioactive molecules including cytokines and proteases which are implicated in the regulation of MMP (Salamonsen and Lathbury, 2000).

We hypothesize that MMP, TIMP and leukocytes are involved in the pathogenesis of abnormal uterine bleeding in women using progesterin-only contraceptives. Thus the aim of this study is to extend our previous studies and determine whether MMP-9, TIMP and leukocytes are present within the endometrium of women using DMPA and the correlation with abnormal uterine bleeding.

Materials and methods

Subjects and tissue collection

Subjects were recruited after informed consent as previously described (Vincent et al., 2000) and included 19 Indonesian women who presented to the Klinik Raden Salah in Jakarta, Indonesia for the administration of DMPA (150 mg i.m. injection every 90 days) and four Australian women who presented to Family Planning, Victoria for administration of DMPA (same regimen as the Indonesian women). After DMPA administration, subjects recorded a daily menstrual diary from the day of DMPA administration to the day of endometrial biopsy. The total duration of implant use was recorded for each subject. Menstrual bleeding charts were analysed by calculating the total number of bleeding days (any bleeding or spotting) in the 90 day reference period prior to endometrial biopsy. Where endometrial biopsy was performed before 90 days of treatment had elapsed, the ratio of number of bleeding + spotting days/total duration was calculated and multiplied by 90 to obtain an equivalent bleeding/spotting days per 90 day reference period (Hourihan et al., 1991).

Immunohistochemical staining

Immunohistochemical analysis of each antigen was performed on a single section per subject selected at random. All incubations were performed in a humid chamber. Each incubation was performed at room temperature and was followed by three Tris-buffered saline (TBS) washes over a 10 min period unless otherwise stated.

MMP-9 immunohistochemistry was performed using a monoclonal mouse anti-human MMP-9 antibody (Insight Biotechnology Ltd, Middlesex, UK) and the alkaline phosphatase–anti-alkaline phosphatase (APAAP) technique as described previously (Vincent et al., 1999). Briefly, following inhibition of non-specific binding (NSB) [30 min incubation with TBS containing 10% normal goat serum (NGS)] the primary antibody (used at a concentration of 2 µg/ml in 10% NGS/TBS) was applied and the sections incubated overnight at 4°C. MMP-9 was visualized using goat anti-mouse IgG (Dako, Glostrup, Denmark) followed by mouse APAAP complex (Dako), repeated twice and with New Fuchsin (Dako) as the chromogen. Endogenous alkaline phosphatase was blocked with 1 mmol/l levamisole.

Immunolocalization of macrophages was performed using the mouse monoclonal anti-human CD68 antibody clone KP-1 (Dako) diluted 1:50 in 10% normal horse serum (NHS)/TBS. Following inhibition of endogenous peroxidase activity (0.3% H2O2 v/v in methanol for 30 min) and NSB (10% NHS/TBS for 30 min), a 2 h primary antibody incubation was performed. Incubations with biotinylated horse anti-mouse IgG (1:200 v/v in 1% fetal calf serum, 1% NHS, 5% normal human serum/TBS for 1 h) (Vector Laboratories, Burlingame, CA, USA) followed by the StreptABC–horse-radish complex (Dako) (used according to the manufacturer’s instructions) preceded colour development with diaminobenzidine (DAB).
T lymphocytes were identified using a polyclonal rabbit anti-human CD3 antibody (Dako) and the EnVision Peroxidase DAB kit (Dako). Immunostaining consisted of sequential applications of peroxidase blocking agent for 5 min, primary antibody (1:100 diluted in 10% NGS/TBS) for 30 min, EnVision polymer for 30 min and DAB plus kit with 5 min TBS washes between each step.

Detection of uterine natural killer (uNK) cells was performed using a microwave antigen retrieval method (Crichtley et al., 1998b) adapted for our study and the APAAP method described above for MPP-9 immunohistochemistry. The tissue sections were initially dewaxed, rehydrated, microwaved at high power in 0.01 mol/l sodium citrate buffer (pH 6.0) for 15 min and then allowed to stand for 20 min. Following microwave antigen retrieval and NSB blocking (10% NGS/TBS), mouse monoclonal anti-human CD56 (diluted 1:150 in 10% NGS/TBS) (Zymed, San Francisco, CA, USA) was applied and the sections incubated overnight at 4°C. Incubations for 45 min with goat anti-mouse IgG (1:25 in 1% fetal calf serum, 1% NGS, 5% normal human serum/TBS) (Dako) and then mouse APAAP (1:50 in primary antibody diluent) (Dako) were performed and then repeated for 15 min. New Fuchsin (Dako) was used as the chromogen with colour development proceeding for 30 min.

Immunohistochemistry for TIMP-1, TIMP-2 and TIMP-3 was performed as previously described (Zhang and Salamonsen, 1997), using sheep anti-human TIMP-1 (a gift from Dr Hideaki Nagase, Kansas City, KS, USA), rabbit anti-human TIMP-2 (Triple Point, Forest Grove, OR, USA) and rabbit anti-human TIMP-3 (Triple Point) as the primary antibodies. The subsequent detection methods used were the Dako StreptABC kit (TIMP-1) and the StrAviGen (Biogenex Laboratories, San Ramon, CA, USA) supersensitive immunostaining system (TIMP-2 and TIMP-3) with New Fuchsin (Dako) as the chromogen.

For each tissue, a second section on the same slide was used as a negative control with an irrelevant α-lactalbumin monoclonal IgG antibody (MPP-9, CD68 and CD56), rabbit IgG (CD3, TIMP-2 and TIMP-3; Dako) or normal sheep IgG (TIMP-1; Serotec, Oxford, UK) substituted at the same concentration as the primary antibody. Positive controls appropriate for each antibody were also included in each series of sections examined and included proliferative phase human endometrium (MPP-9), human synovium and endometrium (CD3), human endometrium and decidua (CD56; kind gift of Dr E.Wallace, Department of Obstetrics and Gynaecology, Monash University, Clayton, Victoria, Australia) and human fetal kidney (TIMP-1, TIMP-2 and TIMP-3). All tissue sections were counterstained with Harris’ haema(toxylin, dehydrated and cleared in xylene then mounted in DPX. Photography was performed using an Olympus BX50 microscope with filter sets for fluorescein isothiocyanate (FITC) and Texas Red fitted with a digital camera (Videoecam Fujix HC-2000; Fuji Photo Film Co., Tokyo, Japan) coupled to a Compucron Pentium PC computer using Analytical Imaging Station (Imaging Research Inc., USA) and Adobe Photoshop software.

Immunolocalization of neutrophils and eosinophils in Carnoy’s fixed tissue using the antibodies, mouse monoclonal anti-neutrophil elastase and anti-eosinophilic cationic protein clone EG1 respectively, proved to be unreliable (A.J.Vincent, unpublished observations) and as DMPA samples fixed in 10% buffered formalin were not available in sufficient numbers, further immunohistochemical assessment and analysis of these leukocyte subtypes was not performed.

Dual immunofluorescence
Dual immunofluorescent staining was used on selected specimens to identify the cellular source of the MPP-9 using the amplification technique described previously (Vincent et al., 1999). MMP-9 anti-serum, 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 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 second antibody conjugated to Texas Red (Amersham Life Science, Little Chalfont, Buckinghamshire, UK) was then performed. The second primary antibodies used were (i) mouse monoclonal anti-mast cell tryptase (Dako), (ii) mouse monoclonal anti-CD56 (Zymed), (iii) mouse monoclonal anti-CD68 (clone KP1) (Dako), (iv) rabbit polyclonal anti-CD3 (Dako), (v) mouse monoclonal anti-neutrophil elastase (Dako) and (vi) mouse monoclonal anti-eosinophilic cationic protein clone EG1 (Pharmacia Ltd, Milton Keynes, Bucks, UK). No detectable signal was observed with conventional immunofluorescent staining using the same MPP-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 immersion mount (Dako) and photographed using the Olympus photomicroscope described above with filter sets for FITC and Texas Red. As the antibodies, mouse monoclonal anti-neutrophil elastase and anti-eosinophilic cationic protein clone EG1 do not reliably detect antigen in Carnoy’s fixed tissue (A.J.Vincent, unpublished observations), 10% buffered formalin-fixed biopsies obtained from two Australian DMPA users were included in this experiment.

Assessment of immunostaining
Quantitative analysis of positive MPP-9 and leukocyte immunostaining was performed as described previously (Vincent et al., 1999) using an Olympus BX-50 microscope and a ×40 objective. The image was captured using a Pulinel 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, Birkerød, 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 and glandular lumen cells) in ≥15 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. Quantitative analysis was performed by the same observer, with no knowledge of the clinical characteristics of the patient donor.

TIMP immunostaining intensity in each endometrial compartment (including luminal and glandular epithelium, stroma, endothelium, vascular smooth muscle cells, and decidual cells) was assessed using a published method (Zhang and Salamonsen, 1997). All endometrial samples were compared with the fetal kidney positive control (included in each immunostaining run) and the negative control section (included on each endometrial sample slide). Immunostaining was scored on a scale from 0 (no staining) to 4 (equivalent to the maximal staining intensity observed on the fetal kidney positive control).

Statistical analysis
Comparisons between groups were made using either the Mann–Whitney test (clinical characteristics) or the Kruskal–Wallis test (immunostaining results). Multiple comparisons were made after statistical significance was found at the 5% level using a published method (Conover, 1980). Correlations between variables were calculated via Spearman’s rank correlation.
Results

Histological assessment

The histological analysis of these tissues has previously been reported (Vincent et al., 2000) and demonstrated that of the 17 endometrial biopsies containing adequate tissue for histological assessment (2/4 Australian and 15/19 Indonesian samples), 12 samples displayed a progesterin-modified appearance (although the grade of pseudo-decidualization varied), three were atrophic and two biopsies showed a shedding morphology.

Clinical characteristics

As described previously, the duration of DMPA use varied from 19 to 376 days (Vincent et al., 2000). Dividing the subjects into three groups according to duration of DMPA use: (I) duration <31 days (n = 7), (II) duration 80–110 days (n = 4) and (III) duration >300 days (n = 5) revealed that the shedding morphology was only observed in association with a shorter duration of use and the atrophic morphology associated with a longer duration of use. The progesterin-modified morphological appearance was observed in all groups. The median number of bleeding days ratio in the 90 day reference period prior to biopsy was greatest in group I (36 days, range 20–50) compared with group II (median 17 days, range 9–28) and group III (median 26 days, range 21–30). However, there were no statistically significant differences in the bleeding ratios between the three groups.

Immunohistochemical staining of endometrial biopsies

No specific positive staining was observed in any of the sections where the primary antibody was substituted with the appropriate negative control antibody.

MMP-9

Immunoreactive MMP-9 was observed in all DMPA biopsies (Figure 1A) and in those from normal cycling women during the perimenstrual period. In endometrial samples from women using DMPA, MMP-9 immunolocalization was mainly confined to either intravascular leukocytes where intracellular staining was observed or in association with leukocytes in areas of tissue lysis where both intracellular and adjacent extracellular immunostaining was noted. There was no glandular epithelial, endothelial or stromal cell expression of MMP-9.

The MMP-9 immunoreactive leukocytes were identified as predominantly neutrophils and lymphocytes on morphological criteria, with macrophages and eosinophils more difficult to discern. Thus, dual immunofluorescent techniques were used to confirm the identity of the MMP-9 immunopositive cells in the endometrium of DMPA users. CD3+ T cells and neutrophil polymorphs were detected with Texas Red fluorescent immunostaining and each of these markers co-localized with MMP-9 positive cells detected with FITC immunofluorescence (Figure 1B and B’; C and C’ respectively). Importantly, the dual immunofluorescent staining also revealed evidence of phenotypic variation in leukocyte MMP-9 immunostaining; immunoreactive MMP-9 positive and negative subgroups were observed within each cell type in a single biopsy sample (Figure 1B and B’). Although mast cells, uNK cells, macrophages and eosinophils were demonstrated using Texas Red immunofluorescence, no co-localization with MMP-9 was observed (Figure 1D and D’).

Quantitative analysis demonstrated that MMP-9 immunostaining was similar in DMPA users and premenstrual and menstrual controls (Figure 1E). There was no correlation between the number of MMP-9 immunopositive cells and the number of leukocytes or bleeding patterns as recorded in menstrual diaries.

TIMP

Immunoreactive TIMP-1 was demonstrated in all major tissue compartments (luminal and glandular epithelium, stroma, endothelium, vascular smooth muscle and decidual cells) in both perimenstrual controls and DMPA users (Figure 1E). Importantly, significantly less TIMP-1 immunostaining was observed in multiple endometrial compartments including luminal epithelium (P = 0.007), glandular epithelium (P = 0.006), stroma (P = 0.002), endothelium (P = 0.003) and vascular smooth muscle (P = 0.005) in biopsies obtained from DMPA users compared with premenstrual or menstrual controls (Figure 3). No correlation between stromal TIMP-1 immunostaining intensity and bleeding parameters was observed (data not shown).

As noted with TIMP-1, immunoreactive TIMP-2 was localized to multiple endometrial compartments including epithelial, endothelial, vascular smooth muscle, decidual and stromal cells but not leukocytes in biopsies from both progestin contraceptive users (Figure 1F) and perimenstrual control women. Significantly less TIMP-2 immunostaining intensity was observed in the luminal epithelial (P = 0.004), stromal (P = 0.04) and endothelial compartments (P = 0.004) compared with perimenstrual control subjects (Figure 3). No correlation between stromal TIMP-2 immunostaining intensity and bleeding parameters was observed (data not shown).

Biopsies obtained from women using DMPA and perimenstrual control subjects revealed positive TIMP-3 immunostaining in the same endometrial components as TIMP-1 and TIMP-2 (Figure 1G). As demonstrated with TIMP-1 and TIMP-2, significantly less immunoreactive TIMP-3 was observed in luminal epithelial (P = 0.01) and stromal (P = 0.02) endometrial compartments of DMPA users compared with perimenstrual control subjects (Figure 3). However, in contrast to TIMP-1 and TIMP-2, TIMP-3 immunostaining intensity, although low, was significantly higher in endometrial endothelium of women using DMPA compared with perimenstrual control subjects (P = 0.007). No correlation between stromal TIMP-3 immunostaining intensity and bleeding parameters was observed (data not shown).

Leukocytes

T lymphocytes, macrophages and uNK cells were identified in all endometrial biopsies from DMPA users (Figure 1L, M and N respectively) and control women (not shown). CD3+ T lymphocytes were widely distributed throughout the stroma including areas of tissue breakdown, with occasional cells infiltrating the luminal and glandular epithelium and present intravascularly. Periglandular aggregates of T lymphocytes
Figure 1. Immunolocalization of matrix metalloproteinase (MMP)-9 (A; indicated by arrows), CD3+ T lymphocytes (L), CD68+ macrophages (M) and CD56+ uterine natural killer (uNK) cells (N) positive cells in the endometrial biopsies of women using depot medroxyprogesterone acetate (DMPA). Positive cells are shown as either pink New Fuchsin chromogen (A and N) or brown diaminobenzidine (L and M) with haematoxylin counterstain. Dual immunofluorescence studies of selected DMPA endometrial tissues demonstrating the production of MMP-9 (B′ and C′) by neutrophils (B) and CD3+ T cells (C), although phenotypic variation was observed with not all leukocytes of one type MMP-9 immunopositive. However, as shown here for mast cell tryptase (D), co-localization of MMP-9 (D′) with other leukocyte types including macrophages, eosinophils, uNK cells and mast cells was not observed. Identical fields were immunostained for cell markers using conventional immunofluorescence with Texas Red and MMP-9 using the amplification technique with fluorescein isothiocyanate. Positive cells are shown as red and green immunofluorescence respectively (arrows). Immunolocalization of tissue inhibitor of matrix metalloproteinase (TIMP)-1, TIMP-2 and TIMP-3 (E, F and G respectively) in endometrial biopsies obtained from women using DMPA was observed in epithelial, stromal, decidual, endothelial and vascular smooth muscle cells but not leukocytes. Representative positive control sections (fetal kidney) for TIMP-1, TIMP-2 and TIMP-3 (H, I and J respectively) demonstrating maximal TIMP immunostaining intensity (designated as an immunostaining score of ‘4’) and a negative control section (K) are shown. Scale bar shown in A = 10 μm and applies to A–D′. Scale bar shown in E = 10 μm and applies to E–K. Scale bar shown in L = 25 μm and applies to L–N. le = luminal epithelium.
Figure 2. Quantitative analysis of the number of matrix metalloproteinase (MMP)-9, uterine natural killer (uNK) cells (CD56+), T lymphocytes (CD3+) and macrophages (CD68+) immunopositive cells per 1000 stromal cells according to the use of depot medroxyprogesterone acetate (DMPA) or stage of the idealized 28 day normal menstrual cycle. Results are given as mean ± SEM. Significantly greater (*P < 0.01) numbers of CD3+ cells were observed in biopsies obtained from women using DMPA compared with perimenstrual control women. Numbers of samples represented by the histograms are given in parentheses.

were commonly observed. CD56+ uNK cells displayed a similar pattern to T cells. Macrophages were also dispersed throughout the stroma and areas of tissue lysis. Infiltration of luminal and glandular epithelium was prominent with CD68+ cells observed in the gland lumens. Macrophages were occasionally noted in association with the lymphoid aggregates. Quantitative analysis of the numbers of positive cells revealed significantly more (P < 0.01) CD3+ T cells in endometrial biopsies from DMPA users compared with premenstrual or menstrual controls (Figure 2). No significant difference was

Figure 3. Cellular localization and relative intensity (mean ± SEM) of immunostaining for tissue inhibitor of matrix metalloproteinase (TIMP)-1, TIMP-2 and TIMP-3 in the endometrium of depot medroxyprogesterone acetate (DMPA) users and perimenstrual control women (day 1–3 and day 26–28) of the idealized 28 day menstrual cycle (Normal cycle). Cellular compartments include luminal epithelium (LE), glandular epithelium (GE), stroma (Stroma), endothelium (Endo), vascular smooth muscle (VSM) and decidual cells (Decidual). Significantly less TIMP-1 immunostaining intensity was observed in all endometrial compartments except decidual cells in biopsies obtained from women using DMPA compared with controls. Significantly less TIMP-2 and TIMP-3 immunostaining intensity was also observed in the luminal epithelial and stromal endometrial compartments of DMPA users compared with perimenstrual control women. As observed with TIMP-1, immunoreactive TIMP-2 was significantly decreased in endothelial cells in endometrial samples from DMPA users. However, TIMP-3 immunostaining intensity, although low, was significantly higher in endometrial endothelium of women using DMPA compared with perimenstrual control subjects. *P < 0.01.
observed between the number of uNK cells and macrophages in control endometria compared with endometrial samples obtained from women using DMPA (Figure 2). However, the number of macrophages was positively correlated ($P = 0.01$) with the morphological grade of decidualization of the progestin-modified histological group (data not shown). There was no correlation between leukocyte numbers and duration of DMPA use or bleeding patterns reported by women.

**Discussion**

This study has demonstrated that leukocyte infiltration of uNK cells, macrophages and CD3+ T lymphocytes including MMP-9 immunopositive leukocytes occurs in the endometrium of women using the progestin-only contraceptive, DMPA. This reflects the state in premenstrual and menstrual tissue in normal women, the time of the cycle when leukocyte numbers are maximal and endometrial breakdown occurs. Importantly, decreased immunoreactive TIMP intensity was observed in various endometrial compartments of DMPA users indicating a potential alteration in MMP/TIMP balance favouring MMP activation and endometrial degradation.

In agreement with previous investigations of the normal menstrual cycle (Hampton and Salamonsen, 1994; Rodgers et al., 1994; Zhang and Salamonsen, 1997), immunoreactive TIMP-1, TIMP-2 and TIMP-3 were observed in all endometrial compartments except leukocytes in both progestin contraceptive users and perimenstrual controls. However, differences in endometrial TIMP immunostaining intensity were observed between DMPA users and control subjects. In comparison with perimenstrual controls, the extent of TIMP-1 and TIMP-2 immunostaining intensity was decreased in the endometrium of DMPA users. Importantly, the endometrial compartments in which altered TIMP-1 and TIMP-2 immunoreactivity was observed included endothelium, stroma and luminal epithelium which are important for the maintenance of endometrial integrity. MMP are inhibited by TIMP with a 1:1 stoichiometry which are important for the maintenance of endometrial tissue lysis and the perimenstrual-like extent and widespread localization of MMP-9 immunopositive leukocytes to areas of tissue lysis and the perimenstrual-like extent and widespread distribution of immunoreactive MMP-1 (Vincent et al., 2000; Barbaix et al., 2000). In comparison with the findings of the current study, endometrial MMP-9 immunostaining was increased in women using Norplant whereas TIMP-1 immunoreactivity was similar to perimenstrual controls (Vincent et al., 1999). Such differences in the pattern of MMP and TIMP immunostaining observed between the different progestins may relate to the differences in bleeding patterns observed between the different agents. However, despite a heterogeneous response observed in the endometrium following treatment with different progestins, alteration in the MMP/TIMP balance in favour of MMP activity and subsequent ECM degradation is a common theme.

The increased endothelial TIMP-3 immunostaining observed in women using progestin-only contraceptives was unexpected and may relate to (i) sampling at non-bleeding endometrial sites, (ii) subject amenorrhoea or (iii) alternative functions of TIMP-3 such as mitogenesis or induction of apoptosis, which have been described in other tissues (Woesnner and Nagase, 2000) but not as yet described in the endometrium. Apoptosis has been proposed as a mechanism involved in the regulation of endometrial endothelial cell numbers (Rogers, 1996).

As reported previously in Norplant users (Vincent et al., 1999), this study demonstrates that MMP-9 is present within endometrial leukocytes in women with abnormal bleeding associated with the use of DMPA. These MMP-9 positive cells were identified as CD3+ T cells and neutrophils, although not all cells of any one type were MMP-9 positive. Prior immunohistochemical (Jezierska et al., 1996; Vincent et al., 1999) and zymography studies (Shi et al., 1995) have also identified endometrial macrophages, eosinophils and uNK cells as a source of MMP-9, although human endometrial mast cells do not appear to produce MMP-9 (Jezierska et al., 1996; Zhang et al., 1998). The observed differences in this study may relate to the small sample size available for experimentation (e.g. eosinophils) and phenotypic variation where only a subset of each type of leukocyte is MMP-9 positive. Both of these are particularly relevant for those cells in very low numbers. Variation in leukocyte phenotype associated with different clinical settings, as observed with endometrial mast cells in control women compared with progestin-only contraceptive users (Vincent et al., 2000), may be an alternative explanation. We therefore cannot exclude endometrial macrophages, eosinophils and uNK cells as a potential source of MMP-9 in women treated with DMPA. Immunoreactive MMP-9 has also been localized to endometrial glandular, stromal, endothelial and perivascular cells in women using the levonorgestrel-releasing intrauterine system (LNG-IUS) (Skinner et al., 1999) and these differences may relate to immunohistochemical technique or the different clinical context.

**Progesterone regulation of endometrial MMP production**

In vitro is well recognized (Salamonsen and Woolley, 1996). Alteration in endometrial progesterone receptor (PR) expression has been observed in response to exposure to progestin-only contraceptives (Crichtley et al., 1993, 1998a; Lau et al., 1996; Mangal et al., 1997) with differences demonstrated in relation to PR isoform, type and duration of the contraceptive agent, potentially contributing to the observed changes in endometrial MMP expression in women using these agents.
One group (Critchley et al., 1998a) reported decreased prostaglandin dehydrogenase immunostaining and activity as well as diminished endometrial PR (PR-B more suppressed than PR-A) following the insertion of the LNG-IUS, concluding that the down-regulation of endometrial PR was associated with a functional response. PR-B expression, identified using Western analysis, was reduced in the endometrium of two subjects using DMPA (Mangal et al., 1997).

The pattern of leukocyte response to the use of exogenous progestins varies with the type of progestin used, duration and pattern of administration (cyclical or continuous), route of administration, presence of bleeding and endometrial morphological appearance (Vincent and Salamonsen, 2000). Consistent with the endometrial leukocyte influx observed in DMPA users in this study, focal and diffuse infiltration of leukocytes, predominately lymphocytes and monocytes, were observed in a light and electron microscopic study of endometrial biopsies obtained from women using long-term, low dose oral progestins or DMPA (Ludwig, 1982). In the current study, the ~6-fold increase in CD3+ T lymphocytes observed is consistent with an immunohistochemical study (Song et al., 1996) which reported increased numbers of leukocytes in the endometrium of women treated with high dose oral progestins compared with secretory phase controls. These included CD3+ T cells, leukocyte common antigen positive cells, neutrophils, CD68+ macrophages and phloxine-positive uNK cells. In the present study, endometrial macrophage and uNK cells numbers were similar between DMPA users and perimenstrual controls, the time of the normal menstrual cycle when leukocyte numbers are maximal (Salamonsen and Lathbury, 2000). These results also concur with the observations of Song et al. (1996) above and those of another study (Critchley et al., 1998b) which reported the presence of CD56+ uNK cells and CD68+ macrophages in the endometrium of women using the LNG-IUS. Interestingly, in the current study a positive correlation was observed between macrophage numbers and the grade of pseudo-deciudalization. In this context, increased macrophage numbers and immunoreactive stromal granulocyte-macrophage colony stimulating factor (GM-CSF), a macrophage chemokine, were observed in the endometrium of LNG-IUS users (in whom significant stromal pseudo-deciudalized change occurs) (Critchley et al., 1998b). In contrast, decreased macrophage numbers were demonstrated in morphologically atrophic endometrium in Norplant users (Clark et al., 1996). Chemokines, including monocye chemotactic protein-1 (MCP-1) (Arici et al., 1995; Hampton et al., 2000), MCP-2 (Hampton et al., 2000), GM-CSF (Critchley et al., 1998b), interleukin-8 (Jones and Critchley, 2000) have been identified in the endometrium of women using progestin-only contraceptives; however, their contribution to leukocyte infiltration or other functions remains unclear.

Endometrial leukocytes do not express PR (King et al., 1996; Salamonsen et al., 2002); however, alteration in endometrial epithelial and stromal cell PR expression (as described above) may result in changes in the endometrial chemokine and cytokine milieu leading to leukocyte influx and activation. Leukocytes, as well as providing a source of MMP-9, produce a variety of enzymes, cytokines and other bioactive mediators. These molecules, in addition to involvement in MMP and TIMP regulation (Salamonsen and Lathbury, 2000), may further influence the endometrial micro-environment thereby contributing to endometrial breakdown.

The association between bleeding patterns and leukocytes remains unclear. Endometrial leukocyte infiltrations reported by Ludwig were observed more frequently closer to the time of last reported bleeding episode and were seen less frequently in morphologically atrophic endometrial samples or those from amenorrhoeic patients (Ludwig, 1982). It was reported that the number of CD68+ macrophages was significantly increased in women using Norplant who reported irregular bleeding compared with non-bleeders (Clark et al., 1996). More recently, endometrial mast cells were increased in women using the LNG-IUS who reported breakthrough bleeding compared with non-bleeders (Milne et al., 2001). In contrast, as observed in the current study, no correlation was observed between bleeding patterns and endometrial MMP-9 or leukocyte immunostaining in Norplant users (Vincent et al., 1999) following the insertion of LNG-IUS (Critchley et al., 1998b). This lack of correlation may reflect the complexity of the endometrial micro-environment and the likelihood that the pathogenesis of menstrual bleeding disturbance associated with progestin-only contraceptives is multifactorial and not dependent upon a single variable. It may also be related to the difficulty in obtaining tissue at the time and site of bleeding. It is often difficult to obtain sufficient biopsy sample for analysis; as reported previously (Vincent et al., 2000) approximately half the DMPA samples collected could not be used for analysis. Similar findings have been reported together with an increased number of bleeding days reported by women in whom a successful biopsy was obtained (Hadisputra et al., 1996). Thus the results of the current study may only apply to a subgroup of DMPA users.

Previous studies have demonstrated temporal variation in endometrial vascular membrane components following Norplant exposure (Hickey et al., 1999a,b) and leukocyte distribution following LNG-IUS insertion (Critchley et al., 1998b) indicating the importance of the timing of sampling in relation to the administration of progestin. No difference in MMP-9, TIMP or leukocytes was observed according to long (>300 days) or short (<31 days) duration of DMPA use; however, the sample size of each group was small.

Comparison between the Indonesian women treated with DMPA and Australian controls (predominately Caucasian women) was a potential source of error in this study as differences in bleeding patterns between women of different ethnicity have been described (Gao et al., 1987). However, it was not possible to obtain control tissue from Indonesian women for this study. Furthermore, no difference was observed between the four DMPA endometrial biopsies obtained from Australian women and the Indonesian DMPA samples. No difference in endometrial endothelin or neutral endopeptidase immunostaining was observed between Australian and Indonesian control subjects (Marsh et al., 1995).

The findings of this study provide further evidence linking MMP to endometrial breakdown associated with abnormal uterine bleeding, although a causal relationship remains to be
established. These investigations also emphasize the variation in endometrial response to different progestins and indicate the importance of the MMP/TIMP balance in determining potential loss or maintenance of endometrial integrity. Such variability may relate to the different patterns of bleeding disturbance reported with different agents (Odlind and Fraser, 1990) and indicates similarities and differences in the potential mechanisms underlying breakthrough bleeding and the process of normal menstruation (Fraser et al., 1996).

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