Circulating sex hormones and endometrial stromelysin-1 (matrix metalloproteinase-3) at the start of bleeding episodes in levonorgestrel-implant users

E. Marbaix¹,²,², M. Vekemans³,⁵, C. Galant¹,², V. Rigot¹, P. Lemoine¹, D. Dubois², C. Picquet¹, P. Henriët¹, P. Twagirayezu³, S. Sufi⁴, Y. Eeckhout¹ and P. J. Courtoy¹

¹Cell Biology Unit, Christian de Duve Institute of Cellular Pathology and ²Department of Pathology, Saint-Luc University Clinics, Medical School of the Université catholique de Louvain, B-1200 Bruxelles, ³Department of Obstetrics and Gynaecology, Centre Hospitalier Universitaire Saint-Pierre, Medical School of the Université libre de Bruxelles, B-1000 Bruxelles, Belgium and ⁴World Health Organization Collaborating Centre for Research and Reference Services in the Immunoassay of Hormones in Human Reproduction, Queen Charlotte’s and Chelsea Hospital, London W6 0XG, UK
⁵Present address: PRIME/INTRAH, University of North Carolina School of Medicine, Chapel Hill, USA
⁶To whom correspondence should be addressed at: Cell Biology Unit, Universite Catholique de Louvain, 75 Avenue Hippocrate, B-1200 Brussels, Belgium. E-mail: marbaix@cell.ucl.ac.be

Unpredictable endometrial bleeding is the major side-effect of levonorgestrel-releasing s.c. implants (Norplant®), otherwise a method of choice for long-term contraception. The mechanisms responsible for bleeding are still unknown and no reliable treatment is available. Several matrix metalloproteinases (MMP) are expressed and activated in human endometrium only at menstruation and specific synthetic inhibitors of MMP fully prevent the tissue breakdown that occurs in menstrual-like endometrial explants. To investigate whether MMP are inappropriately expressed and activated in Norplant-treated endometria during bleeding episodes, volunteers were recruited to provide blood and endometrial biopsies at the start of bleeding episodes and during non-bleeding intervals. Whereas serum concentrations of levonorgestrel and sex hormones showed no change at bleeding, except for a slight decrease of oestradiol concentration, the expression and activation of stromelysin-1 released by explants cultured for 1 day were consistently increased at the start of bleeding episodes. Furthermore, stromelysin-1 was immunolocalized in stromal cells within breakdown areas of several bleeding endometria, but not in non-bleeding endometria. These observations suggest that the expression and activation of stromelysin-1 participate in the initiation of bleeding episodes upon Norplant contraception. New strategies in the prevention and treatment of abnormal bleeding based on MMP control should be envisaged. Key words: contraception/endometrium/ levonorgestrel/matrix metalloproteinase

Introduction

Irregular and unpredictable bleeding is the main side-effect of progestogen-only treatment, e.g. with levonorgestrel-releasing s.c. implants (Norplant®), otherwise a method of choice for long-term contraception (Vekemans, 1995; Vekemans et al., 1997; Fraser et al., 1998). The mechanisms leading to bleeding episodes remain to be elucidated.

The key role of matrix metalloproteinases (MMPs) in the endometrial breakdown that triggers menstruation is being increasingly appreciated (e.g. Marbaix et al., 1996a; Salamonsen and Woolley, 1996). MMP are generally secreted as latent zymogens (proMMP), and, once activated, are able to degrade most extracellular matrix proteins (Nagase and Woessner, 1999). Tissue inhibitors of metalloproteinases (TIMP) and α2-macroglobulin abrogate their activity by forming stoichiometric complexes.
Circulating hormones in Norplant® users

with the active forms of MMP. Many MMP are produced in the endometrium where the expression and activation of several of them, including interstitial collagenase-1 (MMP-1), gelatinase B (MMP-9) and stromelysins-1 (MMP-3) and -2 (MMP-10), are tightly controlled by ovarian steroids and restricted to the perimenstrual period at sites of matrix degradation (Marbaix et al., 1992, 1995; Rodgers et al., 1994; Hampton and Salamonsen, 1994; Kokorine et al., 1996). Moreover, menstrual-like endometrial breakdown, reproduced in explants cultured in the absence of ovarian steroids, is fully and selectively prevented by synthetic inhibitors of MMP (Marbaix et al., 1996b).

Since these MMP are implicated in the normal endometrial tissue breakdown and shedding at menstruation, we investigated whether they could also be involved in the triggering of irregular bleeding upon progestogen-only contraception. If MMP were indeed key factors in the occurrence of bleeding, their activity, an integrated result of expression, activation and/or decreased inhibition by TIMP, should be restricted to bleeding episodes. To test this hypothesis, volunteers bearing s.c. levonorgestrel-releasing implants (Norplant) were recruited for a prospective study to provide two blood and endometrial tissue samples, one at the start of any bleeding episode and another one during a non-bleeding interval, as a control. This investigation, part of which will be reported elsewhere (Galant et al., submitted for publication), demonstrated a clear relationship of bleeding with focal occurrence of tissue breakdown and local expression and activation of MMP-1, activation of MMP-9, increased expression and activation of gelatinase A (MMP-2), and decreased production of TIMP-1.

The present complementary report extends the analysis of the same material by focusing on bleeding patterns in these volunteers, on their serum levels of levonorgestrel and sex hormones, as well as on the endometrial expression and activation of stromelysin-1 (MMP-3) in relation with tissue breakdown at the start of bleeding episodes. Indeed, MMP-3 can degrade many constituents of the extracellular matrix, in particular type IV collagen, laminin, fibronectin and other components of the basement membranes (Nagase, 1998). Moreover, it is also able to activate other proMMP, including proMMP-1 and proMMP-9, regarded as other key proteinases in menstrual tissue breakdown (Marbaix et al., 1996a; Salamonsen and Woolley, 1996).

Materials and methods

Volunteers

Twenty-three healthy volunteers using long-term contraception with s.c. Silastic rods releasing levonorgestrel (Norplant; gifts from Leiras Oy, Turku, Finland) provided blood and endometrial biopsies, sampled at the first (n = 14) or the second day (n = 2) of bleeding episodes, occurring after at least 15 days without bleeding, and during non-bleeding intervals lasting for at least 15 days (n = 23). Two blood samples were not available, one at the first day of a bleeding episode and the other one during a non-bleeding interval in another woman. Volunteers were asked to keep a menstrual diary for the period of study, extending up to at least 2 weeks after the second biopsy, and to mention the use of any drugs. The study was approved by the Ethical Committees of the two involved institutions.

Oestradiol, progesterone, prolactin, FSH, LH and levonorgestrel assays

Blood was collected in glass tubes and serum was frozen at −20°C until analysed. LH, FSH, prolactin and oestradiol were measured using an Abbott AxSYM analyser (Abbott Diagnostics, Maidenhead, UK) in accordance with the manufacturer’s instructions. Progesterone was measured using an Immulite immunoassay analyser (Euro/Diagnostic Products Corporation, Caernarfon, UK) in accordance with the manufacturer’s instructions. Levonorgestrel was measured using reagents produced for the WHO Matched Reagent Programme (Immunometrics Ltd, London, UK). This assay is a first generation steroid radioimmunoassay involving ether extraction of standards and samples, overnight incubation with antibody and tritium-labelled levonorgestrel, followed by separation with dextran-coated charcoal (Ahsan et al., 1998). These reagents and protocols have been extensively used worldwide by the World Health
Organization and other bodies for analysis of samples from clinical trials. Analytical sensitivity of the assay is ~20 pmol/l. Variation between batches at 0.4, 2.0 and 4.0 nmol/l is 12.0, 9.0 and 9.7% respectively.

**Organ culture**

Biopsies were performed with Novak or mini-Novak curettes, immediately immersed in ice-cold saline phosphate buffer, pH 7.4, transferred to the laboratory and part of each was processed for organ culture within 2 h. Groups of six explants of roughly similar size, each one ~1 mm width, were cultured for 2 days without addition of oestradiol or progesterone, as described (Marbaix et al., submitted for publication). Release of enzymes into the conditioned media was measured during the first day of culture, a time period during which explant behaviour is similar to the in-vivo status of the tissue (Marbaix et al., 1996b). N-Acetyl-β-hexosaminidase and TIMP-2 releases did not vary significantly in media conditioned by explants from bleeding and non-bleeding endometria (Galant et al., submitted for publication), indicating the absence of systematic size variation between the two conditions. Moreover, the good viability of the explants was confirmed (i) by their histological appearance at the end of the culture, and (ii) by their capacity to respond to the absence of ovarian steroid by an increased release of several MMP during the second day of culture, irrespective of the level of MMP expression in the tissue before culture (not shown).

**Casein zymography**

Conditioned media were separated by electrophoresis in the presence of 0.1% sodium dodecyl sulphate (SDS) through 10% polyacrylamide gels, copolymerized with 0.5 mg/ml casein (Merck, Darmstadt, Germany). After 1.5 h of electrophoretic migration at 25 mA, SDS was removed by rinsing the gels with 2.5% Triton X-100. The gels were then incubated overnight at 37°C in 50 mmol/l Tris–HCl buffer, pH 7.5, containing 5 mmol/l CaCl₂, 3 mmol/l Na₃, 1% (vol/vol) Triton X-100 and 1 µmol/l ZnCl₂, stained with 0.25% (wt/vol) Coomassie Brilliant Blue R (Sigma, St. Louis, MO, USA), and destained with 7% (vol/vol) acetic acid. Purified human (pro)MMP-1 and (pro)MMP-3 (gifts from H. Nagase, Kansas University, Kansas City, KS, USA) were added as references. The proteinase inhibitors dichloroisoumarin, 1,10-phenanthroline, E64 (all from Sigma) and pepstatin (Boehringer Mannheim, Mannheim, Germany) were used to identify the classes of proteinases responsible for the observed caseinolytic activities. Glycosylated forms of (pro)MMP-1 and (pro)MMP-3 were identified by digesting samples of two conditioned media with 0.25 U/ml N-glycosidase F (Boehringer) for 2 h at 37°C, prior to electrophoretic migration (Wilhelm et al., 1987).

**Western immunoblotting of stromelysin-1**

Proteins were resolved by 0.1% SDS–10% polyacrylamide gel electrophoresis, then electrotransferred onto a nitrocellulose sheet (Hybond-C extra; Amersham, Little Chalfont, UK) during 1 h at 100 V in a 100 mmol/l Tris–HCl buffer, pH 8.3, containing 20% methanol and 16 mmol/l glycine. Non-specific protein binding sites were blocked by a 2 h incubation in 50 mmol/l Tris–HCl buffer, pH 7.5, containing 150 mmol/l NaCl, 5% non-fat dried milk (Gloria®; Nestlé, Vevey, Switzerland) and 0.1% Tween-20 (blocking buffer). The nitrocellulose sheet was then incubated overnight at 4°C with either 0.5 µg/ml monoclonal anti-human MMP-3 antibody (IgGl, clone 55-2A4, a gift from K. Iwata, Fuji Chemical Industries Ltd, Toyama, Japan), a 2000-fold dilution (final concentration, 0.75–1.5 ng/ml) of rabbit polyclonal antibodies RP2S1 or RP3S1 that were affinity-purified on the immunogenic peptide (Triple Point Biologies, Forest Grove, OR, USA), or a 5000-fold dilution of a sheep anti-human MMP-3 antiserum (gift from H. Nagase). After washes, blots were incubated with a 2000-fold dilution of anti-mouse or anti-rabbit immunoglobulins secondary antibodies conjugated to horseradish peroxidase (both from Amersham), or a 10 000-fold dilution of anti-sheep immunoglobulins rabbit antibodies conjugated to peroxidase (Dako A/S, Glastrup, Denmark). Immunoreactive proteins were visualized using the enhanced chemiluminescence system (Amersham). All dilutions and washing steps were performed in the blocking buffer. ‘Rainbow’ molecular weight markers (Amersham) were run in a lane of each gel.
Circulating hormones in Norplant® users

Figure 1. Characterization of various anti-stromelysin-1 antibodies by Western blotting. Twenty µl of medium conditioned by endometrial explants from an untreated woman, during the second day of culture without ovarian steroids (cm; lanes 1, 3, 5 and 7) and 55 ng of purified human (pro)matrix metalloproteinase (MMP)-3 standard (pur; lanes 2, 4, 6 and 8) were blotted and incubated with either a sheep anti-MMP-3 antiserum (lanes 1 and 2), a mouse anti-MMP-3 monoclonal antibody (lanes 3 and 4), or two different rabbit anti-MMP-3 polyclonal antibodies (RP2S1, lanes 5 and 6; RP3S1, lanes 7-9). Lane 9 corresponds to ‘rainbow’ molecular weight markers (MW), in which carbonic anhydrase indicates 30 kDa. This band is spuriously labelled by RP3S1. The glycosylated (gly) and non-glycosylated latent forms of stromelysin-1 (proMMP-3) migrate at Mr of 59 and 57 kDa, respectively, and the corresponding active forms at Mr of 47 and 45 kDa. The catalytic domain of MMP-3 (‘frgt.’) migrates at Mr of 30 kDa.

Specificity of the antibodies was verified on purified human (pro)MMP-3 standard and on a medium conditioned by explants from an unrelated premenstrual endometrium (Figure 1). The same bands were immunolabelled by the monoclonal antibody, the sheep antiserum and the RP2S1 rabbit polyclonal antibodies. Bands migrating at 59 and 57 kDa correspond respectively to the glycosylated (gly. proMMP-3) and non-glycosylated forms of latent MMP-3 (proMMP-3), whereas bands migrating at 47 and 45 kDa correspond to its glycosylated (gly. MMP-3) and non-glycosylated active forms (MMP-3). The truncated 30 kDa catalytic domain of MMP-3 (MMP-3 frgt.) was also immunolabelled with those antibodies (Nagase, 1998). Differences in the affinity of these antibodies for the various molecular species of MMP-3 can be readily seen in Figure 1. The monoclonal antibody also faintly immunolabelled additional high molecular weight bands in purified (pro)MMP-3 standard, presumably corresponding to multimeric forms of (pro)MMP-3 (lane 4 in Figure 1). The RP3S1 rabbit polyclonal antibodies immunolabelled only active MMP-3, not its latent forms (lanes 7 and 8 in Figure 1). However, RP3S1, but none of the other anti-MMP-3 antibodies tested (not shown), also recognized a 30 kDa protein among the molecular weight markers, presumably carbonic anhydrase (lane 9 in Figure 1). Indeed, Western blotting of purified carbonic anhydrase confirmed that RP3S1 cross-reacted with type B, but not type A, carbonic anhydrase isozyme (both from Sigma; not shown).

Immunoreactive (Western blots) and caseinolytic bands (zymograms) were quantified by densiometric analysis using the NIH Image 1.59 software (National Institutes of Health, Bethesda, MD, USA; free software available at http://rsb.info.nih.gov/nih-image/). For the accuracy of comparisons, blots or zymograms were performed simultaneously and each zymogram contained as internal standard an aliquot of purified (pro)MMP-3. Furthermore, conditioned media from paired biopsies were run on the same gel. Scanned photographs of blots and zymograms were digitized, the intensity of each band was measured by multiplying its area by its average density, and finally normalized to the corresponding value of the (pro)MMP-3 standard in the same gel.

Immunohistochemical staining
Upon arrival at the laboratory, a part of each biopsy was fixed overnight in freshly prepared 4% formaldehyde, pH 7.4, and embedded in paraffin.
In addition, four menstrual endometria and two non-menstrual endometria from spontaneously cycling women unrelated to this study were used as controls. Histological sections were stained with haematoxylin and eosin for routine analysis, and processed for MMP-3 immunolocalization.

Immunostaining was performed overnight at 4°C with either 10 μg/ml mouse anti-human MMP-3 monoclonal antibody, a 1/1500 dilution (final concentration, 1–2 ng/ml) of the rabbit anti-human MMP-3 polyclonal antibodies RP2S1 or RP3S1, or a 1/8000 dilution of the sheep anti-human MMP-3 antiserum. Bound mouse and rabbit antibodies were revealed with Envision™ system, according to the manufacturer’s instructions (Dako). Bound sheep antibodies were demonstrated by a 30 min incubation at room temperature with 2.4 μg/ml biotinylated donkey anti-sheep immunoglobulin antibodies (Jackson Immunoresearch Laboratories, West Grove, PA, USA), followed by 30 min with peroxidase-conjugated streptavidin (Dako). Immunolabelling was stained in brown following incubation with diaminobenzidine, and sections were further incubated with Perls’s solution to distinguish the Prussian blue-stained haemosiderin from the immunostaining (Gomori, 1936).

Specificity of the immunolabelling was checked on adjacent histological sections, by using an irrelevant antibody or non-immune sheep serum instead of the primary anti-MMP-3 antibodies. Control antibodies were a mouse anti-leukocyte common antigen monoclonal antibody (IgG1, Dako), a mouse anti-Epstein-Barr virus monoclonal antibody (IgG1, Dako) and rabbit anti-glial fibrillary acidic protein polyclonal antibodies (Biogenesis, Poole, UK).

In cases where MMP-3 was immunolabelled, the negative control adjacent sections were further immunolabelled with 14 μg/ml mouse anti-CD68 monoclonal antibody (IgG1, Dako) to identify macrophages.

**Statistical analysis**

The non-parametric two-tailed Wilcoxon rank-sum test was used for statistical comparisons between the whole groups of bleeding versus non-bleeding samples. Paired samples were compared by the Wilcoxon matched-pairs test. Correlation coefficients were shown to differ significantly from 0 by using a table of the significance limits of the normal distribution of the correlation coefficient (Diem and Seldrup, 1982).

**Results**

**Bleeding charts and serum concentrations of levonorgestrel and hormones**

The median age of the 23 volunteers was 30 years (25th–75th percentiles, 29–35 years; range, 22–42 years) and the median duration of Norplant treatment was 32 months (25th–75th percentiles, 16–47 months; range, 2–74 months). Fifteen volunteers (65%) provided the required bleeding chart, which covered more than 90 consecutive days in 10 cases (Figure 2). The number of bleeding episodes, and of days of bleeding (amount similar to menstrual loss) and/or spotting (lighter loss than during menstruation, as subjectively appreciated by each volunteer) during the 15 available periods of 90 consecutive days are shown in Table I. Bleeding patterns appeared highly variable and unpredictable, except for menstrual-like bleeding recurring every 20–22 days in one case (volunteer ‘c’, second chart in Figure 2). Biopsies of this volunteer showed an hypoplastic and poorly secretory endometrium, suggesting no follicular maturation, and serum progesterone concentrations were 9.3 and 3.6 nmol/l at the times of biopsies.

Serum concentrations of levonorgestrel, oestradiol, progesterone, prolactin, FSH and LH are represented in Figure 3 for all blood samples, and in Figure 4 for the 13 pairs of blood, sampled during a non-bleeding interval and at the start of a bleeding episode in the same volunteer. In general, the levonorgestrel concentrations were quite similar between bleeding episodes and non-bleeding intervals. Oestradiol and progesterone concentrations were low in most bleeding or non-bleeding samples, and oestradiol concentrations were significantly lower at the start of bleeding than during non-bleeding intervals ($P < 0.05, n = 37$, Figure 3), although the difference was not significant when analysing the paired samples ($P < 0.20, n = 2\times13$, Figure 4). No significant differences were found between the concentrations of levonorgestrel, of progesterone, of their combined concentrations, or when calculating the ratios.
Circulating hormones in Norplant® users

Figure 2. Bleeding charts. Bleeding charts covering more than 3 months were available from 10 volunteers. Volunteers 'b', 'c' and 'd' of Figure 6 are identified. The bleeding chart of volunteer 'a' was interrupted during 11 months and does not cover 90 days, but is included for the reader's convenience. Days of spotting are represented as thin vertical lines and days of bleeding as rectangles. Norplant insertion is indicated by triangles (N1, first insertion; N2, second insertion). The duration of treatment with Norplant at the start of the study is indicated on the left side of each chart (in months), and the duration of the bleeding chart is indicated on the right side (in days). Endometrial biopsies are indicated by arrows. The histological appearance was classified as hypoplastic due to progestogen treatment (T), proliferative, suggesting ovarian follicle maturation (P), or secretory, suggesting ovulation (S). In four cases, the amount of tissue was insufficient for histological analysis (I).

Table I. Bleeding patterns during 15 periods of 90 consecutive days in 10 Norplant users

<table>
<thead>
<tr>
<th>Bleeding or spotting episodes</th>
<th>Bleeding days</th>
<th>Spotting days</th>
<th>Bleeding or spotting days</th>
<th>Bleeding or spotting days per episode</th>
</tr>
</thead>
<tbody>
<tr>
<td>Median</td>
<td>3</td>
<td>9</td>
<td>10</td>
<td>21/5</td>
</tr>
<tr>
<td>25th–75th percentiles</td>
<td>3–5</td>
<td>0–18</td>
<td>2–15</td>
<td>14–28/3–8</td>
</tr>
<tr>
<td>Range</td>
<td>1–6</td>
<td>0–44</td>
<td>0–34</td>
<td>7–44/1–31</td>
</tr>
</tbody>
</table>

The 15 periods of 90 consecutive days derive from the 10 bleeding charts represented in Figure 2, some women contributing to more than one period. Notice in Figure 2 differences of bleeding patterns among such periods for the same volunteer.

between oestradiol and combined levonorgestrel and progesterone concentrations. Prolactin showed a non-significant trend towards a decreased concentration at bleeding whereas FSH concentrations showed an inverse trend (P < 0.10 for paired or non-paired comparisons). LH concentrations were highly variable between individuals and showed no consistent change with the occurrence of bleeding.

Histological appearance of the endometrium

Suitable biopsies were obtained in 30 cases (77%), with only nine biopsies providing insufficient tissue for histological analysis (referred to as 'I' in Figure 2). Twenty biopsies showed a histological pattern characteristic of progestin treatment ('T' in Figure 2), i.e. a hypoplastic endometrium with rarefied and poorly secretory glands. In seven biopsies,
the tissue was proliferative, suggesting sustained oestrogenic production by ovarian follicles (‘P’ in Figure 2). However, serum concentrations of oestradiol at sampling of these seven biopsies were not different from the 20 other samples (median 0.27 nmol/l, range 0.10–1.51 nmol/l, see Figure 3).

Three biopsies showed a well-developed, secretory endometrium, suggesting endogenous production of progesterone (‘S’ in Figure 2). Two of these biopsies were from the same woman (volunteer ‘a’ in Figures 2 and 6), who did not bleed for at least 17 days before the first biopsy was sampled (corresponding to lane 2 in Figure 6). However, spotting occurred immediately after the biopsy and was followed by menstrual-like bleeding 2 days later. Since the histological appearance of the tissue was that of a late secretory, premenstrual

Figure 3. Serum concentrations of levonorgestrel and endogenous hormones in all samples. Concentrations of levonorgestrel and of endogenous hormones were measured in sera of volunteers sampled during non-bleeding intervals (Bleeding —; n = 22) or at the start of bleeding episodes (Bleeding + ; n = 15). Whiskers-boxes representation is used, with horizontal lines as medians, boxes as 25th–75th percentiles and vertical lines as ranges. Concentrations of oestradiol were significantly lower at the start of bleeding episodes (*P < 0.05) whereas differences in prolactin and FSH concentrations were close to significance (P < 0.10).

Figure 4. Serum concentrations of levonorgestrel and endogenous hormones in paired samples. Concentrations of levonorgestrel and endogenous hormones are indicated in paired samples from 13 volunteers. Six volunteers with peculiar concentrations of a given compound are represented by defined symbols, so as to identify the corresponding concentrations of the other compounds. Volunteers ‘b’ (●), ‘c’ (□), and ‘d’ (○) of Figures 2 and 6 are identified. Volunteer ‘a’ did not provide blood at the bleeding episode and is thus not included. Only differences in prolactin and in FSH concentrations were close to significance (P < 0.10), based on paired comparisons for each panel.
endometrium, the bleeding episode was interpreted as menstruation. Serum progesterone concentration was 4.5 nmol/l. This volunteer provided a second biopsy at the start of another bleeding episode, which showed a histological pattern of progestin-treated endometrium (lane 3 in Figure 6). She agreed to a third biopsy during a non-bleeding interval 1 year later, which showed a late secretory appearance (lane 1 in Figure 6). Bleeding occurred 2 days after that biopsy, without spotting in the meantime. Progesterone concentration was 17.0 nmol/l. The bleeding chart of the other volunteer who provided a biopsy showing a secretory endometrium was not available. Serum concentration of progesterone was 9.5 nmol/l at biopsy.

Whereas no correlation could be made between the occurrence of bleeding and any one of these histological patterns (Figure 2), bleeding episodes were strongly associated with foci of menstrual-like tissue collapse and fragmentation, referred to as stromal breakdown, as well as with the expression and activation of interstitial collagenase (MMP-1), the activation of gelatinases A (MMP-2) and B (MMP-9), and with a decreased production of tissue inhibitor of metalloproteinases-1 (TIMP-1) but not of TIMP-2 (Galant et al., submitted for publication). Since stromelysin-1 (MMP-3) can degrade many extracellular matrix constituents and, moreover, activate proMMP-1 and proMMP-9, its involvement in the process of stromal breakdown was investigated in the present complementary study.

**Identification of stromelysin-1 in media conditioned by endometrial explants**

When analysed by casein zymography, media conditioned by endometrial explants contained several bands of caseinolytic activity (Figure 5). All bands migrating at $M_r$ lower than 70 kDa were inhibited by 1,10-phenanthroline but not by E64, dichloroisocoumarin or pepstatin, thus defining these bands as (pro)metalloproteinases. Latent forms of MMP are artificially activated when exposed to SDS used for electrophoresis and are therefore evidenced by zymography, as with spontaneously activated MMP. However, activity of these latent forms could be underestimated as some refolding of the prodomain masking the catalytic site could occur after removal of SDS. Several bands of caseinolytic activity contained in the conditioned media were identified by comparison with purified human (pro)MMP standards. ProMMP-3 and active MMP-3 migrate at 57 and 45 kDa, respectively, whereas proMMP-1 and MMP-1 migrate at 55 and 43 kDa. The 30 kDa proteolytic fragment of MMP-3 is also disclosed on casein zymograms. In addition, glycosylated forms of both MMP migrate at slightly higher $M_r$, which was confirmed by digestion with $N$-glycosidase of several conditioned media (not shown). Thus, glycosylated (pro)MMP-1, as well as stromelysin-2 (MMP-10) which is also produced in human endometrium (Rodgers et al., 1994), could overlap with (pro)MMP-3 (Figure 5). Therefore, (pro)MMP-3 released in conditioned media was specifically identified and quantified by Western blotting.

**Stromelysin-1 release by cultured explants from Norplant-treated endometria**

Figure 6 compares MMP-3 release by explants from paired biopsies, obtained from four women upon Norplant treatment during a non-bleeding interval and at the start of a bleeding episode, as revealed by casein zymography (panel A) and Western blotting (panel B). The relative intensity of caseinolytic and immunoreactive bands was determined by densitometry and is reported in Table II for all available explant cultures (23 samples could be analysed by casein zymography and 14 by Western blotting). For the sake of simplicity, values of glycosylated and non-glycosylated forms were combined in the measurements. In general, the two alternative methods detected the same bands of proMMP-3 and MMP-3 with a comparable relative intensity (for proMMP-3, $r = 0.83$, $n = 13$, $P < 0.001$; for MMP-3, $r = 0.95$, $n = 13$, $P < 0.001$). The release of proMMP-3 and its level of activation were both clearly increased when bleeding endometria were compared with non-bleeding ones, in total groups as well as in paired samples. The 30 kDa fragment of MMP-3 was found in media conditioned by five bleeding endometria by zymography and/or immunoblotting, but not in non-bleeding endometria. However, there was no consistency between zymograms and blots regarding this 30 kDa band...
Figure 5. Identification of caseinolytic activities by zymography. Casein zymograms of 80 ng of purified matrix metalloproteinase (MMP)-1 (lane 1), of 55 ng of purified (pro)MMP-3 (lane 2) and of 10 μl of a representative medium. This medium was conditioned during the second day of culture of explants from an endometrium of a Norplant-treated volunteer, sampled at the start of a bleeding episode (lanes 3–7). After electrophoretic migration, lanes were separated and incubated overnight, prior to staining, either in buffer alone (lanes 1–3) or in buffer supplemented with either 1 mmol/l 1,10-phenanthroline, an inhibitor of metalloproteinases, 60 μmol/l E64, an inhibitor of cysteine proteinases, 100 μmol/l dichloroisocoumarin, an inhibitor of serine proteinases, or 15 μmol/l pepstatin, an inhibitor of aspartic proteinases. All activities, except the one migrating at >70 kDa, were abrogated by incubating the gel in presence of 1,10-phenanthroline, but not in the presence of inhibitors of other classes of proteinases.

(e.g. see lane 9 of Figure 6), either because it was further degraded in the interval between the two assays or because another metalloproteinase, such as promatrilysin (proMMP-7), contributed to this caseinolytic activity in some cases. Accordingly, this band was not taken into account for the quantification of MMP-3 in zymograms.

Interestingly, the caseinolytic signal of the combined latent and active forms of MMP-3 correlated with that of combined latent and active forms of MMP-1 (densitometric data not shown) \((r = 0.71, n = 23, P < 0.001)\). In addition, the caseinolytic level of combined latent and active forms of MMP-3 inversely correlated with the serum concentration of oestradiol \((r = -0.51, n = 22, P < 0.05)\), but not with any other hormonal concentration.

**Immunohistochemical labelling of stromelysin-1**

MMP-3 has been immunolocalized in stromal cells of endometrial explants from untreated women, when cultured for 2 days in the absence of hormones, so that it is no longer repressed by ovarian steroids, and in the presence of monensin that blocks exocytosis and thereby increases the intracellular concentration of newly synthesized proteins (Kokorine et al., 1996). *In vivo*, however, MMP-3 is likely to be rapidly secreted and diluted in the extracellular space, where weak immunostaining was reported (Jeziorska et al., 1996). To validate our immunolabelling procedure on non-cultured tissue, endometria from spontaneously cycling patients were tested with the four anti-MMP-3 antibodies available to us. A granular cytoplasmic staining was detected with all these antibodies in a limited number of stromal cells in all four menstrual, but not in the two non-menstrual, endometria tested (Figure 7). The RP3S1 rabbit polyclonal antibody, which cross-reacts with carbonic anhydrase by Western blotting (see Figure 1, lane 9), was the only one to label in addition the cytoplasm of epithelial cells (Figure 7 D). We therefore questioned the validity of this epithelial staining and, accordingly, omitted these antibodies in subsequent analyses. Except for the anti-leukocyte common antigen, which, as expected, specifically immunolabelled the plasma membrane of
Circulating hormones in Norplant® users

Volunteers

Bleeding -

A

97 kDa - 45 kDa - 30 kDa -

B

97 kDa - 45 kDa - 30 kDa -

y-gly. proMM-1 proMMP-3 - proMMP-1 - gly. MMP-3 MMP-3 MMP-1

Figure 6. Comparison of casein zymograms (A) and stromelysin-1 Western blots (B) in paired endometria. Media conditioned during the first day of culture of explants from paired endometria sampled during non-bleeding intervals (Bleeding -) or at the start of bleeding episodes (Bleeding +) in four volunteers (‘a’–’d’) were analysed by casein zymography (A; 10 µl per lane) and by Western immunoblotting using the mouse anti-matrix metalloproteinase (MMP)-3 monoclonal antibody (B; 20 µl per lane). Volunteer ‘a’ (lanes 1–3) provided three biopsies, one at the start of a bleeding episode (lane 3; second biopsy at Figure 2) and two during non-bleeding intervals. These showed the histological appearance of a late secretory endometrium (lane 1; third biopsy at Figure 2) and of a premenstrual one (lane 2; first biopsy at Figure 2, see ‘Histological appearance of the endometrium’ in Results).

Table II. Densitometric measurements of stromelysin-1 caseinolytic activity and immunoblotting

<table>
<thead>
<tr>
<th></th>
<th>Non-bleeding endometria</th>
<th>Bleeding endometria</th>
<th>Difference between bleeding and non-bleeding in paired samples</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Zymograms</td>
<td>Western blots</td>
<td>Zymograms</td>
</tr>
<tr>
<td>ProMMP-3</td>
<td>16 ± 3</td>
<td>15 ± 7</td>
<td>49 ± 11***</td>
</tr>
<tr>
<td>MMP-3</td>
<td>10 ± 3</td>
<td>2 ± 1</td>
<td>40 ± 14*</td>
</tr>
<tr>
<td>30 kDa frgt</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
<td>5 ± 3†</td>
</tr>
<tr>
<td>All forms</td>
<td>26 ± 5</td>
<td>18 ± 8</td>
<td>89 ± 23**</td>
</tr>
<tr>
<td>n</td>
<td>13</td>
<td>6</td>
<td>10</td>
</tr>
</tbody>
</table>

Caseinolytic and immunolabelled bands of proMMP-3 (combined glycosylated and non-glycosylated latent forms) and MMP-3 (combined glycosylated and non-glycosylated active forms), and of its 30 kDa fragment (frgt), were quantified by densitometry. Values are the means ± SEM in arbitrary units.

*P = £ 0.05, **P = £ 0.02 and ***P = £ 0.005 by the two-tailed Wilcoxon rank-sum test or by the Wilcoxon matched-pairs test.
†The 30 kDa form was not included in total zymographic activities (see text), but was included in total Western blot intensities.

leukocytes, the other control antibodies and non-immune sheep serum produced no immunostaining (Figure 7H).

Immunolocalization of MMP-3 with the mouse monoclonal antibody and with the RP2S1 rabbit polyclonal antibodies was then carried out in all 29 endometria from Norplant-treated volunteers for which enough tissue was available. Staining was restricted to some stromal cells, and these were only found in limited foci within areas showing stromal breakdown and tissue shedding. The granular cytoplasmic immunostaining pattern was identical to that observed in menstrual endometria, and the immunostained cells were decidualized.
Figure 7. Stromelysin-1 immunolocalization in endometria sampled during spontaneous menstrual cycles and upon Norplant treatment. Stromelysin-1 was immunolocalized by the mouse monoclonal antibody (A, E and F), the sheep antiserum (B) and rabbit polyclonal antibodies RP2S1 (C and G) and RP3S1 (D). Upper panels show a menstrual endometrium from a spontaneously cycling patient unrelated to this study, used as positive control (A–D). Whereas a granular immunostaining is detected with all antibodies in the cytoplasm of some stromal cells, the cytoplasm of epithelial cells is additionally labelled only with RP3S1 antibodies (right side of D). Lower panels illustrate endometrial biopsies from three volunteers upon Norplant treatment, one sampled during a non-bleeding interval (E) and two at the start of bleeding episodes (F and G). Notice the haemosiderin deposits stained by Prussian blue, indicative of previous focal bleeding (arrows), in epithelial and stromal cells in the non-bleeding endometrium (E) and next to the cluster of MMP-3-labelled stromal cells in the bleeding endometrium (F, arrowhead). As negative control (H), irrelevant rabbit polyclonal antibodies (anti-glial fibrillary acidic protein) were used on an adjacent section of the bleeding endometrium illustrated in G. This control section, which was totally devoid of immunostaining, was further incubated with the anti-CD68 monoclonal antibody, clearly labelling macrophages in other areas of the biopsy (inset), but not in the focus where MMP-3 was immunodetected. Bar = 50 \mu m.

in one case (not shown). Using one random section per biopsy, labelling was disclosed in five out of 14 bleeding endometria (36%) with the monoclonal antibody, and in the same foci of four of these with the polyclonal antibodies (Figure 7). This proportion is similar to that found for MMP-1 mRNA in active endometriotic tissues, where the analysis of multiple sections increased the percentage of positive cases (Kokorine et al., 1997). Macrophages were easily identified by CD68 immunolabelling in adjacent sections (Figure 7H, inset), but not in foci where MMP-3 was immunolocalized (Figure 7H). No MMP-3 immunostaining was detected in the 15 endometrial tissues sampled during non-bleeding intervals. The difference in MMP-3 immunolabelling between bleeding and non-bleeding endometria was significant by the 4-fold table test ($P = 0.05$).

Perls’s reaction was finally used to distinguish the brown immunostaining from haemosiderin (Figure 7E and F). Haemosiderin deposits were identified in stroma as well as in epithelium of most bleeding and non-bleeding endometria.

Discussion

Previous studies strongly supported a key role of matrix metalloproteinases (MMP) in menstrual tissue breakdown. In the normal untreated endometrium, interstitial collagenase (MMP-1), stromelysin-1 (MMP-3) and gelatinase B (MMP-9) are expressed only at menstruation, whereas gelatinase A (MMP-2) expression is increased at that time (Rodgers et al., 1994; Hampton and Salamonsen, 1994; Marbaix et al., 1995, 1996a; Kokorine et al., 1996; Irwin et al., 1996). In addition, the menstrual breakdown of endometrial tissue can be reproduced by culturing explants from non-menstrual endometrium in the absence of oestradiol and progesterone,
and is fully prevented by specific inhibitors of MMP but not by those of other classes of proteinases (Marbaix et al., 1996b). Focal expression of MMP-1 has also been found in active (i.e. bleeding), but not in inactive, endometriotic lesions (Kokorine et al., 1997).

This prospective study, in which volunteers using Norplant provided endometrial biopsies at the start of bleeding episodes and during non-bleeding intervals, demonstrates that the occurrence of endometrial bleeding is closely associated with the expression and activation of MMP-3 in foci of tissue breakdown. We report elsewhere on the expression of proMMP-1 and increased production of proMMP-2, their activation as well as that of proMMP-9, and the decreased production of TIMP-1 in the same foci (Galant et al., submitted for publication). Thus, regulation of the expression, activation, and inhibition of several menstrual-specific MMP is altered, resulting in increased activity at the start of bleeding upon progestogen-only contraception. In addition, another study reported an influx of MMP-9-containing inflammatory cells in Norplant-treated endometria showing breakdown areas (Vincent et al., 1999). Altogether, these observations strongly suggest a key role for several MMP in the initiation of abnormal endometrial bleeding upon Norplant.

As reported in another study (Rogers, 1996), the bleeding patterns were highly variable and unrelated to the duration of treatment. The sampling procedure provided suitable tissue in a high percentage of cases, compared to other biopsy techniques (Hadisaputra et al., 1996). Identification of haemosiderin deposits in most bleeding or non-bleeding endometria demonstrated that these areas were not shed when previous endometrial bleeding occurred, thus confirming the focal nature of endometrial breakdown and shedding during bleeding episodes.

Although variable between individuals, the production of MMP-3 during the first day of culture of explants from bleeding endometria was consistently more abundant than in non-bleeding endometria, when analysed by casein zymography and immunoblotting both in total groups and in paired samples. Correlation between the two assays was excellent. The slight differences observed could be explained by a possible underestimation of the caseinolytic activity of latent forms by zymography, further degradation of the 30 kDa zymographic fragment between the two assays, and/or unequal affinities of the antibodies to the various species of MMP-3. Indeed, the four antibodies used in the present study showed variations in the immunoblotting signals for the glycosylated and unglycosylated, latent and active forms of MMP-3 as well as the 30 kDa catalytic domain.

MMP-3 was disclosed by immunohistochemistry in foci of stromal breakdown in 36% of the bleeding but in none of the non-bleeding endometria. In view of the focal signal, the proportion of labelled bleeding endometria could be expected to increase by the analysis of multiple sections from each biopsy, as was found for MMP-1 mRNA in bleeding endometriotic tissue (Kokorine et al., 1997). In addition, it is likely that the immunohistochemical analysis underestimates the number of cells producing MMP-3 since this proteinase, being directly secreted once synthesized, should be detected only in highly expressing cells. MMP-3 was produced by stromal cells, as in menstrual endometrium or upon explants culture of untreated non-menstrual endometrium without ovarian steroids (Rodgers et al., 1994; Jeziorska et al., 1996; Kokorine et al., 1996). In contrast to a previous study (Jeziorska et al., 1996), MMP-3 could not be disclosed in the extracellular space, possibly due to different fixation procedures.

To exert its effects, proMMP-3 must be activated. In media conditioned by explants from bleeding endometria, released MMP-3 was indeed found to be largely activated, but this was rarely the case in media conditioned by explants from non-bleeding endometria. A large number of proteinases, such as plasmin, kallikrein, leukocyte elastase and mast cell chymase are able to activate proMMP-3 (Lees et al., 1994; Nagase, 1998). Their involvement should be investigated by further studies. Once activated, MMP-3 is able to digest various extracellular matrix proteins, including type IV collagen, laminin and fibronectin of the basement membranes (Nagase, 1998), and is thus likely to play a major role in the focal tissue and vascular breakdown that occurs at menstruation and at bleeding upon Norplant treatment. Moreover,
MMP-3 can activate other proMMP, including proMMP-1 and proMMP-9, which are indeed activated in endometrial bleeding in patients using Norplant (Galant et al., submitted for publication). In all nine conditioned media that were found to contain active MMP-1 and/or MMP-9, active MMP-3 was identified by casein zymography and/or immunoblotting. These observations are in agreement with MMP-3 being an in-vivo activator of endometrial proMMP-1 and proMMP-9.

The control of MMP-3 expression remains unclear. Potential mechanisms include levels of progesterone and oestradiol as well as their receptors, paracrine control by local cytokines, and even mechanical factors such as tissue fragilization. In cultured endometrial explants and purified stromal cells, progesterone and levonorgestrel largely inhibit, but do not suppress, MMP-3 expression; oestradiol on its own has no direct effect but potentiates the inhibition of MMP-3 expression by progesterone (Osteen et al., 1994; Schatz et al., 1994; Hampton et al., 1999), probably by promoting progesterone receptor expression (Nardulli et al., 1988). Conceivably, transient decreases in the serum concentrations of levonorgestrel and/or progesterone during the course of Norplant treatment could thus lead to inappropriate expression of MMP-3. However, there was no change of levonorgestrel and progesterone concentrations upon bleeding and no correlation was found between the total release of MMP-3 and the serum concentration of levonorgestrel or progesterone. In contrast, the average concentration of oestradiol was lower at the start of bleeding episodes than during non-bleeding intervals, and the total release of MMP-3 inversely correlated with serum concentration of oestradiol.

Although generally considered to be responsible for prolonged amenorrhoea due to endometrial atrophy in Norplant-treated women (Fraser et al., 1996), very low serum concentrations of oestradiol were associated with bleeding episodes (Faundes et al., 1998), a finding confirmed in the present study when analysing all samples together. However, this association was not confirmed with paired samples. Unless trivially reflecting different power of statistical analysis because of inequal population size, these discrepancies may be due to differences in the timing of blood sampling, and could be explained if rises and falls in oestradiol concentration triggered the endometrial bleeding. In Norplant-treated endometria, oestrogen receptor-α immunostaining is low, whereas progesterone receptor immunostaining is increased compared to the menstrual cycle (Critchley et al., 1993). However, immunostaining of both oestrogen and progesterone receptors does not correlate with oestradiol serum concentrations (Critchley et al., 1993). Furthermore, endometrial thickness, as measured by ultrasounds, correlates poorly with oestradiol concentrations (Faundes et al., 1998) and no correlation was found between endometrial proliferation and oestradiol concentrations in our study. These data suggest altered oestrogenic effects on Norplant-treated endometria.

In the present study, oestrogen and progesterone receptor immunolabelling was lowered in endometria sampled at the time of bleeding, and they were generally not detected in areas of stromal breakdown (Galant et al., submitted for publication). In agreement, in-situ hybridization has shown a decreased progesterone receptor mRNA amount in Norplant-treated endometria, in particular from women who had recent bleeding episodes (Lau et al., 1996). This lowered content of ovarian steroid receptors could be responsible for a weakened inhibition of MMP-3 expression by levonorgestrel and progesterone. However, because of its unclear relationship with oestradiol and progesterone serum concentrations, the deregulated expression of MMP-3 may be due to other factors, such as paracrine interactions.

MMP-3 expression is induced by interleukin (IL)-1α and tumour necrosis factor (TNF)-α in cultured endometrial stromal cells (Rawdanowicz et al., 1994) and was reported to be inhibited by transforming growth factor (TGF)-β in endometrial explants and in cocultures of purified epithelial and stromal cells (Osteen et al., 1995). Release of IL-1α, assayed in our material, strongly correlated with the release of collagenase activity (r = 0.92; Galant et al., submitted for publication), confirming its potential role as inducer of the expression of endometrial MMP-1 (Singer et al., 1997, 1999), and with that of MMP-3 as measured by densitometry of immunoblots (r = 0.72, n = 10, P < 0.05).
Due to the limited amount of conditioned medium per culture dish, TNF-α and TGF-β could not be measured in our material, and further investigations are clearly needed to unravel the control mechanisms of MMP-3 expression in Norplant-treated endometria.

In conclusion, this prospective study demonstrates that, in addition to several other MMP, MMP-3 is focally expressed and activated in areas of endometrial tissue breakdown at the start of bleeding episodes upon Norplant treatment. Together with a previous study showing that MMP inhibitors fully prevent endometrial stromal breakdown in menstrual-like cultured explants (Marbaix et al., 1996b), these results strongly suggest a pivotal role for MMP in the triggering of abnormal endometrial bleeding upon Norplant. Whereas selective or topical synthetic MMP inhibitors could therefore be considered as a therapeutic prospect against abnormal endometrial bleeding, the molecular mechanisms leading to the deregulated expression of proMMP-3 and other proMMP, as well as to their activation, need to be further investigated.

Acknowledgements

We thank the 23 volunteers who took part in the study, C.d’Arcanguès (WHO) for encouraging the investigation, H. Nagase and K. Iwata for donated reagents, Leiras Oy company for donated Norplant®, M. Berlière, P. Cornet and C. Singer for helpful discussions, and N. Delflasse, S. Rutens, P. Vanden Bergh and L. Wenderickx for practical assistance. We gratefully acknowledge financial support by WHO (project 96391), the Belgian Fonds de la Recherche Scientifique Médicale, Interuniversity Fonds de la Recherche Scientifique of the Université catholique de Louvain. A travel support from N.V. Organon, The Netherlands, was also appreciated.

References


E. Marbaix et al.


