Trimegestone differentially modulates the expression of matrix metalloproteinases in the endometrial stromal cell

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Matrix metalloproteinases (MMP) are considered to be of critical importance in the initiation of menstruation where MMP protein levels are reciprocally modulated by the actions of the gonadal steroid hormones, estradiol (E₂) and progesterone (P₄), with P₄ being considered the principal suppressor of endometrial MMP expression. Trimegestone (T) is a novel progestagen that tightly controls menstruation timing and duration through mechanisms that might involve MMP suppression. Endometrial stromal cells treated with 10⁻⁶ M E₂, P₄ or T in the presence and absence of 10⁻⁶ M RU486 showed that both T and P₄ suppressed the expression of MMP-1 and MMP-3 transcripts and secreted protein, whereas MMP-9 was not produced in culture. The suppressive effect of T or P₄ on MMP-1 and MMP-3 transcript levels was enhanced in the presence of E₂ and attenuated in the presence of RU486, although MMP-1 proteins were unaffected by the presence of RU486, which alone acted as a partial progesterone agonist in these cultures. Immunohistochemistry with MMP-1, MMP-3 and MMP-9-specific antibodies performed on endometrial biopsies obtained from non-treated, LH-dated, normally cycling women and endometrial biopsies obtained from postmenopausal women treated with 10⁻⁶ M E₂, P₄ or T in the presence and absence of 10⁻⁶ M RU486 showed that both T and P₄ suppressed the expression of MMP-1 and MMP-3 transcripts and secreted protein, whereas MMP-9 was not produced in culture. These data suggest that T acts in a similar manner to P₄, but causes subtle differences in expression patterns of MMPs that may explain the different clinical effect that this progestagen has on endometrial behaviour compared to P₄.

Key words: endometrium/HRT/metalloproteinases/progesterone/trimegestone

Introduction

During the menstrual cycle, the gonadal steroid hormones estradiol and progesterone control the ordered growth and differentiation of uterine cells. This remodelling process is critical for implantation of the developing embryo, the formation of the placenta and the maintenance of pregnancy (Pilka and Hrachovec, 2003). To achieve this remodelling process the extracellular matrix (ECM) needs to be modulated to allow cell movement and proliferation (Tabibzadeh, 1996). Indeed, the ECM components themselves are known to not only play important roles in the maintenance of tissue morphology, but also influence cell proliferation, differentiation and local apoptosis (Wang and Passaniti, 1999; Sivridis and Giatromanolaki, 2004).

Limited degradation of ECM is important to these remodelling processes and a major controlling factor is the expression of different endometrial metalloproteinases (MMPs). In the absence of blastocyst implantation, degradation of the ECM is considered to be of critical importance in the initiation of tissue breakdown that leads to menstruation (Marbaix et al., 1996; Salamonsen and Woolley, 1996).

During the proliferative phase of the natural cycle, MMP-1, MMP-3 and MMP-9 are thought to be expressed at low levels in the stroma (Hulboy et al., 1997), presumably to allow limited cell movement within the ECM. The expression of MMPs then decline in the early secretory phase, when the most stable endometrial structure is required, and then increase during the late secretory phase in anticipation of the next proliferative phase. These data are the reciprocal of ovarian follicular and luteal phase serum progesterone levels, which has led to the suggestion that endometrial expression of MMPs is under gonadal steroid hormone control.

Critically, MMP-9 expression is highest in the menstrual phase of the endometrium when tissue breakdown occurs and this is thought to be due to the fact that MMP-9 is expressed mainly in polymorphs, monocyte-macrophages and eosinophils (Hulboy et al., 1997) that infiltrate the endometrium at this critical phase of the menstrual cycle. What role/ effect MMP-1 and MMP-3 might have on MMP-9 expression in the endometrium is unknown although they are clearly implicated in the implementation of the menstrual phase of the cycle.

Progesterone is considered to be the principal suppressor of MMP expression in the endometrium, but the mechanism involved is not clearly understood, especially as the tissue expression of MMPs in the normal menstrual cycle declines before the serum progesterone levels reach physiological significance. Many theories have been proposed for the suppressive effects of progesterone including the activation of PAI-1 (Casslen et al., 1992), increased expression of TGF-β (Bruner et al., 1995) or the stimulation of TIMP-1 expression (Marbaix et al., 1992) all of which are modulated during the menstrual cycle. However, progesterone may itself have a direct suppressive effect that does not involve these intermediary molecules. Thus, if the onset of menstrual bleeding is a function of differential MMP expression, then this...
hypothesis could be tested using a model where menstrual bleeding patterns differ. Postmenopausal sequential combined HRT with a progestagen that has a different pharmacological profile to that of progesterone would provide such a model.

Trimegestone (RU27987) is a novel progestin that has a 6-fold high-affinity for human PR than P4 (Robin-Jagerschmidt et al. 2000; Winneker et al., 2003) and has a relatively long biological half-life of approximately 17 h (range 7–37 h; Bouchoux et al., 1995). This compound when administered in a sequential, combined manner with estrogen to post-menopausal women results in predictable menstruation and reduced menstrual periods, and as such is as good if not better than other similar regimens (Al-Azzawi et al., 1999). To understand why this might be so, we have undertaken a detailed examination of the expression patterns of MMP in endometrial samples from women treated with either 0.05 mg or 0.5 mg sequentially administered trimegestone, who demonstrated different bleeding patterns and compared that MMP expression pattern to the pattern of expression obtained from the normal menstrual cycle (Al-Azzawi et al., 1999).

We have also examined the MMP expression patterns of trimegestone- and progesterone-stimulated stromal cell cultures that mimic this sequential combined treatment regimen and show differences in MMP-1 and MMP-3 expression profiles.

Materials and methods

Materials

The gonadal steroids 17β-estradiol (E2) and progesterone (P4) were from Sigma (Poole, Dorset, UK). Trimegestone (RU27987) and mifepristone (RU486) were kind gifts from Hoechst Marion Roussel (Romianville, France). The First Response® Urinary LH kits were from Carter Wallace Limited (Folkestone, UK).

Stromal cell culture

Stromal cells were isolated from late proliferative phase endometrium obtained from hysterectomy specimens as described (Viganò et al., 1993; Hatayama et al., 1994). Briefly, finely minced endometrial tissue was incubated in 20 ml of 0.25% bacterial collagenase type I (Gibco) in Hank’s buffered salt solution (Gibco), for 2 h at 37°C. Non-digested material was collected at unit gravity for 5 min, the supernatant removed to a fresh tube and cells collected at 350 x g for 5 min. The cell pellet was then washed six times in growth medium; phenol red-free DMEM/F-12 medium (Gibco) supplemented with 10% charcoal-dextran treated fetal calf serum (HyClone, Logan, Utah, USA), antibiotics/antimycotic solution (Sigma; Penicillin 10,000 units/ml, streptomycin 10 mg/ml, Amphotericin B 25μg/ml and 1.25mg/ml sodium bicarbonate (Sigma). The cellular pellet was resuspended in growth medium and seeded into a 25 mm² flask (NUNC, Fisher Scientific, Loughborough, UK) and incubated at 37°C in 5% CO2 in humidified air until the culture reached confluence. The cells were then removed with trypsin-EDTA solution (Gibco), washed twice with growth medium and plated into six-well plates at a density of 0.8–1.0 x 10⁵ cells/ml. Cells were also seeded on polytetrafluoroethylene-coated multiwell glass slides (C.A.Hendley Ltd., Loughton, UK) for vimentin immunocytochemical staining. After 24 h, non-adherent cells were removed by gentle washing with pre-warmed growth medium and the adherent cells used in steroid-treatment experiments.

Immunocytochemistry for vimentin

To confirm that each culture was of stromal origin, cultures grown on glass coverslips were stained with mouse monoclonal anti-vimentin antibody (1 : 100; Novacстра, Vector Laboratories, Peterborough, UK) as previously described (Viganò et al., 1993; Laird et al., 1996; Shiokawa et al., 1996). In each culture more than 95% of the stromal cells were positive for vimentin (Figure 1).

Effect of estradiol on progesterone receptor expression

To confirm the steroid responsiveness of our stromal cell cultures, 0.8–1.0 x 10⁵ cells were plated to six-well plates in triplicate and cultured for 4 days in the presence of 0.1% ethanol until the cultures reached confluence. The culture medium was then changed for one containing either 10⁻⁶ M E₂ or vehicle for 4 days in the presence or absence of 10μg/ml cycloheximide (Fisher Scientific).

The medium was changed every 48 h. Cellular mRNA was then extracted using Trizol (Invitrogen, Paisley, UK) according to the manufacturer’s instructions and treated with DNase I and phenol–chloroform extraction prior to reverse transcription. Total cellular protein was produced using procedures described previously (Taylor et al., 2002) and pooled. After concentration of proteins with 30,000 Dalton cut off concentrators (Pierce, Perbio Science, Crumlington, UK), protein concentration was determined by Bradford assay (Bradford, 1976; Bio-Rad Laboratories, Hemel Hempstead, UK). Proteins (100 μg) were resolved on 7.5% SDS-PAGE and analysed for the presence of progesterone receptor isoforms as described (Taylor et al., 2006).

Steroid treatment of stromal cell cultures

Two separate treatment protocols were used to mimic the normal menstrual cycle and trimegestone HRT treatment regimens (Figure 2). In one set of experiments, stromal cells cultured in triplicate wells were exposed to 10⁻⁶ M E₂ dissolved in growth medium or an equivalent amount of solvent (0.1% ethanol) for 4 days, with the medium being changed on day 2. Next, medium containing either 10⁻⁶ M progesterone alone, 10⁻⁸ M RU486 alone or a combination of these steroids in the presence of E₂ was added to the cultures for an additional 4 or 6 days with the medium being changed on days 6 and 8 (Figure 2). In another set of experiments, 10⁻⁶ M trimegestone was used instead of progesterone. At the end of the culture period, the medium was removed, centrifuged at 10,000 g for 30 s to remove cells and debris, and the supernatants stored at −80°C for MMP measurement by specific ELISA. The cells that remained attached to the plasticware were then processed for cellular mRNA.

Extraction of mRNA for MMP analysis

Cellular mRNA was extracted and processed using oligo-dT-linked Dynabeads® (Dynal, Bromborough, UK), as described previously (Bicknell et al., 1996). Briefly, cells in each well were lysed in 500μl lysis/binding buffer (100 mM Tris-HCl (pH 8), 0.5 M LiCl, 10 mM EDTA, 5 mM dithiothreitol, 1% lithium dodecylsulphate (LiDS)) and incubated with 50 μg/ml proteinase K (Roche Diagnostics Ltd., Lewes, UK) for 1 h at 37°C. DNA was sheared by passing the preparation through a 21G and then a 25G needle 5 times. The lysate was centrifuged for 30 s at 10,000 g and the supernatant mixed with oligo-dT-linked Dynabeads® (Dynal). The mRNA was allowed to anneal to the Dynabeads® for 10 min at room temperature. After collecting the beads on a magnet, mRNA-linked Dynabeads® were washed twice in a buffer that consisted of 10 mM Tris-HCl (pH 8), 150 mM LiCl, 1 mM EDTA, 0.1% LiDS (Dynal) and then washed twice in the same buffer but without LiDS. The mRNA-linked Dynabeads® were finally resuspended in 30 μl of DEPC-treated de-ionized water and used immediately in the reverse transcriptase reaction.

Reverse transcriptase reaction (RT)

The mRNA mixture was reverse transcribed at 42°C for 1 h as a 25μl mixture containing 10μl of mRNA-Dynabead® preparation or 1μg of total cellular RNA, 1μl DMSO, 2.5μl of 5 x AMV-RT buffer (Promega, Southampton, UK), 0.5μl 10 mM dNTPs (Roche), 0.25μl RNase inhibitor (25U, Promega), 10.25μl DEPC-treated

Figure 1. Immunohistochemical staining of primary stromal cell cultures with anti-vimentin antibodies. Bar =100μm.
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de-ionized water and 0.5 μl AMV-RT (SU, in the +RT reaction, Promega). The –RT control for each sample was achieved by omitting the AMV-RT enzyme.

**PCR**

PCR was performed using a hot-start procedure for GAPDH and MMP-1, MMP-3, MMP-9 and PR using mRNA-specific primers (Table I). Each PCR (50 μl) contained 1 μl of the +RT or –RT mixture, 42 μl DEPC-treated de-ionized water, 10 pmol of the forward and reverse primers and 5 μl of 10x AJ Buffer (450 mM Tris-HCl (pH 8.8), 110 mM NH₄SO₄, 45 mM MgCl₂, 2 mM dNTPs, 1.1 mg/ml acetylated BSA (Roche), 110 mM β-mercaptoethanol, 4.4 μM EDTA). After an initial denaturation step at 94°C for 2 min, the samples were heated at 95°C for 30 s, and then held at the annealing temperature for the specific mRNA target; GAPDH (60°C), MMP-1 (62°C), MMP-3 (56°C), MMP-9 (62°C), PR (55°C) for 10 min. At this point, one unit of Taq polymerase (Promega) was added and the PCR cycles initiated. The PCR conditions were

![Figure 2](https://academic.oup.com/molehr/article-abstract/12/3/157/999472/1204157788972)

**Table I.** Oligonucleotide primer sequences and expected amplicon sizes

<table>
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<th>mRNA species</th>
<th>Oligonucleotide sequence</th>
<th>Product size (bp)</th>
<th>Annealing temperature (°C)</th>
<th>Accession number</th>
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<tr>
<td>GAPDH 5’</td>
<td>AGAACATCATCCCTGCCTC689</td>
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<td>60</td>
<td>X01677&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td>3’</td>
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<td>MMP-1 5’</td>
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<td>402</td>
<td>62</td>
<td>NM-002421.1</td>
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<td></td>
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<tr>
<td>MMP-3 5’</td>
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<td>287</td>
<td>56</td>
<td>J03209</td>
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<td>65</td>
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<tr>
<td>PR 5’</td>
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<td>284</td>
<td>55</td>
<td>M15176&lt;sup&gt;b&lt;/sup&gt;</td>
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30 s at 94°C, 30 s at the annealing temperature, 45 s at 70°C, followed by an extended extension period of 10 min at 70°C. The number of cycles were 28, 32, 23, 35 and 35 for GAPDH, MMP-1, MMP-3, MMP-9 and PR, respectively which were all within the exponential phase of amplification (data not shown).

The PCR products were separated on 3% agarose gels, stained with ethidium bromide (2μg/ml) and the images captured under UV transillumination with a digital camera (UVG, Cambridge, UK) using an UV image store 5000 system (UVG, Cambridge, UK). Densitometry measurements of the amplicon densities were performed using the Scion Image analysis software (version beta 4.0.2, Scion Associates, Frederick, Maryland, USA) using KODAK normal density step tablets to calibrate the densitometry images.

**MMP-1 and MMP-3 ELISA**

MMP-1 and MMP-3 ELISA were performed using specific ELISA kits (total MMP Biotrak® Assay, Amersham Biosciences, Chalfont St Giles, UK) according to the manufacturer’s instructions. The samples were analysed in duplicate with the MMP-3 samples needing to be diluted 1:250 with sample diluent to fit on the standard curve. The data were corrected for cell number and are presented as amount of MMP in ng/ml.

**Patient groups**

**Trimegestone-treated group**

The Leicestershire Local Ethics Committee approved the clinical trial and research protocol for this part of this study and all patients signed informed consent. The details of these patients are described in detail elsewhere (Al-Azzawi et al., 1999; Wahab et al., 1999a,b). Briefly, endometrial biopsies were obtained using vabra curette on day 24 of the last treatment cycle. Of the 176 women recruited in Leicester, those who received the highest (0.5 mg, n = 19) and the lowest (0.05 mg, n = 21) doses of trimegestone were included in the present study due to their marked contrast in bleeding patterns (Al-Azzawi et al., 1999).

**Normal group**

The normal endometrial group consisted of 35 healthy, regularly menstruating women aged 27–50 (38 ± 6.1, mean ± SD), undergoing laparoscopic sterilization using sharp curette or hysterectomy for cervical intraepithelial neoplasia, premenstrual tension syndrome or benign ovarian cysts. None of these women had received any hormonal treatments for 2 months prior to specimen procurement. All women were given urinary LH surge detection kit tests (First Response, Carter Wallace limited, Folkstone, UK), which were used during the month preceding the endometrial biopsy or hysterectomy. The normal control samples were deep endometrial biopsies (which included functionalis and basalis layers), obtained by opening the uterus by coronal section and by the slicing of the posterior wall of the uterine cavity vertically from fundus to isthmus, each slice being 1 cm wide. These were coded alphabetically A–E from centre to edge. Our study materials were from slice B.

The specimens were fixed in 10% formal-saline, embedded in paraffin wax, and 5 μm sections stained with haematoxylin and eosin for histological assessment of the phase of the menstrual cycle. These biopsies were then dated both by LH surge data and the date of the last menstrual period, were examined by two independent pathologists who were blinded to the LH surge and menstrual dates and where all agreed, the specimen was included as a control sample. The histological diagnoses were proliferative (n = 8), early secretory (n = 8), late secretory (n = 11) and menstrual (n = 8).

**MMP immunohistochemistry**

Sections measuring 5 μm in thickness were dried to silane-coated slides for 48–72 h before use. After dewaxing in xylene and re-hydration through graded doses of alcohol to water, antigens were retrieved using microwaving at 750W power in 10mM citrate buffer at pH 6.0, for 30 min. Endogenous peroxidase activity was then blocked by incubation with 6% hydrogen peroxide for 10 min. Normal goat serum (NGS; DAKO, Glostrup, Denmark) diluted in 10% NGS for 18 h at 4°C, followed by an incubation with the primary mouse monoclonal antibody (MMP-1: 2μg/ml, MMP-3 or MMP-9: 3μg/ml; Chimicon International Ltd, Harrow, UK) diluted in 10% NGS for 18 h at 4°C. After washing with PBS for 20 min, sections were then incubated with biotinylated goat anti-mouse immunoglobulins (1:400, DAKO), washed with PBS for 20 min, then with Vectastain ABC-linked horseradish peroxidase reagent (ABC Elite: Vector Laboratories, Peterborough, UK) for 30 min, washed again with PBS for 20 min, incubated with 3,3′-diaminobenzidine (DAB substrate; Vector Laboratories, Peterborough, UK) for 5 min, lightly counterstained with Mayer’s haematoxylin (Sigma) and permanently mounted in XAM mountant (BDH, Poole, UK). Controls included the use of mouse IgG (Sigma) in the place of the primary antibody or the omission of the primary antibody. The positive controls (placenta and breast carcinoma) were stained with MMP-1, MMP-3 and MMP-9, and there was no background reaction, while the negative control was devoid of any immunoreactivity (data not shown).

**Assessment of MMP immunoreactivity**

An observer blinded to the clinical data performed the qualitative and quantitative histomorphometric analyses of the positively stained cells. Histomorphometric analysis was performed on the stromal compartment by counting the number of positively stained stromal cells per field from 10 randomly selected fields (Hamilton, 1995) per slide captured (x1000) under oil immersion from the functionalis layer. Images were captured using an Axiosplan microscope (Carl Zeiss, Welwyn Garden City, UK) and a colour video camera (Sony CCD/RGB). The cells counted were evaluated using the KS300 image analysis computer program (Kontron Imaging Systems, Thame, UK). The data presented are number of positive cells per unit area and the corresponding areas of the images captured at 1000 magnification were 0.0046 mm² using a measurement graticule.

**Statistical analysis**

PR transcript and protein levels were compared using one-way ANOVA with Tukey’s HSD test. MMP transcript levels from primary stromal cell cultures were analysed using a mixed model of the log of the ratios of MMP-GAPDH densitometric values that incorporates both subjects and treatments. The Wald test was used to compare treatment effect. Non-parametric Kruskal–Wallis one-way analysis of variance with Dunn’s post-test was used to compare treatment effect in the MMP protein ELISA measurements. The immunohistochemical data of endometrial sections did not fulfil the assumptions necessary for using analysis of variance and t-test; therefore, the non-parametric Kruskal–Wallis and Mann–Whitney tests were used.

**Results**

**The effect of P4 and T on MMP transcript levels**

Stromal cells in culture expressed transcripts for MMP-1 and MMP-3 mRNA (Figure 3A) but not for MMP-9 (Figure 3B). Using experimental protocol number 1, where stromal cells were stimulated with E2 for a total of 8 days, E2 suppressed MMP-1 and MMP-3 mRNA...
levels compared to the vehicle control, but only MMP-1 transcript levels were significantly suppressed by E2 treatment (Figure 4). P4 alone or in combination with E2 significantly suppressed MMP-1 and MMP-3 transcript levels ($P<0.01$ and $P=0.001$, respectively). The antiprogestin, RU486, suppressed MMP-1 and MMP-3 expression by 1.69- and 1.48-fold, respectively, but these data did not reach statistical significance. The combination of E2 and P4 caused a significant 2.88-fold decrease in MMP-1 mRNA levels ($P<0.01$) and an 8.13-fold decrease in MMP-3 mRNA levels ($P<0.01$). When RU486 was combined with E2 and P4, RU486 completely ablated the E2/P4-regulated suppression of both MMP-1 and MMP-3 transcripts (Figure 4).

In this experiment, trimegestone alone had a significant suppressive effect on MMP-1 ($P<0.01$) and MMP-3 ($P<0.05$) transcript levels but was not as effective as P4 alone (Figure 4). The combination of E2 and T further suppressed MMP-1 and MMP-3 mRNA levels from 1.51-fold to 4.46-fold for MMP-1 and 2.24-fold to 3.55-fold for MMP-3, and the suppression of MMP-1 was significantly more pronounced where in the shorter protocol they were not (Figure 4). The extra 2 days of stimulation also enhanced the suppressive effects of T in the presence of E2, although these data did not reach significance ($P=0.39$).

**The effect of P4 and T on secreted MMP protein levels**

Stromal cell cultures produced and released between 100- and 167-times more MMP-3 than MMP-1 (Figure 6). Progesterone, RU486 and trimegestone reduced the secreted levels of MMP-1 in line with mRNA levels (Figures 4 and 5), but these protein data did not reach statistical significance. However, MMP-1 protein levels were significantly suppressed by the E2/T combination ($P<0.05$) and the addition of RU486 to the cultures did not attenuate these effects with the levels of MMP-1 remaining suppressed (Figure 6).

Similar observations were made with the measurement of secreted MMP-3 protein levels in that E2, P4, RU486 and T all suppressed MMP-3 protein levels but these data did not reach significance. However, the combination of E2 and P4 significantly reduced MMP-3 protein levels and that level was not reversed by the addition of RU486. Similarly, the addition of E2 to T-stimulated cultures caused a significant ($P<0.05$) decrease in MMP-3 levels, but in this case the addition of RU486 attenuated T actions and the levels of MMP-3 returned to control values.
and significantly \((\text{Figure 7C and D})\). The addition of the protein synthesis inhibitor cycloheximide had no effect on either basal or E2-stimulated PR transcript levels (Figure 7A and B) but had a minor non-significant effect on GAPDH transcript levels (Figure 7A). MMP immunoreactivity was present in all samples assessed. The immunoreactive staining pattern for MMP-1 in endometrial stromal cells was scattered throughout the functionalis and in common with MMP-1 staining appeared to be perinuclear (Figure 7B). The cell counts for MMP-3+ stromal cells in different phases of the normal menstrual cycle and in the trimegestone treated groups were found to be approximately 10-fold lower than either MMP-1+ or MMP-9+ cell numbers (Figure 9). MMP-3 immunoreactivity was not modulated by the phase of cycle despite a 20% increase in mean MMP-3+ cell count from the late secretory to menstrual phases of the cycle (Figure 9B). The immunoreactive profile of stromal MMP-3 immunoreactivity for the 0.05 mg and 0.5 mg trimegestone HRT groups did not differ from each other or with the normal menstrual cycle samples, despite there being an apparent T-dependent dose-effect. MMP-3 immunoreactivity was absent from glandular epithelial cells (Figure 8B).

**Comparison of MMP-9 staining**

There was a statistically significant difference in MMP-9 expression between the phases of the menstrual cycle \((P=0.03, \text{Figure 9C})\), with the highest expression of MMP-9 in the late secretory phase compared to the proliferative or menstrual phases (Figure 9C; \(P=0.02, P=0.03\), respectively). By contrast, trimegestone-treated endometria had significantly lower levels of MMP-9+ cells compared to early secretory and late secretory phase endometria, presumably indicating the absence of large numbers of infiltrating plasma cells (Figure 8C). In common with MMP-1 staining, MMP-9 immunoreactivity in the glandular epithelial cells was 100% positive throughout the menstrual cycle and the T-treated endometria (data not shown). Again staining was observed throughout the cytoplasm and not confined to the perinuclear space as it is in the stromal cell (arrow in Figure 8C).

**Discussion**

In a previous study (Al-Azzawi et al., 1999) we reported that patients treated with 0.5mg trimegestone/day over a 14 day period, as part of a sequential combined HRT, displayed better control of the day of onset of withdrawal bleeding and a much lower incidence of breakthrough bleeding than women on low dose trimegestone or normally menstruating women. Additionally, the pattern of endometrial histomorphometric features exhibited by the trimegestone-treated groups differed to those obtained from normally menstruating women (Wahab et al., 1999b). These data suggested that trimegestone differed from proges-terone in its actions in the regulation of menstruation.

The mechanisms governing endometrial tissue shedding are not well understood, but it is becoming clear that MMPs play a key role in the cyclical breakdown of ECM that ultimately leads to menstruation. Because MMPs are able to digest matrix proteins under physiological conditions, activation and inhibition of MMP expression must be tightly controlled to prevent inappropriate or untimely tissue destruction. In this regard the ovarian steroid hormones appear to play a key role with focal steroid receptor expression being paramount (Kokrine et al., 1996).

In this study, we found that MMP-1 and MMP-3 mRNA and protein were differentially regulated by P4 and T through an E2-dependent pathway. MMP-1 and MMP-3 transcript levels were suppressed in stromal cells treated with P4 or T, and this suppression was more
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profound when either steroid was combined with E2. The mechanism involved increased expression of stromal cell progesterone receptor(s) by E2 (Figure 7) as previously reported (Eckert and Katzenellenbogen, 1981; Marbaix et al., 1995), but not de novo synthesis of factors involved in PR mRNA synthesis as evidenced by a lack of effect by cycloheximide on PR transcript levels. An interesting observation was that the levels of MMP-1 and MMP-3 mRNA and protein were consistently higher in the untreated samples suggesting that MMP-1 and MMP-3 are readily produced by stromal cells, either inherently in the absence of gonadal steroids, or as a result of the culture conditions.

Figure 7. Effect of E2 on stromal cell progesterone receptor transcript and protein levels. Representative ethidium bromide-stained agarose gels (A) showing PR and GAPDH transcript levels obtained from cultures incubated with vehicle (lanes 1 and 2) or with $10^{-8}$M E2 for 4 days (lanes 3 and 4) either in the presence (lanes 2 and 4) or absence of 10μg/ml cycloheximide (lanes 1 and 3). Densitometric analysis of the data shown in panel A was converted into PR:GAPDH ratios (B) and are vehicle alone (c), cycloheximide alone (cyclo), estradiol (E2) alone, or both E2 and cycloheximide (E2+cyclo). Representative immunoblots (C) for PR protein obtained from (1) T47D breast carcinoma cell extracts, extracts from stromal cells incubated with 0.1% ethanol (2) or $10^{-8}$M E2 for 4 days (3). Relative molecular mass markers are indicated on the left and the identities of the different PR isoforms are shown on the right. Densitometric analysis of the data shown in panel C is shown in panel D. Data are the mean ± SD for three experiments performed in triplicate. *P<0.05, **P<0.01 compared to the untreated control.
Whatever the mechanism, it is clear from these observations that stromal cells in vivo are likely to be activated by E2 and P4 in the luteal phase of the normal cycle, suppressing MMP-1 and MMP-3 expression. Indeed, these observations are supported by other studies where other progestagens inhibited MMP expression (Salamonsen et al., 1997; Schatz et al., 1997; Hampton et al., 1999).

In experiments where stromal cells were subjected to T or P4 in combination with E2, the E2/T combination consistently led to a greater suppression of MMP-1 mRNA and protein levels when compared to that of the E2/P4 combination, and this is probably due to the much higher relative binding affinity and protracted ligand-receptor occupancy (Winneker et al., 2003) of trimegestone (330%) compared to progesterone (50%) or promegestone (100%) to the progesterone receptor (Wiegart and Kuhl, 2004).

Similarly, levonorgestrel (LNG) and norethindrone (NET), which are characterized by both high progesterone and androgen receptor binding affinities (Sitruk-Ware, 2004b), suppressed MMP levels to that of P4 (Hampton et al., 1999) indicative of a possible androgen

Figure 8. Immunohistochemical staining of endometrial sections for MMP-1, MMP-3 and MMP-9. Representative MMP-1 (A) MMP-3 (B) and MMP-9 (C) staining of a late secretory endometrium specimen; MMP9+ glandular epithelial cells are indicated by the arrow. Bar = 100μm.

Figure 9. Histomorphometric analysis of the number of stromal cells positive for MMP-1 (A), MMP-3 (B) and MMP-9 (C). P= Proliferative (n=8), ES=Early secretory (n=8), LS=Late secretory (n=11), M=Menstrual (n=8), 0.05 and 0.5 = 0.05mg (n=21) and 0.5mg (n=19) of trimegestone, respectively. Data are mean ± SE. *P<0.05 compared to ES (MMP-1) or compared to P (MMP-9). **P<0.05, *P<0.05 compared to ES and LS, respectively (MMP-9).
receptor (AR) mediated effect. To test this idea, the potent type I anti-progestin RU486 was used to mimic the effect of progesterone withdrawal by competition for, and conformational alteration of, progesterone receptor binding sites (Baulieu et al., 1987). The data indicated that RU486 blocked the actions of both P4- or T-induced suppression of MMP-1 and MMP-3 expression by increasing both MMP-1 and MMP-3 mRNA and protein levels in agreement with the published literature (Schatz et al., 1997). However, RU486 did not return protein and mRNA levels back to baseline values indicating that equimolar concentrations (10^{-6} M) of RU486 was insufficient to prevent P4- and T-induced MMP-1 and MMP-3 repression, suggesting that a type II anti-progestin would have been more useful, or that some of the effects on stromal cell MMP-1 and MMP-3 repression are mediated through glucocorticoid or androgen receptors. Alternatively, since RU486 can act as a partial agonist/antagonist in several tissues (Gompel et al., 1986; Robbins and Spitz, 1996; Dai et al., 2003), including the endometrial glandular epithelial cell (Taylor et al., 1998), then part of the observed MMP-1 and MMP-3 repression could be directly related to RU486 agonist activity. Indeed, RU486 alone partially inhibited MMP-1 and MMP-3 transcript levels and produced differential effects on T-induced MMP-1 and MMP-3 protein levels, where it blocked T-induced MMP-3 suppression, but not T-induced MMP-1 suppression, suggesting other non-PR mediated pathways for T. The differential action of RU486 on T-induced MMP-3 suppressive action but not MMP-1 suppression might be due to the differential inhibitory actions of P4 and T, or binding potentials at different PR isoforms. Alternatively, RU486 has recently been documented as having inhibitory effects through the androgen receptor (Agoulnik et al., 2003; Song et al., 2004), and as RU486 binds to the androgen receptor whereas progesterone and trimegestone do not, despite trimegestone having significant anti-androgenic activity (Winneker et al., 2003); perhaps this may explain the discrepancy between P4 and T in the presence of RU486? Alternatively, the discrepancy may be due to the relative stabilities of the MMP-1 and MMP-3 proteins in culture, as is shown for MMP-9 (Singer et al., 1999).

The differential regulation of MMP-1 and MMP-3 are unlikely to be due to the phase of cycle chosen to produce the stromal cell cultures because basal MMP expression levels are not affected by phase of the menstrual cycle from which the cultures arise (Rawdanowicz et al., 1994). Additionally, vimentin immunocytochemical staining (Kamelle et al., 2002) for stromal cells indicated that the cultures were >95% vimentin positive demonstrating that contaminating endometrial epithelial cells were not the source of the MMP-1 and MMP-3 differential expression patterns.

Our primary stromal cell cultures did not express MMP-9 mRNA (Figure 3) indicating that stromal cells in culture lack the ability to express MMP-9, because MMP-9 is considered to be confined to infiltrating leukocytes and epithelial cells that were removed in the process of generating the cell cultures. Indeed, stromal cells in culture can only transiently express MMP-9 transcripts after tumour necrosis factor alpha (TNF-α) stimulation and only at the 6 h time-point (Singer et al., 1999). The clinical or physiological relevance of such transient expression is not clear since there was a clear dissociation from transcript levels at the 6 h period and protein levels that were sustained for up to 48 h (Singer et al., 1999).

To apply this new knowledge to the in vivo situation, we examined the MMP expression patterns in normal and T-treated endometria by immunohistochemistry. The data showed a high degree of inter-patient variability within a phase of the cycle and within the trimegestone treatment groups. Variability of the results between patient samples is a common feature of clinical material (Hampton et al., 1999), when obtained from women who demonstrate different functional capacities to activate nuclear receptors, as recently demonstrated for ERα (Kitawaki et al., 2001) and for PR (Treloar et al., 2005) in the progression of endometriosis.

Given that no statistically significant difference in the cycle modulation of MMP-1 and MMP-3 protein expression was noted, we elected to examine the potential effect of a 10-fold dose differential of women treated with 0.05 mg trimegestone dose to 0.5 mg. No dose-response effect of trimegestone on the staining patterns of MMP-1 or MMP-3 was observed, although the number of MMP-1+ stromal cells was observed to be significantly higher in the 0.05mg trimegestone group when compared to the early secretory group of the normal menstrual cycle. Therefore, there appears to be no correlation between the pattern of bleeding in the trimegestone-treated women and the expression of MMP-1, MMP-3 in their endometrial biopsies. This is in general agreement with other reports on the effect of different progestagens (Vincent et al., 1999; Hickey and Fraser, 2001; Perrot-Applanat, 2004). Hence, the expression of these MMP-1 and MMP-3, per se, does not explain the different bleeding pattern with different doses of progestagen (Oliveira-Ribeiro et al., 2004).

In the endometrium of the normal menstrual cycle, MMP-1 and MMP-3 proteins were expressed in all phases and were highest in the menstrual phase, as previously reported (Goldberg et al., 1986; Smith, 1996; Hampton et al., 1999; Vincent and Salamonsen, 2000; Vincent et al., 2000); however, they are also at variance to those studies since our data did not show statistically significant differences. The reason for the discrepancy between the present study and others is not readily apparent although differences in methodology could account for this. For example, we used different antigen retrieval steps and antibodies, which may have identified more MMP+ cells in relation to those previous studies.

Two studies (Skinner et al., 1999; Vincent and Salamonsen, 2000) reported a differential effect of LNG on the MMP protein expression compared to the normal menstrual cycle, while in our study, there was no difference in the immunoreactivity of MMP-1, MMP-3 and MMP-9 between the T-treated endometrium and that of the normal menstrual cycle, suggesting a fundamental difference between T and other progestagens, such as LNG. For example, T has minimal androgen receptor affinity compared to that of LNG and androgen receptor expression changes markedly just prior to menstruation (Taylor et al., 2005). Alternatively, these progestagens may exhibit differential binding for PR isoforms (Sitruk-Ware, 2004a; Sitruk-Ware, 2004b) inducing the differential MMP expression patterns demonstrated in the present study.

MMP-9 immunoreactivity, by contrast, was expressed in the endometrial stroma in all phases of the normal menstrual cycle with highest expression in the late secretory phase and a decrease in the menstrual phase, agreeing with a previous report (Vincent et al., 1999; Cornet et al., 2005), which in turn activates MMP-9 (Vincent and Salamonsen, 2000). It is not clear whether the lower count is due to a limited role of this MMP in the process of endometrial degradation, or is because MMP-3 acts indirectly through activation of other MMPs such as MMP-7 (Osteen et al., 2005), which in turn activates MMP-9 (Vincent and Salamonsen, 2000). This study supports a role for a subset of stromal cells producing large quantities of MMP-3 from a small number of cells (Figure 8).

Co-ordinated expression of the MMPs, 1, 3 and 9 are thus important for endometrial stability at crucial points in the normal menstrual cycle such that a pliable proliferative phase endometrium allows cell
migration and expansion due to proliferation, whereas a secretory phase endometrium remains stable under the influence of P4; hence the progesteragenic inhibition of MMP-1 and MMP-3 expression observed in this study. During pregnancy increased serum P4 levels stabilize the endometrial ECM (Seval et al., 2004) and allows for limited expansion of the decidualized stromal cell. Failure to implant the blastocyst leads to luteolysis of the corpus luteum and progesterone withdrawal leading to restoration of proliferative phase MMP-1 and MMP-3 levels allowing MMP-9 leukocytes to infiltrate the endometrium and cause the tissue instability that leads to menstruation. Because of the longer receptor occupancy of T compared to P4 (Winneker et al., 2003), MMP-3 levels remain slightly elevated preventing the recruitment of MMP-9 cells to the endometrium. This could explain the reduced and less severe menstrual flows experienced by post-menopausal women on the sequential combined tri-megestone HRT regimen (Grubb et al., 2003; Al-Azzawi et al., 2004).

Although there was no dose–response effect of T on the expression of MMP-1, MMP-3 and MMP-9, the lower number of MMP-9 cells in the endometrial stroma of T-treated women suggest that MMP-9 is critical in the control of menstruation, although it does not explain the different bleeding patterns experienced by women on the different doses of T-based HRT (Grubb et al., 2003; Al-Azzawi et al., 2004).

In summary, T behaviour in primary stromal cell culture was similar to P4 in suppressing the expression of MMP-1 and MMP-3 mRNA and protein, which appears to be mediated through binding to PR, since this action was partially mimicked and reversed by the addition of RU486 and was enhanced in the presence of E2, which increased PR expression. The differential effect of T and P4 on MMP-1 protein expression in the presence of RU486 suggests that T and P4 bind to or activate different PR isoforms or that these two progesteragens differentially regulate the levels of MMPs through a mechanism involving differential activation of the AR. The data suggest that T acts in a similar manner to P4, but causes subtle differences in MMP expression patterns that go towards explaining the tighter control that T exerts on menstruation compared to P4.

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