Matrix metalloproteinases in normal menstruation

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Matrix metalloproteinases (MMP) are strongly implicated in menstruation. Messenger RNA for proMMP-1 and -3 was detectable in normal cycle endometrium only peri-menstrually and menstrually, although mRNA for their tissue inhibitors, TIMP-1 and TIMP-2, was present throughout the cycle. MMP-1, -3 and -9 were demonstrated immunohistochemically to be specifically associated with degraded tissue in menstrual endometrium. Activated mast cells and eosinophils, which release regulators of MMP expression and activators of latent enzymes, were also a marked feature of menstrual endometrium. Cultured endometrial stromal cells released MMP-1, -2, -3 and -9 and TIMP-1 and -2, whereas production by epithelial cells was minimal. Progesterone withdrawal from stromal cell cultures (for the final 4 days of a 10 day culture) increased the release of all four enzymes: all but MMP-2 were also stimulated by interleukin-1 or tumour necrosis factor α added to short-term stromal cultures. We postulate that an alteration in the balance of MMP and their inhibitors and the activation of MMP are prerequisites for tissue degradation at menstruation, and that this is regulated by a combination of progesterone withdrawal and paracrine factors from epithelial and stromal cells and from mast cells and eosinophils.

Key words: endometrium/mast cells/menstruation/progesterone/remodelling

Introduction

Normal menstruation is defined as uterine bleeding associated with endometrial breakdown following a normal ovarian cycle. The loss of blood is accompanied by shedding of cell debris from partial degradation of the functional layer of the endometrium (Fraser, 1990; Smith and Hairing, 1992). Little is known about the biochemical mechanisms of normal menstruation or the perturbations resulting in abnormal uterine bleeding. The classic study of menstruation in rhesus monkeys (Markee, 1940) demonstrated rapid regression of endometrial thickness as the first event preceding menstruation, this being followed by intense vasoconstriction and subsequent bleeding from focal points. However, largely because of the paucity of models for menstruation, information on the causative mechanisms remains rudimentary. Scanning electron microscopy of the endometrial surface reveals that, even before bleeding commences, small lesions are apparent in the luminal epithelium, and that there is then very rapid but incomplete degeneration of the functionalis layer, exposing open blood vessels and glands (Ludwig and Spornitz, 1991). During the late luteal phase of the menstrual cycle, when oestrogen and progesterone concentrations are falling, widespread degeneration is seen in the basal lamina surrounding the decidualized endometrial stromal cells and underlying the endothelium of the blood vessels (Roberts et al., 1992), and this is most likely a result of substantial degradation of the extracellular matrix (ECM). The mechanisms by which the ECM (both the interstitial matrix and the basal lamina underlying blood vessels, epithelium and decidual cells) is degraded in the human endometrium are largely unknown, but recent data from our laboratory and others (Hampton and Salamonsen, 1994; Rodgers et al., 1994; Marbaix et al., 1995)
have identified a close association between menstruation and the endometrial expression of matrix metalloproteinases (MMP).

MMP or matrixins are the enzymes which degrade components of both interstitial and basement membrane ECM. They are a family of secreted zinc proteinases which are active at the neutral pH of the extracellular space. The synthesis of most MMP is negligible in normal connective tissue. MMP can be classified into major subfamilies which together are capable of degrading the major macromolecular components of the ECM, the collagens, proteoglycans, fibronectin and laminin (Woessner, 1991). In particular, MMP-1 (interstitial collagenase) degrades collagens I-III, VII and XI; MMP-2 (gelatinase A) degrades gelatins and collagens IV, V, VII and XI; MMP-3 (stromelysin) degrades proteoglycans, fibronectin and laminin; and MMP-9 (gelatinase B) degrades gelatins and collagens IV and V (Nagase et al., 1992).

Characteristically, each MMP is secreted as a latent zymogen which can be activated in vitro by a number of natural proteases [including MMP-3, MMP-7 (matrilysin) and the recently described membrane-associated MMP (MT-MMP; Sato et al., 1994)], or by treatment with organomercurial compounds. Each MMP can be inhibited by specific inhibitors of metalloproteinases (TIMP) by the formation of 1:1 complexes (Nagase, 1991) or, less specifically, by α2-macroglobulin. The genes for MMP and those for TIMP and matrix proteins are regulated by a number of steroid hormones (corticosteroids, progesterone), growth factors, cytokines and other regulatory molecules, but this varies between cell types, in some cases being coordinated while in other cases the regulation of different genes is independent. These mechanisms have been described in a range of tissues and cells in culture (reviewed by Birkedal-Hansen et al., 1993).

**MMP in the endometrium**

There is considerable evidence from our laboratory and others that MMP are produced in the endometrium and that their expression is correlated closely with the process of normal menstruation. Messenger (m)RNA for proMMP-1 and -3 is detectable by Northern blot analysis in endometrium from women with normal menstrual cycles and without evidence of endometrial disorder, only during the peri-menstrual and menstrual phases of the cycle (Hampton and Salamonsen, 1994; Figure 1). Although mRNA for TIMP-1 and -2 was present in all endometrial samples regardless of the day of the cycle, it also increased, but to a lesser extent, during menstruation on days 1 and 2 (Hampton and Salamonsen, 1994). Given that the inhibition of MMP requires 1:1 stoichiometry with TIMP, and provided that the stability of the mRNA is equivalent, the much greater increase in the abundance of mRNA for the MMP (from undetectable to strongly positive) than TIMP (an increase of ~5-fold for TIMP-1 and less for TIMP-2) in menstrual tissue suggests a major alteration in the balance between inhibitors and enzymes in favour of tissue degradation. These data are supported by in-situ hybridisation studies (Rodgers et al., 1994), which defined cell type and cycle-specific patterns of expression for a number of MMP: all the MMP were present during the menstrual phase.

An immunohistochemical analysis of menstrual endometrium confirms that translation of the mRNA results in MMP protein in the tissue. Figure 2 shows the immunohistochemical staining of a single sample of normal menstrual tissue for MMP-1, -3 and -9. The tissue was collected at curettage and contained areas of intact tissue (Figure 2A), along with other areas in which extensive tissue degradation was occurring (Figure 2B–D). The focal nature of MMP production and action is emphasized by the lack of immunostaining for any of the enzymes in the intact tissue, while in the areas of tissue breakdown, considerable positive immunoreactivity for all three enzymes can be seen. Although the antisera used in this study do not differentiate between latent and active enzymes, activation of the MMP can be assumed because of the spatial relationship of the immunostaining to endometrial degradation. Furthermore, the presence of active MMP-1 has been demonstrated in tissue explants from menstrual endometrium but not in those taken during other phases of the cycle (Marbaix et al., 1995).

A number of different cell types contribute to MMP production in the endometrium. At menstruation, most MMP are produced by stromal cells
Figure 1. Diagrammatic representation of the expression of mRNA for pro-matrix metalloproteinase (MMP)-1, proMMP-3, tissue inhibitor of metalloproteinase (TIMP)-1 and TIMP-2, and the activation of mast cells and appearance and disappearance of eosinophils in human endometrium throughout the menstrual cycle. For proMMP expression, each circle represents an individual histologically dated endometrial sample. (+) and (−) represent the presence or absence respectively of mRNA detected by a Northern blot analysis. For TIMP expression, Northern blots were analysed by densitometry. Key to histograms: light grey, white and dark grey bars represent the relative abundance (compared with the abundance for glyceraldehyde phosphate dehydrogenase) of mRNA for TIMP-1 (0.9 kb) and TIMP-2 (3.5 and 1.0 kb transcripts) respectively, expressed as mean ± SEM within each group (n in parentheses). The extent of mast cell activation and eosinophil infiltration across the menstrual cycle is represented diagrammatically, with the width of the bands representing the relative mast cell activation or number of eosinophils in the endometrium on any cycle day. a–d represent significant differences between groups within a transcript size for TIMP-2 mRNA. Derived from data in Hampton and Salamonsen (1994) and Jeziorska et al. (1995).
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Figure 2. Immunohistochemical detection of matrix metalloproteinase (MMP)-1, -3 and -9 in a single sample of menstrual endometrium. (A) Undegraded tissue reacted with anti-MMP-1. (B–D) Degraded tissue reacted with anti-MMP-1, -3 and -9 respectively. Arrowheads in (C) define areas of positivity for MMP-3.

(Rawdanowicz et al., 1994; Rodgers et al., 1994): whether decidualization of these cells in vivo alters their ability to produce MMP has not yet been clearly defined, although stromal endometrial cells which have been decidualized in vitro produce at least proMMP-3 (Schatz et al., 1994). MMP-7 is an exception, being produced by the glandular and luminal epithelium (Rodgers et al., 1993; Bruner et al., 1995), while MMP-9 is immunolocalized to neutrophils in menstrual tissue (Jeziorska et al., 1994).

Regulation of MMP activity at menstruation

MMP are likely to be regulated at a number of levels in the endometrium. These include transcriptional control by steroid hormones (particularly progesterone) or paracrine regulators, activation by other proteases including some MMP, and control of extracellular activity by inhibitors. Enzymes produced by migratory cells (e.g. proMMP-9 from neutrophils) are subject to regulation of their release from secretory granules.

**Regulation of MMP production**

*By progesterone withdrawal*

Both circulating progesterone and the numbers of endometrial progesterone receptors (Lessey et al., 1988) are at very low levels at the time of menstruation, and the resultant loss of progesterone action on the endometrium is likely to be linked directly or indirectly to the endometrial production of MMP at menstruation. Acute progesterone withdrawal in vitro induces both the secretion and the activation of MMP-1, -3 and -9 from endometrial explants (Marbaix et al., 1992) and their production by cultured endometrial stromal cells (Hammond and Salamonson, 1994; Schatz et al., 1994). However, there are a number of observations that argue against progesterone withdrawal as the major deter-
mining factor. In vivo, progesterone withdrawal occurs too early in the cycle to fully explain the temporal rise in expression of mRNA for the MMP, which is detectable only peri-menstrually. Furthermore, menstruation is a characteristic only of women and of old-world primates (in which the terminal differentiation of the stromal cells into decidual cells occurs in the absence of a conceptus), whereas withdrawal of progesterone associated with the demise of the corpus luteum occurs in non-fertile cycles in all mammalian species. Finally, connective tissue degradation at menstruation occurs at focal points over a period of several days; the direct regulation of degradative enzymes by a circulating hormone would be expected to result in more generalized breakdown. Therefore we suggest that the absence of progesterone (which has the potential to down-regulate the gene expression of a number of MMP) provides an environment which is permissive for MMP synthesis, secretion and activation.

The exact intracellular events associated with the actions of progesterone on MMP transcription are not fully understood. Glucocorticoids, whose receptors share a gene family with progesterone and other steroid hormones, have an inhibitory action on collagenase expression which is mediated via binding of the glucocorticoid–receptor complex with components of the AP-1 complex, either unligated or DNA bound, in such a way as to inhibit the functional activity of AP-1 by blocking its capacity to bind to a specific sequence at the 5' end of the procollagenase gene (the TRE sequence) which normally results in gene activation (Jonat et al., 1990; Yang-Yen et al., 1990). Such a mechanism raises the possibility of interactions between glucocorticoid action and those of the large number of cytokines and growth factors which also regulate the MMP genes. Should progesterone act on collagenase or other MMP via similar molecular mechanisms, its absence may be permissive for other regulatory molecules to act via the AP-1 site. When acting by binding of the progesterone–receptor complex directly to steroid response elements on DNA, progesterone stimulates the production of a range of progesterone-dependent secretory proteins, and it is also likely that some of these proteins have paracrine roles related to MMP gene expression or enzyme activation.

By locally produced cytokines and growth factors

Endometrial expression of a number of growth factors and cytokines and/or their receptors displays considerable variation throughout the menstrual cycle, and the abundance of mRNA or protein for a number of these reaches a peak during the peri-menstrual and menstrual phases, thus positioning them as potential regulators of MMP expression associated with menstruation (Table I; reviewed in Tabibzadeh, 1991; Guidice, 1994). We have demonstrated that the production of latent MMP-1, -3 and -9, but not MMP-2, from endometrial stromal cells in culture is stimulated in a concentration-dependent manner by both tumour necrosis factor α and interleukin (IL)-1α (Figure 3), confirming the likely importance of such paracrine regulators at menstruation. Furthermore, in coculture experiments with endometrial stromal and epithelial cells, transforming growth factor α of endometrial stromal origin inhibited the epithelial production of MMP-7 (Bruner et al., 1995). Several cytokines are also known for their chemotactic effects, and may facilitate focal infiltration of the lymphoid cells into human endometrium (Wahl et al., 1987; Morzycki et al., 1990).

By products of migratory or resident cells

The presence of migratory cells in the endometrium is well established (Clark, 1992), although in most instances their spatial and temporal relationship to menstrual bleeding has not been clearly defined. Endometrial granular lymphocytes are increased at this time (Bulmer et al., 1987). These CD56+ cells represent the largest population of leukocytes in late secretory endometrium and decidua, and their granular contents include perforin, granzyme A and TIA-1 (an RNA binding protein capable of inducing DNA fragmentation; King et al., 1993). In vitro, these cells release both proMMP-2 and proMMP-9 (Shi et al., 1995), but whether this also occurs in vivo is not yet established. Mast cells are clearly identifiable in the endometrium throughout the menstrual cycle, and their numbers (expressed in relation to stromal cell numbers per mm²) do not change significantly during the cycle. However, there are well-defined periods of mast cell activation commonly associated with local
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Table I. Potential paracrine regulators of matrix metalloproteinases/tissue inhibitors of metalloproteinases in human endometrium

<table>
<thead>
<tr>
<th>Regulator</th>
<th>Cellular source</th>
<th>Phase of peak production</th>
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<tbody>
<tr>
<td>Transforming growth factor β</td>
<td>Epithelial, stromal</td>
<td>Secretory (M, L)</td>
</tr>
<tr>
<td>Tumour necrosis factor α</td>
<td>Epithelial, stromal</td>
<td>Secretory (M, L)</td>
</tr>
<tr>
<td>Leukaemia-inhibiting factor</td>
<td>Epithelial</td>
<td>Secretory (M, L)</td>
</tr>
<tr>
<td>Relaxin</td>
<td>Decidual, epithelial</td>
<td>Secretory (M, L)</td>
</tr>
<tr>
<td>Prolactin</td>
<td>Decidual, epithelial</td>
<td>Secretory (M, L)</td>
</tr>
<tr>
<td>Endothelin</td>
<td>Epithelial</td>
<td>Secretory, menstrual</td>
</tr>
<tr>
<td>Prostaglandins E2F-α</td>
<td>Stromal/epithelial</td>
<td>Menstrual</td>
</tr>
<tr>
<td>Interleukin-I-18</td>
<td>Stromal/epithelial</td>
<td>Secretory (M, L)</td>
</tr>
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M = mid-secretory phase; L = late secretory phase.

*Kaura et al. (1990).
†Hunt et al. (1992).
‡Vogias et al. (1996).
§Bryant-Greenwood et al. (1993).
¶Salamonson et al. (1992).
‖Tabibzadeh and Sun (1992).

Table II. Secretory products of mast cells

<table>
<thead>
<tr>
<th>Mast cell</th>
<th>Secretory product</th>
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<tbody>
<tr>
<td>Cytokines</td>
<td></td>
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<tr>
<td>Tumour necrosis factor α</td>
<td></td>
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<tr>
<td>Interleukin-1</td>
<td></td>
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<tr>
<td>Interleukin-3</td>
<td></td>
</tr>
<tr>
<td>Transforming growth factor β</td>
<td></td>
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<tr>
<td>Proteases</td>
<td></td>
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<tr>
<td>Tryptase</td>
<td></td>
</tr>
<tr>
<td>Chymase</td>
<td></td>
</tr>
<tr>
<td>Other mediators</td>
<td></td>
</tr>
<tr>
<td>Histamine</td>
<td></td>
</tr>
<tr>
<td>Heparin</td>
<td></td>
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</table>

connective tissue disruption and oedema, with the most marked being just prior to and during menstruation (Figure 1). The mast cell-derived proteinase tryptase can be clearly identified at extracellular locations in menstrual endometrium, particularly in areas of stromal oedema and tissue breakdown. The endometrial mast cells in the functionalis layer are of the MC,T phenotype and do not release the other mast cell-derived proteolytic enzyme, chymase. Endometrial mast cells expressing chymase (MC, phenotype) are found only in the basalis layer at the endometrium-myometrium interface (Jeziorska et al., 1995). Eosinophils are also present in the endometrium just prior to (days 26–28) and during menstruation, often appearing as local extravascular accumulations with a localized release of eosinophil cationic protein indicative of activation. Eosinophils are not detectable at any other time of the cycle (Figure 1; Jeziorska et al., 1995). Each of these migratory/resident cell types produce an array of regulatory molecules, including cytokines and other proteases which may stimulate MMP production and facilitate their activation at menstruation (Tables II and III). In addition, some of their products (e.g. histamine and heparin from mast cells and prostaglandins from eosinophils) may contribute to uterine bleeding by other mechanisms within the micro-environment, such as induction of vasoconstriction, changes in vasopermeability and anticoagulation.

Activation of MMP

At this time, little is known of the mechanisms by which MMP are activated at menstruation. High on the list of contenders is mast cell tryptase which activates proMMP-3 (Lees et al., 1994). MMP-3 is thought to be a key enzyme in the proteolytic cascade of MMP because it is capable of activating both proMMP-1 and proMMP-7, while active MMP-7 can also activate proMMP-1 and proMMP-9. In addition, mast cell chymase can activate latent MMP-1 (Saarinen et al., 1994), and thus the limited endometrial production of this protease at the myometrium-endometrium interface may be important (Jeziorska et al., 1995). Plasmin is the activator with the broadest specificity for MMP
**Figure 3.** Matrix metalloproteinase (MMP)-1 activity in culture medium from endometrial stromal cells cultured without (shaded bar) or with increasing doses of interleukin (IL)-1α (A) and tumour necrosis factor (TNF) α (B). Values are the mean ± SEM (n = 3). Collagenolytic activity was measured without aminophenyl mercuric acetate (APMA) treatment. Difference from control (shaded bar) is P < 0.05 (*).

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in vitro, and most of the components of the urokinase cascade (which results in plasmin production) have been defined in the endometrium. In particular, at menstruation, urokinase plasminogen activator concentrations are higher than at any other time of the cycle (Koh et al., 1992). Furthermore, progesterone increases plasminogen activator inhibitor-1 production from human endometrial stromal cells; thus progesterone withdrawal during the late secretory phase would result in a decrease of this inhibitor (Casslén et al., 1992). The net result of these changes would favour plasmin production and hence the activation of proMMP.

<table>
<thead>
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<th>Table III. Secretory products of eosinophils</th>
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<tr>
<td><strong>Secretory products</strong></td>
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<tr>
<td><strong>Preformed proteins</strong></td>
</tr>
<tr>
<td>Non-enzymatic</td>
</tr>
<tr>
<td>Major basic protein (activates mast cells)</td>
</tr>
<tr>
<td>Cationic protein (activates mast cells)</td>
</tr>
<tr>
<td>Protein X</td>
</tr>
<tr>
<td>Enzymatic</td>
</tr>
<tr>
<td>Peroxidase (activates mast cells)</td>
</tr>
<tr>
<td>Collagenase (hydrolyses collagens I and III)</td>
</tr>
<tr>
<td>Aryl sulphatase B (hydrolyses proteoglycans, glycosaminoglycans)</td>
</tr>
<tr>
<td>De-novo generated mediators</td>
</tr>
<tr>
<td>Lipids</td>
</tr>
<tr>
<td>Prostaglandins, leukotriene, platelet activating factor, eosinophil chemotactic lipid</td>
</tr>
<tr>
<td>Oxygen metabolites</td>
</tr>
<tr>
<td>Cytokines</td>
</tr>
<tr>
<td>Interleukin-3, granulocyte macrophage colony-stimulating factor, transforming growth factor β1</td>
</tr>
<tr>
<td>Neuropeptides</td>
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<tr>
<td>Vasoactive intestinal peptide, substance P</td>
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**Inhibitors of MMP**

mRNA for both TIMP-1 and TIMP-2 is expressed throughout the menstrual cycle. There is a significant increase in TIMP-1 on days 1–2 compared with all other days, and in the 1.0 kb, but not the 3.5 kb, transcript of TIMP-2 at the same time (Figure 1; Hampton and Salamonsen, 1994). TIMP proteins can be detected in culture medium from endometrial stromal cells in culture; while TIMP-1 predominates, TIMP-2 and TIMP-3 can also be identified. Much lesser amounts of these inhibitors are present in culture medium from endometrial epithelial cells (L.A.Salamonsen and A.R.Butt, unpublished observations). In the ovine endometrium, TIMP-1 is localized to both stromal cells and epithelium, and is often seen to be concentrated around blood vessels, suggesting a protective effect on the integrity of vessel membranes (Hampton et al., 1995). However, at menstruation in the human, great regional differences of expression of TIMP-1 mRNA within the tissue have been reported (Rodgers et al., 1994). It is therefore likely that the various TIMP will be subject to local regulation in much the same way as is being established for the MMP.

The broad spectrum plasma protease inhibitor α2-macroglobulin is an effective inhibitor of active
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MMP and may also bind, and thus modulate the functions of, some cytokines. This protein is present in human endometrium throughout the menstrual cycle, predominantly in the stroma, but immunostaining is more intense in the secretory phase where it is localized very strongly in the spongiosum layer (Sayegh et al., 1995). As α2-macroglobulin is also present in high concentrations in blood, menstrual bleeding could itself provide an abundance of this inhibitor and hence protect the endometrium from excessive tissue degradation.

In summary, the regulatory mechanisms governing the balance of MMP and their inhibitors, and the activation of MMP which is a prerequisite for their action in tissue breakdown at menstruation, are clearly multifactorial. A postulated scenario for the regulation of MMP production at menstruation is given in Figure 4. The challenge is to ascertain the relative contribution from the various components of this system to the rapid cyclic tissue degeneration and regrowth that is peculiar to the endometrium.

It remains to be established whether or not the perturbation of MMP production is associated with any or all situations of abnormal uterine bleeding, including menorrhagia and the abnormal bleeding associated with the use of steroid contraceptives, particularly the long-acting progestins. We postulate that in such situations a small perturbation in the expression of MMP could result in breakdown of the basement membranes of some of the capillaries in the subepithelial plexus, leading to breakthrough bleeding at focal points. Such studies are currently in progress and may prove of importance to our understanding of the mechanisms of abnormal uterine bleeding.

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