The role of matrix metalloproteinases and leukocytes in abnormal uterine bleeding associated with progestin-only contraceptives

A.J.Vincent and L.A.Salamonsen¹

Prince Henry’s Institute of Medical Research, Clayton, Victoria, 3168, Australia

¹To whom correspondence should be addressed at: Prince Henry’s Institute of Medical Research, P.O.Box 5152, Clayton, Victoria, 3168, Australia. E-mail: lois.salamonsen@med.monash.edu.au

Progestin-only contraceptives are associated with menstrual bleeding disturbances; a major reason why these agents are discontinued. The pathogenesis of abnormal uterine bleeding associated with progestin-only contraceptives remains ill-defined. Matrix metalloproteinases (MMPs) and leukocytes are postulated to be involved in the process of normal menstruation. Immunolocalization of MMPs and leukocytes in endometrium from women using the progestin-only contraceptives, Norplant® or depot medroxyprogesterone acetate (DMPA) compared with normal controls, revealed foci of positive MMP-1 and -3 immunostaining in stromal cells and adjacent extracellular matrix, the presence of MMP-9 in various subtypes of leukocytes and alterations in mast cell phenotype. In women using progestin-only contraceptives, the extent of endometrial MMP, neutrophil and eosinophil immunolocalization and the mast cell activation state was similar to or greater than that observed in perimenstrual control women. However, differences in MMP immunostaining were observed in endometrial samples from women using different progestin-only contraceptive agents; in particular, significantly higher MMP-1 immunostaining was observed associated with the use of Norplant compared with DMPA. No correlation was observed with the number of bleeding days recorded. These results suggest that MMP and leukocytes may be involved in endometrial breakdown in women using progestin-only contraceptives.

Keywords: contraceptive/leukocytes/matrix metalloproteinases/Norplant/progestin

Introduction

Progestin-only contraceptives, including long-acting subdermally implanted levonorgestrel (Norplant®), and injectable depot medroxyprogesterone acetate (DMPA), are widely used, safe and effective. However, the use of these agents is limited by the disruption to menstrual bleeding patterns which commonly occurs, especially in the first year of use, and constitutes the major reason for discontinuation of these agents (Odlind and Fraser, 1990). The degree of menstrual bleeding disturbance varies with the contraceptive agent used; however the mechanisms and factors involved in the pathogenesis of abnormal uterine bleeding associated with contraceptive steroids remain ill-defined.

Matrix metalloproteinases (MMPs) are a family of zinc-dependent proteases which degrade specific components of the extracellular matrix (ECM) (Birkedal-Hansen et al., 1993). MMPs may contribute to abnormal uterine bleeding by contributing to endometrial tissue loss and vascular fragility through the degradation of the ECM. Regulation of MMPs is complex and occurs at multiple levels, including gene transcription, a cascade of activation in which proteases, including MMP-3, are capable of activating proMMPs and inhibition by specific tissue inhibitors of metalloproteinases (TIMPs).
Studies from our laboratory and others have demonstrated that MMPs are present in the endometrium and that they display spatial and temporal variation throughout the endometrium, with an increase observed perimenstrually. MMPs are postulated to be responsible for the endometrial breakdown observed at menstruation (Marbaix et al., 1995; Kokorine et al., 1996; Salamonsen and Woolley, 1996, 1999). Regulation of endometrial MMPs involves the actions of circulating steroids such as progesterone and interactions between factors produced by endometrial epithelial and stromal cells and leukocytes. These include cytokines such as tumour necrosis factor-α and interleukin (IL)-1 and proteases including neutrophil elastase (Salamonsen and Woolley, 1999; Salamonsen et al., 2000).

Leukocytes are an integral component of the endometrium and display variation in type, number and site across the menstrual cycle (Salamonsen and Lathbury, 2000). It has been postulated that menstruation occurs as a result of an inflammatory process in which leukocytes play a key role (Finn, 1984; Salamonsen and Woolley, 1999; Salamonsen and Lathbury, 2000). Endometrial leukocytes produce a range of regulatory molecules including cytokines and proteases and are likely to respond to chemokines elaborated by endometrial epithelial, endothelial and stromal cells (Salamonsen and Lathbury, 2000). Alteration in the pattern of endometrial leukocytes has been observed in response to treatment with exogenous progestins. However, the role of leukocytes in the pathogenesis of abnormal uterine bleeding observed in steroid contraceptive users remains unclear.

Our laboratory has conducted immunohistochemical studies investigating the presence of MMPs, including MMP-1, -3 and -9, and leukocytes, including neutrophils, eosinophils and mast cells (MC) in endometrial samples obtained from Indonesian and Australian women during the first year of use of the progestin-only contraceptives, Norplant or DMPA, compared with endometrial biopsies from premenstrual or menstrual phase Australian control women [the phases of the normal menstrual cycle when MMP are either only or maximally present (Figure 1)]. This paper summarizes and reviews these findings.

![Figure 1](image.png)

**Figure 1.** Diagrammatic summary of the relative distribution of matrix metalloproteinases and leukocyte subtypes in endometrial stroma across the idealized 28 day menstrual cycle. N = neutrophils; E = eosinophils; MC = mast cells; ø = macrophages; T cells = T lymphocytes; B cells = B lymphocytes; eGL = endometrial granulated lymphocytes. Derived from Jeziorska et al. (1995, 1996), Kokorine et al. (1996) and Salamonsen and Woolley (1999).

**Matrix metalloproteinases**

In endometrial tissue obtained from women using Norplant or DMPA, MMP-1 positive immunostaining was localized to focal areas of endometrial stromal cells and adjacent ECM, although not specifically to sites of tissue breakdown (Figure 2) (Vincent et al., 2000). However, in endometrial samples from perimenstrual controls, MMP-1 was predominately associated with tissue breakdown sites although not perivascularly. Quantitative analysis revealed significantly higher MMP-1 positive staining in biopsies from Norplant users compared
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Figure 2. Immunolocalization of matrix metalloproteinases (MMP) and leukocytes in endometrial biopsies obtained from women using progestin-only contraceptives. Foci of immunopositive stromal cells and adjacent extracellular matrix including (a) MMP-1, (b) MMP-3, (c) MMP-9 positive leukocytes in an endometrial biopsy displaying a shedding morphology. (d) Immunolocalization of eosinophilic cationic protein EGI, a specific marker for eosinophils. (e) Immunolocalization of neutrophil elastase, a specific marker for neutrophils (f). Mast cells (MC) are demonstrated by the presence of MC tryptase with extracellular MC tryptase indicating activated MC. (f') Dual immunolocalization studies indicate that <25% of MC display a MC tryptase (pink chromogen)–MC chymase (black chromogen) phenotype. Immunolocalization of (g) tissue inhibitor of metalloproteinase (TIMP)-1 and (h) TIMP-2 are shown. Representative negative controls for sections (a–f) and (g–h) are indicated in (i) and (i') respectively. Haematoxylin counterstain was used in (a–i) Dual immunofluorescence studies of selected Norplant endometrial tissues demonstrating the production of MMP-9 (j'–n') by different types of leukocytes including (j) eosinophils, (k) CD3+ T cells, (l) neutrophils, (m) macrophages and (n) negative control. Identical fields are shown in which immunostaining for MMP-9 using an amplification technique with fluorescein isothiocyanate (lower panel; green immunofluorescence) was followed by immunostaining for specific leukocyte markers (j–m) or negative control (n) using conventional immunofluorescence with Texas Red (upper panel; red immunofluorescence) (Vincent et al., 1999) with positive cells shown by arrowheads. g = gland; le = luminal epithelium; s = stromal cell; d = pseudo-decidualized stromal cell; b = breakdown site; bv = blood vessel. Bar = 10 μm. Panels c–e, g, h, j–n, j'–n' reproduced with permission (Vincent et al., 1999).
with DMPA users or premenstrual and menstrual controls (Figure 3) (Vincent et al., 2000).

In contrast, MMP-3 positive immunostaining was present in less than 50% of the biopsies and there was no difference between the treatment groups and controls. The pattern of MMP-3 immunolocalization was similar to MMP-1 (Figure 2) (Vincent et al., 2000).

In endometrial biopsies from women using Norplant/DMPA, positive MMP-9 immunostaining was present intracellularly in stromal and intravascular leukocytes and extracellularly adjacent to leukocytes in areas of tissue breakdown (Figure 2) (Vincent et al., 1999a and unpublished observations). These MMP-9 positive leukocytes were identified as neutrophils, eosinophils, macrophages and CD3+ T cells on the basis of dual immunofluorescence (Figure 2) (Vincent et al., 1999a). However, phenotypic variation was observed with only some leukocytes of any one type positive for MMP-9. This may relate to functional differences including maturation and activation. Variation in the number of MMP-9 positive leukocytes was observed in endometrial biopsies from Norplant users displaying different histological appearances. In particular, there was a significant increase in biopsies displaying a shedding morphology, similar to that of menstrual controls, compared with biopsies displaying an atrophic appearance (Figure 3) (Vincent et al., 1999). The number of MMP-9 positive cells was similar in DMPA users and menstrual controls (A.J.Vincent et al., unpublished observations). There was no correlation between bleeding patterns and MMP immunostaining.

**Tissue inhibitors of metalloproteinases**

TIMP-1 and -2 positive immunostaining was observed in endometrial epithelial, endothelial and stromal cells but not in leukocytes in both Norplant and DMPA users (Figure 2) (A.J.Vincent et al., 1999; unpublished observations). The pattern of staining intensity was similar to that seen in the normal menstrual cycle (Zhang and Salamonsen, 1997).

**Leukocytes**

The presence of eosinophils and neutrophils in endometrial biopsies from women using Norplant was assessed immunohistochemically using antibodies directed against eosinophil cationic protein EG1 and neutrophil elastase, specific markers for these cell types respectively. Eosinophils and neu-
MMP, leukocytes and steroid-associated uterine bleeding

Eosinophils

Neutrophils

Mast cells

Figure 4. Number of (a) eosinophil cationic protein EGl or (b) neutrophil elastase immunopositive cells/1000 stromal cells in endometrial biopsies from Norplant users (■) or normal cycling women (□) classified according to histological group or stage of the idealized 28 day menstrual cycle. (c) Number of mast cells (MC)/1000 stromal cells as identified by immunolocalization of MC tryptase and (d) the percentage of these cells demonstrating extracellular MC tryptase indicating activation grouped according to type of progestin-only contraceptive used or stage of the idealized 28 day menstrual cycle. Numbers in parentheses denote number of endometrial samples. Results expressed as mean ± SEM. *P < 0.05; **P < 0.01. Reproduced with permission (Vincent et al., 1999, 2000).

trophils were observed in the stroma or intravascularly and were concentrated at sites of tissue breakdown (Figure 2) (Vincent et al., 1999). As observed with MMP-9 immunostaining, the highest number of positively stained cells (both eosinophils and neutrophil polymorphs) was detected in biopsies from Norplant users displaying a shedding morphology and was similar to the number detected in endometrial samples from menstrual controls (Figure 4) (Vincent et al., 1999).

Endometrial mast cells (MC) were identified using immunolocalization of MC tryptase and chymase and were present in biopsies obtained from Norplant and DMPA users (Figure 2). The MC were distributed in the stroma and were observed at the periphery of tissue breakdown sites. This study showed that the majority of MC were activated, as demonstrated by the presence of extracellular MC tryptase, with no difference in the numbers of MC between treatment and control groups (Figure 3) (Vincent et al., 2000). Dual immunolocalization of MC tryptase and MC chymase revealed that MC tryptase was the predominant MC phenotype, less than 25% of MC were combined MC tryptase-chymase positive or MC chymase positive (Figure 2) (Vincent et al., 2000).

Discussion

These studies demonstrate that MMP-1, -3 and -9, proteases involved in endometrial breakdown at menstruation, and leukocytes, including neutrophils, eosinophils and MC (each of these having a range of regulatory actions in normal endometrium including a potential role in MMP production and activation) (Salamonsen et al., 2000), were present in the endometrium of women using progestin-
only contraceptives. Importantly, the pattern of immunoreactive MMPs and leukocytes displayed both similarities and differences when compared with perimenstrual control endometria (the time of the normal menstrual cycle associated with tissue breakdown) supporting previous research indicating similarities and differences in the potential mechanisms underlying breakthrough bleeding and the process of normal menstruation (Fraser et al., 1996).

The endometrial response to exogenous progestins is variable depending on dose, type, method of administration and duration of exposure. Morphological changes involve surface epithelium, glands, vascular structures, leukocytic infiltration and stroma (Ludwig, 1982; Johannisson, 1990; Clark et al., 1996; Rogers, 1996; Song et al., 1996) while functional differences such as the expression of insulin-like growth factor-binding protein-1 (Pekonen et al., 1992), prolactin (Critchley et al., 1998b) and sex steroid receptors (Critchley et al., 1993, 1998a) have also been described. Such variability may relate to the different patterns of bleeding disturbance reported with different agents (Odlind and Fraser, 1990).

Differences in the pattern of MMP immunolocalization with the use of different progestin-only contraceptives is consistent with these previous studies. Positive MMP-1 immunostaining was higher in endometrial biopsies from Norplant users compared with DMPA users (Vincent et al., 2000). MMP-9 positive immunostaining was only observed in leukocytes in endometrial biopsies from women using Norplant (Vincent et al., 2000) whereas MMP-9 positive decidual cell and perivascular staining was observed in women using the levonorgestrel-releasing intrauterine device (LNG-IUD) (Skinner et al., 1999). Although no difference in MMP production in vitro was observed in response to direct treatment with different progestins (Hampton et al., 1999), it is probable that in vivo the effect of different progestins is mediated indirectly through alteration of the endometrial cytokine/chemokine environment and thus leads to the observed differences in MMP immunolocalization observed in these studies. It is important to remember that immunohistochemical studies detect both inactive and active MMP. The results of these studies indicate the need for further research regarding MMP activity, MMP mRNA expression and the use of models which more approximate the in-vivo environment.

The pattern of leukocyte response to the use of exogenous progestins also varies with the type of progestin used, route of administration, presence of bleeding and endometrial morphological appearance. Increased neutrophils and eosinophils were observed in endometria displaying a shedding morphological appearance compared to an atrophic appearance (Vincent et al., 1999). Focal and diffuse infiltrations of leukocytes, predominately lymphocytes and monocytes, were observed in a light and electron microscope study of endometrial biopsies obtained from women using low dose oral progestins or DMPA (Ludwig, 1982). The endometrial leukocyte infiltrations were observed more frequently closer to the time of last reported bleeding episode and were seen less frequently in morphologically atrophic endometrial samples or those from amenorrhoeic patients. Increased numbers of leukocytes including CD3+ T cells, leukocyte common antigen positive cells, neutrophils and CD68+ macrophages, identified using immunohistochemistry, and phloxine positive endometrial granulated lymphocytes (eGL) were observed in the endometrium of women treated with high dose oral progestins compared with controls (Song et al., 1996). CD3+ T cells were the most numerous subtype observed and correlated with the duration of progestin exposure. There was no correlation with dose of progestin and association with bleeding patterns was not reported. Leukocyte numbers also varied with duration of use of the LNG-IUD (Critchley et al., 1998b) although no correlation with menstrual bleeding patterns was observed. An initial increase in macrophages, assessed semiquantitatively, was observed in endometrial biopsies 1 month post insertion of the LNG-IUD followed by a decline over the next 12 months. In addition, CD56+ (identifying eGL) immunostaining scores in endometrial biopsies obtained from women 1–3 months post LNG-IUD insertion were similar to pre-insertion secretory phase controls samples with a subsequent decrease noted at 12 months post-insertion. In contrast to endometrial tissue obtained from Norplant users (Vincent et al., 1999), positive neutrophil elastase immunostaining was negligible in post-insertion.
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LNG-IUD biopsies (Critchley et al., 1998b). However, Clark and co-workers (1996) reported overall decreased immunolocalization of CD3+ T cells, CD68+ macrophages and eGL in morphologically atrophic endometrial biopsies obtained from women using Norplant, although the number of CD68+ macrophages was significantly increased in women who reported irregular bleeding compared with non-bleeders. No difference in MC numbers, identified by metachromatic granule staining, was observed in endometrial samples obtained from women using a levonorgestrel-releasing vaginal ring and control women (Hourihan et al., 1991). In addition, endometrial mast cell numbers, as determined by MC tryptase immunolocalization, were not increased in women using subdermally implanted levonorgestrel (Norplant) compared with perimenstrual controls (Vincent et al., 2000). In contrast, significantly increased numbers of endometrial mast cells, identified using Toluidine Blue, were observed in samples obtained from women using the LNG-IUD compared with pre-insertion control samples (Yin et al., 1993). In this study, the mean number of mast cells in women with abnormal bleeding was higher than in non-bleeders but the result was not statistically significant due to small sample sizes. Alteration in MC phenotype has also been observed in endometrial samples obtained from women using DMPA or Norplant (MC tryptase, MC tryptase–chymase and MC chymase phenotype) compared with controls (MC tryptase alone) (Jeziorska et al., 1995; Vincent et al., 2000) (Figure 2). Alteration in MC phenotype has been observed in mice in response to changes in the microenvironment (Galli, 1993). It is unlikely that these observed differences could be entirely due to immunohistochemical staining method, sampling technique or quantification, but more likely reflect differing endometrial responses.

A number of studies have demonstrated the importance of leukocytes in normal endometrial function including their role in MMP regulation and menstruation (Salamonsen et al., 2000; Salamonsen and Lathbury, 2000). The papers described above indicate that changes in numbers/ proportions of leukocytes occur in the endometrium of women treated with exogenous progestins. However, only a minority of these studies report any correlation with breakthrough bleeding patterns. Studies describing functional changes in leukocytes are lacking and thus the mechanisms by which leukocytes contribute to the pathogenesis of abnormal uterine bleeding remain unclear. The question remains: are the leukocytes a result of pathological changes or do they have a direct causal role?

In many of these studies, the lack of correlation with bleeding patterns is probably related to the smaller than desired sample sizes and sampling at non-bleeding times. Insufficient biopsy material for histological and immunohistochemical analysis resulting in a smaller sample size available for analysis is a problem that has been experienced by our laboratory and other investigators (Hadisputra et al., 1996; Rogers, 1996). Indeed, Mehra et al. (1970) reported increased numbers of mast cells during the bleeding phase but not during the bleeding-free interval in endometrial biopsies from women using an IUD who develop abnormal uterine bleeding. Given the very focal nature of both MMP and leukocyte localization, it is likely that analysis of bleeding sites would have provided better correlation between bleeding and these parameters. In addition, the failure to detect an association with bleeding patterns may reflect the complexity of the endometrial response to exogenous steroids, and the likelihood that multiple factors are involved in the pathogenesis of abnormal uterine bleeding rather than a single variable.

The occurrence of breakthrough bleeding implies egress of blood beyond the vascular compartment and loss of epithelial integrity. Hysteroscopic examination demonstrated increased endometrial vascular fragility in Norplant users (Hickey et al., 1996). Although the bleeding patterns of the women were not described, ultrastructural changes in the epithelium, basal lamina and underlying matrix have been reported in the endometrium of women using the LNG-IUD (Pakarinen et al., 1998) and other progestational agents (Ludwig, 1982). MMPs, by degrading mature collagen (the only enzymes able to do so) and collagen IV (a key component of basement membranes) could thus contribute to vascular fragility and loss of epithelial integrity and therefore the pathogenesis of breakthrough bleeding. Leukocytes, through their role in the regulation of MMPs, elaboration of various cytotoxic mediators, phagocytotic and chemotactic functions and
Figure 5. Postulated interactions between different endometrial constituents in women using progestin-only contraceptives leading to an alteration in the matrix metalloproteinase (MMP)/tissue inhibitors of metalloproteinase (TIMP) balance and subsequent extracellular matrix (ECM) breakdown contributing to abnormal uterine bleeding. Different progestins may exert differing effects on endometrial epithelial and stromal cells changing the chemokine/cytokine environment thereby contributing to the different endometrial morphological and functional differences observed between different contraceptive agents. The changes in the chemokine/cytokine milieu is likely to result in the infiltration and activation of leukocytes, production and activation of MMPs by both leukocytes and stromal/epithelial cells and resultant degradation of the ECM contributing to endometrial tissue loss and vascular fragility. Modified from Salamonsen and Woolley (1996).

References
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