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Endometrial leukocytes and menstruation

Lois A.Salamonsen[1] and Louise J.Lathbury

Prince Henry's Institute of Medical Research, PO Box 5152, Clayton, Victoria 3168, Australia

This review examines evidence supporting the concept that menstruation occurs as a result of an inflammatory process. In the endometrium, leukocyte numbers rise in the late secretory phase following the fall in serum progesterone concentrations. It is postulated that products released following activation of these leukocytes are critically important for menstruation. Mast cells, eosinophils, neutrophils and macrophages in particular are involved. Endometrial granular lymphocytes may also play a role, although their increase in numbers is somewhat earlier during the menstrual cycle than that of the others, suggesting perhaps a primary role in embryo implantation. Leukocyte products include a range of proteases, chemokines and cytokines which in concert result in focal production and activation of matrix metalloproteinases by endometrial cells and the subsequent breakdown of tissue that characterizes menstruation. Regulation of leukocyte entry, proliferation, differentiation and activation within the endometrium is not yet well understood, although both chemokines and cytokines produced locally by endometrial cells are clearly implicated. The role of progesterone in regulating these events is still not understood although the lack of progesterone receptors on endometrial leukocytes suggests indirect actions.

Key words: leukocyte/menstruation/chemokine/matrix metalloproteinase/progesterone

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Menstruation

Menstruation is the shedding of cell debris accompanied by uterine bleeding which occurs in the endometrium at the end of each normal menstrual cycle. It arises from the disintegration of the superficial or functionalis layer of the endometrium and results in almost complete loss of the functionalis over a period of 5–6 days. Re-epithelialization occurs simultaneously with the tissue destruction and these processes are followed by regeneration of the stromal components. This remarkable tissue remodelling in an adult tissue is unparalleled in any other organ.

The entire cyclical series of events in the endometrium is orchestrated by the ovarian steroid hormones. Menstruation is initiated by the fall in oestrogen and progesterone that results from the demise of the corpus luteum at the end of a non-fertile cycle. It can be mimicked by withdrawal of exogenous hormones (as in the case of oral contraceptive use) or by administration of progesterone receptor (PR) antagonists, e.g. mifepristone at the appropriate time of the cycle (Fraser, 1997). However, although oestrogen and progesterone fall rapidly with corpus luteum degeneration in all mammals with oestrous cycles, only women and some Old World primates menstruate. Thus, there must be features peculiar to the primate endometrium that regulate menstruation.

Classic concepts of the mechanism of menstruation, which remain the current dogma, originate from the work of Markee (1940) who examined the morphological changes in autologous endometrium transplanted to the anterior eye of the rhesus monkey. He observed that prior to menstruation there was vasoconstriction of the spiral arterioles followed by vasodilatation

[1]To whom correspondence should be addressed at: Prince Henry's Institute of Medical Research, PO Box 5152, Clayton, Victoria 3168, Australia. Phone: 61 3 9594 4373; Fax: 61 3 9594 6125; Email: lois.salamonsen@med.monash.edu.au

and postulated that the tissue destruction occurred by necrosis resulting from anoxia. However, overall endometrial blood flow is not significantly reduced just prior to or during menstruation (Fraser and Peek, 1992), and the occurrence of widespread anoxia has not been substantiated in human endometrium. In addition, it has been demonstrated that although the onset of bleeding is the first outward sign of menstruation, it is clearly not the first important event in the process. During the late luteal phase of the cycle, widespread degeneration in the basal lamina supporting the decidualized endometrial cells and the endothelium of blood vessels has been reported (Roberts *et al.*, 1992). Furthermore, scanning electron microscopy has revealed small lesions in the luminal epithelium on day 28 of the cycle, the appearance of these being followed by very rapid but incomplete degeneration of the functionalis layer (Ludwig and Spornitz, 1991).

The concept that menstruation arises as a result of an inflammatory process was first postulated by Finn (1986) and has gained considerable credibility from a large body of data published during the past decade. The hypothesis was originally based on a number of features of endometrium during the late secretory phase: the presence of tissue oedema, the influx of migratory cells and the presence of decidual cells which have some features representative of granulation tissue fibroblasts. The entry of inflammatory cells into the endometrium, their release of potent mediators, possible control mechanisms for these cells, and the potential for the mediators to initiate tissue destruction at menstruation will be reviewed in this article.

Cellular composition of human endometrium

The cellular composition of the human endometrium is dynamic, altering almost on a daily basis in parallel with the hormonal changes during the normal menstrual cycle. The first event following menstruation is re-epithelialization, initiated from the stem cells in the glands in the basalis layer and possibly also from residual rafts of luminal epithelium. Subsequently, throughout the proliferative phase of the cycle, both stromal fibroblasts and cells associated with blood vessels undergo mitosis until the full thickness of the endometrium is restored. During the secretory phase of the cycle, differentiative changes occur in many of these cell types including the decidualization of some stromal cells. In the absence of an embryo, and following the demise of the corpus luteum, there is a diminution in the thickness of the endometrium, thought to result from loss of water, and menstruation follows. Discussion of endometrial cell biology generally ignores the population of lymphomyeloid cells. The total and relative numbers of these cells vary with the different phases of the cycle and are at greatest abundance during the premenstrual phase (Klentzeris *et al.*, 1992; Loke and King, 1995; Salamonsen and Woolley, 1999).

Endometrial leukocytes

Leukocytes in the endometrium have been largely identified by immunostaining or fluorescence-activated cell sorting (FACS) analysis of specific surface antigens although some histochemical stains (e.g. phloxine tartrazine for granular lymphocytes) are still widely used. Caution must be applied when using these techniques as variations in fixation procedures can substantially alter the numbers of cells staining and probably account for differences between reports from individual laboratories. Such discrepancies may relate also to the difficulties in cell counting when cells are not distributed evenly through the tissue (for example, T cells; Starkey *et al.*, 1991), where cells are so numerous that it is difficult to score each separately, and where there is variability in the morphology of the tissue, particularly the degree of oedema and the density of the glands in the section. There may also be variability in cell distribution in the basalis compared with the functionalis. Studies in our laboratory and others correct for at least some of these anomalies by expressing leukocyte numbers per 1000 stromal cells and by assessing distribution between cellular compartments (Vincent *et al.*, 1999). Evidence is also emerging that at any one time, subsets of each cell type, represented by different phenotypes, are present in the endometrium and that apparently similar cells in the endometrium can be functionally different from those in the blood of the same individual (Shi *et al.*, 1995; White *et al.*, 1997). Thus at least some of the cells which traffic into the endometrium must undergo phenotypic changes in response to the new local environment. Most of the phenotypic changes so far recorded relate to regulatory molecules rather than surface proteins and are detailed in Table I.

Neutrophils

Neutrophils are the most abundant leukocytes in the human immune system. They contain specific secretory granules which store a heterogeneous range of regulatory molecules and which differ with the stage of neutrophil maturation. These cells are closely associated with tissue damage in inflammatory disorders. Neutrophils have been identified in endometrial tissue by their morphology and also by immunolocalization of the neutrophil-specific protease, elastase. Endometrial neutrophils have also been defined as $CD11b^{bright}$, $CD66b^{+}$ and $CD16^{+}$ (Yeaman *et al.*, 1998). During most of the cycle, neutrophils are barely detectable in normal endometrium but the numbers rise dramatically perimenstrually, making up 6–15% of the total cell number in the tissue at this time (Salamonsen and Woolley, 1999). Neutrophils have also been identified in increased numbers in areas of endometrial breakdown in patients treated with high-dose oral progestins (Song *et al.*, 1998) or implanted levonorgestrel (Norplant®; The Population Council, New York, NY, USA) (Vincent *et al.*, 1999), where they reach densities similar to those seen in menstrual endometrium. There appears to be more than one neutrophil phenotype in endometrium as

Table I. Endometrial leukocyte phenotype

Leukocyte	Abundance: days 26–28[a]	Surface phenotype	Enzyme content	Other proteins	Actions
Neutrophil	6–15%	$CD11b^{bright}$ $CD66b^{+}$ $CD16^{+}$	elastase, MMP-9, MT1-MMP	IFN-γ, inhibin βA	U
Eosinophil	3–5%	U	ECP-1, ECP-2, MMP-9	eotaxin	U
Macrophage	6–15%	$CD68^{+}$	MMP-9	IL1-receptor antagonist, inhibin βA	U
Mast cell	3–5%	U	tryptase, chymase	U	U
T lymphocyte	1–2%	$CD3^{+}$, $CD4^{+}$/ $CD8^{+}$ICAM-1^{+}, VLA-1^{+}, $CD69^{+}$HLA-DR,DP,DQ^{+}	U	IFN-γ	cytolytic
eGL	6–5%	$CD56^{bright}$, CD16-, $CD2^{+}$, $CD122^{+}$, CD49a,$d^{+}CD69^{+}$, HLA-DR^{+}, LFA-1^{+}, CD3-	perforin, granzyme A, MT1-MMP	TIA-1	cytolytic, proliferate in response to IL-2

MMP-9 = matrix metalloproteinase-9; MT1–MMP = membrane type-1–matrix metalloproteinase; IFN-γ = interferon γ; ECP = eosinophil cationic protein; IL-1 = interleukin-1; TIA-1 = T cell intracytoplasmic antigen-1; eGL = endometrial granular leukocyte; U = Unknown.

[a]Expressed as a percentage of total endometrial cells at this time in the normal menstrual cycle. From Salamonsen and Woolley (1999).

some, but not all of the neutrophils are immunopositive for matrix metalloproteinase (MMP)-9, (Vincent *et al.*, 1999), activin β_A, which has likely functions in cellular differentiation or apoptosis (Leung *et al.*, 1998) and membrane-type (MT)1–MMP which activates latent MMP-2 (Zhang *et al.*, 2000a). Interferon (IFN)-γ has also been identified in intraepithelial neutrophils in human endometrium (Yeaman *et al.*, 1998). IFN-γ has a role in macrophage activation (Haddad *et al.*, 1997), suggesting at least one potential interaction between adjacent leukocytes at menstruation.

Eosinophils

Eosinophils function as effector cells that play an important role in the pathogenesis of late-type inflammatory reactions (Bousquet *et al.*, 1990). Eosinophils have been detected in human endometrium by immunolocalization of eosinophil cationic proteins (ECP1 and ECP2). Like neutrophils, they are absent from normal endometrium during most of the cycle but immediately prior to menstruation there is a dramatic increase in their numbers in the tissue. Most of them are found as aggregates and the extracellular location of the ECP suggests activation of the cells (Jeziorska *et al.*, 1995). Some, but not all of the eosinophils are positive for MMP-9 (Jeziorska *et al.*, 1995) (Vincent *et al.*, 1999). As expected, endometrial eosinophils immunostain positively for both the chemokine eotaxin and for its receptor CCR3 (Zhang *et al.*, 2000b). These cells are negative for both MT1–MMP (Zhang *et al.*, 2000a) and activin β_A (Leung *et al.*, 1998).

Monocytes/macrophages

Resident macrophages in tissues arise by differentiation from monocytes which have migrated from the blood. They display marked phenotypic heterogeneity and respond to a wide range of signals depending on the immediate tissue microenvironment. These cells are an integral part of the immune response in chronic inflammatory lesions and are found at sites of active tissue remodelling. Macrophages ($CD68^{+}$) have been detected in endometrium throughout the menstrual cycle with an increase in numbers from the proliferative through to the menstrual phase (Bonatz *et al.*, 1992). On days 27–28, their numbers are similar to those of neutrophils, comprising between 6–15% of the total cells in the functionalis at this time (Salamonsen and Woolley, 1999). The cells are scattered throughout the tissue, with some aggregates and some concentration around endometrial glands (Song and Fraser, 1995). Increased numbers of macrophages are also found in endometrium from Norplant users (Clark *et al.*, 1996) particularly in tissue from those women with abnormal uterine bleeding. Endometrial macrophages show phenotypic differences, and subtypes have been described with respect to expression of MMP-9 (Jeziorska *et al.*, 1995), activin β_B (Leung *et al.*, 1998) and MT1-MMP (Zhang *et al.*, 2000a). Interleukin (IL)-1 receptor antagonist, a component of the IL-1 system that prevents binding of IL-1 to its type 1 receptor, has also been detected in these cells (Tabibzadeh and Sun, 1992).

Mast cells

Mast cells secrete many vasoactive, nociceptive and proinflammatory molecules and participate in inflammation through vasodilation and enhancing leukocyte infiltration as well as causing direct tissue damage by their release of proteases. Mast cells are present in the endometrium throughout the menstrual cycle. They are detected by immunostaining for the two mast cell-specific serine proteinases, tryptase and chymase, and the extracellular location of the enzymes is indicative of activation.

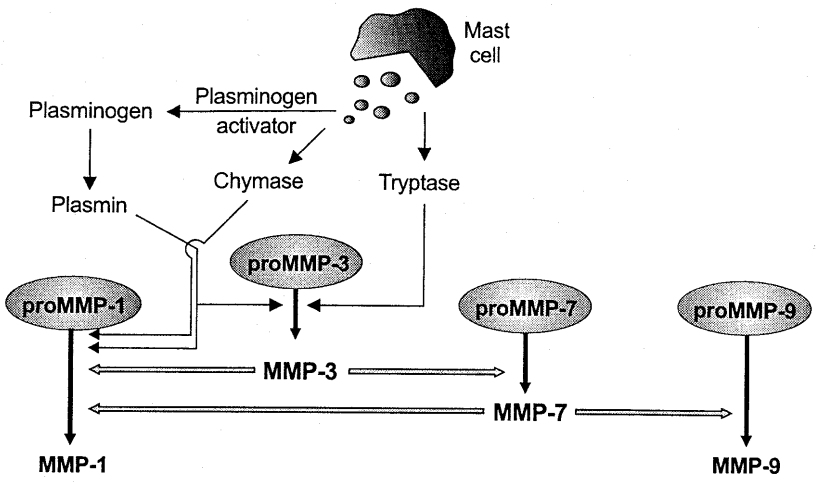

Figure 1. Cascade of matrix metalloproteinase (MMP) activation postulated to be initiated by mast cell degranulation. (Reprinted from Salamonsen and Woolley 1996, with permission).

Endometrial mast cell activation corresponds closely to the phases of tissue oedema and is most marked immediately prior to menstruation (Jeziorska *et al.*, 1995). Mast cells fall into two well-known phenotypic subtypes. Mucosal mast cells are positive for tryptase but not chymase, whereas connective tissue mast cells contain both enzymes. Both of these subtypes have been detected in endometrium, with regional differences in location. Those in the basalis region express both enzymes whereas those in the functionalis are positive only for tryptase (Jeziorska *et al.*, 1995). While it is the functionalis that is shed at menstruation, there is considerable tissue destruction at the basalis–functionalis interface and it is likely that both enzymes contribute to menstruation. Tryptase, and to a lesser extent chymase, can play an important role in establishing a cascade of MMP activation (Figure 1) and this could represent a critical function for these cells at menstruation. Mast cells also produce histamine, heparin, arachidonate products and a variety of multifunctional cytokines and growth factors, all of which have marked effects on endothelial cell function and locally induced oedema. The biological consequence of mast cell degranulation within the endometrium is likely to be extremely complex because it appears that the synthesis and release of specific cytokines and arachidonate products may occur at specific stages post activation. Thus a complex sequence of signals can be produced within the localized domain of the activated mast cell (Norrby and Woolley, 1993).

B and T lymphocytes

$CD3^+$ T cells are present in the endometrium throughout the cycle and their numbers increase prior to menstruation. However, their total numbers are much less than those of other leukocytes (only 1–2% of the total lymphomyeloid cells) (Salamonsen and Woolley, 1999). They are found in three sites: as basal lymphoid aggregates, scattered throughout the stroma and in intraepithelial sites (Loke and King, 1995; Song and Fraser, 1995). Interestingly, the CD4 (T helper phenotype) to CD8 (cytotoxic T cell phenotype) ratio in endometrium is inverted when compared with peripheral blood T cells (66% $CD8^+$ to 33% $CD4^+$ in endometrium). The cells are cytolytically active during the proliferative phase of the cycle but this activity is diminished during the secretory phase. Therefore, progesterone may down-regulate this property (White *et al.*, 1997), although whether this activity is restored when progesterone concentrations fall or whether it exists at menstruation remain to be established. Neither is it known whether the alteration in phenotype around mid-cycle results from a change in the activity of existing cells, the deletion of a minor subset of these cells or the selective migration of specific cell subsets into or away from the endometrium. The observation that peripheral blood $CD3^+$ cells remain cytolytically active throughout the menstrual cycle supports the concept of differentiation within the tissue. Subsets of endometrial T cells express MMP-9 (Vincent *et al.*, 1999) and a proportion of the intraepithelial $CD3^+$ T cells are IFN-γ positive (Yeaman *et al.*, 1998).

$CD45RA^+$ B lymphocytes are also detected in low numbers during the entire cycle and are present in peri-menstrual tissue in clusters among the stromal cells (Salamonsen and Woolley, 1999). Whether or not these cells produce immunoglobulin within the tissue is not known.

Endometrial granular lymphocytes (NK cells)

Granulated cells were recognized in non-pregnant endometrium as early as 1921 (Weill, 1921). Subsequently, these phenotypically unusual $CD56^+$, $CD2^{+/-}$, $CD38^+$, CD16–, and CD3– lymphocytes, commonly known as endometrial granular lymphocytes (eGL) (Bulmer *et al.*, 1991; King *et al.*, 1989;

Klentzeris *et al.*, 1992) have been shown to be among the most numerous haematopoietic cells in perimenstrual endometrium. They have been identified by staining with phloxine–tartrazine and by immunolocalization. Importantly, the CD56 antigen is not readily detectable in formalin-fixed tissue, unless an antigen retrieval step is used to expose the epitope. The CD43 antigen which survives this fixation is often utilized as an alternative, although it also detects some macrophages and T cells. Very few eGLs are present during the proliferative phase but numbers increase during the secretory phase and have been assessed variously as being up to 15% (Salamonsen and Woolley, 1999) or 25% (Bulmer *et al.*, 1991) of the total number of cells in the stroma perimenstrually. The cells are found scattered throughout the stroma and in intraepithelial locations (Song and Fraser, 1995). Due to their expression of CD56, these cells are said to be of the natural killer (NK) lineage (King *et al.*, 1991) but their exact relationship to the $CD56^+$ $CD16^+$ NK cells in peripheral blood is not yet clear (King *et al.*, 1991). From the late proliferative phase on, eGL have cytotoxic activity comparable to that of peripheral blood NK cells (Jones *et al.*, 1998b), suggesting they may be involved in protecting against infection. In support of this notion, the proportion of eGL expressing the activation antigens CD69 and human leukocyte antigen (HLA)-DR was highest in the proliferative phase and decreased during the menstrual phase (Kodama *et al.*, 1998). Further, this suppression of activation antigens in secretory endometrium suggests local functional regulation at this time. eGL contain perforin, granzyme A and T cell intracytoplasmic antigen (TIA)-1 granules (King *et al.*, 1993) and some stain positively for MT1–MMP (Zhang *et al.*, 1999a). All of these factors could contribute to cell and tissue degradation. Whether eGLs have a role in menstruation is not known: it may be that their role at the end of the cycle is one of preparation for pregnancy rather than one specifically directed towards the process of menstruation. This hypothesis was tested in part in a recent in-vivo study in women to whom progesterone was administered and then withdrawn; the eGL numbers in endometrium did not alter following withdrawal (Critchley *et al.*, 1999). However, additional studies are required to finally support or to disprove this concept.

Regulation of leukocyte entry into endometrium

It is not yet known whether in-situ proliferation or migration from the peripheral circulation is responsible for the increase in leukocyte subpopulations in the late luteal phase but it is likely that both of these processes are relevant. Whether these processes are driven directly by ovarian hormones or whether they are mediated via other factors such as chemokines or adhesion molecules is still not clear.

Steroid hormones

The female steroid hormones, oestrogen and progesterone, act via specific receptors (ER and PR respectively) that belong to a large family of nuclear transcription factors which regulate the expression of numerous genes. Two forms or subtypes of each receptor have been identified with the physiological significance of each form currently unknown. The predominant form of ER in all cell types in the uterus throughout the menstrual cycle is ERα, although weak expression of mRNA for the β-subtype is also detectable (Matsuzaki *et al.*, 1999). At the time of menstruation, expression of ERα in endometrial cells is at a nadir. The isoforms of PR are known as PRA and PRB. In human endometrium, PRA is present in glandular and stromal cell nuclei during the proliferative phase but is present only in stromal cells by the end of the secretory phase (Wang *et al.*, 1998). PRB is also present in both cellular compartments in the proliferative phase and absent in the late secretory phase. Thus those PR which have been localized in cells around the vessels in the secretory phase are probably PRA and are likely to mediate any effects of progesterone in the endometrium during the late secretory phase.

Whether or not human endometrial leukocytes express either ER or PR has been a subject of some controversy, not helped by the discovery of the different receptor subtypes. The most recent immunohistochemical data shows that uterine $CD45^+$ leukocytes (including endometrial lymphocytes, macrophages and T cells) express neither ER nor PR (King *et al.*, 1996; Stewart *et al.*, 1998) and thus the effects of oestrogen and progesterone on these cells are likely to be indirect. In rodents, steroid hormone receptors have been demonstrated in a variety of immune cells. Despite their very low binding affinities, numerous effects of the steroids on these cells in rodents support direct actions (for review see Miller and Hunt, 1996). It still remains to be conclusively established whether or not any of the subsets of human endometrial leukocytes express low concentrations of ER or PR and whether there are any direct responses of these cells to the hormones.

Chemokines

The influx of migratory cells into the endometrium is likely to be regulated by chemokines, potent chemoattractant cytokines that promote the recruitment of multiple lineages of leukocytes and which act via specific receptors on attracted cells (Kunkel *et al.*, 1995). The chemokine superfamily has been divided into four groups, CC, C, CXC and CXXXC. The CC and CXC chemokines are the best studied and are differentiated both structurally and by a general distinction in biological properties: most CXC chemokines are chemoattractants for neutrophils (and to some extent lymphocytes) but not monocytes, whereas CC chemokines appear to attract monocytes, lymphocytes, basophils and eosinophils but not neutrophils.

Chemokines mediate their effects via interactions with G protein-linked receptors on the surface of target cells. There are a large number of these receptors with most being promiscuous in their ligand binding. A relatively new concept is that leukocytes can respond sequentially to chemokines in a multi-step

Table II. Chemokines in human endometrium

Chemokine	Chemoattractant for:	Cellular location	Reference
RANTES	Eosinophils, basophils, monocytes, activated T cells, NK cells	stroma	Hornung *et al.*, 1997
IL-8	Neutrophils, T cells, basophils	epithelium, perivascular cells	Arici *et al.*, 1998 Critchley *et al.*, 1994; Jones *et al.*, 1997
MCP-1	Monocytes, activated T cells, NK cells, basophils	epithelium stroma perivascular cells	Jolicoeur *et al.*, 1998 Hampton and Salamonsen, unpublished observations Jones *et al.*, 1997
MCP-2	monocytes,activated T cells, NK cells, basophils, eosinophils	epithelium stroma perivascular cells	Hampton and Salamonsen, unpublished observations
eotaxin	eosinophils, basophils, T lymphocytes that co-localize with eosinophils	epithelium eosinophils perivascular cells, decidualized cells	Zhang *et al.*, 2000b

RANTES = regulated upon activation, normal T cell expressed and secreted; IL-8 = interleukin-8; MCP = monocyte chemotactic protein.

navigation mode with a corresponding sequential expression of different chemokine receptors (Foxman *et al.*, 1997).

Regulation of chemokine production is still only partly understood but typical members of the major groups are stimulated by IL-1 and inhibited by glucocorticoids (Ben-Baruch *et al.*, 1995). Given the structural similarity between progesterone and glucocorticoids and their receptors, it is highly likely that progesterone could act in progesterone-dependent tissues in a similar manner to glucocorticoids in other tissues. Certainly, in mice with targeted disruption of the progesterone receptor, homozygous offspring have inflammatory abnormalities of the uterus (Lydon *et al.*, 1995).

The chemokines which have been examined in the human endometrium are summarized in Table II. In some cases, data regarding the cellular localization is somewhat conflicting, possibly reflecting variability between recognition sequences of the different antisera used for immunolocalization or differences between the sensitivity of different immunohistochemical protocols.

RANTES (regulated upon activation, normal T cell expressed and secreted), acts as a chemoattractant for several leukocyte subsets including monocytes and activated T cells. mRNA and protein for this chemokine have been localized primarily to the stromal compartment of human endometrium, with glandular epithelium appearing mostly free of the antigen (Hornung *et al.*, 1997). However, premenstrual endometrium was not examined. *In vitro*, both IL-1 and tumour necrosis factor (TNF)α, but not oestrogen or progesterone stimulated RANTES production by purified endometrial stromal cells.

IL-8 has neutrophil chemoattractant/activating and T cell and fibroblast chemotactic activities. In one study of endometrial tissue this chemokine was localized to perivascular cells of blood vessels, increasing significantly in the late secretory phase (Critchley *et al.*, 1994; Jones *et al.*, 1997). However, in data from another laboratory, the strongest staining was detected in the surface epithelium and glands, with less intense staining in the walls of blood vessels and no staining in the stroma (Arici *et al.*, 1998). The epithelial staining intensity increased across the cycle from proliferative to late secretory endometrium. Of particular relevance is a study of in-vivo progesterone withdrawal in women, in which endometrial IL-8 protein rose significantly at 48 h after withdrawal (Critchley *et al.*, 1999), suggesting that the fall in progesterone late in the cycle could contribute to a rise prior to menstruation. Cell culture studies using passaged endometrial stromal cells demonstrated increased production of IL-8 in the presence of IL-1 and TNFα (Arici *et al.*, 1996) and a slight decrease with transforming growth factor (TGF)β (Arici *et al.*, 1996). As stromal staining is not observed *in situ*, whether in-vitro stromal production of IL-8 is an artefact of the cell culture conditions remains to be established.

The major known biological actions of the monocyte chemotactic proteins (MCP) are monocyte activation and recruitment of these cells into sites of inflammation. Four structurally related molecules (MCP-1, MCP-2, MCP-3, MCP-4) act via different receptors and may have some functional differences (Van Coillie *et al.*, 1997). MCP-1 at least can also activate basophils, mast cells and NK cells (Allavena *et al.*, 1994; Feliciani *et al.*, 1995). MCP-1 has been immunolocalized in human endometrium in several studies. In one study, it was localized to perivascular cells with intense immunostaining in the early–late proliferative and late secretory phases (Jones *et al.*, 1997). In contrast, other investigators have localized MCP-1 primarily in epithelial cells (Jolicoeur *et al.*, 1998). In our laboratory, MCP-1 immunostaining was extremely variable between individual endometrial samples even on the same day of the cycle, with only 77% of tissues showing epithelial staining, 52% of tissues

demonstrating staining in the stroma and 34% of the tissues with vascular staining. No variation across the menstrual cycle was observed (A.L.Hampton and L.A.Salamonsen, unpublished observations). Secretion studies on stimulated endometrial stromal and epithelial cells in culture (Arici *et al.*, 1995) and in-situ hybridization studies (Jolicoeur *et al.*, 1998) also support a primary epithelial source of MCP-1. MCP-2, a related chemokine, is likewise expressed in human endometrium with a predominantly epithelial localization. This chemokine is also detectable in uterine secretions, suggesting a potential role other than the attraction of monocytes (A.L.Hampton and L.A.Salamonsen, unpublished observations). In other tissues, production of MCP-1 is inducible and can be stimulated *in vitro* by a number of different cytokines including IL-1, TNFα and interferon γ, with combinations of stimuli resulting variously in additive or synergistic induction of MCP-2 compared with MCP-1. When synergy occurs, MCP-1 and MCP-2 expression concentrations can reach a comparable maximum (Struyf *et al.*, 1998).

Eotaxin is one of the most selective of the chemokines, acting primarily on eosinophils through the CCR3 receptor. In the endometrium, eotaxin was detected in perivascular cells in the late secretory phase (Zhang *et al*., 2000b), a time when eosinophils are found in the tissue (Jeziorska *et al.*, 1995; Poropatich *et al.*, 1987). However, this chemokine was also strongly expressed by both luminal and glandular epithelial cells throughout the entire menstrual cycle, and was detected in epithelial and stromal endometrial cells in culture (Zhang *et al*., 2000b). CCR3 is also strongly expressed in human endometrium, predominantly in eosinophils, but also in epithelium at all stages of the cycle and in perivascular cells in a proportion of blood vessels (Zhang *et al*., 2000b). As CCR3 is expressed strongly by the same cells that produce eotaxin, this chemokine may act as a paracrine growth factor. It is important to note that although eotaxin is relatively specific for eosinophils, CCR3 also binds RANTES, MCP-3 and MCP-4.

The overall picture emerging from chemokine studies in the endometrium is that chemokines could be involved in controlling the influx of leukocytes prior to menstruation. Once the cells have entered the tissue the epithelial chemokines may attract them to the subepithelial space and could also 'prime them' for subsequent responses. The experimental data reviewed here also strongly suggests additional paracrine roles for these pleiotropic molecules in endometrial remodelling. Known chemokine actions of possible relevance include regulation of precursor cell cycling and differentiation, and growth factor and angiogenic activities (Schall, 1994). Further studies are required to elucidate the precise biological activities of the chemokines of epithelial cell origin in this tissue and their paracrine targets.

Adhesion molecules

Adhesion molecules are necessary for the attachment of leukocytes to endothelium and for their extravasation and trafficking through tissues. Considerable heterogeneity has been shown in the vasculature of the endometrium with respect to the expression of various adhesion molecules (Tabibzadeh *et al.*, 1994). The intercellular adhesion molecule (ICAM)-1 has been identified in the stroma of the functionalis during the menstrual phase of the cycle ((Tawia *et al.*, 1993; Thomson *et al.*, 1999) and evidence from in-vitro studies suggests this molecule may be constitutively shed from the surface of endometrial stromal cells (Somiglianna *et al.*, 1996). ICAM-1 was also expressed by $CD3^+$ cells in the lymphoid aggregates in the basalis layer and was present on vascular endothelium throughout the cycle with an overall peak in expression at menstruation (Tawia *et al.*, 1993) although this was not consistent across all vessel types. In contrast, ICAM-2 expression was restricted to the vascular endothelium, without change across the menstrual cycle (Thomson *et al.*, 1999). Platelet endothelial cell adhesion molecule (PECAM) was also intensely stained in the stroma during menstruation and in endothelial cells of all vessel types (Tawia *et al*., 1993). VCAM-1 and E-selectin appeared in the stromal cells in the upper functionalis in the secretory phase (Tabibzadeh *et al*., 1994). Furthermore, immunoreactivity for VCAM-1 was the distinguishing feature that separated aggregated lymphoid cells in the tissues from non-aggregated cells, suggesting a function in adhesion of these cells to one another. It is likely that unique cell and site-specific expression of adhesion molecules in the endometrium may account at least in part for the distinct distribution of leukocytes in this tissue

Leukocyte proliferation

Lymphoid cells may enter the endometrium from the blood as appears likely for such cells in the decidua of pregnancy (Marzusch *et al.*, 1993). However, there is some evidence that their increase in the late secretory phase is due to proliferation *in situ*. $CD45^+$, $CD3^+$ T cells, $CD11c^+$ macrophages and $CD56^+$ eGLs express the proliferation markers Ki67 and/or BrdU within endometrium throughout the menstrual cycle with a marked increase in the secretory phase (Pace *et al.*, 1989; Tabibzadeh, 1990; Jones *et al.*, 1998a). However, in perimenstrual tissue eGLs undergo morphological changes indicative of apoptosis, although their high expression of bcl-2 (which inhibits apoptosis) at this time suggests they are still functional (Jones *et al.*, 1998a). eGLs are particularly numerous in decidual tissue in pregnancy (Ritson and Bulmer, 1989; King *et al.*, 1991) and these display a repertoire of killer inhibitory and activatory receptors (Hilby *et al.*, 1997). Both decidual eGLs and those in cycling endometrium proliferate in response to IL-2 (Jones *et al.*, 1998b; Searle *et al.*, 1999): however, there is no evidence for the presence of IL-2 within normal endometrium (Jokhi *et al.*, 1994; King *et al.*, 1995; Saito *et al.*, 1993). In-vitro studies have demonstrated that $CD56^+$ cells also proliferate in the presence of endometrial stromal cells and progesterone (Inoue *et al.*, 1996), suggesting that progesterone-dependent stromal factors may be mitogenic for these cells. Furthermore, proliferation of peripheral blood monocytes in culture was influenced by a soluble

factor produced by uterine epithelial cells in response to cytokines (Prabhala *et al.*, 1998). Thus, in-situ proliferation, regulated by locally produced factors, is apparently one mechanism by which the pre-menstrual increase in these cells is achieved.

Leukocyte activation

The presence of leukocytes in a tissue does not necessarily mean that they continually contribute to the function of that tissue. Many leukocytes can remain inactive in a tissue; their activation results in the release of a plethora of bioactive regulators. For example, mast cells within the endometrium are inactive during most of the cycle, and phases of activation (as detected by extracellular tryptase) coincide with tissue oedema, particularly immediately prior to menstruation (Jeziorska *et al.*, 1996). Likewise, eosinophil activation (as evidenced by extracellular eosinophil cationic proteins) also occurs during the perimenstrual phase of the cycle (Jeziorska *et al.*, 1996). T cell activity may be related to local secretion of cytokines such as IFNγ, which is apparent in lymphoid aggregates in the stratum basalis but less consistently in functionalis throughout the cycle (Stewart *et al.*, 1992). Other markers of T cell activation (CD69 and DR) are similar in both the proliferative and secretory phases and suggest that in the endometrium, T lymphocytes are in a state of recent and persistent activation (Chen *et al.*, 1995). Neutrophil elastase has also been found extracellularly, indicating neutrophil activation prior to menstruation, although extracellular IFNγ originating from neutrophils was located immediately beneath the luminal epithelium through all stages of the cycle (Yeaman *et al.*, 1998). There is no information regarding macrophage activation at menstruation though this might be expected. Evidence is now emerging that the regulation of leukocytes can also occur via the inhibition of activation, and multiple inhibitory receptors have been identified on mast cells, macrophages and NK cells (Katz and Austen, 1997). The mechanisms by which cells are either activated or inhibited from activation in the endometrium are completely unknown, although it is likely that there are considerable interactions between cell types.

Steroid hormones may act directly on leukocyte differentiation and activation (Miller and Hunt 1996). For example in mouse uterine mast cells oestrogen promotes, and progesterone inhibits production of inducible nitric oxide synthase and TNFα while oestrogen has no effect on macrophages, in which progesterone inhibits production of the same two substances (for review, see Hunt *et al.*, 1997). Indirect actions of steroid hormones via production of paracrine mediators by endometrial epithelial and stromal cells are also likely. For example, IFNγ which can modulate haematopoietic cell maturation, differentiation, activation and apoptosis is produced by mouse uterine epithelial cells during oestrus, when oestrogen is predominent. In human endometrium, many cytokines, including IL-1 and TNFα are produced in endometrial stromal or epithelial cells reaching peak production during the mid- to late secretory phases, coincident with the increased numbers of leukocytes in the endometrium (reviewed in Tabibzadeh and Sun, 1992; Salamonsen and Woolley 1996) and are likely to effect leukocyte differentiation and activation. Other local endometrial mediators, whose production appears to be steroid-hormone modulated in the human and which have potential but as yet undefined interactions with immune cells in the endometrium, include leukaemia inhibitory factor, prostaglandins, TGFβs, endothelin and nitric oxide.

Role of leukocytes in menstruation

Leukocytes have the potential to contribute both directly and indirectly to menstruation. A number of their products may directly promote tissue degradation, while others may contribute indirectly through the activation of latent enzymes produced by adjacent cells, or by stimulating adjacent cells to produce other biologically active molecules. Some of the proteolytic enzymes produced by leukocytes that may play a role in menstruation are listed in Table III and are discussed in further detail below.

Matrix metalloproteinases

Matrix metalloproteinases (MMP) are enzymes that degrade components of both interstitial and basement membrane extracellular matrix, their synthesis being negligible in normal connective tissue (Birkedal-Hansen *et al.*, 1993). The MMP can be divided into four major subfamilies, collagenases (MMP-1, MMP-8), gelatinases (MMP-2, MMP-9), stromelysins (including MMP-3) and MT-MMP. Most MMP are secreted as latent zymogens that can be activated *in vitro* by a number of natural proteases including other MMP. Their expression is differentially regulated by tissue and cell-type specific mechanisms involving cytokines, steroid hormones (including progesterone) and other regulatory molecules. There is now strong evidence for a role for these enzymes in menstruation (Salamonsen and Woolley, 1996; Salamonsen and Woolley, 1999). Most of the MMP so far examined are produced by endometrial stromal cells, although MMP-7 and MMP-2 are epithelial cell products. Furthermore, a number of leukocytes

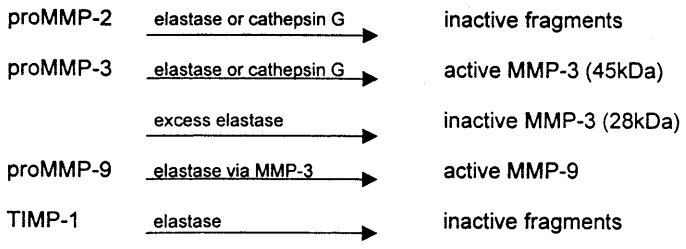

Figure 2. Actions of neutrophil enzymes on matrix metalloproteinases (MMPs)/tissue inhibitors of matrix metalloproteinase (TIMPs) in solution.

Table III. Leukocyte proteases of potential relevance to menstruation

Leukocyte	Protease	Potential substrate
Mast cell	tryptase	activates proMMP-3, uPA
	chymase	activates proMMP-1, proMMP-3, inactivates bradykinin
	chymotrypsin	broad spectrum
	plasminogen activator	plasminogen
Neutrophil	elastase	elastin, proteoglycans, collagens III, IV
	MMP-8	collagens,I,III,VII, VIII, X
	MMP-9	collagen IV, V, VII elastin, FN, LN
	MT1-MMP	activates proMMP-2, proMMP-13, degrades FN, tenascin, nidogen, perlecan, collagen I,III
	heparanase	heparan sulphate proteoglycans
	cathepsin G	elastin, proteoglycans collagen III, IV
Eosinophil	MMP-1	collagen I, III, VII, VIII, X
	MMP-9	as above
	β glucuronidase	proteoglycans
	aryl sulphatase	proteoglycans, GAG
Macrophage	MMP-9	as above
	metalloelastase	elastase, collagen IV, LN, FN, VN, heparin, chondroitin sulphates
	MT1–MMP	as above
	Plasminogen activator	as above
T lymphocyte	MMP-2	as above
	MMP-9	as above
eGL	MT1-MMP	as above

MMP = matrix metalloproteinase; MT1–MMP = membrane type-1–matrix metalloproteinase; FN = fibronectin; LN = laminin; VN = vitronectin; GAG = glycosaminoglycan

produce MMP (see Table III). These and other factors produced by leukocytes have the potential to stimulate the production of latent MMP produced by adjacent cells or participate in MMP activation. In particular, once active MMP-3 is present, a cascade of MMP activation can be initiated, as seen in the case of activation by mast cell tryptase (Figure 1) or neutrophil elastase (Figure 2). The recent finding that MT1-MMP, which activates latent MMP-2, is present in membranes of neutrophils and eGL (Zhang *et al*., 2000b), would provide for local activation of MMP-2 around these cells. The actions of MMPs are apposed by the binding of their active forms by specific tissue inhibitors of MMPs, the TIMPs, which are abundant in human endometrium (Zhang and Salamonsen, 1997).

Studies in our laboratory have examined the regulation of matrix metalloproteinase production and activation by endometrial stromal cells *in vitro*. The human mast cell line (HMC-1) produces the mast cell products tryptase, IL-1 and TNFα. When co-cultured with endometrial stromal cells, HMC-1 cells stimulated stromal cell proMMP-1 and proMMP-3 and to a lesser extent proMMP-2 production, with increasing stimulation as mast cell numbers increased (Zhang *et al*., 1998). These effects were largely abrogated by pre-adsorption of the mast cell conditioned medium with antisera against both IL-1 and TNFα. Further, medium from mast cells activated latent MMP-3 and MMP-1 in stromal cell conditioned medium in the presence of heparin (which stabilizes tryptase activity). Other experiments examined interactions within co-cultures of freshly prepared peripheral blood neutrophils and endometrial stromal cells (Lathbury and Salamonson, 1998). Conditioned medium from neutrophils alone contained proMMP-9 and elastase, while conditioned medium from co-cultures contained active MMP-9 and MMP-3 and fragments of degraded MMP-2. MMP-3 activation and MMP-2 degradation were not observed when the neutrophil conditioned medium was pre-treated with anti-elastase. Therefore it is likely that elastase is involved in the activation of MMP in the endometrium in the vicinity of activated neutrophils.

Other degradative enzymes

Many of these enzymes are listed in Table III and only some will be discussed here. The activation of MMP-3 is a critical step in the cascade of MMP activation and any leukocyte products which facilitate this process will aid in the generation of a full complement of enzymes, capable of degrading all the components of the extracellular matrix. Plasminogen activator, produced by mast cells and macrophages, converts plasminogen to plasmin, which activates both MMP-1 and MMP-3. These two latent enzymes can also be activated by the mast cell-specific enzymes tryptase and chymase. The neutrophil-specific

enzymes elastase and cathepsin G can activate MMP-3. Elastase can also degrade elastin, proteoglycans and type III and type IV collagens, all of which are found within endometrium. Macrophages at sites of inflammation produce a variety of cysteine, serine and metalloenzymes which may be involved in tissue degradation.

Other regulatory molecules with potential roles at menstruation

Description of the plethora of regulatory molecules produced by leukocytes is beyond the scope of this article and has been reviewed by other authors, particularly with respect to mast cells (Galli, 1993; Schwartz, 1994), macrophages (Nathan, 1987) and eosinophils (Kroegel *et al.*, 1994). They include cytokines, vasoactive substances such as nitric oxide and prostaglandins and reactive oxygen species, all of which may play important roles at menstruation (Salamonsen *et al.*, 1999).

Leukocytes and tissue regeneration

Repair of the endometrium begins as early as 36 h after the onset of menstrual bleeding, while tissue breakdown is still in progress, emphasizing the very focal nature of the degradative and repair processes (Ludwig *et al.*, 1990). Regeneration of epithelium from stumps of glands occurs first, followed by thickening of the stroma and mitosis in the endothelium which begins around days 4–5. Given the abundance of leukocytes in the tissue at this time it is highly likely that they contribute to the process of regeneration. Certainly, macrophages are responsible to some extent for the removal of detritus (Ludwig *et al.*, 1990). The process of wound healing, in general, appears to be initiated by a wealth of cytokines and growth factors, and platelets recruit inflammatory cells, particularly neutrophils and macrophages (for review, see Martin, 1997), which produce these necessary factors. In particular, macrophages at wound sites express TGFα, fibroblast growth factors (FGFs), platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF), TGFβ and activin β_B (Martin, 1997), while products of other leukocytes can activate the macrophages. Eosinophils in healing wounds express TGFα and $TGF\beta_1$ (Todd *et al.*, 1991; Wong *et al*., 1993), while lymphocytes are capable of producing the growth factors bFGF and leukocyte-derived growth factor (Blotnick *et al*., 1994; Iida *et al.*, 1996). Isoforms of activin are involved in the proliferation of fibroblasts at a wound margin (Hubner *et al.*, 1996), while TGFβ encourages the synthesis of new collagen-rich matrices (Eckes *et al.*, 1996) and mast cell tryptase can act as a mitogen for epithelial cells (Cairns and Walls, 1996). Endometrial repair is likely to have similarities to tissue repair in embryos, in which scarring is not found and which uses many different regulatory molecules. Almost nothing is known at this time of the cellular and molecular mechanisms of endometrial repair. There is a need to understand this process, given that much abnormal uterine bleeding results from fragile endometrium which is predisposed to bleed, presumably as a result of inadequate repair mechanisms. It is likely that leukocytes will play an important role in this process as we now understand they do in the corollary, menstruation.

Conclusions

There is now strong evidence for a role for leukocytes in the normal biology of human endometrium, particularly in endometrial remodelling, implantation and menstruation. Due to the marked differences in endometrial tissue and leukocytes between species, and the difficulty in performing other than correlative studies in the human, the critical studies providing evidence for the contribution of these cells to the above functions are not possible. Further information is required regarding control of entry of the cells and/or their proliferation in the tissue, regulation of their phenotype and activation, and the means by which the changing steroidal milieu exerts its effects on these parameters. While animal models may provide some information, experimentation on human material will ultimately be required to provide answers relevant to the human and which may assist in treatment of endometrial disorders in women.

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