Chemokine and cyclooxygenase-2 expression in human endometrium coincides with leukocyte accumulation

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The endometrium contains a resident population of leukocytes, the number and subtype of which vary throughout the menstrual cycle and in early pregnancy. Factors controlling these fluctuations are unknown, but a combination of proliferation in situ and migration from the vasculature has been proposed. Locally acting inflammatory mediators, including specific chemokines and prostaglandins, have been implicated in these processes. Interleukin-8 (IL-8) and monocyte chemoattractant protein-1 (MCP-1) are potent chemoattractants and activators for neutrophils and monocytes respectively. Locally acting prostaglandins modulate vascular permeability, and a synergistic action of prostaglandin E (PGE) with IL-8 has been described. In the present study IL-8, MCP-1 and cyclooxygenase-2 (COX-2), the inducible isoform of prostaglandin synthase, were all localized in the endometrium by immunohistochemistry throughout the menstrual cycle and in early pregnancy. All three inflammatory mediators were localized to the perivascular cells of blood vessels in endometrium and decidua, and additional immunoreactivity for COX-2 was identified in the glandular epithelium. The intensity of immunostaining was reduced in the periovulatory, early and mid-secretory phases and significantly increased premenstrually. These results further support the hypothesis that there is a premenstrual migration of leukocytes into the endometrium mediated by chemokines.

Key words: cyclooxygenase-2/endometrium/interleukin-8/ leukocytes/monocyte chemoattractant protein-1

Introduction

The human endometrium undergoes rapid proliferation and differentiation under the control of the ovarian steroid hormones oestrogen and progesterone. Associated with the progesterone mediated decidualization in the mid–late secretory phase is the accumulation of leukocytes in the endometrial stroma (Bulmer et al., 1991a; Starkey et al., 1991). These comprise macrophages and the uterine specific large granular lymphocytes (LGLs), and both subtypes persist in early pregnant decidua (Loke and King, 1995). In the absence of pregnancy, a distinct influx of neutrophils is observed in the immediate premenstrual phase (Poropatich et al., 1987).

The exact function of the endometrial population of leukocytes is unknown, although a role in the regulation of implantation and placentation and also in the initiation of menstruation has been suggested (Bulmer et al., 1991b; King and Loke, 1991). The factors controlling their mode of appearance and fluctuation throughout the menstrual cycle are similarly uncertain. The cyclical pattern of leukocyte presence is suggestive of steroidal control, but the recent demonstration that they do not possess either oestrogen or progesterone receptors implies that this regulation is exerted indirectly (King et al., 1996). Co-localization studies have revealed that the stromal leukocytes express the cell proliferation marker Ki67 (Pace et al., 1989; King et al., 1991), indicating that their increase in numbers is due at least in part to in-situ proliferation. It is likely, however, that the migration of leukocytes from the peripheral circulation also contributes to leukocyte accumulation.

Migration is a multistep process involving both the simultaneous expression of leukocyte adhesion molecules and their corresponding receptors on endothelial cells, and additionally the production of specific chemotactic agents (Springer, 1990; Schall and Bacon, 1994). A family of cytokines exhibiting chemotactic activity for specific leukocytes are the chemokines (for reviews see Matsushima et al., 1992; Bagnoli et al., 1994). Interleukin-8 (IL-8), a potent chemoattractant and activator of neutrophils, has been detected in the human cervix (Barclay et al., 1993), endometrium (Arici et al., 1993; Critchley et al., 1994), chorio-decidua (Dudley et al., 1993) and the placenta (Saito et al., 1994). Increased production of IL-8 in the cervix coincides with neutrophil influx prior to ripening at term (Kelly et al., 1992; El Maradny et al., 1996). IL-8 has been localized to the perivascular cells of blood vessels in non-pregnant and pregnant endometrium (Critchley et al., 1994), where it could participate in recruitment of neutrophils and additionally in the process of angiogenesis (Koch et al., 1992).

A suppression of IL-8 production by the endometrium (Kelly et al., 1994) and cervix (Ito et al., 1994) in vitro by progesterone has been demonstrated, acting either through the glucocorticoid response element in the promoter region of the gene or directly through progesterone receptors present within the cell. If a similar downregulation by progesterone occurs in vivo, the withdrawal of progesterone premenstrually would remove the inhibitory effect, resulting in an increased production of IL-8 and accompanying neutrophil influx.

A closely related chemokine is monocyte chemoattractant protein-1 (MCP-1). MCP-1 displays no chemotactic activity
for neutrophils; instead it is a potent attractant and activator for macrophages, T cells (Cai et al., 1995; Roth et al., 1995), basophils, mast cells (Feliciani et al., 1995) and also natural killer cells (Allavena et al., 1994). It is positively regulated by pro-inflammatory stimuli, such as interleukin-1 (IL-1) and tumour necrosis factor (TNF)-α, as is IL-8 (Baggionini et al., 1994), but is additionally induced by platelet derived growth factor (PDGF) in fibroblasts (Yoshimura and Leonard, 1990). This growth factor has no effect on IL-8 production, indicating that regulatory differences in the gene expression of these chemokines exist. Little is known about the downregulation of MCP-1, with the exception of an inhibition by glucocorticoids in certain cell lines (Brach et al., 1992; Shy et al., 1995).

MCP-1 production by endometriotic cells has recently been described (Akoum et al., 1996), but a role for MCP-1 in leukocyte recruitment to the normal endometrium has not been investigated.

The infiltrate of leukocytes into tissue is frequently accompanied by the leakage of plasma producing stromal oedema. Permeability of blood vessels is modulated by vasoactive substances including prostaglandins (PGs). Prostaglandin E (PGE), a potent vasodilator, and the antagonist vasoconstrictor prostaglandin F(2a) (PGF(2a), have been implicated in the modulation of blood vessel tone prior to and during menses (Baird et al., 1996). A role in the initiation of menstruation is further reinforced by the observed synergism between PGE and IL-8 in the infiltration of neutrophils from the peripheral circulation (Colditz, 1990). Prostaglandins are synthesized by the cyclooxygenase (COX) enzyme, which exists in two differentially regulated isoforms (Goppelt-Streube, 1995). COX-1 is constitutively expressed in most cell types, whilst COX-2 is induced transiently in response to inflammatory stimuli (Zweifel et al., 1995). Decidual cells have been demonstrated to produce COX-2 in response to IL-1β, and this induction can be inhibited by dexamethasone and progesterone (Ishihara et al., 1995). This finding reinforces the observation that prostaglandin release from proiferative and secretory endometrium is decreased by progesterone (Abel and Baird, 1980; Kelly and Smith, 1987).

The present study has investigated the localization and temporal expression of the chemokines IL-8 and MCP-1 and also COX-2 in the endometrium throughout the menstrual cycle and in early pregnancy.

### Materials and methods

#### Tissue collection

Endometrial biopsies (n = 44) were collected by Pipelle suction curette (Laboratoire CCD, Paris, France) from women undergoing minor gynaecological procedures (Table I). All women reported normal regular menstrual cycles (25–35 days) and had not received any form of hormonal treatment for the preceding 3 months. Biopsies were dated from the patient’s last menstrual period (LMP) and tissue sections were examined to ensure that histological appearances were consistent with the date of LMP. In addition, first trimester decidua (n = 17) collected away from the implantation site was biopsied by curettage prior to suction termination of pregnancy. Decidua parietalis, without trophoblast invasion, was confirmed by cytokeratin immunostaining. Written informed consent was obtained prior to tissue collection and ethical approval was received from Lothian Research Ethics Committee. Tissue samples were immediately placed in OCT embedding medium (Tissue-Tek, Miles Inc., Elkhart, USA) and snap frozen in iso-pentane precooled with dry ice. Additionally, a sample was immersion fixed in 10% neutral buffered formalin (NBF) overnight at 4°C, prior to paraffin embedding. Subsequently, 5 μm sections were cut from both the frozen and paraffin blocks for immunohistochemical investigation.

#### Immunohistochemistry

Immunohistochemical protocols for the detection of MCP-1 and COX-2 were optimized to determine the correct conditions for maximal specific staining. Methodology for the immunolocalization of the chemokine IL-8 has previously been reported (Critchley et al., 1994).

#### MCP-1

Immediately prior to staining, frozen sections were lightly fixed in 10% NBF for 10 min at room temperature. Sections were then washed in 0.1 M phosphate-buffered saline (PBS) pH 7.4–7.6. Endogenous peroxidase activity was quenched by immersion of the slides in 3% hydrogen peroxide (H2O2, BDH Laboratory Supplies, Poole, UK) in distilled water for 5 min at room temperature. Following 10 min washing in PBS, diluted non-immune goat serum (Vectorstain Elite PK-6101; Vector Laboratories, Peterborough, UK) was applied to each slide and the slides were incubated for 20 min in a humidified chamber. The excess serum was carefully blotted off and 50 μl of MCP-1 antibody (raised in rabbits using a chemically synthesized 77 amino acid MCP-1 as the antigen) was applied at a dilution of 1:400 in PBS. Coverslips were placed over the tissue sections to minimize antibody evaporation and the slides were incubated overnight (17 h ± 1) at 4°C. Following primary antibody binding, an avidin–biotin peroxidase detection system was utilized, involving the sequential application of biotinylated goat anti-rabbit IgG (Vectastain Elite PK-6101; Vector Laboratories) and a complex of avidin and biotin with horseradish peroxidase (ABC Vectorstain 4002; Vector Laboratories). The positive binding was then identified by the application of the peroxidase substrate 3, 3’-diaminobenzidine (DAB, Vector SK-4100; Vector Laboratories) which produces a brown stain representing the presence of MCP-1. Sections were then washed in distilled water and lightly counterstained with Harris’s haematoxylin (Pioneer Research Chemicals Ltd, Colchester, UK), a non-specific purple nuclear stain. Following washing in tap water, the sections were dehydrated in ascending grades of ethanol and mounted from xylene with Pertex mounting medium (Cellpath plc, Hemel Hempsted, UK).

#### IL-8

A similar protocol was used for IL-8 immunolocalization, with the exception of an incubation period of 60 min at 37°C with the primary

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**Table I. Stages of menstrual cycle at time of biopsy collection**

<table>
<thead>
<tr>
<th>Stage of menstrual cycle</th>
<th>Number of biopsies</th>
</tr>
</thead>
<tbody>
<tr>
<td>Menstrual (days 1–4)</td>
<td>6</td>
</tr>
<tr>
<td>Early proliferative</td>
<td>3</td>
</tr>
<tr>
<td>Mid-proliferative (days 5–7)</td>
<td>4</td>
</tr>
<tr>
<td>Late proliferative (days 11–13)</td>
<td>5</td>
</tr>
<tr>
<td>Peri-ovulatory (day 14)</td>
<td>5</td>
</tr>
<tr>
<td>Early secretory (days 15–18)</td>
<td>10</td>
</tr>
<tr>
<td>Mid-secretory (days 19–24)</td>
<td>9</td>
</tr>
<tr>
<td>Late secretory (days 25 to menstruation)</td>
<td>7</td>
</tr>
<tr>
<td>Decidua (8–10 weeks)</td>
<td>24</td>
</tr>
</tbody>
</table>

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rabbit polyclonal antibody raised against the IL-8 peptide, at a dilution of 1:500.

**COX-2**

Immunostaining for COX-2 was conducted on paraffin sections. These were dewaxed in Histoclear (National Diagnostics, Atlanta, Georgia, USA), and rehydrated through descending grades of ethanol. Prior to endogenous peroxide blocking, a microwave antigen retrieval technique was utilized, whereby the sections were heated in sodium citrate buffer (pH 6.0) for 10 min at high power followed by a 20 min incubation in the oven. A similar protocol as for MCP-1 was then continued, with the exception of 60 min incubation periods with the primary antibody (PG27, rabbit polyclonal; Oxford Biomedical, Biogenesis, Poole, UK) diluted 1:250 at 37°C, the secondary antibody and the tertiary ABC Elite (Vectastain Elite PK-6101; Vector Laboratories), both at room temperature.

**Controls**

Frozen tonsil tissue sections were included as a positive control for both chemokine immunostaining procedures, and were treated in an identical fashion. COX-2 immunoreactivity was confirmed by the inclusion of third trimester fetal membranes in each staining run. To assess the specificity of the immunoreactivity, serial sections of representative slides were also included and either non-immune rabbit IgG or primary antibody preabsorbed with the appropriate synthetic peptide (IL-8 or MCP-1) (100 µg/ml) was substituted for the primary antibody.

**Scoring and analysis of immunostaining**

A semi-quantitative scoring system was employed for assessment of intensity and localization of immunoreactivity in the entire tissue section, where a score of 0 indicates an absence of immunoreactivity, 1 faint immunoreactivity, 2 strong immunoreactivity and 3 very intense immunoreactivity. Scoring was conducted blind to the stage of the cycle, randomly by one observer. Analysis of variance (ANOVA) with Fisher’s PLSD to assign significance was used to evaluate whether or not there were significant differences in the expression of the mediators.

**Results**

The local mediators, MCP-1, IL-8 and COX-2, were detected by immunohistochemistry in samples of endometrium from non-pregnant and pregnant women (see Figure 1). Immunostaining was primarily present in the perivascular cells around all blood vessel types in the endometrial stroma for MCP-1 and IL-8, although weaker immunoreactivity was identified in the glandular epithelium. Additional staining was detected for MCP-1 in the cytoplasm of a subpopulation of stromal cells in occasional aggregates in some decidual biopsies. Intense immunoreactivity for COX-2 was detected in the epithelial glands and perivascular cells. In the menstrual phase and in some of the decidual sections examined, a degree of stromal staining was observed.

Tonsil sections included as a positive control for MCP-1 and IL-8 immunostaining also exhibited perivascular immunostaining. Immunoreactivity for COX-2 in third trimester fetal membranes was detected primarily in the amniotic epithelium and chorion laeve trophoblast, with slight immunostaining in the attached decidual cells, in agreement with published data (Gibb and Sun, 1996). Antibody preabsorbed with the appropriate peptide, or non-immune rabbit IgG applied to serial tissue sections to act as a negative control, resulted in an absence of immunoreactivity.

**MCP-1**

MCP-1 immunoreactivity was detected in a cyclical pattern during the menstrual cycle (Figure 2). Throughout the proliferative phase MCP-1 immunostaining was relatively intense. A distinct reduction in staining intensity was observed in the periovulatory phase and levels remained low in the early and mid-secretory stages. In the premenstrual or late secretory phase, however, immunostaining increased significantly ($P < 0.05$) compared with the periovulatory stage. First trimester decidua exhibited reduced immunoreactivity with respect to premenstrual levels, comparable with mid-secretory immunostaining.

**IL-8**

The intensity of IL-8 immunostaining also varied throughout the menstrual cycle (Figure 3). Low levels were detected in the perivascular cells in the menstrual and proliferative phases of the menstrual cycle. As for MCP-1, there was a clear reduction in staining intensity in the periovulatory phase, although this did not reach significance. Immunostaining levels increased slightly in the early and mid-secretory phase, but a highly significant increase ($P < 0.001$) in staining intensity was apparent in the late secretory phase in comparison with the ovulatory and early secretory phase. In the decidua, lower levels of IL-8 immunoreactivity compared to MCP-1 immunostaining levels were observed. The average immunoreactivity was significantly ($P < 0.01$) lower than that observed premenstrually and was consistent with the expression of IL-8 in the mid-secretory phase.

**COX-2**

COX-2 immunoreactivity was observed at all stages of the menstrual cycle (Figure 4). Both glands and vessels exhibited intense immunostaining in the menstrual phase. In the proliferative stage, levels of immunoreactivity decreased slightly and lower levels still were detected in the ovulatory phase. This pattern continued through the early and mid-secretory phases and a significant increase in COX-2 immunoreactivity was observed in the late secretory phase (glandular $P < 0.01$, vessels $P < 0.05$). Glandular immunoreactivity remained high in early decidua whilst perivascular staining appeared to be reduced (not significant).

**Discussion**

MCP-1, IL-8 and COX-2 were localized in the endometrium and decidua by immunohistochemistry. The localization of these chemotactic agents to the perivascular cells in both pregnant and non-pregnant endometrium is consistent with a putative role in the recruitment of specific leukocytes from the vasculature. Elevated levels of chemokine immunoreactivity were detected in the premenstrual phase of the menstrual cycle. These findings support the hypothesis that migration of leukocytes from the peripheral circulation contributes to the
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Figure 1. Immunohistochemical localization of IL-8, MCP-1 and COX-2 in human endometrium. (A) Periovulatory endometrium immunostained for IL-8. Faint immunoreactivity is apparent around small vessels. (B) Increased intensity of perivascular IL-8 immunoreactivity in late secretory endometrium. (C) MCP-1 immunoreactivity in periovulatory endometrium, demonstrating an absence of immunostaining. (D) First trimester decidua exhibits a marked increase in MCP-1 immunostaining intensity, in the perivascular cells of all vessels. (E) COX-2 immunoreactivity in periovulatory endometrium. Faint immunostaining is observed in the glandular epithelium and vessels. (F) Premenstrual endometrium exhibits intense immunostaining for COX-2 in both epithelial glands and perivascular cells. (G) Additional stromal immunoreactivity is observed in menstrual endometrium. (H) Negative control for MCP-1, decidual section treated with antibody preabsorbed with MCP-1 peptide. Scale bars, 50 µm, V = vessel.

significant leukocyte accumulation observed premenstrually. Furthermore, the co-localization of COX-2 in the perivascular cells reinforces the proposed interaction of prostaglandins and chemokines in the local inflammatory response.

The exact cell types in the perivascular region which exhibit immunoreactivity have not been characterized. Endothelial cells, fibroblasts and smooth muscle cells are present in this location, and all have been demonstrated to be capable of chemokine production (see reviews by Matsushima and Oppenheim, 1989; Bagnoli et al., 1994). Additional immunoreactivity was identified in a subpopulation of stromal cells in some decidual sections. These observations may represent a population of leukocytes with the potential for chemokine production.
Figure 2. MCP-1 immunoreactivity throughout the menstrual cycle and in early pregnancy. A significant increase in immunoreactivity levels is apparent in the premenstrual phase with respect to the ovulatory and early secretory phases (P < 0.05). Significant differences between groups are denoted by matching letters. Y-axis error bars represent the standard error of the mean (SEM). ME, menstrual; EP, early proliferative; MP, mid-proliferative; LP, late proliferative; OV, periovulatory; ES, early secretory; MS, mid-secretory; LS, late secretory; DE, decidua.

Figure 3. IL-8 immunoreactivity throughout the menstrual cycle and in early pregnancy. A highly significant increase in immunostaining occurs in the premenstrual phase compared to levels in the ovulatory and early secretory phases (P < 0.001) and the mid-secretory phase (P < 0.01). In early pregnancy, significantly lower levels of immunoreactivity were detected than premenstrually (P < 0.01). Significant differences between groups are denoted by matching letters. Y-axis error bars represent the standard error of the mean (SEM). Abbreviations as in Figure 2.

Examination of tissues obtained throughout the menstrual cycle and in early pregnancy revealed a cyclical pattern in the intensity of immunoreactivity for all three mediators. Most noticeable was the distinct reduction in staining intensity around the time of ovulation, extending into the early secretory phase. This coincides with the initiation of progesterone production from the corpus luteum and peak oestrogen production. At this stage the endometrium is most responsive to the effects of oestrogen and progesterone due to maximal steroid receptor expression (Snijders et al., 1992). Furthermore, the perivascular cells identified as the site of chemokine production strongly express progesterone receptors throughout the cycle (Perrot-Applanat et al., 1988; Bouchard et al., 1991). Experimental evidence has demonstrated that MCP-1 secretion by monocytes and fibroblasts in culture is inhibited by oestrogen (Kovacs et al., 1993, 1996). A similar regulation may occur in vivo, although the levels of MCP-1 immunoreactivity detected during the proliferative phase would not support this. The levels of chemokine and COX-2 immunoreactivity detected remain low through the early and mid-secretory phases, until the late secretory phase, when a significant elevation in expression is apparent. At this stage in the non-pregnant cycle, regression of the corpus luteum results in a drop in progesterone concentrations. If pregnancy occurs, progesterone values are maintained and this corresponds to a lower level of immunoreactivity for all three mediators. Thus, it appears that the staining intensity for all three mediators is significantly lower levels of immunoreactivity were detected than premenstrually (P < 0.01). Significant differences between groups are denoted by matching letters. Y-axis error bars represent the standard error of the mean (SEM). Abbreviations as in Figure 2. This would represent an anti-inflammatory action of progesterone, in the suppression of a local inflammatory response at the time when fertilization and implantation may occur. The method by which progesterone may achieve this downregulation is as yet unknown, but this immunohistochemical evidence of a co-localisation of responsive cells and progesterone receptors suggests a receptor mediated indirect effect of progesterone in leukocyte migration.

Supporting evidence for a downregulation of chemokine expression by progesterone is the increase in the numbers of macrophages in first trimester decidua, following the pharmacological withdrawal of progesterone by administration of the antigestogen mifepristone (Critchley et al., 1996). This observation may be related to an increase in chemokine expression. The phenotypically unique natural killer-like LGLs (CD56+CD16-) are the predominant leukocyte subtype in the
late secretory endometrium and first trimester decidua. Their origin is uncertain, but it has been suggested that a precursor form may be recruited from peripheral blood into the endometrial stroma, where a subsequent activation/differentiation occurs (King et al., 1991). A putative precursor cell type are the CD56+CD16–agranular cells which comprise ~1% of the circulating NK cells (Lanier et al., 1986). Chemotactic activity for NK cells has been attributed to MCP-1 (Allavena et al., 1994). Further investigation is necessary to determine whether MCP-1 may play a role in the accumulation of LGLs in the mid–late secretory phase and in the first half of pregnancy. IL-8 has also been implicated in the chemotaxis of lymphocytes (Larsen et al., 1989), and this has been linked to the LGL infiltrate in the endometrium (Casey and MacDonald, 1993). However, it has recently been proposed that only the C-C chemokines (including MCP-1) and not the C-X-C subfamily, which includes IL-8, possess chemotactic ability for T lymphocytes (Roth et al., 1995).

The co-localization of IL-8 and COX-2 in the endometrium reinforces the synergistic action of PGE in the IL-8 stimulated recruitment of neutrophils (Colditz, 1990). Furthermore, as MCP-1 and IL-8 are closely related chemokines, it is possible that PGE may also have an enhancing effect on monocyte infiltration. Prostaglandin activity is also regulated by the locally produced metabolizing enzyme prostaglandin dehydrogenase (PGDH). A positive regulation by progesterone has been demonstrated by the reduced immunoreactivity for PGDH following antigenostogen administration in early pregnancy (Cheng et al., 1993b). Also observed was increased immunoreactivity for PGE2 around the small blood vessels in the endometrium (Cheng et al., 1993a). Thus progesterone may affect local prostaglandin concentrations by modulating the enzymes responsible for both their production and metabolism.

The localization of COX-2 primarily to the glandular epithelium supports the in-vitro evidence that glands are the major site of prostaglandin synthesis in the endometrium (Lumsden et al., 1984; Smith and Kelly, 1988). Furthermore, whilst the endometrium retains the ability to synthesize prostaglandins in response to stimuli throughout the menstrual cycle, higher levels of prostaglandins are released from isolated glands in the proliferative than in the secretory phase and early pregnancy (Smith and Kelly, 1988). This is consistent with a stimulation of prostaglandin production by oestrogen (Abel and Baird, 1980), potentially through cyclooxygenase upregulation (Jun et al., 1996), and a downregulation by progesterone.

The present study has therefore identified and immunolocalized the inflammatory mediators MCP-1, IL-8 and COX-2 to the perivascular cells in endometrium and decidua. A cyclical pattern of immunoreactivity has been observed, with maximum expression of MCP-1, IL-8 and COX-2 occurring premenstrually and coinciding with the large increase in leukocytes in the endometrial stroma at this time. These observations support a role for steroid-regulated chemokines in the mechanisms determining leukocyte accumulation in endometrium and decidua.

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References


