Steroids Regulate CXCL4 in the Human Endometrium During Menstruation to Enable Efficient Endometrial Repair

Jacqueline A. Maybin,* Uma Thiruchelvam,* Mayank Madhra, Philippa T.K. Saunders, and Hilary O.D. Critchley

1MRC Centre for Reproductive Health, The University of Edinburgh, Queen’s Medical Research Institute, Edinburgh EH16 4TJ, United Kingdom; and 2MRC Centre for Inflammation Research, The University of Edinburgh, Queen’s Medical Research Institute, Edinburgh EH16 4TJ, United Kingdom

Context: Repair of the endometrial surface at menstruation must be efficient to minimize blood loss and optimize reproductive function. The mechanism and regulation of endometrial repair remain undefined.

Objective: To determine the presence/regulation of CXCL4 in the human endometrium as a putative repair factor at menses.

Patients/Setting: Endometrial tissue was collected throughout the menstrual cycle from healthy women attending the gynecology department. Menstrual blood loss was objectively measured in a subset, and heavy menstrual bleeding (HMB) was defined as >80 mL per cycle. Monocytes were isolated from peripheral blood.

Design: CXCL4 messenger RNA (mRNA) and protein were identified by quantitative reverse transcription polymerase chain reaction and immunohistochemistry. The function/regulation of endometrial CXCL4 was explored by in vitro cell culture.

Results: CXCL4 mRNA concentrations were significantly increased during menstruation. Intense staining for CXCL4 was detected in late secretory and menstrual tissue, localized to stromal, epithelial and endothelial cells. Colocalization identified positive staining in CD68+ macrophages. Treatment of human endometrial stromal and endothelial cells (hESCs and HEECs, respectively) with steroids revealed differential regulation of CXCL4. Progesterone withdrawal resulted in significant increases in CXCL4 mRNA and protein in hESCs, whereas cortisol significantly increased CXCL4 in HEECs. In women with HMB, CXCL4 was reduced in endothelial cells during the menstrual phase compared with women with normal menstrual bleeding. Cortisol-exposed macrophages displayed increased chemotaxis toward CXCL4 compared with macrophages incubated with estrogen or progesterone.

Conclusions: These data implicate CXCL4 in endometrial repair after menses. Reduced cortisol at the time of menses may contribute to delayed endometrial repair and HMB, in part by mechanisms involving aberrant expression of CXCL4. (J Clin Endocrinol Metab 102: 1851–1860, 2017)

The human endometrium displays a remarkable ability to break down and fully repair each month in the absence of pregnancy or lactation. Menstruation is triggered by the withdrawal of the ovarian steroid hormones estrogen and progesterone as the corpus luteum regresses. This results in a local inflammatory response,

*These authors contributed equally to this study.
Abbreviations: HEEC, human endometrial endothelial cell; hESC, human endometrial stromal cell; HMB, heavy menstrual bleeding; MBL, menstrual blood loss; MMP, matrix metalloproteinase; mRNA, messenger RNA; NMB, normal menstrual bleeding; PBMC, peripheral blood monocyte-derived macrophage; PCR, polymerase chain reaction; RT-qPCR, quantitative reverse transcription polymerase chain reaction.
including leukocyte influx and edema, which culminates in tissue breakdown by matrix metalloproteases and bleeding (1). Much less is known about the mechanisms and regulation of endometrial repair, but the processes involved appear to be similar to those seen with classic wound healing. These involve temporally overlapping phases of inflammation, resolution of inflammation, tissue formation, tissue remodeling, and angiogenesis. In the endometrium, this repair process appears to occur in areas of endometrium adjacent to those where breakdown is in progress (2). Delayed repair of the endometrium at menstruation may cause prolonged heavy menstrual bleeding (HMB), which negatively affects quality of life for many women.

Macrophages have a well-established role in the repair process at multiple tissue sites (3). They engulf foreign or apoptotic material as part of their phagocytic role, and they also secrete many proteases, angiogenic factors, and growth factors (4). Macrophage depletion results in defective repair of skin wounds in the guinea pig (5) and of myocardial injury in mice (6). Endometrial macrophages are present throughout the menstrual cycle but display a substantial increase in number during the perimenstrual phase (7). This increase in the number of tissue resident macrophages is thought to depend on the increase in concentrations of endometrial cytokines that occurs in response to progesterone withdrawal. Cytokines have been implicated in both the recruitment of monocytes into the endometrium and increased proliferation of macrophages in situ (7–9). Recent insights into the phenotype of tissue resident macrophages have revealed that both their plasticity and the prevailing tissue microenvironment influence the ability to adopt pro-wound-healing, pro-resolving, and tissue-regenerating phenotypes after injury [reviewed by Wynn and Vannela (10)].

CXCL4 (PF4) is a member of the CXC family that has a role in chemotaxis of neutrophils and monocytes (11, 12). It is unknown whether CXCL4 is an active chemoattractant within human endometrium, but both neutrophils and monocytes are implicated in endometrial repair (13). CXCL4 induces differentiation of peripheral blood monocytes, characterized by prevention of spontaneous apoptosis and promotion of differentiation into macrophages in a tumor necrosis factor-α and granulocyte macrophage colony-stimulating factor–independent fashion (14). CXCL4-stimulated differentiation appears to generate a different macrophage phenotype to the classic M1/M2 subtypes (15). Notably, these macrophages lack expression of the scavenger receptor CD163 (15), cannot upregulate heme-oxygenase 1 (15), and do not express the HLA-DR antigen (14) but produce more matrix metalloproteinase (MMP)-7 and MMP-12 protein than other macrophage subtypes (14).

In addition, CXCL4 is known to be an angiostatic factor, implicated in inhibition of endothelial cell proliferation (16, 17). CXCL4 has been detected at high concentrations at sites of vascular injury (18) and has downregulated expression of MMP-1 and MMP-3 in human vascular endothelial cells, which may contribute to resolution and repair (19).

Because CXCL4 is thought to have a key role in the regulation of angiogenesis, recruitment of monocytes, and wound healing, we hypothesized that it has a key role in endometrial repair at the time of menstruation (20). Therefore, we conducted a comprehensive analysis of human endometrial biopsy samples and used in vitro cell models to examine the regulation of CXCL4 by steroid hormones, including cortisol, because this steroid is thought to play a key role in regulating the local endometrial environment during menstruation. Next, we investigated the effect of CXCL4 on endometrial cells and macrophages. Our results highlight a potential role for this cytokine in the physiologic processes of menstruation and endometrial repair.

Methods

Human endometrial tissue collection

Endometrial biopsy specimens (n = 61) were collected with a suction curette (Pipelle; Laboratorie CCD, Paris, France) from women (median age, 42 years; range, 22 to 50 years) attending gynecological outpatient departments across the National Health Service Lothian in Scotland. Participants provided written consent and the Lothian Research Ethics Committee (LREC 07/S1103/29) granted ethical approval. All women reported regular menstrual cycles (21 to 35 days) and no exogenous hormone exposure for 2 months before biopsy. Women with large fibroids (>3 cm) or endometriosis were excluded. Tissue was divided and (1) placed in the RNAlater RNA stabilization solution (Ambion [Europe] Ltd., Warrington, UK), (2) fixed in neutral buffered formalin for wax embedding, and (3) placed in phosphate-buffered saline for in vitro culture. Cycle stage was determined by (1) histologic dating [criteria of Noyes et al. (21)], (2) reported last menstrual period, and (3) serum progesterone and estradiol concentrations at time of biopsy (Table 1). Samples not consistent for all three criteria were excluded (n = 5).

Objective measurement of menstrual blood loss

A subset of the participants with biopsy specimens collected in the perimenstrual phase agreed to collect their sanitary ware to allow objective quantification of their menstrual blood loss (MBL) (n = 23). Women were provided the same brand of tampon/pad (Tampon®/Always®, P&G, Weybridge, UK) and verbal and written instructions on collection. Blood loss was measured by using a modified alkaline-hematin method as previously described (22, 23). A measured MBL of >80 mL was classified at HMB and <80 mL as normal menstrual bleeding (NMB). This method was validated in our laboratory by using time-expired whole blood applied to the same sanitary products given to participants.
Table 1. Classification of Endometrial Biopsy Specimens

| Stage of Cycle | Mean Estradiol (pmol/L) | Mean Progesterone (nmol/L) | NMB MBL (Mean; mL) | HMB MBL (Mean; mL) |
|----------------|------------------------|---------------------------|--------------------|--------------------|
| Proliferative   | 410 (167–679)          | 2.8 (1.4–4.6)             | N/A                | N/A                |
| Early secretory | 439 (289–664)          | 55.4 (26.6–89.9)          | N/A                | N/A                |
| Mid secretory   | 585 (301–691)          | 81.8 (16.1–246.4)         | N/A                | N/A                |
| Late secretory  | 275 (59–819)           | 7.5 (1.1–17.0)            | 48 (35–62)         | 200 (85–488)       |
| Menstrual       | 174 (50–514)           | 3.4 (1.2–10.6)            | 40 (26–66)         | 180 (91–287)       |

Values in parentheses are the minimum–maximum.
Abbreviation: N/A, not available.

Immunohistochemistry for CXCL4

Paraffin sections, 5 μm, were dewaxed and rehydrated. Antigen retrieval was by pressure cooker in sodium citrate (pH, 6) antigen retrieval buffer. Endogenous peroxidase activity was blocked by 3% hydrogen peroxide. Sections were sequentially incubated in avidin and biotin (Vector Laboratories, Burlingame, CA) and protein block (Dako, Cambridge, UK). Rabbit polyclonal CXCL4 antibody (20 μg/mL; ab9561; Abcam, Cambridge, UK) was applied overnight at 4°C. Negative controls were incubated with rabbit IgG (Dako, Cambridge, UK) at the same concentration as the primary antibody. Biotinylated goat anti-rabbit secondary antibody was used at 1:200 (Vector Laboratories, Burlingame, CA). Avidin-biotin-peroxidase complex (ABC-Elite; Vector Laboratories) was applied for 30 minutes, and a liquid diaminobenzidine kit (Zymed Laboratories, Burlingame, CA) was applied overnight at 4°C. Goat anti-rabbit secondary antibody (Abcam) was applied at 1:2000 dilution for 1 hour at 37°C; omission of primary antibody provided negative controls. Goat anti-rabbit secondary antibody (Abcam) was applied before incubation with TSA fluorescein tyramide system for 10 minutes. BOND wash was followed by a BOND epitope retrieval system (Leica Microsystems, Wetzlar, Germany), block with normal goat serum and incubation with mouse monoclonal CD31 (Novacstra, Leica Biosystems, Newcastle, UK) at a 1-in-600 dilution for 1 hour. Goat anti-mouse secondary antibody (Abcam, Cambridge, UK) was applied, followed by a 4',6-diamidino-2-phenylindole stain (Sigma-Aldrich, Dorset, UK) for 10 minutes. All sections were mounted with Permafluor (Thermo Fisher Scientific, Waltham, MA) and analyzed on a Zeiss LSM710 confocal microscope system (Zeiss, Cambridge, UK).

Semiquantitative immunoscoring

Localization and intensity of immunostaining were evaluated in late secretory and menstrual endometrial samples from women with objectively measured HMB and NMB by two independent, masked observers. The intensity of staining was graded with a three-point scale (0 = no staining, 1 = mild staining, 2 = strong staining). This was applied to the stromal compartment and endothelial cells. The percentage of tissue in each intensity scale was recorded (24). A value was derived for each cellular compartment by using the sum of these percentages after multiplication by the intensity of staining. Average scores are reported unless a discrepancy of >50 points occurred between observers; in these cases, the tissue was examined together and a consensus score determined.

Dual immunofluorescence

Endometrial sections were dewaxed, rehydrated, exposed to antigen retrieval, and treated with 3% hydrogen peroxidase as above. For CD68/CXCL4 dual immunofluorescence, normal donkey serum was used as a protein block and the sections were incubated with mouse monoclonal CD68 (macrophage marker) antibody (Dako) at a 1 in 1000 dilution at 4°C. Donkey anti-mouse peroxidase secondary antibody (Abcam) at a 1:750 dilution was applied for 30 minutes, followed by incubation with TSA fluorescein tyramide system (Perkin Elmer, Waltham, MA) for 10 minutes. The sections were incubated with normal donkey serum for 10 minutes, followed by 20 μg/mL rabbit polyclonal CXCL4 antibody (Abcam) overnight at 4°C. Alexa 546 donkey anti-rabbit secondary antibody (Invitrogen, Paisley, UK) was applied at 1:200 for 1 hour, followed by a 4’,6-diamidino-2-phenylindole stain (Sigma-Aldrich, Dorset, UK) for 10 minutes.

CXCL4 immunofluorescence used Novocastra epitope retrieval solution Ph6 (Novacstra, Leica Biosystems, Newcastle, UK) and the Leica Bond-Max automated immunostainer (Leica Microsystems, Wetzlar, Germany). Normal goat serum was used as a protein block before incubation with CXCL4 antibody (Abcam) at a 1:2000 dilution for 1 hour at 37°C; omission of primary antibody provided negative controls. Goat anti-rabbit secondary antibody (Abcam) was applied before incubation with TSA fluorescein tyramide system for 10 minutes. BOND wash was followed by a BOND epitope retrieval system (Leica Microsystems; Wetzlar, Germany), block with normal goat serum and incubation with mouse monoclonal CD31 (Novacstra, Leica Biosystems) at 1-in-600 dilution for 1 hour. Goat anti-mouse secondary antibody (Abcam, Cambridge, UK) was applied, followed by a 4',6-diamidino-2-phenylindole stain (Sigma-Aldrich, Dorset, UK) for 10 minutes. All sections were mounted with Permafluor (Thermo Fisher Scientific, Waltham, MA) and analyzed on a Zeiss LSM710 confocal microscope system (Zeiss, Cambridge, UK).

Cell culture

Primary human endometrial stromal cells (hESCs) were isolated from midsecretory endometrial tissue (n = 3) by enzymatic digestion as previously described (25). hESCs at passage <6 were plated at a density of 10^6 cells per well in six-well plates in RPMI medium. Cells were serum starved for 24 hours before treatments. Cells were treated with (1) 10 nM estradiol for 48 hours, (2) 1 μM cortisol for 48 hours, (3) 1 μM progesterone for 6 days, or (4) 1 μM progesterone for 6 days, followed by serum-free media for 48 hours to mimic progesterone withdrawal.

Human endometrial endothelial cells (HEECs) were a gift from Yale School of Medicine (26). Their isolation (27) and phenotype (28) have been previously described. Serum-starved HEECs were treated in an identical manner to hESCs, described earlier.

Quantitative reverse transcription polymerase chain reaction

Concentrations of messenger RNAs (mRNAs) encoded by CXCL4 were determined by quantitative reverse transcription polymerase chain reaction (RT-qPCR) (Taqman) analysis. Total RNA from cells and endometrial biopsy samples was extracted by using the RNeasy Mini Kit (Qiagen Ltd, Sussex, UK) according to manufacturer’s instructions: 100-ng RNA samples were reverse transcribed according to standard
laboratory protocols (29). A tube with no reverse transcription and a further tube with water were included as controls. RT-qPCR mixtures were prepared containing Taqman buffer (5.5 mM MgCl₂, 200 μM deoxyadenosine triphosphate; 200 μM deoxyctydine triphosphate; 200 μM deoxyguanosine triphosphate; 400 μM deoxyuridine triphosphate), ribosomal 18S primers/probe (Applied Biosystems, Warrington, UK), and specific forward and reverse primers and probes (CXCL4 forward primer agcctggaggtgatcaagg, reverse primer cccattctttgacccgggact, universal probe library number 43; all from Roche Applied Science, Penzberg, Germany) were added for each RT-qPCR reaction. Negative controls (water instead of complementary DNA) were included in each run. RT-qPCR was carried out by using ABI Prism 7900 (Applied Biosystems, Foster City, CA). Forty cycles were completed (3 seconds at 95°C, 30 seconds at 60°C). Samples were analyzed in triplicate by using Sequence Detector, version 2.3 (PE Biosystems, Foster City, CA) with the comparative threshold method. Expression of target mRNA was normalized to RNA loading for each sample using the 18S ribosomal RNA as a reference.

Results

CXCL4 mRNA concentrations were increased in menstrual phase human endometrium and CXCL4 localized to epithelial, stromal, and endothelial cells and macrophages

CXCL4-encoded mRNAs were detected in human endometrial tissue biopsy samples throughout the cycle [Fig. 1(a)]. CXCL4 mRNA concentrations were significantly higher in menstrual biopsy specimens than those from the proliferative (P < 0.05), early secretory (P < 0.01), and mid-secretory (P < 0.05) phases.

Immunohistochemistry detected CXCL4 protein in the cytoplasm of epithelial and stromal cells throughout the menstrual cycle, with an increase in staining intensity noted in endometrium collected from women during the secretory and menstrual phases [Fig. 1(b)]. Dual immunofluorescence revealed positive CXCL4 staining in CD31+ endometrial endothelial cells during the late secretory/menstrual phase [Fig. 1(b, xi)]. We observed intense immunostaining of occasional cells within the stromal compartment throughout the cycle. Dual immunohistochemistry revealed CXCL4 was present in the cytoplasm of CD68+ macrophage cells throughout the menstrual cycle [Fig. 1(c)].

Endometrial CXCL4 was regulated by progesterone withdrawal and cortisol

After confirming the presence of CXCL4 in endometrial stromal and endothelial cells, we examined its regulation by steroids using primary hESCs and an HEEC cell line. Treatment with 10 nM estradiol mimicked the proliferative phase; 1 μM progesterone, the secretory phase; and sequential progesterone treatment and subsequent removal, the late secretory/menstrual phase. Mounting evidence suggests that cortisol has an important role in regulating the expression of CXCL4.
Figure 1. (a) CXCL4 in whole human endometrial biopsy specimens from across the menstrual cycle reveals maximal expression during the menstrual phase. Each box represents lower quartile, median, and upper quartile. Whiskers display minimum and maximum values. *P < 0.05; **P < 0.01. (b) CXCL4 protein in the human endometrium was localized to the cytoplasm of a few stromal cells during the proliferative phase (i and ii). During the early (iii and iv), mid (v and vi), and late (vii and viii) secretory phases of the menstrual cycle, immunostaining for CXCL4 progressively increased in intensity in both the stroma (St) and secretory glandular epithelium (GE). CXCL4 protein was present throughout the endometrium during the menstrual phase (ix and x) and CXCL4 (green) colocalized to CD31+ endothelial cells (red) in the late secretory phase (xi). Insets depict negative controls. Arrows indicate CXCL4+ cells. Scale bar = 50 µm. (c) CXCL4 (green) colocalizes to CD68+ (red) macrophage cells within the endometrium throughout the menstrual cycle. Proliferative (i and ii), late secretory (iii and iv), and menstrual (v and vi) phases are shown. Insets show negative controls. Arrows indicate colocalized cells.
role in the local endometrial environment at menses (9, 20); therefore, additional cells were treated with 1 μM cortisol. hESCs undergoing progesterone-withdrawal treatments showed a significant increase in concentrations of CXCL4 mRNA compared with those treated with vehicle, estradiol, or cortisol [Fig. 2(a)]. Progesterone withdrawal also significantly increased CXCL4 protein in hESCs compared with vehicle-treated cells [Fig. 2(b) and 2(c)].

Interestingly, CXCL4 regulation in HEECs was different from that detected in hESCs. Cortisol treatment of HEECs displayed maximal increases in concentrations of CXCL4 mRNA [Fig. 3(a)] and protein [Fig. 3(b) and 3(c)], which were significantly greater than treatment to mimic progesterone withdrawal (P < 0.01).

**CXCL4 was significantly decreased in endometrial endothelial cells from women with HMB during menstrual phase**

Because CXCL4 mRNA was maximal in endometrium from the late secretory and menstrual phases of the cycle, we compared mRNA concentrations in endometrial tissue homogenates from these two phases, taken from women with objectively measured MBL. Using a blood loss of >80 mL to define HMB, we found no significant differences in mRNA concentrations when comparing women with HMB and NMB [Fig. 4(a)].

Because we determined that regulation of CXCL4 varied in stromal and endothelial cells *in vitro*, we hypothesized that cellular levels of CXCL4 may differ in women with HMB and NMB, despite no significant differences in global endometrial CXCL4 mRNA concentrations. We examined CXCL4 protein by immunohistochemistry in the endometrium of the late secretory and menstrual phases from women with NMB and HMB. Semiquantitative immunoscopy of the stromal compartment and endothelial cells revealed no significant changes during the late secretory phase between women with NMB and HMB [Fig. 4(b)]. However, menstrual-phase endometrium from women with HMB had significantly decreased CXCL4 staining of endothelial cells compared with tissue from women with NMB (P < 0.05) [Fig. 4(b) and 4(c)]. In contrast, menstrual stromal compartment staining was not significantly different in endometrium from women with NMB and those with HMB.

**CXCL4 had augmented chemotactic action on macrophages pre-exposed to cortisol**

Because CXCL4 increased at menses and colocalized to macrophage cells, we investigated the effect of CXCL4-induced chemotaxis on different macrophage subtypes. Peripheral macrophages were pretreated to induce different subtypes: M0 (macrophage colony-stimulating factor–pretreated), M1 (granulocyte macrophage...
colony-stimulating factor– and interferon γ–pretreated), and M2 (cortisol-pretreated) macrophages or macrophages exposed to a proliferative phase environment (estradiol-pretreated). These pretreated macrophages were plated into wells opposite CXCL4 on a multicchanneled microslide. Cortisol-exposed macrophages migrated toward CXCL4 at a significantly higher rate than any of the other macrophage subtypes [Fig. 5(a) and 5(b)].

Discussion

This report details the presence of CXCL4 in the human endometrium across the menstrual cycle and reveals that maximal levels are present during menstruation. Steroid regulation of CXCL4 occurs in hESCs, with significant increases after withdrawal of progesterone. In contrast, endometrial endothelial cells do not display an increase in CXCL4 on progesterone withdrawal but do demonstrate significant increases in response to cortisol treatment. Furthermore, we reveal that women with HMB have significantly reduced CXCL4 in endothelial cells in the menstrual phase, consistent with a defective cortisol response at menses (20). Macrophages pretreated with cortisol to induce an M2 phenotype migrate significantly faster toward CXCL4 than M0 and M1 subtypes. These data are consistent with CXCL4 having a key role in endometrial breakdown and repair at menstruation.

CXCL4 is present in the human endometrium during menstruation, with both RT-qPCR and immunohistochemistry being consistent with maximal detection during the menstrual phase. The functional layer of the endometrial breaks down during menses, with repair occurring simultaneously in adjacent areas (2). Therefore, maximal CXCL4 within the endometrium at this time is consistent with involvement in breakdown and repair of the tissue. Expression of the CXCL4 receptor, CXCR3, has been identified as necessary for efficient wound healing (30). Mice lacking CXCR3 had significantly delayed re-epithelialization and delayed repair of the basement membrane following excisional wounds.

Next, we investigated the regulation of CXCL4 in the human endometrium. Because of the dramatic variations observed across the menstrual cycle, we examined steroid regulation of this cytokine. A series of in vitro studies revealed that progesterone withdrawal resulted in a significant increase of CXCL4 expression within endometrial stromal cells, consistent with maximal detection during the menstrual phase. HEECs, however, do not express the progesterone receptor (31); hence, it was unsurprising that treatment conditions using progesterone or progesterone withdrawal had no profound effects. However, HEECs are known to express the glucocorticoid receptor.

Figure 3. Steroid regulation of CXCL4 in HEECs. Treating HEECs with estrogen, progesterone, progesterone withdrawal (P withdrawal), and cortisol showed that CXCL4 was significantly upregulated by treatment with cortisol at the (a) mRNA level (n = 4) and (b) protein level (n = 4), quantified by densitometry (c). Green, CXCL4; red, β-tubulin. In (a) and (c), each box represents lower quartile, median, and upper quartile. Whiskers display minimum and maximum value. **P < 0.01.
and treatment of these cells with cortisol resulted in a significant increase of CXCL4 expression. We have previously shown that local levels of cortisol regulating enzymes increase in human endometrial tissue during menstruation (33). Therefore, two different steroid hormones have the ability to regulate CXCL4 in endometrial cells to increase concentrations of this putative wound repair factor during menstruation.

Because CXCL4 is a putative endometrial repair factor, we examined mRNA concentrations in endometrial tissue sample homogenates from women with HMB and NMB. We hypothesized that women with HMB would have reduced CXCL4 induction during menstruation, leading to inefficient endometrial repair and prolonged, HMB. However, no significant differences in CXCL4 mRNA concentrations were detected between these two groups of women during the late secretory or menstrual phases.

There are two potential explanations for these findings. First, there may be no deregulation of CXCL4 in women with HMB. However, our results suggested an alternative explanation: that different cell types within the human endometrium have differential regulation of CXCL4 induction, with progesterone withdrawal having a substantial impact on stromal cells and cortisol regulating CXCL4 in endothelial cells. Examination of homogenized whole endometrial biopsy specimens may mask differential expression of CXCL4 within different cell types in women with heavy versus normal menstrual blood loss. Therefore, we examined CXCL4 protein in stromal cells and endothelial cells in women with NMB and HMB during the late secretory and menstrual phases. This revealed that endothelial cell CXCL4 protein was significantly reduced in women with HMB versus NMB during menses, which might be consistent with a defective cortisol microenvironment (33).

Our laboratory has previously revealed that the cortisol-inactivating enzyme 11β-hydroxysteroid dehydrogenase-2 is significantly increased in endometrium from women with HMB versus NMB, thereby creating a local glucocorticoid deficiency (20). Therefore, we propose that women with HMB have reduced endometrial cortisol

Figure 4. (a) CXCL4 in late secretory and menstrual endometrial biopsy specimens from women with objectively measured NMB (<80 mL, white bars) and HMB (>80 mL, gray bars). (b) Immunoscoring of CXCL4 staining of the stromal (St) compartment and endothelial cells in late secretory (LS) and menstrual (M) endometrium from women with NMB and HMB. (c) Immunohistochemistry staining of CXCL4 in menstrual endometrium from women with NMB and HMB. Inset shows immunoglobulin G–matched negative control. Arrow, endothelial cells; NS, nonsignificant. In (a) and (b), each box represents lower quartile, median, and upper quartile. Whiskers display minimum and maximum value. *P < 0.05.
leading to decreased CXCL4 in endothelial cells, which may contribute to increased MBL. CXCL4 is known to have angiogenic properties (18, 19), but its functional role in the endometrium remains to be determined.

CXCL4 is known to be a chemoattractant in many tissues, triggering migration of monocytes and macrophages to sites of inflammation (34, 35). Herein we show that cortisol-treated, M2-like macrophages exhibit increased chemotaxis toward CXCL4 compared with other steroid treated macrophages. This suggests that the microenvironment created by synthesis of CXCL4 may alter immune cell components. It is also notable that cortisol-treated macrophages have been documented to take part in the resolution of inflammation, including removal of apoptotic cells (36). Taken together, these data suggest that CXCL4 may act as a chemoattractant at focal points within the human endometrium that require repair.

In summary, we have identified that CXCL4 is increased in the human endometrium during menstruation, a time consistent with involvement in endometrial repair. Mechanistically, we have revealed that endometrial CXCL4 is regulated by progesterone withdrawal and cortisol. In addition, we reveal that CXCL4 is reduced in endothelial cells of women with HMB at menses. Functionally, CXCL4 appears to have an important role as a macrophage chemoattractant, particularly for macrophages pre-exposed to cortisol. These data implicate CXCL4 as a key player in the physiologic process of endometrial repair after menses.

Acknowledgments

We are grateful to Sheila Milne for assistance with manuscript preparation; Ronnie Grant for assistance with figure preparation; Catherine Murray and Sharon McPherson for help with patient recruitment; Moira Nicol, Hazel Murray, and Alison Murray for technical support; and Dr Erin Greaves for advice on endothelial cell cultures.

U. Thiruchelvam’s current affiliation is GlaxoSmithKline, Brentford, Middlesex TW8 9GS, United Kingdom.

Address all correspondence and requests for reprints to: Hilary O.D. Critchley, MD, MRC Centre for Reproductive Health, The University of Edinburgh, Queen’s Medical Research Institute, 47 Little France Crescent, Edinburgh EH16 4TJ, United Kingdom. E-mail: hilary.critchley@ed.ac.uk.

This work was undertaken in the MRC Centre for Reproductive Health, which is funded by MRC Centre Grants G1002033 and MR/N022556/1; MRC Grant G0600048 (H.O.D.C.), Wellcome Trust Grant 100646/Z/12/Z (JAM), and MRC Programme Grant G1100356/1 (P.T.K.S.).

Disclosure Summary: H.O.D.C. has clinical research support for laboratory consumables and staff from Bayer Pharma Ag and provides consultancy advice (but with no personal remuneration) for Bayer Pharma Ag, PregLem SA, Gedeon Richter, Vifor
The remaining authors have nothing to disclose.

References

1. Jabbour HN, Kelly RW, Fraser HM, Critchley HO. Endocrine regulation of menstruation. *Endocr Rev*. 2006;27(1):17–46.
2. Garry R, Hart R, Karras A, Burke C. A reappraisal of the histological changes within the endometrium during menstruation: a hysteroscopie, histological and scanning electron microscopic study. *Hum Reprod*. 2009;24(6):1393–1401.
3. Ricardo SD, van Goor H, Edery AA. Macrophage diversity in renal injury and repair. *J Clin Invest*. 2008;118(11):3522–3530.
4. Wynn TA, Vannella KM. Macrophages in tissue repair, regeneration and fibrosis. *Semin Liver Dis*. 2010;30(3):245–257.
5. Leibovich SJ, Ross R. The role of the macrophage in wound repair. A study with hydrocortisone and antimacrophage serum. *Am J Pathol*. 1975;78(1):71–100.
6. van Amerongen MJ, Harmens MC, van Rooijen N, Petersen AH, van Luyn MJ. Macrophage depletion impairs wound healing and increases left ventricular remodeling after myocardial injury in mice. *Am J Pathol*. 2007;170(3):818–829.
7. Thiruchelvam U, Dransfield I, Saunders PT, Critchley HO. The importance of the macrophage within the human endometrium. *J Leukoc Biol*. 2013;93(2):217–223.
8. Guo Y, He B, Xu X, Wang J. Comprehensive analysis of leukocytes, vascularization and matrix metalloproteinases in human menstrual xenograft model [published correction appears in PLoS One. 2011;6(3). doi: 10.1371/annotation/77d64f53-b6fe-442d-9c29-97b74a99ddcf1993;22(9):1490]. PLoS One. 2011;6(2):e16840.
9. Thiruchelvam U, Maybin JA, Armstrong GM, Greaves E, Saunders PT, Critchley HO. Cortisol regulates the paracrine action of macrophages by inducing voascope gene expression in endometrial cells. *J Leukoc Biol*. 2016;99(6):1165–1171.
10. Wynn TA, Vannella KM. Macrophages in tissue repair, regeneration, and fibrosis. *Immunity*. 2016;44(5):450–462.
11. Baltus T, von Hulndellsen H, Mause SF, Buhre W, Rossaint R, Weber C. Differential and additive effects of platelet-derived chemokines on monocyte arrest on inflamed endothelium under flow conditions. *J Leukoc Biol*. 2005;78(2):433–441.
12. Houard X, Touart Z, Ollivier L, Louedec L, Philippe M, Sebagg U, Meilhac O, Rossignol P, Michel JB. Mediators of neutrophil recruitment in human abdominal aortic aneurysms. *Cardiovasc Res*. 2009;82(3):532–542.
13. Maybin JA, Critchley HO, Jabbour HN. Inflammatory pathways in endometrial disorders. *Mol Cell Endocrinol*. 2011;335(1):42–51.
14. Scheurer B, Ernst M, Dürrbaum-Landmann I, Fleischer J, Grege-Griebenow E, Brandt E, Flad HD, Petersen F. The CX3C-chemokine platelet factor 4 promotes monocyte survival and induces monocyte differentiation into macrophages. *Blood*. 2000;95(4):1158–1166.
15. Gleissner CA. Macrophage phenotype modulation by CXCL4 in atherosclerosis. *Front Physiol*. 2012;3:1.
16. Dudek AZ, Nesmiova I, Mayo K, Verfaille CM, Pfitzder S, Slungaard A. Platelet factor 4 promotes adhesion of hematopoietic progenitor cells and binds IL-8: novel mechanisms for modulation of hematopoiesis. *Blood*. 2003;101(12):4687–4694.
17. Zucker MB, Katz IR. Platelet factor 4: production, structure, and physiologic and immunologic action. *Proc Soc Exp Biol Med*. 1991;198(2):693–702.
18. Aidoudi S, Bifkalvi A. Interaction of PF4 (CXCL4) with the vascular: a role in atherosclerosis and angioinvasion. *Thromb Haemost*. 2010;104(5):941–948.
19. Klein-Soyer C, Duhaime-Clérion E, Ravanat C, Orvain C, Lanza F, Caenave JP. PF4 inhibits thrombin-stimulated MMP-1 and MMP-3 metalloproteinase expression in human vascular endothelial cells. *C R Acad Sci III*. 1997;320(11):857–868.
20. Rae M, Mohamad A, Price D, Hadoke PW, Walker BR, Mason JJ, Hillier SG, Critchley HO. Cortisol inactivation by 11beta-hydroxysteroid dehydrogenase-2 may enhance endometrial angiogenesis via reduced thrombospondin-1 in heavy menstruation. *J Clin Endocrinol Metab*. 2009;94(4):1443–1450.
21. Noyes RW, Herrig AT, Rock J. Dating the endometrial biopsy. *Fertil Steril*. 1950;13:1–25.
22. Hallberg I, Nilsson L. Determination of menstrual blood loss. *Scand J Clin Lab Invest*. 1964;16:244–248.
23. Warner PE, Critchley HO, Lumsden MA, Campbell-Brown M, Douglas A, Murray GD. Menorrhagia II: the 80-mL blood loss criterion useful in management of complaint of menorrhagia? *Am J Obstet Gynecol*. 2004;190(3):1224–1229.
24. Aasmundstad TA, Haugen OA, Johannesen E, Hve AL, Kvinsland S. Oestrogen receptor analysis: correlation between enzyme immunosassay and immunohistochemical methods. *J Clin Pathol*. 1992;45(2):125–129.
25. Kane N, Jones M, Brosses JJ, Saunders PT, Kelly RW, Critchley HO. Transforming growth factor-beta1 attenuates expression of both the progestosterone receptor and Dickkopf in differentiated human endometrial stromal cells. *Mol Endocrinol*. 2008;22(3):716–728.
26. Krikun G, Schatz F, Finlay T, Kadner S, Mesia A, Gerrets R, Lockwood CJ. Expression of angiopoietin-2 by human endometrial endothelial cells: regulation by hypoxia and inflammation. *Biochem Biophys Res Commun*. 2000;275(1):159–163.
27. Schatz F, Soderland C, Hendricks-Muñoz KD, Gerrets RP, Lockwood CJ. Human endometrial endothelial cells: isolation, characterization, and inflammatory-mediated expression of tissue factor and type 1 plasminogen activator inhibitor. *Biol Reprod*. 2000;62(3):61–69.
28. Greaves E, Collins F, Critchley HO, Saunders PT. ERβ-dependent effects on uterine endothelial cells are cell specific and mediated via Sp1. *Hum Reprod*. 2013;28(9):2490–2501.
29. Maybin JA, Battersby S, Hirani N, Nikitenko LL, Critchley HO, Jabbour HN. The expression and regulation of adrenomedullin in the human endometrium: a candidate for endometrial repair. *Endocrinology*. 2011;152(7):2845–2856.
30. Yates CC, Whaley D, Hoods S, Hebd A, Bodnar RJ, Wells A. Delayed reepithelialization and basement membrane regeneration after wound healing in mice lacking CXCR3. *Wound Repair Regen*. 2009;17(1):34–41.
31. Critchley HO, Brenner RM, Henderson TA, Williams K, Nayak NR, Slayden OD, Millar MR, Saunders PT. Estrogen receptor beta, but not estrogen receptor alpha, is present in the vascular endothelium of the human and nonhuman primate endometrium. *J Clin Endocrinol Metab*. 2001;86(3):1370–1378.
32. Henderson TA, Saunders PT, Moffett-King A, Groome NP, Critchley HO. Steroid receptor expression in uterine natural killer cells. *J Clin Endocrinol Metab*. 2003;88(1):440–449.
33. McDonald SE, Henderson TA, Gomez-Sanchez CE, Critchley HO, Mason JL. 11beta-hydroxysteroid dehydrogenases in human endometrium. *Mol Cell Endocrinol*. 2006;248(1–2):72–78.
34. Koenen RR, van Hendelmersen P, Nesmelova I, Zenecke A, Liehn EA, Sarabi A, Kramp BK, Piccinni AM, Paludan SR, Kowalska MA, Kungl AJ, Hackeng TM, Mayo KH, Weber C. Disrupting functional interactions between platelet chemokines inhibits atherosclerosis in hyperlipidemic mice. *Nat Med*. 2009;15(1):97–103.
35. Sarabi A, Kramp BK, Drecshler M, Hackeng TM, Soehlein O, Weber C, Koenen RR, Von Hendelmersen P. CXCL4/L1 inhibits angiogenesis and induces undirected endothelial cell migration without affecting endothelial cell proliferation and monocyte recruitment. *J Thromb Haemost*. 2011;9(1):209–219.
36. Liu Y, Cousin JM, Hughes J, Van Damme J, Seckl JR, Haslett C, Dransfield I, Savill J, Rossi AG. Glucocorticoids promote non-phlogistic phagocytosis of apoptotic leukocytes. *J Immunol*. 1999;162(6):3639–3646.