Endometrial chemokines, uterine natural killer cells and mast cells in long-term users of the levonorgestrel-releasing intrauterine system

Alessandra Peloggia¹, Carlos A. Petta¹,³, Luis Bahamondes¹, Marilia Oliveira-Ribeiro¹, Jin Zhang² and Lois Salamonsen²

¹Human Reproduction Unit, Department of Obstetrics and Gynaecology, School of Medicine, Universidade Estadual de Campinas (UNICAMP), Campinas, SP, Brazil and ³Prince Henry’s Institute of Medical Research, Clayton, Victoria, Australia

OBJECTIVE: The objective was to assess endometrial chemokines in users of the levonorgestrel-releasing intrauterine system (LNG-IUS) and correlate them with leucocyte populations, uterine natural killer cells (uNK) and mast cells (MCs). MATERIALS AND METHODS: Endometrium was obtained from two groups of women who had been using LNG-IUS for 3 years or more: 11 amenorrhoeic women formed the non-bleeding group and 15 women who maintained some form of cyclic bleeding comprised the bleeding group. Specific antibodies were used for the assessment of neutrophils, uNK cells and MCs. Immunohistochemistry was performed to locate the chemokines 6CKine and interleukin-8 (IL-8). RESULTS: Neutrophils were few and without differences between the two groups. uNK cells were significantly higher in the bleeding group (P < 0.0001). There was no difference between the total number of MCs and activated MCs, but there was a greater extracellular area stained for MC tryptase (P < 0.05). Chemokines 6CKine and IL-8 were abundant in the stroma and in the epithelium, and there was no difference between the groups. CONCLUSIONS: We observed more uNK cells in users with bleeding and a greater extracellular area stained for MC tryptase, although there were no differences between the number of MCs and activated MCs or the chemokines 6CKine and IL-8. uNK cells and MC products may play a role in provoking breakthrough bleeding in long-term users of the LNG-IUS.

Key words: chemokines/leucocytes/LNG-IUS/mast cells

Introduction

The levonorgestrel-releasing intrauterine system (LNG-IUS) releases 20 μg/day of LNG into the uterine cavity. Its high contraceptive efficacy is related principally to an alteration in endometrial receptivity (Nilsson et al., 1978), suppression of endometrial proliferation, inhibition of sperm migration and thickening of cervical mucus (Luukkainen et al., 1990; Barbosa et al., 1995). The introduction of the LNG-IUS increased the range of contraceptive options available to women and reduced some of the side effects suffered by some users of the copper IUDs, such as pelvic infection and ectopic pregnancy (Toivonen et al., 1991; Odlind, 1998).

In addition to the high contraceptive effectiveness of the LNG-IUS, this device also offers many non-contraceptive benefits such as the treatment of menorrhagia through a dramatic reduction in menstrual bleeding (Andersson and Rybo, 1990; Monteiro et al., 2002; Hurskainen et al., 2004), relief of endometriosis-associated pain (Lockhat et al., 2005; Petta et al., 2005) and endometrial protection during hormone therapy in the post-menopause (Andersson et al., 1992; Suhonen et al., 1995).

The LNG-IUS induces some bleeding disturbances including unexpected breakthrough bleeding (BTB) (Odlind and Fraser, 1990), and this is the main reason given by women for discontinuation of the method. The severity of menstrual bleeding disturbances varies according to the contraceptive method (Vincent and Salamonsen, 2000); however, the mechanisms involved in the pathogenesis of BTB are not fully understood.

One common consequence of all progestin-only (p-only) contraceptive methods, including the LNG-IUS, is an abnormal leucocyte infiltration (Salamonsen and Lathbury, 2000). The normal endometrium possesses an active and tightly regulated immune environment with significant immune cell populations that are associated with specific endometrial events (Bulmer et al., 1991). In the late-secretory phase of the menstrual cycle, there is an increase in the number of leucocytes associated with decidualization and implantation (Bulmer et al., 1988). Leucocyte products include a range of proteases,
chemokines and cytokines, which together result in focal production and activation of matrix metalloproteinases (MMPs) by endometrial cells, with the subsequent breakdown of tissue that characterizes menstruation (Salamonsen and Lathbury, 2000).

Leucocyte infiltration, particularly in the form of neutrophils, eosinophils, macrophages and uterine-specific natural killer cells (uNK), along with changes in the activation state of mast cells (MCs), appears to undergo changes in women using p-only contraceptive methods. Leucocyte subpopulations such as neutrophils and eosinophils are found predominantly in the shedding endometrium, whereas very few of these subtypes are present in atrophic or progestin-modified endometria (Vincent et al., 1999). However, in the decidualized endometrium, there is a great number of uNK cells (Crichtley et al., 1998).

In humans, these uNK cells are present before implantation, but they are also present in the non-pregnant endometrium (Jones et al., 2004). At the end of a non-pregnant cycle, approximately 2 days before menstruation, uNK cells undergo a nuclear change resembling apoptotic cell death (Trundley and Moffett, 2004). In addition, the MCs secrete many vasoactive and proinflammatory molecules and participate in inflammation by enhancing leucocyte infiltration, as well as causing direct tissue damage by their release of proteases. They are detectable by immunostaining for the MC-specific serine proteinase, tryptase, which plays an important role in MMP activation. This may represent a critical function for these cells at menstruation or in abnormal uterine bleeding (Salamonsen and Lathbury, 2000).

Many studies have focused on the local uterine environment and were conducted with the objective of understanding why the use of p-only contraceptives sometimes leads to BTB. The aim of this study was to assess the production of certain endometrial chemokines in long-term users of the LNG-IUS with and without endometrial bleeding and correlate these chemokines with the presence of leucocyte populations, with particular emphasis on the role of the uNK cells and MCs.

Subjects and methods

Characteristics of subjects and tissue collection

The study was conducted at the Department of Obstetrics and Gynecology, School of Medicine, Universidade Estadual de Campinas (UNICAMP), Campinas, Brazil and at Prince Henry’s Institute of Medical Research, Clayton, Victoria, Australia. The research protocol was approved by the ethical committees of the institution, and all women signed an informed consent form before being admitted to the study. Endometrial tissue was obtained from women using the LNG-IUS (Mirena®, Leiras Oy, Turku, Finland) as a contraceptive method by using Pipelle suction curette (Pipelle de Cornier, Neully-in-Thele, France). A single endometrial biopsy was taken from each of the 26 women who had been using the LNG-IUS for contraception for more than 3 years. These women were divided into two groups according to whether or not they were in amenorrhea: 11 amenorrhoeic women formed the non-bleeding group and 15 women who maintained some form of cyclic bleeding comprised the bleeding group.

The women included in this study answered a questionnaire regarding their menstrual pattern following the insertion of the LNG-IUS. Women were considered to belong to the group of non-bleeding women if they had not had any uterine bleeding within the 3 months before biopsy. The women assigned to the bleeding group were those who had had some uterine bleeding in the 3 months before joining the study, but who were not bleeding at the time the endometrial biopsy was taken (Oliveira-Ribeiro et al., 2004).

The endometrial samples were fixed in 10% buffered formalin and dehydrated. They then underwent routine histological processing to paraffin blocks. Sections measuring 5 μm were cut on a Leica RM 2135 microtome and placed on Super Frost® Plus glass slides (Menzel-Glasser, Strasburg, Germany). Paraffin sections were dewaxed in Histosol (Sigma-Aldrich, St. Louis, MO, USA) and rehydrated through descending grades of alcohol to dH2O. A standard haematoxylin and eosin section of each biopsy sample was submitted to a pathologist for classification according to Noyes’ criteria (Noyes et al., 1950).

Immunohistochemistry of leucocyte subtypes

Immunohistochemistry was performed on endometrial biopsies using specific antibodies for neutrophils [monoclonal mouse anti-human NEU elastase (DAKO Cytomation, Glostrup, Denmark, Cat M0752, clone NP57) IgG concentration 70 μg/ml], uNK (CD56 monoclonal mouse anti-human small cell lung cancer (SCLC) (Zymed, San Francisco, CA, USA, Cat# 18-0152)] and MCs [monoclonal mouse anti-human tryptase (DAKO, Clone AA1, Cat M-7052) IgG1 concentration 80 μg/ml] and eosin section of each biopsy sample was submitted to a pathologist for classification according to Noyes’ criteria (Noyes et al., 1950).

Chemokine immunohistochemistry

Immunohistochemistry was performed to locate two chemokines [6Ckine and interleukin-8 (IL-8), which are chemotactants for uNK cells and neutrophils, respectively] in formalin-fixed endometrial biopsies. Purified goat anti-human polyclonal antibodies [for IL-8: C-19, Cat SC 1269 and for 6Ckine: C-15, Cat SC 5808 (Santa Cruz Biotechnology, Santa Cruz, CA, USA)] were used, each at 200 μg/ml. Immunohistochemistry for both chemokines was performed following antigen retrieval by microwaving as above. Primary antibodies and negative controls (goat IgG at the same concentration) were applied and incubated overnight (16–18 h) at 4°C. Detection of positive localization was performed using sequential application of biotinylated horse anti-goat IgG 1/200, Catalog BA 9500 (Vector Laboratories) and Strept ABC/HRP 1/100 (DAKO), followed by 3,3-diaminobenzidine (DAB) (DAKO). Sections were counterstained following the protocol described above.
Analysis of immunostaining

Staining was assessed with the use of an Olympus CH30 microscope (Olympus, Melville, NY, USA), and high-resolution images were captured with an Hc-2000 digital camera (Fujix, Tokyo, Japan). A microcomputer imaging device (AIS) from Imaging Research (Brock University, St Catherine, Ontario, Canada) was used to assess the relative number of uNK cells and MCs in the intact pieces of tissue present in the tissue blocks. The investigators (J.Z. and A.P.) were blinded to the identity of the sections. Since the uNK cells were often clustered and thus not readily countable, data are expressed for each tissue as percentage of total tissue area strongly stained. For MCs, non-activated and activated cells were counted separately, depending on whether staining was intracellular or extracellular. Furthermore as an estimate of MC activation, the percentage of the total area that was stained diffusely with MC tryptase (MCT) is expressed as a percentage of the total area. Statistical analysis was performed to compare differences between bleeding and non-bleeding groups using Student’s t-test for unpaired samples, and significance was established at $P < 0.05$. Data are presented graphically as mean ± SEM.

Results

Leucocytes in bleeding and non-bleeding samples

Neutrophils

Since all the samples were collected from women who had been using Mirena for more than 3 years, all endometria were highly decidualized. Immunohistochemistry showed very few neutrophils in the tissues and no significant difference between the two groups (Figure 1A and B).

Uterine natural killer cells (uNK)

CD56-positive uNK cells were highly abundant (3% of total area stained) in the bleeding group compared to the non-bleeding group (0.05% of total area stained) (Figure 1C and D). This difference was highly significant ($P < 0.0001$) (Figure 2).

Mast cells (MC)

Evaluation of all MCs was performed. These may be either non-activated or activated. Positive staining for MCT was seen in most tissues (Figure 3). In each tissue block, there were some portions of tissue that had more MCT than others. MCT was either contained within the cell (representing a non-activated MC) or extracellular (representing activated MCs), in each tissue. The total number of MCs in each tissue, expressed as $MC/\mu m^2$, and the number of activated MCs in each tissue, expressed as $MC/\mu m^2$, were assessed, and the proportion of total tissue area ($\times 100$) that was MCT stained was also measured.

Figure 4 shows that there were no significant differences in either the total number of MCs (22 versus 12%) or the number of activated MCs (8 versus 3%) in bleeding versus non-bleeding groups. However, a greater proportion of the total tissue area was stained in samples from the bleeding group (100%) than in samples from the non-bleeding group (Figure 3) (20%) ($P < 0.05$), indicating that there was increased extracellular tryptase in the tissue and hence MC activation.

Chemokines

Chemokine immunostaining after insertion of LNG-IUS was consistent with the highly decidualized nature of the tissue. Immunoactive 6Ckine and IL-8 were both abundant in the decidualized stromal cells, but there were no significant differences
between bleeding and non-bleeding groups (Figure 5A and B). IL-8 was also strongly stained in the epithelium: once again, there was no difference between the groups (Figure 5C and D).

Discussion
Leucocyte populations and other endometrial parameters have been previously analysed in endometrial samples of women using the LNG-IUS (Critchley et al., 1998; Oliveira-Ribeiro et al., 2004). This study used two cohorts of LNG-IUS users, those who had no bleeding and those who reported bleeding in the previous 3 months. We examined endometrium from these women for subsets of leucocytes and the chemokines that may act as specific chemoattractants for these cells. P-only contraceptive methods, induce different effects on endometrial morphology (Vincent et al., 2000), and these different morphologies contain different populations of immune cells. In our study, we found few neutrophils in any of the tissues from LNG-IUS users. However, there were significantly more uNK cells along with evidence for increased MC activation in the bleeding group compared with the women who were not bleeding.

In many LNG-IUS users, the endometrium is highly decidualized, with macrophages and uNK cells being predominant (Critchley et al., 1998). This phenomenon is normally seen in decidual samples taken in early pregnancy when uNK cells comprise 40% of all cells in the stromal compartment (Moffett-King, 2002). In the absence of pregnancy, an increase in the number of neutrophils and eosinophils is detectable in the uterine stroma (Poropatic et al., 1987; Jeziorska et al., 1995), and MCs become highly activated: these differing cohorts seem to differentiate between tissue that is prepared for implantation or that in which the inflammatory processes associated with menstruation and tissue repair have been initiated (Kamat and Isaacs, 1987; Bulmer et al., 1988; Salamonsen, 1998). It is therefore interesting to observe the presence of increased uNK cell numbers and one of premenstrual tissue (highly activated MCs), both a feature of early pregnancy, in users of the LNG-IUS in which local levels of progesterin are very high, but particularly in those who are experiencing uterine bleeding. This finding was also observed in a study first reporting the uNK cells and their association with regulating cytokines in post-menopausal endometrium, demonstrating a possible mechanism by which the hormonal therapy might induce the irregular bleeding (Hickey et al., 2005).

Leucocyte subpopulations were previously examined in the LNG-exposed endometrium (Critchley et al., 1998; Vincent et al., 2000), and in accord with the present study, an elevated number of large granulated lymphocytes and macrophages were found 1 month after the insertion of the LNG-IUS. Clark et al. (1996) reported a negligible neutrophil influx in the endometrial samples exposed to local action of LNG in Norplant users. In contrast, Vincent et al. (1999) reported increased numbers of neutrophils and eosinophils in the endometrium of Norplant users in those samples that were
decidualized or showing signs of tissue breakdown. However, much higher levels of LNG are present in the endometrium in the case of LNG-IUS users than in users of contraceptive implants (Vincent and Salamonsen, 2000). Indeed, in users of the LNG-IUS, intrauterine levels of the steroid are 1000-fold greater than serum levels, and it is therefore not surprising that the effect of LNG on the endometrium is different in users of the two types of contraceptives (Nilsson et al., 1982; Pekonen et al., 1992).

The mechanisms involved in the influx of leucocytes in the endometrium are not fully understood. A large number of chemokines are produced in the endometrium, and their cyclical variation is correlated, at least to some extent, to the leucocyte subsets found during each phase; however, redundancy clearly exists (Jones et al., 1997). Since such chemokines are important during the menstrual cycle, they may represent potential candidates as mediators related to BTB provoked by the action of LNG on the endometrium. For this reason, the two chemokines, 6Ckine and IL-8, which are related to uNK cell and neutrophil recruitment, respectively, were chosen for evaluation in this study.

The cellular localization of these chemokines is critical since the endometrium is heterogeneous, particularly at the time of menstruation and decidualization. Among the nine chemokines abundantly expressed in the human endometrium at different stages of the menstrual cycle (Jones et al., 2004), 6Ckine was expressed at moderate/low levels in menstrual, proliferative and mid-secretory stages, whereas IL-8 was highly expressed only in the menstrual phase. The immunoeexpression of IL-8 in users of the LNG-IUS has been previously described by Jones et al. (1997), who detected high immunoreactive IL-8 levels 1 month after insertion of the LNG-IUS, with a subsequent decrease after 3 and 6 months. Furthermore, a number of other chemokines, including 6Ckine, are up-regulated in the decidualized stroma in LNG-IUS users (Jones et al., 2005), although these did not correlate with bleeding. In our study, we found no difference between the two groups, and this may be explained by the fact that all users had been using LNG-IUS for more than 3 years. It is well established that the percentage of highly decidualized samples in the endometrium of LNG-IUS users increases as the duration of use of the device increases (Luukkainen et al., 1990). Decidual derived chemokines such as 6Ckine have also been implicated in the recruitment of uNK cells to the implantation site (Jones et al., 2004). The high chemokine production and the higher number of uNK cells in the study group that maintained some form of bleeding pattern seem to be a consequence of the extensive decidualization of the endometrium.

An important finding of this study was that there was a significantly higher area of extracellular tryptase in the samples obtained from women with bleeding than from non-bleeding samples. Extracellular tryptase is one of many regulatory factors released from MCs upon their activation: these include cytokines, and a range of enzymes, some of which can degrade extracellular matrix. Tryptase itself is an important activator of MMP-3, an enzyme that is central to an activation cascade for other MMPs, and which along with these other MMPs is important for tissue breakdown in endometrium (Salamonsen and Lathbury, 2000). Although Drudy et al. (1991) related no significant differences in the MC count at different phases of the menstrual cycle and in women with dysfunctional uterine bleeding, this study did not take account of the activation state of the MCs.

It was not possible in this study to identify precisely whether the increased uNK cells and increased MC activation are responsible for BTB. It has been proposed that BTB is most likely caused by focal alterations in the endometrium, and this is confirmed by the hysteroscopic visualization of the dilated bleeding vessels (Hickey et al., 2000) and histologically by focal areas of leucocyte infiltration in tissue breakdown, surrounded by unaltered atrophic endometrium (Clark et al., 1996; Song et al., 1996).

In a previous report (Oliveira-Ribeiro et al., 2004) using the same endometrial samples, we identified significantly more immunoreactive MMP-3 in the women who maintained some form of bleeding pattern. Importantly, this enzyme is directly activated by MCT. Thus, focal action of MMP-3 could provide an explanation for the bleeding in those users. The lack of correlation between some of the other endometrial parameters evaluated in the present study and the presence or absence of a bleeding pattern could be explained by the fact that our study sample was composed of women who had used the LNG-IUS for a long time and mainly because the group with bleeding patterns maintained some form of bleeding pattern but not prolonged bleeding or menorrhagia. It is possible to identify the mediator and effector cells involved in BTB by carrying out a detailed analysis of bleeding sites versus non-bleeding areas in the same patient (Lockwood et al., 2000), as identified by hysteroscopy. Unfortunately, this is an invasive procedure that causes discomfort to the patients, that is not widely used and not performed in our clinic.

In conclusion, we have demonstrated that neutrophils are not abundant in the endometrium of long-term users of the LNG-IUD and that while both IL-8 and 6Ckine are highly expressed in the decidualized stromal cells, they did not differ between women with and without uterine bleeding. Importantly, uNK cells were more abundant in long-term users of the LNG-IUS who maintained some bleeding patterns, and the level of MC activation was also higher in these women than in those who do not bleed, suggesting that these factors may play a role in the uterine bleeding in long-term users of the LNG-IUD.

Acknowledgements
This study received partial financial support from the Fundação de Amparo a Pesquisa do Estado de São Paulo, Brazil, award 03/083917. L.A.S. is supported by the National Health and Medical Research Council of Australia (143798, 241000) the NIH (grant HD43192) and the World Health Organisation Human Reproduction Program (15208).

References


Submitted on October 26, 2005; resubmitted on December 1, 2005; accepted on December 6, 2005.