Endometrial dendritic cell populations during the normal menstrual cycle

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BACKGROUND: Dendritic cells (DCs) are specialized antigen presenting cells that are highly involved in the stimulation and modulation of the immune response within mucosal surfaces, including the female reproductive tract. DCs have been poorly characterized in the non-pregnant endometrium. METHODS: Hysterectomy specimens were obtained from premenopausal women (n = 49) with histologically normal endometrium. Endometrial sections were stained immunohistochemically using antibodies for monoclonal mouse anti-human CD1a and CD83, two markers which are specific for populations of immature and mature DCs, respectively. RESULTS: There was a significantly higher density of endometrial CD1a⁺ DCs than CD83⁺ DCs throughout the menstrual cycle (P < 0.001). The density of CD1a⁺ and CD83⁺ DCs did not vary between the fundus and isthmus of the uterus. There was a significant increase in the density of CD1a⁺ DCs, but not CD83⁺ DCs, in the basal layer of the endometrium through the phases of the menstrual cycle. The density of CD83⁺ was significantly greater in the basal layer compared with the functional layer during both the proliferative (P = 0.004) and secretory phases (P = 0.001), whereas for CD1a⁺ DCs, the greater density in the basal layer was only observed in the secretory phase (P < 0.001). CONCLUSIONS: The highly coordinated cyclical changes in DC populations during the normal menstrual cycle reported in this study may be important for local regulatory mechanisms relevant to menstruation and implantation; alterations in this normal profile may contribute to the development of disturbances of function, fertility and even benign gynaecological disease.

Keywords: dendritic cells; endometrium; menstrual cycle; CD1; CD83

Introduction

Dendritic cells (DCs) are a heterogeneous and dynamic population of leukocytes that play an essential role in the initiation and modulation of the immune response throughout the human body, including the female reproductive tract. DCs can be broadly classified according to their developmental pathway; plasmacytoid (lymphoid) and myeloid DCs develop from different haematopoietic progenitors and are proposed to differ in terms of function, tissue distribution, cytokine production and markers. Plasmacytoid DCs are mainly involved in the recognition of viruses and the production of type 1 interferon, whereas myeloid DCs, which are the focus of this study, are known for their role in the initiation of the T-cell response (Ueno et al., 2007).

In addition to the diversity in DC developmental pathways, these cells are adaptive to pathogens and other environmental signals and undergo phenotypic changes during their normal lifespan. Myeloid DCs are integral to the maintenance of peripheral immunity and can be classified in terms of maturation status, which is associated with a specific functional profile (Adams et al., 2005). Immature DCs patrol peripheral tissues where they capture and process foreign antigens. In response to danger signals such as foreign antigens or inflammatory signals, DCs undergo maturation and travel to the lymph nodes via the afferent lymphatics where antigens are presented to T-cells in conjunction with major histocompatibility complex (MHC) molecules. DCs can also regulate the development and maintenance of peripheral tolerance through the uptake of self-antigens (Banchereau and Steinman, 1998).

Uterine leukocytes play a crucial role in the maintenance of the normally sterile uterine environment during the menstrual cycle. Numerous studies have examined lymphocyte populations within the normal endometrium during the menstrual cycle, but an examination of DCs has been overlooked in this research (Kamat and Isaacson, 1987; Bulmer et al., 1988; Starkey et al., 1991; Flynn et al., 2000). As in other tissues, the uterine immune system is dependent on antigen presenting cells (APCs) to initiate and stimulate a
co-ordinated immune response. DCs communicate both directly and indirectly with many other types of leukocytes, including T cells, B cells, natural killer cells and macrophages, thereby shaping the immune response in its entirety (Banchereau and Steinman, 1998). Despite the importance of DCs as antigen-presenting cells, there is a scarcity of research examining DCs in the non-pregnant uterus during the normal menstrual cycle.

The human uterus is unique in its ability to support the semi-allograft fetus, while maintaining the immune protection that is normally associated with other mucosal barriers within the human body (Robertson, 2000). Previous studies have focused on DCs in the decidualized endometrium and their possible role in early spontaneous abortion (Kammerer et al., 2000; Gardner and Moffett, 2003; Askeland et al., 2004). DCs are crucial mediators of the immune response and tolerance, and are likely to be involved in this balance between immune defence and fetal tolerance.

Menstruation has been described as an inflammatory event, which is accompanied by an increase in the number of leukocytes, such as natural killer cells, macrophages and neutrophils, and their associated pro-inflammatory products (Salamonsen and Lathbury, 2000). Although steroid hormones are largely responsible for the transformation of the endometrium during the menstrual cycle, these immune cells and their secreted products are potential mediators of local endometrial changes (Jones et al., 2004). Chemokines and cytokines, which are secreted by DCs and other leukocytes, have been implicated in the processes of menstruation, tissue repair, angiogenesis, embryo implantation and parturi- tion (Kayishli et al., 2002). The potential involvement of DCs in the inflammatory environment of the uterus in the perimenstrual period is unknown. Alterations in the DC populations may disrupt the normal co-ordination of uterine leukocytes contributing to menstrual disturbances, or to the development of pathology or infertility.

Our aim in this study was to characterize myeloid DCs within the normal premenopausal endometrium during different phases of the menstrual cycle. We used the immunohistochemical markers CD1a and CD83, two markers which are specific for immature and mature DCs, respectively, to quantify DCs during the proliferative, secretory and menstrual phases of the menstrual cycle. CD1a, a cell surface glycoprotein, that is structurally related to the MHC molecules and which mediates a MHC-independent antigen presentation pathway, is a highly specific and sensitive marker of immature DCs and Langerhans cells, a population of DCs found in the skin (Krenacs et al., 1993). CD83 is a glycoprotein member of the immunoglobulin superfamily that is strongly up-regulated during DC maturation making it a robust marker for mature DCs (Lechmann et al., 2002). In addition, we examined the density of DCs in different regions of the uterus, the fundus and isthmus, and in different layers of the endometrium, the functional and basal. An understanding of uterine DC populations may reveal important information about their role in maintenance of the normal immune protection of the uterus during the menstrual cycle, as well as in the processes of menstruation, implantation and in the development of disease or infertility.

Materials and Methods

Collection of tissue

This study was approved by the Human Ethics Committees of the Southwest Sydney Area Health Service and the University of Sydney. Hysterectomy samples were obtained from 49 premenopausal women (mean age 45.0, range 36–51) with histologically normal endometrium and pathology which was expected to have minimal effect on endometrial immune function. Paraffin embedded full-thickness hysterectomy samples, containing both endometrium and myometrium were obtained from recent surgical pathology archive blocks. Indications for hysterectomy in these women included uterine prolapse, heavy menstrual bleeding, small intramural fibroids and minimal adenomyosis. None of the patients with uterine prolapse had a complete prolapse (procidentia).

Menstrual cycle staging, using an idealized 28 day cycle, was determined by a blinded specialist gynaecological pathologist for all samples. Uterine samples were collected from both the fundus and isthmus of the uterus. Of the 28 normal samples collected from the fundus, 6 were in the menstrual phase (days 1–7), 12 were in the proliferative phase (days 8–14), and 10 were in the secretory phase (days 15–28). Of the 21 normal samples collected from the isthmus, 6 were in the menstrual phase, 6 were in the proliferative phase, and 9 were in the secretory phase. Paired samples, collected from fundus and isthmus of the same patient during the proliferative (six pairs), secretory (nine pairs) and menstrual phases (six pairs) were used for the analysis of the effect of region. Additional samples were obtained from the fundus only and were combined with paired samples in subsequent analyses.

Immunohistochemistry

Tissue sections were cut at 4 µm and immunostained using monoclonal antibodies for mouse anti-human CD1a (dilution 1:100; Dako Cytomation, Carpinteria, CA, USA) and mouse anti-human CD83 (dilution 1:50; Serotec, Raleigh, NC, USA) for 30 min at room temperature. Antigen retrieval was performed prior to immunostaining for all sections using a pH 9 target retrieval solution (Dako Australia) for all CD1a slides and Proteinase K (Dako Australia) was used for all CD83 slides. Dual endogenous enzyme block (Dako Australia) was applied to all sections for 10 min prior to incubation with the primary antibody. Following the incubation with the primary antibodies, sections were washed in Tris Buffer and incubated with REAL Link Biotinylated Secondary antibody (Dako Australia) for 15 min. Slides were then washed and incubated with streptavidin alkaline phosphatase (Dako Australia) for 15 min and stained with permanent fast red chromogen (Dako Australia) for 10 min. Slides were counterstained with Mayers haematoxylin prior to evaluation. Negative and positive control slides were incorporated into each slide run. Skin and tonsil were used as positive control tissues for CD1a and CD83, respectively. The negative control slides were processed using the same protocol as sample slides, except the primary antibody was omitted.

All immunostaining was carried out on a Dako Autostainer Model S3400 (Dako USA). Images of the sections were captured using an Olympus BX51 microscope and DP70 digital camera (Olympus, Tokyo, Japan). DC staining was assessed using the Image Pro Plus Discovery software (MediaCybernetics, MD, USA). Images were obtained from the functional and basal layers of the endometrium. Twenty 250 µm² sections from both the functional and basal layers were captured for each tissue section. As the functional layer of the endometrium is partially shed during menstruation, data were only obtained from the basal layer for all samples for the menstrual phase. Cells were only counted as positive if they demonstrated both positive staining and
DC morphology. DC morphology was defined nucleated cells of appropriate size with visible processes extending from the cell body. A visible DC process without an apparent cell body was not counted as a DC. The density of CD1a+ and CD83+ DCs per mm² of endometrium was calculated for each specimen.

Statistical analysis

Results were expressed as the mean ± SE of CD1a+ or CD83+ DCs per mm². SPSS 13.0 (SPSS Inc., Chicago, IL, USA) was used to perform all statistical analyses and to calculate means and standard deviations.

The distribution of the data was examined prior to analysis and the Student's t-test was used to compare two normally distributed groups. The Mann–Whitney U-test was used to compare two groups that were not normally distributed. The Kruskal–Wallis test was used to compare three skewed groups. The Paired t-test was used to compare paired, normally distributed data. The Wilcoxon signed ranks test was used for paired, skewed data. Statistical significance was established at P-values of <0.05.

Results

Hysterectomy samples from 49 premenopausal women were obtained for this study. CD1a+ and CD83+ DCs were present in the functional and basal layers of the endometrium in all examined specimens (Fig. 1). There was a significantly higher density of CD1a+ DCs than CD83+ DCs in both layers of the endometrium throughout the menstrual cycle (P < 0.001) (Table I).

Paired samples were collected from the fundus and isthmus in order to examine the effect of region of the uterus on DC density (Fig. 2). The density of CD1a+ DCs did not vary between the fundus and the isthmus during the proliferative, secretory and menstrual phases. There were also no significant differences in the density of CD83+ DCs between the fundus and isthmus of the uterus across all phases of the menstrual cycle. Data from the paired fundal and the isthmic samples were combined with unpaired samples for all further analyses.

The mean density of DCs in the functional and basal layers was compared during the proliferative and secretory phases of the menstrual cycle (Fig. 3). During the proliferative phase, the concentration of CD1a+ DCs was similar in the functional and basal layers of the endometrium. The total number of CD1a+ DCs per mm² was significantly higher in the basal layer than in the functional layer during the secretory phase (P < 0.001). The mean density of CD83+ DCs was significantly greater in the basal layer than in the functional layer during both the proliferative (P = 0.004) and secretory phases (P = 0.001).

The number of CD1a+ DCs significantly increased across the phases of the menstrual cycle in the basal layer (Fig. 4). The density of CD1a+ DCs increased in the basal layer between the proliferative phase and the secretory phase (P < 0.001), with a large influx of CD1a+ DCs occurring during the menstrual phase (P = 0.001). In the functional

Figure 1: CD1a+ and CD83+ DCs in the endometrium. (A) CD1a+ DCs in the endometrium. (B) CD83+ DCs in the endometrium. All images are shown at 40× magnification and stained with the chromogen permanent fast red.

Table 1. Mean density of CD1a+ and CD83+ DCs in the fundus and isthmus of the uterus and functional and basal endometrial layers during the menstrual cycle.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Region</th>
<th>Proliferative Functional</th>
<th>Basal</th>
<th>Secretory Functional</th>
<th>Basal</th>
<th>Menstrual Basal</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD1a</td>
<td>Fundus</td>
<td>18.5 ± 1.8</td>
<td>18.9 ± 1.8</td>
<td>16.2 ± 2.2</td>
<td>29.0 ± 4.4</td>
<td>74.1 ± 10.2</td>
</tr>
<tr>
<td></td>
<td>Isthmus</td>
<td>17.9 ± 3.4</td>
<td>19.3 ± 4.2</td>
<td>15.6 ± 2.4</td>
<td>36.8 ± 3.6</td>
<td>60.8 ± 15.2</td>
</tr>
<tr>
<td>CD83</td>
<td>Fundus</td>
<td>7.9 ± 1.2</td>
<td>12.3 ± 1.2</td>
<td>9.4 ± 1.2</td>
<td>15.1 ± 1.5</td>
<td>16.7 ± 2.9</td>
</tr>
<tr>
<td></td>
<td>Isthmus</td>
<td>10.9 ± 1.8</td>
<td>14.9 ± 1.5</td>
<td>10.8 ± 1.5</td>
<td>15.6 ± 2.0</td>
<td>21.6 ± 4.8</td>
</tr>
</tbody>
</table>

Data are represented by mean density (cells/mm²) ± SEM.
layer, the number of CD1a+ DCs remained stable between the proliferative and secretory phases. There was a small and progressive increase in CD83+ DCs during the menstrual cycle, but this increase was not statistically significant.

Discussion

This study utilized immunohistochemical methods to demonstrate that uterine DC populations vary by phase of the menstrual cycle and layer of the endometrium, but not by region of the uterus. Overall, the endometrial density of both CD1a+ and CD83+ DCs was relatively low compared with other types of leukocytes, such as macrophages and natural killer cells (Bulmer et al., 1988). Only a small number of DCs are necessary to patrol peripheral tissues and trigger a strong T-cell response in the presence of foreign antigen (Banchereau and Steinman, 1998). Previous research found few to no DCs in the endometrium using early DC markers.
The relative rarity of DCs and the lack of robust immunohistochemical markers made it previously difficult to quantify uterine DCs. We also found a significantly lower density of mature uterine DCs than immature uterine DCs which supports previous findings (Rieger et al., 2004). DC maturation is linked with migration out of peripheral tissues to secondary lymphoid organs, so the number of mature DCs in the uterus at any one time is likely to be small (Banchereau et al., 2000). The density of CD83+ DCs was higher in the basal layer during both the proliferative and secretory phases, which is also consistent with the concept that these mature DCs are transiting out of the uterus.

CD1a+ immature DCs increased in density throughout the menstrual cycle in the basal layer of the endometrium, whereas the number of CD83+ mature DCs remained relatively constant throughout the menstrual cycle. The increase in immature CD1a+ DCs, but not mature CD83+ DCs throughout the menstrual cycle suggests that the influx of DCs into the uterus may be hormonally regulated, but that DC maturation is not influenced by hormones. King et al. (1996) evaluated the expression of estrogen and progesterone receptor in CD45+ endometrial lymphocytes and found that neither receptor was expressed by these cells. It is likely that the influence of steroid hormones on uterine leukocytes including DCs is indirect via actions on cytokines and chemokines, a family of cytokines whose major known function is chemotaxis of leukocytes (McColl, 2002).

We found that the density of immature CD1a+ DCs in the basal layer of the endometrium increased throughout the menstrual cycle, presumably as DCs migrated into the uterus. The withdrawal of progesterone at the end of the luteal phase coincides with an uterine inflammatory response, which is accompanied by an increase in other types of leukocytes, such as macrophages, neutrophils and natural killer cells (Salamonson and Lathbury, 2000). This study indicated that immature DCs, like other uterine leukocytes, respond to the hormonal changes within the uterus and migrate into the uterus during the secretory and menstrual phases.

Using a different marker for immature DCs, Rieger et al. (2004) found that the density of immature DCs positive for DC-SIGN (DC-specific ICAM-grabbing nonintegrin, classified as CD209) did not change during the proliferative and secretory phases of the menstrual cycle. Hysterectomy samples from the menstrual phase were not included in this analysis and the density of DC-SIGN+ DCs may not have increased until the menstrual phase. It is also possible that the influx of certain immature DC populations into the uterus is hormone dependent, whereas the migration of other types of DCs is regulated by other mechanisms. Further studies utilizing additional DC markers are required to fully characterize this heterogeneous population of uterine DCs.

The exact role of DCs during the peri-menstrual period is unknown, but DCs can produce matrix metalloproteinases (MMPs), cytokines and chemokines, which are likely to influence the local uterine environment in the period leading up to menstruation or implantation. DCs in the skin were found to produce MMP-9 and MMP-2, which likely play a functional role in DC migration (Osman et al., 2002; Ratzinger et al., 2002). MMPs produced by uterine DCs could be involved in tissue breakdown at menstruation.

DCs can also produce cytokines and chemokines in response to environmental triggers, such as inflammatory stimuli (Bengtsson et al., 2004). Gene expression profiling studies

(Kamat and Isaacson, 1987; Bulmer et al., 1988).
have identified a number of cytokines and chemokines produced by DCs during different stages of maturation, including the interleukins, IL-10, IL-12, IL-6, TNF-α (tumour necrosis factor-α), RANTES (regulated upon activation normal T-cell expressed and secreted protein) and MCP-1 (monocyte chemoattractant protein-1) (Nagorsen et al., 2004). The production of cytokines and chemokines by DCs not only regulates the uterine local environment, but also other leukocyte populations and their secreted products. The careful co-ordination of these cytokines and chemokines is important in the regulation of uterine function and disturbances in the normal profile may contribute to implantation defects or infertility (Kayisli et al., 2002).

The mucosal lining of the female reproductive tract depends on the innate immune system to maintain a barrier against encountered foreign antigens, such as micro-organisms and semen. DCs can directly recognize specific pathogens using toll-like receptors, a class of pattern-recognition receptors and initiate an immune response, providing an important link between adaptive and innate immunity (Pasare et al., 2004). The need for immune protection against microbial infection and the acquisition of sexually transmitted disease is more substantial in the lower genital tract than in the uterus, and correspondingly, there is a high mean density of immature DCs in the lower genital tract, specifically in the vagina (Pudney et al., 2005), ectocervix (Morelli et al., 1992) and uterine cervix (Poppe et al., 1998). Therefore, it would be expected that the density of uterine DCs would be greater in the isthmic region than in the fundus. Surprisingly, we found no significant differences between the density of CD1a+ and CD83+ DCs in the fundus and isthmus of the uterus. DCs are highly motile and migrate in response to local signals; it is likely that under normal conditions uterine DCs are evenly distributed throughout the uterus and density can be altered in the presence of pathogens or other signals.

This study utilized tissue samples from women undergoing hysterectomy for a number of indications, but who had histologically normal endometrium. All women who have a hysterectomy are likely to have some uterine abnormality that precludes them from being completely ‘normal’. Also, women undergoing hysterectomy are likely to be in their late 30s or 40s, at the end of their reproductive lifetime. An attempt was made to exclude any patients with any conditions or pathologies that affect the endometrium or immune system. Although this study was not designed to examine DC populations in a number of minor pathologies, statistical analysis revealed no significant or suggestive differences between the subgroups.

In conclusion, we demonstrated that the density of immature CD1a+ DCs in endometrium increased throughout the menstrual cycle and is likely to be directly regulated by steroid hormones. Variation in the density of mature CD83+ DCs does not appear to be hormone dependent, and the overall number of these mature DCs is small, presumably due to their continual transit out of the endometrium upon maturation. The endometrium is a highly dynamic tissue where changes occur not only across the phases of the menstrual cycle, but on a daily or even hourly basis especially around the time of menstruation. DCs may be involved in the signalling and regulation of some of these local changes in the uterus. CD1a+ and CD83+ DCs represent only a portion of the entire uterine DC population and further studies are necessary to identify and characterize all uterine DCs. The alteration of uterine DC populations may contribute to the menstrual disturbances, implantation defects, infertility or other pathologies which characterize endometrial disease and further investigation is needed to examine the possible role of uterine DCs in these conditions.

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