Cells with haematopoietic stem cell phenotype in adult human endometrium: relevance to infertility?

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BACKGROUND: Uterine lymphoid cell repertoires are specialized in order to meet the twin demands of successful pregnancy and local immunosurveillance. The possibility that some of these populations might differentiate locally from progenitor cells has been proposed. METHODS: Endometrial tissue from women with a history of infertility as well as fertile controls was examined for haematopoietic stem cells (HSCs) and lymphoid progenitors using three-colour flow cytometry. RESULTS: Significant populations of phenotypic HSCs (CD34+ CD45+) were detected in all samples, a high proportion of which co-expressed the differentiation marker CD45RA (45.7%), indicating ongoing differentiation. Almost 30% of uterine HSCs co-expressed CD56 and 44% co-expressed CD7, suggesting the presence of lymphoid progenitors. Small proportions expressed CD127 and CD122, receptors for interleukin (IL)-7 and IL-15, respectively. HSC numbers were similar in the endometrial samples from fertile and infertile women. However, the proportion co-expressing the natural killer (NK) antigen CD56 was significantly increased compared with HSCs found in the endometrium of fertile controls (P = 0.002). CONCLUSIONS: This is the first demonstration of cells with an HSC phenotype in the human endometrium, and increased proportions of NK progenitors in endometrium of women with infertility suggests a dysregulation of this pathway that may contribute to infertility.

Key words: endometrium/haematopoiesis/human/infertility/stem cells

Introduction

The human endometrium has unique immunological requirements. In pregnancy, the endometrium must tolerate the invading embryo which will express both paternal and maternal antigens, although local immunity is critical to defence against pathogenic challenge and malignancy. Uterine natural killer (uNK) cells and T cells have critical roles in meeting both demands (Bulmer and Lash, 2005). While their exact role is unknown, it has been suggested that uNK cells may control implantation through recognition of non-classical major histocompatibility complex (MHC) antigens expressed by fetal trophoblasts and the production of suppressive cytokines (Moffett-King, 2002). It has also recently been shown that NK/NKT cells may be involved in the Th1/Th2 hypothesis which has dominated reproductive immunology for several years. Borzychowski et al. (2005) have shown that the type-2 shift during pregnancy was predominantly in the NK-cell (CD56bright and CD56dim) and NKT-cell (CD56+ CD3+ ) populations instead of in the Th or cytotoxic T-cell populations. Elevated NK cell activity has been implicated as a risk factor for infertility, failure of IVF treatment and early pregnancy loss (Fukui et al., 1999; Matsubayshi et al., 2001; Yamada et al., 2001).

Endometrial T-cell populations also play a role in successful pregnancy. Some decidual CD3+ cell populations appear to be specialized for their location (Flynn et al., 2000; Trundley and Moffett, 2004) and include a significant population of γδ T cells, regulatory T cells and NKR+ T cells. Local γδ T cells have been implicated to have a role in successful pregnancy and infertility (Mincheva-Nilsson, 2003). In addition, regulatory T (Treg) cells, a population with an important role in tolerance, are abundant in the human decidua, and have been shown to be decreased in decidua from spontaneous aborters (Aluvihare et al., 2004; Sasaki et al., 2004). NKR+ T cells which co-express NK cell receptors and a functional T cell receptor (TCR) are also increased in decidua (Tsuda et al., 2001). Their ability to mediate lysis in both a T and NK cell manner, their restriction by non-classical MHC molecules and production of both Th1 and Th2 cytokines suggest that NKR+ T cells have important immunoregulatory significance (Doherty et al., 1999; Abo, 2001). However, the role of NKR+ T positive cells in pregnancy outcome remains relatively unexplored.
Given that lymphoid populations are important for successful pregnancy, how do these specialized populations arise in the endometrium? Regulation of leucocyte homing, proliferation and differentiation within the endometrium is poorly understood. Certainly, there is evidence that specialized mechanisms that control uterine lymphocyte homing correlate with phases of the cycle (Kammerer et al., 2004). However, in addition to induced homing from the periphery, it is also possible that lymphoid cells differentiate locally in adult endometrium. Although the evidence over the last decade favours the former, extrathymic lymphoid development in situ may influence the specialized lymphoid repertoire in human endometrium. In humans, it has been proposed that uNK cells may be renewed from a precursor within the non-cycling portion of the endometrium (Wira et al., 2005). A recent study has also shown that when T, B and NK cell deficient NOD/SCID/gamma-null mice, which are deficient in T and B cells and NK activity, are transplanted with human endometrium, there is an increase in T and NK cell levels by day 28. As the mice originally lacked lymphocytes, they could not have been derived from the periphery and must have arisen from the human transplanted endometrium (Matsuura-Sawada, 2005). Proliferation of uNK cells from a local progenitor population may therefore account for the dramatic increase in number seen at the proliferative phase of the menstrual cycle and during pregnancy (Bulmer and Lash, 2005). Indirect evidence also suggests that local production of T lymphocyte subpopulations in the endometrium is possible. Yeaman et al. (2001) showed CD8+ T cell division by staining for Ki-67 staining in the endometrium, albeit in low levels. Recombinase-activating gene (RAG) transcripts, the molecular machinery required for T-cells development, have also been detected in the human decidua (Hayakawa et al., 1994; Mincheva-Nilsson et al., 1997).

If lymphoid differentiation does occur, haematopoietic stem cells (HSCs) and lymphoid progenitors should be present in the endometrium. HSCs are defined as clonogenic cells capable of self-renewal and multilineage differentiation (Till and Mc Culloch, 1961). All mature circulating blood cells are derived from self-renewing multipotent HSCs (Krause et al., 1996). Stem cells of haematopoietic origin are identified by the co-expression of CD34 and CD45, as CD34 is also expressed by some non-haematopoietic cells (Lin et al., 1995). Early on, in the haematopoietic pathway, CD34+ stem cells acquire CD45. As HSCs mature and begin to differentiate, CD34 is gradually down-regulated with the concomitant up-regulation of activation markers, CD38 and CD45RA. Haematopoietic precursors destined for different cell lineages are identified by co-expression of lineage-specific markers. CD34+CD45+ cells committed to myeloid differentiation co-express CD33, whereas immature lymphoid precursors co-express CD56 (NK), CD7 (T cells) and CD19 (B cells) (Figure 1). Expression of receptors for interleukin (IL)-7 and IL-15, cytokines required for T and NK cell development, indicates responsiveness to these cytokines (Civin and Gore, 1993).

Previous studies of endometrial lymphoid development have mainly focused on the pregnant decidua. This study aims to examine the human endometrium for the presence of phenotypic HSCs and to determine the impact of the menstrual cycle and other parameters on these cell populations. It also further characterizes the phenotype of these cells in both fertile and infertile women to assess whether a dysregulation in this pathway could be a factor in some cases of previously unexplained infertility.

Materials and methods

Patients

Nineteen premenopausal women admitted for operative procedures under general anaesthetic at the National Maternity Hospital, Dublin, were studied (Table I). Fertile women (n = 7) were attending for a range of procedures, mainly tubal ligation, whereas women with infertility (n = 12) were attending for tubal patency assessment via laparoscopy and hydrodistention, having had a normal reproductive hormone profile and semen analysis prior to this assessment. Of the infertile women, eight had primary infertility and four had secondary infertility. Patient histories were analyzed in detail and any women with factors that could interfere with normal endometrial physiology were excluded. Stage of menstrual cycle was determined for each patient by histological assessment (Noyes et al., 1950). The study was approved by the hospital’s Ethics Committee and written informed consent was obtained from all participants.

Endometrial samples

Endometrial biopsies were obtained by sharp curettage and were collected in ion-free (calcium and magnesium free) Hanks balanced salt...
solution (HBSS) (GIBCO BRL, Paisley, Scotland) supplemented with antibiotics (100 U/ml penicillin, 100 mg/ml streptomycin) and 5% fetal calf serum (FCS), (GIBCO), transported to the laboratory, drained and weighed. The samples were retained for flow cytometric analysis, which was performed immediately.

Preparation of single cell suspensions

Single cell suspensions of endometrial cells were prepared as previously described (Flynn et al., 1999). In brief, the tissue was washed thoroughly with HBSS to remove residual blood, minced finely with opposing scalpels and placed in 5 ml of enzyme solution containing RPMI, 20 mM HEPES buffer, 1% FCS, 1% bovine serum albumin (BSA) (GIBCO BRL) 200 U/ml collagenase IV (Worthington Biochemicals, NJ, USA) and DNase I (Sigma, Poole, UK). In general, 5 ml of enzyme solution is required per 1 g (or less) of tissue. The digestion mix was rotated for 20 min at 37°C. The suspension was then passed through a 30 μm gauze to remove tissue fragments. The resulting suspension was washed twice with HBSS at 400 g for 10 min and resuspended in 1 ml complete RPMI (plus 20 mM HEPES, antibiotics and 10% FCS). Cell yields and viability were assessed by Ethidium Bromide/Acridine Orange staining. The cell suspension was adjusted to $1 \times 10^6$ cells/ml in complete RPMI.

Blood samples

Five millilitres of venous blood was collected in heparinized tubes concurrently in all 19 participants in the study. Peripheral blood mononuclear cells were prepared by standard density gradient centrifugation over Lymphoprep (Nycomed, Oslo, Norway) at 400 g for 25 min. Cells were then washed twice with HBSS supplemented with HEPES buffer solution (GIBCO BRL) and antibiotics. Cell pellets were resuspended in 1 ml complete RPMI, and cell yields and viability were assessed as before. The cell suspension was adjusted to $1 \times 10^6$ cells/ml in RPMI.

Cell surface staining for flow cytometric analysis

Aliquots of 100 μl ($1 \times 10^5$) cells were labelled with monoclonal antibodies (mAbs) directed against cell surface markers classically associated with differentiating haematopoietic progenitor cells. The appropriate mAbs (0.3 μg/ml final concentration) were added to cells, which were incubated in the dark at 4°C for 30 min. Cells were then washed twice with 1 ml PBS–BSA Azide. Labelled cells were fixed in 0.5 ml 1% paraformaldehyde (Sigma Aldrich, Ireland). mAbs used in this study

For the identification/detection of cells of haematopoietic origin, fluorescein isothiocyanate (FITC)-labelled anti-CD45 mAb (Becton Dickinson, UK) was included in all tubes. For the identification of stem/progenitor cells, peridinin chlorophyll protein (PerCP)-labelled anti-CD34 mAb (clone 8G12; Becton Dickinson) was used. In addition to anti-CD45-FITC and anti-CD34-PerCP, one of the following phycoerythrin (PE) labelled anti-differentiation/lineage marker mAbs was also used in each tube: anti-CD117 (c-kit; early stem cell marker), anti-CD45RA (activation, naive T-cell marker, B cells and monocytes; both from Pharmingen, San Diego, CA, USA), anti-CD33 (myeloid progenitor marker), anti-CD56 (NK cell) anti-CD127 (IL-7 receptor; all from Becton Dickinson, UK) was included in all tubes. For the identification of monocytes and macrophages, the anti-CD14-FITC mAb (clone HCD14; Becton Dickinson) was used.

mAbs used in this study

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Table I. Relevant clinical details and history of participants in this study

Statistical analysis

The median and range were used to express results. Differences between groups were assessed using the Mann–Whitney U-test, or ANOVA, for comparisons of three or more groups. P < 0.05 was considered significant.
Results

Cell yields and viability
Endometrial curettings were collected from each patient. The average weight of the endometrial sample was 1.51 g (range 0.5–4.28 g). The mean mononuclear cell yield obtained from the endometrial single cell suspension was $1.86 \times 10^6$ cells/ml (range 0.4–6.6 $\times 10^6$). The weight of the biopsy did not significantly affect the cell yield. Cell viability in all cases exceeded 80%. Matched peripheral blood (5 ml) was also collected from each patient. The mean mononuclear cell yield obtained from 5 ml of peripheral blood was $7 \times 10^6$ cells/ml. Cell viability in all cases exceeded 85%.

Detection of phenotypic endometrial HSCs
The gating method for the detection and characterization of phenotypic HSC cells is outlined in Figure 2. Significant populations of phenotypic CD34$^+$ CD45$^+$ HSCs were detected in all endometrial biopsies studied (median 3.15% of CD45$^+$ cells; range 1.2–6.29; $n = 19$). The majority of endometrial HSCs express low levels of CD34 and high levels of CD45, indicating a more differentiated phenotype than the primitive multipotent CD34$^{\text{high}}$ HSCs found in the bone marrow (Figure 3A). The endometrial samples had significantly larger populations of phenotypic HSCs (3.15%) than matched peripheral blood (0.35%; 0.08–1.4; $n = 19$, $P < 0.0001$; Figure 3B).

Effect of menstrual cycle stage on phenotypic HSC levels in the endometrium
Menstrual data were collected for all women and correlated with histology to determine cycle stage. Seven women were in the proliferative phase of their menstrual cycle, 10 women were in the secretory phase and 2 were in the early proliferative phase. Endometrial cells phenotypic of HSCs were found in all endometrial biopsies and menstrual cycle stage was not found to influence HSC levels (early proliferative versus secretory, $P = 0.6$; early proliferative versus proliferative, $P = 0.6$; secretory versus proliferative, $P = 0.975$; Figure 4).

Early stem cell antigen expression
Three colour analyses were used to determine the co-expression of the cell surface markers classically associated with immature, differentiating and lineage restricted phenotypic HSCs as well as cytokine receptors on endometrial CD34$^+$ CD45$^+$ cells. Figure 5 illustrates the representative plots of co-expression markers on CD34$^+$ CD45$^+$ in the endometrium and blood and a graph of the various marker median levels. The early stem cell marker, c-kit, was found to be co-expressed on only a small proportion of the phenotypic endometrial HSCs (median: 2.57%, range 0–9%) compared with a much higher proportion of phenotypic HSCs from matched peripheral blood (7.96%; *$P < 0.05$) (Figure 5). This suggests that the population, phenotypic to HSCs in the endometrium, is more mature than its equivalent circulating population. The activation antigen (CD45RA) was expressed on 45.7% of endometrial HSCs, an indication of active differentiation. There was no significant
difference between CD45RA co-expression on HSCs in matched peripheral blood (60.67%) and endometrium. The high level of CD45RA in the periphery suggests that CD45RA may be associated with mobilization of HSCs from bone marrow (Figure 5).

Lineage-specific antigen expression
To further characterize endometrial phenotypic HSCs, the co-expression of lineage-specific antigens, CD33 which identifies myeloid progenitors, CD56 which identifies NK cell progenitors and CD7 which identifies T cell progenitors, was also investigated. Approximately 20% endometrial HSCs co-expressed the myeloid antigen (CD33), suggesting that production of myeloid cells is not the primary function of endometrial HSCs. Of particular interest, however, was the co-expression of the lymphoid antigens CD56 and CD7, which were co-expressed on 30% and 45% of endometrial HSCs, respectively. This suggests that the CD34+ CD45+ cells of the endometrium are primarily involved in NK, T or NKT cell production. This phenotype is in contrast to the HSC population of normal bone marrow (median: CD7, 9.9%; CD56, 3.73%; CD33, 54.3%) examined in a previous study (Lynch et al., 2006). Differences were observed in the proportions of peripheral blood HSCs co-expressing a proportion of these lineage markers (CD56, 43%; CD7, 70%), although this was not significant. Lymphopoietic cytokine receptors IL-7R-α and, to a lesser extent, IL-2/15R-β were also detected on endometrial HSCs (Figure 5), suggesting that these cells are responsive to IL-7 and IL-15, cytokines with key roles in lymphoid differentiation.

Phenotypic HSCs in the infertile endometrium
Within the endometrial samples collected, 12 patients had infertility (eight primary and four secondary). To investigate if there was any difference in the cells with HSC phenotype populations between normal and infertile endometrium, HSC levels and lineage marker expression were analysed in all patients. No significant difference between levels of HSCs (CD34+ CD45+ as percentage of total CD45+ cells) within normal and infertile endometrium cell preparations was seen (Figure 6). In addition, there was no significant difference in phenotypic HSC levels in the peripheral blood between fertile and infertile women (data not shown).

Although levels of phenotypic HSCs were similar in fertile and infertile patients, significant differences were seen in their lineage marker expression. Although these are preliminary results from a small cohort, significantly higher
expression of the NK marker CD56 by HSCs was found in the infertile group (*P < 0.02; Figure 7A). Although the mean level of CD7 expression was higher in CD34+ CD45+ cells from endometrium of fertile women compared to infertile women, this was not significant. In addition, there were significantly lower levels of CD127 co-expression by phenotypic HSCs in the infertile endometrium compared to normal endometrium (*P = 0.04; Figure 7B). CD127, the IL-7 receptor, is essential for TCR-γδ development (Laky et al., 1998).

Discussion
Much focus has been given to the study of lymphocyte populations in the endometrium and decidua. Although there is evidence of fluctuating levels of these cells through the stages of the menstrual cycle and pregnancy, it is still not fully understood where these cells originate. Clearly trafficking from the periphery may play a role. However, in situ proliferation from a local pool of lymphoid progenitors may also be important. In this study, using flow cytometry, a population phenotypic of HSCs was detected for the first time in the endometrium of healthy women. Indeed, the adult endometrium is enriched with this HSC population when compared with matched blood.

The concept of extrathymic lymphocyte development predicts that lymphoid progenitors and all factors required for their maturation are present at extrathympic sites. Previous studies have provided indirect evidence that the normal human decidua and endometrium may support lymphopoiesis, such as the presence of RAG transcripts in decidua (Hayakawa et al., 1994; Mincheva-Nilsson et al., 1997) stromal mitoses in endometrium (Bulmer and Lash, 2005) and early pregnant human decidua (Pace et al., 1989; Mincheva-Nilsson et al., 1997). In addition, proliferation of uNK cells in late secretory endometrium and decidua has been demonstrated by the detection of the Ki67 proliferation antigen (Jones et al., 1998; Trundley and Moffett, 2004). Collectively, these studies support the hypothesis that lymphoid development may occur in situ in the endometrium and decidua.

Although all haematopoietic stem and progenitor cells express CD34 (Krause et al., 1996), not all CD34+ cells are HSCs, as CD34 is also an endothelial antigen. In this study, we used co-expression of CD45 on endometrial CD34+ cells to identify HSCs. However, considerably more than half of the CD34+ population detected in the endometrium did not express CD45 (Figure 3, endometrium; upper left-hand quadrant), suggesting that they were not of haematopoietic origin. It is unlikely that all CD45− CD34+ cells are mature endothelial cells, as the gating technique used would have excluded the majority of endothelial cells on the basis of size. A previous study has shown that CD34+ cells, along with other stem cell markers, are present in the stroma of the basalis layer (Cho et al., 2004). It is thus possible that this is a mixed population containing non-cycling HSCs that have yet to up-regulate CD45, stromal cells, endometrial epithelial and stromal stem cells (Schwab et al., 2005).

In this study, the presence of CD45RA, the sparse expression of c-kit and high levels of lineage antigen expression on phenotypic endometrial HSC populations indicate that the endometrial HSCs are a relatively mature, lineage-committed population. Almost half of the CD34+ CD45+ HSCs in the endometrium co-expressed the lymphoid antigens CD7, whereas approximately 20% co-expressed the myeloid antigen CD33. This endometrial HSC phenotype is in stark contrast to the HSC population of normal human bone marrow (Lynch et al., 2006), where the majority of CD34+ CD45+ cells co-expressed CD33 (54.3%) with T-cell progenitors comprising only a small proportion of HSCs (9.9%). Approximately 30% of phenotypic endometrial HSCs co-expressed the NK cell antigen (CD56), an almost 10-fold increase than that of bone marrow HSCs (3.73%). This is a distinctive progenitor population, previously only described in the

Figure 7. Co-expression of lineage markers on normal and infertile endometrial HSCs (CD34+ CD45+). There was significantly more CD56 co-expression (*P < 0.05) on HSCs from infertile (n = 10) than normal (n = 7) endometrium (A). There was less CD127 co-expression (*P = 0.04) on HSCs from infertile endometrium (n = 6) than normal endometrium (n = 6) (B).
human intestine, liver and lymph nodes (Golden-Mason et al., 2000; Freud et al., 2005; Lynch et al., 2006) and other sites where local lymphopoiesis is thought to occur. These results suggest that the phenotypic HSC population of the endometrium is a different population to bone marrow HSCs and, unlike bone marrow HSCs, endometrial phenotypic HSCs appear to be preferentially focused on lymphoid differentiation indicating a possible priority for T and NK/NKT cell differentiation in the endometrium. The presence of CD34+ CD45+ CD56+ cells in adult human endometrium suggests a role for this organ in NK cell production.

It is also worth noting that the majority of CD56+ cells co-expressed by CD34+ CD45+ cells were CD56bright. This was true for all endometrial samples, with the exception of one individual which expressed significant populations of both CD56bright and CD56dim cells. In contrast, the majority of CD34+ CD45+ cells were CD56low/medium in the peripheral blood. It is known that the uNK population is functionally and phenotypically different from peripheral NK cells, being predominantly CD56bright in contrast to CD56dim cells in the blood (Dosiou and Giudice, 2005). There is also a significantly larger population of NKt cells in the decidua compared with matched blood, a population that has important immunomodulatory role (Tsuda et al., 2001). This evidence combined with the presence of NK/NKT cell progenitors (CD34+ CD45+ CD56+) supports the possibility of local differentiation of NK+ cells in the human endometrium.

Few endometrial HSCs expressed e-kit in contrast to matched peripheral blood (P = 0.05), indicating that the peripheral blood phenotypic HSC population are more immature than those in the endometrium. There was no significant difference in expression of other lineage markers (CD7, CD56, CD33) by peripheral and endometrial HSCs, even though the median levels of CD7 and CD56 were considerably higher in peripheral blood. This may be due to the high variance in CD7 and CD56 expression in the endometrium. The high level of CD7 expression on peripheral blood HSCs is consistent with circulation of T-cell progenitors prior to their homing to the thymus and other organs which might support T-cell differentiation.

Small proportions of endometrial phenotypic HSCs co-express the IL-2/15 receptor CD122 (~5%), and approximately 10% co-express the IL-7 receptor CD127. The IL2/15 receptor is essential for NKT cell development (Ohteki et al., 1997) and IL-7 for TCR-γδ development (Laky et al., 1998). The presence of these lymphopoietic cytokine receptors on the phenotypic endometrial HSC population is further evidence of local lymphoid progenitors, which may be responsive to IL-7 and IL-15. IL-15 is a cytokine which has been detected in the endometrium (Kitaya et al., 2000).

Age appeared to have no effect on phenotypic HSC levels as our study group ranged from 22 to 54 years. However, all of the women involved in this study were premenopausal and so the presence and levels of HSCs in post-menopausal women remain undetermined. Phenotypic HSC populations were also unaffected by stage of menstrual cycle. Although this is merely speculative, it would suggest that the HSCs are located in the basal layer of the endometrium, which is not shed during menstruation, rather than in the more superficial functional endometrium, which is shed when menses supervenes.

Phenotypic populations of HSCs were found in all endometrial samples studied, regardless of fertility status, although the infertile endometrial phenotypic HSC population was significantly different from that of the fertile women with regard to CD56 and CD127 co-expression. Although one must be cautious about interpreting data from a small cohort (12 infertile women, 7 fertile women), the results were particularly interesting. The CD34+ CD45+ population in infertile women co-expressed more CD56 than the corresponding population in fertile controls. Although the possible causes of infertility are numerous, and a larger patient cohort is needed to verify the results, this finding may reflect dysregulation of the haematopoietic pathway, contributing to infertility as elevated NK-cell activity has been implicated as an independent risk factor for infertility, failure of IVF treatment and early pregnancy loss (Fukui et al., 1999; Yamada et al., 2001). The lower co-expression of CD127 on infertile endometrial CD34+ CD45+ cells may also have implications for women with infertility, given that IL-7R (CD127) is essential for TCR-γδ development, a cell population present in the endometrium and during pregnancy (Suzuki et al., 1995; Mincheva-Nilsson, 2003).

The results of this study show, for the first time, that the normal human endometrium harbours a population phenotypic of lineage-committed haematopoietic progenitors throughout the menstrual cycle. These cells differ significantly from their circulating and bone marrow counterparts. The majority of these progenitors express lymphoid-associated antigens and are thus likely to have a role in the emergence of lymphoid repertoires important to local immunoregulation and function. Furthermore, the HSC phenotype found in endometrium of infertile women differed from that of normal controls, suggesting that a dysregulation of the normal haematopoietic pathway might impact on human infertility; however, further studies are necessary to investigate the functional role of these cells in the human endometrium.

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