Early pregnancy Measurements of CD56+ cells in peripheral blood and endometrium by flow cytometry and immunohistochemical staining in situ

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BACKGROUND: CD56+ cells in peripheral blood or the endometrium may be increased in women with reproductive failure. However, the relationship between numbers of peripheral blood CD56+ and endometrial CD56+ cells is uncertain. The aim of this study was (i) to compare the numbers of CD56+ cells in peripheral blood and endometrium in samples taken simultaneously and (ii) to compare measurements by flow cytometry and immunohistochemistry of CD56+ cells in the same endometrial biopsy.

METHOD: Endometrial biopsies and blood were obtained from women with recurrent miscarriage (n = 25) on days LH+7–LH+9 of the cycle. The total number of CD56+, CD56+CD16− and CD56+CD16+ cells in blood was measured by flow cytometry; the number of CD56+ cells in the endometrium was determined by immunohistochemistry. Endometrial samples were also obtained from fertile women (n = 20) and used to measure CD56+ and CD45+ cells, by both flow cytometry and immunostaining.

RESULTS: There was no correlation between the numbers of total CD56+, CD56+CD16− or CD56+CD16+ in peripheral blood and the number of endometrial CD56+ cells in the same women. In endometrium from fertile women, a significant correlation was found between the numbers of CD56+ cells measured by flow cytometry and immunohistochemistry (correlation = 0.497, P = 0.026, when expressed as % total cells; correlation = 0.570, P = 0.009 when expressed as % CD45+ cells).

CONCLUSIONS: Measurements of CD56+ cells in peripheral blood do not correlate with endometrial CD56+ cell numbers and therefore should not be extrapolated to events in the endometrium. In contrast, measurements of endometrial CD56+ cells by flow cytometry and immunostaining correlate well.

Key words: uNK cells / endometrium / recurrent miscarriage / CD56+

Introduction

Natural killer (NK) cells, which form part of the innate immune system, are found in peripheral blood and in the endometrium of humans. However, the characteristics and possible functions of peripheral blood and endometrial NK cells are very different. The majority (90%) of the peripheral blood NK cells express the characteristic NK cell markers, CD56, CD16 and CD3. In contrast, the majority (90%) of NK cells found in the endometrium (uNK cells) express high levels of CD56, but are CD16 and CD3 negative. The remaining 10% of uNK cells resemble peripheral blood NK cells and are CD16+. Expression of NK cell receptors also differs between peripheral blood and uNK cells within any one individual. The numbers of uNK cells in the endometrium increase markedly during the secretory phase of the menstrual cycle and remain high during the first trimester of pregnancy (Bulmer et al., 1991; Laird et al., 2003).

The presence of increased numbers of uNK cells in the endometrium and peripheral blood of women with recurrent miscarriage is well established (Coulam et al., 1995; Kwak et al., 1995; Quenby et al., 1999; Tuckerman et al., 2007; King et al., 2010), although one study has shown no differences in levels of peripheral blood NK cells or uNK cells in women with recurrent miscarriage (Shimada et al., 2004). However, a study from our laboratory has shown that the measurement of uNK in non-pregnant endometrium of women with recurrent miscarriage is not predictive of pregnancy outcome (Tuckerman et al., 2007). More recently, an increased number of uNK cells has been reported in endometrium and peripheral blood.
of women undergoing IVF (Ledee-Bataille et al., 2004; Thum et al., 2004; Tuckerman et al., 2010), but again numbers in peripheral blood were not predictive of pregnancy outcome (Thum et al., 2005). The lack of predictive value of these measurements may reflect a lack of understanding of the subsets of NK cells in endometrium or peripheral blood that are increased in women with recurrent miscarriage or undergoing IVF treatment.

An understanding of the role of uNK cells in reproductive failure is also hampered by the differences in the methods used to study NK cells in different compartments. Measurements of NK cells in blood are traditionally measured by flow cytometry, while those in endometrial tissue may be measured by immunohistochemistry (Lachapelle et al., 1996) or immunohistochemical staining in situ (Quenby et al., 1999; Tuckerman et al., 2007) and these measurements are beginning to be used in clinical practice (Quenby et al., 2005). The advantage of flow cytometry is that it can be used to detect the subpopulations (e.g. CD16- and CD16+ cells) and activation state of CD56+ cells. However, for endometrial tissue, this requires obtaining a larger biopsy sample, which needs to be processed immediately upon collection. In contrast, immunohistochemistry can be carried out on small biopsies, which once fixed can be processed at the convenience of the laboratory. There is also the reassurance of seeing the positive staining cells in situ.

Although a recent study has shown a correlation between numbers of peripheral blood NK cells and uNK cells from decidual tissue (Park et al., 2010), there are no studies to date that have correlated peripheral blood NK cells with uNK cells in non-pregnant endometrium or that have correlated measurement of endometrial uNK cells by flow cytometry and immunostaining. The aim of this study was to (i) measure peripheral blood NK cells by flow cytometry and compare their numbers with those in the endometrium collected from the same women at the same time and (ii) measure endometrial uNK cells in the same endometrial biopsy sample by flow cytometry and immunostaining.

**Materials and Methods**

**Human subjects**

Endometrial biopsies were obtained from women attending the Jessop Wing, Sheffield Teaching Hospitals. All samples were collected after obtaining informed consent from patients, and ethical committee approval was obtained for the study. Two groups of women were recruited. The first group (n = 25) were women attending the recurrent miscarriage clinic, with unexplained recurrent miscarriage defined according to our established protocol (Li, 1998). As part of their ongoing investigation, a timed endometrial biopsy was taken. Daily measurements of LH in 1 ml cold fluorescence-activated cell sorting (FACS) buffer and then embedded in wax using an automated process. A 10 ml and a 5 ml and LH timed endometrial biopsy was taken. Daily measurements of LH in established protocol (Li, 1998). As part of their ongoing investigation, a clinic, with unexplained recurrent miscarriage defined according to our least 2 months prior to the study. Samples obtained from the operating theatre were divided into two parts: one part was fixed and embedded in wax to detect CD56+ cells by immunohistochemistry, while the other part was used for flow cytometry. Menstrual cycle dating of these biopsies was carried out from the date of the last menstrual period and by the morphological appearance of the immunostained sections. They were divided into proliferative (Days 1–14) (n = 5), early secretory (Days 15–19) (n = 4), mid secretory (Days 20–23) (n = 6) and late secretory (Days 24–29) (n = 5) according to last menstrual period and morphological appearance. In all cases, the groupings based on last menstrual period and morphological appearance agreed.

**Hormone assays**

Serum was immediately separated from cells by centrifugation and stored at −20°C until assay. Levels of estradiol and progesterone were determined by a chemiluminescent assay (Abbott Axsttm analyser; Abbott diagnostics). Levels of LH and FSH were measured using the Advia Centaur assay system (Bayer-Siemens plc). The intra- and inter-assay coefficients of variation were: estradiol: 7.9 and 11.9%; progesterone: 6.8 and 12.4%; LH: 4.5 and 10.4%; FSH: 5.9 and 8.0%.

**Immunohistochemistry for CD56 and CD45**

Five micro meter sections were dewaxed in xylene, rehydrated through descending alcohols to tris-buffered saline pH 7.6 (TBS) and quenched in 0.3% hydrogen peroxide in methanol for 20 min. A negative control in which the specific antibody was replaced by an equivalent concentration of mouse immunoglobulin G was included for each marker. After washing, unmasking was performed in an 800 W microwave oven in 10 mmol/l citrate buffer pH 6.0. The buffer was heated in the microwave oven until boiling. Slides were added to the buffer, and left covered at a high heat for 3 min. Slides were further incubated for 12 min on medium heat and allowed to cool for 20 min. An ABC kit (Vector Laboratories, UK) was used according to the manufacturer’s instructions and the following adaptations. Slides were washed in TBS and blocked in blocking buffer containing 250 μl avidin/ml (Vector Laboratories) for 1 h at room temperature, and incubated overnight at +4°C with either a mouse monoclonal primary anti-CD56 antibody (NCL-C-DS6-504 Novacastra Laboratories Ltd, UK) diluted 1:50 (1 μg/ml), or a mouse monoclonal anti-CD45 antibody (Dako M0701) diluted 1:50 as per the manufacturer’s instructions in antibody buffer containing 250 μl/ml biotin. The addition of avidin in the blocking buffer and biotin with the antibody blocks endogenous activity. Slides were washed in TBS throughout, and after application of secondary antibody and vectorstain, positive cells were visualized by incubation with peroxidase substrate (3.3′diaminobenzidine terahydrochloride; Vector Laboratories). Slides were washed in distilled water and counterstained with 20% haematoxylin for 30 min, differentiated, dehydrated through ascending alcohols, cleared in xylene and mounted in Vectormount (Vector Laboratories).

**Flow cytometry**

For comparisons between methods of analysis of CD56+ cells in the same endometrial biopsy, the part of the sample used for flow cytometry was collected into 1 ml of RPMI 1640 medium containing 100 IU/ml penicillin, 100 mg/ml streptomycin and 10% heat-inactivated pooled human serum. Preparation of tissue for flow cytometry was carried out using a method based on that described by Manaster et al. (2008). The tissue was chopped into small pieces and strained through a cell strainer (70 μm). The tissue that passed through the strainer was incubated with red blood cell lysing buffer for 5 min and then the cell suspension was centrifuged at 300g for 5 min. The cell pellet was washed in phosphate-buffered saline (PBS) and, after a further centrifugation at 300g, was re-suspended in 1 ml cold fluorescence-activated cell sorting (FACS) buffer. The total
number of cells was counted using a haemocytometer. Cell suspensions (200 μl) were then incubated with either no antibodies (negative control), or 5 μl anti-CD45 fluorescein isothiocyanate (FITC), 5 μl anti-CD56 allophycocyanin (APC), 5 μl anti-CD45-FITC plus anti-CD56-APC or isotype controls in the dark, on ice for 30 min. All antibodies were obtained from BD Biosciences, Oxford, UK and used without further dilution. After incubation, cold FACS buffer (500 μl) was added to each cell suspension, which were pelleted at 400 g for 4 min. The cell pellet was washed again in FACS buffer and re-suspended in 300 μl of 1% parafomaldehyde and kept in the dark until analysed using a BD Biosciences FACs Calibre flow cytometer.

For measurements of CD56+ cells in peripheral blood, leukocytes were prepared from the 5 ml blood sample using Lymphoprep 1.077. The cells at the interface were washed in PBS and re-suspended in PBS prior to counting. Cells (1 x 106) were incubated with either 20 μl of anti-CD56-APC, 20 μl anti-CD4-FITC, 20 μl anti-CD16 phycoerythrin, 20 μl of all antibodies, 20 μl of isotype control antibodies or no antibody (negative control). After incubation, the cells were fixed and analysed as described already.

Analysis

For immunohistochemistry, the numbers of CD56 positive, CD45 positive and total number of negative stromal cells were counted visually in 10 x 400 magnification microscopic fields for each biopsy. Cell counting was carried out by one individual (N.M. for the sections from normal fertile women and L.W. for sections from recurrent miscarriage women). Both observers had undergone a period of training and a quality control of their counting had been undertaken and shown to be <5% different from others in the laboratory. The number of CD56+ cells was expressed as a percentage of all stromal cells or as a percentage of CD45+ cells.

For flow cytometry, the numbers of CD56+ cells were calculated as either a % of total cells in the sample or as a % of CD45+ cells found in the sample.

The biopsies from normal control women were divided into proliferative (n = 5), early secretory (n = 4), mid secretory (n = 6) and late secretory (n = 5). Non-parametric Mann–Whitney tests were used to determine statistical differences in the number of endometrial CD56+ cells at different times in the cycle.

The relationships between the numbers of endometrial cells measured by flow cytometry and immunohistochemistry in normal control women and the numbers of peripheral blood CD56+ cells measured by flow cytometry and endometrial CD56+ cells measured by immunohistochemistry in women with recurrent miscarriage were determined using the Pearson correlation test.

Results

The age and peripheral blood levels for LH, FSH, progesterone and estradiol for the 25 recurrent miscarriage women are shown in Table I. The concentrations of each hormone are within the normal range.

Figure 1 shows the immunohistochemical staining for CD45+ and CD56+ cells in the proliferative and secretory endometrium from the group of normal fertile women. In proliferative phase endometrium, CD45+ and CD56+ cells were present as individual cells scattered throughout the stroma, while secretory phase endometrium contained more CD45+ and CD56+, which were also present as clumps of cells (Fig. 1a–d). Figure 1e and f shows immunohistochemical staining in secretory phase endometrium from women with recurrent miscarriage. It shows CD56+ cells present as individual or groups of cells.

Figure 2 shows the relationship between endometrial CD56+ cells and peripheral blood CD56+ cells in the group of recurrent miscarriage women. There was no significant correlation between numbers of endometrial CD56+ cells and total peripheral blood CD56+, or the CD56+CD16+, CD56+CD16− subsets of CD56+ cells.

There was also no correlation between the numbers of endometrial CD56+ cells or peripheral blood CD56+ cells (or subsets) and the concentration of plasma LH, FSH, estradiol or progesterone (data not shown).

Table I shows the numbers of CD56+ cells in the endometrium obtained during the proliferative, early secretory, mid secretory and late secretory phases in the menstrual cycle from individual normal fertile women, measured by immunohistochemistry and flow cytometry. Although there are some variations, the numbers are low during the proliferative and early secretory phase of the cycle and start to increase only from the mid–late secretory phase. The numbers of CD56+ cells in endometrium obtained during the late secretory phase of the cycle was significantly higher (P < 0.05) than the numbers in endometrium obtained at other stages when measured by both immunocytochemistry and flow cytometry.

Figure 3 shows the relationship between numbers of endometrial CD56+ cells measured by flow cytometry and by immunohistochemistry in the same endometrial biopsy samples obtained from normal fertile women throughout the menstrual cycle. When expressed as a percentage of all cells, the measurements of CD56+ cells by flow cytometry were lower (0.1–18%) than measurements obtained by immunohistochemistry (3.8–52.8%), whereas when expressed as a percentage of CD45+ cells, both methods gave more comparable results (range 3.8–61% for flow cytometry and 10.8–70% for immunohistochemistry). There was a significant correlation between the measurement of cells by flow cytometry and immunohistochemistry both when the cell numbers were expressed as a percentage of total cells (correlation = 0.497, P = 0.026) or as a percentage of CD45+ cells (correlation = 0.570, P = 0.009).

Discussion

Previous studies on the possible role of uNK cells in pregnancy outcome have produced contradictory results; some studies have shown that women with reproductive failure such as repeated implantation failure or recurrent miscarriage have increased numbers of uNK cells, while others have suggested that there is no difference or...
reduced numbers (Moffat et al., 2004; Thum et al., 2004; Bulmer et al., 2010; King et al., 2010). These differences may be due to the involvement of specific subsets of uNK cells in reproductive failure and may even be due to differences in the subsets associated with recurrent miscarriage and repeated implantation failure. The understanding of the role of these different subsets has not been helped by comparisons of uNK cell numbers in different compartments (e.g. blood, endometrium or decidua) or by different methodologies (e.g. immunohistochemistry or flow cytometry) (Laird et al., 2003).

In this study, we have investigated numbers of NK cells in the endometrium and peripheral blood in samples obtained at the same time from the same individual woman in a group of women undergoing investigation for recurrent miscarriage. We found no correlation between numbers of total CD56+, CD56+CD16− or CD56+CD16+ and endometrial CD56+ cells in these women. This is in contrast to the study of Park et al. (2010), who showed a correlation between the numbers of CD56+ and CD56+CD16+ peripheral blood cells and decidual NK cells in samples obtained from the same women during early pregnancy. However, the samples in this study were collected after miscarriage and therefore may reflect inflammatory responses associated with miscarriage.

A limitation of our study is that it was carried out in women with recurrent miscarriage; a comparable study in control fertile women would also be of interest. However, recruitment of large numbers of fertile women to studies that require taking simultaneous blood and biopsy samples is difficult. There are very few studies comparing numbers of CD56+ endometrial and peripheral blood cell numbers even in patients with reproductive disorders attending clinics for

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**Figure 1** Photomicrograph of immunostaining for (a) CD45 in proliferative endometrium from a fertile woman (b) CD56 in proliferative endometrium from a fertile woman (c) CD45 in secretory endometrium from a fertile woman (d) CD56 in secretory endometrium from a fertile woman (e) and (f) CD56 in secretory endometrium from two different women with recurrent miscarriage. Positive staining cells indicated by arrows. Scale bar = 10 μm.
We also compared the numbers of endometrial uNK cells measured by immunohistochemistry and flow cytometry in endometrial biopsy samples obtained at the same time from individual fertile control women. Our study showed a correlation between the numbers of CD56\(^+\) cells measured by the two different methods. We expressed these measurements as CD56\(^+\) cell numbers as a percentage of all cells and CD56\(^+\) cells as a percentage of CD45\(^+\) cells for both methodologies. When expressed as a percentage of all cells, the measurements of CD56\(^+\) cells by flow cytometry were lower (0.1–18\%) than measurements obtained by immunohistochemistry (3.8–52.8\%), whereas when expressed as a percentage of CD45\(^+\) cells, the methods gave more comparable results (range 3.8–61\% for flow cytometry and 10.8–70\% for immunohistochemistry). This means that care should be taken in comparing absolute numbers of uNK cells using the two different methodologies either within the same study or between studies. It also means that before measurement of uNK cells is translated into clinical practice, a decision needs to be made about the chosen methodology of measurement.

Although absolute values of CD56\(^+\) cells measured in each biopsy differed using the different methods there was a correlation in the cell numbers obtained, meaning that biopsies in which there were high numbers of CD56\(^+\) cells were at the high end of the range in each method used. This means that in studies comparing numbers of CD56\(^+\) cells in two different groups of women or before and after treatments differences in results may not be due to differences in the methods used to detect the cells as long as the same method is used for all groups in the same study.

The samples obtained from fertile women were not precisely timed according to the LH surge as were the samples from women with recurrent miscarriage and this is a limitation of the study. However, these samples were obtained throughout the whole menstrual cycle,
which allowed comparison of the data obtained by both methods in samples that contained a large variation in the numbers of CD56+ cells. The results showed that significantly more cells were obtained in endometrium during the late secretory phase compared with all other stages in the cycle, and this was seen when samples were analysed by either immunohistochemistry or flow cytometry. Although this was not an aim of this study, it is reassuring that the data are in agreement with previous more comprehensive studies. In a previous study, we reported that the numbers of CD56+ cells in endometrium obtained from a small group of control women (n=10) on days LH+7–LH+9 of the menstrual cycle was 2.2–13.9% (Tuckerman et al., 2007). In the current study, the numbers ranged from 3.9 to 52.8%. A massive increase in CD56+ cells in the endometrium at the end of the cycle just prior to menstruation has been reported in a previous work (Salmensen and Lathbury, 2000) and very high numbers of CD56+ cells were also found in the endometrium only in samples obtained during the late secretory phase of the cycle in this study. Of the seven samples obtained in the mid secretory phase of the cycle (Days 19–23) in this study, only one was slightly (14.6%) above the 13.9% upper value reported in the previous study.

The fact that there is no correlation between peripheral blood NK cell number and uNK cell number raises questions about the role of studies and measurements of peripheral blood NK cells in reproductive medicine. Measurements of peripheral blood NK cells by flow cytometry are considerably easier, as they do not require obtaining an endometrial biopsy sample, which is invasive. However, the numbers of peripheral blood NK cells may not relate to those seen in the endometrium and therefore it may be inappropriate to extrapolate results from peripheral blood to what is happening in the endometrium. In addition, a recent study has shown that peripheral blood NK cells are increased at times of stress and that the stress of obtaining a blood sample may artificially increase peripheral blood NK cells (Shakhar et al., 2006)—again question the use of peripheral blood NK cell measurements in the diagnosis and treatment of recurrent miscarriage and other reproductive disorders.

Peripheral blood and endometrial and decidual uNK cells are in contact with different areas of the developing placenta; the peripheral blood bathes the syncytiotrophoblasts, while endometrial and decidual cells are in contact with the invading trophoblast. It is thought that the endometrial and decidual NK cells are more likely to be involved in reproductive pathologies because of their proximity to the implantation process. Peripheral blood NK and uNK cells are likely to have different functions. Peripheral blood NK cells are part of the innate immune system and are involved in the protection against infection. The precise role of uNK cells in the implantation process is unknown, but could include a direct interaction with major histocompatibility complex molecules on the invading trophoblast, production of chemokines and cytokines, angiogenesis and spiral artery remodelling or innate immunity (Bulmer and Lash, 2005; Quenby et al., 2009).

In conclusion, we have shown that there is no correlation between numbers of CD56+ cells in endometrium and peripheral blood in a group of women with recurrent miscarriage, suggesting that it is inappropriate to extrapolate findings in peripheral blood to the endometrium and decidua. In addition, we have shown a correlation between measurements of endometrial NK cells by flow cytometry and immunohistochemistry, which suggests that differences in results seen in some studies cannot be solely attributed to the different methodologies used.

**Authors’ roles**

S.M.L. supervised and provided advice on the work and wrote the paper. N.M. carried out the flow cytometry and immunohistochemistry on endometrium from normal fertile women. L.W. carried out flow cytometry and immunohistochemistry on peripheral blood and endometrium from women with recurrent miscarriage. T.C.L. (principal investigator) provided the tissue, supervised the work and drafted the paper.
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**References**


