Uterine natural killer cells and angiogenesis in recurrent reproductive failure

Siobhan Quenby, Helena Nik, Barbara Innes, Gendie Lash, Mark Turner, Jo Drury, and Judith Bulmer

1School of Reproductive and Developmental Medicine, University of Liverpool, First Floor, Liverpool Women’s Hospital, Crown Street, Liverpool L8 7SS, UK
2Uterine Cell Signalling Group, Institute of Cellular Medicine, Newcastle University, Framlington Place, Newcastle upon Tyne NE2 4HH, UK
3Correspondence address. E-mail: squenby@liv.ac.uk

BACKGROUND: Increased numbers of phenotypically unusual CD56bright CD16- uterine natural killer (uNK) cells have been associated with recurrent reproductive failure. uNK cells produce angiogenic growth factors and are potential regulators of decidual angiogenesis in early pregnancy. The final common mechanism for early pregnancy loss is thought to be early onset of the maternal circulation and excessive placental oxidative stress. We tested the hypothesis that increased uNK cells in preimplantation endometrium are associated with altered angiogenesis.

METHODS: Women with recurrent reproductive failure (n = 122) were investigated with uterine artery Doppler and endometrial biopsy. Immunohistochemistry was used to identify uNK, endothelial and vascular smooth muscle cells and image analysis was used to assess location, density and differentiation.

RESULTS: uNK cell density was positively correlated with the formation of blood (P = 0.005, r = 0.5) and lymphatic vessels (P = 0.0001, r = 0.6), spiral arteriole smooth muscle differentiation (P = 0.01, r = 0.5) and endometrial oedema (P = 0.004). The functional effect of this was a reduced uterine artery resistance to blood flow.

CONCLUSIONS: These data suggest that uNK cells may regulate angiogenesis in non-pregnant endometrium. The mechanisms of reproductive failure associated with increased uNK cell density appear to be increased angiogenesis and peri-implantation blood flow, which may lead to early maternal circulation and hence pregnancy failure due to excessive oxidative stress.

Key words: recurrent miscarriage / uNK cells / angiogenesis / recurrent implantation failure / endometrium

Introduction

Recurrent reproductive failure includes recurrent implantation failure (no pregnancies despite transfer of five good quality embryos into the uterus) and recurrent miscarriage (three or more consecutive pregnancy losses). Currently, ~50% of cases of recurrent reproductive failure are of unknown aetiology (Quenby and Farquharson, 1993; Rai and Regan, 2006), and an altered endometrial environment is thought to be associated with this idiopathic condition.

Embryo implantation and early placental development occur in a relatively hypoxic environment (2–3% O₂) (Yedwab et al., 1976; Rodesch et al., 1992). Oxygen levels in the intervillous space rise...
between 10 and 12 weeks gestation as extravillous trophoblast plugs within the lumen of the uterine spiral arteries dissipate (Jauiaux et al., 2000), allowing blood flow into the intervillous space; this is associated with a minor degree of oxidative stress damage to the placental syncytiotrophoblast, which then adapts to the higher oxygen levels (Hung et al., 2001). Histological studies have reported the disruption of the trophoblast shell and reduced plugging of spiral arteries by endovascular trophoblast in miscarriage (Hustin et al., 1999), and Hempstock et al. (2003) demonstrated increased placental levels of oxidative stress markers in miscarriage compared with gestationally matched controls undergoing termination of pregnancy. Inappropriate maternal blood flow to the intervillous space may have several different underlying causes and may be a common pathway in both sporadic and recurrent miscarriage.

The endometrium undergoes regular cycles of growth and breakdown and is one of the few adult tissues in which significant angiogenesis occurs on an ongoing, physiological basis. It has been proposed that endometrial angiogenesis progresses from vessel stumps remaining after menstruation (Rogers, 1996). Endometrial vessels acquire more vascular smooth muscle cells (VSMCs) as the menstrual cycle progresses, although the origin of these VSMCs and the molecular triggers for these events are not yet clear (Girling and Rogers, 2005). As new blood vessels develop, the VSMCs differentiate from a proliferative to a contractile phenotype acquiring different markers; for example smooth muscle actin is an early marker (proliferative phenotype), although smooth muscle myosin is a late marker of VSMCs (contractile phenotype). Transvaginal pulsed-Doppler ultrasonography allows non-invasive evaluation of the uterine circulation. Reduced resistance to flow has previously been reported in the early proliferative phase in women with menorrhagia who, in separate studies, have reduced resistance to flow of new blood vessels. These studies, however, have not detected significantly high uNK cell density in endometrium compared with increased and normal uNK cell density. We hypothesized that uNK cells mediate angiogenesis in secretory-phase endometrium and pregnant decidua (Li et al., 1999, 2005). Furthermore, uNK cells form aggregates around spiral arteries and endometrial glands in late secretory-phase endometrium and early pregnancy decidua (Bulmer et al., 2001; Lash et al., 2005). Studies of pregnant transgenic mice deficient in uNK cells found abnormally straight and narrow uterine spiral arteries, suggesting that uNK cells are important for spiral artery remodelling (Croy et al., 2002; van den Heuvel et al., 2005). Furthermore, in vivo studies in murine pregnancy have shown that uNK cell-derived interferon-γ modifies gene expression in the uterine vasculature and stroma, thereby initiating vessel instability and facilitating pregnancy-induced remodelling of decidual arteries (Ashkar et al., 2000). The potential role of uNK cells in spiral artery remodelling is supported by the high levels of angiogenic growth factors [vascular endothelial growth factor (VEGF)-C, placental growth factor, angiopoietin (Ang)-1, Ang-2 and transforming growth factor-β1] secreted by uNK cells from both non-pregnant endometrium and early pregnancy decidua (Li et al., 2001; Lash et al., 2006). Furthermore, Hanna et al. (2006) demonstrated increased endothelial cell angiogenesis (tubule formation) in both in vitro and in vivo models in response to uNK cells from early pregnancy. It is not known whether uNK cells play a role in the preimplantation endometrial angiogenesis.

We hypothesized that uNK cells mediate angiogenesis in secretory-phase endometrium. Thus we proposed that uNK cells increase endometrial vessel formation, thereby increasing uterine artery blood flow and increasing oxidative stress in the early placenta. In order to test this hypothesis, we investigated whether increased endometrial uNK cell density positively correlated with increased numbers of endometrial vessels, VSMC differentiation and oedema. We investigated endometrium from women with recurrent reproductive failure and compared groups with increased and normal uNK cell density. We have not detected significantly high uNK cell density in endometrium from fertile controls (Quenby et al., 1999, 2005). We demonstrated the functional importance of the newly formed blood vessels by relating them to uterine artery blood flow resistance.

Materials and Methods

Subjects

Local ethical committee approval was obtained and all women included in this study gave written informed consent. Women were recruited from...
24 h, routinely processed and embedded in paraffin wax. Three-

Endometrial biopsies were fixed in 10% neutral-buffered formalin for

Immunohistochemistry waves and averaged. The mean value of the vascular resistance from

The pulsatility index (PI) was calculated using three consecutive uniform

waveform was obtained by placing the Doppler gate on the target vessel.

Nemio) with a 6 MHz transvaginal transducer. Uterine vessels were visu-

sonography and colour Doppler, using ultrasound equipment (Toshiba

The evaluation of uterine artery blood flow was performed by transvaginal

Doppler measurement

Endometrial sampling

Women were asked to use commercially available urine LH kits and to tel-

ephone the hospital at the time of their LH surge. Six to 8 days after the

LH surge, an endometrial biopsy was collected with a Wallach endocell

sampling device.

Good-quality embryos transferred

<table>
<thead>
<tr>
<th>Sample Type</th>
<th>Recurrent miscarriage, n = 98</th>
<th>Recurrent implantation failure, n = 24</th>
<th>Subgroup for detailed vessel analysis, n = 28 (all had recurrent miscarriage)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>36 (23–40)</td>
<td>37 (33–40)</td>
<td>36 (27–40)</td>
</tr>
<tr>
<td>First trimester miscarriages</td>
<td>5 (2–16)</td>
<td>1 (0–8)</td>
<td>4 (0–10)</td>
</tr>
<tr>
<td>Ectopic pregnancies</td>
<td>0 (0–2)</td>
<td>0 (0–2)</td>
<td>0 (0–2)</td>
</tr>
<tr>
<td>Second trimester miscarriages</td>
<td>0 (0–1)</td>
<td>0 (0–0)</td>
<td>0 (0–1)</td>
</tr>
<tr>
<td>Still births</td>
<td>0 (0–1)</td>
<td>0 (0–0)</td>
<td>0 (0–0)</td>
</tr>
<tr>
<td>Live births</td>
<td>0 (0–1)</td>
<td>0 (0–1)</td>
<td>0 (0–1)</td>
</tr>
<tr>
<td>Oocyte collections</td>
<td>0 (0–0)</td>
<td>4 (1–5)</td>
<td>0 (0–0)</td>
</tr>
<tr>
<td>Good-quality embryos transferred</td>
<td>0 (0–0)</td>
<td>8 (5–12)</td>
<td>0 (0–0)</td>
</tr>
</tbody>
</table>

Medians (ranges). 

throughout the UK via referrals from general practitioners or local hospital consultants. Women were included in the study if they had recurrent miscarriage (three or more consecutive miscarriages) or recurrent implantation failure (five or more good-quality embryos replaced with no pregnancy success). Women were excluded if one of the following screening investigations revealed a possible contributing factor for their pregnancy losses: antiphospholipid syndrome [lupus anticogulant tested for using the dilute Russell viper venom time and immunoglobulin (Ig)G and IgM anticardiolipin antibodies], thrombophilia (activated protein C resistance, Leiden factor V mutation, prothrombin gene mutation, protein C and S deficiency and antithrombin III deficiency), uterine anomaly (transva-
ginal ultrasonography), polycystic ovarian syndrome (transvaginal ultrasonography), diabetes (fasting blood glucose), abnormal thyroid function tests or parental balanced translocations (leucocyte culture). Table I shows the demographic details of the 122 women included in this study for Doppler studies and uNK cell assessment and the subset of the first 28 women recruited whose samples were examined in greater detail for vessel development.

Endometrial sampling

Women were asked to use commercially available urine LH kits and to tel-
ephone the hospital at the time of their LH surge. Six to 8 days after the

LH surge, an endometrial biopsy was collected with a Wallach endocell

sampling device.

Doppler measurement

The evaluation of uterine artery blood flow was performed by transvaginal sonography and colour Doppler, using ultrasound equipment (Toshiba Nemio) with a 6 MHz transvaginal transducer. Uterine vessels were visu-

alized with colour Doppler technique, and the ascending branch was identi-

fied lateral to the cervix, at the level of the internal os. The blood velocity waveform was obtained by placing the Doppler gate on the target vessel. The pulsatility index (PI) was calculated using three consecutive uniform waves and averaged. The mean value of the vascular resistance from both ascending uterine artery branches was used in calculations.

Immunohistochemistry

Endometrial biopsies were fixed in 10% neutral-buffered formalin for 24 h, routinely processed and embedded in paraffin wax. Three-
micrometre-thick sections were mounted onto APES (3-aminopropyltriethoxysilane, Sigma Chemical Co., Poole, UK) coated slides. Serial sections were stained with markers for endothelial cells (factor 8-related antigen, F8; ulex europaeus agglutinin 1, Ulex), smooth muscle cells (α smooth muscle actin: αSMA; myosin heavy chain: SMM), lymphatic endothelial cells (D2-40), uNK cells (CD56) and endometrial stromal cells (CD10) using the antibodies and antigen-retrieval methods detailed in Table II. With the exception of Ulex, an avidin biotin peroxidase technique (Vectastain Elite ABC kit, Vector Laboratories, Peterborough, UK) was used as described previously (Pongcharoen et al., 2004). All primary antibodies were incubated for 60 min. The reaction was develop-

ed with 3,3-diaminobenzidine (DAB; Sigma Chemical Co.) containing 0.01% H2O2 to give a brown reaction product. Sections were lightly counter-

stained with Mayer’s haematoxylin, dehydrated, cleared and mounted with DPX synthetic resin (Raymond A. Lamb Ltd, London, UK). Appropriate positive controls were performed in each staining run, and negative controls were performed for each sample by replacing the primary anti-

body with mouse IgG.

Ulex staining was performed on 3-μm paraffin sections pretreated with trypsin (Sigma Chemical Co.) (0.1% trypsin in 0.1% CaCl2) at 37°C for 10 min, as described previously (Thrower et al., 1991). Sections were treated with normal lamb serum (Sigma Chemical Co.) for 10 min and washed twice (5 min each) in Tris-buffered saline (TBS) before incubation in Ulex antibody (1:100; Sigma Chemical Co.) for 30 min. After two further 5-min TBS washes, sections were incubated in streptavidin-horse-radish peroxidase (1:100 dilution, Dako) for 30 mins. After two further 5-min TBS washes, the reaction was developed in DAB as described earlier. Sections were then counterstained with haema-

toxylin, dehydrated, cleared and mounted in DPX as described earlier.

Analysis

uNK cell density was assessed using an image analysis technique which was adapted for this purpose. Digital images of 10 randomly selected high-

power (×400) fields were captured on Eclipsenet software (Nikon). Glands were then removed from the images using the ‘Lasso’ tool on Adobe Photoshop CS2 software (Adobe, San Jose, CA, USA). ImageJ free-

ware (NIH, USA) was then used to convert the image to eight-bit grey-

scale. The total number of stromal cells was determined using a manual threshold followed by the application of the ‘watershed’ function, which generated a visual array in which each cell was black and the non-cellular material was white. Cells were then automatically counted with the ‘analyse particles’ function. Finally, a visual check was performed utilizing the ‘colour merge’ function in ImageJ. The numbers of CD56
Table II Details of primary antibodies used for immunohistochemistry and antigen retrieval methods

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Company</th>
<th>Clone</th>
<th>Working dilution</th>
<th>Pretreatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alpha smooth muscle actin (αSMA)</td>
<td>Novocastra, Newcastle upon Tyne, UK</td>
<td>αsm-1</td>
<td>1:75</td>
<td>Trypsin pH7.8</td>
</tr>
<tr>
<td>Heavy chain myosin (SMM)</td>
<td>Sigma, Missouri, USA</td>
<td>Hsm-v</td>
<td>1:600</td>
<td>Pressure cooker Citrate pH6.0</td>
</tr>
<tr>
<td>Von Willebrand factor, factor B-related antigen (F8)</td>
<td>Dako, Glostrup, Denmark</td>
<td>F8/86</td>
<td>1:50</td>
<td>Trypsin pH7.8</td>
</tr>
<tr>
<td>CD56</td>
<td>Novocastra</td>
<td>Eric-1</td>
<td>1:100</td>
<td>Pressure cooker Citrate pH6.0</td>
</tr>
<tr>
<td>Ulex europaeus aggultinin 1, (Ulex)</td>
<td>Sigma, Dorset, UK</td>
<td>Uea 1</td>
<td>1:1000</td>
<td>Trypsin pH7.8</td>
</tr>
<tr>
<td>D2-40</td>
<td>Abcam, Cambridge, UK</td>
<td>Ab 11 842</td>
<td>Bought pre-diluted</td>
<td></td>
</tr>
<tr>
<td>CD10</td>
<td>Novocastra</td>
<td>CD10-270</td>
<td>1:40</td>
<td>Pressure cooker Citrate pH6.0</td>
</tr>
</tbody>
</table>

All pretreatments are pressure-cooked for 1 min.

immunopositive uNK cells in each field were then counted using the manual pick tool in the ImageJ software and the percentage of uNK cells per field determined as a function of the total number of stromal cells. This technique has been shown to have good inter and intra-observer reliability (Drury et al., 2007).

Vessels were assessed in serial sections, immunostained for F8, Ulex, D2-40, SMM and αSMA (Fig. 1). For each patient’s sample, low-power fields (× 100) were identified in all sections that contained an epithelial surface. For each antibody/lectin, the number of positive vessels per low-power field was assessed by two observers blinded to the origin of the sample. Only vessels with a visible lumen were analysed. Vascular smooth muscle differentiation was assessed by adapting a method described previously (Rogers, 1996; Rogers and Abberton, 2003). In sections immunostained with SMM and αSMA, vessels were assessed as completely surrounded by smooth muscle (Fig. 1) (‘complete’) or partially surrounded by smooth muscle (‘partial’). A calculation of complete / (complete + partial) × 100 was made to determine the extent of vascular smooth muscle differentiation in each sample.

Endometrial stromal cells were delineated by immunostaining for CD10, which is strongly expressed by stromal cells in non-pregnant endometrium (Klemmt et al., 2006). To quantify endometrial oedema, the number of stromal cells per high-power field (×400) was calculated using the modified image analysis described earlier.

All analyses were performed using GraphPad Prism software for personal computers (San Diego, CA, USA, www.graphpad.com). The data were not normally distributed. Correlation between two variables was performed using the Spearman rank correlation coefficient. Differences between two groups were analysed using Mann–Whitney U-test and between multiple groups using Kruskall–Wallis test. A value of P < 0.05 was considered significant.

Results

Subjects
We recruited women with extreme phenotypes for this study; all had suffered many miscarriages and/or repeated failure of assisted reproduction techniques following many years of infertility (Table I). This group of women are inevitably at the older end of the reproductive age group (Table I). Hence, we initially examined the data to determine whether there was any relationship between uNK cell density, uterine artery pulsatility and age, but none existed (data not shown).

uNK cell density and identification of vessels
The uNK cell density (uNK cells as a percentage of total stromal cells) ranged from 0.3 to 19.9% of stromal cells in the recurrent miscarriage group (n = 98) and from 0.3 to 17.1% of stromal cells in the recurrent implantation failure group (n = 24). Fig. 1a and d demonstrates an example of high (11.8% of stromal cells) uNK cell density. On review of our previous studies, very few cells stained positive for CD16 (<1% stromal cells) and <10% of the CD56+ cells could have been CD16+, and this proportion remained constant in women with low and high NK cell density (Quenby et al., 1999, 2005). As CD56+ CD16+ cells are CD56bright whereas the CD16+ cells are CD56dim, it is reasonable to assume that the majority of the CD56+ cell population that we studied was CD56brightCD16+.

Endometrial blood vessels were initially identified by immunostaining for endothelial cells using anti-F8 and Ulex (Fig 1b and c). F8 detected more vessels than Ulex but there was a positive correlation between the number of vessels identified with both markers (P = 0.007, r = 0.5, n = 28).

VSMCs surrounding endometrial arterioles were identified by immunostaining for αSMA (early VSMC marker, Fig. 1f) and SMM (late VSMC marker). There was a positive correlation between the number of vessels identified with endothelial markers and those containing VSMC (Ulex and F8) (αSMA: Ulex P = 0.004, r = 0.5; F8 P = 0.0001, r = 0.7; SMM: Ulex P = 0.002, r = 0.6; F8 P = 0.0007, r = 0.6; n = 28). D2-40 was used to identify endometrial lymphatics; vessels that immunostained for D2-40 did not immunostain for any of the other vessel markers used (Fig. 1e).

Correlation of endometrial vessel number with uNK cells
There was a positive correlation between the number of endometrial arterioles stained with F8, Ulex and the density of
CD56-immunopositive uNK cells ($P = 0.005$, $r = 0.5$, $P = 0.008$, $r = 0.5$, $n = 28$; Fig. 2a and 2b). There was also a positive correlation between the number of endometrial lymphatics (D2-40 positive) and uNK cell density ($P = 0.0001$, $r = 0.6$, $n = 28$; Fig. 2c). There was no correlation between the proportion of arterioles completely surrounded by VSMC-expressing aSMA and the density of uNK cells (Fig. 3a). However, there was a positive correlation between the proportion of arterioles completely surrounded by VSMC-expressing SMM and uNK cell density ($P = 0.011$, $r = 0.51$, $n = 28$; Fig. 3b). These data suggest that the increased numbers of arterioles observed in the presence of higher density of uNK cells are also more highly muscularized and differentiated.

**Stromal oedema**

Areas of endometrium with a high density of uNK cells (>5%) had a lower density of stromal cells than those with low uNK cell density (<5%) ($P = 0.004$, $n = 20$) (Fig. 4). However, as the image analysis technique counts nuclei, this increased spacing between nuclei could be due to either increased stromal oedema or increased cytoplasmic volume. The delineation of endometrial stromal cells with anti-CD10 (Fig. 1g–i) demonstrated that the decreased stromal cell density associated with increased numbers of CD56+ cells per stromal cell was due to increasing spaces between the stromal cells, and was hence due to stromal oedema (Fig. 1g–i).

**Uterine artery pulsatility studies**

The higher the PI the greater the resistance to blood flow and therefore potentially decreased flow. Conversely, the lower the PI the lower the resistance to flow and potentially increased blood flow. Women with lower uterine artery resistance to blood flow had significantly higher density of uNK cells than those with greater resistance to blood flow in both the recurrent miscarriage ($P = 0.002$, $n = 98$, ...
Fig. 5a) and recurrent implantation failure ($P = 0.04$, $n = 24$, Fig. 5b) groups. This finding was not simply due to increased blood vessel density in women with high uNK cell density, as there was no correlation between uterine artery PI and arteriole density (data not shown). The relationship between uterine artery resistance to flow and uNK cell density was explained by the high degree of VSMC differentiation in the group of women with low uterine artery resistance to flow ($\alpha$SMA $P = 0.02$, $n = 28$; SMM $P = 0.01$, $n = 28$; Fig. 5c). The relationship between blood flow and uNK cell density was similar in women with both recurrent miscarriage and recurrent reproductive failure (Fig. 5a and b), and there was no significant difference in the uNK cell density results between these different subject groups with similar uterine artery Doppler readings.

Figure 2 (a) Correlation between percentage of uNK cells per total stromal cell count and number of factor 8-positive vessels per low-power field ($\times 100$) ($P = 0.005$, $r = 0.5$). (b) Correlation between percentage of uNK cells per total stromal cell count and number of ulex-positive vessels per low-power field ($P = 0.008$, $r = 0.5$). (c) Correlation between percentage of uNK cells per total stromal cell count and number of lymphatics per low-power field ($P = 0.0001$, $r = 0.6$) ($n = 28$).

Figure 3 (a) Correlation between percentage of uNK cells per total stromal cell count and percentage of vessels completely surrounded by smooth muscle when stained for $\alpha$SMA ($P = 0.2$, $r = 0.27$). (b) Correlation between percentage of uNK cells per total stromal cell count and percentage of vessels completely surrounded by smooth muscle when stained for SMM ($P = 0.011$, $r = 0.51$) ($n = 28$).

Figure 4 Areas of endometrium with high density of uNK cells (>5%) had a lower density of stromal cells than those with low uNK cell density (<5%). ($P = 0.004$).
Uterine natural killer cells and reproductive failure

Discussion

In the current study, we have demonstrated that in groups of women with idiopathic recurrent miscarriage or recurrent implantation failure, there is positive correlation between the density of uNK cells and the density of endometrial vessels including lymphatics, as well as in the vascular smooth muscle differentiation status of endometrial arterioles. We have detected only these high levels of uNK cell density in mid-luteal-phase endometrium obtained from women with recurrent miscarriage and recurrent implantation failure (Quenby et al., 1999, 2005). Hence, these findings have implications for both the function of uNK cells in normal preimplantation endometrium and the etiology of recurrent reproductive failure. In addition, increased uNK cell density and arteriole differentiation was associated with reduced uterine artery resistance and hence increased potential endometrial blood flow. This implies a functional significance to increased uNK cell density not reported previously. It is, however, also possible that all the parameters measured are affected by the same upstream signal, which may or may not be associated with the progression of the menstrual cycle and/or pregnancy loss. Our finding of a similar relationship between uNK cells and uterine artery Doppler in women with both recurrent miscarriage and recurrent implantation failure suggests a similar underlying endometrial pathology in these two conditions.

The current study used human tissues ex vivo and hence ethical limitations allowed limited sampling opportunities. One potential explanation for the current data is that increased endometrial angiogenesis allowed increased trafficking of uNK cells from the peripheral circulation into the uterus (van den Heuvel et al., 2005). However, this explanation was not supported by the finding that uterine artery resistance to blood flow did not correlate with endometrial vessel density but rather negatively correlated VSMC development and differentiation. Therefore, increased blood flow to the uterus with its potential to deliver more cells for trafficking is not a plausible explanation for our data. A second, more plausible explanation is that uNK cells, already resident in the non-pregnant uterus, promote angiogenesis and lymphangiogenesis within the endometrium. uNK cells are a major source of angiogenic growth factors in both pregnant and non-pregnant women (Li et al., 2001; Moffett-King, 2002; Hanna et al., 2006; Lash et al., 2006; Le Bouteiller and Tabiasco, 2006). The role of uNK cells in vessel muscularization is not clear; indeed few of the molecular triggers for this process in endometrium are known.

The presence of lymphatics within the endometrium has only recently been described (Donoghue et al., 2007) using an antibody specific for lymphatic endothelium (D2-40). The use of another lymphatic endothelial monoclonal antibody (LYVE-1) did not demonstrate any endometrial lymphatics in two previous studies (Koukourakis et al., 2005; Red-Horse et al., 2006). Whether these two antibodies distinguish between lymphatics in different stages of development is not clear. In the current study, we confirmed the findings of Donoghue et al. (2007) using D2-40. A novel finding was the association of lymphatics with aggregates of uNK cells, as well as a positive correlation between the number of lymphatics and uNK cell density. One of the most abundant cytokines secreted by uNK cells in early pregnancy decidua is VEGF-C (Lash et al., 2006), which has a more significant effect on lymphangiogenesis, rather than on arteriogenesis (Koukourakis et al., 2005; Red-Horse et al., 2006; Donoghue et al., 2007). However, we also demonstrated increased stromal oedema in areas of increased uNK cells, and increased lymphatic vessels. It is possible, therefore, that these vessels may not yet be functioning fully to clear the tissue of excess fluid and maintaining extracellular fluid homeostasis. Stromal oedema is a feature of the luteal phase of the menstrual cycle and is maximal at LH +7 days after which the excess fluid decreases rapidly over days, presumably as the lymphatics start to function. The development of endometrial lymphatics in early pregnancy would allow antigen-presenting cells and uNK cells to travel to lymph nodes following contact with semi-allogenic fetal trophoblast. The maternal lymph nodes have the necessary immunocompetent cells to develop maternal tolerance to fetal cells and activate NK cells (Moretta et al., 2008) for their important role in...
promoting implantation and early placentation (Le Bouteiller and Tabiasco, 2006).

In the current study, endometrium from women with recurrent reproductive failure and low uNK cell density was compared with samples from women who are similar in every clinical respect but have high uNK cell density. As reproductive failure is a multi-factorial problem, our patient groups have allowed us to concentrate on the role of uNK cells in reproductive failure. Several previous studies have shown that women with recurrent miscarriage have higher uNK cell density in luteal-phase endometrium than fertile controls, implicating these cells in reproductive failure; until now, the functional significance of this observation was unknown (Clifford et al., 1999; Quenby et al., 1999, 2005, 2006; Laird et al., 2005; Tuckerman et al., 2007). Whether high numbers of uNK cells in luteal-phase endometrium in women with recurrent miscarriage predict miscarriage in a subsequent pregnancy is controversial; one small study suggested that they do (Quenby et al., 1999), but another slightly larger study refuted this (Tuckerman et al., 2007). Neither of these studies was sufficiently powered, nor had the rigorous methodology needed to accurately assess whether endometrial uNK cell density predicts reproductive failure, as both studies had significant numbers of women lost to follow up. An alternative approach would be to compare women with a past history of successful pregnancy with those with a past history of reproductive failure but such comparison can be affected by multiple confounding factors. Furthermore, the vast majority of women who have had several successful pregnancies use hormonal methods of contraception and therefore their endometrium cannot be accessed during a natural menstrual cycle.

The human endometrium contains a vascular tree of spiral arterioles that grows during each menstrual cycle. During this process of arteriogenesis, the capillaries acquire a vascular smooth muscle coat, thereby gaining the ability to regulate blood flow. VSMCs differentiate from mesenchymal cells in a spatially regulated fashion, expressing a series of characteristic markers with a progressive increase in cytoskeletal complexity (Kohnen et al., 2000; Rogers and Abberton, 2003). Two cytoskeletal markers were investigated: aSMA, an early marker of smooth muscle differentiation, and SMM, which is expressed later (Kohnen et al., 2000; Rogers and Abberton, 2003). The quantification of VSMCs development and differentiation was first reported by Rogers and Abberton (2003) in the context of pathological endometrial bleeding; endometrial arterioles were described as initially partially coated with VSMC before being completely surrounded. A similar quantification scale of partially and completely coated arterioles was used in the current study to assess VSMC development and differentiation. We are the first group to compare VSMC in controlling uterine artery blood flow. This contrasts with the role of VSMCs during menstruation when they need to constrict to prevent excessive blood loss (Rogers and Abberton, 2003) and later pregnancy when their disappearance is associated with reduced uterine artery resistance. Hence this, the first report of increased muscularization and differentiation of spiral arterioles in women with increased uNK cell density and reduced uterine artery resistance, has highlighted the multifunctional importance of VSMC in controlling uterine artery blood flow.

Previous authors have reported increased mid-luteal-phase uterine artery resistance in women with recurrent miscarriage (Habara et al., 2002), recurrent implantation failure (Goswamy et al., 1988) and unexplained infertility (Steer et al., 1994). Furthermore decreased uterine artery PI on the day of embryo transfer did not predict subsequent pregnancy outcome in one study (Isaksson et al., 2000) but did in another (Chien et al., 2004). Thus the finding of decreased uterine artery resistance in association with uNK cells in both types of recurrent reproductive failure is unique and is unlikely to be due to any other confounding variables.

At the end of the first trimester of pregnancy, the feto-placental unit is exposed to a three-fold increase in prevailing oxygen concentrations due to the onset of maternal blood flow (Jauniaux et al., 2000). First trimester placental tissues are highly sensitive to oxygen due to low concentrations of the principal antioxidant enzymes. In miscarried pregnancies, the onset of the circulation is both premature and disorganized, and levels of placental oxidative stress and trophoblast degeneration are much higher than normal (Jauniaux et al., 2003). The increased number and the differentiation of spiral arterioles described in the current study may lead to inappropriate blood flow to the developing feto-placental unit, causing oxidative stress and consequent miscarriage as described earlier. Indeed, increased blood vessel density has been described in the decidua parietalis of women who miscarried (Vailhe et al., 1999).

In conclusion, in this study, we found that uNK cell density was positively correlated with endometrial angiogenesis, oedema and a decreased uterine artery resistance to blood flow. An interpretation of these data is that uNK cell function in preimplantation endometrium is to promote angiogenesis and thus endometrial blood flow. Thus, these data provide a potential mechanism by which the increased endometrial uNK cell density noted in multiple studies of recurrent reproductive failure contributes to increased preimplantation angiogenesis leading to early maternal circulation and miscarriage by the final common pathway of excessive oxidative stress.

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