Levels of circulating angiogenic cells are not altered in women with endometriosis

K.E. Webster*, S.H. Kennedy, and C.M. Becker

Nuffield Department of Obstetrics and Gynaecology, University of Oxford, Women’s Centre, John Radcliffe Hospital, Oxford OX3 9DU, UK

*Correspondence address. E-mail: katie.webster@doctors.org.uk

Submitted on September 24, 2012; resubmitted on November 22, 2012; accepted on November 29, 2012

STUDY QUESTION: Are levels of circulating angiogenic cells (CACs) affected by the presence of endometriosis?

SUMMARY ANSWER: Levels of CACs are equivalent in women with and without endometriosis.

WHAT IS KNOWN ALREADY: Murine models have suggested a role for CACs in the development of endometriosis, but their levels in humans have not yet been studied.

STUDY DESIGN, SIZE, DURATION: Eighty-seven women participated in this study. Recruitment took place from July 2010 to May 2012.

PARTICIPANTS/MATERIALS, SETTING, METHODS: All women underwent laparoscopy for investigation of symptoms suggestive of endometriosis. Thirty women had no evidence of endometriosis, and 47 women were found to have endometriosis at laparoscopy. CAC levels were determined in peripheral blood by flow cytometry in 64 women. Colony forming unit (CFU) analysis was conducted in 30 women. A separate group of 10 healthy, asymptomatic women donated blood at four time points to assess the effect of the menstrual cycle on CAC levels.

MAIN RESULTS AND THE ROLE OF CHANCE: For the whole sample, CAC levels (0.0797 ± 0.0052%) and CFU number (10.68 ± 1.98) were equivalent in women with and without endometriosis. CAC levels and CFU number were also unaffected by the stage of disease. No changes in CACs were detected during the menstrual cycle.

LIMITATIONS, REASONS FOR CAUTION: A difference of at least one standard deviation between the groups would be required to detect a difference with this sample size. Therefore, while CAC levels are not a useful biomarker of disease it is still possible that they are modestly altered by the presence of endometriosis. We did not describe specific types of lesion and it is possible that CAC elevation only occurs when vessel development is at its most prolific. Furthermore, although signals from endometriotic lesions may recruit CACs from blood, this may be insufficient to alter peripheral levels.

WIDER IMPLICATIONS OF THE FINDINGS: These data show that CACs are not a useful biomarker of endometriosis and indicate that they may be unaffected by the presence of this disease.

STUDY FUNDING/COMPETING INTEREST(S): This work was supported by grants from the MRC (New Investigator Award, G0601458 to C.M.B.), the Oxford Partnership Comprehensive Biomedical Research Centre with funding from the Department of Health’s NIHR Biomedical Research Centres Scheme and the Oxfordshire Health Services Research Committee (OHSRC). There are no conflicts of interest to be declared.

TRIAL REGISTRATION NUMBER: N/A.

Key words: endometriosis / endothelial progenitor cells / circulating angiogenic cells / biomarkers / angiogenesis

Introduction

Angiogenesis, the development of blood vessels from pre-existing vasculature, is thought to play a major role in the development of endometriosis (May and Becker, 2008; Taylor et al., 2009) as elevated pro-angiogenic factors are found in the peritoneal fluid (Oosterlynck et al., 1993) and eutopic endometrium (Donnez et al., 1998) of affected women.

The process of angiogenesis is believed to involve a population of circulating stem cells with the capacity to differentiate into endothelial
cells. These are known as endothelial progenitor cells (EPCs) and were first identified in a seminal paper by Asahara et al. (1997). It was hypothesized that EPCs are recruited to sites of tissue damage or ischaemia where they facilitate new vessel formation. Much of the early research focused on EPCs as putative disease biomarkers in cardiovascular medicine and oncology, as well as potential therapeutic targets to enhance or prevent neovascularization.

Recent studies have questioned whether these ‘progenitor’ cells genuinely have the capacity to differentiate into endothelial cells and form stable, long-lasting vessels, despite their undoubted ability to promote angiogenesis (Zentilin et al., 2006; Sieveking et al., 2008).

The varied cell-surface markers and culture assays used to identify EPCs contribute to this confusion regarding their precise role. Hence, we prefer to use the term circulating angiogenic cells (CACs) as it encompasses the diverse cell types that play a role in enhancing neovascularization (Rehman et al., 2003; Alev et al., 2011). CAC identification has been performed using a variety of methods, including flow cytometry and cell culture, which appear to quantify different subpopulations of pro-angiogenic cells.

Two studies have suggested that CACs play a role in a murine model of endometriosis. The first showed CAC incorporation into ‘endometriotic’ lesions, but was unable to identify a change in peripheral cell levels in affected mice (Laschke et al., 2011). The second identified CACs in ‘endometriotic’ lesions, and demonstrated that peripheral CAC levels were increased in mice with severe disease (Becker et al., 2011). A recent international consensus statement emphasized the need to identify biomarkers of endometriosis to improve diagnosis (Rogers et al., 2009). However, no clinically useful biomarkers have been identified to date (May et al., 2010). If CAC levels are sufficiently increased in human endometriosis, they have potential as a surrogate marker of the disease.

Physiological growth and remodelling of the endometrium also requires angiogenesis (Rees and Bicknell, 1998; Rogers and Gargett, 1998). Some authors have identified fluctuations in CAC levels across the menstrual cycle, suggesting possible involvement of CACs in endometrial angiogenesis (Fadini et al., 2008; Lemieux et al., 2009; Robb et al., 2009).

In view of these data, we sought to investigate the relationship between CACs and the presence of endometriosis in women, so as to determine whether CACs could be used as a disease biomarker. We also studied the effect of hormonal fluctuations during the menstrual cycle on CAC levels in a separate group of healthy women.

Materials and Methods

Subject recruitment

Women scheduled for laparoscopy for symptoms or signs suggestive of endometriosis (including chronic pelvic pain, subfertility or ovarian cysts) were invited to participate in the study. All women were free of exogenous hormones in the preceding 3 months. Peripheral blood was collected on the day of surgery, prior to inducing anaesthesia. The surgeon, who was blinded to the laboratory results, noted the presence or absence of endometriosis. Disease stage, where appropriate, was assessed using the revised American Society for Reproductive Medicine (ASRM) classification system.

A separate group of 10 healthy controls without symptoms of endometriosis were recruited through advertisements to assess fluctuations in CAC count through the menstrual cycle. None of the women had taken exogenous hormones in the 3 months prior to recruitment. Samples were collected during the menstrual (Day 1–3), follicular (Day 6–9), periovulatory (Day 13–16) and luteal (Day 18–23) phases of the cycle (normalized to a 28 day cycle where necessary). Ovulation was confirmed with luteal progesterone levels.

This study was approved by the local ethics committee (Oxfordshire REC A 09/H0604/58) and all women provided written, informed consent prior to participation.

CAC identification by flow cytometry

An established protocol for identifying viable CD34brightCD133CD31+CD45dim cells (Duda et al., 2007) was used for CAC enumeration. Briefly, 25 ml peripheral blood was collected, anticoagulated with EDTA (BD Bioscience, Oxford, UK) for mononuclear cell isolation and processed immediately. Peripheral blood mononuclear cells (PBMCs) were isolated using density-gradient centrifugation on Ficoll-Paque PLUS as per the manufacturer’s instructions (GE Healthcare, UK). Following isolation, cells were washed twice and incubated with Fc Receptor blocking agent (Miltenyi Biotec, UK) to minimize non-specific antibody binding. 2 × 10^6 cells were then incubated with antibodies to CD34-PECy7 (BD Biosciences, UK), CD133-PE (Miltenyi Biotec), CD45-PerCP-Cy5.5 (eBioscience, UK), CD31-APC-Cy7 (BioLegend, UK) and a viability dye (YO-PRO 1, Life Technologies, UK) for 30 min on ice. Fluorescence minus one (FMO) control tubes were run for all antibodies except CD45 (cells of interest were identified using FMO controls). FMO control tubes contain all antibodies bar one and are used to identify positive events for each fluorophore. Cells were then washed twice, prior to analysis on a BD LSR II flow cytometer. 1 × 10^6 events were captured for each sample processed, and sample tubes were analysed in duplicate for each subject.

CAC gating strategy

The gating strategy used to identify CACs is shown in Supplementary data, Fig. S1. Positive staining boundaries were identified using FMO controls. Briefly, PBMCs with low forward and side scatter (a typical lymphocyte gate) were identified. From this gate, viable cells were then selected and identified as CD34+ and, from this population, CD31+ and CD45dim− cells were identified. Finally, CD31+ cells were selected. All CAC levels are expressed as a percentage of viable, low forward/side scatter PBMCs. The mean CAC count from the duplicate sample tubes was calculated for each subject.

Colony forming unit analysis

In a subgroup of women, CAC levels were also assessed using a colony forming unit (CFU) assay. PBMCs were isolated as above and then cultured using a commercial kit for CFU formation (Stem Cell Technologies, France). In brief, 5 × 10^6 cells were cultured in a single well of a fibronectin-coated 6-well plate, in duplicate. After 48 h, non-adherent cells were removed and replated at a density of 1 × 10^6 well in two wells of a fibronectin-coated 24-well plate in duplicate. After a further 72 h, cells were fixed with 3% paraformaldehyde and CFU were counted manually using a light microscope (Leica Microsystems, Germany). A typical colony is shown in Supplementary data, Fig. S2. The mean number of colonies per well was recorded for each subject.

Estradiol and progesterone measurements

Blood (5 ml) was collected from the separate group of 10 asymptomatic controls into a serum separating tube (BD Bioscience) and centrifuged at 1800g for 10 min at room temperature. Aliquots of serum were...
stored at −80 °C. Estradiol (E2) and progesterone concentrations were measured using a chemiluminescence immunoassay on a Siemens ADVIA Centaur analyser (Siemens Healthcare Diagnostics Limited, UK).

**Statistical analysis**

Our primary aim was to assess the possible use of CACs as an endometriosis biomarker. A power calculation was performed to identify a difference of at least one standard deviation (SD) between the groups of women (Stage I–II endometriosis patients, Stage III–IV endometriosis patients and unaffected controls). Differences <1SD would lead to a test with insufficient specificity and sensitivity to be of clinical use. For 90% power at the 5% level this required 21 patients in each arm of the study.

Data were confirmed to be normally distributed using the Kolmogorov–Smirnov test. A two-tailed unpaired t-test was then used to identify significant differences between endometriosis cases and controls. As phase of menstrual cycle was found not to influence CAC or CFU levels, cycle phase was not accounted for in the comparison between control subjects and women with endometriosis. Therefore data from women at all cycle phases were pooled for the analysis.

One way analysis of variance (ANOVA) was used to analyse the differences between endometriosis stage I–II, Stage III–IV and unaffected controls. Repeated measures ANOVA was used to analyse the variation in CAC count across the menstrual cycle, and multiple regression to assess the association between CACs and E2 and progesterone levels. Results are expressed in the text as mean (±SEM). Statistical significance was set at P < 0.05. SPSS version 19 was used for all statistical analysis.

**Results**

**Subject demographics**

**Flow cytometry group**

Samples from 64 women were used (Stages I/II = 21; Stages III/IV = 21; unaffected controls = 22). Demographics are shown in Table I. There were no significant differences in age, BMI, smoking status, presenting features or cycle phase between the groups. Findings in the control group included: a normal pelvis (n = 11); adhesions (n = 5); ovarian cysts (n = 2); polycystic ovary syndrome (n = 1); pelvic inflammatory disease (n = 2) and hydrosalpinx (n = 1).

**CAC levels through the menstrual cycle**

All asymptomatic controls donated blood at all four time-points in the menstrual cycle. While CAC levels varied considerably between individual women, there was no consistent fluctuation in CAC levels across the four time-points (P = 0.279, F = 1.359, 3 df repeated measures ANOVA; Fig. 1). No correlation was found between CAC count and serum E2 level (P = 0.322, Fig. 2) or progesterone level (P = 0.704, data not shown).

**CAC levels in endometriosis**

There was no statistically significant difference (P = 0.320, Fig. 3a) in the mean CAC levels between the endometriosis patients [all stages; 0.084% (±0.007) and unaffected controls 0.072% (±0.009)]. CAC levels in women with Stage I–II disease were 0.0897% (±0.009) compared with 0.0774% (±0.009) in women

### Table I: Demographics for women in flow cytometry study of CACs in peripheral blood.

<table>
<thead>
<tr>
<th>Demographics</th>
<th>Controls (n = 22)</th>
<th>Endometriosis (n = 42)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean age in years (SD)</td>
<td>32.9 (7.3)</td>
<td>35.6 (5.0)</td>
<td>0.136</td>
</tr>
<tr>
<td>Mean BMI (SD)</td>
<td>24.8 (4.4)</td>
<td>25.8 (3.7)</td>
<td>0.330</td>
</tr>
<tr>
<td>Number of smokers (%)</td>
<td>8 (36%)</td>
<td>13 (31%)</td>
<td>0.781</td>
</tr>
<tr>
<td>Presenting features (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pain</td>
<td>13 (59%)</td>
<td>31 (74%)</td>
<td>0.228</td>
</tr>
<tr>
<td>Subfertility</td>
<td>14 (64%)</td>
<td>22 (52%)</td>
<td>0.389</td>
</tr>
<tr>
<td>Ovarian cyst</td>
<td>5 (23%)</td>
<td>10 (24%)</td>
<td>0.923</td>
</tr>
<tr>
<td>Phase of cycle (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Menstrual</td>
<td>3</td>
<td>6</td>
<td>0.680</td>
</tr>
<tr>
<td>Follicular</td>
<td>8</td>
<td>13</td>
<td></td>
</tr>
<tr>
<td>Peri-ovulatory</td>
<td>3</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>Luteal</td>
<td>8</td>
<td>14</td>
<td></td>
</tr>
<tr>
<td>Not available</td>
<td>0</td>
<td>4</td>
<td></td>
</tr>
</tbody>
</table>

Age and BMI compared with Student’s t-test, smoking status, presenting features and cycle phase compared with χ² test.

*Some women presented with more than one symptom/sign.

### Table II: Demographics for women in CFU study.

<table>
<thead>
<tr>
<th>Demographics</th>
<th>Controls (n = 10)</th>
<th>Endometriosis (n = 20)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean age in years (SD)</td>
<td>33.6 (7.0)</td>
<td>34.3 (6.1)</td>
<td>0.796</td>
</tr>
<tr>
<td>Mean BMI (SD)</td>
<td>25.0 (3.6)</td>
<td>24.8 (3.8)</td>
<td>0.860</td>
</tr>
<tr>
<td>Number of smokers (%)</td>
<td>3 (30%)</td>
<td>8 (40%)</td>
<td>0.702</td>
</tr>
<tr>
<td>Presenting features (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pain</td>
<td>3 (30%)</td>
<td>13 (65%)</td>
<td>0.122</td>
</tr>
<tr>
<td>Subfertility</td>
<td>8 (80%)</td>
<td>10 (50%)</td>
<td>0.235</td>
</tr>
<tr>
<td>Ovarian cyst</td>
<td>2 (20%)</td>
<td>5 (25%)</td>
<td>1.000</td>
</tr>
</tbody>
</table>

Age and BMI compared with Student’s t-test, smoking status and presenting features compared with Fisher’s exact test.

*Some women presented with more than one symptom/sign.
with Stage III–IV disease. Comparing levels in controls against different stages of disease again showed no statistically significant difference between the groups ($P = 0.390, F = 0.956, 2 \text{ df}$, Fig. 3b).

**CFU in endometriosis**

There was no statistically significant difference ($P = 0.776, \text{ Fig. 4a}$) in the mean number of CFU between the endometriosis patients (all stages; $10.28 \pm 2.61$) and unaffected controls ($11.5 \pm 3.03$).

Mean CFU number in women with Stage I–II disease was $15.42 \pm 4.43$ compared with $5.13 \pm 1.77$ in women with Stage III–IV disease. Again, no significant difference was found when comparing controls to women with Stage I–II or Stage III–IV disease ($P = 0.098, F = 2.538, 2 \text{ df}$, Fig. 4b).

**Discussion**

The demonstration of CAC involvement in murine models of endometriosis suggested that these cells may play a role in the development of the disease. However, our study indicates that peripheral CAC levels are not affected by the presence of endometriosis, implying that these cells cannot be used as a disease biomarker.

There are several reasons why CAC levels may be unaffected by endometriosis. First, in humans, vessel growth may rely only upon the proliferation and migration of existing endothelial cells rather than the involvement of circulating progenitors. The endometrium undergoes significant angiogenesis and vessel remodelling as part of the menstrual cycle (Gargett and Rogers, 2001) and ectopic endometrial tissue may, therefore, be rich in pro-angiogenic factors and activated endothelial cells able to participate in vessel development.

Secondly, angiogenesis may predominate in certain types of endometriosis. We studied women with different stages of disease based on the revised ASRM classification system; we did not describe other features, such as whether red, white or black lesions were present. These varied lesions may display different features of angiogenesis (Fujishita et al., 1999). The involvement of CACs in endometriosis is likely to occur when vessel development is at its most prolific. Hence, it is possible that CAC elevation only occurs in women with red, vascular lesions and not in other forms of the disease. Similarly, if lesions are static, then angiogenesis will be complete and CACs are likely to return to normal levels. As a consequence, elevated CAC levels may only occur during the early stages of disease or whilst new lesions are forming.

Thirdly, vessel development in endometriosis may be minimal compared with other sites of angiogenesis, such as tumour growth or wound healing. CACs may also be recruited to the eutopic endometrium during physiological endometrial remodelling. This may result in higher CAC levels in women of reproductive age which could mask any small elevation related to endometriosis. Previous studies have identified a significant increase in CAC levels in premenopausal women—when compared with age-matched men or post-menopausal women—indicating a role for these cells in the female reproductive tract (Fadini et al., 2008; Rousseau et al., 2010). It is possible that the relatively high levels of CACs related to endometrial angiogenesis masks the involvement of these cells in endometriosis.

Further, the peripheral levels of CACs may not accurately reflect the activity of these cells in angiogenesis. Specific signals derived from developing tissues (including endometriotic lesions) may stimulate the recruitment of CACs from blood. However, this recruitment may not significantly alter the peripheral levels of these cells. Consequently, the direct study of endometriotic lesions may reveal the involvement of CACs in lesion formation without a demonstrable effect on peripheral CAC levels (Laschke et al., 2011).

Finally, our study may be underpowered to identify subtle differences in CAC levels between women with and without endometriosis. As our primary aim was to assess the use of CAC levels as a possible biomarker of disease, we were looking for a large difference in cell numbers between the two groups reflected in our power calculation. Although there is no suggestion that CACs can be used as a disease biomarker, the results of our study suggest that further work is required to understand the role of CACs in the development of endometriosis.
biomarker, it is still possible that low-level changes could be biologically significant.

Our study did not identify significant fluctuations in CACs across a menstrual cycle. This contrasts with the work of other groups that have shown alterations in CAC levels throughout the cycle. Robb et al. (2009) found no change in CFU numbers across the cycle, but did identify a follicular phase increase in CACs using flow cytometry. Interestingly, cell numbers did not correlate with E2 levels (Robb et al., 2009). Lemieux et al. (2009) also found significant variation in CAC levels across the cycle, identifying a peri-ovulatory peak in cell numbers. Finally, a third study identified pre-ovulatory increases in CAC levels (Fadini et al., 2008).

Notably, previous papers considering the effect of the menstrual cycle on CACs have all used vascular endothelial growth factor receptor (VEGFR)-2 as a marker of endothelial-like properties. We and others have identified significant concentration-dependent staining with commercially available VEGFR-2 antibodies, limiting their use for flow cytometry (Estes et al., 2010). Instead, we include CD31 (PECAM-1), an established endothelial marker (van Mourik et al., 1985; Muller et al., 1989). Other authors have employed this flow cytometry panel to show the use of CACs as a biomarker in multiple myeloma (Bhaskar et al., 2012) and paediatric solid tumours (Pradhan et al., 2011). The subtle change in antibody panel may result in the identification of different cell populations, accounting for the difference seen in menstrual cycle variation.

Furthermore, developments in multi-colour flow cytometry over recent years may also contribute to our different results. It is now increasingly recognized that FMO controls provide the optimum gating strategy for multi-colour experiments and this technique is replacing the use of isotype control antibodies to identify positive populations (Roederer, 2001; Herzenberg et al., 2006). While isotype control antibodies may account for non-specific antibody staining they are unable to correct adequately for fluorescence spillover between channels. This represents a major source of background staining in multi-colour experiments, and can only be accounted for with FMO controls. This is the first study to utilize FMO controls when assessing CAC levels through a menstrual cycle.

Figure 3: CAC levels in women with and without endometriosis. Boxes show median, 25th and 75th percentiles. Whiskers show minimum and maximum values with statistical outliers shown as filled circles (values falling between 1.5 and 3 inter-quartile ranges from the end of the box). (a) Controls versus women with all stages of endometriosis (P = 0.320). (b) Controls versus women with Stage I–II and Stage III–IV disease (P = 0.390).

Figure 4: Number of CFUs in women with and without endometriosis. (a) Comparing controls to women with all stages of endometriosis (P = 0.776). (b) Comparing controls to women with Stage I–II and Stage III–IV disease (P = 0.098). Bars show mean ± standard error.
Finally, the number of events acquired for flow cytometry analysis is critical to ensure accuracy when assessing rare populations of cells (Donnenberg and Donnenberg, 2007; Roederer, 2008). Previous studies have acquired fewer events (Robb et al., 2009) or have relatively high coefficients of variation in their data (Fadini et al., 2006, 2008), suggesting limited reproducibility of the protocol. To improve the reproducibility of the results from our assay, we routinely stain samples in duplicate and use the mean CAC count for analysis. Furthermore, we acquire $1 \times 10^6$ events for each sample and control tube to improve the accuracy of our counts.

In summary, our data demonstrate that peripheral CAC levels are unaffected by the presence of endometriosis and therefore have no potential as a disease biomarker. They also indicate that CACs are not affected by the phase of the menstrual cycle. Further work is needed to identify whether CACs participate in angiogenesis within endometriotic lesions and have any role in the pathogenesis of this challenging disease.

**Supplementary data**

Supplementary data are available at http://humrep.oxfordjournals.org/.  

**Acknowledgements**

We are grateful to all the women who participated in this study. We are also grateful to Dr Rebecca Dragovic and Dr Jennifer Southcombe for helpful discussions regarding flow cytometry, as well as Dr Tim James for arranging the hormone assays.

**Authors’ roles**

K.E.W. carried out the experimental work, analysed the data and drafted the manuscript. S.H.K. participated in the study design and edited the manuscript. C.M.B. designed the study, edited the manuscript and was involved in critical discussion regarding the results.

**Funding**

This work was supported by grants from the MRC (New Investigator Award, G0601458 to C.M.B.), the Oxford Partnership Comprehensive Biomedical Research Centre with funding from the Department of Health’s NIHR Biomedical Research Centres Scheme and the Oxfordshire Health Services Research Committee (OHSRC).

**Conflict of interest**

None.

**References**


Zentilin L, Tafuro S, Zacchigna S, Arsic N, Pattarini L, Sinigaglia M, Giacca M. Bone marrow mononuclear cells are recruited to the sites of VEGF-induced neovascularization but are not incorporated into the newly formed vessels. *Blood* 2006;107:3546–3554.