The distribution of immune cells and macrophages in the endometrium of women with recurrent reproductive failure. II: adenomyosis and macrophages

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A B S T R A C T

Adenomyosis, a condition usually associated with multiparity, is not generally seen as a cause of infertility. However, recent studies have reported a reduction in IVF implantation rates and a link with miscarriage, suggesting that adenomyosis may interfere with successful implantation. To investigate this hypothesis, the clinical records and laboratory results, which routinely include immunohistochemical examination of a late luteal phase endometrial biopsy for leukocytes, were retrospectively reviewed for 64 women with implantation failure and who previously had been screened for the presence of adenomyosis by pelvic MRI.

The presence of either diffuse or “adenomyoma” type of adenomyosis was associated with a marked increase (\(p=0.004\)) in the density of macrophages and natural killer cells in the endometrial stroma, compared to those women with mild focal adenomyosis or no disease. These findings point to an immunological mechanism by which adenomyosis might interfere with successful embryo implantation.

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1. Introduction

Adenomyosis, a disorder related to endometriosis (Kunz et al., 2005; Leyendecker et al., 2006), is characterised by heterotopic endometrial glands and stroma in the myometrium, and is an established cause of menorrhagia, dysmenorrhoea and pelvic pressure symptoms (Peric and Fraser, 2006). Most commonly it is diagnosed in multiparous women in their 5th decade of life and therefore is not traditionally associated with an inability to conceive (Vercellini et al., 2006). Adenomyosis is primarily diagnosed at pathological examination of the uterus following hysterectomy, a sterilising procedure unlikely to be undertaken in an infertile patient. Since as many as one third of women with pathologically confirmed adenomyosis are symptom-free (Peric and Fraser, 2006), it is likely that adenomyosis in infertile women is frequently missed.

Recent studies have suggested that adenomyosis may be associated with impaired reproduction. A study of 46 women undergoing resection of bowel endometriosis reported that the subsequent natural and IVF assisted pregnancy rate in those with co-existing adenomyosis was very significantly reduced (Ferrero et al., 2009). Furthermore, two small case series have linked the presence of adenomyosis with early miscarriage (Kano et al., 1997; Olive et al., 1982). All of these studies suggest that adenomyosis may interfere with successful implantation.

Pelvic Magnetic Resonance Imaging (MRI) is the radiological investigation of choice for the diagnosis of
adenomyosis, with a sensitivity and specificity approaching 90% (Reinhold et al., 1998; Tamai et al., 2006), now enabling the non-invasive examination of the relationship between adenomyosis and infertility. Two prospective MRI studies (Kissler et al., 2006; Maubon et al., 2010) and a more recent case-series reported from our group (Tremellen and Russell, 2011) have confirmed a link between failure of successful implantation of good quality embryos during IVF treatment and adenomyosis. However, other investigators have not confirmed a significant correlation between MRI evidence of adenomyosis and implantation failure (Kunz and Beil, 2010; Turnbull et al., 1994). Therefore we sought to examine in a large study cohort if adenomyosis may produce implantation failure, by investigating differences in endometrial function between women with and without adenomyosis detected by MRI scan.

Whilst the exact mechanisms by which adenomyosis may limit fertility are still under debate, alterations in the endometrial immune environment were suggested as a possible cause in a small case series of patients with adenomyosis, who exhibited excessively high endometrial macrophage density compared to published normal ranges (Russell et al., 2011; Tremellen and Russell, 2011). Since it is well recognised that macrophages have the capability of releasing embryo-toxic cytokines and reactive oxygen species (Agarwal et al., 2005; Haddad et al., 1995; Lee et al., 2004), we specifically examined the relationship between MRI diagnosed adenomyosis and endometrial immune cell populations in a larger cohort of women experiencing IVF implantation failure.

2. Materials and methods

2.1. Study population

Investigation of recurrent implantation failure in Repromed (Adelaide, South Australia) generally consists of an assessment for thrombophilias (lupus anticoagulant, anticardiolipin antibody, factor V Leiden mutation, prothrombin mutation, MTHFR genotype, homocysteine), evidence for autoimmunity (thyroid antibodies, ANA), coeliac antibody screen and an endometrial biopsy in the late luteal phase (endometrial morphology and assessment of selected immunocompetent cells). A diagnostic hysteroscopy and laparoscopy are performed if any abnormalities are found on ultrasound or symptoms suggest pelvic pathology such as endometriosis. Patients with submucous or cavity-distorting intramural fibroids or pelvic pathology such as hydrosalpinx were excluded from the study as these conditions are already known to alter endometrial immune cell populations (Copperman et al., 2006; Miura et al., 2006).

Since late 2009, we have routinely performed a pelvic MRI in patients with recurrent IVF failure, and therefore we were able to retrospectively correlate the MRI diagnosis of adenomyosis with changes in the numbers of selected endometrial immunocompetent cells. This study was approved by the local scientific advisory committee and did not require formal ethics committee review due to its purely retrospective nature, in line with Australian National Health and Medical Research Council guidelines.

![Fig. 1. Immunostain of CD163+ macrophages in the superficial endometrial stroma (dark brown) in a background of non-reactive stromal cells (pale blue nuclei). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)](image)

2.2. Endometrial tissue collection and processing

Endometrial biopsies were conducted between 8 and 12 days post ovulation, corresponding to days 22 through to 26 of a standardised 28-day menstrual cycle. In patients with irregular cycles, serum hormone profiles were used to time biopsies. Anovulatory patients were given clomiphene citrate to produce ovulation or an artificial hormone replacement cycle (oral oestradiol valerate for a minimum 2 weeks before commencing vaginal progesterone). All patients had the endometrial sample dated according to the time-honoured criteria of Noyes et al. (1975). An additional six cases where histological dating of the endometrium, using these traditional morphological criteria, was outside the day 22–26 period of interest, were omitted from the study.

All endometrial samples were fixed in 10% neutral buffered formalin, processed within 48 h into paraffin, had sections cut at 4 μm and stained with haematoxylin and eosin according to a standard protocol. Serial sections were also cut at 4 μm and immunostained in a Ventana Benchmark XT using ULTRA view® DAB detection kit (Ventana Medical Systems, Oro Valley, USA), using commercially available monoclonal antibodies, for natural killer (NK) cells (CD56) and macrophages (CD163), as previously published by our group (Russell et al., 2011). All histological studies were performed by one specialist pathologist (PR). The only information provided to the pathologist before reporting of the biopsy was the date of the last menstrual period and a clinical history of recurrent implantation failure. The pathologist was blinded to the MRI result so as to remove any chance of bias in the report findings. CD56+ NK cell and CD163+ macrophage numbers were quantified both as immunopositive cells per mm² of endometrial stroma in the superficial functional layer of the endometrium, and as a percentage of stromal cells in the same area (Fig. 1). Stromal macrophage counts were performed on 5 high power fields (40x objective), devoid of endometrial glands. Percentage counts were performed on
Fig. 2. Immunostain of CD163+ macrophages within the gland lumens of the superficial endometrium (dark brown) – this case corresponding to +++ in severity. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)

a single oil immersion field (100× objective) deemed to be representative of the cell density, as has been previously reported by our group (Russell et al., 2011). In addition, the presence of CD163+ macrophages within the glandular lumens were semi-quantified on a +, ++, +++ and ++++ grading scale, when present (Fig. 2).

2.3. Pelvic MRI protocol

All pelvic MRIs were performed on a Philips Eclipse 1.5 T (Philips, Netherlands) MRI scanner, with a 1.5 T magnet and 27-mT/m gradients. Sequences included T2-weighted sagittal, frontal and axial sequences. Patients were advised to preferably have their scan in the second half of the menstrual cycle when adenomyosis is most prominent.

Patients found to have a junctional zone less than 8 mm in thickness were considered to be normal with no evidence of adenomyosis (Reinhold et al., 1998; Tamai et al., 2006). Patients identified as having a thickened junctional zone ≥12 mm were considered to have sufficient MRI evidence of adenomyosis, irrespective of whether or not they had additional features of adenomyosis such as the presence of sub-mucosal cysts (Reinhold et al., 1998). Those patients with features of adenomyosis localised to a small area of the uterus less than 2 cm in width were classified as having focal “mild” adenomyosis, whilst those patients exhibiting widespread junctional zone thickening of the majority of the anterior and/or posterior uterine wall, or a nodular “adenomyoma” type lesions producing distortion of the endometrium, were considered to have “severe” adenomyosis.

2.4. Statistical analysis

Statistical analysis was performed using the Graphpad Prism 5 software (San Diego, USA). Of all the continuous variables recorded, only maternal age and CD56 density were normally distributed and therefore expressed as a mean (±SEM) and analysed using the non-paired t-test. The remaining continuous variables were expressed as medians with inter-quartile (25th–75th percentile) ranges and analysed by the non-parametric Mann Whitney test. Categorical variables such as infertility diagnosis were analysed by a Chi-square test. The differences between comparison groups were considered to be statistically significant at \( p < 0.05 \).

3. Results

3.1. Clinical data

Sixty-four women with recurrent implantation failure were identified as having had a pelvic MRI and late luteal phase endometrial biopsy between January 2010 and March 2011. Their baseline clinical data are given in Table 1. In summary, the mean age of the adenomyosis group (n = 31) was slightly older than women found not to have adenomyosis (n = 33), but this difference did not reach statistical significance (\( p = 0.061 \)). All women had undergone a significant number of unsuccessful embryo transfers, and the majority were experiencing primary rather than secondary infertility (Table 1). Laparoscopically confirmed endometriosis was present in five of 31 (16%) adenomyosis cases and in three of 33 (9.1%) women with no evidence of adenomyosis on MRI (\( p = 0.46 \)). Other aetiological factors for infertility were shared equally between the two groups.

3.2. Immune cell populations

Fig. 1 shows the typical representative section displaying the immunohistochemistry stain patterns of CD163+ macrophages in the superficial endometrial stroma, with Fig. 2 being an example of a marked infiltration of CD163+ macrophages into the superficial endometrial gland lumens. The differences in median (inter-quartile range) CD163+ density in the endometrial stroma and

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Subject baseline characteristics.</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Adenomyosis positive</td>
</tr>
<tr>
<td>Number subjects</td>
<td>31</td>
</tr>
<tr>
<td>Maternal age (years)</td>
<td>37.3 ± 4.4</td>
</tr>
<tr>
<td>Number prior miscarriages</td>
<td>1 (0–2)</td>
</tr>
<tr>
<td>Number of prior live births</td>
<td>0 (0–1)</td>
</tr>
<tr>
<td>Total number of embryos transferred during IVF</td>
<td>7 (4–12)</td>
</tr>
<tr>
<td>Aetiology infertility</td>
<td></td>
</tr>
<tr>
<td>1. Male</td>
<td>22.6%</td>
</tr>
<tr>
<td>2. Female</td>
<td>29%</td>
</tr>
<tr>
<td>3. Combined</td>
<td>29%</td>
</tr>
<tr>
<td>4. Unknown</td>
<td>19.4%</td>
</tr>
</tbody>
</table>

Maternal age is expressed as mean ± SEM. Other variables are expressed as median (inter-quartile range).

* Unpaired t-test.

b Mann Whitney test.

c Chi square analysis.
Table 2

Immunohistochemistry analysis.

<table>
<thead>
<tr>
<th></th>
<th>Adenomyosis positive</th>
<th>Adenomyosis negative</th>
<th>p-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day of biopsy</td>
<td>24 (23–24)</td>
<td>24 (23–24)</td>
<td>0.321</td>
</tr>
<tr>
<td>Stromal CD 163 density (mm²)</td>
<td>280 (190–360)</td>
<td>210 (170–245)</td>
<td>0.013</td>
</tr>
<tr>
<td>Superficial glandular CD163 density (0 to 4+)</td>
<td>1 (0–2)</td>
<td>1 (0–2.5)</td>
<td>0.418</td>
</tr>
<tr>
<td>Stromal CD 56 density (mm²)</td>
<td>1160 ± 124</td>
<td>913 ± 93</td>
<td>0.112</td>
</tr>
</tbody>
</table>

Macrophages quantified using CD163 and uterine Natural Killer cells with CD56 immunostains. CD163 data were not normally distributed and therefore are presented as median (inter-quartile range) and analysed with Mann Whitney test. CD56 data were normally distributed and expressed as a mean (±SEM) and analysed by an unpaired t-test.

The superficial glands observed between patients with mild focal adenomyosis, severe diffuse adenomyosis and those with no MRI evidence of adenomyosis is recorded in Table 2, and is represented in graphical form in Fig. 3. The median day of sampling was the same in the adenomyosis and non-adenomyosis groups, enabling legitimate comparisons between these two groups.

The median density of CD163+ stromal macrophages (Table 2) was greatly increased in the adenomyosis group compared with the non-adenomyosis group (median 280/mm² vs 210/mm², p = 0.013). However, if the adenomyosis population is divided into those with “mild” focal adenomyosis and those with “severe” diffuse or adenomyoma type of adenomyosis, the above difference is almost entirely due to increased macrophage populations in the severely affected patients (Fig. 3). The density of stromal macrophages does not significantly differ between subjects with no MRI evidence of adenomyosis (median 210/mm², IQR 170–245/mm²) compared with those women with mild adenomyosis (median 220/mm², IQR 172–310/mm²). However, women with severe adenomyosis have a statistically significant greater stromal macrophage density (median 335/mm², IQR 263–560/mm²) than both non-adenomyosis controls and mild focal adenomyosis cases (p = 0.004). Analysis of macrophage numbers in the superficial glandular lumens (Table 2) using the semi-quantitative 0 to 4+ scale revealed no significant differences between the adenomyosis and non-adenomyosis groups.

Whilst less pronounced than changes seen in macrophage density, significant differences were also observed in CD56+ uNK cell densities (Fig. 4) between the severe adenomyosis and the non-adenomyosis group.

4. Discussion

This study is the first to report an increased stromal macrophage population in the functional layer of the endometrium in patients experiencing implantation failure with co-existing “severe” diffuse or the “adenomyoma” variant of adenomyosis. A previous histological study has described such an increase within the myometrium of women with adenomyosis, but did not comment on endometrial macrophage numbers (Ota et al., 1991). A more recent histological study also identified significant numbers of macrophages in the eutopic endometrium of patients with endometriosis, myomas and adenomyosis, but the authors did not comment on any significant difference in endometrial macrophage density between these pathological groups (Khan et al., 2010). Interestingly, several investigators have reported a link between the presence of endometriosis on laparoscopy and an increase in macrophage density in the eutopic endometrium (Berbic et al., 2009; Khan et al., 2004; Leiva et al., 1994). Only one study to date has reported a significant decrease in macrophage density in the eutopic endometrium of women with endometriosis (Braun et al., 2002).
As both endometriosis and adenomyosis have been linked to impaired implantation potential (Barnhart et al., 2002; Ferrero et al., 2009; Maubon et al., 2010; Tremellen and Russell, 2011), it is possible that the observed increase in macrophage density may represent a common underlying pathological mechanism by which these cells contribute to a hostile immune environment for the implanting embryos. Whilst not all subjects in this study underwent laparoscopy to identify the presence of endometriosis, those patients with symptoms suggestive of endometriosis usually did. Furthermore, pelvic MRI is a relatively good imaging modality to non-invasively diagnose severe endometriosis (endometriomas, thick peritoneal deposits). Taken together, it is unlikely that we have significantly under-estimated the existence of significant endometriosis disease co-existing with adenomyosis. Finally, the observation that only severe adenomyosis, not mild focal disease, is linked with an increased endometrial macrophage density makes it less likely that mild undiagnosed endometriosis is primarily responsible for the observed macrophage infiltration.

It is presently unknown how adenomyosis stimulates an increased endometrial macrophage density or whether, indeed, these phenomena are related or merely co-incidental. A recent study has reported that long term GnRH agonist down-regulation therapy can produce a very significant fall in endometrial macrophage numbers, with a co-existent decline in endometrial tissue production of MCP-1, a cytokine known to be chemotactic for macrophages (Khan et al., 2010). Furthermore, this study reported that down-regulation therapy produced a marked reduction in endometrial capillary density, a parameter that is known to be significantly increased in the adenomyotic endometrium (Ota and Tanaka, 2003). Taken together, these studies suggest that under local control of oestrogen there is an increase in endometrial capillary density and production of cytokines chemotactic for macrophages, both likely to result in a net influx of macrophages into the endometrium. Adenomyosis is known to be characterised by a local state of oestrogen dominance (Kitawaki, 2006). Whilst serum levels of oestrogen do not differ between women with or without adenomyosis, menstrual blood oestradiol levels have been reported to be raised in the adenomyosis group, reflecting an increased level of aromatase expression in the adenomyotic endometrium (Kitawaki, 2006; Takahashi et al., 1989). An excess of oestrogen action in the endometrium may result in an increase in capillary density and production of pro-inflammatory cytokines, resulting in an elevated endometrial macrophage density.

When appropriately activated, macrophages are known to have the capacity to secrete cytokines such as TNFα and IL-1, plus reactive oxygen and nitrogen species, all potentially toxic to embryos (Agarwal et al., 2005; Haddad et al., 1995). The endometrial environment of patients with adenomyosis has been reported to contain elevated levels of nitric oxide (Ota et al., 1998), a “free radical” chemical linked with impaired embryo development and poor pregnancy rates (Lee et al., 2004). Furthermore, endometrial biopsies taken from patients with adenomyosis contain elevated amounts of anti-oxidant enzymes such as catalase, superoxide dismutase and glutathione peroxidase, a clear sign of local oxidative stress caused by excessive reactive oxygen species production (Agarwal et al., 2005; Ota et al., 2002). These reactive oxygen and nitrogen species will directly damage embryos and have been reported to increase local production of prostaglandin F2α, leading to an increase in myometrial contractility that is likely to further impede the implantation process (Agarwal et al., 2005).

Whilst the cause and effect relationship, if any, between severe adenomyosis and increased macrophage populations in the endometrium requires elucidation, our novel observation now provides a pathological mechanism by which adenomyosis may impede successful implantation of an embryo. However, as these observations are only preliminary findings of a small retrospective case series, they must be verified in a larger prospective study before firm conclusions can be made. Furthermore, in the future it would be useful to correlate the immunohistochemistry defined macrophage infiltrations seen with adenomyosis with changes in endometrial inflammatory mediators (cytokines and prostaglandins) and the production of reactive oxygen species, as these observations would help strengthen the causative link between adenomyosis and failure of implantation of good quality embryos.

References

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