Endometrial integrin expression in women undergoing IVF and ICSI: a comparison of the two groups and fertile controls

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BACKGROUND: Integrins are thought to play a vital role in implantation. Three integrins in particular (α⁴β¹, α⁵β³ and α¹β¹) are all present during the implantation window. Defects in their expression have been linked to tubal disease, unexplained infertility and endometriosis. Hence, a reduced endometrial integrin expression would be expected in women attending for IVF due to these causes of infertility when compared with those with male factor infertility attending for ICSI. METHODS: Women attending for IVF (n = 25) and ICSI (n = 25) treatment were recruited, and timed endometrial biopsies were taken during the ‘implantation window’ (cycle day 20–24). A group of fertile women (n = 15) attending for sterilization was used as controls. RESULTS: There was no significant difference in integrin expression between patients undergoing IVF or ICSI. Neither did these groups differ from the control group. CONCLUSIONS: The endometrium in patients undergoing ICSI treatment is sometimes thought to be more receptive, as the infertility might be due to a male factor. This study shows that there is no significant difference in integrin expression between patients attending for IVF or ICSI and the control group. These data add to the increasing uncertainty about the clinical value of assessing the endometrium with only one marker, in this case integrins.

Key words: endometrium/implantation/integrins/IVF

Introduction

Assisted conception techniques have improved dramatically since the birth of the first baby after IVF treatment in 1978 (Steptoe and Edwards, 1978). The introduction of ICSI, pioneered by Palermo et al. in Brussels in the early 1990s, revolutionized options for the treatment of male infertility (Palermo et al., 1993). Standard IVF techniques are still used when the semen analysis is normal, mainly because of the continued uncertainty about fetal and childhood development after ICSI (Sutcliffe et al., 1998; Aboulghar et al., 2001). Couples undergoing IVF either have ‘unexplained’ infertility or a condition known to affect female fertility, such as endometriosis or tubal disease. It is unclear if there is any difference in the uterine receptivity between patients undergoing IVF and ICSI, and in addition how these patients compare with fertile controls.

Neither endometriosis nor tubal damage, per se, should affect the endometrium as they are both conditions that essentially take place outside the lining of the uterine cavity. However, it has been suggested that the endometrium may be affected by the presence of endometriosis (Yovich et al., 1988), and both structural (Fedele et al., 1990) and functional (Weed and Arquembourg, 1980) abnormalities of the endometrium have been described in this condition. With regards to tubal disease, hydrosalpinx fluid is thought to have a detrimental effect on implantation in human reproduction (Anderson et al., 1994; Kassabji et al., 1994). Success rates in IVF have been shown to be greater after the removal of hydrosalpinges, suggesting that this leads to an improvement in endometrial receptivity (Vandromme et al., 1995).

The human endometrium undergoes changes that are vital if implantation is to take place. Although a classic method reported in 1950 (Noyes et al., 1950) has now been used for over half a century to date, and therefore assess, receptivity of the endometrium, recent attention has focused on the expression of integrins within the endometrium, and their relation to infertility. Certain integrins have been shown to undergo specific changes within the endometrium during different phases of the menstrual cycle and, therefore, are thought to play an integral role in implantation (Lessey et al., 1996). The endometrium is generally hostile to implantation outside a critical period of time during the mid-secretory phase. This period—otherwise known as the ‘implantation window’—coincides with the appearance of three integrins; α₅β₃, α₁β₁
and α4β1. The expression of all three integrins occurs only during the 4-day period of days 20 to 24 of the menstrual cycle. Due to the temporal pattern of their expression around the time of implantation and their absence in conditions related to infertility, these integrins might have an important role as a potential marker of uterine receptivity (Lessey et al., 1994a; Tabibzadeh, 1997). Aberrant expression of these integrins has been found to be associated with endometriosis (Lessey et al., 1994b), hydrosalpinges (Meyer et al., 1997) and unexplained infertility (Lessey et al., 1995).

The first aim of the present study was to compare integrin expression between fertile controls and those patients attending for assisted conception treatment. Second, the groups of patients undergoing IVF and ICSI were compared. The hypothesis was that integrin expression would be reduced in patients attending for IVF when compared with the other two groups, as the IVF group comprised patients with endometriosis, tubal disease and unexplained infertility, all of which are associated with reduced integrin expression.

Materials and methods

Patients

Ethical approval for this project was granted by the Liverpool Local Research Ethics Committee. Women attending the Reproductive Medicine Unit (RMU) at the Liverpool Women’s Hospital for IVF and ICSI treatment were recruited into the study. The control patients were recruited from those women attending for laparoscopic sterilization. An information sheet was given to all women, and informed consent obtained from those agreeing to take part. All participating patients were aged <37 years and had a regular menstrual cycle.

Control group

A total of 15 patients was recruited when they attended the preoperative clinic prior to their sterilization. All had at least one full-term pregnancy and had been using only barrier methods of contraception in the previous 2 months. No endometriosis or hydrosalpinges were seen in any of the control patients at laparoscopy.

ICSI group

Prior to treatment, all male partners underwent a repeat semen analysis. A decision to perform ICSI was taken if: (i) the sperm migration test (SMT) was <2×10⁶/ml; or (ii) if the SMT was between 2–5×10⁶/ml and the sperm motility or morphology was judged to be low as defined previously (Biljan et al., 1994). In the 25 women recruited to the ICSI group, male factor was the only identifiable cause of infertility. Couples in which there was also co-existing endometriosis or tubal disease in the female partner, and those having ICSI treatment for previous poor fertilization, were excluded.

IVF group

Among the 25 women in the IVF group, primary causes of infertility were tubal disease (n = 12), endometriosis (n = 6) and unexplained infertility (n = 7). All male partners had normal semen analysis, with an SMT >5×10⁶/ml.

Investigations

The case notes and referral letters of all women were reviewed to ascertain if the women had undergone tubal assessment by either laparoscopy or hysterosalpingogram (HSG). Although a higher proportion of women in the IVF group had undergone a laparoscopy ± HSG (n = 17 and n = 12 for IVF and ICSI patients respectively), as opposed to HSG alone (n = 8 and n = 12 respectively), all women in the ICSI group except one had undergone one of these investigations. This woman’s partner had undergone a failed reversal of vasectomy and remained azoospermic. As the couple required surgical sperm retrieval and ICSI, it was not felt appropriate to investigate tubal patency in the female partner as assisted conception treatment would be required in any case. In this case the woman had no symptoms of endometriosis and no history of pelvic inflammatory disease.

In all patients an endometrial biopsy was performed 7–8 days after the detection of the mid-cycle urinary LH surge (Clearplan™; Unipath, Bedford, UK). Although ultrasonographic monitoring of ovulation has been shown to be the ideal method of detecting ovulation when dating the endometrium (Shoupe et al., 1989), ovulation is known to occur in nearly 95% of women within 2 days of a positive urinary LH surge (Behre et al., 2000). The use of Clearplan was therefore considered adequate and less invasive for the patients. Biopsies were taken using an endometrial sampler (Wallace Ltd, Hythe, Kent, UK). The biopsies were immediately frozen in liquid nitrogen and stored at −70°C until use. In those patients attending for IVF or ICSI the biopsies were performed at least two menstrual cycles prior to the commencement of treatment.

Immunohistochemistry and monoclonal antibodies

Cryostat sections of 5 µm thickness were cut and mounted onto glass slides. After drying overnight at room temperature, slides were wrapped in aluminium foil and frozen at −20°C until immunostaining. Sections were then removed and allowed to reach room temperature. Staining was performed using the alkaline phosphatase-anti-alkaline phosphatase (APAAP) immunoalkaline phosphatase technique (Mason, 1985). The slides were first fixed in acetone for 10 min and, after washing in Tris-buffered saline (TBS 0.05 mol/l, pH 7.6), were incubated with the appropriate diluted monoclonal antibody for 30 min in a humidified chamber. Details of the primary antibodies used in the study are given in Table 1. The antibody to the β3 integrin was used to check with the α4β1 integrin and ensure that it was not α4β3 or α4βα. Staining for this antibody was used to ensure that uptake was seen at the same areas as the α4 and α4β integrin subunits. Mouse IgG was used in place of the primary antibody as a negative control. After 2×5 min washes in TBS, bound antibodies were detected by the APAAP method using rabbit anti-(mouse IgG) IgG (diluted 1:25; Dako Ltd, High Wycombe, UK) for 30 min, washed in TBS and then incubated with a pre-formed complex of calf intestinal alkaline phosphatase and mouse monoclonal anti-(alkaline phosphatase) (diluted 1:50; Dako Ltd) for a further 30 min. Staining was developed with Naphthol AS-MX phosphate and Fast Red (Sigma, Poole, Dorset, UK) with the inclusion of 1 ml of levamisole to block any endogenous

<table>
<thead>
<tr>
<th>Integrin</th>
<th>Clone</th>
<th>Host</th>
<th>Company</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>α6β1</td>
<td>LM609</td>
<td>Mouse</td>
<td>Chemicon</td>
<td>1 in 50</td>
</tr>
<tr>
<td>β3</td>
<td>PM6b13</td>
<td>Mouse</td>
<td>Chemicon</td>
<td>1 in 50</td>
</tr>
<tr>
<td>α1</td>
<td>FB12</td>
<td>Mouse</td>
<td>Chemicon</td>
<td>1 in 100</td>
</tr>
<tr>
<td>β1</td>
<td>DF58</td>
<td>Mouse</td>
<td>Serotec</td>
<td>1 in 100</td>
</tr>
<tr>
<td>α4</td>
<td>B-5H10</td>
<td>Mouse</td>
<td>Dr Martin Hemler</td>
<td>1 in 100</td>
</tr>
</tbody>
</table>

Table 1. Primary antibodies used in this study

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Chemicon, Harrow, UK.


Dr Martin Hemler, Dana Faber Institute, Boston, USA.
alkaline phosphatase. Slides were counterstained with haemalum and mounted in Aquamount (BDH, Poole, Dorset, UK).

The slides were evaluated using a Nikon microscope. First, the endometrium was dated according to published criteria (Noyes et al., 1950), and the staining was then analysed. All slides were analysed blind by a single examiner (K.T.). Five sets of slides were also checked by an independent observer to ensure minimal inter-observer error. The intensity of staining of the antibodies was analysed in both the glandular and luminal epithelium, and assessed by using the HSCORE. This semiquantitative form of analysis (Budwit-Nowotny, 1986) has been shown to have a low intra- and inter-observer error (Lessey et al., 2000). The HSCORE was calculated using the following equation: HSCORE = Σ Pi(i + 1), where i = 1, 2 or 3 and is the intensity of the stained epithelium, while Pi is the percentage of stained epithelial cells for each intensity and ranges from 0 to 100%.

An HSCORE ≤0.7 was not clearly distinguishable from no staining, and was used as the cut-off for a negative test. Colour prints were made using Kodak 100 ASA film.

Statistical analysis
As the data comprised continuous, non-normally distributed variables, statistical analysis was performed using the Mann–Whitney U-test. A commercial statistical package (SPSS®) was used.

Results
The mean age in the IVF group was 31.4 (range 28–35) years, compared with 29.3 (range 27–35) years in the ICSI group. Among participants, the body mass index (BMI) ranged from
especially that of unexplained infertility all reduce integrin expression, and which have shown that tubal disease, endometriosis and cause of the infertility did not affect integrin expression. Both analysis of the different subgroups within the IVF group, the control groups (all luminal or glandular epithelium, between the ICSI, IVF and difference in values for any of the integrins, in either the still strongest in the glandular epithelium.

The receptivity of the endometrium is vital to the process of implantation. Good quality embryos and ease of transfer will improve the success rates of IVF/ICSI treatment, but if the endometrium is un receptive then the treatment is likely to fail (Paulson et al., 1990). ICSI treatment is mainly used in cases of male factor infertility. In the majority of these cases, the woman is thought to be fertile and the endometrium assumed receptive. In those patients undergoing IVF, the cause of the infertility may be unexplained or due to conditions affecting the female reproductive tract (e.g. endometriosis or tubal disease). These conditions have been shown in previous studies to be associated with aberrant endometrial integrin expression within the endometrium (Lessey et al., 1994a; Meyer et al., 1997).

It is therefore surprising that the present results showed there to be no significant difference in integrin expression between the three groups studied. Clearly, the IVF group comprising patients with tubal disease, endometriosis and unexplained infertility who have reached the point of assisted conception treatment should, based on previous data, have shown reduced integrin expression. Data have been reported however showing that integrin expression is not altered by diseases such as endometriosis (Creus et al., 1998), and the present results add weight to the uncertainty of the role of integrins in uterine receptivity.

What is unclear in the literature is the effect of the severity of tubal disease, and the influence of its treatment, on integrin expression. Tubal disease is thought to affect the endometrium due to a detrimental affect of hydrosalpinx fluid (Anderson et al., 1994). Others (Meyer et al., 1997) showed a reduced expression of the $\alpha_\beta_3$ integrin in these patients which improved after corrective surgery, and this was in agreement with the findings of other studies which showed improved pregnancy rates after repair (Vandromme et al., 1995). Therefore, a decrease in integrin expression would be expected in patients undergoing IVF treatment, and in theory this could lead to an impaired receptivity of the endometrium and a decreased pregnancy rate.

In the present study, none of the patients with tubal disease had identifiable hydrosalpinges on ultrasound before their treatment started. The significance of being able to visualize the hydrosalpinx on ultrasound remains controversial. One group (De Wit et al., 1998) found implantation rates to be significantly lower in the presence of an ultrasound-visible hydrosalpinx, suggesting that it is the amount of hydrosalpinx fluid that seems to cause the reduced IVF outcome. A large prospective, randomized multicentre trial conducted in Scandinavia supported the argument that only those patients with ultrasound-visible hydrosalpinges benefited from prophylactic salpingectomy (Strandell et al., 1999). The two groups where there were significant differences, in both implantation and clinical pregnancy rates after surgery, were the women with bilateral hydrosalpinges (25.6 versus 12.3%, $P = 0.038$) and in those patients with ultrasound-visible hydrosalpinges. Patients with bilateral hydrosalpinges visible on ultrasound had a 3.5-fold increase in delivery rates after treatment. If, therefore, only these patients have impaired endometrial receptivity, secondary to the effect of the hydrosalpinx fluid, then this would help to explain the similarity in integrin expression seen in the present study.

### Table II. HSCOREs (mean and interquartile range) for staining in the glandular epithelium

<table>
<thead>
<tr>
<th>Integrin</th>
<th>Group 1 (IVF)</th>
<th>Group 2 (ICSI)</th>
<th>Group 3 (Control)</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\alpha_\beta_3$</td>
<td>1.3 (0.9–1.7)</td>
<td>1.6 (0.95–1.95)</td>
<td>1.7 (1.3–2.1)</td>
<td>1 versus 2 NS</td>
</tr>
<tr>
<td>$\alpha_\gamma_1$</td>
<td>2.9 (2.5–3.45)</td>
<td>2.7 (2.0–3.2)</td>
<td>2.7 (2.2–3.3)</td>
<td>1 versus 2 NS</td>
</tr>
<tr>
<td>$\alpha_\beta_1$</td>
<td>2.6 (2.2–3.0)</td>
<td>2.6 (1.65–3.2)</td>
<td>2.9 (2.5–3.5)</td>
<td>1 versus 3 NS</td>
</tr>
</tbody>
</table>

### Table III. HSCOREs (mean and interquartile range) for staining in the luminal epithelium

<table>
<thead>
<tr>
<th>Integrin</th>
<th>Group 1 (IVF)</th>
<th>Group 2 (ICSI)</th>
<th>Group 3 (Control)</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\alpha_\beta_3$</td>
<td>1.3 (0.9–1.7)</td>
<td>1.0 (0.4–1.7)</td>
<td>1.3 (1.1–1.8)</td>
<td>1 versus 2 NS</td>
</tr>
<tr>
<td>$\alpha_\gamma_1$</td>
<td>0.5 (0.4–0.8)</td>
<td>0.4 (0.3–0.7)</td>
<td>0.6 (0.45–1.0)</td>
<td>1 versus 3 NS</td>
</tr>
<tr>
<td>$\alpha_\beta_1$</td>
<td>0.5 (0.4–0.7)</td>
<td>0.4 (0.2–0.6)</td>
<td>0.4 (0.3–0.7)</td>
<td>1 versus 2 NS</td>
</tr>
</tbody>
</table>

18 to 30 kg/m$^2$. There were no inter-group statistical differences in either age or BMI.

Staining of the endometrium using the various antibodies is shown in Figure 1. Antibodies were used against the different subunits in each integrin. The $\beta_1$ subunit is known to combine with a wide variety of $\alpha$ chains. The staining of each subunit was assessed using the HSCORE for the luminal and glandular epithelium. Stromal staining was not assessed in this study. Staining patterns varied from patient to patient, but the $\beta_1$ subunit showed strong expression in all patients due to the fact that it combines with such a wide variety of $\alpha$ subunits. Expression of the $\alpha_1$ and $\alpha_4$ subunits was greater in the luminal epithelium than the glandular epithelium (see Figure 1B; the arrowheads indicate the luminal epithelium). The $\alpha_3\beta_3$ integrin showed more uptake in the luminal epithelium than either the $\alpha_1$ or $\alpha_4$ subunits, but staining was still strongest in the glandular epithelium.

On analysis of staining using the HSCORE, there was no difference in values for any of the integrins, in either the luminal or glandular epithelium, between the ICSI, IVF and control groups (all $P > 0.05$) (Tables II and III). Also, on analysis of the different subgroups within the IVF group, the cause of the infertility did not affect integrin expression. Both of these findings were surprising in view of previous studies which have shown that tubal disease, endometriosis and unexplained infertility all reduce integrin expression, and especially that of $\alpha_3\beta_3$.

### Discussion

The receptivity of the endometrium is vital to the process of implantation. Good quality embryos and ease of transfer will

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With regards to endometriosis, a lack of $\beta_3$ expression was shown to be closely related to the diagnosis of endometriosis, and the assay for endometrial $\alpha_\beta_3$ integrin has a high positive predictive value for the condition (Lessey et al., 1994b). Although others (Hii and Rogers, 1998) reported that the $\alpha_\beta_3$ integrin was present in the glandular epithelium throughout the cycle, and was unaffected by endometriosis, the report by these authors has been criticised because formalin-fixed preparations were used (Lessey, 1998). However another study showed no difference in the $\alpha_\beta_3$ expression between patients with and without endometriosis (Crews et al., 1998).

The association between minimal or mild endometriosis and infertility is far from conclusive (Bancroft et al., 1989). At least two meta-analyses have shown that no treatment appears to be as effective as treatment (Hughes et al., 1993; Adamson and Pasta, 1994), and that approximately 50% of women will become pregnant without any treatment (Seibel et al., 1982). These findings suggest that endometrial receptivity may not be impaired in cases of minimal endometriosis. Also, hormonal treatment available for the treatment of endometriosis and its symptoms could have an effect on integrin expression. In the UK, the waiting lists for IVF treatment on the National Health Service (NHS) are invariably longer than 3 years, and a large number of women would receive treatment during this time, perhaps improving the severity of the disease and therefore, in theory, the integrin expression. It is also recognized that a number of women who had an HSG as an investigation for tubal patency may have minimal, asymptomatic endometriosis.

The process of controlled ovarian stimulation (COS) has been shown to affect integrin expression in the endometrium (Thomas et al., 2002), and it may be this process that leads to poor implantation rates. Hence, a much better understanding of the endometrium during a normal cycle is needed, taking into account the effect of the implanting embryo. Only then will it become possible to examine in more detail the effect of COS on the endometrium as to how a more receptive endometrium can be created during this process.

In summary, the results of the present study suggest that integrin expression is not reduced in patients undergoing IVF treatment when compared with controls and with women undergoing ICSI for purely male factor purposes. It has been postulated that this might be due to the severity of the disease or to the time between referral and treatment. If integrins are good markers of uterine receptivity, then it could be concluded that there is no difference in uterine receptivity between these groups, and this would be clinically valuable. However, the present results add to the ongoing uncertainty about the value of integrins alone in assessing endometrial receptivity. If the endometrium is affected by tubal disease and endometriosis, then this is not reflected in integrin expression in this study. Implantation is a complex multi-factorial process that is unlikely to be controlled by one factor or gene. Although integrins probably play a role, their use alone in assessing receptivity, in either a research- or clinic-based situation, should be questioned.

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