Expression pattern of osteopontin and αvβ3 integrin during the implantation window in infertile patients with early stages of endometriosis

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Background: To study endometrial receptivity in terms of osteopontin (OPN) and αvβ3 integrin expression and co-expression in infertile women with early stages of endometriosis.

Methods: We investigated the immunohistochemical expression and co-expression of OPN and αvβ3 integrin in the endometrium of 20 infertile patients with Stage I or II endometriosis as the only detectable cause of infertility, 20 infertile patients with unexplained infertility and 20 fertile women undergoing tubal sterilization. Two endometrial biopsies were performed during a single menstrual cycle (postovulatory Day +7 to +8 and 4 days later) in each subject.

Results: No statistically significant differences regarding OPN and αvβ3 integrin expression were found between infertile patients with endometriosis and the two control groups. There was no significant correlation between OPN and αvβ3 integrin staining intensity in the mid-luteal phase biopsies in any of the groups studied.

Conclusions: Endometrial OPN and αvβ3 integrin expression or co-expression is not impaired during the window of implantation in patients with Stage I–II endometriosis. Further studies are needed to determine whether these results imply normal endometrial receptivity in such patients or add to the increasing uncertainty about the clinical value of assessing the endometrium with these markers of implantation.

Key words: endometrium / endometriosis / implantation / integrins / osteopontin

Introduction

A significant association between infertility and minimal-to-mild endometriosis has been reported in the literature (Trinder and Cahill, 2002). Numerous investigations have been performed to study fertility impairment in patients with endometriosis and different mechanisms have been proposed to explain why fertility is affected in these women: altered folliculogenesis, poor oocyte quality, reduced fertilization, abnormal embryogenesis and also decreased embryo implantation capacity (Gupta et al., 2008).

The reduced implantation capacity in endometriosis patients is difficult to explain because of a lack of understanding about the normal physiologic mechanisms of embryo implantation. Moreover, successful implantation depends both on a good quality embryo and a receptive endometrium and, at present, there is controversy as to whether reduced implantation in patients with endometriosis is due to altered oocyte/embryo quality or endometrial inadequacy (Garrido et al., 2002). Interestingly, a previous meta-analysis (Barnhart et al., 2002) suggested that the potential negative effect of endometriosis on IVF is not exclusively on the receptivity of the endometrium but also on the development of the oocyte and embryo.

One of the mechanisms proposed for impaired implantation in endometriosis patients involves the abnormal expression of cellular adhesion molecules in the eutopic endometrium. αvβ3 Integrin and its extracellular matrix ligand, osteopontin (OPN) are two of the best-characterized endometrial receptivity biomarkers. These two glycoproteins have been found to be co-ordinately expressed in the human endometrium throughout the menstrual cycle in normally...
cycling fertile women (Apparao et al., 2001). The maximal expression of these two molecules during the implantation window in human endometrial epithelial cells and secretion of OPN into the uterine cavity suggests a role of both factors in the regulation of endometrial function and embryo implantation (Apparao et al., 2001; Von Wolff et al., 2001). For this reason, the study of these two markers has been proposed as a means of distinguishing receptive from non-receptive endometrium in clinical practice and as a new method to investigate the impaired endometrial receptivity in certain groups of infertile patients (Lessey, 2002; Makker and Singh, 2006; Strowitzki et al., 2006).

Different authors (Lessey et al., 1994; Ordi et al., 2003; Odagiri et al., 2007; Cho et al., 2009; Wei et al., 2009) have studied the endometrial expression of αvβ3 integrin or OPN in endometriosis patients with controversial results. On the other hand, to our knowledge there are no previous controlled investigations on the simultaneous expression of these two endometrial receptivity markers in endometriosis patients. Therefore, the present study was aimed to investigate endometrial expression and co-expression of αvβ3 integrin and OPN in women with early stages of endometriosis.

**Materials and Methods**

**Patients and study cycle**

We investigated the expression of αvβ3 integrin and OPN in the endometrium of 20 infertile patients diagnosed by laparoscopy with Stage I or II endometriosis (American Fertility Society, 1985) as their sole detectable cause of infertility (END group). We included two control groups: 20 infertile patients with unexplained infertility (UNEX group) and 20 fertile women who were undergoing tubal sterilization and had no evidence of endometriosis (FERT group). This latter group were healthy women who had at least one child and had no history of infertility or miscarriage. Unexplained infertility patients had a normal infertility work-up including, in addition to endometrial biopsy, a semen analysis, mid-luteal serum progesterone and prolactin determination, an hysterosalpingogram and laparoscopy. According to the ESHRE Guidelines (1996), standard investigations aimed to evaluate infertility in a couple include laboratory assessment of ovulation, evaluation of tubal patency and semen analysis. Thus, unexplained infertility is a term applied to an infertile couple whose standard investigations (semen analysis, tubal patency and laboratory assessment of ovulation) yield normal results (ESHRE, 1996). However, for the specific purpose of this study couples were diagnosed as having unexplained infertility only once laparoscopy was performed and the presence of endometriosis excluded. The mean age of the women with endometriosis, unexplained infertility and those who were fertile was 31.5 ± 0.7, 32.1 ± 0.5 and 34.9 ± 0.9 years (mean ± SEM), respectively. All the women (both fertile and infertile) included in our study had regular menstrual cycles (27–32 days) and were taking no medication. A number of both infertile patients and controls had participated in previously published studies (Casals et al., 2008, 2010). The sample size was decided arbitrarily but in keeping with previous studies on the subject (Odagiri et al., 2007; Wei et al., 2009).

In all women, basal body temperature, luteal serum concentrations of estradiol and progesterone and endometrial biopsies were obtained in the same cycle to assess luteal function according to a previously reported (Casals et al., 2008) scheme of evaluation. Commencing on Days 8–10 of the study cycle (depending on the cycle length of the woman) patients underwent daily transvaginal ultrasonographic evaluation of follicular growth using a Toshiba Ecocceee SAA-340A/EF unit (Toshiba Co., Tokyo, Japan) equipped with a 5–7 MHz endo-vaginal probe (PVF-641VT). The maximum follicular diameter was measured in all patients. Both ovaries were identified and the largest diameter was measured in both the longitudinal and transverse dimensions in all follicles. The day of ovulation was designated as the day of maximum follicular enlargement, which was followed the next day by sudden disappearance or filling in of this follicle showing loss of clear demarcation of its walls and intrafollicular echoes (Shoupe et al., 1989; Peters et al., 1992). We used ultrasonographic monitoring of ovulation because previous studies have shown that the accuracy of histological endometrial dating is best determined when ovulation is detected by that method (Shoupe et al., 1989; Peters et al., 1992).

Two endometrial biopsies were performed during a single menstrual cycle in each subject. The chronological day of each patient was determined by counting forward from the ovulation day as detected by ultrasonographic scans. The early biopsy (mid-luteal) was performed on ovulation Day +7 to +8, whereas the second biopsy (late luteal) was always performed 4 days after the first biopsy. For the specific purpose of this study, endometrial evaluation was performed in all women as a part of a routine infertility work-up and always before laparoscopy. It should be stressed that a study by Daya (1996) on pregnancy loss showed that a relationship between miscarriage and endometriosis was only found in the patients studied before laparoscopy not after. The use of human tissue for research was based on informed consent and was approved by the Ethics Committee of our hospital. Hormones in serum were quantified on the same day as endometrial sampling. All samples were obtained in the fasting state between 08.00 and 10.00 h corresponding to the period of minimal progesterone variability in spontaneous menstrual cycles, thereby providing added accuracy to the measurement (Flicicori et al., 1984).

**Endometrial samples**

Endometrial samples were divided into two parts: one part was fixed in 10% formalin and embedded in paraffin and the second tissue sample was snap frozen in methylbutane (Merck, Darmstadt, Germany) immersed in liquid nitrogen and stored at −70°C until immunolabelling.

**Endometrial dating**

For endometrial dating, 4-µm sections stained with haematoxylin and eosin and periodic acid-Schiff stain were evaluated according to the histopathological criteria of Noyes et al. (1950). All endometrial biopsies were evaluated by the same experienced gynaecological pathologist (I.O.) who was blinded with regard to the ultrasonographically detected ovulatory day and the study group. Endometrial biopsy interpretation was performed using a single-day evaluation whenever possible and when the traditional 2-day spread evaluation method (i.e. Day 20–21) was provided, the latter day was used for comparison with immunohistochemical assays. An out-of-phase biopsy was defined as a lag of ≥3 days between the chronological and the histological day (Creus et al., 2002; Ordi et al., 2002).

**Immunohistochemistry**

Immunohistochemical studies were performed with the automated immunohistochemical system TechMate 500™ (Dako Co., Carpinteria, CA, USA), using the EnVision system (Dako) as previously reported (Creus et al., 2002; Casals et al., 2008, 2010). Integrin αvβ3 was detected in frozen tissue using a monoclonal antibody (clone LM609, dilution 1:200; Chemicon Int., Temecula CA, USA) and OPN in formalin-fixed, paraffin-embedded tissue, using a polyclonal antibody (Chemicon). Frozen sections (4 mm thick) were fixed for 10 min in acetone at 4°C and dried. Paraffin sections were deparaffinized and rehydrated in xylene and graded alcohols. Peroxidase was blocked for 7.5 min in
ChemMate peroxidase-blocking solution (Dako). Then the slides were incubated with the primary antibodies for 30 min and washed in ChemMate buffer solution (Dako). The peroxidase-labelled polymer was applied for 30 min. After washing in ChemMate buffer solution, the slides were incubated with the diaminobenzidine substrate chromogen solution, washed in water, counterstained with haematoxylin, washed, dehydrated and mounted. As previously reported by our group and others (Von Wolff et al., 2001; Creus et al., 2002), a negative control was performed in every case by omission of incubation with the primary specific antibody. The reactivity of each monoclonal antibody with endometrial glands and surface epithelium, stromal cells and vessels was assessed. The intensity of staining of the endometrial components was evaluated by a semi-quantitative scoring system (0–4) used in our previous publications (Creus et al., 2002; Ordi et al., 2002): absent (−), weak or focal (+), moderate (+++) and strong (+++; Fig. 1). As there is frequent expression of OPN and αvβ3 integrin in the endometrial stroma with minor variations, the intensity of the staining was evaluated as the intensity observed in the epithelial surface and glands where variations were evident. Endometrial samples were considered as expressing αvβ3 integrin and/or OPN when these glycoproteins were detected in both endometrial glands and luminal surface epithelium with the intensity of the reaction ranging from weak/focal to strong (Creus et al., 2002; Ordi et al., 2002; Casals et al., 2008, 2010). The whole biopsy was analysed in each sample for immunohistochemical expression. As some variation was observed in intensity between different areas in a single biopsy, each biopsy was scored as the highest intensity observed in at least 30% of the glands.

To further evaluate OPN and αvβ3 integrin expression in the endometrium, the H-Score method was also used as recommended by others (Lessey et al., 1995a,b). This immunohistochemical semi-quantitative method consists of a sum of the percentages of positively stained cells multiplied by a weighted intensity of staining: H-Score = ΣPi(i+1), where Pi is the percentage of stained cells in each intensity category (0–100%), and i is the intensity indicating weak (i = 1), moderate (i = 2) or strong staining (i = 3; Budwit-Novotny et al., 1986; Lessey et al., 1995b). A large study of endometrial adenocarcinoma have previously reported a low intraobserver (r = 0.983; P = 0.00001) and interobserver (r = 0.994; P = 0.00001) differences for H-Score method (Budwit-Novotny et al., 1986).

The correlation between the H-Score method and the scoring system used in our previous studies on the subject was also analysed in the current investigation.

**Hormone assays**

Hormones were measured using commercially available kits. Estradiol and progesterone concentrations in serum were estimated by a competitive chemiluminescent assay (ADVIA Centaur CP System; Siemens Healthcare Diagnostics, Tarrytown, NY, USA). The sensitivity was 10 pg/ml for estradiol and 0.15 ng/ml for progesterone and the inter-assay CVs were 5 and 5.4%, respectively.

**Figure 1** Immunohistochemistry of OPN in endometrial specimens: (A) no expression detected in epithelial cells; (B) focal immunostaining detected in the glandular epithelium; (C) moderate immunostaining and (D) strong immunostaining.
Statistical analysis

Data were analysed with SPSS statistical software (Release 15.0, SPSS, Inc., Chicago, IL). The χ² test and Kruskal–Wallis test were used as appropriate. The correlation between histological dating, ανβ3 integrin and OPN expression and between the H-Score method and our previously used scoring system was evaluated using the Spearman rank correlation coefficient. Results are expressed as means ± SEM. The level of significance was set at P ≤ 0.05.

Results

All menstrual cycles studied in the current investigation were ovulatory according to ultrasonographic criteria and mid-luteal serum progesterone > 10 ng/ml. The endometrial specimens were noted to be clearly progestational fundal samples in all instances. A late-luteal endometrial biopsy could not be done in four patients in the END group, in four in the UNEX group and in one in the FERT group because menses had commenced at the time of the second endometrial sampling. No inflammatory or reactive change related to the first sampling was detected in the second biopsy in any patient.

Tables I and II summarize the data related to endometrial histology and ανβ3 integrin and OPN expression in the mid-luteal and late-luteal phase endometrial biopsies carried out in the END, UNEX and FERT groups, as well as mid-luteal and late-luteal serum hormone concentrations. No statistically significant differences were found between the three groups studied with respect to histology, expression of endometrial markers evaluated with both staining intensity evaluation methods or hormonal parameters in either the mid- or the late-luteal phase. Histological dating, ανβ3 integrin expression and OPN expression in endometrial samples in the three groups are presented in Fig. 2 (mid-luteal biopsy) and Fig. 3 (late-luteal biopsy).

When the whole group of endometrial samples included in this study were considered, the H-Score method and our previously reported scoring system were highly correlated in evaluating the immunohistochemical staining intensity: r = 0.96 for integrin and r = 0.91 for OPN in the mid-luteal phase, r = 0.71 for integrin and r = 0.84 for OPN in the late-luteal phase (P < 0.0001 in all cases). This provides support to our previous studies.

Endometrial co-expression of ανβ3 integrin and OPN during the implantation window is shown in Table I. The simultaneous presence or absence of both markers was observed in only 60% (36/60) of mid-luteal biopsies with no differences between the three groups studied. There was no significant correlation between ανβ3 integrin and OPN staining intensity in the mid-luteal phase biopsies in any of the groups studied (Fig. 4).

Discussion

OPN is related to cell adhesion and inflammation and, therefore, this molecule and its ligand ανβ3 integrin may be involved in the genesis of endometriosis. On the other hand, OPN has been found to be consistently up-regulated in the endometrium during the window of implantation in different studies of the transcriptome (Kao et al.,

| Table I | Endometrial biopsy and epithelial quantitative and qualitative ανβ3 integrin and OPN expression and their coexpression in the three groups studied in the mid-luteal phase. |
|-----------------|---------------------------------|---------------------------------|---------------------------------|-----------------|
| Parameter | Endometriosis (n = 20) | Unexplained infertility (n = 20) | Fertile controls (n = 20) | P value |
| Endometrial biopsy | | | | |
| Chronological dating | 7.3 ± 0.2 | 7.3 ± 0.1 | 7.5 ± 0.1 | NS |
| Histological dating | 5.6 ± 0.4 | 5.4 ± 0.4 | 5.6 ± 0.4 | NS |
| In-phase endometria | 14 (70.0) | 11 (55.0) | 12 (60.0) | NS |
| ανβ3 integrin expression | | | | |
| Positive samples | 10 (50.0) | 8 (40.0) | 7 (35.0) | NS |
| Mean staining score | 0.7 ± 0.2 | 0.5 ± 0.2 | 0.6 ± 0.2 | NS |
| H-Score | 0.5 ± 0.1 | 0.4 ± 0.2 | 0.6 ± 0.2 | NS |
| OPN expression | | | | |
| Positive samples | 14 (70.0) | 13 (65.0) | 14 (70.0) | NS |
| Mean staining score | 1.3 ± 0.2 | 1.3 ± 0.2 | 1.1 ± 0.2 | NS |
| H-Score | 1.1 ± 0.2 | 0.9 ± 0.2 | 0.8 ± 0.2 | NS |
| OPN/ανβ3 coexpression | | | | |
| OPN (−)/ανβ3 (−) | 4 (20.0) | 6 (30.0) | 5 (25.0) | NS |
| OPN (+)/ανβ3 (−) | 6 (30.0) | 6 (30.0) | 8 (40.0) | NS |
| OPN (−)/ανβ3 (+) | 2 (10.0) | 1 (5.0) | 1 (5.0) | NS |
| OPN (+)/ανβ3 (+) | 8 (40.0) | 7 (35.0) | 6 (30.0) | NS |
| Hormone concentrations | | | | |
| Estradiol (pg/ml) | 153.2 ± 13.9 | 143.3 ± 14.5 | 139.3 ± 12.9 | NS |
| Progesterone (pg/ml) | 20.2 ± 2.8 | 19.2 ± 1.6 | 17.8 ± 1.6 | NS |

Values are expressed as mean ± SEM or n (%).
Table II  Endometrial biopsy and epithelial quantitative and qualitative αvβ3 integrin and OPN expression and their coexpression in the three groups studied in the late-luteal phase.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Endometriosis (n = 16)</th>
<th>Unexplained infertility (n = 16)</th>
<th>Fertile controls (n = 19)</th>
<th>P value</th>
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<tr>
<td>Endometrial biopsy</td>
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<tr>
<td>Chronological dating</td>
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<td>11.3 ± 0.1</td>
<td>11.5 ± 0.1</td>
<td>NS</td>
</tr>
<tr>
<td>Histological dating</td>
<td>11.3 ± 0.2</td>
<td>11.2 ± 0.2</td>
<td>11.7 ± 0.2</td>
<td>NS</td>
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<tr>
<td>In-phase endometria</td>
<td>16 (100)</td>
<td>16 (100)</td>
<td>19 (100)</td>
<td>NS</td>
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<tr>
<td>αvβ3 integrin expression</td>
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<tr>
<td>Positive samples</td>
<td>16 (100)</td>
<td>16 (100)</td>
<td>18 (94.7)</td>
<td>NS</td>
</tr>
<tr>
<td>Mean staining score</td>
<td>2.6 ± 0.2</td>
<td>2.9 ± 0.1</td>
<td>2.6 ± 0.2</td>
<td>NS</td>
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<tr>
<td>H-Score</td>
<td>3.2 ± 0.2</td>
<td>3.4 ± 0.2</td>
<td>3.2 ± 0.3</td>
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<td>OPN expression</td>
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<tr>
<td>Positive samples</td>
<td>16 (100)</td>
<td>16 (100)</td>
<td>19 (100)</td>
<td>NS</td>
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<tr>
<td>Mean staining score</td>
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<td>2.1 ± 0.1</td>
<td>2.3 ± 0.2</td>
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<tr>
<td>H-Score</td>
<td>2.4 ± 0.2</td>
<td>2.2 ± 0.1</td>
<td>2.6 ± 0.2</td>
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<td>OPN/αvβ3 coexpression</td>
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<td>NS</td>
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<tr>
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<tr>
<td>Hormone concentrations</td>
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<tr>
<td>Estradiol (pg/ml)</td>
<td>122.2 ± 11.1</td>
<td>125.9 ± 15.6</td>
<td>93.0 ± 13.7</td>
<td>NS</td>
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<tr>
<td>Progesterone (pg/ml)</td>
<td>10.9 ± 1.2</td>
<td>11.6 ± 1.0</td>
<td>8.8 ± 1.2</td>
<td>NS</td>
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Values are expressed as mean ± SEM or n (%).

Figure 2  Box-and-whisker plot showing histological dating and αvβ3 integrin and OPN expression in patients with endometriosis, unexplained infertility and fertile women in the mid-luteal phase. Each box represents the middle 50% of the data (25–75% range). The central horizontal line represents the median. Vertical lines represent the 10–90% range of data, as indicated by the small horizontal lines. No significant differences were found among the three groups studied.

Figure 3  Box-and-whisker plot showing histological dating and αvβ3 integrin and OPN expression in patients with endometriosis, unexplained infertility and fertile women in the late-luteal phase. Each box represents the middle 50% of the data (25–75% range). The central horizontal line represents the median. Vertical lines represent the 10–90% range of data, as indicated by the small horizontal lines. No significant differences were found among the three groups studied.

For these reasons, in recent years OPN and its ligand αvβ3 integrin have been intensively studied in reproductive medicine in general (Quenby et al., 2007; Franchi et al., 2008; DuQuesnay et al., 2009) and in endometriosis in particular (Odagiri et al., 2007; Cho et al., 2009; Wei et al., 2009).
Endometrial expression of OPN has been studied by immunohistochemistry in a rat endometriosis model and in human endometriosis patients and controls (Odagiri et al., 2007). This study showed that, in humans, the staining pattern of OPN in endometriotic lesions was similar to that in eutopic endometrium in the secretory phase of control specimens. Other investigators demonstrated that OPN mRNA expression in eutopic endometrium was significantly increased in patients with endometriosis compared with that in controls (Cho et al., 2009). In contrast with these findings, a recent immunohistochemical study revealed decreased OPN expression in the late secretory phase endometrium in patients with endometriosis. However, OPN expression was similar in endometriosis and control groups in the mid-secretory phase (Wei et al., 2009). On the other hand, an earlier report on patients with endometriosis indicated that αvβ3 integrin expression appeared to be reduced while OPN expression remained unaffected. This author suggested reduced OPN binding to the surface epithelium, but only in cases where αvβ3 integrin was decreased (Lessey, 2002). Finally, our immunohistochemical analysis of OPN and its ligand αvβ3 integrin did not show different expression of these two markers between eutopic endometrial specimens of endometriosis patients and control groups without endometriosis, either when these two markers were studied alone or in combination. Discrepancies between different studies could be explained by the following facts.

First, discordant results are often obtained in endometriosis investigations because of the heterogeneity of this disorder, with patients with different stages of endometriosis often being included in the same study group (Gupta et al., 2008). To avoid this circumstance, the inclusion of patients in the current study was restricted to patients with early stages of endometriosis. Second, it has been stressed that appropriate controls without the disease are required to study endometrial receptivity in endometriosis (Garrido et al., 2002). This was done in our study which included fertile women without endometriosis as well as a second control group of patients with unexplained infertility. On the contrary, patients with pathologies such as cervical carcinoma in situ (Odagiri et al., 2007), myomas or benign ovarian cysts (Cho et al., 2009) have been included as controls in some cases, although the authors stressed this limitation in their studies. Third, endometrial samples obtained at different times during the menstrual cycle have been included in the same study group in some investigations (Odagiri et al., 2007; Cho et al., 2009), but we and others have previously demonstrated the cyclic changes in the endometrial expression of integrins and OPN along the menstrual cycle (Lessey et al., 1992; Creus et al., 1998; Apparao et al., 2001; Von Wolff et al., 2001; Casals et al., 2008). Finally, the analysis of mRNA or protein could produce discrepant results because all the mRNA expressed may not always be translated into protein (Cho et al., 2009).

Although immunohistochemical studies have low sensitivity to detect minor variations and semi-quantitative scoring systems used to evaluate these studies have some limitations, they have been extensively used not only in reproductive medicine studies but also in many other areas. On the other hand, both intra- and inter-observer validation may be necessary in studies using immunohistochemical

Figure 4 Correlation between staining intensity for OPN and αvβ3 integrin expression in epithelial cells of mid-luteal endometrial biopsies in patients with endometriosis, unexplained infertility and fertile women.
evaluation of the endometrial markers investigated. In this respect, it is to note that the H-Score used in this investigation has been reported as having low intra-observer and inter-observer differences (Budwe-Novotny et al., 1986).

In addition, as recently stressed, the simplest and most efficient way to study gene expression is the identification of specific proteins with antibodies applied in capture assays, flow cytometry, immunocytochemistry and immunohistochemistry (Uhlen and Ponten, 2005; Serrafin et al., 2009), as mRNA recomposition, modification and processing alters functional protein production (Azad et al., 2006). Immunohistochemistry enables the pathologist to extract additional information from fixed, deparaffinized specimens and to provide data critical to optimal clinical management of the patient. There is currently a wealth of applications of this technique to gynaecologic pathology, in lesions that include neoplastic and non-neoplastic conditions (Yaziji and Gown, 2001). Immunohistochemistry, as used in this study, allows the analysis of protein expression, providing information on the histological and subcellular distribution of the protein (i.e. glandular epithelium or stroma) and is particularly appropriate for clinically based studies. In fact, as previously stressed (Pei et al., 2007), immunohistochemistry staining is necessary to access the clinicopathological characteristics of proteins identified by proteomic and genomic analysis.

It could be argued that repetitive endometrial biopsies may have an impact on subsequent endometrial findings. Thus, recent data suggest that injury to the endometrium causes biological events that appear to return the endometrium to a more normal and fertile state (Almeg et al., 2010). In addition, an early paper by Castelbaum et al. (1994) using two biopsies in the same month also showed correction of a histologic delay, possibly illustrating catch-up, following an initial out of phase biopsy. Although a mechanical effect of the first biopsy in inducing endometrial differentiation in the second biopsy cannot be completely excluded, it is unlikely. The following facts support this contention. First, we and others (Castelbaum et al., 1994; Creus et al., 1998; Acosta et al., 2000; Ordi et al., 2002, 2003) have found no inflammatory or reactive changes consistent with a previous biopsy site when performing two endometrial biopsies during a single menstrual cycle for luteal phase evaluation. Second, we have previously reported (Creus et al., 1998; Ordi et al., 2002, 2003; Casals et al., 2008) that normal or aberrant integrin expression is not associated with specific aetiologies of infertility, mainly endometriosis and unexplained infertility. Third, we have also shown that the expression of the OPN-αvβ3 integrin complex is closely correlated with histological maturation of the endometrium evaluated by histological dating, but neither OPN nor αvβ3 alone nor in combination are useful markers of endometrial functional receptivity (Casals et al., 2008, 2010). In fact, neither mid-luteal histological evaluation nor αvβ3 integrin expression in mid- or late-luteal endometrial biopsy specimens correlated with outcome for subsequently untreated infertile women (Ordi et al., 2002). Finally, the normal pattern of pino-pode expression, one of the most cited markers postulated to frame the window of implantation, has been established on the basis of sequential endometrial biopsies performed in normal menstruating women (Nikas, 1999a,b).

It could be claimed that if biomarkers like integrins and OPN are evidence of a biochemical defect, only a subset of infertile or endometriosis patients would appear to be affected. Given the high rate of out-of-phase endometrium in both fertile and infertile women (Coutifaris et al., 2004; Murray et al., 2004), inclusion of these samples would tend to dilute the true negative biopsy (those that were in phase). For this reason Lessey et al. (1994) studied αvβ3 integrin expression in endometriosis patients including only in phase biopsies. However, no differences were detected in integrin and/or OPN expression between the three groups of patients in our study irrespective of considering in phase mid-luteal biopsies alone, out-of-phase mid-luteal biopsies alone, in phase late-luteal biopsies alone, out-of-phase late-luteal biopsies alone (data not shown) or the whole group of histological samples investigated.

Studies such as ours, which showed no impairment of potential markers of endometrial receptivity, could be in line with different authors suggesting that infertility in endometriosis patients is not related to an inadequate endometrial environment affecting endometrial receptivity but is due to decreased oocyte quality (Pellicer et al., 1994; Sung et al., 1997). The oocyte donation model has been used for this purpose in several studies. A large retrospective analysis compared reproductive outcomes in oocyte recipients with and without endometriosis and demonstrated no adverse effects of this disease on implantation rates, even when recipients were subdivided by stage of endometriosis (Sung et al., 1997). Other authors prospectively compared oocyte donors with endometriosis with recipients who had endometriosis and found reduced pregnancy and implantation rates when the oocytes came from donors with endometriosis but normal rates when only the recipients had endometriosis (Pellicer et al., 1994). The same group confirmed these findings in recipients with Stage III–IV endometriosis (Diaz et al., 2000).

According to our results, it may be concluded that there is no impairment in endometrial receptivity markers in patients with mild stages of endometriosis if OPN and αvβ3 integrin are proved to be accurate markers of uterine receptivity. However, we and others have reported data providing uncertainty about the value of integrins and OPN in assessing endometrial receptivity in the clinical setting (Creus et al., 2002, 2003; Ordi et al., 2002; Thomas et al., 2003; Casals et al., 2008, 2010). On the other hand, the number of patients in our study is relatively low, and thus a type II error cannot be excluded.

In conclusion, the results of the present study show that OPN and αvβ3 integrin expression or co-expression during the window of implantation are not impaired in patients with Stage I–II endometriosis. Whether these results imply normal endometrial receptivity markers in such patients or add to the increasing uncertainty about the clinical value of assessing the endometrium with these markers of implantation remains to be shown.

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Authors’ roles

G.C., J.O. and J.B. conceived and designed the study; G.C., M.C. and F.F. contributed to patient recruitment, ultrasonographic monitoring of ovulation and endometrial biopsies; J.O. performed the histopathological and immunohistochemical analysis; R.C. performed the
References


