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Endometrium & Endometriosis

Endometrial Pinopode and $\alpha v\beta 3$ Integrin Expression Is Not Impaired in Infertile Patients with Endometriosis

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***Purpose*:** To investigate endometrial receptivity in terms of pinopode formation and $\alpha v\beta 3$ integrin expression in infertile women with endometriosis during natural cycles.

***Methods*:** We investigated the expression of $\alpha v\beta 3$ integrin and pinopode formation in the endometrium of 12 infertile patients with stage I or II endometriosis as the only cause of infertility, 12 infertile patients having unexplained infertility, and 12 fertile women who were undergoing tubal sterilization. Two endometrial biopsies (postovulatory day +7 to +8 and 4 days later) were performed during a single menstrual cycle in each subject.

***Results*:** No statistically significant difference regarding $\alpha v\beta 3$ integrin expression and pinopode formation was found between infertile patients with endometriosis and the two control groups.

***Conclusion*:** $\alpha v\beta 3$ integrin expression and pinopode formation are not reduced during the window of implantation in patients with stage I–II endometriosis. 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 those markers of implantation, warrants further studies.

KEY WORDS: Endometrial receptivity; endometriosis; implantation; integrins; pinopodes.

INTRODUCTION

A significant association between minimal to mild endometriosis and infertility is shown by prevalence studies (1). However, the exact mechanisms by which endometriosis affects fertility is unknown (2). Virtually every step in reproduction has been investigated and purported to be impaired in the presence of endometriosis. In vitro fertilization (IVF) provides an opportunity to study the impact of endometriosis on the critical steps that are involved in reproduction. Thus, a recent meta-analysis investigating the IVF outcome for patients with endometriosis-associated infertility concluded that such patients respond with significant decreased levels of all markers of reproductive process, resulting in implantation and pregnancy rates that are almost one half those of women with other indications for IVF (3).

Successful embryonic implantation is dependent upon both a good quality embryo and a receptive endometrium. At present, controversy exists as to whether reduced implantation in patients with endometriosis is due to hampered oocyte/embryo

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quality or endometrial inadequacy (4,5). Investigation of endometrial function has been traditionally made by dating pre-menstrual endometrial biopsy according to the morphological criteria reported 50 years ago by Noyes *et al.* (6). Over the past decade, however, the relationship between histological changes and endometrial receptivity has been seriously questioned (7–11). Recently, midluteal endometrial evaluation of the so-called markers of implantation has been proposed as a means of distinguishing receptive endometrium from nonreceptive in clinical practice, thus offering new directions for a better understanding of occult causes of infertility in women. In this regard, $\alpha v\beta 3$ integrin expression and pinopode formation are the two most cited markers postulated to frame the window of implantation (12,13).

Integrins have been proposed to be sensitive indicators of endometrial receptive status and specifically $\alpha v\beta 3$ integrin expression has been reported to be reduced in women with endometriosis; thus, that integrin has been proposed as a useful marker of the disease (14,15). However, other authors did not confirm these findings (16–18). On the other hand, according to a recent report (19) endometrial receptivity in terms of pinopode expression is not impaired in women with endometriosis undergoing oocyte donation under hormone replacement therapy. However, it has been recently stressed that no data exist in the literature regarding pinopode expression in women with endometriosis during natural cycles (5,19). Therefore, on the above evidence, this study was undertaken to investigate both $\alpha v\beta 3$ integrin expression and pinopode formation in women with early stages of endometriosis. Thus, a feature of the present study is that we investigated both markers in the same endometrial sample during natural cycles.

MATERIAL AND METHODS

Patients and Study Cycle

We investigated the expression of $\alpha v\beta 3$ integrin and pinopode formation in the endometrium of 12 infertile patients undergoing a routine workup and being diagnosed by laparoscopy as having stage I or II endometriosis (20) as their sole cause of infertility (Group END). Twelve infertile patients having unexplained infertility (Group UNEX) and 12 fertile women who were undergoing tubal sterilization and had no evidence of endometriosis (Group FERT) were used as control groups. Unexplained infertility was defined as a normal infertility workup including, in addition to endometrial biopsy, a semen analysis, a midluteal serum progesterone and prolactin determination, a postcoital test, a hysterosalpingogram, and laparoscopy. The use of human tissue for research was based on informed consent and was approved by the Ethics Committee of our hospital. The mean age of endometriosis and unexplained infertility patients was 32.3 ± 1.5 and 30.8 ± 1.2 (mean $\pm$ SEM) years, respectively. All of them had regular menstrual patterns every 27–32 days. Healthy control women had a mean parity of 1.4 (range 1–4) and were aged 29–41 years (mean age 33.8 ± 1.1). These control women had regular menstrual cycles (27–32 days) and were taking no medication.

In all women, basal body temperature, luteal serum concentrations of estradiol and progesterone, and endometrial biopsies were used in the same cycle to assess luteal function according to a scheme of evaluation previously reported (17,21). Commencing on days 8–10 of the study cycle (depending on the cycle length of the woman) patients underwent daily transvaginal ultrasonographic evaluation of the follicular growth using a 5 MHz vaginal transducer attached to an Aloka scanner (Model SSD-620; Aloka Co. Ltd, Tokyo, Japan). The maximun 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 (22,23). 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 (22,23).

Two endometrial biopsies were performed during a single menstrual cycle in each subject. The patient's chronological day was determined by counting forward from the ovulation day as detected by ultrasonographic scans. The early biopsy (midluteal) was performed on ovulation day +7 to +8 whereas the second biopsy (late luteal) was always performed 4 days after the first biopsy.

Hormones in serum were quantified on the same days as endometrial sampling. All samples were obtained in the fasted state between 08.00 and 10.00 h which corresponded to the period of minimal progesterone variability in spontaneous menstrual cycles, and added to the accuracy of the measurement (24).

Endometrial Samples

Biopsies were taken from the uterine fundus using the Pipelle (Laboratoire CCD, Paris, France). Endometrial samples were divided in three parts. One of them was fixed in 10% formalin and embedded in paraffin for light microscopy. The second portion of the tissue was snap frozen on methylbutane (Merck, Darmstadt, Germany) immersed in liquid nitrogen and stored at -70°C until immunolabelling for integrin determination. The remaining portion was fixed in glutaraldehyde for scanning electron microscopy investigation. The use of separate endometrial portions for light microscopy study and scanning electron microscopy investigation was necessary considering a recent study (25) concluding that scanning electron microscopy but not light microscopy remains the only conclusive tool for the evaluation of the stage of pinopode formation. One observer, gynecological pathologist (J.O.), who was blinded to the identity of the slides as well as with regard to the ultrasonographically detected ovulatory day, performed all the assessments.

Endometrial Dating

For endometrial dating 4 μm sections stained with hematoxylin and eosin and PAS stain were evaluated. All endometrial biopsies were evaluated according to the histopathological criteria of Noyes *et al.* (6) using a single-day evaluation whenever possible and when the traditional 2-day spread evaluation method (i.e., day 20–21) was provided, the later day was used for comparison to immunohistochemical assays. An out-of-phase biopsy was defined as $\geq$ 3 day lag between the chronological and the histological day.

Immunohistochemistry

$\alpha v\beta 3$ integrin was detected in frozen sections using the EnVision system (Dako Co., Carpinteria, CA, USA) as previously reported (17,21,26). Briefly, 4 μm sections were fixed 10 min in acetone at 4°C and dried. After washing in PBS for 5 min the peroxidase was blocked for 5 min in 0.03% H_2O_2 containing sodium azide. Then the slides were incubated with the primary antibody for 40 min and washed in TBS (Dako). The monoclonal antibody LM609 (Chemicon, Temecula, CA, USA, dilution 1:200), which recognizes the complete $\alpha v\beta 3$ heterodimer (27) and is being widely applied by us (17,21,28,29) and others (14,30–32) was used. The peroxidase labelled polymer was then applied for 40 min. After washing in TBS, the slides were incubated with the diaminobenzidine substrate chromogen solution, washed in distilled water, counterstained with hematoxylin, and washed, dehydrated, and mounted. In every case a negative control was performed by omission of incubation with the primary specific antibody. As $\alpha v\beta 3$ is consistently expressed in vascular endothelia, positive staining of endometrial vessels was considered as internal positive control (27).

The reactivity in the endometrial glands epithelium and luminal surface epithelium of the endometrium, stromal cells, and vessels was assessed. The intensity of staining of the endometrial components was evaluated by a semiquantitative scoring system (0–3) as follows (17,21,27,28): absent (0), weak or focal (+), moderate (++), and strong (+++) (Fig. 1). As in previous work it was found that the expression of $\alpha v\beta 3$ in the luminal surface epithelium starts abruptly on day 19–20 of the cycle, thus opening the window of implantation, and only staining in the glands seems to be clinically relevant (9,33,34); for the specific purpose of this study, endometrial samples were considered as expressing $\alpha v\beta 3$ integrin when this integrin was detected in endometrial glands and luminal surface epithelium with any intensity of the reaction ranging from weak/focal to strong.

Scanning Electron Microscopy

As previously reported (21), endometrial tissue was fixed for at least 24 h in phosphate buffered (0.1 mol/L, pH 7.4) 2.5% glutaraldehyde and postfixed for 1 h in 1% osmium tetroxide. The samples were dehydrated in a graded series of ethanol, critical point dried with a Polaron CPD 7501 system (VG Microtech, U.K.), and mounted and coated with gold in a Bio-Rad SC510 sputter coater (VG Microtech, U.K.). All samples were observed under the same KV and electron beam current conditions in a Zeiss DSM940A scanning electron microscope (Carl Zeiss, Oberkochen, Germany). For each biopsy three to nine fragments 2 mm each were evaluated and at least 4 mm^2 of well-preserved epithelial luminal surface was required to be available for evaluation. A thorough examination of the complete surface was conducted. Digital micrographs were taken with the computer program Quartz PCI (Quartz Imaging Co., Vancouver, BC, Canada), and were evaluated independently by two observers. As previously reported by others and ourselves (13,21,34), pinopodes were defined as spherical protrusions without microvilli on the apical surface of

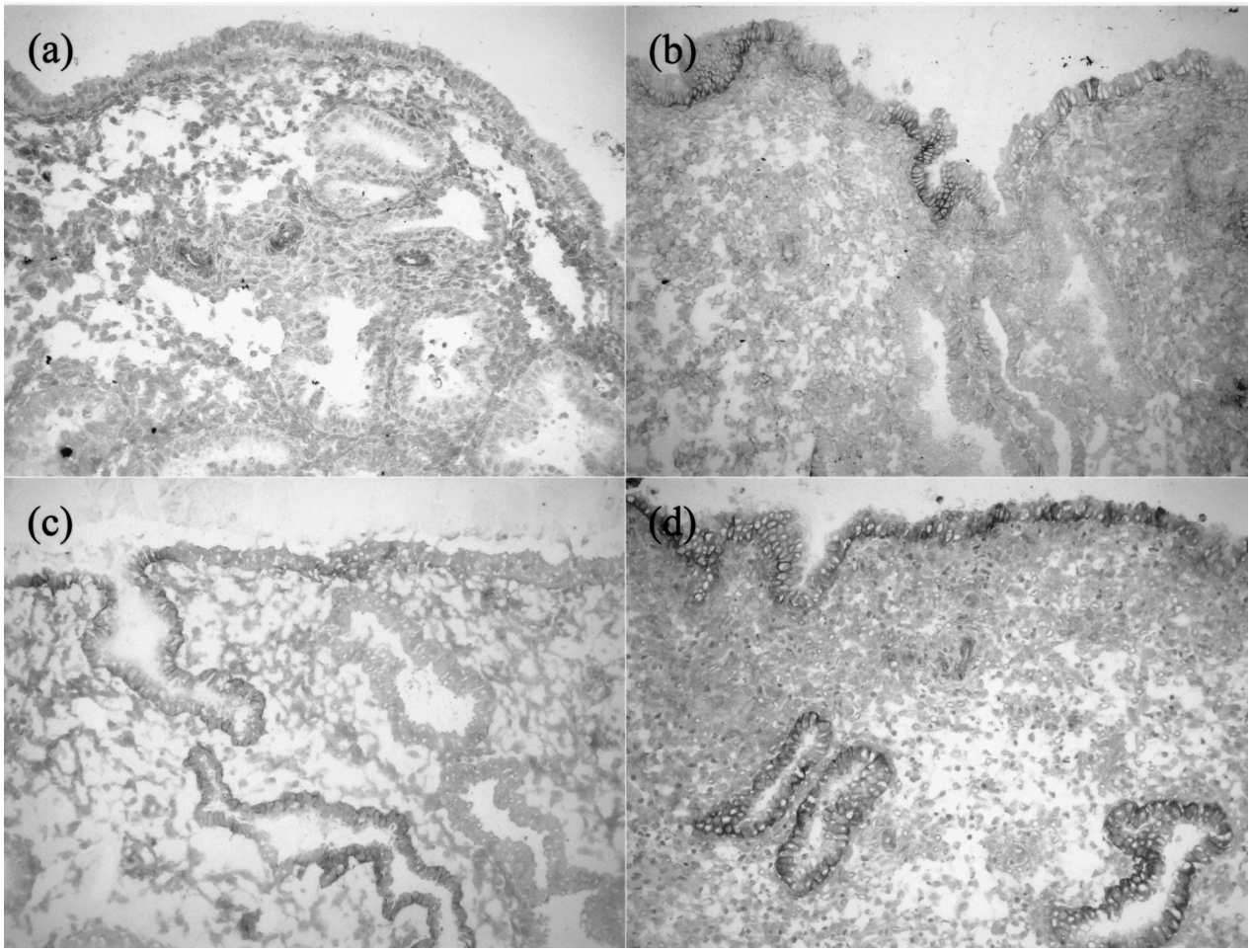

Fig. 1. Immunohistochemistry of $\alpha v\beta 3$ integrin in endometrial specimens. (a) score 0: no expression detected in epithelial cells. (b) score +: focal immunostaining detected in both the surface and the glandular epithelium. (c) score ++: moderate immunostaining. (d) score + + +: strong immunostaining.

the luminal uterine endometrium and were semiquantitatively evaluated as absent (0), isolated pinopodes (+), small groups of pinopodes (++), and confluent pinopodes (+ + +) (Fig. 2).

Hormone Assays

Hormones in serum were measured using commercially available kits as previously reported (Creus *et al.*, 2002). Estradiol was measured by a competitive immunoenzymatic assay (Immuno 1, Bayer, Tarrytown, NY, USA). The sensitivity of the assay was 10 pg/mL and the interassay coefficient of variation 5%. Progesterone was determined by a competitive chemiluminiscent immunoassay (Immulite, DPC, Los Angeles, CA, USA). The sensitivity of the method was 0.2 ng/mL and the interassay coefficient of variation was 6.7%. Blood was allowed to clot, and serum was separated and stored at -20°C until assayed. Samples from each subject were analyzed in a single assay.

Statistics

Data were analyzed by SPSS statistical software (Release 10.0, SPSS Inc., Chicago, IL). The Mann–Whitney *U*-test and Wilcoxon matched-pairs signed-ranks test were used as appropriate. Results are expressed as means ± SEM. The level of significance was set at $P \leq 0.05$.

RESULTS

All menstrual cycles included in the present investigation were ovulatory according to ultrasonographic

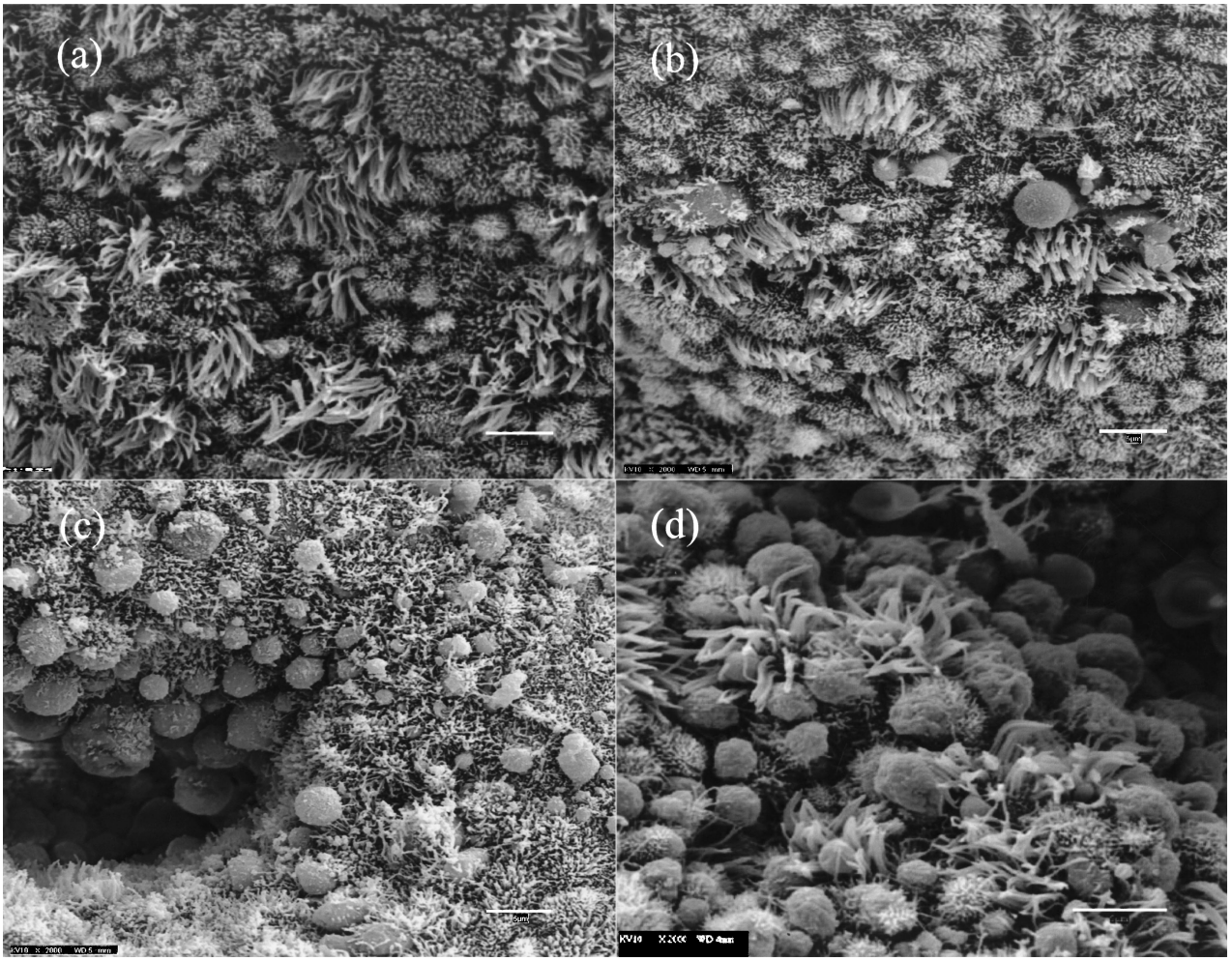

Fig. 2. Scanning electron microscopy showing the apical surface of the luminal uterine epithelium in endometrial samples. (a) score 0: only ciliated and microvillous cells are seen. (b) score +: few isolated pinopodes with smooth surface area. (c) score ++: moderate numbers of well-developed pinopodes surrounding the opening of an endometrial gland. (d) score + + +: fully developed pinopodes covering the surface.

criteria and midluteal serum progesterone concentration > 10 ng/mL. A late luteal endometrial biopsy could not be carried out in 2 and 1 patients in groups END and UNEX, respectively, as well as in 1 of the fertile controls because menses had commenced at the time of the second endometrial sampling. In all instances the endometrial specimens were noted to be clearly progestational fundal samples. No inflammatory or reactive change related to the first sampling was detected in any late luteal biopsy.

Histological dating, $\alpha v\beta 3$ integrin expression, and pinopode formation in midluteal endometrium specimens in the three groups studied are presented in Fig. 3. No differences were found between group END and groups UNEX and FER with respect to the three parameters of endometrial morphology and function investigated. Midluteal hormonal levels are presented in Table I. Ovarian steroid hormones were similar in the three groups studied.

No significant differences were found between the three groups of patients investigated with respect to the expression of endometrial markers in the late luteal phase biopsy (Fig. 4). Hormones were also similar in the three groups of patients (Table II).

No differences either in midluteal or late luteal serum concentrations of estradiol and progesterone were detected among groups when estratified by the expression or not of $\alpha v\beta 3$ integrin, and by the presence or absence of pinopodes (data not shown).

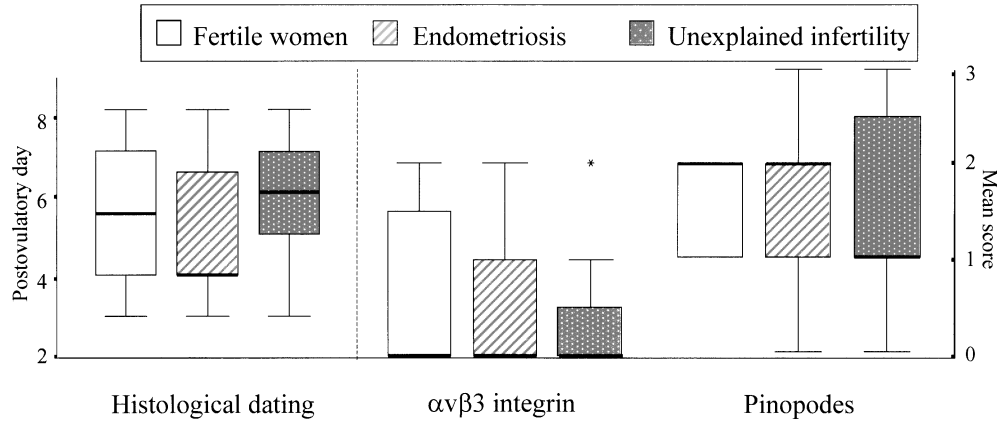

Fig. 3. Box-and-whisker plot showing histological dating, $\alpha v\beta 3$ integrin expression, and pinopode formation in patients with endometriosis-associated infertility, unexplained infertility, and fertile women in the midluteal phase. Each box represents the middle 50% of the data (25–75% range). The central horizontal line represents the median. Vertical lines represent the 10–90% range of data, as indicated by the small horizontal lines. Observed points more extreme than these values, if any, are individually plotted (*).

DISCUSSION

The human endometrium undergoes changes that are vital if implantation is to take place. Implantation is an extremely coordinated event requiring the presence of developing embryos with the ability to induce the appropriate changes in the endometrial mucosa, together with the presence of an endometrium able to receive these signals and to act in consequence (35). Therefore, any endometrium unable to answer properly, without the correct timing in the functional changes, would be adversely affecting the reproductive process (5).

It has been recently stressed that, to address this matter in endometriosis, the suitable design of any study should compare eutopic endometrium of women with endometriosis with appropriate controls without the disease (5). This was done in the current study where fertile women were used. On the other hand, it has been suggested that a hostile endometrial environment in women with endometriosis could not be related to the endometriosis itself, but to the infertility associated with the disease (4). Thus, we used a second control group of patients having unexplained infertility. In contrast, the vast majority of studies regarding endometrium and endometriosis are focused on the differences between eutopic and ectopic endometrium in such patients and are hence concerned with molecules presumably implicated in the origin of disease (5). They are mainly focused on molecules related to the attachment and growth of the ectopic tissue, but some efforts have been dedicated to molecules previously related to implantation and infertility (5).

Table I. Hormonal Levels in the Three Groups Studied in the Midluteal Phase

	Estradiol (pg/mL)[a]	Progesterone (ng/mL)[a]
Endometriosis ($n = 12$)	150.1 ± 16.7	16.7 ± 2.0
Unexplained infertility ($n = 12$)	129.1 ± 11.8	17.9 ± 1.9
Fertile women ($n = 12$)	137.5 ± 18.3	16.7 ± 2.0

Note. Values are mean ± SEM.
[a] No significant differences between groups studied.

It has been suggested that eutopic endometrium of women with endometriosis behaves different from the endometrium of women without the disease and this would explain reduced implantation in endometriosis patients (4). This is not supported by the present report where there was no significant difference in $\alpha v\beta 3$ integrin expression or pinopode formation, the two most cited markers framing the window of implantation, between patients having endometriosis-associated infertility, unexplained infertility, and fertile controls. There are three possible explanations to our findings.

First, if integrins and pinopodes are good markers of uterine receptivity, then it could be concluded that there is no difference in endometrial receptivity between the three groups studied, and this would be clinicallly valuable. However, we and others have reported data providing uncertainty about the value of integrins and pinopodes in

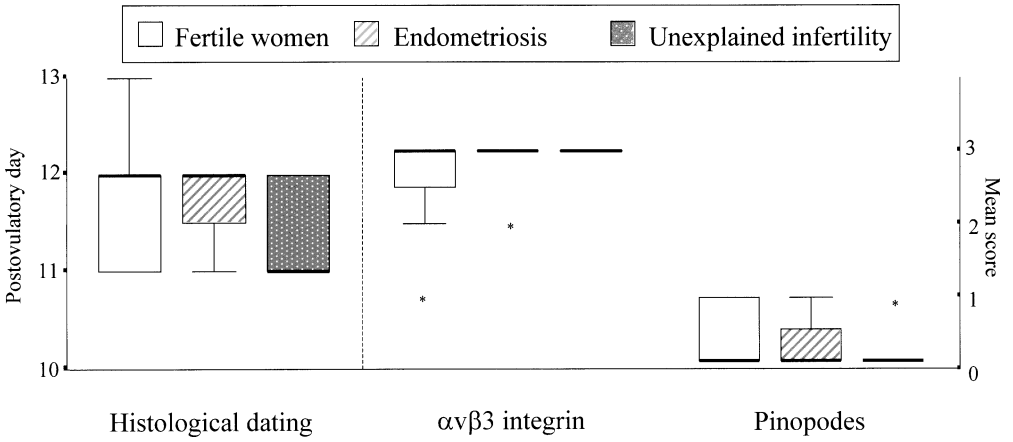

Fig. 4. Box-and-whisker plot showing histological dating, $\alpha v\beta 3$ integrin expression, and pinopode formation in patients with endometriosis-associated infertility, unexplained infertility, and fertile women in the late luteal phase. Each box represents the middle 50% of the data (25–75% range). The central horizontal line represents the median. Vertical lines represent the 10–90% range of data, as indicated by the small horizontal lines. Observed points more extreme than these values, if any, are individually plotted (*).

assessing endometrial receptivity in the clinical setting (17,21,26–28,36).

Second, an alternative explanation is simply that implantation rates in patients with endometriosis are decreased because oocyte/embryo quality is impaired (2). In order to investigate this possibility, several studies have been performed on oocyte donor cycles. Thus, in 1994 Simón *et al.* (37) compared oocyte donors who had 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. In 2000, the same group confirmed this finding in recipients with stage III–IV endometriosis (38). These studies complemented a large retrospective analysis of 239 oocyte recipients (39), which demonstrated no adverse effects on implantation rates, even when recipients were subdivided by stage of endometriosis. From these data one may conclude that endometriosis does not affect implantation rates in oocyte recipients pretreated with gonadotropin-releasing hormone (GnRH) agonists. It remains to be settled whether this is because endometriosis does not significantly impair the endometrial environment, or because this impairment is overcome by good quality oocytes or pretreatment of recipients with GnRH agonists (2).

Finally, the association between minimal or mild endometriosis and infertility is far from conclusive (2,40). Thus, several studies have shown that no treatment appears to be as effective as treatment and that approximately 50% of women will become pregnant without any treatment (41). On the other hand, a prospective cohort study showed that the fertility of infertile women whose condition is diagnosed as minimal or mild endometriosis was not significantly lower than that of women in whom infertility remains unexplained after a laparoscopy (42). These findings suggest that endometrial receptivity and implantation may not be impaired in cases of minor endometriosis.

In conclusion, the results of the present study show that $\alpha v\beta 3$ integrin expression and pinopode formation are not reduced during the window of implantation in patients with stage I–II endometriosis. 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 those markers of implantation, remains to be shown. This study, however, may have a significant type II statistical error because the number of patients included is limited. Considering the

Table II. Hormonal Levels in the Three Groups Studied in the Late Luteal Phase

	Estradiol (pg/mL)[a]	Progesterone (ng/mL)[a]
Endometriosis ($n = 12$)	119.4 ± 16.2	10.7 ± 2.0
Unexplained infertility ($n = 12$)	109.3 ± 17.6	13.4 ± 2.9
Fertile women ($n = 12$)	106.2 ± 19.02	8.3 ± 1.4

Note. Values are mean ± SEM.
[a] No significant differences between groups studied.

differences obtained in statistical comparisons conducted between different groups in the present investigation, a sample size ranging between 52 and 122,270 patients per group would be necessary to provide an 80% statistical power of avoiding a type II error, and 5% chance of making a type I error.

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