Differential expression of endometrial integrins and progesterone receptor during the window of implantation in normo-ovulatory women treated with clomiphene citrate

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Objective: To assess the effect of clomiphene citrate (CC) on endometrial epithelial integrins and P receptors (PR) during the window of implantation.
Design: Controlled, prospective, clinical study.
Setting: Teaching hospital and university research laboratory.
Patient(s): Thirty-one fertile, normo-ovulatory women participated in this trial. Thirteen women exhibited a CC-stimulated cycle with 50 mg on days 5–9, and 18 women with spontaneous menstrual cycles served as controls.
Intervention(s): Endometrial biopsies in the midluteal phase.
Main Outcome Measure(s): Immunohistochemical determination and endometrial cellular localization of α_1, αv, β_3, and α_4 epithelial integrins and PR during the window of implantation. The staining intensity was assessed by a semiquantitative index (HSCORE) and compared by nonparametric Mann-Whitney test.
Result(s): Higher plasma levels of P and E_2 and delayed histologic dating of the endometrium (38%) were features of CC-treated women. In addition, a low epithelial β_3 integrin expression and persistent PR were observed in glandular epithelial cells of "out-of-phase" endometrial biopsies from CC-treated women. In contrast, in "in-phase" biopsies, neither epithelial PR nor β_3 integrin were different from spontaneous control cycles. There was no difference in the expression of α_1, αv, and α_4 between the groups studied.
Conclusion(s): The administration of clomiphene produces aberrant endometrial β_3 integrin expression in conjunction with a failure in the down-regulation of PR during the window of implantation in a significant number of normo-ovulatory women, notwithstanding the higher plasma P levels. Therefore, CC might affect the expression of endometrial receptivity markers. (Fertil Steril® 2005;83:587–93. ©2005 by American Society for Reproductive Medicine.)

Key Words: Integrins, progesterone receptors, clomiphene citrate, endometrial receptivity

Integrins belong to the cell–cell adhesion family of molecules, which might be involved in implantation mechanisms and have been suggested as molecular markers of endometrial receptivity (1, 2). A number of investigators have demonstrated that glandular and stromal endometrial integrins are expressed in a predictable and cyclical manner during the luteal phase. The α_1, αv, and β_3 integrins are co-expressed in the epithelial endometrium only during days 20–24 of the menstrual cycle, supporting the concept that these cycle-dependent proteins, expressed within the putative implantation window, are potential biochemical markers of uterine receptivity (3–5). Moreover, we and others have demonstrated that certain infertility conditions, such as unexplained infertility (6, 7), endometriosis (8), hydrosalpinx (9), and luteal phase deficiency (LPD) (10, 11), are associated with an aberrant pattern of integrin expression. It seems that physiologic epithelial P receptor (PR) down-regulation and $\alpha v\beta_3$ integrin expression are critical steps in the implantation process. For example, LPD is characterized by inadequate ovarian P secretion, leading to delayed endometrial maturation and a failure in the down-regulation of PR. Both of these events are closely associated with an aberrant low expression of $\alpha v\beta_3$ epithelial integrin (12).

Clomiphene citrate (CC) is a compound widely used in ovulation induction. Clomiphene citrate is a mixture of cis and trans isomers with estrogenic and antiestrogenic effects. It binds to the estrogen receptors (ER), leading to the misinterpretation of the E_2 feedback mechanism at the hypothalamic level. The administration of this drug results in increased pituitary gonad-

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otropin release and enhanced follicular development and ovarian response (13, 14).

The inconsistency between high ovulation (60%–85%) and low (30%–40%) pregnancy rates characteristic of CC (15) in anovulatory women has been partially explained by its antiestrogenic effect on cervical mucus and the endometrium. These effects are morphologic, morphometric, and biochemical in nature (16–18).

The endometrial antiestrogenic effect of CC has been proposed as a possible explanation for abnormal endometrial maturation. It is, therefore, tempting to hypothesize that long-lasting ER occupancy by CC (19) might alter the endometrial cell function, thus affecting the expression of proteins related to uterine receptivity.

To investigate whether CC provokes changes in the pattern of endometrial receptivity, endometrial samples were obtained during the midsecretory phase from normoovulatory women treated with CC to test the expression of integrin subunits and PR.

MATERIALS AND METHODS

Subjects

Endometrial samples were obtained from 31 regularly cyclical parous women (mean parity range 2–5) requesting tubal ligation, who were invited to participate in the study. Only women with regular menstrual cycles were recruited; participants with a history of miscarriage were not included. Thirteen women received CC (50 mg orally) on days 5–9 of the menstrual cycle, and 18 women with spontaneous ovulatory cycles served as controls. Surgery and endometrial biopsies were scheduled at the midsecretory phase, within the 6th to 8th postovulatory day. Ovulation was assessed by the surge of LH in urine (Clear-plan ovulation test; Unipath Limited, Betford, United Kingdom) and by serial ultrasonography with a 7.5-MHz transvaginal probe (Model SA 6000; Medison, Seoul, Korea). The detection of urinary LH surge was considered as day 0, and ovulation (follicular rupture) was confirmed by loss of clear demarcation of its walls and intrafollicular echoes on vaginal ultrasound.

The Ethics Committee of the San Borja-Arriarán Clinical Hospital, Santiago, Chile, approved the investigation protocol, and all women participating in the study provided written informed consent.

Radioimmunoassay

Progesterone and E_2 concentrations were determined from plasma samples at the time of the endometrial biopsy and measured by specific RIA, as previously reported (20).

Tissue Preparation

Endometrial tissue was obtained through a sampling device (Pipelle de Cornier, Paris, France). A portion of tissue samples was immediately embedded in a Tissue-Tek Optimal Cutting Temperature compound (Miles, Elkhard, IN), frozen in liquid nitrogen, and stored at −80°C until used to determine the integrin expression in a cryostat section. A second portion was fixed in 4% formaldehyde in phosphate-buffered saline (PBS) and embedded in paraffin at 60°C to form paraffin blocks, which were used for PR immunostaining. Sections of 4 μm were stained with hematoxylin-eosin and evaluated for endometrial dating according to Noyes's criteria (21). The dating of endometrial samples was evaluated independently by two pathologists (F.G. and P.P.) blind to the clinical treatment.

Immunohistochemistry

Immunostaining of endometrial epithelial integrins was conducted as previously reported, with minimal modifications (22). Briefly, the localization of epithelial integrins was assessed in serial cryostat sections (4–5 μm) on silanized slides fixed in acetone at −20°C for 20 minutes. The immunostaining was performed with the streptavidin-biotin-peroxidase method (DAKO, Carpinteria, CA), and 3-amino-9-ethylcarbazole (AEC [DAKO]) was used as a chromogen. The following specific monoclonal antibodies targeted to integrins were used diluted in PBS–bovine serum albumin (BSA): anti-α_1 (clone TS 2/7, 1:2000), anti-α_4 (clone B5G10, 1:1000), and anti-β_3 (clone SSA6, 1:500). The monoclonal antibodies were generously donated by Professor B. Lessey (University of North Carolina, Chapel Hill, NC), and anti-αv (clone VNR 147, 1:200) was obtained from GIBCO, BRL Products, Gaithersburg, MD. Nonspecific staining was minimized by incubation with a blocking solution (LSAB Kit system, DAKO).

Primary antibodies were incubated at 4°C overnight. Negative controls were incubated with irrelevant mouse monoclonal antibodies instead of primary antibodies. A secondary antibody (biotinylated goat antimouse Ig, diluted 1:500 in PBS-BSA 1%) was added after three PBS rinses and followed by 30 minutes of incubation.

The sections were then incubated with streptavidin-biotinylated horseradish peroxidase macromolecular complex for 30 minutes at room temperature, followed by the addition of AEC for 10 minutes to complete the reaction. Finally, samples were washed in water, counterstained with hematoxylin, and mounted in a glycerol-based medium.

Immunohistochemistry for PR was performed on 5–6-μm sections of formalin-fixed, paraffin-embedded endometrial biopsies according to the labeled streptavidin-biotin method (DAKO ER/PR system), as previously described (23). 3,3′-diaminobenzidine (DAB) was used as a chromogen. Tissue sections were deparaffinized in xylene and hydrated in a series of graded ethanols. After heat induction in a water bath (95°C–99°C) for 20 minutes in a target retrieval solution (diluted 1:10 with distilled water), samples were incubated with 3% hydrogen peroxide for 5 minutes to block endogenous peroxidase activity.