Prokineticin 1, homeobox A10, and progesterone receptor messenger ribonucleic acid expression in primary cultures of endometrial stromal cells isolated from endometrium of healthy women and from eutopic endometrium of women with endometriosis

Federica Tiberi, B.S.,[a] *Anna Tropea, M.D.,*[b] *Federica Romani, M.D.,*[b] *Rosanna Apa, M.D.,*[b] *Riccardo Marana, M.D.,*[a] *and Antonio Lanzone, M.D.*[c]

[a] Istituto Scientifico Internazionale "Paolo VI," Università Cattolica del Sacro Cuore (UCSC), Rome; [b] Cattedra di Fisiopatologia della Riproduzione Umana, UCSC, Rome; and [c] Istituto di Ricerca "Associazione OASI Maria SS ONLUS," Troina, Italy

Objective: To examine prokineticin 1 (PROK1), homeobox (HOX) A10, and P receptor (PR) messenger ribonucleic acid (mRNA) expression in primary cultures of endometrial stromal cells (ESC) obtained from eutopic endometrial samples of patients with endometriosis and to clarify whether in vitro steroid hormone dependence of PROK1 gene expression is altered in endometriosis.
Design: Prospective laboratory study.
Setting: Tertiary university hospital.
Patient(s): Twelve normal women (controls) and 12 patients affected by moderate to severe endometriosis in the midsecretory phase of the menstrual cycle.
Intervention(s): Endometrial specimens were obtained from control women and from women affected by endometriosis; ESC were isolated from endometrial biopsies, and primary cultures were established.
Main Outcome Measure(s): Real-time polymerase chain reaction analysis of PROK1, HOXA10, and PR mRNA expression in ESC after 1–4 days of steroid hormone treatment and after decidual differentiation.
Result(s): Contrary to ESC from control women, in ESC obtained from women affected by endometriosis PROK1 and PR mRNA expression was not induced by 1–4 days of treatment with steroid hormones. Nevertheless, when ESC from both groups of women were differentiated to decidual phenotype, PROK1 mRNA was up-regulated and PR and HOXA10 mRNA were down-regulated to the same extent.
Conclusion(s): Our results provide additional evidence for P resistance in endometriosis. (Fertil Steril® 2010; 94:2558–63. ©2010 by American Society for Reproductive Medicine.)

Key Words: Prokineticin 1, progesterone receptor, HOXA10, eutopic endometrium, endometriosis

Prokineticin 1 (PROK1) is an angiogenic and permeability-enhancing factor expressed in the endometrium of women of reproductive age, with increased expression in the secretory phase of the menstrual cycle (1). It is well known that either in vivo or in vitro endometrial PROK1 messenger ribonucleic acid (mRNA) expression is P dependent (1, 2). We have demonstrated that in isolated glands from eutopic endometrium PROK1 mRNA expression is more rarely detected in patients with endometriosis than in a control group. Moreover, when comparing the positive samples from the two groups, detectable PROK1 mRNA levels were 10 times lower in samples obtained in patients with endometriosis than in healthy women (3).

On the basis of this evidence, in the present study we investigated whether in vitro steroid hormone dependence of PROK1 mRNA regulation is maintained in the eutopic endometrium of patients with endometriosis by using cultured endometrial stromal cells (ESC). Actually, the relative balance of estrogen (E) and P activity governs endometrium function throughout the menstrual cycle, and a reduced responsiveness to P in eutopic endometrium of women with endometriosis has been implicated in the pathogenesis of this disease (4, 5).

Moreover, because ESC are involved in embryo implantation, in the present study we evaluated PROK1 expression in ESC during in vitro differentiation to decidual phenotype, because decidualization is a critical process for successful establishment and maintenance of pregnancy (6). Over the years, the decidualization process has been extensively studied, and the critical genes identified to be involved in this process include P receptors (PR) and homeobox (HOX) A10 (7).

Indeed, PR seems to play the role of repressor of decidual transformation, the expression of decidual-specific genes being inversely correlated with cellular PR levels. Moreover, HOXA10, a member of the homeobox gene family, is well known to be essential to embryo development (8, 9), and it seems to be an essential regulator of endometrial receptivity and decidualization (10, 11).

Dysregulation of various P target genes during the implantation window in women with endometriosis has been widely reported

Received October 14, 2009; revised February 27, 2010; accepted March 2, 2010; published online April 18, 2010.
F.T. has nothing to disclose. A.T. has nothing to disclose. F.R. has nothing to disclose. R.A. has nothing to disclose. R.M. has nothing to disclose. A.L. has nothing to disclose.
Reprint requests: Federica Tiberi, B.S., Istituto Scientifico Internazionale "Paolo VI," Università Cattolica del Sacro Cuore, 00168 Rome, Italy (FAX: 39-06-30155205; E-mail: fm1810@inwind.it).

0015-0282/$36.00
doi:10.1016/j.fertnstert.2010.03.006

(12–16), and an endometrial microenvironment characterized by attenuated P response may be inhospitable to embryonic implantation (4).

Several studies have analyzed HOXA10 expression in the endometrium of women with endometriosis. Northern blots as well as immunohistochemical studies show down-regulation of HOXA10 in the eutopic endometrium of women with endometriosis during the window of implantation (17–19).

To better clarify the mechanisms underlying the in vitro modulation of PROK1 expression by steroid hormones in the endometrium of women affected by endometriosis and the role of HOXA10 and PR in this benign gynecologic disease, we investigated in the same in vitro conditions PROK1, HOXA10, and PR mRNA expression on cultured ESC.

MATERIALS AND METHODS

Chemicals

Collagenase type IA and penicillin–streptomycin solution, E_2, and 6α-methyl-17α-hydroxyprogesteroneacetate (MAP) were purchased from Sigma Aldrich (Milan, Italy). Dulbecco's modified essential medium (DMEM) and Phenol Red-free DMEM were obtained from GIBCO (Invitrogen, Milan, Italy). Fetal bovine serum (FBS) was purchased from EuroClone (Milan, Italy) and charcoal/dextran-stripped FBS from HyClone (Thermo Fisher Scientific, Waltham, MA). TRIzol, oligo dT primers, first strand buffer, 1,4-dithiothreitol, 2′-deoxynucleoside 5′-triphosphates, and Moloney murine leukemia virus reverse transcriptase were purchased from Invitrogen and iQ Supermix from Bio-Rad Laboratories (Hercules, CA). Primers and probes for real-time polymerase chain reaction (PCR) were obtained from Sigma-Genosys (Sigma-Aldrich).

Samples

All samples were collected from women (n = 32) of reproductive age (26–40 years) who underwent laparoscopy for evaluation of infertility or evaluation and possible treatment of endometriosis (3). All patients had a history of regular menstrual cycles and did not receive any hormonal treatment in the 3 months before surgery. Women with endometrial pathology or with any hormonal alteration were not included. Informed written consent was obtained from all participants, and the present protocol was approved by the institutional review board of the Università Cattolica del Sacro Cuore.

Uterine curettage was performed during the putative window of implantation (days 19–24 of the menstrual cycle) according to the last menstrual period and confirmed by histologic assessment according to Noyes' criteria (20).

Endometriosis was excluded in 20 patients. Eight of these women, who had laparoscopic diagnosis of nonendometriotic ovarian or paraovarian cysts, were excluded from the study. The remaining 12 women were included in the control group.

According to the results of laparoscopy, 12 women had documented moderate to severe endometriosis according to the Revised American Fertility Society scoring system (21).

Isolation of ESC from Control Women and from Patients with Endometriosis

Histologic criteria of Noyes (20) were used to confirm the secretory phase of each control (n = 12) and eutopic endometriosis (n = 12) specimen.

The separation of ESC from endometrial glands was obtained according to the procedures described by Satyaswaroop et al. (22), with minor modifications (2, 3). Cell viability was assessed by Trypan Blue exclusion (approximately 85.8%).

Establishment of Primary ESC Cultures

Purified ESC were suspended in DMEM (supplemented with 10% heat-inactivated FBS and 50 μg/mL penicillin–streptomycin), plated into 25-cm^2 tissue culture flasks, and cultured until confluence was reached (37°C, 5% CO_2).

Once grown to confluence (2 to 3 days later), ESC from controls and from women with endometriosis were detached by trypsin treatment, seeded into 24-well plates (10^5 cells per well) for 24 hours, and used for the following experiments.

Short-Time Treatment of ESC with Steroid Hormones

Endometrial stromal cells obtained from 6 control women and 6 patients with endometriosis were treated with medium alone (Phenol Red-free DMEM supplemented with 10% heat-inactivated charcoal/dextran-stripped FBS and 50 μg/mL penicillin–streptomycin; control, CTR) or with a physiologic dose of MAP (5×10^{-8} M) alone or with E_2 (4×10^{-10} M) for 24, 48, 72, or 96 hours (2). Each treatment was carried out in triplicate.

In Vitro Decidualization of ESC

Endometrial stromal cells obtained from 6 control women and from 6 patients with endometriosis were treated with medium alone (DMEM supplemented with 10% heat-inactivated FBS and 50 μg/mL penicillin–streptomycin; CTR) or with MAP (2×10^{-7} M) and E_2 (10^{-8} M) for 16 days (MAP/E_2). Decidualization was obtained and confirmed as previously described (23–26). Each culture of ESC was carried out in triplicate.

Total RNA Extraction and cDNA Synthesis

At the end of the incubation period, total RNA extraction was performed using a standard TRIzol extraction method according to the manufacturer's instructions (Invitrogen). Ribonucleic acid purity and integrity were checked spectroscopically and by gel electrophoresis. Total RNA concentration was quantified using 260-nm absorbance.

According to the manufacturer's instructions, cDNA was synthesized using Moloney murine leukemia virus reverse transcriptase starting from 1 μg of total RNA.

Real-Time PCR Analysis

To analyze PROK1, PR, and HOXA10 mRNA expression in short-time-treated as well as in decidualized ESC, a CTR sample of each experimental setting was used as calibrator.

Messenger RNA expression of PROK1, PR, and HOXA10 was evaluated using the i-Cycler iQTM system (Bio-Rad Laboratories) as previously described (3). For the target genes and the endogenous housekeeping gene—encoding for glyceraldehyde-3-phosphate dehydrogenase—a primer pair and Taqman probe, which hybridizes the region between primers, were designed using Beacon Designer 2 v. 3.00 software (Premier Biosoft International, Palo Alto, CA) and synthesized by Sigma-Genosys (Table 1).

Real-time PCR for PROK1, PR, and HOXA10 mRNA expression semiquantification was performed as previously described (3). Glyceraldehyde-3-phosphate dehydrogenase and the CTR samples were used as normalizer for the comparative cycle time method described in Applied Biosystems User Bulletin 2 (PN 4303859) and according to the literature (27). Samples were considered to be negative for PROK1, PR, and HOXA10 expression when no amplification was detected after 40 cycles (3).

Statistical Analyses

For statistical data analysis, continuous variables were expressed as mean ± SD, categoric variables were displayed as frequencies, and the appropriate parametric or nonparametric test was used to assess significance of the differences between subgroups. Unpaired Student's t test was used to compare the average of PROK1, PR, and HOXA10 mRNA expression levels. Values with $P<.05$ were considered statistically significant.

RESULTS

PROK1 mRNA Expression in Short-Time-Treated ESC from Controls and Patients with Endometriosis

In ESC obtained from both groups, PROK1 mRNA expression was undetectable in CTR samples after 24, 48, 72, and 96 hours of culture.