P_{450}Arom induction in isolated control endometrial cells by peritoneal fluid from women with endometriosis

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Objective: To study the effect of peritoneal fluid from women with (PF-E) and without (PF-C) endometriosis on P_{450}Arom expression in endometrial cells.
Design: Experimental study.
Setting: University research unit.
Patient(s): Forty women of reproductive age with (n = 22) or without (control; n = 18) endometriosis.
Intervention(s): Peritoneal fluid and eutopic endometrial samples were obtained during surgery from women with (n = 13 and 9, respectively) and without (n = 4 and 14, respectively) endometriosis.
Main Outcome Measure(s): Expression study for P_{450}Arom, steroid factor 1 (SF-1), chicken ovalbumin upstream transcription factor I (COUP-TFI), and COUP-TFII messenger RNA (reverse transcriptase–polymerase chain reacion) and/or protein (immunoblot) in isolated endometrial epithelial cells transfected or not with expression vector containing SF-1, COUP-TFI, or COUP-TFII complementary DNAs.
Result(s): Basal messenger RNA and/or protein expression of P_{450}Arom and SF-1 were augmented in endometriosis, and that of COUP-TF was diminished. In control cells, $(Bu)_2$cAMP and PF-E increased P_{450}Arom and SF-1 expression (but not COUP-TF expression) in a dose-dependent way, an effect not observed with PF-C, adsorbed PF-E, or 10^{-5} M indomethacin. Transfected cells confirmed these results. Any treatments modified the studied molecules in endometriosis cells.
Conclusion(s): These data indicate that molecules contained in PF-E favor an estrogenic microenvironment, suggesting a role in the etiopathogenesis of endometriosis enabling the survival, maintenance, and growth of endometrial implants in the ectopic locations. (Fertil Steril® 2010;94:2521–7. ©2010 by American Society for Reproductive Medicine.)

Key Words: P_{450}Arom, SF-1, endometriosis, peritoneal fluid, cell culture, eutopic endometrium

Endometriosis is an estrogen-dependent pathology characterized by the presence of a functional endometrium outside the uterine cavity (1, 2). Because almost all women have retrograde menstruation (3), the coexistence of a menstrual efflux removal defect in the peritoneal cavity and an aberrant expression of some molecules in eutopic and ectopic endometria has been postulated (4). The peritoneal fluid is increased in endometriosis and contains high concentrations of macrophages and monocytes, which secrete many molecules that favor the angiogenesis, viability, and adhesion of the implant (5–7).

The enzyme P_{450}Arom, which catalyzes the conversion of androgen to estrogen, is coded by *Cyp19A1*, a gene regulated by 10 tissue-specific promoters (8–10). Its abnormal expression in eutopic and ectopic endometria of women with endometriosis (11–13) is regulated in a similar manner as the ovarian gene through promoter II by steroid factor 1 (SF-1), a 67-kDa monomer that positively regulates steroidogenic genes, and negatively by the 90-kDa homodimer chicken ovalbumin upstream transcription factor (COUP-TF) (14, 15). Steroid factor 1 competes with higher affinity for the same upstream site on promoter II than COUP-TF (14). Two isoforms of COUP-TF are described: COUP-TFI mediates process on organogenesis, cellular differentiation, and homeostasis, and COUP-TFII is highly expressed in uterine epithelial cells (16).

Chicken ovalbumin upstream transcription factor I is more expressed in normal endometrium, whereas SF-1 is mainly expressed in eutopic and ectopic endometria of women with endometriosis (11, 17). Therefore, the aberrant expression of P_{450}Arom described in ectopic and in eutopic endometria would depend on the transcription factors available in the tissue (4, 9, 12, 14, 17–23).

Prostaglandin E_2 (PGE_2) is also an inducer of P_{450}Arom expression in endometriotic cells. Estrogen increases PGE_2 formation by the stimulation of cyclooxygenase type 2 (COX-2), which catalyzes

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prostanoid synthesis in uterine endothelial cells (24). Thus, a positive feedback between prostaglandins and local estrogen production is established, favoring the cell proliferation and inflammation that characterizes this disease (11, 24, 25).

Here, we study the effect of peritoneal fluid from women with endometriosis on *Cyp19A1* expression through SF-1, COUP-TFI, and COUP-TFII regulation in epithelial cell cultures from eutopic endometrium of women with and without endometriosis.

MATERIALS AND METHODS

Subjects

Eutopic endometrium was obtained by laparoscopy with a Pippelle suction curette from 9 women age, 35.3 ± 6.4 years) for peritoneal endometriosis diagnosis (endometriosis group) and 14 women without endometriosis (age, 39.2 ± 5.1 years) for tubal ligation or hysterectomy for subserous or intramural myomas (control group); the women were without hormonal treatment in the last 3 months. A piece of tissue was fixed in formalin for dating (26), and the endometria were classified as proliferative (days 5–14; 3 control and 3 endometriosis samples) or secretory (days 15–28; 11 control and 6 endometriosis samples); the rest were used for cell preparation. Peritoneal fluid (PF) was obtained during surgery from 4 women without (PF-C, one pool) or 13 with (PF-E, three pools) peritoneal endometriosis, with each pool having 4 to 5 women in the proliferative phase of the menstrual cycle. Each PF pool was aliquoted and frozen at −80°C until use. To eliminate small molecules, some PF-E aliquots were adsorbed with activated charcoal and dextran (Sigma, St Louis, MO).

This study was approved by the institutional boards of University of Chile and the Metropolitan Central Health Service of Chile, and each patient provided written, informed consent before surgery.

Cell Culture

The endometrium was washed, minced, and digested as described by Pino et al. (27). The glands were obtained as indicated previously (28). Cells of second passages were cultured until subconfluence, incubated for 24 hours in defined medium (27), and treated (24 hours) with PF-E, PF-C, adsorbed PF-E, N6-2′-O-dibutyryl cyclic adenosine 3′,5′-monophosphate [$(Bu)_2$cAMP, 10^{-6} mol/L], or indomethacin (10^{-7}–10^{-5} mol/L, COX inhibitor) added 30 minutes before the PF-E addition.

Cell Transfection

Cells were transfected with the vectors pcDNA2, pCR3.1, and pcDNA3.0 containing SF-1 (pSF-1), COUP-TFI (pCOUP-TFI), and COUP-TFII (pCOUP-TFII) complementary DNAs (cDNAs), respectively (kindly donated by Drs. Ming-Jer Tsai and Keith L. Parker). The cells, cultured as above until 60% confluence, were transitorily transfected using 6 μL GeneJammer Reagent (Stratagene, La Jolla, CA) and 2 μg of plasmid DNA mixed to up 100 μL sterile Dulbecco's Modified Eagle Media (DMEM) for 10 minutes at room temperature. The volume was completed to 1 mL of culture medium and further incubated for 5 hours at 37°C. Later, another 1 mL was added, and the incubation continued for 24 hours. As transfection control, the cells were transfected with the vector cytomegalovirus containing β-galactosidase cDNA or with empty vector p-Cis (Promega, Madison, WI). After 48 hours the cells were cultured for 24 hours in defined medium and then treated for 24 hours with 10% PF-E, $(Bu)_2$cAMP, or 10% PF-E plus 10^{-5} mol/L indomethacin.

RNA isolation and RT-PCR

Total RNA and cDNA were obtained as indicated previously (27). The primers for COUP-TFI (NM005654; 380 bp) were upstream 5′-AAGCACTACGGCCAATTCAC-3′ and downstream 5′-GCGTTCATCCTCATCGAAGT-3′; for SF-1, P_{450}Arom, and COUP-TFII amplifications were previously reported (14, 21, 29).

One microliter of cDNA was added to 24 μL of a reaction mix that included 0.625 U of Taq DNA polymerase (Invitrogen Life Techonologies, Bethesda, MD), 0.4 mmol/L of each primer, 0.25 mmol/L 2′-deoxynucleoside 5′-triphosphate mix (0.20 mmol/L for SF-1), and 3 mmol/L $MgCl_2$ (1.5 mmol/L for P_{450}Arom). The amplification was at 94°C for 60 seconds, 50°C (P_{450}Arom), 51°C (COUP-TFII), 53.3°C (SF-1), or 55°C (COUP-TFI) for 60 seconds, and 72°C for 90 seconds and repeated for 30 (COUP-TFI and COUP-TFII), 33 (SF-1), or 35 (P_{450}Arom) cycles. As internal control, 18S ribosomal RNA (30) was amplified as previously described (31). Semiquantitation of PCR products was performed by image analysis (Kodak 1D Image Analysis; Rochester, NY), and their identity was confirmed by sequencing (Macrogen, Seoul, Republic of Korea).

Protein Homogenate Preparation and Immunoblot Analysis

Cytoplasmic protein homogenized in 50 μL lysis buffer A and nuclear proteins suspended in buffer B (1:1) were prepared as described previously (32). Thirty micrograms of cytoplasmic or nuclear proteins were resolved and electrotransferred into nitrocellulose membranes (BioRad, Hercules, CA). The immunoblot was done using antibodies against SF-1 (polyclonal, 1:800; ABR Affinity BioReagents, Golden, CO.), COUP-TFI (monoclonal; 1:1,000; R&D Systems, Minneapolis, MN), P_{450}Arom (monoclonal; 1:600; Serotec, Oxford, United Kingdom), TFIIB (monoclonal, 1:500; BD Biosciences Pharmingen, Chicago, IL), and β-actin (monoclonal, 1:15,000; Sigma). The bands were visualized, captured, and analyzed as indicated previously (27) and normalized with β-actin or TFIIB for cytosolic or nuclear extracts, respectively.

Statistical Analysis

The Mann-Whitney test for continuous variables and the Kruskal-Wallis test for discrete variables when comparing more than two categories were used. A P value of $<.05$ indicates statistical significance. Results are expressed as mean ± SEM and patient ages as mean ± SD.

RESULTS

Basal mRNA and Protein

Chicken ovalbumin upstream transcription factor II messenger RNA (mRNA) was lower in the endometriosis cells than in the control cells. In contrast, the SF-1 and P_{450}Arom mRNA levels, almost undetectable in control cells, were 93% and 305% higher in endometriosis cells (Table 1).

The nuclear COUP-TFI protein content was 50% higher in control compared with endometriosis cells; in contrast, the nuclear SF-1 and the cytoplasmic P_{450}Arom proteins were almost undetectable in control cells but strongly expressed in endometriosis cells ($P<.01$) (Fig. 1).

Effect of Treatments on mRNA and Protein Expression

Only in the control cells, SF-1 mRNA levels were increased 52% and 79% by PF-E (1% and 10%, respectively), 41% by $(Bu)_2$cAMP, and 152% by the combination with 10% PF-E compared with basal levels. Similarly, P_{450}Arom mRNA levels were increased 150% and 255% by PF-E (1% and 10%), and 246% and 582% by $(Bu)_2$cAMP alone or with 10% PF-E compared with basal levels, respectively. Chicken ovalbumin upstream transcription factor I and COUP-TFII were not affected (Table 1). Control PF or adsorbed PF-E did not modify any molecule studied in either cell culture group (Table 1).

The PF-E stimulatory effect on the mRNA of SF-1 and P_{450}Arom was reduced by indomethacin in a dose-dependent way only in control cells (10^{-7} mol/L: 48% and 11%; 10^{-6} mol/L: 67% and 36%; 10^{-5} mol/L: 99% and 90%, respectively) without affecting COUP-TFI and COUP-TFII, shown only for 10^{-5} mol/L dose in Table 1.