Concurrent estrogen action was essential for maximal progestin effect in oral contraceptives

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Objective: To investigate the impact of estrogen contained in oral contraceptives (OCs) on the action of progestin on ovarian endometrioma epithelial cells.
Design: Experimental in vitro study and immunohistochemical analysis.
Setting: University hospital.
Patient(s): Patients who underwent surgery due to ovarian endometrioma.
Intervention(s): Not applicable.
Main Outcome Measure(s): Telomerase-immortalized epithelial cells derived from ovarian endometrioma were treated with norethindorone (NET; 80 nmol/L) or levonorgestrel (LNG; 20 nmol/L) with or without 17β-ethynylestradiol (EE; 0.6 nmol/L) for 96 hours, and the cell growth was monitored. Estrogen receptor (ER) α, progesterone receptor (PR) A, and PRB expressions in clinical samples of ovarian endometrioma epithelial cells were analyzed with the use of immunohistochemistry.
Result(s): NET or LNG effectively suppressed cell growth, and addition of EE significantly enhanced the growth suppression. This EE-mediated enhancement of cell growth suppression was observed only in cells that expressed ERα and therefore was ERα dependent. Western blot analysis revealed that expression of PRB was significantly induced by the addition of EE. Immunohistochemical analysis confirmed that ERα expression and PRB expression are significantly correlated, indicating that progestin-sensitive cells with PRB expression are predisposed to react with estrogen stimulation.
Conclusion(s): These findings suggest that EE contained in OCs plays a supportive role in progestin-induced growth inhibition of ovarian endometrioma epithelial cells. In the absence of estrogen priming, concurrent estrogen action was essential for rapid induction of PR to achieve maximal progestin effect. (Fertil Steril® 2014;101:1337–43. ©2014 by American Society for Reproductive Medicine.)
Key Words: Estrogen, progestin, oral contraceptive, ovarian endometrioma

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Endometriosis is a common gynecologic disorder associated with dysmenorrhea, pelvic pain, and subfertility; it is also a leading cause of disability and loss of productivity in women of reproductive age (1). Ovarian endometrioma is a subtype of endometriosis; this subtype arises in ovaries, forms a pelvic mass, and induces adhesion and dysfunction of ovary, causing sterility. There are multiple treatment options for ovarian endometrioma, depending on tumor size, severity of symptoms, and patient age, among other factors. Hormonal therapy is a popular treatment option when surgical treatment is not recommended. Endometriosis is defined as an estrogen-dependent disease, and decreased estrogen levels result in regression of endometriotic lesions; consequently, medical therapies have focused on lowering estrogen levels; such therapies include use of GnRH analogues, which suppress estrogen levels and effectively promote endometriotic lesion regression (2). However, the

Received November 28, 2013; revised and accepted February 5, 2014; published online March 19, 2014.
Y.B. has nothing to disclose. S.K. has nothing to disclose. T.K. has nothing to disclose. Y.M. has nothing to disclose. M.N. has nothing to disclose. Y.M. has nothing to disclose. M.T. has nothing to disclose. H.F. has nothing to disclose.
Supported in part by a Grant-in-Aid for Scientific Research from the Japan Society for the Promotion of Science (23390387 to S.K.).
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Fertility and Sterility® Vol. 101, No. 5, May 2014 0015-0282/$36.00

http://dx.doi.org/10.1016/j.fertnstert.2014.02.005

hypoestrogenic state caused by these drugs can lead to severe side effects, such as pseudomenopause; therefore, these remedies are not appropriate for prolonged use. Alternatively, oral contraceptives (OCs) or various progestins have both been in wide use; they are safe, effective, and appropriate for long-term use (3–7). They are also used to prevent or delay recurrence of endometriomas after surgery (8–11). The mechanistic basis of these contraceptives as treatments for ovarian endometrioma is that progestin acts to inhibit cellular growth, to prevent implantation, and to induce cellular differentiation (decidualization) of endometriotic cells (12, 13), but the pharmacologic action is still not understood in detail.

Estrogen promotes growth of endometriotic lesions (14, 15). In patients with endometriosis, E_2 can be synthesized locally in endometriotic lesions from inactive adrenal precursors via the aromatase pathway (1, 16, 17). Expression of aromatase, which catalyzes conversion of ovarian or adrenal T to E_2 and conversion of androstenedione to estrone (E_1), increases in endometriotic lesions (18, 19). E_1 formed via the aromatase pathway is converted into E_2 by the action of reductive 17β-hydroxysteroid dehydrogenase type 1 (HSD17B1), but this E_2 can be inactivated by the action of the oxidative 17β-hydroxysteroid dehydrogenases type 2 (HSD17B2). Reportedly, expression of HSD17B1 increases in endometriotic lesions, and expression of HSD17B2 decreases (18, 20). Such estrogen-dominant hormonal conditions may support overall growth of endometriotic lesions; nevertheless, whether or how estrogen contained in OCs affects growth of endometriotic lesions is not known. Clinicians are concerned about the undesirable action of estrogen contained in OCs because estrogen action sometimes antagonizes progestin action. A current topic in the field of endometriosis research is the malignant progression of benign ovarian endometrioma to ovarian cancer, especially to clear cell or endometrioid cancer subtypes (21). Very little information on whether continuous and long-term treatment of ovarian endometrioma with contraceptives triggers carcinogenesis in epithelial cells is available.

Previously, we created immortalized epithelial cells, designated EMOsis-CC/TERT1, from ovarian endometrioma by introducing *cyclinD1*, *cdk4*, and *human TERT* expression vectors into purified epithelial cells from surgically removed ovarian endometrioma (22). We have tested the responsiveness of these cells to various kinds of progestin in vitro, and found that medroxyprogesterone acetate (MPA), P, and dienogest each effectively inhibited growth of these cells. The present study expanded those analyses and investigated the impact of estrogen contained in OCs on the growth of ovarian endometrioma.

MATERIALS AND METHODS

Cell Culture

Immmortalized epithelial cells from ovarian endometrioma (EMOsis-CC/TERT1 cells) were created previously; *cyclinD1*, *cdk4*, and *human TERT* expression vectors were introduced into purified epithelial cells from surgically removed ovarian endometrioma (22). estrogen receptor (ER) α–expressing EMOsis-CC/TERT cells (EMOsis-CC/TERT1/ER) were generated by introducing an ERα expression vector into EMOsis-CC/TERT1 cells (22). These immortalized epithelial cells were usually maintained in Dulbecco modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum in an atmosphere of 5% CO_2 at 37°C.

In Vitro Growth Assay

The proliferative activity of cells treated with progestins, estrogen, or both was examined by counting cells. Briefly, the cells were seeded at a density of 15–20 $\times$ 10^4 cells/well in 6-well flat-bottomed plates; cells were then grown overnight in phenol red–free DMEM supplemented with charcoal-treated 10% fetal calf serum (FCS) for 24 hours. Next, the cultures were treated with one of three progestins—norethindorone (NET) at 80 nmol/L, levonorgestrel (LNG) at 20 nmol/L, or dienogest at 120 nmol/L—in the presence or absence of 17β-estradiol (E_2) at 0.2 nmol/L or 17β-ethynylestradiol (EE) at 0.6 nmol/L. Cells were counted 72 hours after steroid hormone treatments. Data are presented as mean $\pm$ SD of three independent experiments. A P value of $<.05$ was considered to indicate statistical significance.

Western Blot Analysis

Whole-cell extracts were prepared with RIPA lysis and extraction buffer (Thermo Fisher Scientific). A 30-μg sample of each extract was run through a sodium dodecyl sulfate–polyacrylamide gel via electrophoresis; the resolved proteins were then transferred to polyvinylidine difluoride membranes. Membranes were blocked by immersion in Tris-buffered saline solution with Tween-20 (TBST; 150 mmol/L NaCl, 20 mmol/L Tris-Cl, pH 7.5, 0.1% Tween) containing 5% nonfat dried milk, and then incubated sequentially with specific antibodies; anti–progesterone receptor (PR; H-190, dilution 1:1,000; Santa Cruz Biotechnology), anti-PRB (C1A2, dilution 1:1,000; Cell Signaling Technology), and antiactin (C-11, dilution 1:1,000; Santa Cruz Biotechnology) antibodies were used; each type of primary antibody was subsequently labeled with anti–rabbit IgG conjugated with horseradish peroxidase. An ECL detection system (GE Healthcare Biosciences) was used according to the manufacturer's protocols to visualize immunoreactive protein bands.

Previously established immortalized endometrial epithelial cells (EM-E6/E7/TERT/PRA or EM-E6/E7/TERT/PRB) were used as positive controls for PRA and PRB; these cell lines were generated by introducing HPV16 *E6*/*E7* and *TERT* as well as PRA or PRB cDNA into primary endometrial epithelial cells (23). Two different antibodies were used to probe the Western blots: one antibody recognizes both PRA and PRB (H190), and the other recognizes only PRB (C1A2). Based on the signal from the anti-PR antibody that recognizes both PRA and PRB, EM-E6/E7/TERT/PRA cells expressed only one band that corresponded to PRA (94 kDa), and EM-E6/E7/TERT/PRB cells exhibited the PRB band (114 kDa) as well as a lower band that was close to the PRA band but was considered to be degraded PRB (23).