Dienogest inhibits aromatase and cyclooxygenase-2 expression and prostaglandin E_2 production in human endometriotic stromal cells in spheroid culture

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Objective: To determine the effect of dienogest (DNG) on the expression of aromatase and cyclooxygenase-2 (COX-2) and the production of prostaglandin E_2 (PGE_2) in human endometriotic stromal cells (ESCs).
Design: Experimental study in vitro.
Setting: University hospital.
Patient(s): Seventeen patients with ovarian endometrioma.
Intervention(s): ESCs from chocolate cyst linings of ovaries were treated with DNG.
Main Outcome Measure(s): Expression of aromatase and COX-2 evaluated in spheroid cultures of human ESCs by real-time quantitative polymerase chain-reaction and immunocytochemistry, production of PGE_2 quantified by enzyme-linked immunosorbent assay (ELISA), and nuclear factor kappa B (NF-κB) DNA-binding examined by ELISA and immunocytochemistry.
Result(s): The pharmaceutical actions of DNG on the expression of aromatase and COX-2 and the production of PGE_2 were examined using spheroid cultures of human ESCs. More aromatase, COX-2, and PGE_2 were expressed in spheroid cultures than in conventional ESCs monolayers. In the spheroid cultures, DNG (10^{-7} M) and progesterone (10^{-7} M) inhibited the expression of aromatase, COX-2, and PGE_2. DNG also inhibited NF-κB DNA-binding activity and reduced the immunocytochemical protein expression of aromatase, COX-2, and NF-κB p50 nuclear localization.
Conclusion(s): Because DNG inhibits aromatase and COX-2 expression as well as PGE_2 production in ESCs, these pharmacologic features might contribute to a therapeutic effect of DNG on endometriosis. (Fertil Steril® 2012;97:477–82. ©2012 by American Society for Reproductive Medicine.)
Key Words: Aromatase, cyclooxygenase-2, dienogest, endometriotic stromal cells, spheroid culture

Endometriosis is characterized by endometrium-like lesions growing outside the uterine cavity. The main symptoms are pelvic pain, including dysmenorrhea, dyspareunia, and infertility (1). Endometriosis is a multifactorial disease and both hormone and inflammatory factors contribute to the growth of lesions and pelvic pain. Endometriosis is therefore considered as an estrogen-dependent inflammatory disease. A positive feedback cycle indicates that aromatase, an enzyme responsible for estrogen biosynthesis, and cyclooxygenase-2 (COX-2), a prostaglandin E_2 (PGE_2) synthase, are responsible for the continuous local formation of estrogen and PGE_2 in endometriotic stromal cells (ESCs) (2). Prostaglandin E_2 is a major mediator of the pain resulting from the pathophysiology of endometriosis. Aromatase expression might be induced by PGE_2 in endometriotic cells (3). Aromatase produces E_2, which consequently promotes PGE_2 production by inducing COX-2 expression (3). On the other hand, the transcription factor nuclear factor-kappa B (NF-κB) is implicated in the pathophysiology of endometriosis by inducing COX-2 expression in ESCs (4–6). Therefore, drugs that can inhibit estrogen and PGE_2 formation should block this

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positive feedback cycle, and thus contribute to pain relief and suppress the pathological growth associated with endometriosis.

The 19-nortestosterone derivative dienogest (DNG) is a fourth-generation progestin with potent oral progestational activity without systemic androgenic activity (7–9) that directly suppresses the proliferation of stromal (10) and immortalized epithelial cells (11) derived from the human endometrium. DNG also suppresses the proliferation (12) and secretion of IL-8 from ESCs (13). DNG potently relieves pelvic pain associated with endometriosis (14), and clinical trials have shown that while DNG and GnRH agonists are equally effective, the DNG group developed fewer adverse effects caused by hypo-estrogenic states (15–17). We previously demonstrated that DNG inhibits aromatase expression and PGE_2 production in human immortalized endometrial epithelial cells (18). However, whether DNG has similar inhibitory effects on local estrogen and PGE_2 production in endometriotic cells remains unknown. Others have shown that spheroid cell culture systems contain multicellular aggregates composed of cells and extracellular matrices. Spheroid formation is prone to expressing massive proinflammatory, proteolytic and growth factor responses (19) and the underlying mechanism seems to be involved in the NF-κB signaling pathway (20). COX-2 is easily induced in spheroid cultures of various cells (21). Thus, spheroid cultures might serve as an experimental model of endometriosis, which is considered a chronic inflammatory disease. We already showed that spheroid cultures of endometrial epithelial cells are superior to conventional monolayer cultures for higher COX-2 and NF-κB activities (18). Therefore, the present study examines the effects of DNG on the expression of aromatase and COX-2 and PGE_2 production in ESCs in spheroid cultures and investigates the pharmacological mechanisms of DNG that are involved in the treatment of endometriosis.

MATERIALS AND METHODS

Patients and Samples

Ovarian endometrioma specimens from 17 patients (mean age: 34.5 years; range: 23 to 44 years) with endometriosis accompanied by pain and/or infertility were obtained at the time of laparoscopy. All of the women had regular menstrual cycles, the cycle phases of which were determined according to serum estradiol and progesterone (P4) levels. The menstrual phase at the time of the operation was proliferative and secretory in 10 and 7 patients, respectively. None had received hormone treatment for at least 6 months before surgery. The stages of endometriosis were III (n = 5) and IV (n = 12). The institutional review boards of Kyoto Prefectural University of Medicine and Mochida Pharmaceutical Co., Ltd. approved the experimental procedures, and each patient provided written, informed consent to participate in the study.

Isolation and Culture of ESCs

Excised tissues were immediately rinsed with ice-cold Dulbecco's modified essential medium-F12 medium (DMEM-F12; Invitrogen) and minced into small pieces. Tissues were agitated in DMEM-F12 containing 2.5% type I collagenase (Sigma-Aldrich) and 15 IU/mL of deoxyribonuclease I (Takara Shuzo, Tokyo, Japan) for 60 minutes at 37°C, and then they were filtered through a nylon cell strainer. Cell clumps were collected from the filtrate by centrifugation at 100 × g for 5 minutes, and the red blood cells were removed using Histopaque-1077 (Sigma-Aldrich). The ESCs were resuspended and cultured in DMEM-F12 containing 10% fetal bovine serum (FBS; Invitrogen) and antibiotics in a humidified atmosphere at 37°C in 5% CO_2 and 95% air. The >95% purity of the ESC preparations was confirmed by positive staining for CD10 (Neomarkers) as a marker of stromal cells and negative staining for cytokeratin (Santa Cruz Biotechnology) as a marker of epithelial cells.

Cell Cultures and Compounds

The ESCs were seeded with phenol red-free DMEM supplemented with 10% dextran-coated, charcoal-treated FBS (Invitrogen) and antibiotics into six-well culture plates to form monolayer cultures. Dispersed cells were seeded in U-bottom 96-well plates (Sumilon) at a density of 4×10^4 cells per well to generate spheroids. The medium was replaced 72 hours later with the same medium containing DNG (Bayer Schering Pharma), P4, danazol (Dan), mifepristone (Mife), or celecoxib (Celex) (Sigma-Aldrich) dissolved in dimethyl sulfoxide (DMSO) or DMSO alone (control). The final concentration of DMSO in the culture medium was 0.1%. Conditioned media, cells, and cell lysates were collected for the following assays before and after incubating the cells with drugs for 48 hours.

Real-Time Quantitative PCR

The total RNA was extracted from cells using RNeasy Mini kit (QIAGEN) according to the manufacturer's instructions, and then the complementary DNA (cDNA) was prepared using the SuperScript III first-strand synthesis system (Invitrogen). The polymerase chain reaction (PCR) mixture (20 μL) contained 1 × TaqMan fast universal PCR master mix (Applied Biosystems), cDNA, and probes for the target genes as follows: TaqMan assay on demand gene expression primer/probe sets (Applied Biosystems) for COX-2 (assay ID Hs00153133_m1) and aromatase (assay ID Hs00240671_m1), and the human GAPDH endogenous control primer/probe set for human GAPDH (VIC/TAMRA probe, Primer Limited; Applied Biosystems). Real-time quantitative PCR proceeded under cycling conditions of 95°C for 20 seconds, 40 cycles of 95°C for 3 seconds, and 60°C for 30 seconds. Cycle threshold (Ct) values were determined. Target gene messenger RNA (mRNA) Ct values for each sample (Ct (target)) were normalized using the Ct value of the housekeeping GAPDH (Ct (GAPDH)) in the same sample as follows: ΔCt (target) = Ct (target) − Ct (GAPDH). The relative mRNA level was expressed as the value of $2^{-\Delta Ct\ (target)}$.

Measurement of PGE_2 in Culture Media

Concentrations of PGE_2 in conditioned media were measured using Correlate-EIA Prostaglandin E_2 Enzyme Immunoassay Kits (Assay Designs), according to the manufacturer's instructions.