Differences in gene expression in the proliferative human endometrium

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Objective: To use microdissection and DNA microarray technology to demonstrate differences in gene expression between epithelial and stromal areas in the proliferative human endometrium.
Design: Pilot study.
Setting: University hospital.
Patient(s): Patients with normal menstrual cycles and at least one previous intrauterine pregnancy.
Intervention(s): Uterine endometrial biopsy.
Main Outcome Measure(s): Gene expression.
Result(s): From a total of 1,200 genes, 14 were strongly expressed in epithelial areas and 12 were strongly expressed in stromal areas. Among the genes strongly expressed in the stroma, expressions of decorin and discoidin domain receptor were confirmed by real-time polymerase chain reaction. Decorin was localized in the stromal areas by immunohistochemical staining. To confirm the effects of estrogen on gene expression, stromal cells were cultured. When E_2 was added to the culture media, expression of decorin mRNA was increased.
Conclusion(s): The data demonstrated in this study help to understand the physiology of human endometrium. Decorin was strongly expressed in the stromal areas and was regulated by estrogen, and therefore it may be involved in restoration of the endometrium. (Fertil Steril® 2005;83(Suppl 1):1206–15. ©2005 by American Society for Reproductive Medicine.)

Key Words: Human endometrium, microdissection, microarray, decorin

Human endometrium exhibits dramatic remodeling through proliferation and differentiation during the menstrual cycle in response to the rise and fall of ovarian steroids. These characteristic changes in the endometrium are thought to be the result of not only steroids but also locally released factors. Furthermore, stromal-epithelial interactions are recognized as a necessary component in mediating steroid-induced endometrial turnover (1).

The endometrial periodic change has been reported in relation to several factors. Growth factors, receptors and related proteins, such as epidermal growth factor (EGF), platelet-derived growth factor (PDGF), and insulin-like growth factor (IGF) may be involved. EGF is strongly involved in the estrogenic proliferative effect as a local factor (2, 3). Levels of EGF receptor increase during the proliferative phase, reaching a maximum just before ovulation, and decrease thereafter during the secretory phase, reaching a minimum before menses (4). PDGF receptor immunostaining is highest in the proliferative phase and is considerably reduced in the secretory phase. EGF and PDGF are mitogenic for endometrial stromal cells, thus suggesting an autocrine/paracrine role in the modulation of endometrial cell growth and differentiation (3). IGF promotes cellular growth and differentiation and it is reported that IGF-I is expressed during the proliferative phase (5). Tenascin, which increases during the proliferative phase, is an extracellular matrix glycoprotein and may be involved in endometrial proliferation (6).

Although dramatic changes in growth-related factors, particularly in the proliferative phase, have been studied, global gene expression in the proliferative endometrium has not been documented and the regulation mechanisms are not well understood. Studies using cultured cells study may not reflect the in vivo state because stromal-epithelial interactions are not taken into consideration. Therefore, different methodologies may reveal additional information.

In this study, microdissection was used to collect stromal and epithelial areas. RNA was extracted and differences in gene expression between epithelial and stromal areas were analyzed by cDNA microarray. Differential expression of candidate genes was confirmed by real-time polymerase chain reaction (PCR). Stromal cell cultures were then performed sequentially using endometrium obtained from different subjects in the proliferative phase to study the effects of estrogen on gene expression.

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MATERIALS AND METHODS

Materials

Human proliferative-phase endometrium was obtained at day 9–11(mid-proliferative phase) from 17 patients (25–38 years old) with normal menstrual cycles and at least one previous intrauterine pregnancy. Endometrial biopsy specimens were obtained using curetting technique. The day of the menstrual cycle was determined based on patient history, plasma progesterone levels, and the histologic criteria of Noyes et al. (7). Patients did not receive any hormonal therapy.

Informed consent was obtained from all patients who participated in the study. The use of human subjects and the procedures were approved by the Institutional Review Boards of Showa University.

Methods

Laser microdissection and RNA extraction. The experiment was conducted with reference to the method of Wittliff and Erlander (8). Endometrium was embedded in optimal cutting temperature (OCT) compound and frozen in isopentane cooled with liquid nitrogen. The frozen block was sliced by cryomicrotome at a thickness of 8 μm. Frozen sections were fixed in 100% methanol for 3 minutes and stained with 1% toluidine blue. Approximately 20–40 sections were laser-microdissected using a PALM MicroBeam system (PALM Microlaser Technologies, Bernried, Germany).

Total RNA was extracted from tissue sections using a modified acid guanidinium-phenol-chloroform (AGPC) method (9). Samples collected were denatured in denaturing solution (Guanidium thiocyanate + Sarcosyl + sodium citrate + Diethylpyrocarbonate [DEPC]). After denaturing, sodium acetate, phenol and chloroform (Sigma, St. Louis, MO) were added to the medium. Medium was centrifuged and upper layers taken. Isopropanol (Wako Pure Chemical, Osaka, Japan) was added and medium was stored at −80°C overnight. After centrifugation, the supernatant was poured off and ethanol was added, followed by DEPC, and this solution was stored at −80°C.

Microarray. RNA obtained was converted to cDNA using the modified oligo (dT) primers in the BD SMART PCR cDNA Synthesis Kit (BD Biosciences Clontech, Palo Alto, CA). cDNA was PCR amplified for 23–29 cycles according to the manufacturer's instructions (BD Atlas SMART Probe amplification Kit; BD Biosciences Clontech). A DNA sample (550 ng) was then labeled with α32P dCTP (3,000 Ci/mmol) using the Randam primer. Labeled probes were hybridized to a nylon array (BD Atlas Nylon cDNA Expression Array; BD Biosciences Clontech) in ExpressHyb solution at 68°C overnight. After hybridization, the nylon membrane was washed once with 2× standard saline citrate (SSC) + 1% sodium dodecyl sulphate (SDS) (Wako Pure Chemical) and twice with 1× SSC + 0.5% SDS at 68°C (10, 11). The membrane was exposed to a phosphor screen (Fujifilm, Kanagawa, Japan) for 24 hours and was scanned using a STORM 830 Scanner and IMAGEQUANT 4.1-J (Molecular Dynamics, Sunnyvale, CA).

Hybridization signal intensities for individual genes were background subtracted and normalized against signals for the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and beta-actin genes, respectively, using an AIS Array (Imaging Research, St. Catharines, Canada). Each normalized gene expression signal was compared with epithelial and stromal areas and was automatically calculated as a ratio (12, 13).

Isolation and culture of endometrial and stromal cells and RNA extraction. Five samples were used in this study. Human endometrial tissue samples were washed in Hanks' balanced salt solution (HBSS; Gibco BRL, Grand Island, NY) and minced into small fragments. The isolation of stromal cells was performed as previously described (14). Briefly, tissue fragments were centrifuged at 170*g* for 5 minutes and the supernatant was removed. Stromal cells were isolated from epithelial cells by digesting with HBSS containing 0.25% collagenase in a humidified 5% CO_2/95% air atmosphere at 37°C for 90 minutes. After digestion, samples were filtered through a 38-μm steel-mesh filter. Filtrates were centrifuged at 170*g* for 5 minutes to collect stromal cells. The tissue fragment residue on the steel mesh was washed and refiltered. Cell pellets were washed in culture medium containing F-12 Nutrient Mixture, 10% fetal bovine serum (FBS), 100 IU/mL penicillin, and 100 μg/mL streptomycin. Cells were allowed to adhere overnight in a humidified 5% CO_2/95% air atmosphere at 37°C, and the medium was changed every 48 hours.

After the cells reached confluence, they were subcultured in F-12 Nutrient Mixture without FBS for an additional 24 hours. Cells were incubated with 10 nmol/L and 100 nmol/L E_2 for 18 hours. RNA was extracted using Trizol reagent (Life Technologies, Grand Island, NY) according to the manufacturer's instructions. Reverse transcription was performed using a TaKaRa RNA PCR Kit (AMV) ver. 2.1 (Takara Bio, Shiga, Japan) according to the manufacturer's instructions.

Real-time PCR. RNA was reverse transcribed using the oligo (dT) primers in the TaKaRa RNA PCR Kit (AMV) ver. 2.1 (Takara Bio) according to the manufacturer's instructions. PCR was performed using an ABI PRISM 7700 Sequence Detection System. TaqMan Universal PCR Master-Mix and Assay-on-Demand Gene Expression probes (Assay ID Hs00370385, context sequence AGAGCATAAGTA-CATCCAGGTTGTC, Applied Biosystems, Foster City, CA) were used for the PCR step. Primer sequences are not publicly available, although their validity has been established by the manufacturer.

Expression values obtained were normalized against those from the control human GAPDH (15). Statistical signifi-