Protein profiling of human endometrial tissues in the midsecretory and proliferative phases of the menstrual cycle

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Objective: To identify the proteins displaying differential expression in midsecretory phase endometrium as compared with proliferative phase endometrium.
Design: Prospective study with two groups of women in the midsecretory or proliferative phase.
Setting: Clinical research outpatient department.
Patient(s): Healthy, regularly cycling women of proven fertility.
Intervention(s): Collection of endometrial biopsy samples.
Main Outcome Measure(s): Image analysis software was used to compare two-dimensional protein maps of midsecretory phase endometrial tissues (MSE) with maps of proliferative phase endometrial tissues (PROE) and midsecretory phase uterine fluids (MSU). Matrix-assisted laser desorption/ionization time of flight in tandem (MALDI-TOF-TOF) analysis was carried out to identify eight proteins that were differentially expressed between the two phases and also to identify the spots that shared similar coordinates in the two-dimensional maps of MSE and MSU.
Result(s): Densitometry analysis and subsequent MALDI-TOF-TOF analysis revealed up-regulation of calreticulin, the β chain of fibrinogen, adenylate kinase isoenzyme 5, and transferrin in the PROE and of annexin V, α1-antitrypsin, creatine kinase, and peroxidoxin 6 in MSE compared with the other phase. Superimposition of the two-dimensional maps of MSE on those of MSU revealed the presence of heat-shock protein 27, transferrin, and α1-antitrypsin precursor in both endometrial tissues and uterine secretions.
Conclusion(s): Differentially expressed proteins identified in the present study could be of relevance in endowing the endometrium with receptivity. (Fertil Steril® 2009;92:1091–103. ©2009 by American Society for Reproductive Medicine.)

Key Words: Two-dimensional polyacrylamide gel electrophoresis, endometrial tissue, midsecretory phase, protein maps, receptivity

Functional genomics tools have generated a copious amount of information on cycle-dependent molecular phenotypes of the human endometrium (1–5). This information may unravel the specific molecular signatures of the human endometrium during the midsecretory or receptive phase (days 6 to 9 after ovulation), the phase of the menstrual cycle during which the uterus achieves competency for implantation. Keen interest in deciphering the code of endometrial receptivity has stemmed from poor pregnancy outcomes and failures in a significant percentage of cases who opt for assisted reproductive technologies.

Studies on comparative transcriptomes of the human endometrium in different phases of the menstrual cycle have demonstrated differential expression of several genes regulating intracellular signaling, transcription, and metabolism in the midsecretory or receptive phase (3–5). However, consensus on the number, identity, and the expression pattern of the factors associated with endometrial receptivity has yet to be achieved. Of several differentially expressed factors, investigators have found that only few such as osteopontin, claudin-4, and endothelin follow a similar pattern of expression (1–3, 6). Considering that microarrays do not reveal the effect of posttranscriptional or posttranslational regulation on protein expression, few proteomics-based studies have been undertaken (7–9). However, the focus of these investigations was not stringently upon the midsecretory phase protein profile of endometrium; and in some cases, the outcome of the study was restricted by technological limitations.

Our study used two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) to construct a 2D map of

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midsecretory-phase human endometrium and to identify the proteins that are differentially expressed compared with proliferative-phase endometrium. The midsecretory-phase endometrial tissue 2D map was also compared with that of uterine fluid from the same phase. These studies led to identification of polypeptide spots hitherto unknown for their cycle-dependent variations or for their coexistence in endometrial tissue and uterine fluid.

MATERIALS AND METHODS

Participants

Collection of samples from healthy women for our study was approved by the institute's human ethics committee. All women gave informed consent to participate in the study.

Samples were collected from women of reproductive age (25 to 35 years) who had a history of regular monthly menses and had had at least one live birth, as described previously elsewhere (10). The range of menstrual cycle length in these women was 28 ± 2 days. Women using any hormone contraceptive method or who had symptoms of reproductive tract infection, polycystic ovary syndrome, or uterine fibroids were excluded from the study. Endometrial tissue samples were obtained using a Probet (Gynetics Medical Products N.V., Hamont-Achel, Belgium) from six women in their midsecretory phase (on day 6 after ovulation) and from five women during their proliferative phase (on day 2 or 3 before ovulation). Serial ultrasonography was performed to document the ovulation. The first serial ultrasonography was started on day 6 or day 7 of the menstrual cycle, depending on the length of the last menstrual cycle; the second was performed on day 8 or day 9, and then it was done daily until evidence of follicle rupture (i.e., ovulation). Proliferative phase samples were collected on day 2 or 3 before the expected day of ovulation, predicted on the basis of the length of the last menstrual cycle. In these women serial ultrasonography was continued until follicle rupture. This helped to determine retrospectively the actual day of sample collection with respect to the day of ovulation. For this group, samples were collected from more women, and the analysis included only those who had ovulated 2 to 3 days after the sample collection. Before collecting the biopsy samples, we collected uterine fluid samples from women in both groups, as described previously elsewhere (10).

Samples were immediately fixed in 10% buffered formalin for endometrial dating. Blood samples were also collected from the women on the day of biopsy sampling. Progesterone levels in sera were estimated by specific radioimmunoassays. Endometrial tissues fixed in 10% buffered formalin were incubated in 70% vol/vol ethanol for 24 hours and were embedded in paraffin. Paraffin sections were cut at 5 μm and stained with hematoxylin and eosin. Endometrial biopsy samples were dated according to Noyes' criteria (11). The histologic features of the samples were found to be in accordance with the characteristics of the cycle phase during which the samples had been collected.

Cell Lines

The Ishikawa cell line (Sigma-Aldrich, St. Louis, MO) was maintained in phenol red free Dulbecco modified Eagle's minimal essential medium (DMEM) + Ham's F12 (1:1, vol/vol) supplemented with 10% fetal calf serum (FCS) in humidified chambers containing 95% air and 5% CO_2 at 37°C. Two million cells were plated in T75 culture flask 16 hours before the experiment. Cells at 50% to 60% confluency were treated with phenol red free DMEM + Ham's F12 (1:1, vol/vol) containing 5% charcoal stripped FCS with or without steroids. The hormones were added from a 1000-fold concentrated stock prepared in 100% ethanol. Estradiol (10^{-8} mol/L), progesterone (10^{-6} mol/L), or vehicle (ethanol 0.001%) was added to cells as described previously elsewhere (12). Cells were harvested after 2 days for flow cytometry.

Flow Cytometry

Adherent cultures of the Ishikawa cell line treated with steroids or vehicle were rinsed twice with phosphate-buffered saline (PBS) and then incubated with trypsin and ethylenediaminetetraacetic acid (EDTA) (0.025% trypsin in 0.1 mM EDTA) for 5 to 10 minutes to detach the adherent cells. Cells (100,000/100 μL PBS) were fixed in 4% paraformaldehyde for 20 minutes at room temperature and washed twice with PBS for 10 minutes each by centrifugation at 300 × g at room temperature. Cells were then permeabilized with 0.1% NP-40 for 2 minutes at room temperature and washed twice with PBS for 10 minutes at room temperature. Aliquots of 500 μL were resuspended in 300 μL of 4% bovine serum albumin (BSA) in PBS (BPBS) and incubated for 20 minutes at room temperature to prevent nonspecific binding. This was followed by resuspension of cells in primary antibody (Sigma-Aldrich) raised in rabbit against antihuman calreticulin (at 1:100 dilution in BPBS) or antihuman anti-α1-antitrypsin precursor (at 1:50 dilution in BPBS) and overnight incubation at 4°C. Cell pellets were then washed with 1 × PBS at 300 × g for 10 minutes, resuspended in 300 μL BPBS, and incubated with secondary antibody (swine anti-rabbit antibody conjugated to FITC-DAKO) diluted 1:100 in BPBS for 2 hours. Pellets washed with PBS were analyzed using a FACScan with CELLQUEST software for data acquisition and data analysis (Becton Dickinson, San Jose, CA).

Cells labeled with secondary antibody conjugated to fluorescein in the absence of primary antibody were used to obtain the median of background fluorescence (usually 0.5% to 1.0%). This value was subtracted from the median fluorescence of the other samples (labeled with secondary antibody in the presence of primary antibody). The adjusted median of each sample (treated with steroid, progesterone or estradiol) was then divided by the adjusted median of vehicle-treated sample (control) to obtain value X for each stimulated sample. The percentage increase or decrease in the median fluorescence of the stimulated sample relative to the median fluorescence of the control sample was calculated as $(1 - X) \times 100$.