Expression and activation of the membrane-cytoskeleton protein ezrin during the normal endometrial cycle

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Objective: To examine total ezrin expression (ezrin and phospho-ezrin) through the normal endometrial cycle and to correlate ezrin activation and localization with cytologic changes.
Design: Experimental laboratory study.
Setting: University medical centers.
Patient(s): Reproductive-age women.
Intervention(s): A total of 36 samples of normal early, mid-, and late proliferative- and secretory-phase endometrium were studied for immunoreactive total ezrin (ir-T-ezrin) and phospho-ezrin (ir-p-ezrin) expression by histology, immunohistochemistry, and Western blotting.
Main Outcome Measure(s): Total ezrin and phospho-ezrin expressions through the normal endometrial cycle.
Result(s): Throughout the cycle ir-T-ezrin is present in the epithelium. The intensity and localization of both ir-ezrin and ir-p-ezrin vary greatly throughout the cycle. The main findings include the following: lateral localization of ir-ezrin/ir-p-ezrin in association with membrane specializations; dense staining around secretory vacuoles (secretory phase); dense staining of the apical surfaces, including microvilli and pinopodes of epithelial cells, especially during the mid- to late secretory phases; and the presence of ezrin in the glandular secretions. Immunoreactive total ezrin and ir-p-ezrin were not expressed by stromal fibroblasts.
Conclusion(s): Ezrin is a prominent protein in the cycling endometrium. The most striking findings were the gravitation of ir-ezrin/ir-p-ezrin to the periphery of secretory vacuoles, localization on apical surfaces of the luminal epithelium, dense ezrin staining in secretory-phase epithelial cell plumes, and the presence of ir-ezrin/ir-p-ezrin in secretory-phase luminal secretions. These findings may have functional implications, especially for implantation biology. (Fertil Steril® 2012;97:192–9. ©2012 by American Society for Reproductive Medicine.)
Key Words: Ezrin, phospho-ezrin, endometrium, histology, pinopodes, implantation

The human endometrium is specialized for reproductive success. During the ovarian cycle the endometrium first exhibits proliferation, then differentiation into a secretory organ. Finally, depending on the arrival of an embryo, the endometrium undergoes decidualization or tissue breakdown (1, 2). Hormones, growth factors, cytokines, and transcription factors (such as forkhead box protein O1, interferon regulatory factor-1, homeobox10) are involved during the preparation and development of an appropriate endometrium for blastocyst adhesion and implantation (3–6). For example, homeobox10 is a member of a family of genes that serve as transcription factors during development; its transcription product has been shown to be expressed in both epithelial and stromal cells, with increased expression during the window of implantation (7). Estrogen (E) drives the proliferative phase until progestins from the luteinizing follicle curtail proliferation in favor of differentiation. All of these effects are mirrored by cytologic changes that are regulated by the actin cytoskeleton (8). From the mid-secretory phase the gland lumens contain glycoproteins that seem to foster implantation.

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Additionally, the glands express increased adhesion proteins that participate in embryo adhesion and implantation (9). At the peak of the secretory phase (mid-secretory phase; day 21 of a 28-day cycle) the endometrium reaches its maximum thickness, accumulation of secretions, and the development of an inflammatory response of the stroma, all part of the implantation process (10). In the event of a barren cycle, when the corpus luteum expires, there is shedding of the upper endometrium (functionalis) and the next endometrial cycle begins with the glandular cells and stroma of the basalis layer beginning to proliferate and re-epithelize the open wound (11, 12). All of these activities require cellular and tissue remodeling (13).

Ezrin belongs to the ERM (ezrin, radixin, moesin, Merlin; band 4.1) family of proteins that gravitate toward the cell membrane (14, 15). Phosphorylation of ezrin at tyrosine 353 (p-ezrin) is required for cytoskeletal rearrangements (14, 16) that are involved in cell–cell attachments and membrane specializations (15, 17, 18). Ezrin tethers actin filaments to the plasma membrane, facilitating the development of lateral specializations such as zonae adherens. Ezrin also fosters cells' polarization and enables cells to perform specialized functions, motility, and proliferation (15, 19). Ezrin expression is in part regulated by E (20, 21), the main driver of the proliferative phase. Estrogen and growth factors induce ezrin and ezrin activation; this phosphorylation regulates development of specializations such as microvilli, ruffles, brush borders, and junctional complexes (15, 22, 23). These cell–cell interactions likely play roles in embryo adhesion and implantation during the luteal phase (15, 24–27).

In this study, we examined the cyclic expression of total immunoreactive ezrin (ir-ezrin) and phospho-ezrin (ir-p-ezrin) in the normal endometrium. We also correlated ezrin activation and localization with cytologic changes during the normal endometrial cycle.

MATERIALS AND METHODS

Tissue Collection

Endometrial tissues were obtained from hysterectomy specimens for benign conditions or from endometrial biopsies. Endometrial malignancy, polyps, and submucosal fibroids were excluded. The mean patient age was 37.3 years (range, 24–50 years). Written, informed consent approved by institutional review boards of Yale University, New York University, and University of Texas Southwestern Medical Center was obtained from each patient before surgery. The day of the menstrual cycle was established from the patient's menstrual history and was verified by histologic examination of the endometrium (28). A total of 36 endometrial samples from different phases of normal endometrial cycle were classified as early proliferative (days 4–7; n = 6), mid-proliferative (days 8–10; n = 6), late proliferative (days 11–14; n = 6), early secretory and late secretory phase (days 24–28; n = 6). All tissues were fixed in 4% paraformaldehyde for 4–6 hours and embedded in paraffin or placed in Hank's balanced salt solution and transported to the laboratory for Western blot analysis. All studies were performed in triplicate.

Immunohistochemistry

Serial sections were collected on poly-L-lysine–coated slides (Sigma), dewaxed, dehydrated, then placed in citrate buffer (pH 6; 1 L distilled water; 2.1 g citric acid, 15 mL NaOH). Immunohistochemical detection procedures have been described previously (29) and were slightly modified for our antibodies. Initially antigen stabilization was performed by twice-treating the samples in a microwave oven at 750 W for 5 minutes. After cooling for 10 minutes at room temperature, the sections were washed in phosphate-buffered saline (PBS; pH 7.4). To exhaust endogenous peroxidase activity, sections were kept in 3% fresh hydrogen peroxide (Dako) for 30 minutes and then washed three times with PBS. Two primary antibodies were used: monoclonal (mAb) mouse anti-human ezrin IgG1 isotype (1:600; Clone 3C12, Sigma-Aldrich) and rabbit anti-human phospho-ezrin (Tyr353) antibody (1:100; Sigma-Aldrich). Whereas the ezrin antibody binds to total ezrin (T-ezrin; both ezrin and phospho-ezrin), the phospho-ezrin antibody detects ezrin if it is phosphorylated at tyrosine 353 (26, 30). Neither antibody cross-reacts with moesin, radixin, p-moesin, p-radixin, or Merlin (16, 26). After 30 minutes' incubation with blocking solution to inhibit nonspecific binding, the sections were incubated with the primary antibodies for 2 hours at room temperature. This was followed by sequential 30-minute incubations with biotinylated secondary antibodies (Vector Laboratories) and peroxidase-labeled streptavidin. Detection was performed using a horseradish peroxidase–labeled streptavidin–biotin kit (Labvision) that was developed with diaminobenzidine (Labvision). The sections were counterstained with Mayer's hematoxylin solution (Merck) and mounted with glycerol-gelatin (Sigma). Replacing the primary antibodies with the appropriate nonimmune IgG or isotypes served for negative controls. Photomicrographs were taken using Zeiss Axio Observer wide-field microscopy.

Semiquantitative Immunohistochemistry Evaluation by "H-SCORE"

Tissue sections from different menstrual phases were evaluated for localization and intensity of the antigens. Stained cells were assigned to one of four categories of staining density: 0 (no staining), 1+ (weak but detectable staining), 2+ (moderate or distinct staining), or 3+ (intense staining). In each section, for comparison an average density of staining between cell types, an "H-SCORE" value, was calculated by summing the percentages of each type of cells grouped in each intensity category and multiplying this number by the weighted intensity of the staining, using the formula [H-SCORE = $P_i(i + l)$], where i represents the intensity scores, and P_i is the corresponding percentage of the cells. In each slide, five different areas were evaluated under a microscope using 40× original magnification. Two investigators who were blinded to slides and menstrual timing determined the percentage of the cells' staining at each intensity within these areas. The inter- and