A novel in vitro model of the early endometriotic lesion demonstrates that attachment of endometrial cells to mesothelial cells is dependent on the source of endometrial cells

R. Scott Lucidi, M.D., Craig A. Witz, M.D., Michelle Chrisco, M.S., Peter A. Binkley, M.S., Sydney A. Shain, Ph.D., and Robert S. Schenken, M.D.

Division of Reproductive Endocrinology and Infertility, Department of Obstetrics and Gynecology, University of Texas Health Science Center at San Antonio, San Antonio, Texas

Objective: To characterize the source of variability in endometrial stromal cell (ESC) binding to peritoneal mesothelial cells (PMC).
Design: In vitro study.
Setting: University medical center.
Patient(s): Reproductive-age women without endometriosis undergoing surgery for benign conditions.
Intervention(s): None.
Main Outcome Measure(s): Binding of ESCs (n = 9) to PMCs collected from the anterior abdominal wall (AAW) (n = 5), a commercially available mesothelial cell line (LP9) (three different passages) and normal ovarian surface epithelium (NOSE) (n = 5).
Result(s): There were no differences in the binding of same-source ESCs to mesothelial cells obtained from the AAW of different women, to different passages of LP9s or to NOSE of different women. There was a trend toward increased binding of ESCs to NOSE compared to AAW PMCs. In contrast, there were significant differences in the ability of ESCs obtained from different women to bind to same-source PMCs.
Conclusion(s): There is significant variability in ESC binding to PMCs. This variation is dependent primarily on the source of the ESCs. The ESC binding to LP9 PMCs was similar to AAW PMCs and NOSE. (Fertil Steril® 2005;84:16–21. ©2005 by American Society for Reproductive Medicine.)

Key Words: Endometriosis, mesothelium, endometrial stromal cell, attachment

Endometriosis is defined as the ectopic location of endometrial tissue outside the uterine cavity. Sampson's (1) theory proposes that fragments of menstrual endometrium pass retrograde through the fallopian tubes, then attach and grow on peritoneal surfaces. There is mounting evidence that Sampson's theory explains the development of the majority of endometriotic lesions (2). However, this theory fails to explain why only some women develop endometriosis. Although most women (90%) with patent fallopian tubes have reflux menstruation into the peritoneal cavity (3, 4), the incidence of endometriosis is much lower affecting approximately 5%–10% of reproductive-age women and up to 30% of infertile women (5). The specific factors involved in the implantation of refluxed endometrium to the peritoneal surface remain unknown.

Some investigators have questioned whether an intact mesothelial cell layer prevents adhesion of endometrial fragments to the peritoneum (6–8). However, we recently demonstrated that proliferative, secretory, and menstrual endometrial fragments rapidly attach to intact peritoneal mesothelium in culture (9–12). These studies demonstrated that both endometrial stromal cells (ESCs) and endometrial epithelial cells attach to peritoneal mesothelial cells (PMCs) within 1 hour and that transmesothelial invasion rapidly occurs within 12–24 hours. These studies also showed that the percentage of ESCs bound to PMCs varied. The source of this variation, however, is not clear. Because these studies used ESCs and PMCs from different sources, the variability could be due to differences in the ESCs, PMCs, or both.

The purpose of this study was to examine the source of variability in ESC binding to PMCs. Using the model described herein, we compared the attachment of ESCs from several women to PMCs of different sources. The PMC sources used included the anterior abdominal wall (AAW), a commercially available mesothelial cell line (LP9), and normal ovarian surface epithelium (NOSE).

MATERIALS AND METHODS

Approval for this study and collection of endometrium and peritoneum was obtained from the Institutional Review

Received May 25, 2004; revised and accepted October 26, 2004.
Supported by National Institutes of Health grant 1R01 HD044135.
Presented at the 51st Annual Meeting of the Society for Gynecologic Investigation, Houston, Texas, March 24–27, 2004.
Reprint requests: R. Scott Lucidi, M.D., University of Texas Health Science Center at San Antonio, Department of Obstetrics and Gynecology, 7703 Floyd Curl Drive, San Antonio, Texas 78284 (FAX: 210-567-4963; E-mail: lucidi@uthscsa.edu).

0015-0282/05/$30.00
doi:10.1016/j.fertnstert.2004.10.058

Board of the University of Texas Health Science Center at San Antonio.

Endometrial Cell Culture

Proliferative phase endometrium was obtained immediately after hysterectomy or by aspiration biopsy using a Pipelle (Unimar Inc., Prodimed, Neuilly-en-Thelle, France) during surgery performed for benign conditions (such as pelvic organ prolapse, stress urinary incontinence, menorrhagia) in women (n = 9) not taking hormonal medication. Tissue samples were placed in Cellgro (Mediatech, Herndon, VA) complete serum-free medium for transport to the laboratory. We chose proliferative phase endometrium because it is readily available from women undergoing surgery and because it is more easily cultured.

Endometrium was mechanically dispersed with a scalpel. The ESCs were separated from the endometrial samples as previously described by Kirk and Irwin (13). Briefly, endometrium was enzymatically digested with 0.1% collagenase type 1 and 0.05% DNAse. Endometrial epithelial cells were separated from ESCs by gravity sedimentation. The stromal cell-rich supernatant was placed in a culture flask and cells were allowed to adhere for 20 minutes then washed with medium. Adherent ESCs were cultured as monolayers in flasks with Dulbecco's modified Eagle Medium (DMEM)/F-12 (1:1) (Sigma, St. Louis, MO) containing antibiotics/antimycotics, 5 μg/mL insulin (Sigma), and 10% fetal calf serum (HyClone, Logan, UT).

After the second passage, ESCs were cultured on eight-well chamber slides. Purity of cultures was assessed morphologically after hematoxylin and eosin (H & E) staining. Stromal cells were fusiform, but more rounded than fibroblasts. Cells were also evaluated immunohistochemically using monoclonal antibodies to human cytokeratin, vimentin, von Willebrand factor, and CD45. The ESCs expressed vimentin, and did not express cytokeratin, von Willebrand factor, or CD45. Using these techniques of ESC separation, and as defined by these criteria, there is greater than 97% purity of ESCs (14, 15).

Peritoneal Mesothelial Cell Culture

Peritoneum from the AAW was obtained from reproductive-age women (n = 5) without endometriosis undergoing surgery for benign conditions. At the time of surgery, there was no evidence of infection. Peritoneum was obtained from locations free of adhesions. Care was taken not to abrade the mesothelium or allow the tissue to air dry. The tissue was immediately placed in DMEM (Sigma) plus 10% defined fetal calf serum (FCS) and transported to the laboratory.

The PMC cultures were established as previously described (16). Briefly, PMCs were enzymatically dispersed from sections of peritoneum using 0.1% collagenase, type 1 and 0.05% DNAse. Cells were plated in 25-cm^2 tissue culture flasks and grown in MCDB-131/Medium 199 (1:1) (Sigma) supplemented with epidermal growth factor (20 ng/mL), L-glutamine (2 mM), hydrocortisone (400 ng/mL), 1% antibiotic/antimycotic, HEPES buffer, and 15% FCS.

The NOSE was collected from ovaries removed electively from reproductive-age women (n = 5) undergoing surgery for benign disease. Collection and culture of NOSE cells was similar to that described by other investigators (17, 18). Briefly, the ovary was placed in trypsin–EDTA solution (Sigma) for 5 minutes and then transferred to a 6-cm petri dish containing DMEM plus 10% defined FCS. The ovarian surface was gently scraped with a cell scraper (Nunc, Naperville, IL) and the medium containing NOSE cells was transported to the laboratory. The NOSE cells were cultured in the same medium as PMCs from the AAW.

At first passage, AAW PMCs and NOSE were plated on eight-well chamber slides (Nunc). Morphologic assessment of these replicates of monolayer cultures was performed after H & E staining. The AAW PMCs and NOSE were polygonal with irregular borders (16).

Purity of cultures was determined by incubation with monoclonal antibodies to human cytokeratin (Oncogene Science, Uniondale, NY), vimentin (Oncogene Science), CD45 (The Binding Site, San Diego, CA), von Willebrand factor (Dako, Carpinteria, CA) and epithelial membrane antigen (Dako) (16, 18, 19). Immunohistochemistry was performed as described for the ESC culture. The PMCs from the AAW and NOSE expressed cytokeratin and vimentin (19), and did not express CD45 and von Willebrand factor. The NOSE cells did not express epithelial membrane antigen (18).

The LP9 peritoneal mesothelial cell line, obtained from the National Institutes of Health Aging Cell Repository (Coriell Institute for Medical Research, Camden, NJ), was grown in the same media as AAW PMCs and NOSE. Characterization of this cell line by immunohistochemistry was similar to AAW PMCs.

ESC Labeling and Fluorescence Readings

After culture, ESCs were collected using nonenzymatic cell dissociation media (Sigma) and washed with stromal cell complete medium. Cells were centrifuged at 650 $\times$ g for 2 minutes. The pellet was labeled with 5 μM calcein-AM (Molecular Probes, Inc., Eugene, OR) for 20 minutes at 37°C, followed by washing in Hank's balanced salt solution (Invitrogen, Grand Island, NY). Calcein-AM is converted by acetoxymethyl ester hydrolysis in viable cells. Peak absorption and emission for calcein are at the wavelengths 494 nm and 517 nm, respectively. Calcein-AM–treated ESCs were initially plated on 96-well plates at various concentrations obtained by serial dilution. Fluorescence readings were taken for each well using a fluorescence bioassay reader (model HTS-7000, Perkin-Elmer, Norwalk, CT) with filters for absorption and emission of 485 nm and 538 nm, respectively. Fluorescence readings obtained were linearly proportional to the number of cells in each well over a range of 625–300,000