Increased expression of macrophage colony–stimulating factor and its receptor in patients with endometriosis

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Objective: To investigate the expression and regulation of colony-stimulating factor 1 (CSF-1) and its receptor, C-FMS, in endometriosis.
Design: In vivo and vitro study.
Setting: University-based academic medical center.
Patient(s): Reproductive-age women undergoing surgery for benign conditions.
Intervention(s): Peritoneal and endometrial tissue samples were obtained.
Main Outcome Measure(s): CSF-1 and C-FMS expression.
Result(s): Significantly higher CSF-1 levels were found in peritoneal fluid of patients with endometriosis compared with control subjects. Ectopic endometriotic tissue had 3.5-fold and 1.7-fold increases in CSF-1 and C-FMS expression, respectively, compared with eutopic tissue. Coculture of endometrial cells from either established cell lines or patient samples with peritoneal mesothelial cells (PMCs) led to increased expression of CSF-1 and C-FMS. A higher but nonsignificant increase in levels of C-FMS and CSF-1 was found in cocultures of endometrial epithelial cells from patients with endometriosis compared with those without endometriosis.
Conclusion(s): Increased CSF-1 levels may contribute to endometriosis lesion formation and progression. Elevation in CSF-1 after coculture of endometrial cells with PMCs suggests that endometrial tissue may be a source of peritoneal CSF-1. Increased C-FMS expression in endometrial cells from women with endometriosis cocultured with PMCs suggests that endometrial tissue involved in lesion formation is highly responsive to CSF-1 signaling. (Fertil Steril® 2012;97:1129–35. ©2012 by American Society for Reproductive Medicine.)
Key Words: Endometriosis, colony-stimulating factor 1, C-FMS, early lesion development, macrophage, cell culture, peritoneal fluid

Endometriosis affects 10% of reproductive-age women and is associated with infertility and pelvic pain (1, 2). The most widely accepted etiology is Sampson's theory of retrograde menstruation where shed endometrial tissue is refluxed through the fallopian tubes to attach and proliferate within the pelvis (1). Evidence supporting this theory is twofold: First, an increased incidence of endometriosis is seen in women with uterine outflow obstruction; second, placement of endometrial tissue in the peritoneal cavity results in endometriotic lesions in other animals (3). However, both women with and without endometriosis undergo retrograde menstruation, suggesting that other factors may mediate the formation of endometriotic lesions (4, 5). It has been postulated that susceptibility to endometriosis is due to enhanced endometrial cell adhesion to the peritoneum or poor clearance of refluxed endometrial cells by host immune responses. Increased peritoneal fluid leukocytes, cytokines and growth factors have been found in patients with endometriosis, suggesting that abnormalities in immune function may predispose patients to development of endometriosis (4).

Cytokines are synthesized by peritoneal macrophages, lymphocytes, and mesothelial cells as well as by endometriosis implants (6). Colony-stimulating factor 1 (CSF-1) is a cytokine produced by macrophages and has been implicated in the pathogenesis of endometriosis. Acting through its receptor, C-FMS, CSF-1 stimulates growth, recruitment, and differentiation of macrophages (7). Increased levels of CSF-1 and its receptor have

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been detected in gynecologic cancers and may contribute to malignant invasiveness. Cancer cells expressing C-FMS are responsive to growth stimulation by CSF-1 (8). Our previous studies have shown that CSF-1 stimulates endometrial epithelial cells via an autocrine mechanism (9). Knockdown of CSF-1 in endometrial epithelial cells results in decreased proliferation and transmesothelial invasion of endometrial epithelial cells (10). We have also demonstrated that endometrial tissue from CSF-1 knockout mice develop significantly fewer endometriotic lesions than control tissue in a syngeneic mouse model of endometrial tissue transplantation into the pelvic cavity (11).

It is unclear whether an increase in CSF-1 levels in the pelvic cavity of patients with endometriosis is due to inflammation-associated macrophages or whether increased CSF-1 is produced by endometriotic lesions themselves. Furthermore, studies on CSF-1 levels in the peritoneal fluid of women with endometriosis have been inconsistent. Fukaya et al. and Mettler et al. found an increased expression of CSF-1 and C-FMS in peritoneal fluid of patients with endometriosis, whereas Ueki et al. and Weinberg et al. noted no difference in levels of CSF-1 in peritoneal fluid of patients with endometriosis compared with those without (12–15). These inconclusive data on CSF-1 and C-FMS expression hamper our understanding of the physiologic role of CSF-1 signaling in endometriosis. To better define a physiologic context for CSF-1 signaling in endometriosis, we examined CSF-1 levels in the peritoneal fluid of women with endometriosis. Our data showed a significant increase in CSF-1 levels in women with endometriosis. We hypothesized that interaction of endometrial tissue with the mesothelial lining of the peritoneum induces CSF-1 and C-FMS expression in endometrial cells. Our data demonstrated that coculture with peritoneal mesothelial cells (PMCs) leads to elevated induction of CSF-1 and C-FMS expression in endometrial cells from women with endometriosis, suggesting that endometrial tissue may contribute to elevated CSF-1 levels in the pelvic cavity of women with endometriosis and that endometriotic lesions may exhibit heightened responsiveness to CSF-1 stimulation.

MATERIALS AND METHODS

Patient Selection and Tissue Collection

Approval for this study was granted by the Institutional Review Boards of the University of Texas Health Science Center at San Antonio and Emory University. Patients aged $\geq$18 years with a history of pelvic laparoscopy were eligible. Menstrual-phase endometrium was obtained by effluent collection or aspiration biopsy using a Pipelle (Unimar; Prodimed).

Endometrial stroma cells (ESCs) and endometrial epithelial cells (EECs) were separated from the endometrial samples as previously described (16–18). Briefly, endometrium was enzymatically digested with 0.1% collagenase type 1 and 0.05% DNAse. EECs were separated from ESCs by gravity sedimentation. The stroma-rich supernatant was removed from the epithelial-rich pellet, and both fractions were washed, resuspended in Dynabead epithelial-enriched magnetic polystyrene beads coated with mouse IgG1 monoclonal antibody (mAb Ber-EP4) specific for two (34 and 39 kDa) glycopolypeptide membrane antigens expressed on most normal and neoplastic human epithelial tissues, and incubated for 30 minutes. EECs were isolated from the ESCs by placing the tubes on the magnet for 2 minutes.

The ESCs from supernatant were plated in equilibrated (37°C, in 5% CO_2) culture flasks of Dulbecco Modified Eagle Medium (DMEM)/F12 (1:1) (Invitrogen Life Technologies), containing antibiotics/antimycotics, 5 ug/mL insulin, and 10% fetal bovine serum. Cells were allowed to adhere for 20 minutes then washed. Adherent ESCs were primary cultures of monolayers or passaged and frozen in aliquots of passage 1.

The pellets of EECs were plated in equilibrated culture flasks of enriched epithelium medium of MCDM 131/Medium 199/MEMα (40:40:20; Invitrogen) containing antibiotics/antimycotics, 5 ug/mL insulin, 300 ug/mL D-Glucose, and 10% fetal bovine serum. EECs were primary cultures of monolayers or passaged and frozen in aliquots of passage 1.

Flow cytometry was performed to quantitate separation of EECs from ESCs; 98.6% of purified ESCs expressed high levels of the stromal marker vimentin, whereas only 2% expressed the epithelial marker cytokeratin-18 (Fig. 1). More than 98% of purified EECs expressed cytokeratin-18. Our data are consistent with earlier studies showing high purification of EECs and ESCs using the methods developed by Irwin et al. (19).

Paired Ectopic and Eutopic Tissue

Paired eutopic and ectopic endometrial tissue samples were obtained from patients (n = 7) aged $\geq$18 years undergoing scheduled surgery for endometriosis. Follicular-phase endometrium and peritoneal biopsies were collected at the time of laparoscopy. Tissue samples were immediately frozen in liquid nitrogen and stored in −80°C until used for Western blot analysis as described below.

Peritoneal Fluid Collection and CSF-1 ELISA

Patients undergoing laparoscopy for benign gynecologic conditions aged $\geq$18 years were eligible for enrollment. Peritoneal fluid was obtained from women with (n = 72) and without (n = 43) endometriosis and purified by Ficoll gradient purification. CSF-1 ELISA was performed on peritoneal fluid with the use of the R&D M-CSF Quantikine ELISA kit per the manufacturer's directions. CSF-1 levels were calculated in ng/mL.

Western Blot of CSF-1 and C-FMS in Endometrium and Endometriosis Tissues

Protein extracts from endometrial tissue were prepared by homogenizing the tissue in protein lysis buffer containing Triton-X and protease inhibitors. Equal amounts (generally 60–75 μg) of protein from each sample were separated on a denaturing polyacrylamide gel and transfered to nylon membrane. The protein-bound membranes were then incubated for $\geq$4 h at room temperature with Tris-buffered saline solution containing 0.05% Triton X-100 (TBST) and 5% nonfat dry milk to block nonspecific antibody binding. The

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