Regulation and activation of ezrin protein in endometriosis

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BACKGROUND: Ezrin protein and its activated form phospho-ezrin play a role in cell morphology, motility and adhesiveness. In this study, we hypothesized that these proteins play a role in the pathogenesis of endometriosis by promoting adhesion and invasion of endometrial stromal cells (ESCs) in ectopic sites. METHODS: We compared the expression of ezrin and phospho-ezrin in normal endometrium from women without endometriosis with their expression in eutopic and ectopic endometrial tissues from women with endometriosis, using immunohistochemistry and western blot analysis. Paired eutopic and ectopic endometrial tissue samples from women with endometriosis (n = 13) and normal endometrium from women without endometriosis (n = 12) were collected. Invasive potential of ESCs from each of these samples was compared using Matrigel membrane invasion assay. RESULTS: Eutopic and ectopic endometrial tissue samples from women with endometriosis have higher ezrin and phospho-ezrin levels as confirmed by immunohistochemistry and western blot analysis (P < 0.05). The Matrigel membrane invasion assay revealed that ectopic ESCs have more invasive characteristics, more protrusions and higher ezrin staining than normal ESCs (P < 0.05). CONCLUSIONS: Ezrin can be a potential marker for endometrial cell invasion and may play a role in the pathogenesis of endometriosis.

Keywords: endometriosis; endometrium; ezrin; phosphoezrin

Introduction

Endometriosis is a common gynecologic disorder characterized by the presence of endometrial tissue outside the uterine cavity. Although various theories have been put forth to explain the mechanisms for the development of this disease, the retrograde menstruation and implantation hypothesis of Sampson (1927) is the most widely accepted one. This hypothesis suggests that endometriotic implants reach their most common site of implantation, the peritoneal cavity, by traveling through the fallopian tubes during menstrual shedding. Shed menstrual endometrium is viable (Keettel and Stein, 1951) and so are the endometrial cells found in the peritoneal fluid (Kruitwagen et al., 1991). In order to implant and grow, these cells need to establish cell-to-cell or cell-to-extracellular matrix (ECM) interactions with the peritoneal lining. It is likely that ECM is actively involved in the adhesion of endometrial implants to the peritoneum. There is evidence suggesting that early endometriosis lesions invade the ECM of the peritoneum after the initial attachment (Spuijbroek et al., 1992). Interactions between endometrial cells and the ECM are mostly mediated by the integrin family of cell-surface receptors (Juliano and Haskell, 1993), thus related to linker proteins called ERM (i.e. ezrin, radixin, moesin) which could be responsible for the attachment and invasion of the endometrial cells in endometriosis.

Ezrin belongs to ERM family of proteins that are localized just beneath the plasma membrane of cellular protrusions such as microvilli (Bretscher et al., 1997). These proteins act as linkers between the plasma membrane and the actin cytoskeleton and are involved in cell adhesion and membrane ruffling (Tsukita and Yonemura, 1997). Ezrin tethers actin filaments to the plasma membrane on one hand and anchors membrane proteins at specific sites on the other, thus enabling cells to maintain specialized functions in defined surface environment. Many recent studies have investigated the relationship of ezrin with cancer (Makitie et al., 2001; Ohtani et al., 2002; Pang et al., 2004; Slater et al., 2007), but the relationship with endometriosis has not been investigated. Berryman et al. reported that ezrin is located within the cell–cell junctions in which the cytoskeleton networks are being remodeled, thus further implicating that ezrin plays a major role in the regulation of cell morphology, migration and attachment (Berryman et al., 1993). In addition, ezrin interacts with cell-surface adhesion molecules including CD44 and ICAM-1 (Hirao et al., 1996; Tsukita and Yonemura, 1997; Heiska et al., 1998; Legg and Isacke, 1998). In the pathogenesis of endometriosis, endometrial cells need to establish cell to cell interactions with the peritoneal lining to adhere, implant and grow ectopically. Therefore, we hypothesized that ezrin may...
play an important role in promoting cell attachment, invasion and growth in endometriosis.

Ezrin is regulated by growth factor-induced phosphorylation (Bretscher, 1989; Jiang et al., 1995; Crepaldi et al., 1997). Two cortical proteins, ezrin and spectrin, become phosphorylated in a coincident time frame with the remodeling of the cell surface. ERM proteins undergo intra- or intermolecular interaction between their N- and C-terminal domains, existing as inactive, cytosolic monomers or dimers (Mangeat et al., 1999). Phosphorylation at a C-terminal threonine residue, which disrupts the N- and C-terminal association, may play a key role in modulating the conformation and function of ERM proteins (Matsui et al., 1998; Gautreau et al., 2000). Phosphorylation at Thr567 of ezrin is required for cytoskeletal re-arrangements and oncogene-induced transformation (Tran Quang et al., 2000). Ezrin is also phosphorylated upon growth factor stimulation. This is consistent with the notion that phosphorylated ezrin is the activated form of ezrin, which may play a role in the formation of cell-surface projections.

Since ezrin and phosphorylated ezrin (phospho-ezrin) are proteins that participate in cell attachment, migration and invasion, they may have a role in the development of endometriosis.

Our main objective in this study is to investigate the expression pattern of ezrin in endometriosis. Furthermore, we aimed to determine the differences in the ezrin and phosphorylated ezrin expression between normal endometrium, and eutopic and ectopic endometrial tissues of women with endometriosis.

Materials and Methods

Tissue collection

Endometrial tissue samples were collected from women undergoing laparoscopy, laparotomy or hysterectomy. Written informed consent was obtained from each patient before surgery, using consent forms and protocols approved by the Human Investigation Committee of Yale University. The mean age of the patients was 35.6 (range 30–49) years.

Distributions of tissues from patients with endometriosis were as follows: peritoneal implants (n = 6), uterine serosal implants (n = 4), ovarian endometriomas (n = 3). All of them were from proliferative phase according to the last menstrual date. Homologous eutopic endometrial tissues from women with endometriosis were also obtained by endometrial biopsy.

As a control group, proliferative endometrial tissues (normal endometrium) were obtained from 12 fertile women without endometriosis (mean age 36.3, range 30–49) undergoing laparoscopy or hysterectomy for benign gynecological disorders other than endometrial disease. Indications for surgery for the control group were as follows: voluntary sterilization (n = 5) and benign adnexal mass (n = 7). These women had no visible pelvic inflammation or endometriosis at laparoscopy or laparotomy. Patients with leiomyomata were not included in the study since leiomyomata belongs to the class of estrogen-dependent diseases.

Part of the endometrial tissue was fixed in 4% paraformaldehyde for 4–6 h and embedded in paraffin for immunohistochemistry. A further part of the endometrial tissue was placed in Hank’s balanced salt solution (HBSS) and transported to the laboratory for separation and culture of endometrial stromal cells (ESCs) for the invasion assay and western blot analysis. Cells obtained from each patient were considered as separate experiments. Each experimental set-up was repeated on at least three separate occasions using cells obtained from different patients.

Immunohistochemistry

Ectopic and homologous eutopic endometrial samples from patients with endometriosis and normal proliferative endometrium from patients without endometriosis were used. Serial sections were collected on poly-l-lysine coated slides (Sigma, St Louis, MO, USA), dewaxed, dehydrated and placed in citrate buffer (pH 6). Immunohistochemical detection procedures have been described previously (Demir et al., 2002). To unmask antigens, samples were treated twice in a microwave oven at 750 W for 5 min. After cooling for 10 min at room temperature, the sections were washed in phosphate-buffered saline (PBS, pH 7.4). To remove endogenous peroxidase activity, sections were kept in 3% hydrogen peroxide (Dako A/S, Glostrup, Denmark) for 30 min and washed three times with PBS. Slides were then incubated in a humidified chamber with blocking serum (Lab Vision, Fremont, CA, USA) for 10 min at room temperature. The following primary antibodies were used: monoclonal mouse anti-human ezrin IgG1 isotype (1:600; Clone 3C12, Sigma-Aldrich, Inc.) and rabbit anti-human phospho-ezrin antibody (1:800; TYR-353, Cell Signaling Technology, Beverly, MA, USA). Phospho-ezrin (Tyr353) antibody detects endogenous levels of ezrin only when phosphorylated at tyrosine 353. The antibody does not cross-react with phosphorylated moesin or radixin. After 10 min incubation with blocking solution, sections were incubated with the primary antibodies for 1 h at room temperature. Sections were rinsed and washed three times for 5 min with PBS, then biotinylated horse anti-mouse or anti-rabbit antibody (1.5 mg/ml; Vector Laboratories, Burlingame, CA, USA) was added at 1:500 dilution for 45 min at room temperature. The antigen antibody complex was detected by using an avidin–biotin–peroxidase kit (Lab Vision). Diaminobenzidine (3,3-diaminobenzidine tetrahydrochloride dehydrate; Lab Vision) was used as the chromogen, followed by counterstaining with Mayer’s hematoxylin (Merck) solution and mounting with Permount (Fisher Chemicals, Springfield, NJ, USA) on glass slides. The internal quality control procedures were carried out by performing negative controls in which the primary antibodies were replaced with the appropriate non-immune IgG isotypes.

Photomicrographs were taken with an Axioplan microscope (Zeiss, Oberkochen, Germany). Tissue sections from normal and endometriosis patients were evaluated for protein localization and intensity. For each individual antibody, all samples were exposed to the same protocol and were stained using the same duration of staining. The intensity of immunoreactivity was semi-quantitatively evaluated as follows. Positively stained cells were grouped according to the following categories: — (no staining), 1+ (weak but detectable), 2+ (moderate or distinct), 3+ (intense). For each tissue, an HSCORE value was calculated by summing the percentages of cells grouped in one intensity category and multiplying this number with the weighted intensity of the staining, using the formula $HSCORE = P_i(i + 1)$, 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 ×400 original magnification, the percentage of the cells for each intensity within these areas was determined by two investigators who were blinded to slide contents and menstrual timing and the average score was used (Selam et al., 2001).

Isolation and culture of human ESCs

ESCs isolated from normal, eutopic and ectopic endometrium were separated and maintained in monolayer cultures, as described previously (Arici et al., 1993). Briefly, endometrial tissue was rinsed in
HBSS to remove blood and debris, then was digested by incubation of tissue minces in HBSS that contained HEPES (25 mmol; Sigma), penicillin (200 U/ml; Sigma), streptomycin (200 mg/ml; Sigma), collagenase (1 mg/ml, 15 U/ml; Sigma) and deoxyribonuclease (0.1 mg/ml, 1500 U/mg; Sigma) for 30 min at 37 °C with agitation. The dispersed endometrial cells were separated by filtration through a wire sieve (73-µm diameter pore; Sigma). The endometrial glands (largely undispersed pieces) were retained by the sieve, whereas the dispersed stromal cells passed through the sieve into the filtrate. Stromal cells were plated in DME/F12 medium containing 10% FBS and antibiotics–antimycotics (1% v/v Gibco BRL) in T-75 plastic flasks (Falcon, Franklin Lakes, NJ, USA), maintained at 37 °C in a humidified atmosphere (5% CO₂ in air), and allowed to replicate to confluence. Thereafter, the stromal cells were passaged by standard methods of trypsinization and some were plated in six-well culture dishes for treatments, some in chamber slides for immunofluorescence assay and some in cell culture insert plates with 8-µm pores for the invasion assay. ESCs were passaged twice. In previous studies, ESCs after first passage were assayed immunocytochemically using specific cell-surface markers and were found to contain 0–7% epithelial cells, no detectable endothelial cells and 0–2% macrophages (Arici et al., 1993; Kayisli, 2003). Experiments were commenced within 1–3 days after confluence was attained. The confluent cells were harvested for western blot analysis or immunofluorescence or invasion assay.

Western blot analysis

Western blot analysis was performed in cultured normal, eutopic and ectopic ESCs as described previously (Selam et al., 2001). Total protein from the cells was extracted in a lysis buffer (50 mM HEPES, pH 7.4, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1.5 mM MgCl₂–6H₂O, 1 mM EGTA, 100 mM NaF, 10 mM sodium pyrophosphate and protease inhibitors, 1 mM Na₃VO₄, 10 μM leupeptin, 10 μg/ml aprotinin and 4 mM PMSF). The protein concentration was determined by a detergent-compatible protein assay (Bio-Rad Laboratories, Hercules, CA, USA). Samples (15 μg) were loaded and electrophoretically separated by SDS–polyacrylamide gel using 7.5% Tris–HCL Ready Gels (Bio-Rad) and electroblotted onto a Hybond electrochemiluminescent nitrocellulose membrane (Amersham Pharmacia Biotech, Buckinghamshire, UK). The membrane was blocked with 5% non-fat dry milk in PBS-T buffer (0.05% Tween-20 in PBS, pH 7.4) for 1 h to reduce the non-specific binding, incubated for 1 h with mouse anti-human ezrin monoclonal antibody (Sigma) diluted at 1:1000 at room temperature and thereafter washed with Tris-buffered saline [TBS-Tween-20 (TBS-T)]. The membrane was further incubated for 1 h with peroxidase-labeled anti-mouse IgG (Vector Laboratories) diluted at 1:10 000. The immunoblot was developed using a chemiluminescent kit (NEN Life Science Products, Boston, MA, USA). After stripping the membrane with western blot stripping buffer (Pierce, Rockford, IL, USA), the membrane was washed with TBS-T and blocked with 5% non-fat dry milk in TBS-T for 1 h. It was later incubated for 1 h with mouse anti-human actin monoclonal antibody (Sigma) diluted at 1:10 000 to confirm equal loading of proteins in each lane. Thereafter, the same protocol with ezrin immunoblotting was carried out to develop the actin bands. Ezrin expression was then normalized by dividing the arbitrary densitometry units for ezrin by those for actin for each band. Similar experiments were conducted on at least three different occasions with cells prepared from three different endometrial tissues from normal and endometriosis patients.

Invasion assay

For quantitative measurement of ESC invasion, cells (1 × 10⁶ cells/ml) were plated on cell culture inserts with 8-µm pore PET membranes (23.1 mm; Biocoat; Becton Dickinson, Franklin Lakes, NJ, USA) coated with 300 μl of 1.0 mg/ml growth-factor-reduced Matrigel (Becton Dickinson). The FBS was replaced with 1% NuSerum (Collaborative Research, Bedford, MA, USA) to limit the presence of protease inhibitors. Fibronectin (25 μg/ml) was added to the medium in the lower chamber as a chemoattractant. At 24 h, filters were rinsed with PBS and the cells were fixed in methanol:acetone (1:1, at −20 °C) for 20 min at 4 °C. After three 5-min washes in PBS, cells were permeabilized with 25% methanol and stained with ezrin antibody with standard immunocytochemistry techniques (Selam et al., 2001). Filters were gently rinsed with double-distilled H₂O. Each of triplicate filters was cut from the cell culture inserts with a scalpel and mounted on a slide with the underside of the filter facing up. Cell bodies and processes that had migrated through the filter pores and had reached the underside were visualized by light microscopy and counted by two independent investigators blinded to the treatment groups. All assays were repeated at least three times.

Statistical analysis

Differences in ezrin and phospho-ezrin in endometrial stroma and glands HSCORE values between normal endometrium, eutopic endometrium and endometriosis samples were analyzed using one-way analysis of variance (ANOVA) test and post hoc Tukey test for pairwise multiple comparisons. For western blot analysis, each experiment was repeated three times using cells prepared from three independent endometrial tissue specimens. Levels of ezrin and phospho-ezrin in western blot densitometries were normally distributed as tested by the Kolmogorov–Smirnov test. ANOVA and post hoc Tukey test were used for statistical analysis and pair-wise multiple comparisons. A P-value of <0.05 was considered statistically significant. Statistical calculations were performed using Sigmasstat for Windows, version 3.0 (Jandel Scientific Corporation, San Rafael, CA, USA).

Results

Expression of ezrin and phospho-ezrin in endometrium and endometriosis: immunohistochemistry findings

Immunohistochemistry was performed on the endometrial tissues of women without endometriosis and on the eutopic and ectopic endometrial tissues of women with endometriosis. The endometrial tissues of women without endometriosis contained immunostaining for ezrin in both the stroma and glands. However, both the stroma and glands had a stronger ezrin immunostaining in the eutopic and ectopic endometrial tissues of women with endometriosis when compared with that of the endometrium of women without endometriosis (Fig. 1A, C and E). In the eutopic and ectopic tissues (Fig. 1C and E), it is notable that the glandular cells have a relatively more intense ezrin immunostaining than the stromal cells. Ezrin in eutopic endometrium (Fig. 1C) appears to be largely localized beneath the cell membrane surfaces of the glandular cells (shown in the inset of Fig. 1C) and at the apical side of the glands.

Phospho-ezrin expression was observed in all of the endometrial samples (i.e. endometrium of women without endometriosis, eutopic and ectopic endometrial tissues of women with
endometriosis.) However, the stroma has a stronger phospho-ezrin immunostaining in the eutopic and ectopic endometrial tissues of women with endometriosis when compared with that of the endometrium of women without endometriosis (Fig. 1B, D and F). In contrast to the ezrin immunostaining pattern in the eutopic and ectopic endometrial tissues, the phospho-ezrin immunostaining (Fig. 1D and F) was proportionally higher in the stroma than in the glandular cells. The phospho-ezrin staining was concentrated at the apical surface epithelium of the glands with the absence of cytoplasmic ezrin staining.

We also observed that the ezrin immunoreactivity is distributed diffusely throughout the glandular structures in the eutopic and ectopic endometrial tissues in women with endometriosis. This pattern significantly contrasts with the phospho-ezrin staining (Fig. 1D and F) from the serial sections of the same tissues. We found that phospho-ezrin demonstrated a polarized distribution of immunostaining where the immunoreaction was largely concentrated at the apical surfaces of the glandular structures in both the eutopic and ectopic endometrial tissues of women with endometriosis (Fig. 1D and F).

To quantify our immunohistochemistry results, we compared the HSCORE values of the ezrin and phospho-ezrin staining among the normal, eutopic and ectopic endometrial samples. We found a significant increase in the ezrin HSCORE in both stromal and glandular compartments in the eutopic and ectopic endometrial samples of women with endometriosis when compared with normal endometrium ($P < 0.05$) (Fig. 2A). In ectopic endometrium, ezrin HSCORE values were significantly higher than in the eutopic endometrium of women with endometriosis in both stromal and glandular structures ($P < 0.05$). Among all the groups, we observed that the immunostaining intensity for ezrin was significantly higher in the glandular cells than in the stromal cells ($P < 0.05$) (Fig. 2A).

For phospho-ezrin, we observed a higher HSCORE in both stromal and glandular compartments in the eutopic and ectopic endometrial samples of women with endometriosis compared with normal endometrium ($P < 0.05$) (Fig. 2B). Moreover, in the ectopic endometrial tissue, phospho-ezrin HSCORE values were significantly higher than in the autologous eutopic endometrium in stromal cells ($P < 0.05$), but not in the glandular cells (Fig. 2B). In contrast to the ezrin immunostaining pattern, we observed that the HSCORE values for phospho-ezrin was stronger in the stromal cells than in the glandular cells in all types of endometrial tissues ($P < 0.05$) (Fig. 2B).

**Pattern of ezrin and phospho-ezrin expressions in endometriosis implants**

Ezrin and phospho-ezrin immunohistochemistry was performed in serial sections of endometriosis implants. We observed intense ezrin immunoreactivity in the glands of an implant from adipose tissue of the peritoneal cavity (Fig. 3A1 and A2). Using a higher magnification, ezrin immunoreactivity was seen to be diffusely distributed throughout the glands but more at the apical part and to a lower extent in the endometrial stroma (Fig. 3A2). On the other hand, the immunoreactivity for phospho-ezrin was largely limited to the apical part of the gland (Fig. 3B1 and B2).
Immunoreactivity for ezrin and phospho-ezrin was noted in the stroma and glands of an implant close to the intestinal area, but their corresponding distribution was substantially different. We observed that the ezrin immunostaining was diffuse throughout the glands (Fig. 4A and C) but the phospho-ezrin immunostaining was concentrated at the apical part of the glands with absence of immunostaining in the other parts of the glands (Fig. 4B and D). Phospho-ezrin immunoreactivity was generally stronger than that of ezrin in the stroma (Fig. 4A–D). Ezrin staining was more intense in glandular cells than in the stroma, but the stromal cells were more intensely stained than the glandular cells for phospho-ezrin (Fig. 4A–D). There were no overall differences in the immunostaining in the ectopic tissues from the different sites. There appears to be substantial staining for phospho-ezrin in the blood vessels in the tissues. However, this was consistent among the three groups; therefore, it was not included in the stromal staining analysis.

**Ezrin and phospho-ezrin protein expressions in endometrium and endometriosis: western blot analysis**

Western analysis was used to investigate the ezrin and phospho-ezrin protein expressions in eutopic, ectopic and normal ESCs in culture. We have observed that the ectopic and eutopic ESCs from women with endometriosis demonstrated a higher ezrin protein level than the endometrium from women without endometriosis (P < 0.05) (Fig. 5A and B). Between ectopic and eutopic ESCs from women with endometriosis, ectopic implants had the highest amount of ezrin expression (P < 0.05) (Fig. 5A and B). For phospho-ezrin, western analysis revealed that ectopic ESCs from women with endometriosis expressed higher phospho-ezrin protein levels than the endometrium of women without endometriosis (P < 0.05) (Fig. 5C and D).

**Ezrin expression in endometrial stroma and glands**

![Ezrin expression in endometrial stroma and glands](image)

**Phospho-ezrin expression in endometrial stroma and glands**

![Phospho-ezrin expression in endometrial stroma and glands](image)

**Figure 2:** Ezrin and phospho-ezrin expressions in endometrial stroma and glands. The distribution of endometrial (proliferative and secretory phases combined) ezrin and phospho-ezrin immunostaining intensity (HSCORE) in normal endometrium, and eutopic and ectopic endometrial samples of women with endometriosis. Normal: endometrium from women without endometriosis. Eutopic: eutopic endometrium from women with endometriosis. Ectopic: ectopic endometrium from women with endometriosis. Bars represent mean ± SEM. *P < 0.05 significantly different in eutopic and ectopic compared with normal in pair-wise comparison in glandular and stromal cells for ezrin and phospho-ezrin.

**Ezrin and phospho-ezrin immunostaining in endometriosis implants.** Patterns of immunostaining for ezrin (A) and phospho-ezrin (B) in ectopic endometrial implants from an adipose tissue of the peritoneal cavity. These micrographs (A and B) represent an implant that consists of the cross-section of two glandular structures as indicated by Box 1 and Box 2, respectively. (A1) (1 and 2) and (B1) (1 and 2) are the corresponding magnified images that demonstrate the immunostaining for ezrin and phospho-ezrin, respectively. Pezrin: phospho-ezrin. Magnification: (A and B) ×20; (A1 and B1) ×40; (A2 and B2) ×100. Arrows: ESCs. Scale bar (B): 100 μm; (B1): 50 μm; (B2): 20 μm.

**Figure 3:** Ezrin and phospho-ezrin immunostaining in endometriosis implants. Patterns of immunostaining for ezrin (A) and phospho-ezrin (B) in ectopic endometrial implants from an adipose tissue of the peritoneal cavity. These micrographs (A and B) represent an implant that consists of the cross-section of two glandular structures as indicated by Box 1 and Box 2, respectively. (A1) (1 and 2) and (B1) (1 and 2) are the corresponding magnified images that demonstrate the immunostaining for ezrin and phospho-ezrin, respectively. Pezrin: phospho-ezrin. Magnification: (A and B) ×20; (A1 and B1) ×40; (A2 and B2) ×100. Arrows: ESCs. Scale bar (B): 100 μm; (B1): 50 μm; (B2): 20 μm.

**Invasion assay of endometrial and endometriosis stromal cells: analysis of cell morphology and ezrin expression**

Using the Matrigel invasion assay, we evaluated the invasive potential of stromal cells from normal endometrium, and
and more invasive characteristics were noted in the eutopic mainly localized in the protrusions and pseudopodia of the ectopic stromal cells have the highest ezrin expression (Fig. 6A) and with eutopic ESCs (Fig. 6C). Furthermore, the doppia (Fig. 6E) when compared with the normal ESCs (Fig. 6F). These serial sections reveal that the ezrin immunostaining is diffusely distributed throughout the glands, and immunostaining intensity for ezrin was significantly higher in the glandular cells than in the stromal cells (A and C). Phospho-ezrin demonstrated a polarized distribution of immunostaining where the immunoreaction was largely concentrated at the apical surfaces of the glandular structures (B and D). Immunostaining intensity for phospho-ezrin was significantly higher in the stromal cells than in the glandular cells (B and D). Magnification: (A and B) × 60; (C and D) × 80. Scale bar (B): 30 μm; (D): 25 μm.

Figure 4: Ezrin and phospho-ezrin immunostaining in ectopic endometrial implants. Representative micrographs of immunohistochemistry of ezrin (A and C) and phospho-ezrin (B and D) immunostaining in ectopic endometrial implants (A–D) obtained from an intestinal region. These serial sections reveal that the ezrin immunostaining is diffusely distributed throughout the glands, and immunostaining intensity for ezrin was significantly higher in the glandular cells than in the stromal cells (A and C). Phospho-ezrin demonstrated a polarized distribution of immunostaining where the immunoreaction was largely concentrated at the apical surfaces of the glandular structures (B and D). Immunostaining intensity for phospho-ezrin was significantly higher in the stromal cells than in the glandular cells (B and D). Magnification: (A and B) × 60; (C and D) × 80. Scale bar (B): 30 μm; (D): 25 μm.

from eutopic and ectopic endometrial tissues. Isolated stromal cells were plated on a Matrigel-covered multipore membrane. The vast majority (>99%) of cells were found to adhere to the upper surface of the Matrigel within 24 h of incubation. Cells in all groups were capable of invading Matrigel and passing through the 8-μm multipore membrane. Cells that passed to the other side of membrane were counted. The analysis of the invasion assay revealed that the ectopic ESCs from women with endometriosis had the highest capacity to invade through the Matrigel membrane (P < 0.05), followed by eutopic ESCs (P < 0.05) (Fig. 6F). Normal ESCs from women without endometriosis have the least number of cells that passed through the parous membrane (P < 0.05) (Fig. 6F). Analysis of the cell morphology revealed that the normal ESCs (Fig. 6A) that have not invaded the Matrigel express less ezrin and have less invasive characters (i.e. lack of protrusions) than the cells that have penetrated the Matrigel membrane (Fig. 6B). Moreover, the ectopic ESCs have more invasive characteristics such as increased protrusions and pseudopodia (Fig. 6E) when compared with the normal ESCs (Fig. 6A) and with eutopic ESCs (Fig. 6C). Furthermore, the ectopic stromal cells have the highest ezrin expression mainly localized in the protrusions and pseudopodia of the cells (Fig. 6D and E). More intense ezrin immunostaining and more invasive characteristics were noted in the eutopic ESCs from women with endometriosis when compared with cells from normal endometrium (Fig. 6A and 6C).

Discussion

On the basis of the retrograde menstruation and implantation theory, the initial step in the initiation of endometriosis is the attachment of the retrogradely menstruated endometrial cells to the peritoneal surfaces. Ezrin is thought to play a central role in interacting with cell-surface adhesion molecules including CD44 and ICAM-1 (Hirao et al., 1996; Tsukita and Yonemura, 1997; Heiska et al., 1998; Legg and Isacke, 1998), thus suggesting its strong involvement in the cell adhesion events. In this study, we evaluated the expression of ezrin protein and its activated form, phospho-ezrin.

Our in vivo data showed that in the eutopic endometrium of women with endometriosis, ezrin and phospho-ezrin expressions were increased compared with that of normal endometrium. Furthermore, ectopic implants expressed significantly higher amount of ezrin and phospho-ezrin compared with that of normal and eutopic endometrium. It was also interesting to observe that the phospho-ezrin expression was the highest in the ectopic tissue, especially in the stroma. The immunohistochemistry results are consistent with the western analysis results in which the ectopic tissue has the highest ezrin expression. These data lead us to speculate that as ezrin expression is increasing, endometrial cells may be becoming more invasive and gaining the ability to implant outside the uterine cavity. Since the ESCs may be responsible for the initiation of the disease by invading through the peritoneal surfaces, it is possible to claim that ezrin activation may play a role in this process, as we have shown that the highest expression of phospho-ezrin is in the stroma of ectopic implants. Ezrin is a link between the cell membrane and actin cytoskeleton that is held inactive in the cytoplasm through an intramolecular interaction. Fiev et al. (2004) have demonstrated that ezrin binding to phosphatidylinositol 4,5-bisphosphate (PIP2), through its NH2-terminal domain, is required for T567 phosphorylation resulting in the conformational activation of ezrin in vivo. These researchers have also found that PIP2 binding and T567 phosphorylation are both necessary for epithelial cell morphogenesis. Further studies are needed to investigate the causes of ezrin protein activation in endometriotic cells and the regulation of its phosphorylation. Several studies in the past have also suggested that the alterations in the intraperitoneal immune environment may play a role in the pathogenesis of endometriosis (Senturk and Arici, 1999; Starzinski-Powitz et al., 2001; Witz, 2002) in which cytokines are believed to have major regulatory and effector functions (Harada et al., 2001). In particular, monocyte chemotactic protein (MCP-1) is one of the cytokines that play a role in the chemotaxis of macrophages which is elevated in the peritoneal fluid of women with endometriosis (Arici et al., 1997). Some recent data, obtained in murine macrophages, suggest a crucial role of the actin cytoskeleton at different phases of the phagocytosis process, such as actin assembly on secondary phagosome membrane (Defacque et al., 2000) that is caused by an association between cellular membranes and the actin cytoskeleton.
through ERM. This may further suggest that ezrin plays an important role in the motility of macrophages and therefore may have an involvement in the pathogenesis of endometriosis.

Many molecular mediators of endometrial cell attachment to mesothelium were investigated (Garcia-Velasco and Arici, 1999; Dechaud et al., 2001). Several cell adhesion molecules, including integrins, ICAM-1, vascular cell adhesion molecule-1, have been implicated. Recently, hyaluronic acid and CD44 have been implicated in the interaction of peritoneal mesothelium with endometrial cells (Dechaud et al., 2001).
ERM proteins are believed to be membrane organizers and linkers between plasma membrane molecules such as CD44 and ICAM-2 and the cytoskeleton (Yonemura et al., 1999).

To our knowledge, this is the first study to report that ezrin protein is over-expressed in endometriotic tissues. By western analysis, we were able to show that ezrin expression in ectopic tissues is the highest compared with eutopic and normal endometrium. In addition, eutopic endometrium of women with endometriosis has more ezrin protein compared with the normal endometrium. Its greater level of ezrin expression suggests that eutopic endometrium may have more invasive potential than normal endometrium. The ezrin over-expression in the eutopic and ectopic endometrial tissue may cause certain women to be more susceptible to endometriosis. On the other hand, it is not clear at this point of time whether these changes in ezrin and phospho-ezrin expressions are occurring simply in response to the disease.

We have also evaluated the differences in the ability to invade between the normal, eutopic and ectopic ESCs by using an invasion assay. It has been previously demonstrated that ezrin transcription was required for in vitro invasion and was involved in the acquisition of metastatic potential in endometrial cancer cells (Ohtani et al., 2002). Our data revealed that the ectopic implant cells showed much greater ability to invade than eutopic endometrial cells, suggesting there may be an association between increased ezrin expression and increased invasiveness. However future studies using ezrin protein inhibitors or ezrin knock-out mice are needed in order to be able to demonstrate direct relationship between ezrin expression and increased invasiveness in endometriotic implants. The results lead us to believe that ezrin content in endometrial cells may help in defining their ability to adhere and invade in the process of ectopic tissue implantation.

In summary, the present data indicate that there is a strong expression of ezrin cytoskeleton protein in patients with endometriosis. At a clinical setting, ezrin may potentially be one of the prognostic factors for endometriosis. Moreover, at a therapeutic level, blocking the function of ezrin and other ERM proteins may potentially prevent the formation of membrane protrusions and the invasive ability of the cells. Nevertheless, we believe that further experimental studies on the specialized functions of ezrin and its role in endometriotic tissues may shed further light on the underlying mechanism of the pathogenesis of endometriosis.

References


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