

Contents lists available at ScienceDirect

European Journal of Obstetrics & Gynecology and Reproductive Biology

journal homepage: www.elsevier.com/locate/ejogrb

Attachment to extracellular matrices is enhanced in human endometriotic stromal cells: a possible mechanism underlying the pathogenesis of endometriosis

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ARTICLE INFO

Article history:

Received 16 June 2010

Received in revised form 23 September 2010

Accepted 30 October 2010

Keywords:

Endometriosis

Adhesion

Extracellular matrix

Integrin

Peritoneum

Pathogenesis

ABSTRACT

Objective: Endometriosis is characterized by the ectopic growth of endometrial tissue. One of the first steps to the spread of endometriosis in the peritoneal cavity is the attachment of endometriotic cells to peritoneal surfaces after they have been released into the peritoneal fluid from pre-existing endometriotic lesions. The increased adhesive and proliferative potential of endometriotic cells in response to specific extracellular matrix (ECM) components has been suggested to contribute to the pathogenesis of endometriosis.

Study design: Adhesive properties of endometriotic stromal cells (ECSC) and normal eutopic endometrial cells (NESC) to various extracellular matrix proteins were investigated by in vitro cell adhesion assays. The expression levels of integrins in these cells were also examined by Western blot analysis.

Results: Both ECSC and NESC significantly adhered to collagen type I and collagen type IV. ECSC revealed higher adhesive properties to these ECM proteins than NESC did. ECSC, but not NESC, adhered to fibronectin and laminin. Higher levels integrin of $\alpha 1$, $\alpha 2$, αv , $\beta 1$, and $\beta 3$ protein expression were observed in ECSC than in NESC. On the other hand, the levels of integrin $\alpha 3$ and αL proteins were lower in ECSC than in NESC. **Conclusions:** The results suggest that endometriotic cells possess stronger adhesion to ECM proteins, and that increase may be mediated, in part, through integrins. These findings may elucidate one of the mechanisms underlying the formation of peritoneal endometriotic lesions.

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1. Introduction

Endometriosis, a benign estrogen-dependent disease affecting 3–10% of women of reproductive age, is characterized by the ectopic growth of endometrial tissue, and is associated with pelvic pain, dysmenorrhea, and infertility [1]. Histologically, this disease is characterized by the presence of dense fibrous tissue surrounding the endometrial glands and stroma [2,3]. Although endometriosis and normal eutopic endometrium are histologically similar, distinct molecular differences have been noted between them. These include a variety of anomalies in structure, proliferation, immune components, adhesion molecules, proteolytic enzymes and their inhibitors, steroid and cytokine production and responsiveness, gene expression, and protein production [4]. Such anomalies include, for example, the deficient expression of 17-hydroxysteroid dehydrogenase type 2 [5] and interleukin-1 receptor type I [6], decreased

expression of HOXA10 [7], aberrant expression of aromatase P450 [8], enhanced contractility [9], and enhanced expression of both interleukin (IL)-6 [10], and B-cell lymphoma/leukemia-2 (Bcl-2) [11] in endometriosis. All of these differences may promote the development and progression of endometriosis.

One of the first steps to the spread of endometriosis in the peritoneal cavity is the attachment of endometriotic cells to peritoneal surfaces after they have been released into the peritoneal fluid from pre-existing endometriotic lesions [12]. Following the initial attachment to the peritoneum, endometriotic cells rapidly implant, invade the extracellular matrix (ECM) beneath the mesothelium, proliferate and form new peritoneal lesions [13–16]. It has been suggested that endometriotic stromal and glandular epithelial cells have two distinct roles: stromal cells are involved in the initial attachment process, and glandular cells are involved in the growth of the endometriotic lesion [15,16]. As the disease develops, these endometriotic cells need to establish cell–cell and cell–ECM interactions with the peritoneal lining by means of adhesion molecules and their ligands. The ECM of the peritoneum contains collagen types I and IV, tenascin, vitronectin, fibronectin and laminin, all of which may be the potential binding targets of endometriotic cells [16–18].

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Integrins, a ubiquitous class of cell adhesion molecules, seem to be the major receptors by which cells attach to components of the ECM, such as collagen, laminin, fibronectin, and vitronectin [19,20]. Some integrins also mediate important cell–cell interactions [19–21]. Ligand specificity is determined by pairings of noncovalently associated transmembrane α and β subunits, as well as by the membrane composition and other intracellular conditions (“inside out” signaling) [19,20]. Integrin binding leads to the transduction of intracellular signals that contribute significantly to cell phenotype regulation such as cellular differentiation, cell motility, attachment and cell–cell communication as well as to hormonal responsiveness [21,22]. The transmission of integrin-mediated signals in endometriotic cells may be an important step toward determining which cells are going to adhere and thus contribute to the spread of endometriosis.

In the present study, we investigated the adhesive properties of endometriotic cells to various ECM proteins by applying primary cultures of endometriotic cyst stromal cells (ECSC) and normal eutopic endometrial stromal cells (NESC). We also examined the protein expression of integrins in these two cell types. We discuss herein the mechanisms underlying the progression of endometriosis.

2. Materials and methods

2.1. ECSC and NESC isolation procedure and cell culture conditions

ECSC were obtained from premenopausal patients who had undergone salpingo-oophorectomy or cystectomy for ovarian endometriotic cysts ($n = 7$, aged 32–41 years). NESC were obtained from premenopausal patients who had undergone hysterectomies for leiomyoma and had no evidence of endometriosis ($n = 6$, aged 37–43 years). All patients had been free of any hormonal treatments for at least one year prior to the operation. All the specimens were diagnosed as being in the mid to late proliferative phases using a standard histological examination of endometrial tissues. This study was approved by the institutional review board (IRB) of the Faculty of Medicine, Oita University.

ECSC were isolated from ovarian endometriotic tissues by enzymatic digestion as previously described [6]. Briefly, the tissues were minced in Hank's balanced salt solution and digested with 0.5% collagenase (Gibco-BRL, Gaithersburg, MD, USA) in Dulbecco's modified Eagle's medium (DMEM) (Gibco-BRL) at 37 °C for 20 min. The dispersed cells were filtered through a 70 μm nylon mesh to remove the undigested tissue pieces. The filtrated fraction was separated further from epithelial cell clumps by differential sedimentation at unit gravity, as follows. The cells were resuspended in 2 ml of culture medium and layered slowly over 10 ml of the medium in a centrifuge tube. The tubes were placed in an upright position at 37 °C in 5% CO_2 in air for 30 min. After sedimentation, the top 8 ml of medium was collected. Finally, the medium containing stromal cells was filtered through a 40 μm nylon mesh. Final purification was achieved by allowing stromal cells, which attach rapidly to plates, to adhere selectively to culture dishes for 30 min at 37 °C, followed by the removal of nonadhering epithelial cells. NESC were also isolated by digesting the endometrial tissue fragments with 0.5% collagenase as previously described [6]. Isolated ECSC and NESC were cultured in DMEM supplemented with 100 IU/ml of penicillin (Gibco-BRL), 50 mg/ml of streptomycin (Gibco-BRL), and 10% charcoal-stripped heat-inactivated fetal bovine serum (FBS) (Gibco-BRL) at 37 °C in 5% CO_2 in air.

ECSC and NESC in monolayer culture after the third passage were >99% pure, as determined by immunocytochemical staining with antibodies to vimentin (V9; Dako, Copenhagen, Denmark), CD10 (SS2/36; Dako), cytokeratin (Dako), factor VIII (Dako), and

leukocyte common antigen (2B11 + PD7/26, Dako), and were used for the following experiments [6]. Cells isolated from each patient were used for one experiment at a time. Each experiment was performed in triplicate and conducted at least four times.

2.2. Assessment of cell adhesion to ECM protein in vitro

Cell adhesion was evaluated using the CytoSelect™ 48-well cell adhesion assay (ECM Array, Cell Biolabs, Inc., San Diego, CA, USA) according to the manufacturer's protocol. ECSC and NESC were harvested and resuspended in serum-free DMEM containing 0.5% BSA at a concentration of 8×10^5 cells/mL. A cell suspension of ECSC or NESC was added to each well coated with fibronectin, collagen type I, collagen type IV, laminin, fibrinogen, or BSA in a volume of 150 μL . Each plate was then incubated at 37 °C in 5% CO_2 in air for 90 min. The cells were then washed four times with 250 μL of PBS. Then, 200 μL of cell stain solution supplied by the manufacturer (Cell Biolabs) was added to each well and incubated for 10 min at room temperature. The stain was then removed and the cells were washed four times with 500 μL of deionized water. After allowing the cells to air dry for 25 min, 200 μL of extraction solution (Cell Biolabs) was added to each well and placed on an orbital shaker for 10 min. The absorbance of the extracted samples was measured at 560 nm by a plate reader. The results were normalized by subtracting the absorbance of the negative controls.

2.3. Assessment of the expression of integrin subunits in ECSC and NESC

As shown in Table 1, the expression levels of integrin α and β subunits ($\alpha 1$, $\alpha 2$, $\alpha 3$, αL , αv , $\beta 1$, and $\beta 3$) in untreated ECSC and NESC were investigated by Western blot analysis as previously described [6,9]. Most of these integrin subunits examined were chosen because they were recognized as receptors for fibronectin, collagen, laminin, and/or fibrinogen, whereas αL subunit was chosen because it was not related to fibronectin, collagen, laminin, and fibrinogen. ECSC and NESC were cultured on 100 mm dishes until confluence. The cells were then washed with phosphate-buffered saline (PBS), and whole cell extracts were prepared by lysing the cells in lysis buffer (50 mM Tris-HCl, 125 mM NaCl, 0.1% Nonidet P-40, 5 mM ethylenediamine tetraacetic acid, 50 mM NaF, and 0.1% phenylmethylsulfonyl fluoride). The suspension was centrifuged at 15,000 rpm for 15 min at 4 °C, and the supernatant was collected. The total protein concentration was quantified using the Coomassie protein assay reagent (Pierce, Rockford, IL, USA). The whole cell protein extract was resolved with sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using a 10% polyacrylamide gel under reduced conditions. After transfer to Immobilon-P transfer membrane (Millipore, Bedford, MA, USA), the protein was stained with Ponceau S (Sigma-Aldrich, St. Louis, MO, USA) to verify uniform loading and transfer. Membranes were blocked with 5% skimmed milk (Becton-Dickinson, Sunnyvale, CA, USA) in Tris-buffered saline with Tween 20 (50 mM Tris-HCl, 150 mM NaCl, 0.1% Tween 20, pH 7.4) (TBS-T) overnight and subsequently incubated with primary antibodies [integrin $\alpha 1$ (FB12; Millipore), integrin $\alpha 2$ (2/CD49b; BD Biosciences, San Jose, CA, USA), integrin $\alpha 3$ (42; BD Biosciences), integrin αv (21; BD

Table 1

Integrins examined in the present study and their representative ligands [19,20].

Integrin	Ligands
$\alpha 1\beta 1$	Collagen, laminin
$\alpha 2\beta 1$	Collagen, laminin, tenascin
$\alpha 3\beta 1$	Collagen, laminin, fibronectin
$\alpha L\beta 2$	Intercellular adhesion molecule (ICAM)-1, ICAM-2, ICAM-3
$\alpha v\beta 3$	Fibronectin, vitronectin, tenascin, fibrinogen, von Willebrand factor

Biosciences), integrin α L (2D7; BD Biosciences), integrin β 1 (18/CD29; BD Biosciences), integrin β 3 (BV4; Abcam, Tokyo, Japan), and glyceraldehydes-3-phosphate-dehydrogenase (GAPDH) (Ambion, Austin, TX, USA)] at appropriate dilutions for 1 h at room temperature. The membrane was washed three times with TBS-T and incubated with the appropriate horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature. Subsequently, the membrane was washed three times with TBS-T and analyzed by enhanced chemiluminescence (Amersham Pharmacia Biotech, Chicago, IL, USA).

2.4. Statistical analysis

Data are presented as the means \pm SD of representative experiments and were analyzed by the Bonferroni/Dunn test with StatView 4.5 (Abacus Concepts, Berkeley, CA, USA). Values of $p < 0.05$ were considered to indicate statistical significance.

3. Results

The properties of ECSC and NESC adhesion to ECM protein were evaluated by in vitro cell adhesion assays. As shown in Fig. 1, both ECSC and NESC significantly adhered to collagen type I and collagen type IV ($p < 0.0001$). ECSC showed higher adhesive properties to ECM proteins than NESC ($p < 0.0001$). ECSC, but not NESC, adhered to fibronectin and laminin. Neither ECSC nor NESC attached to fibrinogen.

To further assess the underlying mechanisms of these observations, expression levels of integrin α 1, α 2, α 3, α v, α L, β 1, and β 3 subunits in ECSC and NESC were evaluated by Western blot analysis. As shown in Fig. 2, upregulated expression levels of α 1, α 2, α v, β 1, and β 3 proteins were observed in ECSC in comparison with NESC. On the other hand, the levels of α 3 and α L proteins were lower in ECSC than in NESC.

4. Discussion

The interaction between endometriotic tissue and peritoneum is an important aspect in the initiation and progression of the disease. Several in vitro models with tissue fragments and dispersed cells have been described that mimic the initial processes of the development of endometriotic lesions [15,16,23,24]. In these models, whole explants of peritoneum and monolayer cultures of mesothelial cells grown on ECM were used [15,16,23,25]. Previous reports have demonstrated that both eutopic endometrial stromal cells and endometrial epithelial cells

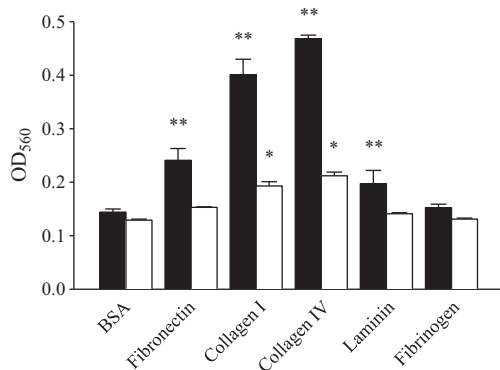


Fig. 1. Adhesive properties of ECSC and NESC to various ECM proteins in vitro. Cell adhesion of ECSC and NESC was evaluated using CytoSelect™ 48-well cell adhesion assay according to the manufacturer's protocol. The data are presented as the absorbance at 560 nm. Representative results are shown. Closed bars, ECSC; open bars, NESC. * $p < 0.0001$ vs. BSA group, ** $p < 0.0001$ vs. BSA group and corresponding NESC group.

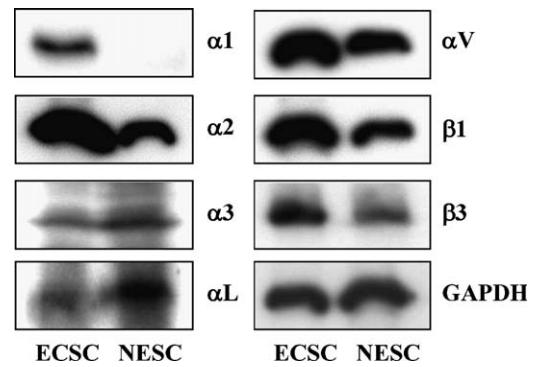


Fig. 2. Protein expression of integrin α 1, α 2, α 3, α L, α v, β 1, and β 3 subunits in ECSC and NESC. The expression levels of these integrins were investigated by Western blot analysis. The expression of GAPDH was also evaluated as internal controls. Representative results are shown.

attach to peritoneal mesothelial cells rapidly within 1 h, and transmesothelial invasion occurs within 18–24 h [15,26–28]. Another report has found that this process is mediated predominantly by endometrial stromal cells [15]. Furthermore, it has been shown that eutopic endometrial cells adhere preferentially to collagen types I and IV compared to fibronectin and laminin [29]; integrins are suggested to be involved in the attachment of endometriotic cells to the peritoneum [13,29,30]. However, all of these previous studies utilized eutopic endometrial cells instead of endometriotic cells. Therefore, it should be addressed whether or not the characteristics of these two cell types differ.

In the present study, we demonstrated that ECSC had higher adhesive properties to ECM proteins in comparison to NESC. Higher levels of α 1, α 2, α v, β 1, and β 3 protein expressions were also observed in ECSC, whereas the levels of α 3 and α L proteins were lower in ECSC than in NESC. Evaluation of a large repertoire of integrin subunits is mandatory for the completion of this study, but our preliminary findings suggest that some integrins along the surface of endometriotic stromal cells may have a role in the initial attachment to the peritoneal lining during the progression of endometriosis. It has been proposed that the increased adhesive and proliferative potential of endometriotic cells in response to specific ECM components may be a key feature in the pathogenesis of endometriosis [12]. It has also been demonstrated that eutopic and ectopic endometrial stromal cells from women with endometriosis have aberrant integrin profiles in vitro compared with stromal cells derived from healthy controls [12], which is consistent with our results. These observations suggest that aberrant integrin expression in ECSC is involved in the pathogenesis of the increased adhesive properties of endometriotic tissues and cells.

Abnormal regulation of cell–cell and cell–matrix interactions, including aberrant integrin expression in endometriosis, has repeatedly been demonstrated from the viewpoint of the development and progression of the disease [13,31–34]. Increased expression of integrin subunits α 4 and α 5 has been reported in endometriotic epithelial cells [33]. Increased expression of integrin subunits α 3 [32], α 5 [33], and β 1 [32] has been reported in ECSC, whereas decreased expression of integrin subunits α 6 has been reported in ECSC [32]. A loss of cyclicity in the expression of epithelial α v β 3 also has been noted [32]. Most of these findings were consistent with our results. The picture that arises from these works is, however, only beginning to become clear and is still far from being consistent or elucidative.

In addition to the integrin family, adhesion molecules, such as cadherin, selectin, the immunoglobulin superfamily and CD44, have been suggested to play roles in the adhesion of endometriotic tissue to develop additional peritoneal lesions [26,33,35–37]. For

example, P-cadherin has been demonstrated to be the predominant cadherin subtype present in the peritoneum [35]. Using immunohistochemistry, Van der Linden et al. [37] demonstrated P-cadherin expression in endometriotic lesions as well as in eutopic endometrium. P-cadherin mRNA levels in endometriotic lesions were significantly greater than those observed in the corresponding eutopic endometrium [35]. On the other hand, N-cadherin was constantly expressed in peritoneal endometriosis and peritoneal mesothelial cells [36]. The expression of P-cadherin and N-cadherin in both endometriotic lesions and peritoneum suggests that these cadherins may play central roles in the development of endometriosis by mediating endometrial–peritoneal cell interactions in a homophilic manner [36,37].

In summary, we demonstrated here that ECSC had higher adhesive properties than NESC to ECM proteins. Aberrant integrin expression in ECSC is thought to be involved in this phenomenon. It is possible that endometriotic cells escape the pre-existing peritoneal lesions and seed new lesions at other sites within the peritoneal cavity through integrin–ECM-mediated cell adhesion. Integrin-mediated ECSC–ECM interactions may trigger the signal transduction pathways for subsequent cell proliferation, activation, differentiation, and invasion that are necessary for disease progression [5,6,8–11]. Alternatively, one way to prevent the progression of peritoneal endometriosis is to minimize the disease dissemination by inhibiting the adherence of endometriotic cells or tissue to the peritoneal lining. Further investigations into the integrin–ECM interactions may provide insights into the crucial pathological steps in the development of endometriosis.

Acknowledgements

This work was supported in part by a Grant-in-Aid for Scientific Research from the Japan Society for the Promotion of Science (no. 20591920 to K. Nasu).

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