Interleukin-8 expression in endometrial stromal cells is regulated by integrin-dependent cell adhesion

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Concentrations of interleukin (IL)-8, a potent chemotactic factor produced by many cell types, are elevated in the peritoneal fluid of women with endometriosis. We investigated whether endometrial stromal cell (ESC) adhesion induces the expression of IL-8 and if this process is integrin-mediated. ESCs were plated onto culture dishes coated with various extracellular matrix (ECM) substrates, such as fibronectin, laminin, collagen IV, and poly-L-lysine, or mouse anti-human integrin β¹ and β² monoclonal antibodies. IL-8 expression was induced by adherence of ESCs to fibronectin or collagen IV, but not to poly-L-lysine, a non-integrin-dependent adhesion matrix. Engagement of β¹-containing integrins was associated with ESC adhesion and resulted in up-regulation of IL-8 mRNA expression and protein secretion. Disruption of the actin cytoskeleton by treating ESC with cytochalasin D completely blocked the increase of IL-8 that was induced in response to integrin activation. These findings indicate a novel mechanism of IL-8 regulation; cell adhesion to ECM is an important event that leads to stimulation of IL-8 expression, and this process is mediated by integrins.

Key words: adhesion/endometrium/extracellular matrix/integrins/interleukin-8

Introduction

Despite all the efforts invested in endometriosis research, with over 4000 articles published over the last 10 years, its pathogenesis remains poorly understood. Retrograde menstruation, a mechanism in which viable endometrial cells are shed into the peritoneal cavity through the Fallopian tubes, with subsequent implantation and growth, is the most widely accepted theory (Sampson, 1927). The plausibility of Sampson’s model for endometriosis is reinforced by the fact that women with Mullerian defects that favour retrograde menstruation have a higher incidence of endometriosis (Sanfilippo et al., 1986). In addition, experimental data obtained from baboons show that the prevalence and recurrence of retrograde menstruation is increased in baboons with spontaneous endometriosis (D’Hooghe et al., 1996). However, we still do not know how and why retrogradely menstruated endometrial cells attach to the pelvic peritoneum in some women and not in others, if up to 90% of women with patent Fallopian tubes have reflux menstruation (Bartosik et al., 1986).

Endometrial cells from menstrual fluid are viable (Keetel 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–cell or cell–extracellular matrix (ECM) interactions with the peritoneal lining. Endometrial stromal cells (ESC) are relevant in stimulating and inhibiting the growth of glandular epithelium (Cooke et al., 1997). It is likely that ESC are actively involved in the adhesion of endometrial implants to the peritoneum. A recent report clearly showed that ESC are the critical cells in endometrial attachment to the mesothelial surface of the peritoneum, and endometrial epithelial cells fail to attach to the mesothelium (Witz et al., 1999).

Previous studies suggest that early endometriosis lesions invade the ECM of the peritoneum after the initial attachment (Spuijbroek et al., 1992), which is consistent with recent in-vitro studies (Witz et al., 1999). The integrin family of cell surface receptors mediates many of these interactions between endometrial cells and the ECM (Juliano and Haskill, 1993). Although they lack the characteristics of signal generating receptors, integrins are able to transduce intracellular signals (Juliano and Haskill, 1993). Adhesion of endometrial cells to the peritoneal ECM may result in the transmission of the necessary signals for cell migration and invasion. Additionally, it has been shown that the mesothelial cells of the peritoneum express integrins in-vitro as well as in-vivo (Witz et al., 1998). Several integrins are expressed by the mesothelium of the peritoneum, with different expression observed in-vivo and in-vitro in monolayer and explant cultures. This recent identification of integrins along the surface of the mesothelium suggests that these mesothelial integrins may have a role in the initial attachment of ectopic endometrium to the peritoneal lining.

Activated leukocytes are a dominant feature of the inflammatory reaction and frequently contribute to the pathogenesis of the underlying disease. Leukocyte chemoattraction in the inflammatory lesion is mediated by several factors. Interleukin (IL)-8 is one of the potent chemotactic/activating factors for granulocytes and T cells (Matsushima et al., 1988). IL-8 is produced by a number of cell types including endometrial...
stomial and glandular cells (Arici et al., 1993, 1998a), and its values in the peritoneal fluid are elevated in women with endometriosis (Ryan et al., 1995; Arici et al., 1996; Iwabe et al., 1998).

We postulated that cell adhesion itself may enhance the expression of IL-8, thus contributing to the inflammation in the peritoneal cavity, and as a consequence, may play a role in the pathogenesis of endometriosis. In the present study, we have investigated the role that the interaction between ESC with ECM components may have in the regulation of IL-8 gene expression and secretion.

Materials and methods

Chemicals and matrix preparation

Fibronectin was acquired from Becton Dickinson (Bedford, MA, USA). Collagen IV, laminin, and poly-L-lysine (molecular weight 70–150 kDa) were purchased from Sigma Chemical Co (St Louis, MO, USA). Mouse anti-human integrin β1 and β2 monoclonal antibodies were purchased from Chemicon International Inc (Temecula, CA, USA). The ECM proteins were dissolved in calcium- and magnesium-free phosphate-buffered saline (PBS) to the concentration of 20 µg/ml fibronectin, 10 µg/ml type IV collagen, and 20 µg/ml laminin. For poly-L-lysine coating, a 0.1 mg/ml solution was applied. Coating of 100 mm Petri dishes and 24-well plates was carried out by overnight incubation at 4°C. Dishes were then incubated for 2 h at 37°C with 1% bovine serum albumin (BSA; wt/vol), denatured at 70°C for 15 min to block the non-specific binding sites on plastic, and washed with sterile PBS before plating. As control, dishes were directly blocked with denatured BSA. Stock cytochalasin D was prepared at a concentration of 5 µg/ml in dimethyl sulfoxide (DMSO) (Baker Inc, Phillipsburg, NJ, USA).

Tissue collection and cell culture

Endometrial tissue samples were obtained from women undergoing diagnostic laparoscopy or hysterectomy for benign disease. Informed consent was obtained from each woman prior to surgery using ESC grown to confluence was obtained from women undergoing diagnostic laparoscopy or hysterectomy for benign disease. Informed consent was obtained from each woman prior to surgery using ESC grown to confluence. Informed consent was obtained from each woman prior to surgery using ESC grown to confluence.

Endometrial cells were dispersed by incubation of minced tissue in HBSS containing HEPES (25 mmol/l), penicillin (200 IU/ml), streptomycin (200 µg/ml), collagenase (2 mg/ml; 15 IU/mg), and DNase (0.2 mg/ml; 1500 IU/mg) for ~20 min at 37°C with constant agitation. The dispersed endometrial stromal cells were separated from glands by filtration through a wire sieve (73 µm diameter pore). Endometrial glands remained largely undispersed and were retained by the sieve, while stroma passed through into the filtrate. Although small fragments of glandular cells may pass, most epithelial cells will be lost during subsequent culture. The endometrial cells were characterized using factor VIII as a marker of endothelial cells, cytokeratin as a marker of epithelial cells, and 3C10 as a marker of macrophages. After second passage endothelial cells are not present, epithelial cells account for <0–7% of the cells, and cells expressing a macrophage-marker antigen accounted for 0.2% of the total, as previously shown (Arici et al., 1993).

Endometrial stromal cells were plated in Dulbecco’s modified Eagle’s medium: Ham’s F12 (DMEM/F12 1:1, vol/vol) containing antibiotics-antimycotics (1% vol/vol) and fetal bovine serum (10% vol/vol) in plastic flasks (75 cm²), maintained at 37°C in a humidified chamber (5% CO₂ in air) and allowed to replicate until confluent in monolayer. Thereafter, the cells were passed by standard methods of trypsin treatment and again allowed to replicate to confluence. After cells reached confluence, cultures were treated with serum-free, Phenol Red-free medium for 24 h.

Northern analysis

The ESC cultures were treated with trypsin, centrifuged, and resuspended in fresh serum-free, Phenol Red-free medium at a concentration of ~500 000 cells/ml. Cell suspension (2 ml) was then seeded onto 100-mm Petri dishes precoated as indicated above, and the cells were incubated for 6 h. The media was then aspirated to remove non-adherent cells. Total RNA was extracted from the adhering cells using Trizol (Gibco BRL, Grand Island, NY, USA), size-fractionated (10 µg per lane) by electrophoresis on 1% formaldehyde-agarose gels, transferred electrophoretically to Hybond-N+ membrane (Amersham, Arlington Heights, IL, USA), and cross-linked to the membrane using UV light. Prehybridization was conducted for 5 h at 65°C in a buffer composed of NaCl (0.9 mol/l), Tris–Cl (90 mmol/l, pH 8.3), EDTA (6 mmol/l), 5× Denhardt’s solution, sodium dodecyl sulphate (SDS; 0.1% wt/vol), sodium pyrophosphate (0.1% wt/vol), and salmon sperm DNA (0.2 mg/ml). Hybridizations were conducted for 16 h at 65°C in a buffer that contained an IL-8-specific oligonucleotide probe (5'-TGG CGC AGT GTG GTC CAC TCT CAA-3') end-labelled with [γ-32P]-ATP; the sequence of this probe corresponds to a portion of exon 2 in the coding region of the IL-8 gene (Matsushima et al., 1988). Thereafter, blots were washed once with 6× standard saline citrate and SDS (0.1% wt/vol) for 15 min at room temperature, once with 2× standard saline citrate and SDS (0.1% wt/vol) for 15 min at room temperature, and once for 20 min at 65°C. Autoradiography of the membrane was performed at ~80°C using Kodak X-Omat AR film (Eastman Kodak, Rochester, NY, USA). The amount of RNA in each lane was normalized by the analysis of glyceraldehyde-3-phosphate dehydrogenase (G3PDH) mRNA. The autoradiographic bands were quantified using a laser densitometer (Molecular Dynamics, Sunnyvale, CA, USA).

IL-8 enzyme-linked immunosorbent assay (ELISA)

ESC grown to confluence and serum-starved for 24 h were treated with trypsin and resuspended in serum-free, Phenol Red-free medium at a concentration of ~100 000 cells/ml. Identical aliquots of cell suspension (400 µl) were then plated into 24-well plates precoated with the appropriate ECM component as indicated above. After 24 h, the conditioned media were collected, briefly centrifuged at 4°C to remove cells, and stored at ~80°C until analysis. Immuno-reactive IL-8 concentration was quantified using a commercial ELISA kit specific for human IL-8 (R&D Systems, Minneapolis, MN, USA). According to the manufacturer, there is no measurable cross-reactivity with other known cytokines in this assay. The sensitivity for IL-8 was 0.47 pg/10⁶ cells.

Statistical analysis

The concentrations of IL-8 mRNA and protein were normally distributed (tested by Kolmogorov–Smirnov test). Analysis of variance (ANOVA) and Scheffé’s correction for pairwise multiple comparisons were used for statistical analysis. P < 0.05 was considered to be significant. Statistical calculations were performed using SigmaStat.
Regulation of IL-8 expression by cell adhesion

Figure 1. Adhesion of endometrial stromal cells to fibronectin or collagen-coated plates induces a significantly higher interleukin-8 (IL-8) mRNA expression compared with BSA (control). Northern blots for IL-8 were performed with 10 µg of total RNA extracted from in-vitro cultures of isolated human endometrial stromal cells, previously treated with trypsin and incubated for 6 h over extracellular matrix-coated plates (n = 5). Glyceraldehyde-3-phosphate dehydrogenase (G3PDH) hybridization was carried out to verify that the same amount of total RNA was loaded into each lane. BSA = bovine serum albumin; F = fibronectin; L = laminin; CIV = collagen IV; P = poly-L-lysine. *P < 0.05 fibronectin and collagen IV versus other groups.

Results

Adhesion to different ECM proteins induces variable IL-8 expression in ESC

To investigate whether adhesion of ESC to different ECM proteins would affect IL-8 expression, cells were plated on various ECM proteins. Cells plated on BSA expressed low values of IL-8 mRNA, whereas cells adhering to fibronectin or collagen IV expressed markedly higher values of IL-8 mRNA (Figure 1). Compared with BSA (control), fibronectin and collagen IV induced 5- and 2-fold increase in IL-8 mRNA values (n = 5) (P < 0.05), respectively. No significant changes were observed when cells adhered to laminin. Interestingly, IL-8 mRNA values in cells plated on poly-L-lysine, a non-integrin-dependent adhesion matrix, were similar to those in controls.

Integrin-dependent cell adhesion up-regulates IL-8 mRNA

To establish whether the effect of ECM on the expression of IL-8 is mediated by interactions with specific integrins, ESCs were plated on dishes coated with anti-human β1 integrin monoclonal antibody. Engagement of β1-containing integrins was associated with ESC adhesion and resulted in up-regulation of IL-8 mRNA expression (4-fold over control, P < 0.05) (n = 5) (Figure 2). This over-expression was 2-fold higher than that induced by fibronectin.

An intact cytoskeleton is required to stimulate IL-8 gene expression in response to integrin activation

In order to demonstrate that the cytoskeleton plays a role in the integrin-mediated signalling process of IL-8 expression on adhesion, ESC were pretreated for 30 min with 5 µg/ml cytochalasin D, which disrupts the actin cytoskeleton. Then ESC were plated on fibronectin coated plates for 6 h. Northern blot analysis showed a complete block of the increase of IL-8 mRNA that is induced in response to integrin activation while no change was observed in G3PDH mRNA (n = 3) (Figure 3). In this experiment, fibronectin caused a 2-fold induction of IL-8 mRNA over the control, compared with a 5-fold induction observed in the previous experiment. This variation is most likely due to differences in experimental conditions.

Adhesion induces IL-8 secretion from ESC

To confirm whether adhesion stimulated both IL-8 mRNA expression and IL-8 protein secretion, we assessed the production of IL-8. Supernatants from ESC plated on different ECM matrices were collected after 6, 12, and 24 h, and then IL-8 values from six replicates were measured by ELISA. Figure 4A shows the time-dependent production of IL-8 from ESC, and differences were statistically significant at 12 and 24 h (n = 3) (P < 0.001, fibronectin versus poly-L-lysine). ESC plated on either collagen IV or laminin also showed a time-dependent increase in IL-8 protein secretion, although they did not reach statistical significance (data not shown). These experiments also confirmed at the protein level that ESC engagement with an ECM protein or with anti-β1 integrin antibody, but not anti-β2 integrin antibody, significantly up-regulates IL-8 secretion, compared with control or poly-L-lysine, a non-integrin dependent adhesion substrate (Figure 4B).

Discussion

Adhesion of endometrial cells to the peritoneal lining is a crucial step in the early stages of endometriosis and endometrial
Figure 2. Engagement of endometrial stromal cells with mouse anti-human integrin β1 monoclonal antibody coated surface induces a significantly higher expression of interleukin (IL)-8 than in the control, and this increase is 2-fold higher than the increase induced by fibronectin. Northern blots for IL-8 were performed with 10 µg of total RNA extracted from in-vitro cultures of isolated human endometrial stromal cells, previously treated with trypsin and incubated for 6 h over BSA, fibronectin or anti-human integrin β1 antibody-coated plates (n = 5). Glyceraldehyde-3-phosphate dehydrogenase (G3PDH) hybridization was carried out to verify that the same amount of total RNA was loaded into each lane. BSA = bovine serum albumin; F = fibronectin; AB = mouse antihuman integrin β1 monoclonal antibody. *P < 0.05 fibronectin versus BSA; P < 0.01 AB versus BSA.

Figure 3. Endometrial stromal cells plated on fibronectin require an intact cytoskeleton to complete the interleukin (IL)-8 signalling process, as pre-treatment with cytochalasin D totally disrupts IL-8 gene expression on adhesion to control levels. Northern blots for IL-8 were compared with 10 µg of total RNA extracted from in-vitro cultures of isolated human endometrial stromal cells, previously treated for 30 min with 5 mg/ml cytochalasin D, treated with trypsin and incubated for 6 h over BSA or fibronectin (n = 3). Glyceraldehyde-3-phosphate dehydrogenase (G3PDH) hybridization was carried out to verify that the same amount of total RNA was loaded into each lane. *P < 0.01 fibronectin with cytochalasin D versus other groups.

cancer invasion. The ECM is required for cell survival and proliferation in response to growth factors and in the absence of ECM cells undergo apoptosis (Meredith et al., 1993; Re et al., 1994). Cell shape and cytoskeletal organization are required for anchorage-dependent cell survival. A physical linkage may be established between the cytoskeletal structure of
peritoneum. Endometrial cells are transported through the α., 1986) of women during menses, and these cells are variable response also may be due to binding to two different
actions during cell adhesion and migration (Juliano and Haskil, ECM proteins that are the ligands for the β–
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1993). We have shown that the adhesion of ESC to different
receptors mediates many of these cell
increments of IL-8 found in the peritoneal fluid of women with endometriosis (Ryan et al., 1995; Arici, 1999). This may imply that the increased
concentrations of IL-8 in the peritoneal fluid of women with endometriosis (Ryan et al., 1995; Arici, 1996; Iwabe et al., 1998) may not only be an initiating factor in the
establishment of endometriosis but also facilitate further adhesion of endometrial cells to the peritoneum, contributing to the development of endometriosis.

Endometrial tissue displays a changing profile of integrin expression throughout the menstrual cycle (Lessey et al., 1992). The only integrin that is constitutively expressed in ESC and persists in endometriotic implants is the so-called ‘classic’ fibronectin receptor, αβ1 (Lessey et al., 1992; Van der Linden et al., 1994). We focused our study on the β-subunits as β-integrins are relevant in mediating the organization of ECM proteins (Shiokawa et al., 1996), and they are part of most of the different integrin dimers. Our main goal was to investigate the potential role of β-integrins in connecting the ECM framework to intracellular cytoskeletal structures. We evaluated IL-8 expression after adhesion of ESC to different ECM proteins that are the ligands for the β1 subunit: fibronectin, laminin, and collagen IV. All but laminin up-regulated IL-8 gene expression and protein secretion. Fibronectin induced a much higher IL-8 expression than did collagen IV, although fibronectin is not the most abundant ECM protein in the mesothelium. Fibronectin, collagen IV and laminin receptors share structural components such as β1 integrin, but differences in the α subunits may account for the observed response since the α cytoplasmic domain regulates the specificity of the ligand-dependent interactions (Sastry and Horwitz, 1993). This variable response also may be due to binding to two different regions within the β1 subunit (Otey et al., 1993). Interestingly, when a non-integrin dependent cell adhesion matrix was tested (poly-L-lysine), it did not induce any change in IL-8 mRNA or protein. The finding that ESC, when seeded on plates coated with the antibody against the β1 subunit, induced an increase
different cell types and ECM proteins, and specific intracellular signalling may be required. The integrin family of cell surface receptors mediates many of these cell–cell, cell–ECM interactions during cell adhesion and migration (Juliano and Haskil, 1993). We have shown that the adhesion of ESC to different ECM proteins induces variable levels of IL-8 gene expression, and this event is integrin-mediated.

According to Sampson’s theory of retrograde menstruation, before endometrial cells implant and proliferate, they have to adhere to the peritoneal mesothelial surfaces. These cells need to establish cell–cell or cell–ECM interactions with the peritoneum. Endometrial cells are transported through the Fallopian tubes to the peritoneal cavity in 76–90% (Bartosik et al., 1986) of women during menses, and these cells are viable (Kruitwagen et al., 1991). Recent evidence suggests that endometrial adhesion to intact mesothelium in a co-culture system is dependent on stromal cells, since no adhesion of endometrial epithelium was identified in the first 24–48 h (Witz et al., 1999). If some of these refluxed cells are able to evade the defence mechanisms of the peritoneal fluid such as macrophages and lymphocytes, they may attach to the ECM and grow. We have shown how ESC adhesion to different ECM proteins induces an up-regulation in IL-8 gene expression and protein secretion. These findings have important implications in the pathogenesis of some gynaecological diseases, e.g. endometriosis or endometrial cancer.

Viable refluxed endometrial cells may survive many days in the peritoneal fluid. These endometrial fragments recovered from the peritoneal fluid are able to establish cell–ECM contacts with the peritoneal lining, as these fragments express integrins and cadherins (Van der Linden et al., 1994). Any condition that may damage the delicate serosal surface of the abdominal organs such as surgery, infections, hypoxia, inflammatory conditions such as in endometriosis, or antibodies may expose the ECM proteins underneath the thin mesothelial layer. It has recently been suggested that cellular adhesion stimulates chemokine expression (Smith et al., 1997). When endometrial cells attach to ECM, they secrete higher values of IL-8 among other substances. Granulocytes are recruited, thus contributing to the inflammatory reaction. In fact, we have recently shown that increasing doses of IL-8 stimulate ESC adhesive capacity to an ECM protein, fibronectin (Garcia-Velasco and Arici, 1999). This may imply that the increased

-Regulation of IL-8 expression by cell adhesion

Figure 4. (A) Interleukin (IL)-8 protein secretion by endometrial stromal cells is up-regulated by adhesion in a time-dependent manner. 24 h after the adhesion process starts, fibronectin induces a significantly higher IL-8 secretion compared with a non-integrin dependent substrate (poly-L-lysine). IL-8 protein expression was studied in culture supernatants obtained after 12 and 24 h incubation over 24-well plates coated with fibronectin and poly-L-lysine. Data were obtained from six replicates for each condition and three different experiment. *P < 0.05 at 12 h; P < 0.01 at 24 h; fibronectin versus poly-L-lysine. (B) Engagement with the β1 but not the β2 integrin subunit up-regulates IL-8 protein secretion in endometrial stromal cells. IL-8 protein expression was studied in culture supernatants obtained after 24 h incubation over 24-well plates coated with BSA, mouse anti-human integrin β1 and β2 monoclonal antibody and poly-L-lysine. Data were obtained from six replicates for each condition and three different experiment. BSA = bovine serum albumin; β1: mouse antihuman integrin β1 monoclonal antibody; β2 = mouse antihuman integrin β2 monoclonal antibody; P = poly-L-lysine. *P < 0.01 β1 versus other groups.
in IL-8 values confirmed that IL-8 secretion on adhesion is an integrin-dependent event. When ESC were attached to anti-β1 or anti-β2 integrin antibody coated plates, the rise in IL-8 expression suggests that it is the engagement of the β1 that induces the IL-8 up-regulation.

The integrins of the β1 subfamily recognize various ECM proteins. These integrins provide a physical linkage between cytoskeletal structure and the ECM. By organizing the cytoskeleton, integrins may transmit signals, regulating cell shape and internal cellular architecture (Aplin et al., 1998). Integrin-mediated anchorage may also be implicated in cell cycle and cell growth control by releasing soluble mitogens, e.g. IL-8, or by cytoplasmic signalling that mediates cell cycle activation (Juliano and Haskill, 1993). We treated ESC with cytochalasin D just before plating the cells. Cytochalasin D disrupts actin filament organization and blocks tyrosine phosphorylation of pp125fak, the focal adhesion kinase. The disruption of the actin cytoskeleton blocked the increase in IL-8 induced by fibronectin, confirming that an intact cytoskeleton is required to stimulate IL-8 gene expression in response to integrin activation. This finding is consistent with the proposed mechanisms of integrin-mediated signal transduction based on the assumption that integrins bind directly to the cytoskeleton, thereby promoting the reorganization of the cell architecture. The newly formed cytoskeleton framework may transduce intricate signals to the cellular nucleus regulating cell cycle and mitogenesis, inducing gene expression and cell differentiation, and influencing apoptosis survival, probably by activating genes that may protect the cell from dying (Juliano and Haskill, 1993; Aplin et al., 1998). This integrin-mediated anchorage may release soluble mitogens, e.g. IL-8, which is known to stimulate ESC proliferation (Arici et al., 1998b). Transmission of the correct signal to endometrial cells may be an important step for determining which cells are going to adhere and contribute to the development of endometriosis.

Our findings suggest that cell adherence to ECM stimulates IL-8 expression and that integrin-mediated adhesion is involved in this process. Further investigation into the molecular mechanisms of adhesion-induced expression of inflammatory mediators should provide insight into the early events of endometriosis.

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