Macrophage derived growth factors modulate Fas ligand expression in cultured endometrial stromal cells: a role in endometriosis

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Fas–Fas ligand (Fasl) interactions play a significant role in the immune privilege status of certain cell populations, and several cytokines and growth factors can modulate their expression. When a Fasl-expressing cell binds a Fas-bearing immune cell, it triggers its death by apoptosis. In this study, we demonstrate that normal human endometrial epithelial but not stromal cells express FasL. Moreover, we showed that macrophage-conditioned media induced FasL expression by endometrial stromal cells in a dose-dependent manner. To elucidate which macrophage product was responsible for the up-regulation of Fasl, endometrial stromal cell cultures were treated with the macrophage products platelet-derived growth factor (PDGF), transforming growth factor (TGF)-β1, and basic fibroblast growth factor (bFGF). The first two (which are known to be elevated in the peritoneal fluid of women with endometriosis) induced a dose-dependent up-regulation of FasL expression, which was specifically inhibited by the antibody. Interestingly, bFGF (which is not elevated in peritoneal fluid of women with endometriosis) did not induce any response. These results suggest that the pro-inflammatory nature of the peritoneal fluid of women with endometriosis induces the FasL expression by regurgitated endometrial cells, and signals Fas-mediated cell death of activated immune cells. This could be a mechanism for endometrial cells to escape immune surveillance, implant and grow.

Key words: bFGF/Fas–FasL system/immunoprivilege/PDGF/TGF-β1

Introduction

Endometriosis, one of the most enigmatic gynaecological diseases, is characterized by the presence of endometrial cells, both epithelial and stromal, outside the uterus (Olive and Schwartz, 1993). Retrograde menstruation has been accepted as a mechanism for transportation of endometrial fragments, their implantation into the pelvis, and their proliferation at the ectopic sites. However, while 76–90% of women experience retrograde menstruation (Bartosik et al., 1986), endometriosis affects only one in 15 women of reproductive age (Olive and Schwartz, 1993). This suggests that other factors, such as an impaired peritoneal environment, may determine a woman’s susceptibility to developing endometriosis (Oral and Arici, 1996).

During the last decade, a growing interest has emerged in the role of the intraperitoneal environment in the pathogenesis of endometriosis (Ramey and Archer, 1993; Oral and Arici, 1996). Although macrophages are present in the peritoneal fluid under physiological conditions, an increased number of macrophages, as well as a higher activation level of these cells, have been reported in women with endometriosis (Haney et al., 1981; Halme et al., 1983, 1984, 1987). This is relevant since we know that activated macrophages express, synthesize, and release a great variety of growth factors, such as transforming growth factor (TGF)-β, platelet-derived growth factor (PDGF) or basic fibroblast growth factor (bFGF) (Nathan, 1987). In addition, some of these macrophage products have been implicated in the pathogenesis of endometriosis (Nathan, 1987). Most recently, increased values of PDGF (Halme et al., 1988) and TGF-β1 (Oosterlynck et al., 1994) have been found in the peritoneal fluid (PF) of women with endometriosis. Other growth factors, such as bFGF, did not show any increase in the PF of women with endometriosis (Seli et al., 1998).

The Fas–Fas ligand (Fas–FasL) system is a major pathway for the induction of apoptosis in cells and tissues (Nagata and Golstein, 1995). Fas, also called APO-1 or CD95, is a type I membrane protein of 45 kDa that belongs to the tumour necrosis factor (TNF)/nerve growth factor (NGF) receptor family (Nagata and Golstein, 1995). FasL, a type II membrane protein of 37 kDa, belongs to the TNF superfamily (Suda et al., 1993; Nagata and Golstein, 1995). Fas is expressed in various tissues such as thymus, liver, heart and kidney (Watanabe-Fukunaga et al., 1992), and its expression in thymus-derived (T) and bone marrow-derived (B) cells is enhanced after lymphocyte activation. In contrast, FasL expression is reported to be more restricted with predominant expression in activated T cells (Nagata, 1994; Suda et al., 1995). The Fas–FasL interaction has been implicated as a mechanism for clonal deletion, the control of T cell expansion during immune responses and in killing by cytotoxic T cells (Dao et al., 1996; Strand et al., 1996). More recently, FasL expression has been reported in non-immune cells, mainly in cells from immune-privileged tissues, suggesting that the Fas–FasL system may play an important role in the mechanism
underlying this immune privileged status. Thus, FasL expression has been detected in stromal cells of the retina (Griffith et al., 1995), as well as in the Sertoli cells in the testis (Bellgrau et al., 1995). We, and others, have also described FasL expression in the human placenta and its role in trophoblast invasion/proliferation (Uckan et al., 1996; Runic et al., 1997; Mor et al., 1998), as well as in tumour cells (Gutierrez et al., 1998; Rabinowich et al., 1998) and in the brain (Bechmann et al., 1998).

Several reports have clearly shown how different cytokines and growth factors may regulate FasL expression (Ohtsuki et al., 1997; Ashley et al., 1998; Sata and Walsh, 1998). Even more, the capacity of human tissue macrophages to express FasL has recently been reported (Dockrell et al., 1998). In the present study, we tested the hypothesis that activated macrophage products induce FasL expression in endometrial stromal cells (ESC), thereby allowing them to escape immune surveillance, implant and grow within the peritoneal cavity.

Using reverse transcriptase–polymerase chain reaction (RT–PCR) and Western blotting, we demonstrated the up-regulation of FasL expression in ESC by PDGF and TGF-β1, macrophage-derived growth factors which are found in increased levels in the peritoneal fluid of women with endometriosis. In contrast, bFGF, found at normal concentrations in the peritoneal fluid of women with endometriosis was found to have no effect on FasL expression by ESC. Thus, these data suggest that the Fas–FasL system may be involved in endometrial cell survival and implantation.

Materials and methods

Reagents

Roswell Park Memorial Institute (RPMI) 1640 and fetal bovine serum were purchased from Life Technologies (Grand Island, NY, USA). PDGF, TGF-β1, bFGF and the anti-human PDGF polyclonal antibody were purchased from R&D Systems (Minneapolis, MN, USA). Other chemicals, unless otherwise specified, were obtained from Sigma Chemical Co (St Louis, MO, USA).

Tissue collection and cell culture

Endometrial cell preparation

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 protocols approved by the Human Investigation Committee of Yale University. Tissues were kept in Hank’s balanced salt solution (HBSS) and transported to the laboratory for culture.

Endometrial cells were dispersed by incubation of tissue minced in HBSS containing HEPES (25 mM), penicillin (200 IU/ml), streptomycin (200 mg/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 ESC 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. Immunohistochemical analyses of isolated cells were conducted using factor VIII as a marker of endothelial cells, cytokeratin as a marker of epithelial cells, vimentin as a marker of stromal cells, and 3C10 as a marker of macrophages. In primary cultures, endothelial cells, epithelial cells, and macrophages accounted for 10, 17, and 2% of the cells respectively, and the remaining were stromal cells.

Stromal cells were plated in Dulbecco’s modified Eagle’s medium (DMEM):Ham’s F12 (1:1 v/v) containing antibiotics-antimycotics (1% v/v) and fetal bovine serum (FBS; 10% v/v) 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 passaged by trypsinization and plated in 6-well dishes for RNA extraction and again allowed to replicate to confluence. At that stage, by immunohistochemistry, endothelial cells were not present, epithelial cells accounted for 0–7% of the cells, and macrophages accounted for 0.2% of the total. After cells reached confluence, cultures were treated with serum-free, Phenol Red-free media for 24 h before the treatment with growth factors was initiated. All treatments were conducted in serum-free, Phenol Red-free media as well. Individual cultures were set up for each patient. All the experiments were conducted on at least three different occasions.

Induction of differentiation of THP-1 cells

The THP-1 myeloid leukaemia cells were induced to differentiate into macrophage-like cells by incubation for 48–72 h with phorbol methyl ester (PMA) at a concentration of 6 ng/ml.

Preparation of conditioned media

Differentially macrophage-like THP-1 cells at a density of 1×10⁶ cells/well were seeded in 6-well plates in Phenol Red-free RPMI 1640 medium with 10% FBS and supplemented with PMA (6 ng/ml). The cells were cultured at 37°C in a CO2 incubator for 24 h. The media was then removed from the plates, centrifuged and stored at 4°C for <48 h. These media were designated THP-1-conditioned media (CM). Non-conditioned media consisted of fresh Phenol Red-free RPMI 1640 medium with 10% FBS. Supplementation of the non-conditioned media was carried out as for the THP-1 CM (Mor et al., 1993, 1998b).

Incubation with macrophage-derived growth factors

ESC were treated with PDGF (10 ng/ml), TGF-β1 (1 ng/ml), and bFGF (10 ng/ml) for 3, 6, 12, and 24 h prior to RNA and protein extraction. Dose–response experiments were performed with PDGF (0.1, 1, and 10 ng/ml) and TGF-β1 (0.01, 0.1, and 1 ng/ml).

Blocking experiments

ESC were treated with PDGF (1 and 10 ng/ml), anti-human PDGF monoclonal antibody (1 μg/ml), and with PDGF at the aforementioned concentrations plus the antibody for 3 h before RNA extraction.

Preparation of total RNA and protein

Total RNA and protein were prepared from endometrial epithelial and stromal cells, and Jar cells using TRIZol® reagent (GIBCO BRL, Gaithersburg, MD, USA) according to the manufacturer’s instructions. The TRIZol® method allowed us to extract RNA and protein from the same culture and, thus, from the same patient. This is an advantage since we were able to study the same samples at both the mRNA and protein level.
Figure 1. Reverse transcription–polymerase chain reaction (RT–PCR) linearity. The Fas ligand (FasL) signal was measured by densitometry and standardized against the actin signal using a digital imaging and analysis system (AlphaEase, Alpha Innotech Corporation; San Leandro, CA, USA). The linearity of the system was determined using serial dilutions of cDNA.

Figure 2. Expression of Fas ligand (FasL) in cultured human endometrial epithelial and stromal cells. (A) Reverse transcription–polymerase chain reaction (RT–PCR) for FasL was performed with total RNA extracted from in-vitro monolayer cultures of isolated human endometrial epithelial and stromal cells. Total RNA from the human choriocarcinoma Jar cell line was used as positive control (lane 1) β-actin housekeeping gene was amplified to verify that the same amount of cDNA was loaded in each lane. (B) Western blotting for FasL was performed with protein samples obtained from the same cell cultures. Proteins were separated by sodium dodecyl sulphate–polymerase chain reaction (SDS–PAGE) using 10% polyacrylamide gels, transferred to nitrocellulose membranes, and immune blotted with FasL monoclonal antibody (clone 33). Results shown are representative of at least three experiments. MM = molecular marker; EEC = glandular epithelial cells; ESC = stromal cells.

RT–PCR analysis

RT–PCR was performed using the RT–PCR kit from Pharmacia BioTech (Piscataway, NJ, USA) according to the manufacturer’s directions. cDNA synthesis was performed with pd(N) 6 0.2 µg and 1 µg total RNA. The primers used for amplification of FasL have been recently described (Strand et al., 1996) and have the following sequence: upstream, 5′-ATAGGATCCATGTTCTCGCTTCACC-TACAGAG-3′; downstream, 5′-ATAGGATCTGACCAAGAGAGAGCTCAGATACGTTGAC-3′. Each PCR cycle consisted of denaturation at 95°C, 1 min; annealing at 56°C, 1 min; and elongation at 72°C, 1 min, for a total of 30 cycles. The PCR products were analysed by gel electrophoresis.

The FasL signal was measured by densitometry and standardized against the actin signal using a digital imaging and analysis system (AlphaEase, Alpha Innotech Corporation; San Leandro, CA, USA). The linearity of the system was determined using serial dilutions of cDNA (Figure 1).

Western blot analysis

Proteins were separated by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) using 10% polyacrylamide gels and transferred to nitrocellulose membranes. Immunoblotting was performed after blocking the membranes with 5% powder milk. The primary antibody (FasL monoclonal antibody, clone 33, Transduction Laboratories, Lexington, KY, USA) was used at 1:1000 dilution. The secondary antibody (peroxidase-labelled horse anti-mouse, Vector, Burlingame, CA, USA), was developed with TMB Peroxidase substrate kit (Vector, Burlingame, CA, USA).

Co-culture DNA fragmentation assay (JAM test)

Target Jurkat cell (Fas-positive) death resulting from the co-culture with effector stromal cells (FasL-positive) was quantified by measurement of target cell DNA fragmentation using the JAM assay. (Matzinger, 1991) Adherent ESC were seeded into the wells of a flat 96-well microtitre plate at a cell number appropriate to give the required E:T ratios. Target Jurkat cell DNA was labelled by prior incubation with 10 Ci/ml of [3H]-TdR at 37°C for 24 h. Labelled Jurkat cells were washed and added to the seeded effector cells in a final volume of 200 µl/well. After co-culture at 37°C for 8–24 h, the cells were removed from the wells and filtrated onto glass fibre filters using an automatic 96 well filtration unit. The cells were then lysed with hypotonic buffer and their DNA was washed through the
Fas ligand modulation by growth factors in endometrial cells

Figure 3. Endometrial stromal cells treated with macrophage-differentiated conditioned media. In-vitro cultured endometrial stromal cells were serum-starved for 24 h, and then treated with macrophage-conditioned media obtained from differentiated macrophages/THP-1 cells. Fas ligand (FasL) expression was analysed after a 24 h incubation using (A) reverse transcription–polymerase chain reaction (RT–PCR) and (B) Western blotting as described before. Results shown are representative of at least three experiments. C = control; CM 50% = macrophage-conditioned media at 50% concentration; CM 100% = macrophage-conditioned media at 100% concentration.

filter by four washes with D/D water. The radioactivity of intact chromosomal DNA retained on each filter was measured by liquid scintillation counting. Specific cell killing was calculated using the following equation (Matzinger, 1991):

% specific killing = (S – E/S)×100

where E (experimental) is cpm of retained (complete) Jurkat cell DNA in the presence of MCF7 effector cells and S (spontaneous) is cpm of retained DNA in the absence of effector cells.

Results

Expression of FasL in human endometrium

Determination of FasL mRNA expression by RT–PCR
To test the possibility that FasL was expressed in endometrial tissue, total RNA was extracted from cultured endometrial epithelial cell (EEC) and ESC cultures and evaluated by RT–PCR. In all cases studied, EEC cultures were found to express FasL mRNA at a level comparable to that of Jar cells. (Figure 2A). However, we found variable FasL expression in different preparations of ESC cultures, 90% of the cases studied had undetectable FasL mRNA expression, while the remaining 10% showed low FasL mRNA levels. The reason for this difference is under investigation.

Western blot analysis
To confirm FasL expression in endometrial cells, we performed Western blot analysis of whole cell lysates from the same patients and cultures from which we had previously obtained mRNA. As seen on Figure 2B, the Western blot confirmed, at the protein level, the RT–PCR results. FasL protein was present in both EEC and ESC, but at a lower extent in the latter.

In-vitro regulation of FasL expression

Effect of macrophage-conditioned media on FasL mRNA expression
To evaluate the effect of activated macrophage secreted soluble products on FasL expression, ESC cultures were incubated with CM obtained from differentiated macrophages/THP-1 cells. FasL expression was analysed after a 24 h incubation using RT–PCR analysis. As shown in Figure 3, CM induced a marked increase in FasL expression detected at both the mRNA and protein level.

PDGF, TGF-β₁, and bFGF modulation of FasL mRNA expression in ESC
To characterize the macrophage-secreted soluble growth factors which could mediate the observed effect of CM on FasL expression, ESC cultures were treated with PDGF, TGF-β₁, and bFGF and the expression of FasL was analysed both at the RNA and protein levels.

Time-course experiments: Endometrial stromal cells were treated with PDGF (10 ng/ml), TGF-β₁ (1 ng/ml) and bFGF (10 ng/ml), and incubated for time intervals of 0, 3, 6, 12, and 24 h, after which the experiment was terminated. Treatment with PDGF or TGF-β₁ induced an early increase in the transcription of FasL observed at 3 h, and which gradually decreased at 6 and 12 h. Interestingly, no up-regulation of
Figure 4. Effect of platelet-derived growth factor (PDGF), transforming growth factor (TGF)-β1, and basic fibroblast growth factor (bFGF) on Fas ligand (FasL) expression in endometrial stromal cells. In-vitro cultured endometrial stromal cells were serum-starved for 24 h, and then treated with (A) PDGF at 10 ng/ml, (B) TGF-β1 at 1 ng/ml, and (C) bFGF at 10 ng/m for 3–24 h. Total RNA was extracted and the FasL expression was analysed using reverse transcription–polymerase chain reaction (RT–PCR). Results shown are representative of at least three experiments.

FasL expression was observed with bFGF, the only growth factor of the three tested which is not increased in the PF of women with endometriosis (Figure 4).

Dose–response experiments: A dose response was performed to assess the dependence of FasL transcription on increasing concentrations of PDGF and TGF-β1. Endometrial cell cultures in 6-well plates were treated for 3 h with PDGF at concentrations ranging from 0 to 10 ng/ml, and with TGF-β1 at concentrations ranging from 0 to 1 ng/ml. Both macrophage-derived growth factors induced a concentration dependent increase in FasL expression (Figure 5).

Blocking experiments: To investigate the specificity of the up-regulation of FasL induced by PDGF, we performed blocking experiments. ESC cultures were treated for 3 h with PDGF at concentrations of 0, 1 and 10 ng/ml, and with a specific anti-human PDGF monoclonal antibody at a fixed dose of 10 μg/ml. A 65% reduction in the induction of FasL gene expression was observed when anti-PDGF was added together with 1 ng/ml of PDGF to ESC (Figure 6). However, when the stimulus was stronger (10 ng/ml), the same concentration of anti-PDGF could only induce a 5% reduction (Figure 6).

Effect of PDGF, and TGFβ1 on FasL expression at the protein level
The FasL protein expression was examined by Western blot analysis of whole cell lysates. As demonstrated in Figure 7, FasL was present in cell lysates obtained from ESC cultures treated for 24 h with either PDGF or TGF-β1, confirming the previous mRNA findings.

Figure 5. Dose–response effect of platelet-derived growth factor (PDGF), transforming growth factor (TGF)-β1 on Fas ligand (FasL) expression in endometrial stromal cells. Endometrial stromal cell cultures were treated for 3 h with (A) PDGF at concentrations ranging from 0 to 10 ng/ml, and (B) TGF-β1 at concentrations ranging from 0 to 1 ng/ml. Total RNA was extracted and reverse transcription–polymerase chain reaction (RT–PCR) was performed to study FasL expression. Results shown are representative of at least three experiments.

Fas-positive Jurkat cells are killed by PDG-treated stromal cells
In order to ascertain whether the FasL present on the stromal cells is functional we established a co-culture system in which
et al. provide evidence that this mechanism is mediated by the Fas–FasL system, allowing endometrial cells to implant and grow. In their attempt to delete the menstrual debris that reached the peritoneal cavity through retrograde menstruation, bind to the endometrial cells in the peritoneal cavity, allowing endometrial cells to implant and grow.

In the present study, we test the hypothesis that macrophages secrete cytokines such as IL-8, capable of promoting endometrial cell growth and proliferation (Arici et al., 1998). We speculated that cytokines released at the micro-environment could up-regulate FasL expression in endometrial cells providing the means to defeat the immune system and escape immune surveillance. Activated T cells expressing Fas, in their attempt to delete the menstrual debris that reached the peritoneal cavity through retrograde menstruation, bind to the FasL expressed by endometrial cells, undergo apoptosis, thus allowing endometrial cells to implant and grow.

Our data strongly support this hypothesis. FasL expression is induced on endometrial cells by conditioned media obtained from in-vitro differentiated macrophages. Similar results are obtained with TGF-β and PDGF, both macrophage-products (Shanker et al., 1995) known to be increased in women with endometriosis when compared to women without endometriosis. This effect is clearly specific since other macrophage-derived growth factors, such as bFGF, had no effect on FasL expression.
Figure 7. Fas ligand (FasL) protein expression in endometrial stromal cells. FasL protein expression was studied in samples obtained from endometrial stromal cell cultures treated for 24 h with either (A) platelet-derived growth factor (PDGF; 10 ng/ml) or (B) transforming growth factor (TGF)-β1 (1 ng/ml), as previously described. Results shown are representative of at least three experiments.

Figure 8. Fas ligand (FasL) induction of apoptosis of Fas bearing cells. Endometrial stromal cells (ESC) were treated with platelet-derived growth factor (PDGF, 10 ng/ml) for 24 h and co-cultured with [3H]-thymidine-labelled Fas-positive Jurkat cells for 12h. The percentage of specific killing was calculated as described in the text. Only PDGF–ESC treated cells induced apoptosis of Fas-positive Jurkat cells. Results shown are representative of at least three experiments.

expression. Worth mentioning is the fact that bFGF is not increased in peritoneal fluid from women with endometriosis (Huang et al., 1996; Seli et al., 1998).

The effect of TGF-β on ESC cultures is relevant in view of its presence in other immune privileged sites. It has been demonstrated that the microenvironment of certain immune privileged sites constitutively contains TGF-β, which is able to inhibit the inflammatory response (Wilbanks and Streilein, 1992; Taylor et al., 1994). Therefore, we propose that one of the mechanisms by which TGF-β and PDGF maintain the immune privileged status could be through the induction of FasL expression by the local cells which may alter the afferent antigenic signals, thus escaping the systemic immune system. Additionally, given the fact that TGF-β and PDGF are chemotactic factors for inflammatory cells, and promote cell proliferation, differentiation and angiogenesis (Surrey and Halme, 1991; Chegini et al., 1992, 1994); they could also have synergistic effects on endometrial cells. Thus, TGF-β and PDGF could help to promote endometriotic lesions, as well as induce FasL-mediated apoptosis in Fas-bearing activated immune cells (Figure 8).

A recent report described the invasive potential of E-cadherin-negative endometrial cells (Gaetje et al., 1997) suggesting that these cells might be the ones that migrate to ectopic locations, thus causing endometriosis. If the E-cadherin status of the endometrial cells is coupled with up-regulation of FasL, we may speculate that the immune escape stage would be set. Perhaps this may partially explain why EEC expressing FasL do not always implant at ectopic sites, if retrograde menstruation is seen in up to 90% of women.

In summary, ESC culture is a useful model in which to study the regulation of the Fas–FasL system. Moreover, our studies demonstrate the interaction between peritoneal macrophages and endometrial cells. Contrary to the expected cytolytic effect of these immune cells, the macrophage products may confer the endometrial cells with the means to escape immune
surveillance. Thus, macrophage-derived growth factors which are elevated in women with endometriosis up-regulate FasL expression, which in turn, may induce apoptosis in activated T cells, permitting endometrial cells to survive in the peritoneal cavity. These findings provide new insights about the pathogenesis of endometriosis. At the same time, it opens the door for a new therapeutic approach, involving the use of immunomodulators.

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