Influence of peritoneal fluid on the expression of angiogenic and proteolytic factors in cultures of endometrial cells from women with endometriosis


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BACKGROUND: Endometriosis, defined as the presence of endometrium outside the uterus, is one of the most frequent benign gynaecological diseases. It has been suggested that both endometrial and peritoneal factors, related to angiogenesis and proteolysis, can be implicated in this disease. The aim of this study was to evaluate the influence of peritoneal fluid on the expression of angiogenic and proteolytic factors in cultures of endometrial cells from women with and without endometriosis.

METHODS: Endometrial cells were isolated, cultured and treated with endometriotic or normal peritoneal fluid. Vascular endothelial growth factor-A (VEGF-A), urokinase plasminogen activator (uPA), matrix metalloproteinase-3 (MMP-3) and their inhibitors including thrombospondin-1, plasminogen activator inhibitor-1 and MMP inhibitor type 1 (TIMP-1) mRNA levels were evaluated by quantitative RT-PCR, and protein levels were quantified by ELISA.

RESULTS: Peritoneal fluid from women with endometriosis induced an increase in VEGF-A and uPA protein and VEGF-A mRNA and uPA mRNA levels in endometrial cell culture from women with (P<0.01) and without endometriosis (P<0.05). The highest levels of VEGF-A and uPA were observed in endometrial cell cultures from patients with endometriosis and treated with peritoneal fluid from women with endometriosis.

CONCLUSIONS: Peritoneal fluid from women with endometriosis induced more VEGF and uPA expression in endometrial cell culture from women with endometriosis than did normal peritoneal fluid. Endometrial–peritoneal interactions increased angiogenic and proteolytic factors in endometrial cells, which could contribute to the development of endometriotic lesions.

Key words: endometriosis / angiogenesis / proteolysis / peritoneal fluid / endometrial cell culture

Introduction

Endometriosis, characterized by the presence of endometrial tissue outside the uterus, represents one of the most frequent benign gynaecological diseases (Giudice and Kao, 2004). Although the pathogenesis of endometriosis remains controversial, Sampson’s (1927) theory of retrograde menstruation is by far the most widely accepted. Despite its high prevalence and incapacitating symptoms, the exact pathogenic mechanisms of endometriosis remain unsolved. It is a multifactorial disease in which both endometrial and peritoneal factors, related to angiogenesis and proteolysis, may be implicated.
It has been reported that angiogenesis may have an important role in the pathogenesis of endometriosis. Similar to tumour metastasis, endometriotic implants require neovascularization to proliferate, invade the extracellular matrix (ECM) and establish an endometriotic lesion (Taylor et al., 2002; Laschke et al., 2006; Laschke and Menger, 2007). Vascular endothelial growth factor (VEGF) represents one of the most potent angiogenic factors (McLaren, 2000; Carmeliet, 2003). Several studies have reported an increase in VEGF levels in endometriosis and it has been suggested that VEGF plays an important role in the progression of the disease (Donnez et al., 1998; Fasciani et al., 2000; Takehara et al., 2004; Girling and Rogers, 2005; Gilabert-Estellés et al., 2007; Cosín et al., 2008). Thrombospondin-1 (TSP-1) is an inhibitor of angiogenesis, and it has been reported that alterations in TSP-1 expression may contribute to many pathologies of the reproductive tract, including endometriosis, in which vessel formation occurs (Tan et al., 2002; Kawano et al., 2005; Gilabert-Estellés et al., 2007).

Furthermore, angiogenesis depends on controlled interactions between cells and ECM, and a controlled extracellular proteolysis is a requirement for new vessel formation. Most of the relevant extracellular enzymes belong to plasminogen activator and matrix metalloproteinase (MMP) systems (Pepper, 2001). The role of these two systems has been studied in endometriosis (Bruse et al., 1998, 2004; Chung et al., 2001; Cox et al., 2001; Gilabert-Estellés et al., 2003, 2005, 2007; Lembessis et al., 2003; Ramón et al., 2005).

We have previously reported an increase in the expression of angiogenic (VEGF-A) and proteolytic factors (urokinase plasminogen activator—uPA; metalloproteinase—MMP-3) in endometrial tissue from patients with endometriosis (Gilabert-Estellés et al., 2003, 2007; Ramón et al., 2005) and we have suggested that this increase might contribute to the invasive potential of endometrial cells. Moreover, we have also observed an increase in VEGF-A, uPA and MMP-3 levels in peritoneal fluid from patients with endometriosis in comparison with women without the disease (Gilabert-Estellés et al., 2007). These results suggest that both endometrial and peritoneal factors of women with endometriosis enhance the angiogenic and proteolytic capability of ectopic tissue facilitating the implantation process.

However, to the best of our knowledge, the role of peritoneal fluid in the expression of angiogenic and proteolytic components in endometrial cell culture has not yet been established. Cell cultures are a useful tool to study tissue interactions under controlled conditions. Moreover, the development of endometrial cell cultures could help in the research of antiproliferative and antiangiogenic products as possible future therapeutic tools.

To evaluate the endometrial—peritoneal interactions controlling the angiogenic and proteolytic systems in endometriosis, we have studied the influence of peritoneal fluid on these systems in endometrial cell cultures from women with and without the disease.

**Materials and Methods**

Women with moderate or severe endometriosis (stages III–IV, revised American Society for Reproductive Medicine classification system, 1997) were studied (n = 14; mean age 31.14 years; range 21–42). Surgery was performed in all cases for pelvic pain and ultrasound suspicion of ovarian endometrioma. A systematic examination of the pelvis and upper abdomen was performed to obtain topography scores and to determine the extent of the disease. Peritoneal fluid was collected immediately after the establishment of the pneumoperitoneum and before any laparoscopic manipulation. Systematic mobilization of the rectosigmoid and the ovaries was performed, and pelvic adhesions were excised to identify the presence of superficial or deep endometriotic lesions. Only patients with at least one ovarian endometrioma and active peritoneal endometriotic implants were eligible for the study. Women with rectovaginal endometriosis or extragenital disease were excluded. A complete excision of the endometriotic tissue was performed. The diagnosis of endometriosis was confirmed by the post-operative pathological examination of all the specimens obtained. Patients with suspected endometriosis without pathological confirmation of the disease were excluded from the study.

Normal endometrial tissues were obtained from fertile women without endometriosis who underwent surgery for tubal sterilization (n = 6; mean age 34.33 years; range 28–40). Peritoneal fluids were also obtained from 10 fertile women without endometriosis (mean age 35.80 years, range 29–41). Peritoneal fluid was also collected immediately after the establishment of the pneumoperitoneum and before any laparoscopic manipulation. The absence of the disease was confirmed after surgical examination of the abdominal cavity. Meticulous examination of the peritoneum, ovaries, intestine and diaphragm was performed to detect any typical or atypical endometriotic lesion. Biopsies of suspicious areas of endometriosis were confirmed to be negative in these women.

Women affected by menorrhagia, hypermenorrhoea or who had been pregnant or breast-feeding in the previous 6 months were also excluded from the study. None of the included women had received hormonal treatment for at least 3 months before the study.

Informed consent was obtained from all patients and controls, and the study was approved by the Ethical Committee of our Institution.

**Tissue samples**

Endometrial biopsies (eutopic endometrium) from women with moderate or severe endometriosis (stages III–IV) (n = 14) were performed by aspiration using the Corringer device (Gynetics, Hamont-Achel, Belgium). Endometrial biopsies from patients without endometriosis (normal endometrium) (n = 6) were obtained using the same procedure.

All samples were rinsed in phosphate-buffered saline (PBS, Dulbecco’s; Gibco BRL, Life Technologies Ltd, Paisley, UK). All tissue samples from endometriotic lesions were also microscopically evaluated to confirm the diagnosis.

The menstrual phase was identified according to the day of the reproductive cycle and the histological analysis of the endometrium. Five (35.7%) women with endometriosis were in the proliferative phase and nine (64.3%) were in the secretory phase. Two (33.3%) controls were in the proliferative phase and four (66.7%) were in the secretory phase of the menstrual cycle.

**Peritoneal fluid**

Peritoneal fluid samples were carefully collected, as described in previous reports (Gilabert-Estellés et al., 2003, 2007), from the pouch of Douglas and the vesicouterine space. The fluid was collected in vacuum tubes with a sterile syringe, which was attached to an endoscopic catheter. Blood-contaminated peritoneal fluids were excluded. No peritoneal washings were performed prior to the collection of peritoneal fluids, and no anticoagulant was used. The peritoneal fluid was immediately cleared of cells and cell debris by centrifugation at 1500g for 30 min at 4°C, filtered through a 0.2 μm pore size membrane and stored at −80°C.

At the time of the experiment, 10 samples of peritoneal fluid from women with endometriosis in the secretory phase of the menstrual cycle (endometriotic peritoneal fluid pool) were thawed and pooled and...
10 samples of peritoneal fluids from women without endometriosis in secretory phase (normal peritoneal fluid pool) were also thawed and pooled.

**Endometrial cell culture**

Primary culture of endometrial cells was prepared as described previously (Ryan et al., 1994) with minor modifications. The endometrial biopsy was collected in PBS containing 50 U penicillin/ml and 50 μg streptomycin/ml (Sigma), stored at 4°C, and processed within 2–18 h. The tissue was cut into 1 mm³ pieces and incubated at 37°C for 60 min in the presence of collagenase (2.5 mg/ml, Sigma). Dissociated tissue was filtered through a nylon sieve to remove undigested material.

The purity of the endometrial stromal cells was evaluated by immuno-cytotoxic staining, using antibodies against cytokeratin, vimentin and CD68, and was >95%.

The cell suspension was centrifuged at 550g for 5 min, and the pellet was resuspended in DMEM-F12 phenol red-free medium containing 10% fetal bovine serum (FBS, Invitrogen), 50 U penicillin/ml and 50 μg streptomycin/ml (Sigma) medium. Cell viability, assessed by trypan blue exclusion test, was >95%. Then,stromal cells were plated on a 12-cm² flask and maintained in a humidified atmosphere of 5% CO₂ and 95% air at 37°C. The culture medium was renewed every 2 days until the cell monolayer reached confluence, and the cells were then subcultured. The cells were detached using 0.25% trypsin/0.02% EDTA (Gibco BRL, Paisley, UK) at 37°C and seeded at a density of 270,000 cells/well in 12-well plates. Confluent passage 2 cultures were used for experimental assays.

We first used a 4-h preconditioning treatment by culturing stromal cells in FBS-free medium. Thereafter, cells were shifted to medium supplemented with increasing concentrations of peritoneal fluid pools (0, 10 or 25% final concentration) from women with or without endometriosis and incubated for another 4-h period.

Finally, cell culture supernatants were collected for protein quantification, and cell culture extracts were used for RNA extraction and mRNA quantification. All the experiments performed in cell cultures from 14 women with endometriosis and in cell cultures from 6 women without endometriosis were performed in triplicate.

**Quantitative real-time RT–PCR**

Briefly, RNA from endometrial cell cultures was extracted using the RNeasy total RNA kit (Qiagen, Inc., Valencia, CA, USA) in accordance with the manufacturer’s instructions. A 1-mg sample of RNA was treated with DNase I (Invitrogen, Carlsbad, CA, USA) and stored at −80°C until use. The concentration and purity of RNA were determined spectrophotometrically. The 1-mg sample of total RNA was reverse-transcribed into first-strand cDNA by using Superscript RNase H⁻ (Invitrogen) with an oligo (d)T₁₅ primer (Promega, Madison, WI, USA). The cDNA was stored at −20°C until use.

cDNA calibrators were prepared by PCR amplification with the appropriate primers. The resulting PCR products were purified by column chromatography (QIAquick PCR purification kit; Qiagen). The samples showed a single band by agarose gel electrophoresis. The amount of DNA was determined by PicoGreen fluorescence (Molecular Probes) (Castello et al., 2002).

Analysis of VEGF, TSP-1, uPA, plasminogen activator inhibitor-1 (PAI-1), MMP3, MMP inhibitor-1 (TIMP-1) and β-actin (control gene) mRNA expression was performed in a LightCycler apparatus, using version 3.5 software (Roche Molecular Biochemicals, Mannheim, Germany). The LightCycler software constructed the calibration curve by plotting crossing point (Cₚ) versus the logarithm of the number of copies for each calibrator. The specific primers used for amplification of VEGF, TSP-1, uPA, PAI-1, MMP3, TIMP-1 and β-actin, the reaction mixture and the PCR conditions were as described previously (Ramón et al., 2005; Gilabert-Estellés et al., 2007).

**Protein quantification**

VEGF-A protein level was measured by a commercially available ELISA (Human VEGF, IBL International, Germany). No cross-reactivity or interference with placental growth factor was observed. This assay recognizes human VEGF-A₁₆₅ and VEGF-A₁₂₅ isoforms (Gilabert-Estellés et al., 2007; Cosín et al., 2008). The intra-assay and interassay variabilities were 4–6 and 7–10%, respectively.

TSP-1 level was quantified by an indirect ELISA, as described previously (Gilabert-Estellés et al., 2007). The coefficient of variation was 4.8%.

uPA protein levels were quantified by commercially available ELISA (Zymutest uPA, Hyphen Biomed, France), which measures single-chain urokinase and the high-weight molecular form of uPA with similar efficiency. The intra-assay and interassay variabilities were 3–5 and 8–11%, respectively.

PAI-1 protein levels were quantified by commercially available ELISA (Imubind tissue PAI-1, America Diagnostica, USA). The assay detects free and complexed PAI-1 and is insensitive to PAI-2. The intra-assay and interassay variation coefficients were 3–4 and 6–8%, respectively.

MMP-3 protein and TIMP-1 protein levels were quantified by commercially available ELISAs (MMP-3 and TIMP-1 ELISA, Oncogene) as described previously (Gilabert-Estellés et al., 2003, 2007). The MMP-3 assay detects MMP-3 but does not recognize MMP-3/TIMP complexes. The intra-assay and interassay variabilities were 4–6 and 7–10%, respectively. The TIMP-1 assay recognizes free and complexed TIMP-1. The intra-assay and interassay variabilities were 3–5 and 6–8%, respectively.

Protein levels of all the parameters studied were determined in culture supernatants and in the peritoneal fluid pools. The protein amounts (VEGF, uPA, MMP3, TSP-1, PAI-1 and TIMP-1), released to the culture medium by cells incubated with peritoneal fluid pools, were calculated by subtracting the VEGF, uPA, MMP3, TSP-1, PAI-1 and TIMP-1 contents of the peritoneal fluid-supplemented media from the total amount of each protein in the culture supernatant after the 4-h incubation period.

**Statistical analysis**

All variables were checked for normal distribution with the Kolmogorov–Smirnov test. All the parameters studied showed a normal distribution. The differences between the means of the variables studied in the cell cultures for the different treatments were analysed by one-way ANOVA test. When significant P-values were determined, post-hoc analyses were performed using Bonferroni test. Differences between the variables studied in the endometrial cells from women with or without endometriosis for the same treatment were analysed by unpaired Student’s t-test. Values are expressed as mean ± standard error of the mean. Levels of significance in correlations between variables were calculated by the bivariate Pearson correlation test. P-values <0.05 (two-tailed) were considered significant. All these tests were performed using the statistical package SPSS Release 11.5 for Windows (SPSS Inc.).

**Results**

**Preconditioning experiments to optimize the concentration of peritoneal fluid to add to endometrial cell cultures**

To optimize the concentration of peritoneal fluid to add to endometrial cell cultures, the effect of peritoneal fluid concentration on
cellular viability was assessed by the trypan blue exclusion test. Peritoneal fluid up to 25% final concentration had no effect on cell viability. As indicated in Materials and Methods, the stromal cells were first pre-conditioned in FBS-free medium for a 4-h period. Thereafter, cells were shifted to medium supplemented with increasing concentrations of peritoneal fluid pools (0, 10 or 25% final concentration) from women with or without endometriosis and incubated for another 4-h period.

When endometrial cells in culture were treated with 0, 10 or 25% peritoneal fluid in serum-free medium, VEGF and uPA levels increased with increasing peritoneal fluid concentrations (Fig. 1). Thereafter, endometrial cells were treated with 25% peritoneal fluid for 4 h in all subsequent experiments.

**Figure 1** Protein levels of VEGF and uPA in endometrial culture supernatants from women with endometriosis treated with endometriotic peritoneal fluid. The purpose of the experiment was to optimize the concentration of the peritoneal fluid added to the cell culture. Data are expressed as mean ± SEM.

Effect of peritoneal fluid on VEGF, uPA and MMP-3 levels in endometrial cell cultures from endometriotic and normal endometrium

Endometrial cells from endometriotic and normal endometrium were treated with serum-free medium containing peritoneal fluid (25% final concentration) for 4 h; the results were compared with endometrial cells cultured in medium without peritoneal fluid (0% final concentration) (Tables I and II).

Endometriotic and normal peritoneal fluids induced a significant increase in VEGF mRNA and protein levels in endometrial culture when compared with the corresponding control medium (Table I). The highest VEGF level was observed in endometrial cell cultures from endometriotic women treated with peritoneal fluid from women with endometriosis (Table I).

Moreover, both normal and endometriotic peritoneal fluids induced an increase in uPA mRNA and protein levels in the endometrial cultures from women with and without endometriosis. Again, the highest level of uPA was observed in endometrial cell cultures from endometriosis patients treated with peritoneal fluid from women with endometriosis (Table I).

Although both types of peritoneal fluid induced a significant increase in MMP-3 protein levels in endometrial cell culture supernatants from women with and without endometriosis, no significant increase in MMP-3 mRNA was observed (Table I).

In response to the same treatments, the levels of VEGF, uPA and MMP3 were higher in eutopic endometrial cells from women with endometriosis than in endometrial cells from control women; however, these differences were not statistically significant.

**Effect of peritoneal fluid on inhibitor levels (TSP-1, PAI-1 and TIMP-1) in endometrial cell cultures from women with and without endometriosis**

In relation to the angiogenic inhibitor, TSP-1, no significant effect was observed in endometrial cell cultures from women with or without endometriosis when normal peritoneal fluid was added. When endometriotic peritoneal fluid was used, an increase in TSP-1 protein was observed in endometrial culture supernatants from women with endometriosis, but no significant differences in TSP-1 mRNA were obtained (Table II).

While both types of peritoneal fluid induced an increase in PAI-1 mRNA and protein levels in endometrial cell culture extracts from women without endometriosis, there was no significant effect on endometrial cell cultures from women with endometriosis (Table II).

Furthermore, peritoneal fluid from both controls and endometriosis patients had no significant effect on TIMP-1 protein and mRNA levels in endometrial cell cultures from women with and without endometriosis (Table II).

**Correlation between changes in angiogenic, fibrinolytic and MMP parameters after treatment with peritoneal fluid**

In endometrial cell cultures from women without endometriosis, a significant positive correlation was observed between percentage changes in VEGF mRNA compared with both uPA and MMP-3 mRNA levels ($r = 0.657$ and $r = 0.778$, $P = 0.020$ and $P = 0.003$, respectively) and in VEGF protein levels compared with uPA and MMP-3 protein levels ($r = 0.745$ and $r = 0.602$, $P = 0.005$ and $P = 0.038$, respectively) after treatment with peritoneal fluid (Fig. 2). In endometrial cell cultures from women with endometriosis, a significant correlation was also observed between increases in VEGF and...
Fluid from women with endometriosis alter the expression of some components of the angiogenesis, plasminogen activator and MMP systems, indicating that these systems may play a role in the pathogenesis of this disease (Donnez et al., 1998; Fasciani et al., 2000; McLaren, 1996; Sharpe-Timms, 2001; Tan et al., 1998). However, no studies have previously been published on the effect of endometriotic and normal peritoneal fluid in relation to the expression and release of several angiogenic and proteolytic components in endometrial cell cultures from women with and without endometriosis.

### Discussion

Several studies suggest that the eutopic endometrium and peritoneal fluid from women with endometriosis alter the expression of some components of the angiogenesis, plasminogen activator and MMP systems, indicating that these systems may play a role in the pathogenesis of this disease (Donnez et al., 1998; Fasciani et al., 2000; McLaren, 1996; Sharpe-Timms, 2001; Tan et al., 2002; Gilbert-Estellés et al., 2003, 2005, 2007; Takehara et al., 2004). However, no studies have previously been published on the effect of endometriotic and normal peritoneal fluid in relation to the expression and release of several angiogenic and proteolytic components in endometrial cell cultures from women with and without endometriosis.

The present study shows that peritoneal fluid from women with endometriosis induces the highest increase in both VEGF and uPA mRNA expression levels and the release of VEGF and uPA protein in endometrial cultures from women with the disease. Peritoneal fluid contains a variety of cytokines, growth factors, steroid hormones, angiogenic and proteolytic factors (Koninckx et al., 1998). Moreover, several cell types, such as macrophages, endometrial and red blood cells, have been detected in peritoneal fluid (Koninckx et al., 1998). It has been reported that activated macrophages secrete interleukin-1β, which increases the expression of VEGF mRNA in cultured human stromal cells from endometriosis patients (Lebovic et al., 2000). In addition, endometriotic peritoneal fluid induces the production and release of VEGF by neutrophils (Na et al., 2006).

Because endometriotic peritoneal fluid induced the highest increase in VEGF mRNA and protein levels in endometrial cell culture extracts...
and supernatants from women with endometriosis, we hypothesize that some cytokines or growth factors present in endometriotic peritoneal fluid might contribute to the increase in angiogenesis in endometrial cells from endometriosis patients.

The angiogenic inhibitor, TSP-1, presented no significant differences in TSP-1 mRNA levels in endometrial cell cultures treated with peritoneal fluid. The increase in VEGF without a significant increase in TSP-1 obtained in the endometrium of women with endometriosis could indicate higher angiogenic activity, which might contribute to the capability of implantation of endometrial cells at ectopic sites.

Extracellular proteolysis is implicated in the initial stages of angiogenesis, and the group of proteinases involved includes components of the plasminogen and MMP systems (Ugwu et al., 1999; Pepper, 2001; Lijnen, 2002; Zorio et al., 2008). Given that VEGF induces uPA expression (Pepper, 2001), it seemed reasonable to simultaneously study angiogenic, fibrinolytic and MMP systems.

In relation to the modifications of fibrinolytic and MMP parameters in endometriosis, we have also published that uPA and MMP-3 mRNA and their protein levels are significantly higher in the endometrium from women with endometriosis than in controls (Gilabert-Estellés et al., 2007), thus confirming previous reports (Osteen et al., 1996; Sillem et al., 2001; Gilabert-Estellés et al., 2003; Ramón et al., 2005). In the present work, significant induction of mRNA and protein levels of uPA and protein levels of MMP-3 in the endometrial cell culture was observed in the presence of peritoneal fluid. Furthermore, the highest levels were observed in endometrial cell cultures from endometriosis patients and treated with peritoneal fluid from endometriosis. Moreover, a significant positive correlation was observed between percentage changes in VEGF levels and in both uPA and MMP-3 levels. The increase in proteolytic factors induced by peritoneal fluid from patients may also favour angiogenesis and the invasive properties of this tissue.

The development of endometriosis explants involves a series of cellular events including proliferation, adhesion, invasion and neovascularization. Some of these events utilize similar processes; for example, invasion and neovascularization both utilize proteolytic processes. Protein and mRNA levels of several angiogenic and proteolytic components may reflect the transcriptional and post-transcriptional regulation of these components and their relationship to these critical events in the development of endometriosis. In the present study, we have observed that peritoneal fluid induces a greater increase in protein levels of VEGF-A, uPA and MMP-3 than in the corresponding mRNA levels. These results suggest that certain proteins in endometriotic peritoneal fluid regulate the angiogenic and proteolytic factors in endometrial cultures from women with endometriosis at the transcriptional level but more so at the post-transcriptional level.

Moreover, high proteolytic and angiogenic activity in the endometrium may not be the only alteration responsible for the implantation process at ectopic sites. An impaired peritoneal environment, with increased capacity to further induce and potentiate alterations in endometrial tissue, may be a condition in the pathogenesis of the disease.

In conclusion, endometriotic peritoneal fluid induced more VEGF and urokinase levels in endometrial cell cultures from women with endometriosis than did normal peritoneal fluid. The highest levels were observed in endometrial cell cultures from endometriosis patients. Thus, endometrial–peritoneal interactions induced an increase in angiogenic and proteolytic factors in endometrial cells and may therefore participate in the establishment and persistence of peritoneal endometriosis. However, the specific mechanisms by which the angiogenic and proteolytic factors are produced by endometrial cell cultures treated with peritoneal fluid will require further investigation.

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