Expression of angiogenic factors in endometriosis: relationship to fibrinolytic and metalloproteinase systems

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BACKGROUND: Endometriosis a highly prevalent, benign disease in which the angiogenic, fibrinolytic and metalloproteinase (MMP) systems may be implicated. The objective of this study is to analyse mRNA expression and protein levels of several angiogenic factors and to correlate them with several components of the fibrinolytic and MMP systems in samples from 71 women with endometriosis and 50 controls. METHODS AND RESULTS: Eutopic endometrium showed higher mRNA expression of vascular endothelial growth factor (VEGF) in patients than in controls. However, ovarian endometrioma had lower VEGF mRNA levels than did the eutopic endometrium of patients. Similar results were obtained for VEGF protein levels. On the other hand, a significant increase in thrombospondin-1 (TSP-1) levels was observed in ovarian endometriosis than in eutopic endometrium. The peritoneal fluid from women with endometriosis showed a significant increase in VEGF, urokinase-type plasminogen activator (uPA) and MMP-3 levels than that of controls. A significant correlation was observed between the levels of VEGF and uPA in endometrium and in peritoneal fluid. CONCLUSIONS: Endometrium and peritoneal fluid from women with endometriosis have increased levels of VEGF, uPA and MMP-3 levels. Therefore, the development of endometriotic implants at ectopic sites may be facilitated, promoting the progress of the endometriosis.

Keywords: endometriosis; angiogenesis; VEGF; fibrinolytic system; metalloproteinase system

Introduction

Endometriosis, defined as the presence of endometrium outside the uterus, is one of the most frequent benign gynecological diseases (Martinez-Roman et al., 1997; Giudice et al., 1998; Pellicer et al., 2001). Despite its high prevalence and the incapacitating symptoms, the exact pathogenetic mechanisms of endometriosis remain unsolved. It is a multifactorial entity in which the angiogenic, fibrinolytic and metalloproteinase (MMP) systems may be implicated. (Donnez et al., 1998; Gilabert-Estelles et al., 2003, 2005; Bruse et al., 2004; Ramón et al., 2005).

Angiogenesis plays a major role in the pathogenesis of cancer, inflammation, rheumatoid arthritis and other diseases (Folkman, 1995). Angiogenesis is an important event for endometrial growth and tissue repair (Carmeliet, 2003), and abnormal angiogenesis may contribute to several endometrial-related pathologies, such as menorrhagia, endometrial cancer and endometriosis (McLaren et al., 1996; Healy et al., 1998; Girling et al., 2005). Several studies have indicated that angiogenesis may have an important role in the pathogenesis of endometriosis. Similarly to tumor metastasis, endometriotic implants require neovascularization to proliferate, invade the extracellular matrix (ECM) and establish an endometriotic lesion (Taylor et al., 2002; Laschke et al., 2005). Moreover, it has been suggested that antiangiogenic therapy may be a novel therapeutic approach to endometriosis (Hull et al., 2003; Nap et al., 2004; Becker et al., 2005; Laschke et al., 2006).

Angiogenesis is under the control of numerous inducers, including the vascular endothelial growth factor (VEGF) family and inhibitors, such as thrombospondin-1 (TSP-1) (McLaren et al., 2000). Endometrium undergoes cyclical growth and regression during the menstrual cycle, which depends on ovarian steroids levels. It is therefore a rich source of angiogenic growth factors, including VEGF. VEGF induces endothelial cell proliferation, migration, differentiation and capillary formation (Breier et al., 1997). Several studies have reported an increase in VEGF levels in endometriosis and it has been suggested that VEGF plays a role in the progression of the disease (Donnez et al., 1998; Fasciani et al., 2000; Takehara et al., 2004).

J. Gilabert-Estellés and L. A. Ramón have contributed similarly in the present article.
Placental growth factor (PIGF) is a member of the VEGF family, with ~50% identity to VEGF in the platelet-derived growth factor-like domain (Iyer et al., 2001). Whereas PIGF was originally identified in the placenta, it has been also detected in other organs such as heart, lung, thyroid gland and skeletal muscle (Carmeliet et al., 2001; Autiero et al., 2003). Despite the evidence for a role of PIGF in the control of angiogenesis, the specific biologic role of increased PIGF concentrations on angiogenesis and its effects on inflammatory reactions have remained unclear. Expression of PIGF mRNA has been found in uterine natural killer cells (Li et al., 2001) and an increase in PIGF antigen levels has been reported in peritoneal fluid of endometriosis when compared with women with cystadenomas (Suzumori et al., 2003). However, to our knowledge, there are no previous studies on the expression of PIGF in tissue from patients affected by endometriosis.

TSP-1 is a multifunctional ECM protein that was formerly identified in platelet α-granules and later shown to be produced and secreted by many cell types (Iruela-Arispe et al., 1996). TSP-1 inhibits adhesion, migration and proliferation of a variety of cell types and inhibits angiogenesis (Iruela-Arispe et al., 1996; Armstrong et al., 2003). However, the precise mechanism that results in angiogenic inhibition is not clearly understood. It has been reported that TSP-1 is expressed in endometrium (Iruela-Arispe et al., 1996; Kawano et al., 2005), and alterations in TSP-1 expression may contribute to abnormalities in the menstrual cycle of women (Kawano et al., 2005). Although Tan et al. (2002) have studied the expression of VEGF and TSP-1 mRNA in endometriosis, no studies have been reported on TPS protein levels in peritoneal fluid and tissue extracts in endometriosis.

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 (PA) and matrix MMP systems (Pepper et al., 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; Lembessis et al., 2003; Ramón et al., 2005). We have previously reported (Gilabert-Estellés et al., 2003; Ramón et al., 2005) increased levels of uPA and MMP-3 in endometrium of women with endometriosis, and suggested that this increase might contribute to the invasive potential of endometrial cells.

Although several studies have pointed out the importance of angiogenesis in endometriosis, simultaneous evaluation of angiogenic and antiangiogenic factors and their relationship with components of the fibrinolytic and MMP systems has not been previously published in relation to this disease.

Therefore, the objective of this study is to analyse mRNA expression and protein levels of VEGF, PIGF and TSP-1 in endometrium and peritoneal fluid of women with and without endometriosis and in ovarian endometrioma of patients. In addition, we studied the relationship of these factors to components of the fibrinolytic and MMP systems in order to have a better understanding of the tissue disorders implicated in this pathology.

### Materials and Methods

#### Clinical groups

Seventy-one women with moderate or severe endometriosis (stages III–IV, revised ASRM classification system, 1997) were studied (mean age: 32.7 years; range 19–46). All the women underwent laparoscopic surgical examination of the abdominal cavity and a complete excision of the endometriotic tissue was performed. The presence of the disease was suspected either clinically or by ultrasoundography, and confirmed by the surgical findings and the postoperative pathological examination.

Fifty women (mean age: 36.5 years; range 20–52) without endometriosis, who underwent surgery for pelvic pain (12%), sterility (18%) or tubal sterilization (70%), were included in the control group. The absence of the disease was confirmed after surgical examination of the abdominal cavity. Biopsies of suspicious areas for endometriosis were confirmed to be negative in these women.

The menstrual phase was identified according to the day of the reproductive cycle and the histological analysis of the endometrium. Thirty-three (46.5%) women with endometriosis were in the proliferative phase, and 38 (53.5%) women were in the secretory phase of the menstrual cycle. Twenty-three (46%) controls were in the proliferative phase and 27 (54%) in the secretory phase of the menstrual cycle.

Women in the menstrual phase were excluded from the study.

Patients with irregular menstruation or women who had been pregnant or breast-feeding in the previous six months were also excluded from the study. None of the women had received hormonal treatment for at least three months before the study. Informed consent was obtained from all patients and controls, and the study was approved by the institutional review board.

#### Tissue extracts

Ovarian endometriomas were obtained from 71 patients with moderate or severe endometriosis (group A). Tissue samples from endometriomas were macroscopically separated from ovarian tissue and peritoneal implants were excised surgically, avoiding the use of electrocautery in the dissection. Endometrial biopsies (eutopic endometrium) (group B) from the 71 patients with ovarian endometriomas and from 50 women without endometriosis (control endometrium, group C) were performed by aspiration using the Cornier device (Gynetics, Hamont-Achel, Belgium). However, in 31 patients with ovarian endometrioma there was insufficient quantity of endometrium for the study.

All the 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. Cytosolic and membrane extracts from endometriotic and endometrial tissues were obtained as previously described (Gilabert-Estellés et al., 2003).

#### Peritoneal fluid

Peritoneal fluid samples were carefully collected, as described in previous reports (Gilabert-Estellés et al., 2003), 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 collection of peritoneal fluids and no anticoagulant was used. The peritoneal fluid was immediately cleared of cells and cell debris by centrifugation at 1500 × g for 30 min at 4°C, and stored at −80°C.

#### Methods

**Quantitative real-time RT-PCR**

Total RNA and cDNA were obtained as previously described (Castelló et al., 2002). In brief, RNA from frozen tissue was extracted with the
RNeasy total RNA kit (Qiagen Inc., Valencia, CA, USA), in accordance with the manufacturer’s instructions. One microgram of RNA was treated with DNase I (Invitrogen, Carlsbad, CA, USA) and stored at −80 °C until used. The concentration and purity of RNA were determined spectrophotometrically. One microgram of total RNA was reverse transcribed into first-strand cDNA by using Superscript RNase H (Invitrogen) with an oligo (dT)15 primer (Promega, Madison, WI, USA). The cDNA was stored at −20 °C until used.

Analysis of VEGF, PIGF, TSP-1, uPA, PA inhibitor I (PAI-1), MMP-3, tissue inhibitor of metalloproteinases I (TIMP-1) and β-actin (control gene) mRNA expression was performed in a LightCycler apparatus, software version 3.5 (Roche Molecular Biochemicals, Mannheim, Germany).

The specific primers used for amplification of uPA, PAI-1, MMP-3, TIMP-1 and β-actin were obtained as previously described (Castelló et al., 2002; Ramón et al., 2005).

Fragments of 265 bp of VEGF and 78 bp of PI GF were amplified using specific primers (VEGF forward: 5'-ATC ACG TGG TGA AGT TC -3' and VEGF reverse: 5'- TGC TGT AGG AAG CTC ATC TC -3'; PI GF forward: 5'- CCT ACG TGG AGC TGA CGT CTT TC -3' and PI GF reverse: 5'- TCC TTT CCG GCT TCA TCT TCT-3'), as previously described (Tsatsaris et al., 2003; Zhou et al., 2003). The VEGF assay was performed using primers that allow detection of all known VEGF-A isoforms.

Fragments of 275 bp of TSP-1 were amplified using specific primers (TSP-1 forward: 5'-TGC AAG ATG AAC ATG GAG TCC TCT GGA GAT GAC-3' and TSP-1 reverse: 5'-TTG TGG CCA ATG TAG TTA GTG-3') which were designed using specific primer analysis software (Primer3). These sequences were analysed by FASTA in the EMBL database (http://www.embl-heidelberg.de/).

The reaction mixture and the PCR conditions for uPA, PAI-1, MMP-3, TIMP-1 and β-actin had already been described (Castelló et al., 2002; Ramón et al., 2005). The assay conditions for VEGF, PI GF and TSP-1 were empirically determined and we observed that those used for uPA, PAI-1, MMP-3 and TIMP-1 could also be appropriated for VEGF, PI GF and TSP-1. Each assay was carried out in a final volume of 10 μl containing 1.5 μl cDNA (1:10), 3 mM MgCl2, 0.5 μM of each VEGF, PI GF, TSP-1, uPA, PAI-1, MMP-3 or TIMP-1 primer, or 0.3 μM of each β-actin primer, 1 μl LC-Fast Start Reaction Mix SYBR Green I and 1 μl LC-Fast Start DNA Master SYBR Green I/Enzyme, including TaqDNA polymerase, reaction buffer and desoxyribonucleotide triphosphate mix (Roche). The amplification program consisted of the following three steps. The first step was an initial heating for 10 min at 95 °C to denature the cDNA and to activate the TaqDNA polymerase. Then, DNA was amplified for 40 cycles of 15 s at 95 °C for β-actin, uPA, PAI-1, MMP-3 and TIMP-1 or 0 s for VEGF, PI GF and TSP-1 (denaturation), 5 s at 60 °C for uPA, PAI-1, MMP-3 and TIMP-1 or at 62 °C for β-actin or 10 s at 60 °C for VEGF and PI GF or at 62 °C for TSP-1 (annealing) and 18 s at 72 °C for β-actin, uPA, PAI-1, MMP-3 and TIMP-1 or 12 s for TSP-1 or 10 s for VEGF and PI GF (extension). Finally, the temperature was raised gradually (0.1 °C/s) from 65 to 95 °C for the melting curve analysis. To verify the melting curve results, representative samples of the PCR products were assayed on 2% agarose gels. Two negative controls were included in each assay: one without a template sample and the another one without reverse transcriptase. The number of sample copies was calculated by setting their crossing points to the standard curve. Data are shown as the ratio target cDNA concentration/β-actin cDNA concentration.

Protein quantification

Total protein assay in both cytosolic and membrane extracts was determined with the BCA protein assay (Pierce, Rockford, IL, USA). Standard bovine serum albumin, fraction V (Sigma) was used for calibration. Samples and standards were tested in duplicate.

VEGF-A antigenic level was measured by a commercially available ELISA (Human VEGF, ELISA Development System, Duoset, RD systems, Minneapolis, MN, USA). It exhibited no cross-reactivity or interference with PI GF. This assay has been shown to recognize human VEGF-A165and VEGF-A121isoforms.

Another commercially available ELISA (Human PI GF, ELISA Development System, Duoset, RD systems, Minneapolis, MN) was used to quantify PI GF antigenic levels. No cross-reaction with VEGF was observed.

TSP-1 level was quantified by an indirect ELISA. Briefly, ELISA plate (MaxiSorp surface, Nunc) was incubated overnight with TSP-1 (20–40 μg/ml) obtained from human platelets. The samples and the TSP-1 standard (Sigma-Aldrich Co) were incubated overnight with specific polyclonal antibody for TSP-1 (TSP Ab-8, NeoMarkers Lab Vision Corporation) and then they were added to the ELISA plate and incubated for 2 h. An antirabbit IgG horseradish peroxidase conjugated from donkey (Amersham) was added, as a second antibody, and incubated for 2 h. A solution of o-phenylenediamine-dihydrochloride (OPD, Sigma-Aldrich Co) was used as substrate. The reaction was stopped with 4 M H2SO4. The optical density was read in a reader of plates for ELISA (Thermo Lab- systems). The coefficient of variation was 4.8%.

uPA antigen and PAI-1 antigen levels were quantified by a commercially available ELISA (Tint Elize uPA and PAI-1, Biopool) as previously described (Estellés et al., 1994).

MMP-3 antigen and TIMP-1 antigen levels were quantified by commercially available ELISAs (MMP-3 and TIMP-1 ELISA, Oncogene) as previously described (Gilabert-Estellés et al., 2003).

Antigenic levels of all the parameters were determined in both cytosolic and membrane extracts.

Statistical analysis

The differences between the means of the studied variables in the tissue extracts of the different groups were analysed by unpaired Student t-test or non-parametric Mann–Whitney U-test. The percentages of the menstrual cycle phases in the different groups were compared by using the Chi-square test. Multivariate analysis was performed among groups in order to detect differences due to the menstrual cycle phase. Simultaneous endometriotic and endometrial tissues were available in 40 patients. In order to analyse the differences found in this subgroup according to the type of tissue, paired t-test and Wilcoxon non-parametric test were performed, depending on the type of the variable assessed. Levels of significance in correlations between variables were calculated by the bivariate Pearson’s correlation. P-values <0.05 (two-tailed) were considered to be significant. All these tests were performed using the statistical package SPSS Release 11.5 for Windows (SPSS Inc.).

Results

Endometrium from women with endometriosis versus endometrium from women without endometriosis

Extracts of eutopic endometrium from women with endometriosis (stages III–IV) (n = 40) showed a significant increase in mRNA (10.803 ± 1.107 versus 6.395 ± 0.538 P < 0.001) and antigenic levels of VEGF (249 ± 43 versus 114 ± 13 ng/ml, P < 0.05) in comparison with the endometrium from controls (n = 50). However, no significant differences were observed in PI GF and TSP-1 levels between endometrium from women with and without endometriosis.

2122

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The expression of angiogenic factors in endometrium from women with endometriosis and in endometrium from controls was also studied according to the cycle phase (Table 1).

In the proliferative phase, we detected significantly higher VEGF mRNA levels ($P < 0.05$) and antigenic levels ($P < 0.05$) in eutopic endometrium from women with endometriosis (group B1) than in controls (group C1) (Table 1). In secretory phase, endometrium from women with the disease (Group C1) also showed higher mRNA levels ($P < 0.05$) and antigenic levels of VEGF ($P < 0.01$) in comparison with endometrium from controls (group C2) (Table 1).

By examining the expression of TSP-1 across the menstrual cycle we found that extracts of endometrium from controls showed significantly higher TSP-1 antigenic levels in the secretory (Group C2) than in the proliferative phase (Group C1) ($P = 0.045$) (Table 1). In contrast, no significant differences were observed in TSP-1 Ag levels when comparing the secretory versus the proliferative phase in extracts of endometrium from women with endometriosis (Group B2 versus Group B1, $P = 0.226$) and in endometrioma (Group A2 versus Group A1, $P = 0.451$). Furthermore, there were no significant differences in TSP-1 mRNA levels when comparing the proliferative versus the secretory phase in extracts of endometrium from women with or without endometriosis and in endometrioma.

In relation to fibrinolytic and MMP parameters, uPA and MMP-3 mRNA and protein levels were significantly higher in the endometrium from women with endometriosis when compared with controls in secretory phase only (Table 2).

### Ovarian endometrioma versus endometrium from women with endometriosis

A significant increase in TSP-1 mRNA and protein levels was observed in ovarian endometrioma when compared with eutopic endometrial tissues from women with endometriosis, in both the proliferative and secretory phases ($P < 0.001$) (Table 1). In relation to fibrinolytic and MMP inhibitors, a significant increase in PAI-1 and TIMP-1 mRNA and protein levels

<table>
<thead>
<tr>
<th>Endometriotic lesions (ovarian endometrioma) (n = 71)</th>
<th>Eutopic endometrium from women with endometriosis (n = 40)</th>
<th>Endometrium from controls (n = 50)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Proliferative (A1) (n = 33)</strong></td>
<td><strong>Secretory (A2) (n = 38)</strong></td>
<td><strong>Proliferative (B1) (n = 18)</strong></td>
</tr>
<tr>
<td>VEGF mRNA*</td>
<td>0.317 ± 0.045</td>
<td>0.348 ± 0.047</td>
</tr>
<tr>
<td>VEGF (ng/mg)</td>
<td>1.98 ± 0.34</td>
<td>2.03 ± 0.24</td>
</tr>
<tr>
<td>PI GF mRNA*</td>
<td>2.148 ± 0.491</td>
<td>1.233 ± 0.195</td>
</tr>
<tr>
<td>PI GF (ng/mg)</td>
<td>17.93 ± 5.11</td>
<td>10.96 ± 3.09</td>
</tr>
<tr>
<td>TIMP-1 mRNA*</td>
<td>0.026 ± 0.006</td>
<td>0.048 ± 0.013</td>
</tr>
<tr>
<td>TIMP-1 (ng/mg)</td>
<td>4.32 ± 0.67</td>
<td>2.60 ± 0.42</td>
</tr>
<tr>
<td>PAI-1 mRNA*</td>
<td>10.025 ± 1.995</td>
<td>0.513 ± 1.987</td>
</tr>
<tr>
<td>PAI-1 (ng/mg)</td>
<td>72 ± 8</td>
<td>112 ± 16</td>
</tr>
</tbody>
</table>

*Data are expressed as means ± SEM. NS, not significant. Ag values represent cytosol extract plus membrane extract.

**Normalized mRNA = 100 × ([target gene mRNA copies/β-actin mRNA copies])**.
was observed in ovarian endometrioma compared with eutopic endometrial tissues from women with endometriosis (Table 2).

When we analysed only the 40 women in whom eutopic endometrium and ovarian endometriomas were simultaneously collected, ovarian endometriomas also showed significantly higher mRNA and antigenic levels of TSP-1, PAI-1 and TIMP-1 than did eutopic endometrium (TSP Ag 1811 ± 185 versus 352 ± 38 ng/mg, P < 0.01; PAI-1 Ag 13.01 ± 2.98 versus 2.298 ± 0.512, P < 0.01; TIMP-1 Ag 1811 ± 185 versus 352 ± 38 ng/mg, P < 0.01; PAI-1 mRNA 2.059 ± 0.480 versus 0.355 ± 0.093, P < 0.01; PAI-1 Ag 13.01 ± 2.98 versus 2.13 ± 0.43 ng/mg, P < 0.01; TIMP-1 mRNA 15.570 ± 2.298 versus 1.630 ± 0.243, P < 0.01 and TIMP-1 Ag 1811 ± 185 versus 59 ± 14 ng/mg P < 0.01).

Peritoneal fluid from women with or without endometriosis

The levels of different parameters in peritoneal fluid are shown in Fig. 1. The concentration of total protein in peritoneal fluid was similar in women with (n = 53) and without endometriosis (n = 35). The peritoneal fluid from women with endometriosis showed a significant increase in VEGF, uPA and MMP-3 levels when compared with controls (VEGF: 317 ± 50 versus 184 ± 30 pg/ml; uPA: 1.76 ± 0.15 versus 1.24 ± 0.08 ng/ml; MMP-3: 51.2 ± 5.2 versus 32.4 ± 5.2 ng/ml, P < 0.05). However, no significant differences were observed in PAI-1, PI GF, TSP-1 and TIMP-1 levels between peritoneal fluid from women with and without endometriosis.

Correlation between angiogenic, fibrinolytic and MMP parameters

In endometrium of women without endometriosis, a significant correlation was observed between VEGF and both uPA and MMP-3 Ag levels (r = 0.609, P < 0.001 and r = 0.329, P < 0.05, respectively). In women with endometriosis, a significant correlation was obtained between VEGF and uPA Ag levels in endometrium and peritoneal fluid (r = 0.328 and r = 0.340, P < 0.05, respectively). In ovarian endometrioma, a significant correlation was obtained between TSP-1 and PAI-1 Ag levels (r = 0.409, P < 0.01).

Discussion

Several studies suggest that the eutopic endometrium from women with endometriosis has altered expression of several components of the angiogenesis, PA 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; Sharpe-Timms et al., 2001; Gilabert-Estelle et al., 2003; 2005; Takehara et al., 2004). However, no studies have previously been published on the simultaneous evaluation of mRNA and protein expression of VEGF, PI GF and TSP-1 and their correlation with some components of fibrinolytic and MMP systems in tissue and peritoneal fluid from women with endometriosis.

Although previous studies have indicated that VEGF mRNA expression is higher in endometrium of women with endometriosis than in controls (Tan et al., 2002; Takehara et al., 2004). Print et al. (2004) found similar VEGF production by cultured endometrium from endometriosis and controls. In the present study, we have observed an increase in VEGF mRNA and antigenic levels in the endometrium of women with endometriosis: a condition that may contribute to the progression of this disease. The discrepancy of these results may not be related to patient selection criteria, as suggested by Print et al. (2004), because the selection criteria are similar in our study. Moreover, cultured endometrium does not exactly reproduce in vivo conditions and this limitation may influence the results.

In relation to endometriotic lesions, Takeara et al. (2004) have reported an increase in VEGF mRNA expression in endometriotic tissue (early stages) as compared to eutopic endometrium. However, in the present study, we did not detect any increase in VEGF mRNA levels in ovarian endometriomas (stages III–IV). The characteristics of the endometriotic samples might be the reason why these results do not coincide. As previously indicated, endometriosis seems to be a progressive disease and a reduction in the angiogenic activity of
endometriotic lesions has been observed in advanced stages (Nisolle et al., 1993). Peritoneal endometriotic implants are lesions with a high potential of angiogenesis and invasion of ECM, while ovarian endometriomas are advanced manifestation of the disease with low capability for remodelling the surrounding tissue.

It has been reported that in addition to VEGF, human endometrium expresses mRNAs encoding other growth factors such as PlGF (Li et al., 2001). However, expression of PlGF in endometriotic tissue of patients with the disease has not been previously reported. Although it has been suggested that PlGF contributes to angiogenesis in pathological conditions (Carmeliet et al., 2001), in the present study we found no significant differences in the antigenic and mRNA PlGF levels in tissue extracts of patients and controls.

It has been indicated that alteration in TSP-1 expression may contribute to many vascular pathologies of the reproductive tract (Kawano et al., 2005). In the present study, an increase in TSP-1 protein and mRNA levels and a decrease in VEGF levels were observed in ovarian endometrioma in comparison with endometrium from patients. Our results on TSP-1 mRNA levels are in agreement with those previously reported by Tan et al. (2002), although no studies of TSP-1 protein levels in endometriosis have been previously reported. The increased levels of TSP-1 may reduce the angiogenic activity in ovarian endometrioma, which may contribute to the low capability of invasiveness of this tissue.

It has been reported that TSP-1 levels in endometrial samples are higher in the secretory phase than in the proliferative phase and that TSP-1 expression is regulated by progesterone (Iruela et al., 1996). According to these results, in the present study we have detected higher TSP-1 Ag levels in endometrium extracts from controls in the secretory phase in comparison with the proliferative phase. However, no significant differences were observed in endometrium from women with endometriosis. Therefore, our results suggest there could be an altered response to progesterone in the eutopic endometrium from women with endometriosis.

Extracellular proteolysis is implicated in the initial stages of angiogenesis, and the group of proteinases involved includes the plasminogen and MMP systems (Ugwu et al., 1999; Pepper, 2001; Lijnen, 2002). Moreover, in the last few years, several papers based on microarray methods have shown differences in gene expression of MMP components, between normal and eutopic endometrium from women with endometriosis (Wu et al., 2006).

In relation to the modifications of fibrinolytic and MMP parameters in endometriosis, uPA and MMP-3 mRNA and their protein levels in the secretory phase were significantly higher in the endometrium from women with endometriosis than that of controls, confirming previous reports (Osteen et al., 1996; Síllem et al., 2001; Gilabert-Estellés et al., 2003; Ramón et al., 2005). Moreover, a significant correlation between uPA and VEGF levels was observed. The high mRNA and protein levels of uPA and MMP-3 in the endometrium from women with endometriosis, observed in the present report, may also contribute to angiogenesis and favour the invasive properties of this tissue.

We have also observed an increase in mRNA and protein levels of PAI-1 and TIMP-1 in endometriotic tissue in comparison with eutopic endometrium. The increase in PAI-1 and TIMP-1 expression in ovarian endometrioma might reduce the invasive potential of the endometriotic tissue in advanced stages of the disease.

Previous studies have suggested that both endometrial and peritoneal factors promote the angiogenesis in endometriosis (Donnez et al., 1998; Kooninckx et al., 1998). In relation to peritoneal fluid, an increase in VEGF levels has been previously reported in women with endometriosis (Shifren et al., 1996; Mahnke et al., 2000; McLaren et al., 2000; Takehara et al., 2004; Na et al., 2006). We have also found higher peritoneal levels of VEGF, uPA and MMP-3 in patients with endometriosis (stages III–IV) than in women without the disease. No significant differences were observed in the rest of parameters studied. An increase in PlGF levels in peritoneal fluid has been reported in endometriosis (stage II) (Suzumori et al., 2003). However, in our study no significant differences were found. This discrepancy may be due to the advanced stages studied in the present report. The increased VEGF may be related with the endometriosis, allowing the ectopic tissue to survive in the peritoneal cavity and to develop an endometriotic lesion.

Hull et al. (2003) reported that antiangiogenic agents inhibited the growth of explants in an in vivo model of endometriosis by disrupting the vascular supply, and this effect is likely to apply to the human disease. These findings suggest that antiangiogenic agents provide a novel therapeutic approach for the treatment of endometriosis.

In conclusion, the increase in VEGF without a significant increase in TSP-1 obtained in the endometrium of women with endometriosis could indicate a higher angiogenic activity, which might contribute to the capability of implantation of endometrial cells at ectopic sites. The increase in VEGF, uPA and MMP3 in peritoneal fluid of women with endometriosis may permit the development of endometriotic lesions. Excessive endometrial and peritoneal angiogenesis suggest that the use of antiangiogenic agents may be a promising therapy for endometriosis.

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