Expression of several components of the plasminogen activator and matrix metalloproteinase systems in endometriosis

J.Gilabert-Estellés1, A.Estellés2,7, J.Gilabert6, R.Castelló2, F.España2, C.Falcó2, A.Romeu1, M.Chirivella3, E.Zorio4 and J.Aznar5

1Maternal Hospital, 2Research Center, 3Anatomopathology, 4Cardiology and 5Clinical Pathology Departments, Hospital Universitario La Fe, and 6Gynecology Service, Hospital Arnau de Vilanova, Valencia, Spain

7To whom correspondence should be addressed at: Hospital Universitario ‘La Fe’, Centro de Investigación. Avda. Campanar 21, 46009 Valencia, Spain. E-mail: estelles_amp@gva.es

BACKGROUND: Endometriosis is considered a benign disease that has the ability to invade normal tissue. As in neoplastic growth, local extracellular proteolysis may take place. The aim of this study is to analyse several components of the plasminogen activator (PA) pathway and the matrix metalloproteinase (MMP) system in endometriotic tissue, endometrium and peritoneal fluid from women with and without endometriosis (controls).

METHODS AND RESULTS: Thirty-nine women with endometriosis and 35 controls were studied. In eutopic endometrium of women with endometriosis, the antigenic levels of urokinase-type PA (uPA) and MMP-3 were elevated when compared with endometrium from controls. Ovarian endometriotic tissues had higher antigenic levels of PA inhibitor type 1 (PAI-1) and tissue inhibitor of metalloproteinases type 1 (TIMP-1) than endometrium. The peritoneal fluid from women with endometriosis showed a significant increase in uPA levels compared with controls.

CONCLUSIONS: The increase in antigenic levels of uPA and MMP-3 in endometrium of women with endometriosis might contribute to the invasive potential of endometrial cells. Once the ovarian endometriotic cyst is developed, an increase in PAI-1 and TIMP-1 is detected and significant proteolytic activity is no longer observed. This increase in inhibitors and decrease in proteolytic activity could explain the frequent clinical finding of isolated endometriotic cyst without invasion of the surrounding ovarian tissue.

Key words: endometriosis/MMP-3/PAIs/plasminogen activators/TIMP-1

Introduction

Endometriosis is defined by the presence of endometrial glands and stroma outside the uterus. It is a disease that affects up to 60% of women with pelvic pain and infertility (Giudice et al., 1998; Pellicier et al., 2001). The aetiology and pathogenesis of endometriosis are far from clear, despite several decades of research in this field. Several factors have been implicated as causes of endometriosis, including immune system disorders, genetic predisposition, altered peritoneal environment and endometrial disorders (Martinez-Roman et al., 1997; Gogushev et al., 2000; Sharpe-Timms, 2001; Stefansson et al., 2002).

Although endometriosis is a benign disease, the endometrial tissue acquires the ability to attach and invade the peritoneum where a local extracellular proteolysis might take place (Spuijbroek et al., 1992; Kobayashi et al., 2000). This process may involve the plasminogen activator (PA) and matrix metalloproteinase (MMP) systems.

The PA system is associated with several processes, including lysis of fibrin clots, tissue remodelling, tumour invasion and also the reproductive process (Loskutoff et al., 1991; Estellés et al., 1994; Gilabert et al., 1995b; Andreasen et al., 1997; Murphy et al., 2000; Castelló et al., 2002). Urokinase-type plasminogen activator (uPA) is a serine protease which catalyses the conversion of plasminogen into plasmin. Plasmin is an active enzyme which degrades a variety of extracellular matrix proteins and activates MMPs and growth factors (Andreasen et al., 1997; Murphy et al., 2000). Another PA is the tissue-type plasminogen activator (tPA), which is released mainly from vascular endothelium.

The activity of the PAs is regulated by specific PA inhibitors (PAIs). The principal PAIs are PAI-1, initially termed the endothelial cell PAI (Loskutoff et al., 1991), PAI-2, historically known as placental-type PAI (Kruithof et al., 1995; Grancha et al., 1996), and PAI-3, which is identical to protein C inhibitor (Heeb et al., 1987). PAI-1 is a multifaceted proteolytic inhibitor (Harbeck et al., 2001). It does not function only as a fibrinolytic inhibitor, but also plays an important role...
in signal transduction, cell adherence and cell migration (Harbeck et al., 2001).

MMPs are involved in both normal and pathological processes in which degradation of the extracellular matrix is a key event. MMP activities are regulated by tissue inhibitors of metalloproteinases (TIMPs) (Matrisian et al., 1990). TIMP-1 is one of the four members identified in this family, which inhibits the collagenase, stromelysin and gelatinase classes of MMPs (Nagase and Woessner, 1999). TIMP-1 is thus considered to inhibit invasion and metastasis of carcinoma, being a potential target for cancer gene therapy (Brand, 2002).

However, other reports suggest that TIMP-1 also possesses a growth-promoting function (Hayakawa et al., 1992).

Conflicting evidence exists regarding the role of the PA system in the pathogenesis of endometriosis. Bruse et al. (1998) have reported an increase in uPA and PAI-1 antigen levels in endometriotic tissue compared with eutopic endometrium. However, a lower basal release of uPA and soluble uPA receptor (uPAR) has been found in endometriotic cells (Guan et al., 2002). Increased expression of uPAR has been reported in endometriotic cells (Kobayashi et al., 2000) and in eutopic endometrial cells of women with endometriosis (Sillem et al., 1997).

The MMP system has been implicated in the establishment of endometriosis in animal models (Sharpe-Timms et al., 1998b; Cox et al., 2001) and in humans (Saito et al., 1995; Sillem et al., 1997; Sharpe-Timms et al., 1998a; Wenzl and Heinzl, 1998; Osteen et al., 1999; Gottschalk et al., 2000; Chung et al., 2001, 2002; Bruner-Tran et al., 2002). An increase in the induced secretion of MMP-3 and TIMP-1 has been found in endometrial cells from patients with endometriosis (Sillem et al., 1997). Other authors have reported a decrease in the expression of TIMP-1 and an increase in MMP-1 in endometriotic tissue in comparison with endometrium (Gottschalk et al., 2000), and a decrease in TIMP-1 levels was found in peritoneal fluid from women with endometriosis compared with disease-free controls (Sharpe-Timms et al., 1998a; Szamatowicz et al., 2002).

Previous studies have measured antigen levels of several components of fibrinolytic and MMP systems only in cytosolic fractions of endometriosis, but none of them has quantified these components in the membrane fraction of endometrial cells. On the other hand, other parameters such as PAI-3 and functional levels of PAs have not been determined previously.

The aim of this study was to analyse antigen and functional levels of several components of the PA and MMP systems in cytosolic and membrane fractions from endometriotic and endometrial tissue samples and also in cell-free peritoneal fluid.

### Materials and methods

#### Patients

Endometriotic tissue samples were obtained from ovarian endometriomas (n = 39 women, mean age 35 years, range 23–47; AFS revised classification system, stages III and IV; American Fertility Society, 1985). Eutopic endometrium was obtained from 21 of these 39 women with endometriosis. In the endometriosis group, 23 of the 39 women with endometriosis were infertile and all had regular menstruations. Twenty-seven of these women underwent laparoscopy, the indications for surgery being pelvic pain (n = 16) and infertility (n = 23) (Table I).

Thirty-five control endometrial biopsies (control endometrium) from women without endometriosis (mean age 40 years; range 29–47) were obtained. The absence of endometriosis was verified by systematic examination of the abdominal cavity with attention for early and atypical lesions. The surgery was performed according to clinical criteria (Table I) by laparotomy (n = 27), laparoscopy (n = 5) or vaginal approach during a laparoscopy-assisted vaginal hysterectomy (n = 3).

Eight of the 39 women with endometriosis were in the proliferative phase of the menstrual cycle, 29 were in the secretory phase and two in the menstrual phase. Eleven of the 35 control women who provided endometrium were in the proliferative phase, two in the secretory phase and two in the menstrual phase.

Peritoneal fluid was obtained from 22 of the 39 women with endometriosis (stages III–IV of AFS) and from 12 control women (mean age 35 years; range 29–47).

None of the women had received hormonal treatment for at least 3 months before the study. Women who were pregnant or breast-feeding in the previous 6 months were excluded from the study.

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

#### Tissue extracts and peritoneal fluid

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

Cytosolic extracts from endometrium tissues were obtained by homogenization of tissue in 10 mmol/l Tris–HCl buffer containing 1.5 mmol/l EDTA and 10% glycerol. The suspension was centrifuged at 100 000 g at 4°C for 15 min to pellet the membrane fraction, and aliquots of the supernatant were taken and stored at −80°C (Bouchet-Bernet et al., 1996).

Membrane extracts were obtained by solubilization of the pelleted membranes in 20 mmol/l Tris–HCl buffer containing 125 mmol/l NaCl and 1% Triton X-100. They were then incubated overnight at 4°C and centrifuged at 100 000 g at 4°C for 15 min and the aliquots of the supernatant (membrane extracts) were taken and stored at −80°C (Bouchet-Bernet et al., 1996).

Peritoneal fluid samples were collected, carefully as described in previous reports (Bouckaert et al., 1986), from the pouch of Douglas and vesico-uterine space. The fluid was collected in vacuum tubes with a sterile syringe, which was attached to an endoscopic catheter in the laparoscopic approach. Blood-contaminated peritoneal fluids were excluded. No peritoneal washings were performed prior to collection.

### Table I. Clinical characteristics of patients

<table>
<thead>
<tr>
<th>Clinical symptoms</th>
<th>Women without endometriosis (n = 35)</th>
<th>Women with endometriosis (n = 39)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Infertility</td>
<td></td>
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<tr>
<td>Primary</td>
<td>2 (5.7)</td>
<td>20 (51.3)</td>
</tr>
<tr>
<td>Secondary</td>
<td>1 (2.9)</td>
<td>3 (7.7)</td>
</tr>
<tr>
<td>Chronic pelvic pain</td>
<td>2 (5.7)</td>
<td>5 (12.8)</td>
</tr>
<tr>
<td>Dysmenorrhea</td>
<td>2 (5.7)</td>
<td>11 (28.2)</td>
</tr>
<tr>
<td>Pelvic floor defect</td>
<td>3 (8.6)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Asymptomatic</td>
<td>25 (71.4)</td>
<td>0 (0)</td>
</tr>
</tbody>
</table>

Values in parentheses are percentages.

*Only the main symptom considered as indication for surgery.

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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**

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

Determination of tPA antigen was performed with a commercially available enzyme-linked immunosorbent assay (ELISA) (Imulysé tPA, Biopool, Sweden). The assay detects free and complexed tPA with similar efficiency. The intra- and inter-assay variabilities were 4 and 6% respectively.

A chromogenic substrate was used as previously described to perform the tPA functional assay (Gilaibert et al., 1995a). The intra- and inter-assay variabilities were 8 and 12% respectively.

uPA antigen was quantified by a commercially available ELISA (Tint Elize uPA, Biopool), which measures single-chain urokinase (scuPA) and the high weight molecular form of uPA (HMW-uPA) with similar efficiency. The low molecular form of uPA is measured with ~40% the efficiency of scuPA and HMW-uPA on a molar basis. The intra- and inter-assay variabilities were 4 and 10% respectively.

Functional levels of uPA and activable scuPA were determined by an immunosorbent activity assay (Chromolize uPA, Biopool). The intra- and inter-assay variabilities were 6 and 11% respectively.

uPAR was determined by a commercially available ELISA (Imunbind, total uPAR, American Diagnostica). Free uPAR as well as uPAR complexes are all recognized by this assay. The intra- and inter-assay variabilities were 5 and 7% respectively.

Another commercially available ELISA (Tint Elize PAI-1, Biopool) was used to quantify PAI-1 antigen levels. It detects active and inactive forms of PAI-1, as well as complexes such as tPA–PAI and uPA–PAI. The intra- and inter-assay variation coefficients were 3 and 7% respectively.

PAI-1 functional levels were determined by a chromogenic assay as previously described (Estellés et al., 1994). One unit of PAI-1 activity is defined as the amount that inhibits 1 IU of single tPA in 15 min at room temperature under the conditions used. The intra- and inter-assay variabilities were 6 and 10% respectively.

PAI-2 antigen was quantified by a commercially available ELISA (Imunbind PAI-2, American Diagnostica). Free PAI-2 and PAI-2–uPA complexes are recognized with equal sensitivity when using this assay. The intra- and inter-assay variabilities were 5 and 7% respectively.

PAI-3 antigen levels were measured by an ELISA, as previously reported (España and Griffiths, 1989; España et al., 1991). The intra- and inter-assay variabilities were 4–8 and 6–9% respectively.

uPA–PAI-3 and tPA–PAI-3 complexes were quantified by ELISAs, as previously described (España et al., 1993). The intra- and inter-assay variabilities were 5–9 and 7–12% respectively.

MMP-3 antigen was quantified by a commercially available ELISA (MMP-3 ELISA, Oncogene). The assay detects MMP-3, but does not recognize MMP-3–TIMP complexes. The intra- and inter-assay variabilities were 5 and 9% respectively.

TIMP-1 antigen was quantified by a commercially available ELISA (TIMP-1 ELISA, Oncogene). The assay recognizes free and complexed TIMP-1. The intra- and inter-assay variabilities were 4 and 7% respectively.

Antigen levels of all the parameters were determined in both cytosolic and membrane extracts. However, PAI-1, uPA and tPA functional levels were determined only in cytosolic extracts, since the solubilization of the pelleted membranes with Triton X-100 decreases or blocks the functional activity of these parameters.

**Statistical analysis**

The differences between the means of the studied variables in the tissue extracts of the different groups were analysed by the one-way ANOVA test and Student–Newman–Keuls multiple range test (when applicable). A two-way ANOVA test was used to detect possible interaction between the presence of the disease and the cycle phases. Paired t-test or Wilcoxon non-parametric test was used in the subgroup of 21 patients in which endometriotic and endometrial tissues were available. Differences between the means of the variables studied in peritoneal fluid were analysed by the Student t-test or by the Mann–Whitney U-test. Levels of significance in correlations between variables were calculated by the bivariate Pearson correlation, partial correlation and multiple linear regression test. P-values <0.05 (two-tailed) were considered to be significant. All these tests were performed using the statistical package SPSS Release 6.0 for Windows (SPSS Inc.).

**Results**

Tables II and III show antigen levels of the parameters measured in tissue extracts (cytosolic and cytosolic plus membrane extracts) as well as PAI-1, uPA and tPA functional levels measured in cytosolic extracts of patients with and without endometriosis.

Since there is a significant amount of the studied components in the pelleted membranes of tissues, the quantification of cytosolic plus membrane extracts provides a better evaluation of the total antigen levels. Meanwhile, the cytosolic fraction is evaluated separately in order to give an estimate of functional levels and to facilitate the interpretation of our results and the comparison with previous reports.

**Endometrium from women with or without endometriosis**

Endometrium from women with endometriosis had higher antigenic levels of uPA and MMP-3, in cytosolic and cytosolic plus membrane extracts, compared with women without endometriosis (Table II). The analysis of the rest of the parameters studied, including tPA and uPA functional levels (Table II), and antigenic levels of PAIs, TIMP-1 or uPA–PAI-3 and tPA–PAI-3 complexes (Table III) showed no significant differences with the one-way ANOVA test applied.

No interactions were detected between the cycle phase and the results obtained in the endometrium from women with endometriosis compared with women without endometriosis, by using the two-way ANOVA test.

**Endometriotic tissue and endometrium from women with endometriosis**

Endometrium from women with endometriosis showed a significant increase in uPA and MMP-3 antigenic levels in cytosolic and cytosolic plus membrane extracts compared with endometriotic tissue (Table II).

Endometriotic tissue had significantly higher antigenic levels of PAI-1 and TIMP-1 and significantly lower antigenic levels of PAI-3 compared with endometrium from women with endometriosis (Table III).
Plasminogen activator and metalloproteinase systems in endometriosis

In 21 of the 39 women with endometriosis, samples of endometriotic and endometrial tissues were collected at the same time. In this subgroup of 21 patients with endometriosis, endometriotic tissue also had significantly higher levels of PAI-1 (10.24 ± 3.00 ng/mg) and TIMP-1 (120 ± 30 ng/mg) than endometrium (1.60 ± 0.42 ng/mg, P < 0.01; and 31 ± 6 ng/mg, P < 0.01).

**Endometriotic tissue and endometrium from women without endometriosis**

Endometriotic tissue showed a significant increase in the antigenic levels of PAI-1 and TIMP-1 and a decrease in PAI-3 antigenic levels in both cytosolic and membrane extracts compared with endometrium from women without endometriosis (Table III).

**Correlations between variables**

A significant positive correlation was observed between PAI-1 antigen and activity levels (r = 0.64, P < 0.005) in cytosolic extracts from endometriotic tissue. No significant correlation was observed in the cytosolic extracts of endometrium from women with or without endometriosis. A significant positive correlation was also found between tPA antigen and activity in endometrium from women with (r = 0.61, P < 0.005) and without endometriosis (r = 0.72, P < 0.001). No significant correlation between tPA antigen and activity was observed in endometriotic tissue.

### Table II. PA and MMP-3 levels in tissue extracts from endometriotic and endometrium tissues from women with and without endometriosis

<table>
<thead>
<tr>
<th></th>
<th>Endometriotic tissue (n = 39) (I)</th>
<th>Endometrium from women with endometriosis (n = 21) (II)</th>
<th>Endometrium from women without endometriosis (n = 35) (III)</th>
<th>ANOVA</th>
<th>I – II</th>
<th>II – III</th>
<th>I – III</th>
</tr>
</thead>
<tbody>
<tr>
<td>tPAag (ng/mg)</td>
<td>2.84 ± 0.26&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.02 ± 0.46</td>
<td>3.78 ± 0.46</td>
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<tr>
<td></td>
<td>9.33 ± 0.69&lt;sup&gt;b&lt;/sup&gt;</td>
<td>12.69 ± 1.52</td>
<td>9.14 ± 1.33</td>
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</tr>
<tr>
<td>tPAag (U/mg)</td>
<td>0.26 ± 0.05&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.41 ± 0.09</td>
<td>0.58 ± 0.10</td>
<td>P &lt; 0.05</td>
<td>NS</td>
<td></td>
<td>P &lt; 0.05</td>
</tr>
<tr>
<td>uPAag (ng/mg)</td>
<td>0.18 ± 0.01&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.70 ± 0.11</td>
<td>0.22 ± 0.03</td>
<td>P &lt; 0.001</td>
<td>NS</td>
<td>P &lt; 0.01</td>
<td>P &lt; 0.001</td>
</tr>
<tr>
<td></td>
<td>1.20 ± 0.12&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.99 ± 0.32</td>
<td>1.22 ± 0.14</td>
<td>P &lt; 0.05</td>
<td>NS</td>
<td>P &lt; 0.05</td>
<td>NS</td>
</tr>
<tr>
<td>uPAag (ng/mg)</td>
<td>0.09 ± 0.02&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.11 ± 0.02</td>
<td>0.06 ± 0.01</td>
<td>NS</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>uPA-R (ng/mg)</td>
<td>0.68 ± 0.10&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.47 ± 0.14</td>
<td>0.69 ± 0.15</td>
<td>NS</td>
<td></td>
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<tr>
<td></td>
<td>3.28 ± 0.30&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.51 ± 0.55</td>
<td>2.90 ± 0.32</td>
<td>NS</td>
<td></td>
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<tr>
<td>MMP-3ag (ng/mg)</td>
<td>0.96 ± 0.15&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.91 ± 0.47</td>
<td>0.73 ± 0.15</td>
<td>P &lt; 0.01</td>
<td>P &lt; 0.01</td>
<td>P &lt; 0.01</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>3.63 ± 0.64&lt;sup&gt;b&lt;/sup&gt;</td>
<td>4.09 ± 0.86</td>
<td>2.07 ± 0.29</td>
<td>P &lt; 0.05</td>
<td>NS</td>
<td>P &lt; 0.05</td>
<td>NS</td>
</tr>
</tbody>
</table>

The data are expressed as mean ± SEM.

<sup>a</sup>Cytosolic extract.
<sup>b</sup>Cytosolic extract plus membrane extract.

tPA, uPA, uPAR and MMP-3 antigen (ag) values were evaluated in cytosolic and membrane extracts, and tPA and uPA functional (fc) levels were determined only in cytosolic extract.

NS = not significant.

### Table III. PAIs, activator–inhibitor complexes and TIMP-1 levels in tissue extracts from endometriotic and endometrium tissues from women with and without endometriosis

<table>
<thead>
<tr>
<th></th>
<th>Endometriotic tissue (n = 39) (I)</th>
<th>Endometrium from women with endometriosis (n = 21) (II)</th>
<th>Endometrium from women without endometriosis (n = 35) (III)</th>
<th>ANOVA</th>
<th>I – II</th>
<th>II – III</th>
<th>I – III</th>
</tr>
</thead>
<tbody>
<tr>
<td>PAI-1&lt;sub&gt;ag&lt;/sub&gt; (ng/mg)</td>
<td>2.68 ± 0.54&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.46 ± 0.18</td>
<td>0.73 ± 0.19</td>
<td>P &lt; 0.001</td>
<td>P &lt; 0.01</td>
<td>NS</td>
<td>P &lt; 0.001</td>
</tr>
<tr>
<td></td>
<td>9.11 ± 1.82&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.60 ± 0.42</td>
<td>1.58 ± 0.43</td>
<td>P &lt; 0.001</td>
<td>P &lt; 0.001</td>
<td>NS</td>
<td>P &lt; 0.001</td>
</tr>
<tr>
<td>PAI-1&lt;sub&gt;ag&lt;/sub&gt; (U/mg)</td>
<td>2.16 ± 0.59&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.64 ± 0.59</td>
<td>1.12 ± 0.35</td>
<td>NS</td>
<td></td>
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<tr>
<td>PAI-2&lt;sub&gt;ag&lt;/sub&gt; (ng/mg)</td>
<td>5.57 ± 1.13&lt;sup&gt;a&lt;/sup&gt;</td>
<td>7.28 ± 2.59</td>
<td>3.99 ± 1.67</td>
<td>NS</td>
<td></td>
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<tr>
<td></td>
<td>6.64 ± 1.29&lt;sup&gt;b&lt;/sup&gt;</td>
<td>10.69 ± 3.33</td>
<td>4.53 ± 1.75</td>
<td>NS</td>
<td></td>
<td></td>
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<tr>
<td>PAI-3&lt;sub&gt;ag&lt;/sub&gt; (ng/mg)</td>
<td>43 ± 3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>86 ± 20</td>
<td>119 ± 15</td>
<td>P &lt; 0.001</td>
<td>P &lt; 0.01</td>
<td>NS</td>
<td>P &lt; 0.001</td>
</tr>
<tr>
<td></td>
<td>171 ± 17&lt;sup&gt;b&lt;/sup&gt;</td>
<td>233 ± 50</td>
<td>340 ± 45</td>
<td>P &lt; 0.005</td>
<td>NS</td>
<td>P &lt; 0.01</td>
<td>NS</td>
</tr>
<tr>
<td>tPA–PAI&lt;sub&gt;3&lt;/sub&gt; (ng/mg)</td>
<td>0.02 ± 0.01&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.16 ± 0.09</td>
<td>0.03 ± 0.02</td>
<td>NS</td>
<td></td>
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<tr>
<td></td>
<td>0.02 ± 0.01&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.20 ± 0.10</td>
<td>0.06 ± 0.02</td>
<td>NS</td>
<td></td>
<td></td>
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<tr>
<td>uPA–PAI&lt;sub&gt;3&lt;/sub&gt; (ng/mg)</td>
<td>0.27 ± 0.15&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.27 ± 0.13</td>
<td>0.23 ± 0.10</td>
<td>NS</td>
<td></td>
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<tr>
<td></td>
<td>0.28 ± 0.14&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.27 ± 0.13</td>
<td>0.36 ± 0.18</td>
<td>NS</td>
<td></td>
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<tr>
<td>TIMP-1 (ng/mg)</td>
<td>30 ± 5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>17 ± 4</td>
<td>15 ± 3</td>
<td>P &lt; 0.05</td>
<td>NS</td>
<td>NS</td>
<td>P &lt; 0.05</td>
</tr>
<tr>
<td></td>
<td>94 ± 15&lt;sup&gt;b&lt;/sup&gt;</td>
<td>31 ± 6</td>
<td>30 ± 5</td>
<td>P &lt; 0.005</td>
<td>NS</td>
<td>NS</td>
<td>P &lt; 0.001</td>
</tr>
</tbody>
</table>

The data are expressed as mean ± SEM.

<sup>a</sup>Cytosolic extract.
<sup>b</sup>Cytosolic extract plus membrane extract.

PAI-1, PAI-2, PAI-3, tPA–PAI-3, uPA–PAI-3 and TIMP-1 antigen (ag) values were evaluated in cytosolic and membrane extracts, and PAI-1 functional (fc) levels were determined only in cytosolic extract.

NS = not significant.
The levels of different fibrinolytic parameters in peritoneal fluid are shown in Table IV. The concentration of total protein in peritoneal fluid was similar in women with and without endometriosis. The peritoneal fluid from women with endometriosis showed a statistically significant increase in uPA antigen levels, but not in the other parameters, compared with controls (Table IV).

**Table IV.** Fibrinolytic and MMP parameters in peritoneal fluid from women with and without endometriosis

<table>
<thead>
<tr>
<th></th>
<th>Women with endometriosis (n = 22)</th>
<th>Women without endometriosis (n = 12)</th>
<th>Statistical significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total protein (ng/ml)</td>
<td>38.7 ± 3.2</td>
<td>38.6 ± 4.1</td>
<td>NS</td>
</tr>
<tr>
<td>PAI-1&lt;sub&gt;ag&lt;/sub&gt; (ng/ml)</td>
<td>20.8 ± 3.9</td>
<td>15.5 ± 4.0</td>
<td>NS</td>
</tr>
<tr>
<td>PAI-1&lt;sub&gt;c&lt;/sub&gt; (U/ml)</td>
<td>5.0 ± 1.9</td>
<td>3.1 ± 2.0</td>
<td>NS</td>
</tr>
<tr>
<td>PAI-2&lt;sub&gt;ag&lt;/sub&gt; (ng/ml)</td>
<td>4.5 ± 1.1</td>
<td>5.0 ± 1.7</td>
<td>NS</td>
</tr>
<tr>
<td>PAI-3&lt;sub&gt;ag&lt;/sub&gt; (ng/ml)</td>
<td>1669 ± 235</td>
<td>1656 ± 407</td>
<td>NS</td>
</tr>
<tr>
<td>tPA&lt;sub&gt;fc&lt;/sub&gt; (U/ml)</td>
<td>19.3 ± 2.6</td>
<td>27.5 ± 6.8</td>
<td>NS</td>
</tr>
<tr>
<td>uPA&lt;sub&gt;fc&lt;/sub&gt; (U/ml)</td>
<td>0.05 ± 0.01</td>
<td>0.19 ± 0.09</td>
<td>NS</td>
</tr>
<tr>
<td>uPA&lt;sub&gt;ag&lt;/sub&gt; (ng/ml)</td>
<td>2.19 ± 0.36</td>
<td>1.13 ± 0.15</td>
<td>P &lt; 0.05</td>
</tr>
<tr>
<td>MMP-3 (ng/ml)</td>
<td>85 ± 23</td>
<td>62 ± 12</td>
<td>NS</td>
</tr>
<tr>
<td>TIMP-1 (ng/ml)</td>
<td>1612 ± 181</td>
<td>1602 ± 262</td>
<td>NS</td>
</tr>
</tbody>
</table>

The data are expressed as mean ± SEM.
NS = not significant.

**Peritoneal fluid from women with or without endometriosis**

The levels of different fibrinolytic parameters in peritoneal fluid are shown in Table IV. The concentration of total protein in peritoneal fluid was similar in women with and without endometriosis. The peritoneal fluid from women with endometriosis showed a statistically significant increase in uPA antigen levels, but not in the other parameters, compared with controls (Table IV).

**Discussion**

In the present report, we have analysed the antigenic and functional levels of several components of the PA and MMP systems. Measurements of antigenic levels were made in cytosolic and membrane extracts from endometriotic tissue and endometrium of women with and without endometriosis. Endometriotic tissue was obtained from ovarian endometriomas in women with advanced disease (stages III and IV, revised AFS classification). In endometrium from women with endometriosis, an increase in antigenic levels of uPA and MMP-3 was observed compared with disease-free controls. Antigenic levels of PAI-1 and TIMP-1 were higher in endometriotic tissue than in endometrium of women with or without endometriosis.

It has been established that ectopic endometrial tissue fragments have the capability to attach to and invade an intact peritoneal surface (Witz et al., 2001). Obviously, products of endometrial and endometriotic tissues, including PAs, MMPs and their inhibitors, may actively participate in the establishment and remodelling of endometriotic lesions. Misregulated expression of PAs, MMPs and their inhibitors is associated with a more aggressive phenotype and facilitates the degradation of the peritoneal extracellular matrix.

We have found an increased level of uPA antigen in the endometrium of women with endometriosis compared with controls. It has been suggested that a higher concentration of uPA in endometrium of women with endometriosis might alter the degree of the endometrium during menstruation, leading to a disturbed shedding process (Bruse et al., 1998; Kobayashi et al., 2000). This alteration might result in endometrial fragments with a higher potential to degrade the extracellular matrix after implantation at ectopic sites.

Previous studies (Bruse et al., 1998) have reported a higher antigen concentration of uPA in endometriotic tissue of women with endometriosis. However, in the present study, no increase in uPA antigenic levels was observed in endometriotic tissue. The characteristics of the endometriotic samples might be the reason for the disagreement between these results. In the present report, all the endometriotic lesions are ovarian endometriomas; however, in the previous report (Bruse et al., 1998), the lesions were not only endometriotic cysts but also peritoneal lesions, vaginal lesions and scars in the abdominal wall.

Our results suggest that uPA expression is significantly increased in the initial stage of endometriosis and, after development of an endometriotic cyst, this proteolytic activity is no longer increased. A similar suggestion has been reported in relation to the expression of MMP-2 in endometriosis (Chung et al., 2002).

We have found higher PAI-1 levels in endometriotic tissue than in endometrium. Higher PAI-1 levels could be implicated in the capability of growth and invasiveness of the lesion. Higher antigen concentrations of PAI-1 in endometriotic tissue than in endometrium have also been reported previously (Bruse et al., 1998). In the present study, endometrium from women with endometriosis showed no increase in PAI-1 antigenic levels compared with controls. These results agree with previous studies (Fernandez-Shaw et al., 1995) where no difference was found in PAI-1 expression with immunohistochemical techniques between endometrium from women with and without endometriosis. Our finding of high levels of PAI-1 in ovarian endometriomas suggests that its expression is correlated with more advanced stages of the disease.

PAI-3 is a protease inhibitor present in plasma, urine, seminal and follicular fluid (Espana et al., 1991, 1993, 1999). Previous studies have suggested that this inhibitor may be involved in human reproduction (Espana et al., 1991, 1993, 1999). In the present report, lower PAI-3 levels have been found in endometriotic tissue than in endometrium, and no significant differences in PAI-3 complexes were detected between the groups. To our knowledge, this is the first report
which evaluates this inhibitor in relation to endometriosis. It has been suggested that PAI-3 protects uPA from the inactivation by PAI-1 (Schwartz and España, 1999). The decreased levels of PAI-3 observed in endometriotic tissue might enhance the inhibition of uPA by PAI-1, which is increased in this tissue, and contribute to the implantation of endometrial cells.

In the present report, high levels of MMP-3 have been found in endometrium of women with endometriosis, and this may enhance the invasion properties of this endometrium. The retrograde migration of the endometrial fragments into the peritoneal cavity could initiate a degradation of extracellular matrix that facilitates tissue invasion. An increase in levels of MMP-3 has also been reported in the endometrial cells of patients with endometriosis (Silmel et al., 2001). However, in a rat model, MMP-3 mRNA was detectable in endometriotic implants but not in eutopic uterine tissues (Cox et al., 2001). Rodent models of surgically induced endometriosis have been developed to study the role of MMPs in the pathogenesis of this disease, but induction of endometriosis through autotransplantation of rodent endometrium is not spontaneous as it is in humans. The differences in eutopic endometrial MMP-3 observed between our study and animal models are important, and support a role for MMP-3 in endometrium from women with endometriosis in the pathogenesis of the disease.

We have also observed an increase in TIMP-1 levels in endometriotic tissue. This increase has been demonstrated previously in vitro (Sharpe-Timms et al., 1995; Sharpe-Timms, 2001). However, other authors have reported a decreased expression of TIMP-1 in endometriotic tissue in comparison with endometrium (Gottschalk et al., 2000). An increase in the induced secretion of TIMP-1 in the endometrial cells of patients with endometriosis has also been detected (Silmel et al., 2001). The increase in TIMP-1 could induce an inhibition of MMP activity, and this would explain the clinical finding of isolated endometriotic cyst without invasion of the surrounding ovarian tissue.

Our results allow us to speculate on the sequence of events leading to the implantation and formation of ovarian cysts. The initially high enzyme activity observed in the endometrium of women with endometriosis may facilitate the attachment of endometrial tissue to the peritoneum and ovarian surface, as well as the invasion of extracellular matrix, leading to the formation of early endometriotic lesions. Once the ovarian endometriotic cyst is developed, an increase in inhibitors is detected and high proteolytic activity is no longer observed. The reason why the proteolytic activity is limited to the initial stages of endometriosis is still unknown, but this behaviour could explain, as indicated above, the frequent clinical finding of isolated endometriotic cyst without invasion of the surrounding ovarian tissue.

When analysing the peritoneal fluid, we have found higher uPA antigen levels in patients with endometriosis (stages III and IV) than in women without endometriosis. No significant differences were observed in the rest of the fibrinolytic parameters studied. Conflicting results in relation to fibrinolytic activity in peritoneal fluid of patients with endometriosis have been reported. PA activity was reported to be similar in peritoneal fluid from patients with or without endometriosis (Batzofin et al., 1985). However, Astedt and Nordenskjold (1984) have found an increase in PA levels in peritoneal fluid of women with endometriosis. Furthermore, an increase in uPA levels in peritoneal fluid has been reported in cases of pelvic adhesions, and a decrease in PAI-2 levels has been found in early stages of endometriosis without visible adhesions (Edelstam et al., 1998). In consequence, the increased uPA level in the peritoneal fluid of women with endometriosis suggests that the fibrinolytic system can be activated in order to restrict further formation of adhesions.

In conclusion, the increase in uPA and MMP-3 levels observed in endometrium from women with endometriosis might contribute to the invasive potential of endometrial cells. Higher levels of inhibitors PAI-1 and TIMP-1 are present in the ovarian endometriomas where proteolytic activity is no longer detected, and could facilitate the implantation of endometrial cells.

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