mRNA analysis of several components of the plasminogen activator and matrix metalloproteinase systems in endometriosis using a real-time quantitative RT–PCR assay

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BACKGROUND: The plasminogen activator (PA) and matrix metalloproteinase (MMP) systems are implicated in the establishment of endometriosis. The mechanisms by which these systems are involved in the pathogenesis of this disease are not well defined and controversial results have been published. The aim of this study was to analyse mRNA and protein levels of several components of the PA and MMP systems in endometriotic tissue and endometrium from women with and without endometriosis.

METHODS and RESULTS: Real-time quantitative RT–PCR assays were developed to quantify mRNA levels of these components in 57 women with endometriosis and 32 controls. Endometrium of women with endometriosis showed higher mRNA and antigenic levels of urokinase type-PA (uPA) and MMP-3 than endometrium from controls. In these patients, ovarian endometriotic tissue had higher mRNA and antigenic levels of PA inhibitor type 1 (PAI-1) and MMP inhibitor type 1 (TIMP-1) than endometrium. CONCLUSIONS: The increase in mRNA and protein levels of uPA and MMP-3 observed in endometrium of women with endometriosis may facilitate the attachment of endometrial tissue to the peritoneum and ovarian surface, as well as the invasion of the extracellular matrix. This process would lead to the formation of early endometriotic lesions. Once the ovarian endometriotic cyst is developed, PAI-1 and TIMP-1 would increase which could explain the frequent clinical finding of an endometrioma without invasion of the adjacent ovarian tissue.

Key words: endometriosis/inhibitors/matrix metalloproteinase/plasminogen activators/quantitative RT–PCR

Introduction

Endometriosis is one of the most frequent benign gynaecological diseases that affects women of reproductive age with pelvic pain or infertility (Martinez-Roman et al., 1997; Giudice et al., 1998; Pellicer et al., 2001). Little is known about the pathogenesis of endometriosis. However, it is thought that retrograde menstruation may transport endometrial tissue to ectopic locations. By degrading extracellular matrix, the ectopic endometrium may be able to implant and invade peritoneum and the surrounding structures (Spuijbroek et al., 1992; Kobayashi, 2000). The plasminogen activator (PA) and matrix metalloproteinase (MMP) systems may be involved in this process.

The PA system includes a broad spectrum of proteolytic enzymes with physiological and pathophysiological functions such as fibrinolysis, tissue remodelling and tumor invasion, and may also be implicated in the reproductive process (Loskutoff, 1991; Estellés et al., 1994; Gilabert et al., 1995a and 1995b; Andreasen et al., 1997; Murphy et al., 2000; Castelló et al., 2002). Plasminogen is activated to plasmin by two types of activators, urokinase-type PA (uPA) and tissue-type PA (tPA). Whereas tPA is involved in the lysis of fibrin clots, uPA is mainly implicated in cellular proteolysis. The activity of the PAs is regulated by specific PA inhibitors (PAIs). The principal PAIs are PAI-1, also named endothelial cell PAI (Loskutoff, 1991), PAI-2, or 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). In addition, PAI-1 plays an important role in signal transduction, cell adherence and cell migration (Harbeck et al., 2001).

Matrix metalloproteinases (MMPs) are a family of enzymes involved in extracellular matrix remodelling (Matrisian, 1992). These proteases have been implicated in
the endometrial remodelling during the menstrual cycle (Salamonsen and Woolley, 1996) and also in the growth of endometriotic tissue outside the uterus associated with endometriosis (Cox et al., 2001). MMP activities are regulated by tissue inhibitors of MMPs (TIMPs) (Matrisian, 1990). TIMP-1, the first identified TIMP, can inhibit in a 1:1 ratio the collagenase, stromelysin and gelatinase classes of MMPs (Nagase and Woessner, 1999).

An interrelation between the PA and the MMP systems has been described. First of all, 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). On the other hand, MMP-3 specifically hydrolyses and inactivates human PAI-1 (Lijnen, 2002) and may regulate cell-associated plasmin activity (Ugwu et al., 1999).

The mechanisms by which the PA and MMP systems are implicated in the pathogenesis of endometriosis are not well defined. Controversial results have been published about gene expression, protein production and localization of the different components of these systems in endometriosis. However, these studies are not homogeneous with respect to the variety of tissues, models, conditions and techniques.

In situ hybridization studies performed by Bruse et al. (2004) showed that uPA mRNA seems to be up-regulated in both endometriotic glands and endometrial stroma from women with endometriosis. On the other hand, Lembessis et al. (2003) reported an increase in uPA mRNA expression in endometriotic lesions compared to eutopic endometrium. However, other authors found a lower basal release of uPA and soluble uPA receptor (uPAR) in endometriotic stromal cells, using an in vitro culture model (Guan et al., 2002). Recently, we have reported an increase in uPA antigenic levels in endometrium from women with endometriosis (Gilabert-Estellés et al., 2003).

In relation to the MMP system, an increase in the induced secretion of MMP-3 and TIMP-1 has been found in endometrial cell cultures from patients with endometriosis (Sillems et al., 1997, 2001). However, in a rat model, MMP-3 mRNA was detectable in endometriotic implants but not in eutopic uterine tissue (Cox et al., 2001). Other authors have reported a decrease in the expression of TIMP-1 in endometriotic tissue in comparison to endometrium (Gottschalk et al., 2000). We have reported high protein levels of MMP-3 in endometrium of women with endometriosis and an increase in TIMP-1 protein levels in ovarian endometriomas (Gilabert-Estellés et al., 2003). Sharpe-Timms et al. (1995) also found increased TIMP-1 production in both rat and human endometriotic tissue.

The real-time quantitative RT–PCR (QRT–PCR) assay is a more sensitive, faster (Ratge et al., 2000) and less expensive method to quantify gene expression than conventional methods, such as northern blotting (Estellés et al., 1994). Therefore, mRNA assays can be extremely useful when dealing with samples with small amounts of tissue. Furthermore, QRT–PCR permits an accurate quantification of mRNA (Wellmann et al., 2001), whereas competitive RT–PCR is a PCR endpoint analysis that permits only semiquantitative analysis at a low sensitivity (Zimmermann et al., 1997).

To our knowledge, real-time QRT–PCR has not been yet employed to evaluate the mRNA expression of the components of the PA and MMP systems.

The aim of the present study was to determine mRNA expression of several components of both the PA and MMP systems in endometriosis by real-time QRT–PCR. We compared mRNA expression and antigenic levels in extracts from endometriotic and endometrial tissue samples from women with endometriosis and women without the disease.

Materials and methods

Clinical groups

Fifty-seven women with endometriosis were studied (mean age: 33.6 years; range 23–45). This group included 50 women with ovarian endometrioma, classified as moderate or severe endometriosis (stages III–IV, Revised American Society for Reproductive Medicine, 1997). Seven women with active peritoneal implants were classified as minimal or mild endometriosis (stages I–II, Revised ASRM classification system, 1997). All the women underwent surgical examination of the abdominal cavity and excision of the endometriotic tissue, either by laparoscopy (75.4%) or laparotomy (24.6%). The presence of the disease was suspected either clinically or by ultrasound exam, and confirmed by the surgical findings and the postoperative pathological study.

Thirty-two women (mean age: 38.4 years; range 29–49) without endometriosis, who underwent surgery for pelvic pain (14%), sterility (17%) or tubal sterilization (69%), were included in the control group. The absence of the disease was confirmed after surgical examination of the abdominal cavity by laparoscopy (53.1%) or laparotomy (46.9%).

The menstrual phase was identified according to the day of the reproductive cycle and the histological analysis of the endometrium using the method of Noyes et al. (1950). Twenty-three (40.3%) of the 57 women with endometriosis were in the proliferative phase of the menstrual cycle, 31 (54.4%) were in the secretory phase and three (5.3%) were in the menstrual phase. Thirteen (40.6%) of the 32 controls were in the proliferative phase, 17 (53.1%) in the secretory phase and two (6.3%) were in the menstrual phase of the menstrual cycle.

Patients with irregular menstruation, steroid treatment or women who were pregnant or breast-feeding in the previous 6 months were excluded from the study. None of the 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 institutional review board.

Tissue extracts

Endometriotic tissue samples were obtained from ovarian endometriomas in fifty patients with moderate or severe endometriosis (stages III–IV) (group A) and from active peritoneal implants of seven women with minimal or mild endometriosis (stages I–II) (group C). Tissue samples from endometriomas were macroscopically separated from ovarian tissue and peritoneal implants were excised surgically. Twenty-six endometrial biopsies (eutopic endometrium) (group B) from the 50 women with ovarian endometrioma and seven biopsies (eutopic endometrium) (group D) from the seven women with endometriosis stages I–II were performed by aspiration using the Cornier device (Gynetics, Hamont-Achel, Belgium).
Thirty-two endometrial biopsies from patients without endometriosis (control endometrium) (group E) were obtained, using the same device. 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).

**QRT–PCR**

Total RNA and cDNA were obtained as previously described (Castello et al., 2002). In brief, RNA from frozen tissue was extracted with the RNeasy total RNA kit (Qiagen, Inc., Valencia, CA), according to the manufacturer’s instructions. One microgram of total RNA was treated with DNase I (Invitrogen, Carlsbad, CA) 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). The cDNA was stored at −20°C until used.

Analysis of uPA, PAI-1, PAI-3, MMP-3, 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, TIMP-1 and β-actin were obtained as previously described (Castelló et al., 2002). Fragments of 332 bp of PAI-3 and 261 bp of MMP-3 were amplified using specific primers (PAI-3 forward: 5′-GCAAGC-GAAGGCCAGAATT-3′ and PAI-3 reverse: 5′-TCCTCAAGCGTTT-TCTCACA-3′; MMP-3 forward: 5′-AGCAAGGACCTCGTTTT-CATT-3′ and MMP-3 reverse: 5′-GTCAATCCCTGGAAAGTC-CTTCA-3′) which were designed using specific primer analysis software (Oligo 4.0, National Biosciences Inc., Plymouth, MN). These sequences were analyzed by FASTA in the EMBL database (http://www.embl-heidelberg.de/).

The reaction mixture and the PCR conditions for uPA, PAI-1, TIMP-1 and β-actin were previously described (Castelló et al., 2002). The assay conditions for PAI-3 and MMP-3 were empirically determined and we observed that those used for uPA, PAI-1 and TIMP-1 were also appropriate for PAI-3 and MMP-3. Each assay was carried out in a final volume of 10 μl containing 1.5 μl cDNA (1:10), 3 mM MgCl$_2$, 0.5 μM of each uPA, PAI-1, PAI-3, MMP-3, and TIMP-1 primers, or 0.3 μM of each β-actin primers, 1 μl LC-Fast Start Reaction Mix SYBR Green I and 1 μl LC-Fast Start DNA Master SYBR Green I/Enzyme, including Taq DNA polymerase, reaction buffer and deoxynucleotide 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 Taq DNA polymerase. In the second step, DNA was amplified for 40 cycles of 15 s at 95°C (denaturation), 5 s at 60°C for uPA, PAI-1, PAI-3, MMP-3 and TIMP-1 or at 62°C for β-actin (annealing), and 18 s at 72°C (extension). Finally, the temperature was raised gradually (0.1 deg C/s) from 65°C 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 second one without reverse transcriptase. The numbers of copies of samples were 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). Standard bovine serum albumin fraction V (Sigma) was used for calibration. Samples and standards were tested in duplicate.

uPA antigen was quantified by a commercially available enzyme-linked immunosorbent assay (ELISA) (Tint Elize uPA, Biopool), which measures single-chain urokinase (scuPA) and the high molecular weight form of uPA (HMW-uPA) with similar efficiency. The low molecular form of uPA is measured with ~40% molar basis of efficiency of scuPA and HMW-uPA. The intraassay and inter assay variabilities were 3–5% and 8–11%, respectively.

Another commercially available ELISA (Tint Elize PAI-1, Biopool) was used to quantify PAI-1 antigenic levels. It detects active and inactive forms of PAI-1, as well as complexes such as tPA/PAI and uPA/PAI. The intraassay and interassay variation coefficients were 2–4% and 6–8%, respectively.

PAI-3 antigenic levels were measured by an ELISA, as previously reported (España and Griffin, 1989; España et al., 1991). The intraassay and interassay variabilities were 4–8% and 6–9%, respectively.

MMP-3 antigen was quantified by a commercially available ELISA (MMP-3 ELISA, Oncogene). The assay detects MMP-3, but does not recognise MMP-3/TIMP complexes. The intraassay and interassay variabilities were 4–6% and 7–10%, respectively.

TIMP-1 antigen was quantified by a commercially available ELISA (TIMP-1 ELISA, Oncogene). The assay recognises free and complexed TIMP-1. The intraassay and interassay variabilities were 3–5% and 6–8%, respectively.

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 analyzed by the one-way analysis of variance test and Student–Newman–Keuls multiple range test (when applicable). The percentages of the menstrual cycle phases in the different groups were compared by using the Chi-square test. Both endometriotic and endometrial tissue were available in 26 patients. In order to analyse the differences found in this subgroup attending to the type of sample, 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 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**

Tables I and II show mRNA and antigenic levels of the parameters measured in tissue extracts. The distribution of the samples according to the phase of menstrual cycle was compared in women with or without endometriosis and showed no statistically significant differences, by using Chi-square test. The menstrual cycle phase from patients with endometriosis was proliferative (40.3%), secretory (54.4%) and menstrual (5.3%), and did not differ from controls (40.6%, 53.1% and 6.3%, respectively).
Plasminogen activator and metalloproteinase systems in endometriosis

Table I. Urokinase plasminogen activator (uPA) and metalloproteinase-3 (MMP-3) mRNA and antigenic levels in tissue extracts from ovarian endometrioma, peritoneal lesions and endometrium from women with and without endometriosis (controls)

<table>
<thead>
<tr>
<th></th>
<th>Endometriotic lesions (III–IV) (ovarian endometrioma) (n = 50) (A)</th>
<th>Eutopic endometrium from women with endometriosis (III–IV) (n = 26) (B)</th>
<th>Endometriotic lesions (I–II) (peritoneal lesions) (n = 7) (C)</th>
<th>Eutopic endometrium from women with endometriosis (I–II) (n = 7) (D)</th>
<th>Endometrium from controls (n = 32) (E)</th>
<th>ANOVA</th>
</tr>
</thead>
<tbody>
<tr>
<td>uPA mRNA*</td>
<td>0.320 ± 0.045</td>
<td>0.515 ± 0.087</td>
<td>0.350 ± 0.070</td>
<td>0.351 ± 0.068</td>
<td>0.313 ± 0.040</td>
<td>P &lt; 0.05</td>
</tr>
<tr>
<td>uPA Ag (ng/mg)</td>
<td>1.36 ± 0.12</td>
<td>1.88 ± 0.29*</td>
<td>2.16 ± 0.30**</td>
<td>2.53 ± 0.44*</td>
<td>1.22 ± 0.10</td>
<td>P &lt; 0.05</td>
</tr>
<tr>
<td>MMP-3 mRNA*</td>
<td>0.056 ± 0.018</td>
<td>0.130 ± 0.059</td>
<td>0.038 ± 0.016</td>
<td>0.042 ± 0.020</td>
<td>0.017 ± 0.007</td>
<td>P &lt; 0.05</td>
</tr>
<tr>
<td>MMP-3 Ag (ng/mg)</td>
<td>3.68 ± 0.50</td>
<td>4.27 ± 0.82*</td>
<td>4.03 ± 0.73</td>
<td>3.87 ± 0.59*</td>
<td>2.03 ± 0.31</td>
<td>P &lt; 0.05</td>
</tr>
</tbody>
</table>

ANOVA: analysis of variance. Data are expressed as means ± SEM (ng target protein/mg total protein). Antigenic (Ag) values represent cytosol extract plus membrane extract.

*Normalized mRNA = 100 × [(target gene mRNA copies/β-actin mRNA copies)].

**P < 0.05 vs endometrium from controls.

Table II. Plasminogen activator inhibitors (PAI-1 and PAI-3) and matrix metalloproteinase inhibitor type 1 (TIMP-1) mRNA and antigenic levels in tissue extracts from ovarian endometrioma, peritoneal lesions and endometrium from women with and without endometriosis (controls)

<table>
<thead>
<tr>
<th></th>
<th>Endometriotic lesions (III–V) (ovarian endometrioma) (n = 7) (A)</th>
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<th>Endometrium from controls (n = 32) (E)</th>
<th>ANOVA</th>
</tr>
</thead>
<tbody>
<tr>
<td>PAI-1 mRNA*</td>
<td>1.450 ± 0.239**</td>
<td>0.938 ± 0.298</td>
<td>0.341 ± 0.124</td>
<td>0.131 ± 0.060</td>
<td>0.429 ± 0.175</td>
<td>P &lt; 0.02</td>
</tr>
<tr>
<td>PAI-1 Ag (ng/mg)</td>
<td>10.48 ± 1.73***#</td>
<td>1.86 ± 0.42</td>
<td>1.21 ± 0.62</td>
<td>1.11 ± 0.46</td>
<td>0.99 ± 0.29</td>
<td>P &lt; 0.0001</td>
</tr>
<tr>
<td>PAI-3 mRNA*</td>
<td>0.987 ± 0.194*</td>
<td>1.989 ± 0.371</td>
<td>0.692 ± 0.285</td>
<td>0.708 ± 0.261</td>
<td>2.564 ± 0.488</td>
<td>P &lt; 0.0003</td>
</tr>
<tr>
<td>PAI-3 Ag (ng/mg)</td>
<td>172 ± 12**</td>
<td>219 ± 37</td>
<td>160 ± 55</td>
<td>244 ± 68</td>
<td>359 ± 39</td>
<td>P &lt; 0.0001</td>
</tr>
<tr>
<td>TIMP-1 mRNA*</td>
<td>6.988 ± 1.165**#</td>
<td>1.467 ± 0.510</td>
<td>2.391 ± 1.025</td>
<td>1.460 ± 0.312</td>
<td>0.976 ± 0.155</td>
<td>P &lt; 0.0001</td>
</tr>
<tr>
<td>TIMP-1 Ag (ng/mg)</td>
<td>83 ± 8*#</td>
<td>28 ± 4</td>
<td>74 ± 16</td>
<td>22 ± 6</td>
<td>34 ± 5</td>
<td>P &lt; 0.0001</td>
</tr>
</tbody>
</table>

Data are expressed as means ± SEM (ng target protein/mg total protein). Antigenic (Ag) values represent cytosol extract plus membrane extract.

*Normalized mRNA = 100 × [(target gene mRNA copies/β-actin mRNA copies)].

**P < 0.01 vs endometrium from controls.

***P < 0.001 vs endometrium from controls.

#P < 0.01 vs endometrium from women with endometriosis (III–IV).

Endometrium from women with endometriosis vs endometrium from women without endometriosis

Extracts of eutopic endometrium from women with endometriosis (stages III–IV) (group B) showed a significant increase in mRNA and antigenic levels of uPA and MMP-3 in comparison to the endometrium from controls (group E) (Table I). uPA and MMP-3 protein levels in the endometrium from women with minimal or mild endometriosis (stages I–II) (group D) were higher than in the endometrium from controls (group E) (Table I).

The expression of PA and MMP systems in endometrium from women with endometriosis (stages III–IV) and in endometrium from controls were also studied according to the cycle phase.

In secretory phase, when comparing eutopic endometrium from women with endometriosis (stages III–IV) versus endometrium from controls, we detected significant higher uPA antigenic levels (2.02 ± 0.30 vs 1.36 ± 0.15 ng/mg, P < 0.05) and higher MMP-3 antigenic levels (4.96 ± 1.22 vs 2.42 ± 0.50 ng/mg, P < 0.05) in patients than in controls.

Endometrium from women with the disease also showed higher mRNA levels of uPA (0.562 ± 0.139 vs 0.152 ± 0.029, P < 0.005) and MMP-3 (0.296 ± 0.133 vs 0.044 ± 0.001, P < 0.05) in comparison to endometrium from controls. However, no significant differences were observed in proliferative phase between endometrium from women with and without endometriosis.

The expression of PA and MMP systems across the menstrual cycle was also studied. Extracts of endometrium from controls showed significantly higher levels of uPA mRNA and MMP-3 mRNA in proliferative than in secretory phase (uPA mRNA: 0.440 ± 0.062 vs 0.152 ± 0.029, P < 0.001; MMP-3 mRNA: 0.015 ± 0.006 vs 0.004 ± 0.001, P < 0.05, respectively). In contrast, no significant differences were observed in uPA mRNA and MMP-3 mRNA when comparing proliferative versus secretory phase in endometrium from women with endometriosis (uPA mRNA: 0.446 ± 0.072 vs 0.562 ± 0.139; MMP-3 mRNA: 0.029 ± 0.009 vs 0.296 ± 0.133, respectively). There were no significant differences in uPA or MMP-3 antigenic levels when comparing proliferative versus secretory phase in endometrium from women with or without endometriosis.

Ovarian endometrioma vs endometrium from women with endometriosis (stages III–IV)

A significant increase in PAI-1 protein levels and TIMP-1 mRNA and protein levels was observed in 50 samples from ovarian endometrioma (group A) compared with 26 eutopic endometrial tissues from women with endometriosis (stages III–IV).
III–IV) (group B) (Table II). However, no significant differences were observed in PAI-1 mRNA levels.

When we analysed only in the 26 women in whom eutopic endometrium and ovarian endometrioma tissues were collected at the same time, ovarian endometriomas also showed significantly higher PAI-1 antigenic levels than eutopic endometrium (10.52 ± 2.44 ng/mg vs 1.86 ± 0.42 ng/mg, \( P < 0.01 \)). However, no significant differences were observed in PAI-1 mRNA levels (1.080 ± 0.215 vs 0.938 ± 0.298). Both TIMP-1 antigenic levels (87 ± 13 ng/mg vs 28 ± 4 ng/mg, \( P < 0.01 \)) and TIMP-1 mRNA levels (7.311 ± 1.294 vs 1.467 ± 0.510, \( P < 0.01 \)) were elevated in endometriomas in comparison to eutopic endometrium.

**Ovarian endometriomas vs endometrium from women without endometriosis**

Ovarian endometriotic tissue (group A) showed a significant increase in mRNA and antigenic levels of PAI-1 and TIMP-1, and a decrease in PAI-3 mRNA and antigenic levels compared to endometrium from women without endometriosis (group E) (Table II).

**Peritoneal endometriotic implants (stages I–II) vs ovarian endometrioma (stages III–IV)**

An increase in uPA antigenic levels was observed in peritoneal lesions (group C) in comparison to ovarian endometrioma (group A) (2.16 ng/mg vs 1.36 ng/mg) (Table I). Meanwhile, ovarian endometriomas had higher PAI-1 antigenic (10.48 ng/mg vs 1.21 ng/mg) and mRNA levels (1.45 vs 0.34) than peritoneal implants.

On the other hand, the ratio of uPA/PAI-1 (antigenic and mRNA levels) was higher in the peritoneal implants than in the ovarian endometriomas (peritoneal lesions vs ovarian endometriomas: antigenic levels: 2.16/1.21 vs 1.36/10.48; mRNA levels: 0.350/0.341 vs 0.320/1.450).

**Correlation between mRNA and protein levels**

In endometrium from women without endometriosis, mRNA levels were significantly correlated to their corresponding protein levels, mainly PAI-1 levels (\( r = 0.831, P < 0.001 \)). However, in ovarian endometrioma, no significant correlation was observed between PAI-1 protein and mRNA levels.

**Discussion**

In the present study we have observed that endometrium from women with endometriosis has an increase in uPA and MMP-3 mRNA and antigenic levels compared to disease-free controls. mRNA and antigenic levels of PAI-1 and TIMP-1 were higher in ovarian endometriomas than in endometrium from women without endometriosis.

Ectopic endometriotic lesions are histologically similar to eutopic endometrial tissue. However, biochemical differences exist between these two tissues. Although little information has been published regarding the differences between endometrium from women with and without endometriosis, evidences suggest that the eutopic endometrium from women with endometriosis has altered expression of several components of the MMP system and may play a role in the pathogenesis of this disease (Sharpe-Timms, 2001).

Obviously, in vitro models lack the environmental modulations of hormones, growth factors or cytokines, which are normally present in in vivo studies. This observation may justify the differences found in both types of studies (Cox et al., 2001). Although the rat model is an in vivo system free from confounding factors (Sharpe-Timms, 2001), the fact that the rodents do not develop spontaneous endometriosis may explain the absence of biochemical alteration in their eutopic endometrium.

In relation to PA levels in eutopic endometrium from women with endometriosis, it has been suggested that a higher concentration of uPA in the endometrium might result in endometrial fragments with a higher potential to degrade the extracellular matrix after the implantation at ectopic sites (Spuijbroek et al., 1992; Bruse et al., 1998, 2004; Kobayashi, 2000). In the present report, an increase in uPA mRNA and antigenic levels has been found in the endometrium of women with endometriosis compared to controls.

The high mRNA and protein levels of MMP-3 in the endometrium from women with endometriosis, observed in the present report, may also favour the invasive properties of this tissue. In previous reports, eutopic endometrium of women with endometriosis showed increased MMP-3 antigen levels (Sillem et al., 2001) and high MMP-9 mRNA levels (Chung et al., 2001), however, in a rodent model, MMP-3 mRNA was detectable in endometriotic implants but not in eutopic uterine tissues (Cox et al., 2001). This would imply that no proteolytic alteration should be present in the endometriotic tissue in rats as it is in women with endometriosis. The differences in eutopic endometrial MMP-3 levels 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.

In vitro studies have shown that progesterone is a potent inhibitor of endometrial MMP mRNA expression and protein secretion (Bruner-Tran et al., 2002). Stromelysins, such MMP-3, are expressed in vivo during proliferation-associated remodelling but not during the progesterone-dominated secretory phase of the cycle (Bruner et al., 1995). In the present study, secretory endometrium from controls showed lower uPA and MMP-3 mRNA levels than proliferative endometrium. However, no differences are observed between proliferative and secretory phase in the endometrium from patients with endometriosis. On the other hand, secretory endometrium from women with endometriosis had higher mRNA and protein levels of MMP-3 and uPA than secretory endometrium from controls. These findings may indicate a failure of progesterone or locally produced factors to suppress these enzymes in women with endometriosis and might facilitate the implantation of endometrial fragments after retrograde pass through the fallopian tubes.

In relation to endometriotic lesions, previous studies (Lembessis et al., 2003) have reported an increase in uPA mRNA expression in endometriotic tissue compared to...
might be an important clue in the invasive potential and in observed in endometrium from women with endometriosis additional inhibition of proteolytic activity in this location. Expression observed in ovarian endometrioma would result in (Sharpe-Timms, 2001). However, other authors have reported decreased PAI-3 antigenic levels in endometriotic tissue in patients and controls. However, the increase in PAI-1 antigenic levels was higher than the increase in PAI-1 mRNA levels. It has been previously reported that MMP-3 specifically hydrolyses human PAI-1 (Lijnen, 2002). A probable explanation of our results could be that the low levels of activators in ovarian endometriomas may lead to less degradation of PAI-1 protein, resulting in higher PAI-1 antigenic levels. The increase in PAI-1 protein expression in ovarian endometriomas might contribute to limit the invasive potential of the endometriotic tissue in advances stages of the disease.

We have reported increased PAI-3 antigenic levels in an ovarian endometriosis compared to endometrium (Gilabert-Estellés et al., 2003). PAI-3 is a protease inhibitor that may be involved in human reproduction (España et al., 1991, 1993, 1999). The precise role of this inhibitor in extracellular proteolysis has not been fully elucidated, but it has been suggested that PAI-3 protects uPA from the inactivation by PAI-1 (Schwartz and España, 1999). In the present study, we have found a reduced mRNA expression of PAI-3 in ovarian endometriomas, which might enhance inhibition of uPA by PAI-1 in ovarian endometriomas, and therefore contribute to the reduced proteolytic activity of this tissue.

We have also observed an increase in mRNA and protein levels of TIMP-1 in endometriotic tissue. This increase has been previously demonstrated (Sharpe-Timms et al., 1995, Sharpe-Timms, 2001). However, other authors have reported a decreased expression of TIMP-1 in endometriotic tissue in comparison to endometrium (Gottschalk et al., 2000). Endometriotic lesions have been found to synthesize and secrete TIMP-1 protein in vitro, while TIMP-1 protein levels are lower in peritoneal fluid of women with endometriosis (Sharpe-Timms et al., 1995). The increased TIMP-1 expression observed in ovarian endometrioma would result in additional inhibition of proteolytic activity in this location.

In conclusion, the up-regulation of uPA and MMP-3 observed in endometrium from women with endometriosis might be an important clue in the invasive potential and in the growth of endometrial tissue outside the uterus. This process would lead to the formation of early endometriotic lesions. Once the ovarian endometriotic cyst is developed, PAI-1 and TIMP-1 would increase and the proteolytic activity would, therefore, decrease. This observation would explain the frequent clinical finding of an isolated endometriotic cyst without invasion of the surrounding ovarian tissue. Quantitative real-time RT–PCR can be a valuable tool in further investigations about the role of the PA and MMP systems in endometriosis.

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