Matrix metalloproteinase-9 and tissue inhibitor of matrix metalloproteinase-1: a possible role in the pathogenesis of endometriosis

J.Szamatowicz1,3, P.Laudański1 and I.Tomaszewska2

1Department of Gynaecology and 2Department of Gynaecological Endocrinology, Białystok Medical University, Białystok, Poland
3To whom correspondence should be addressed at: Department of Gynaecology, Białystok Medical University, ul. Marii Skłodowskiej—Curie 24a, 15–276 Białystok, Poland. E-mail: szamatj@cksr.ac.bialystok.pl

BACKGROUND: Matrix metalloproteinases (MMPs) are a family of endopeptidases which play a role in the degradation and turnover of extracellular matrix proteins. Their action is regulated by specific tissue inhibitors called tissue inhibitors of metalloproteinases (TIMPs). METHODS: We measured the concentrations of total and active MMP-9 in peritoneal fluid of infertile women with mild or moderate endometriosis (n = 22) and compared them with those in a control group of infertile patients (n = 21). RESULTS: We found that the mean (±SD) total concentrations of MMP-9 in the peritoneal fluid of patients with endometriosis was 6.2 ± 1.8 ng/ml, in comparison with 2.9 ± 2.6 ng/ml in the control group (P = 0.001). Concentrations of active MMP-9 did not differ significantly between the groups. The concentrations of TIMP-1, after logarithmic transformation, were significantly lower (P = 0.017) in endometriotic peritoneal fluids than in peritoneal fluid of control women, 1.02 ± 0.21 ng/ml and 1.16 ± 0.18 ng/ml respectively. No correlation between stage of disease, steroid hormone concentration, MMP-9 (total and active) and TIMP-1 was found. CONCLUSIONS: These results suggest that a disturbed equilibrium exists between MMP-9 and TIMP-1 in peritoneal fluid of women with endometriosis. This may play an important role in the pathogenesis of the disease.

Key words: mild-moderate endometriosis/MMP-9/peritoneal fluid/TIMP-1

Introduction

Endometriosis is an invasive but benign gynaecological disease characterized histologically by the presence of endometrial glands and stroma outside the uterine cavity. It is estimated that 10–20% of women of reproductive age (Goldman and Cramer, 1990) are affected and up to 50% of infertile patients suffer from endometriosis (Battista, 1991). Since endometriotic tissue undergoes cyclic changes similar to eutopic endometrium, the resulting local inflammatory processes cause common symptoms of endometriosis such as dysmenorrhoea, dyspareunia and pelvic pain. As regards pathogenesis, the most widely accepted theory is that the disease is caused by retrograde menstruation and subsequent implantation of endometrial glands on the surface of the abdominal cavity (Sampson, 1925). Since retrograde menstruation occurs in many women, it is postulated that endometriosis develops as a consequence of disturbances in the balance between amount of menstrual blood and capacity of the ‘clearance’ system in the peritoneal milieu (Olive and Schwartz, 1993).

Matrix metalloproteinases (MMPs) are a family of endopeptidases that play a role in the degradation and turnover of extracellular matrix (ECM). These zinc-dependent enzymes, which include collagenases, gelatinases and stromelysins, are capable of degrading all components of the ECM. Tissue inhibitors of metalloproteinases (TIMPs), which affect normal and pathologic matrix remodelling, regulate the activity of MMPs (Matrisian, 1992; Salamonsen and Woolley, 1996). It is proposed that MMP may enable endometriotic tissue to digest into peritoneal ECM and underlying connective tissue. It is also well known that endometrial remodelling and MMP expression occur during proliferative and menstrual phases of the cycle and that progesterone is a strong suppressor of MMPs (Osteen et al., 1996). The production of MMPs and their inhibitors takes place in the endometrial stroma and epithelium as well as in polymorphic mononuclear leukocytes. Another important source of the enzymes are macrophages, neutrophils and eosinophils, activated as a consequence of a low grade inflammation state present in the peritoneal cavity of women with endometriosis (Busiek et al., 1995; Jeziorska et al., 1995; Shi et al., 1995; Jeziorska et al., 1996). It is not known whether the concentrations of these enzymes are connected only with laparoscopically visible endometriotic lesions or are also changed in patients with pain and/or infertility but with no clearly seen endometriosis. The aim of the study was to
evaluate the levels of total and active MMP-9 as well as that of TIMP-1 in peritoneal fluid of infertile women with visible endometriotic lesions and in patients with no signs of endometriosis. We also examined the possible correlation between levels of MMP-9, TIMP-1, the stage of the disease and sex steroid hormone levels in peritoneal fluid.

Material and methods

Patients

A total of 43 women who underwent laparoscopy to evaluate infertility at the Gynaecological Clinic of the Medical University in Bialystok, Poland, participated in the study; 22 patients were selected by the existence of visible peritoneal endometriotic lesions (stage II, III) (ASRM, 1997), characterized as red- or gland-like lesions as well as red vesicles. Of these women, 10 (45%) were scored as second and 12 (55%) as third stage. Twenty-one infertile women, without any sign of endometriosis presented at laparoscopy, were used as a control.

The study was approved by institutional ethics and informed consent was obtained from the patients. All laparoscopies were performed in the first phase of menstrual cycle (days 8–12).

Peritoneal fluid

Peritoneal fluid samples were collected, cleared of cells and cell debris by means of centrifugation at 3000 g for 10 min. They were then stored at –80°C. They were tested in one batch for total and active MMP-9 as well as for TIMP-1.

Quantification of MMP-9 in peritoneal fluid obtained from women with and without endometriosis was performed by two kinds of kits. An ELISA kit (R&D Systems, Minneapolis, USA) was used to evaluate total levels of the enzyme (pro and/or active) and Amersham’s activity ELISA assay system was employed to evaluate active MMP-9. The concentrations of TIMP-1 were determined by an immunoassay from R&D Systems. The methods used were those described in the protocols accompanying the ELISA kits. Briefly, for total MMP-9 and TIMP-1, 100 µl peritoneal fluid samples were pipetted in duplicates into the appropriate ELISA microtitre wells and incubated for 2 h at 20–25°C. Wells were washed and incubated with 200 µl of the conjugate for 1 h at 20–25°C and then incubated with 200 µl of the substrate solution. After adding 50 µl of ‘stop solution’ (1 mol/l sulphuric acid) to each well absorbance at 570 nm was measured spectrophotometrically in an automated plate reader.

For the active MMP-9, 100 µl peritoneal fluid samples were plated, in duplicate, and refrigerated at 4°C overnight. Plates were washed in an automated plate washer and 50 µl of a 1 mol/l p-aminophenylmercuric acetate, which activates pro-MMP, was added into wells in which total MMP was to be measured. After 2 h incubation at 37°C, 50 µl detection reagent containing a modified urokinase, and S-2444 peptide substrate, was added to all wells. Plates were read spectrophotometrically at time 0 and after a 2 h incubation at 37°C at an absorbance of 405 nm. These values were compared with a standard curve of serial dilutions of a known concentration of activated enzyme. Normal ranges of values were provided by the manufacturer.

Total protein was quantified as previously described (Lowry et al., 1951).

Radioimmunoassay for estradiol and progesterone

Estradiol and progesterone concentrations in peritoneal fluids were measured in duplicate after 1:1000 dilution in serum-free assay buffer (sodium phosphate 0.1 mmol/l + NaCl 0.3 mol/l + sodium azide 0.9 g/l, pH 7.5) using radioimmunoassay (Orion Diagnostica, Espoo, Finland).

Figure 1. Concentrations of total metalloproteinase-9 (MMP-9) in peritoneal fluid obtained from women with and without endometriosis. Significantly higher (P = 0.001) concentrations of total MMP-9 were found in peritoneal fluid obtained from patients with endometriosis.

Table I. Clinical characteristics of patients

<table>
<thead>
<tr>
<th>Age (years)</th>
<th>Control group (n = 21)</th>
<th>Endometriosis (n = 22)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>29.05 ± 0.29</td>
<td>29.3 ± 2.62</td>
</tr>
<tr>
<td>Duration of infertility (months)</td>
<td>50.2 ± 23.4 (14–130)</td>
<td>56.4 ± 18.3 (23–89)</td>
</tr>
<tr>
<td>Infertility, n (%)</td>
<td>Primary</td>
<td>Secondary</td>
</tr>
<tr>
<td></td>
<td>14 (67.6)</td>
<td>7 (32.4)</td>
</tr>
<tr>
<td></td>
<td>16 (72.7)</td>
<td>8 (27.3)</td>
</tr>
</tbody>
</table>

Data are mean ± SEM. Ranges are provided for ‘duration of infertility’.

Statistical analysis

The statistical analysis was performed using the SAS STAT package. Student’s t-test was used to compare the concentrations of MMP-9, TIMP-1, total protein, estradiol and progesterone. Since the TIMP-1, estradiol and progesterone values in both groups of patients were not normally distributed, logarithmic transformations of the values were carried out. P values < 0.05 were considered statistically significant.

Results

The clinical characteristics of women who participated in this study are summarized in Table I. The mean age of patients in the study and control groups was similar (29.3 ± 2.62 and 29.05 ± 0.29 years old respectively). The mean volume of peritoneal fluid was 5.5 ± 1.8 ml in the study group and 3.5 ± 0.9 ml in controls.

The peritoneal fluid concentrations of estradiol were 73.9 ± 56 pg/ml and 90.8 ± 55 pg/ml and of progesterone 1.9 ± 2.47 pg/ml and 1.17 ± 1.3 pg/ml in endometriotic and disease-free groups respectively.

The concentration of total protein in peritoneal fluid was similar in the study and control groups, 4.8 ± 0.76 and 4.39 ± 0.79 g/dl respectively. Concentrations of total and active MMP-9 in peritoneal fluid of individual patients are shown in Figures 1 and 2. The mean total concentration of MMP-9 in peritoneal fluid of patients with endometriosis was 6.2 ± 1.8 ng/ml and 2.9 ± 2.6 ng/ml in controls respectively (P = 0.001). The levels of active MMP-9 in the endometriosis
The concentrations of the active form of MMP-9 were much lower than those of total MMP-9 in both the group with endometriosis and in the control group. No differences were found between the two groups. This may be due to the presence of a pro-MMP-9 form and possibly attenuation of the pro-MMP-9 activation processes in peritoneal fluid. Higher levels of total MMP-9 do not necessarily imply an increased proteolytic activity since most of the MMPs are secreted as inactive zymogens requiring proteolytic removal of the aminoterminal domain for the expression of activity (Tryggvason et al., 1987). The physiological mechanisms for the control of MMP-9 activity may involve many factors, such as urokinase type plasminogen activator (uPA) and plasmin system or membrane type MMP-1 (MT1-MMP, MMP-14) (Sato et al., 1994; Tokuraku et al., 1995). Recently, it was suggested that human trypsin-2, the main isoenzyme of tumour-associated trypsinogens, is more potent than plasmin and other serine proteinases in activating several MMPs and is the most efficient activator of the 92 kDa gelatinase B (MMP-9) known so far (Sorsa et al., 1997). Interestingly, it was found in cancer cells that down-regulation of trypsin-2 expression and activity is associated with decreased pro-MMP-9 activation (Lukkonen et al., 2000). Taking into account our results, where no differences were found between groups in the concentrations of the active form of MMP-9, one may assume that the pattern of regulation in endometriosis is similar, with down-regulation of trypsin-2 expression. TIMP-1, which we found to be lower in the peritoneal fluid of women with endometriosis, is also possibly involved. Sorsa et al. showed that TIMP-1 reduces, but not abolishes the activation of MMP-9 by trypsin-2 (Sorsa et al., 1997). Alternatively, TIMP-1 is able to form complexes with proMMP-9, thereby inhibiting the process of MMP-9 activation (Goldberg et al., 1992). Despite the fact that we found the concentrations of TIMP-1 to be lower in endometriosis patients, it is likely that the amount of TIMP-1 is sufficient for at least one of these possibilities. It is also feasible that endometriotic tissue itself may show more pronounced activity of MMP-9. Immunohistochemical staining for MMP-1 and MMP-3, performed by Koks et al. was found to be strong in stroma and for MMP-7 in epithelium of antegradely shed endometrium, even after culturing for 24 h (Koks et al., 2000). In contrast MMP-2 and MMP-9 were weakly expressed in stroma. Both TIMP-1 and TIMP-2 were expressed in menstrual endometrium (Koks et al., 2000). In another study, Gottschalk et al. found that there is a high protein expression of MMP-1 and significantly lower TIMP-1 and TIMP-2 protein expression in endometriotic tissue compared with normal endometrium (Gottschalk et al., 2000). Chung et al. found that ectopic and eutopic endometrium from endometriosis patients may be more invasive and prone to peritoneal implantation because of greater MMP-9 and less TIMP-3 mRNA expression than endometrium of women without endometriosis (Chung et al., 2001). They suggested that increased proteolytic activity of endometrial and endometriotic tissue may be one of the reasons for the invasive properties of the endometrium, resulting in the development of endometriosis.

Figure 2. Concentrations of active MMP-9 in peritoneal fluid of women with and without endometriosis. The difference was not found to be statistically significant ($P = 0.39$).

Figure 3. Logarithmic transformation of concentration values of TIMP-1 in peritoneal fluid of women with and without endometriosis. The logarithmic transformation of TIMP-1 concentrations shows significantly lower values in endometriotic group when compared with control ($P = 0.017$).
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(Chung et al., 2001). Having this in mind, one can speculate that endometrium could possibly be a predominant source of MMP-9, total concentrations of which were higher than control in peritoneal fluid. However, ELISA determinations performed by Sillem et al. show very low concentrations of MMP-9 in culture supernatants of endometrial cells, whereas MMP-3 was found in very high but MMP-1 and MMP-2 in moderate concentrations (Sillem et al., 1998). One can assume from those results that the higher concentrations of total (pro and/or active) MMP-9 might be due to the increased production by cellular components of peritoneal fluid.

On the other hand, it remains to be determined whether the concentrations of active MMP-9 are sufficient to proceed to the invasion of endometriotic lesions. Studies on the activity of other MMPs in endometriotic implants are ongoing in our laboratory. There may be different sources of MMP-9 in peritoneal fluid. Zeng and Guillem found over-production of MMP-9 in liver metastasis macrophages (Zeng and Guillem, 1996). Since tumour associated macrophages, when stimulated appropriately, as in endometriosis (Köninckx et al., 1999; Loh et al., 1999), are involved in the development of metastases of some tumour types, it is reasonable to conclude that endometriotic cells may stimulate the migration of endometriosis-associated macrophages to the endometriotic loci, leading to increased local MMP-9 production which may facilitate the progression of the disease.

The role of steroids present in peritoneal fluid on the progression of endometriosis is still unknown. Sharpe-Timms et al. found in a rat model that gonadotrophin-releasing hormone (GnRH) agonist therapy, known to induce a hypo-oestrogenic state, decreased plasminogen activator and MMP but increased plasminogen activator inhibitor and TIMP-1 activities (Sharpe-Timms et al., 1998a). The authors conclude that GnRH agonist therapy induced a shift to a less invasive phenotype by altering fibrinolysis and extracellular matrix remodelling. They suggest that the hormonal milieu is of utmost importance in regulating the proteolytic imbalance associated with peritoneal adhesion formation and endometriosis. In another study, the same group found that TIMP-1 concentrations were significantly lower in peritoneal fluid and sera of women with endometriosis compared with disease-free women, and furthermore, that GnRH agonist therapy restored TIMP-1 concentrations (Sharpe-Timms et al., 1998b). Diagnostic laparoscopy during the first half of the menstrual cycle is routinely carried out in our clinic during infertility investigations. This is why the peritoneal fluid from patients with endometriosis was collected from women at follicular phase of the cycle, the time when oestrogens are considered to be dominant in terms of hormonal activity. Our study, however, fails to show differences in estradiol and progesterone concentrations in both groups of patients. This is in contrast to clear imbalance in total MMP-9 and TIMP-1 concentrations. It seems feasible to conclude that neither estradiol nor progesterone concentrations in peritoneal fluids have any significant impact on MMP-9 expression, at least in the proliferative phase of the cycle, when the peritoneal fluids were obtained. It would therefore be interesting to examine the difference in the concentrations of MMP-9 in peritoneal fluid of the luteal phase.

It is very difficult to prove that our control group was a suitable one, since it has been shown that 6% of cases, where macroscopically normal pelvic anatomy was found, presented microscopic endometriotic lesions (Nisolle et al., 1990). Nevertheless, in our country it is a difficult task to collect patients who undergo diagnostic laparoscopic procedure for other reasons than infertility and/or pelvic pain. As a matter of fact, high levels of total MMP-9 and low TIMP-1 were found in two cases (9.5%) where no visible lesions on the surface of peritoneum were found. To see if endometriosis develops in those individuals in the following period would be of practical use. Performing the evaluation of total MMP-9 in peritoneal fluid, in women diagnosed because of infertility and/or pelvic pain, could possibly provide one of the tools to predict the incidence of endometriosis in cases in which the lesions are not visible.

In conclusion, our study demonstrates the presence of statistically higher total, but not active, MMP-9 levels in endometriotic peritoneal fluids than in controls. Higher total MMP-9 and lower TIMP-1 concentrations do not correlate with steroid hormones levels, which are similar in both groups and it is unlikely that their concentration in peritoneal fluid may be of any significant importance in modulating the MMP-9 function. These results, however, support the notion that MMP-9 and TIMP-1 may both play a role in the pathogenesis of endometriosis.

Acknowledgements

We are grateful to Mrs Malgorzata Kęptowska for her professional help in statistical analysis, Professor Mats Akerlund and Britt-Marie Agnell from Department of Obstetrics and Gynecology, University Hospital, Lund, Sweden for critical reading of the manuscript. This work was supported by KBN grant No. 1–0578.

References


Submitted on November 10, 2000; resubmitted on August 14, 2001; accepted on October 18, 2001