Extracellular matrix remodelling in the endometrium and its possible relevance to the pathogenesis of endometriosis

M.Sillem¹,³, S.Prifti², M.Neher² and B.Runnebaum²

¹Stiftung Deutsche Klinik für Diagnostik GmbH, Wiesbaden and ²Department of Obstetrics and Gynaecology, University of Heidelberg, Germany

TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Introduction</td>
<td>730</td>
</tr>
<tr>
<td>Protease secretion in cell culture</td>
<td>731</td>
</tr>
<tr>
<td>Proteases in patients with and without endometriosis</td>
<td>731</td>
</tr>
<tr>
<td>Role of endometrial proteases and endometriosis</td>
<td>732</td>
</tr>
<tr>
<td>Acknowledgements</td>
<td>734</td>
</tr>
<tr>
<td>References</td>
<td>734</td>
</tr>
</tbody>
</table>

Essential features of endometrial physiology involve the extracellular matrix (ECM). In the pathogenesis of endometriosis, interactions of endometriosis cells with ECM can be postulated. Two systems of secreted proteases in the endometrium, the plasmin(ogen) activator/inhibitor and the matrix metalloproteinases and their inhibitors were examined in cell cultures of uterine endometrial cells from women with and without endometriosis. Soluble urokinase receptor secretion is increased, and mRNA transcription of tissue inhibitor of metalloproteinases-2 (TIMP-2) is upregulated by progestin in endometriosis. These findings are compatible with an altered ECM turnover in the endometrium of these patients that may explain a higher invasive potential of retrogradely menstruated endometrial fragments.

Key words: endometrium/endometriosis/extracellular matrix/pathogenesis/secreted proteases

Introduction

Remodelling of the extracellular matrix (ECM) is an essential process for physiological endometrial functions like secretory transformation, implantation, decidualization and menstruation. According to the theory of retrograde menstruation (Sampson, 1925), which plausibly explains most pathophysiological and clinical aspects in endometriosis, uterine endometrium is the tissue of origin for endometriosis foci. With laparoscopies being increasingly performed, however, it has become evident that retrograde menstruation must be regarded as a normal phenomenon (Halme et al., 1984), and Blumenkrantz et al. (1981) observed blood in peritoneal dialysates of women with patent tubes at the time of menstruation. Even during the remainder of the menstrual cycle, some reflux of endometrial cells into the peritoneal cavity seems to be physiological, as reported by Bartosik et al. (1986). However, these authors found increased amounts of endometrial cells in the peritoneal fluid of endometriosis patients after uterine irrigation. Leyendecker and co-workers (1996) recently demonstrated uterine hyperperistalsis and dysperistalsis to be present in patients with endometriosis, and suggested this phenomenon as a causal factor for both reduced fertility (via an impaired sperm transport) and the development of endometriosis.

Menstruation is initiated by oestrogen and progesterone withdrawal and mediated at the molecular level, mainly by changes in the expression of secreted proteases. Briefly, there are two groups, one being the plasmin(ogen) activator/inhibitor system (also known as serine proteases), and the other being matrix metalloproteinases (which can be activated by plasmin) and their tissue inhibitors (TIMPs; Tabibzadeh, 1996). The hormonal regulation and cell specificity of TIMPs in human endometrium has recently been described in detail (Zhang and Salamonsen, 1997). An endometrial function which is even more important than menstruation, i.e. the implantation of the blastocyst, depends

³To whom correspondence should be addressed at: Fachbereich Gynäkologie, Stiftung Deutsche Klinik für Diagnostik GmbH, Wiesbaden, Germany. Tel: +49-611-577-206; Fax: +49-611-577-580; e-mail: gyn@dkd-weisbaden.de
equally on a regulated matrix metalloproteinase expression (Bjorn et al., 1997). Lastly, all proteases mentioned are involved in the pericellular proteolytic processes associated with tumour cell invasion and metastasis (Mignatti and Rifkin, 1993).

In the pathogenesis of endometriosis, retrograde menstruation may be regarded as a necessary, but not sufficient, prerequisite. Therefore, enhanced adhesion and/or invasion of retrogradely shed endometrial cells or tissue fragments can be postulated, and secreted proteases would be likely mediators for these mechanisms. In fact, an increased concentration of procollagen type III fragments has been observed in the peritoneal fluid of women with early endometriosis (Spuijbroek et al., 1991).

In order to investigate whether or not changes in the integrity of the ECM influence the development of endometriosis, we have previously used an animal model of endometriosis. We were able to demonstrate that the development of severe endometriosis is impaired when the integrity of the endometrial fragments is destroyed enzymatically, or when the proteolytic potential is inhibited pharmacologically (Sillems et al., 1996). Thus, the physical (possibly the mere size) and biochemical properties of endometrial fragments may determine the rate at which endometriosis develops.

The studies presented here were performed in order to identify mechanisms in the ECM turnover in the uterine endometrium of women with and without endometriosis which could affect the adhesive and/or invasive capacity of endometrial fragments that reach the peritoneal cavity retrogradely through the Fallopian tubes. For this purpose, the secretion of components of the plasminogen activator/inhibitor system and of some matrix metalloproteinases (MMP) and their inhibitors by endometrial cells in vitro was investigated.

Protease secretion in cell culture

Endometrial biopsies were obtained from previously untreated regularly cycling women at laparoscopy or laparotomy, at which the presence or absence of endometriosis was noted. Patients with suspected endometrial pathology were not included. All subjects gave their informed consent and the study was approved by the Ethical Committee of the University of Heidelberg Faculty of Medicine.

All media and supplements were from Sigma Chemical Co. (Deisenhofen, Germany). Glutamax was obtained from Carl Roth GmbH (Karlsruhe, Germany) and fetal bovine serum (FBS) from CCPro (Neustadt, Germany). Diethylstilboestrol (DES) was purchased from Sigma, and promegestone (R 5020) from NEN Life Science Products (Cologne, Germany). For urokinase plasminogen activator (uPA), plasminogen activator inhibitor type-1 and type-2 (PAI-1 and -2) and soluble uPA receptor (suPA-R) enzyme-linked immunosorbent assays (ELISAs), commercially available kits were used (American Diagnostica, Greenwich, CT, USA). mRNA extraction was performed using the MicroFast Track kit (Invitrogen, San Diego, CA, USA) and for the reverse transcriptase–polymerase chain reaction (RT–PCR), the Gene Amp RNA PCR kit (Perkin-Elmer/Roche Molecular Systems, Branchburg, NJ, USA) was used.

The tissue was digested enzymatically, and cells were seeded out at a standard density. After confluence, the cells were switched to a serum-free, phenol red-free medium and treatment with DES (10^{-10} M) and R 5020 (10^{-8} M and 10^{-6} M) was initiated. A negative control containing ethanol at 0.1% v/v was included in all cultures. However, these controls were never significantly different from treatment with DES only. uPA, PAI-1 and -2 and suPA-R concentrations were assayed in the conditioned media according to the manufacturer’s instructions.

To demonstrate the activity of the MMP system in the conditioned media of our cultures, we performed a photometric assay using Azocoll as a chromogenic synthetic substrate for these proteases (Chavira et al., 1984).

In order to evaluate the regulation of the system on the mRNA level, an RT–PCR was performed on the cells harvested on day 6. Specific primer pairs were used for stromelysin-1 (MMP 3), matrilysin (MMP 7), and TIMP-1 and -2. The housekeeping gene β actin was used to permit semiquantitative comparison of transcription rates.

Statistical tests were performed using non-parametric comparisons for small sample sizes. The level of significance was set at \( P < 0.05 \).

Proteases in patients with and without endometriosis

uPA and PAI-2 levels were not influenced by steroid treatment and did not differ between women with and without endometriosis, whereas PAI-1 was significantly \( (P < 0.001) \) upregulated by R 5020 in both groups (Figure 1). In contrast, suPA-R expression was not influenced by steroid treatment but was significantly \( (P < 0.05) \) higher in cells from endometriosis patients at all time points (Figure 2).

In the chromogenic assay with Azocoll, an activity in the order of magnitude of 100 ng trypsin/ml was found in all media and the activation by p-aminophenyl mercuric acetate (APMA) and the inhibition by 1,10-phenanthroline...
was typical for MMP. However, with this method we were unable to detect differences between samples from patients with and without endometriosis, or any effect of progestin treatment.

In the RT–PCR experiments, except for TIMP-2, no differences were observed between cells from women with or without endometriosis and for cells treated with oestrogen (DES) alone or with a combination of oestrogen and progestin (R 5020). For TIMP-2, however, a considerable difference in the transcription rate after addition of progestin was seen in cells from endometriosis patients (Figure 3, lanes E25–E32) compared with samples from women without endometriosis (Figure 3, lanes K25–K32).

**Role of endometrial proteases and endometriosis**

For our studies, we have used an experimental model that permits the examination of ECM turnover under controlled conditions. Two interrelated families of secreted proteases were investigated.

Plasminogen, a ubiquitous protein secreted by the liver, is activated to the highly potent protease plasmin by two types of activators, namely, tissue-type plasminogen activator (tPA) and urokinase-type plasminogen activator (uPA), which binds to a cell surface receptor (uPA-R). While the activation by tPA is important for fibrinolysis, uPA triggers localized pericellular proteolysis of the extracellular matrix. The system is tightly controlled by potent inhibitors, at the level of plasminogen activators by PAI-1 and -2, and at the level of plasmin by alpha 2 macroglobulin and alpha 2 antiplasmin (Littlefield, 1991).

As can be expected from the functions mentioned before in the endometrium, a regulation by steroids and local paracrine factors has been reported. Mainly, the activity is decreased under progestin influence. Complexing of uPA to PAI-1 and binding to uPA-R both seem to facilitate clearance of active plasmin (Schatz and Lockwood, 1993). On the other hand, uPA binding to its surface receptor greatly increases its activity (Casslén et al., 1995). Recently, a soluble form of uPA-R has been described which is encoded by the same gene as the surface receptor. The binding domain for uPA is similar, which suggests a retained binding activity (Casey et al., 1994).

Apart from physiological functions, the plasmin(ogen) activator/inhibitor system seems to be involved in the pericellular proteolytic processes occurring during tumour cell migration and metastasis (Mignatti and Rifkin, 1993). In endometriosis, plasminogen and single-chain urokinase have been detected immunohistochemically in higher

---

**Figure 1.** Plasminogen activator inhibitor type-1 (PAI-1) concentrations (ng/ml) in endometrial cell culture supernatants from women without (□) and with (■) endometriosis, secreted by $2.5 \times 10^5$ cells. Squares indicate median values; upper and lower horizontal bars indicate maximum and minimum values, respectively. Cultivated cells were treated with steroids for 2, 4 or 6 days. PAI-1 concentrations were significantly higher after R 5020 treatment ($P < 0.001$).
concentrations in endometriosis biopsies compared with corresponding eutopic endometrium.

Our results for uPA, PAI-1 and PAI-2 confirm previous studies of physiological uterine endometrium, but the finding that these parameters are unaltered in endometriosis is novel. Furthermore, the secretion of soluble uPA receptor by endometrial cells has not been reported previously. This substance can be detected at low concentrations in plasma from healthy volunteers and at high concentrations in plasma from patients with septicaemia or in pleural effusions and ascites from patients with inflammatory or malignant conditions (Mizukami et al., 1995). In-vitro, invasiveness of glioblastoma cells mediated by uPA-R can be inhibited by a specific monoclonal antibody (Mohanam et al., 1993). Recent findings indicate that binding of single-chain uPA to suPA-R retards its inhibition by PAI-1 and also its inactivation and clearance (Al Roof Higazi et al., 1996). Lastly, the integrin-mediated adhesion of cells to vitronectin, an ECM component, was found to be inhibited by suPA-R (Wei et al., 1996). With respect to our findings, this would indicate that an increased concentration of suPA-R, resulting in a higher local proteolysis, may have two consequences: (i) reduced anchoring of endometrial cells and fragments at their site of origin; and (ii) an increased invasiveness at the site of peritoneal attachment. In particular, the first mechanism would fit in well with the observations regarding retrograde seeding by Bartosik et al. (1986) and uterine dysperistalsis by Leyendecker et al. (1996), as discussed in the Introduction.

A second set of secreted proteases are the MMP and their inhibitors (TIMP). MMP are physiologically activated by plasmin; additionally, synthetic activators and inhibitors have been described (Woessner, 1991; Okhuysen et al., 1996). The role of MMP in the female reproductive system, in particular during implantation and menstruation, and their regulation by steroids has recently been reviewed extensively. In general, progestins seem to decrease the activity of these proteases (Hulboy et al., 1997). As with the plasmin system, tumour cell migration and metastasis are associated with activation of this system (Mignatti and Rifkin, 1993). In addition to, and independent of, its inhibitory function in the MMP system, TIMP-2 has been identified as a growth factor. This was observed in normal keratinocytes (Bertaux et al., 1991) as well as in SV40-transformed fibroblasts (Nemeth and Goolsby, 1993; Hayakawa et al., 1994).

To our knowledge, the MMP system has not been examined extensively for its possible role in the pathogenesis of endometriosis. One report (Saito et al., 1995) indicates that MMP3 and TIMP-1 are present in

Figure 2. Soluble urokinase receptor (suPA-R) concentrations (ng/ml) in endometrial cell culture supernatants from women without (□) and with (■) endometriosis, secreted by 2.5×10^5 cells. Squares indicate median values; upper and lower horizontal bars indicate maximum and minimum values, respectively. Cultivated cells were treated with steroids for 2, 4 or 6 days. The suPA-R concentration was significantly higher in the group with endometriosis (P < 0.05).
endometriosis foci and that TIMP-1 is upregulated under danazol, whereas MMP-3 is downregulated. With our substrate-based test we were unable to demonstrate a difference in MMP activity between samples from women with endometriosis and those without, though this may be due to technical shortcomings of the assay system. With regard to mRNA transcription rates, we postulate that an altered regulation of TIMP-2 may lead to the generation of more invasive endometrial fragments and that their ectopic growth may be enhanced in endometriosis. However, more detailed investigations on the protein level are necessary and are presently under way.

In conclusion, alterations in two related systems of secreted proteases were identified that may contribute to an increased availability, adherence and invasiveness of retrogradely menstruated endometrial fragments. Thus, if uterine endometrium is in fact the tissue of origin in endometriosis, these findings corroborate and extend Sampson’s theory of retrograde menstruation.

Acknowledgements

Dr L. Pitzel, University of Goettingen, Germany, kindly provided the primers and specifications for TIMP-2. The authors acknowledge the experimental work of doctorate students Petra Buvari and Usama Shamia. The expert technical assistance of Ms Julia Jauckus is gratefully acknowledged.

References


