Endometrial cathepsin D immunostaining throughout ovulatory and anovulatory menstrual cycles

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The results of histological examination of the endometrium are normal in most patients with unexplained sterility. Cathepsin D is a ubiquitous lysosomal protease regulated by progesterone in the endometrium. Assays of concentrations of cathepsin D might be useful in determining the functional responses of the endometrium to progesterone. To examine this possibility, we quantified immunostaining of endometrial cathepsin D using an image analysis system in women with regular menstrual cycles. An endometrial sample was obtained during the proliferative and luteal phases from 17 women with ovulatory menstrual cycles and at the beginning and during the last 14 days of a cycle from 15 women having anovulatory menstrual cycles. In endometrial glands of ovulatory women, cathepsin D protein immunostaining increased during the cycle and was significantly higher during the luteal than during the proliferative phase [51 ± 38.1 arbitrary units (AU) versus 118.2 ± 58.9 AU; P < 0.01]. This increase was also observed in stromal cells, although to a lesser extent (28.6 ± 26.9 versus 41.5 ± 43.1 AU; P = NS). In the endometrium of women with anovulatory menstrual cycles, cathepsin D staining was high both for the proliferative and the luteal biopsies in glands (respectively 95 ± 43 and 104 ± 51.3 AU) and stromal cells (respectively 61.8 ± 33.8 and 75 ± 32.6 AU). In women with ovulatory cycles, cathepsin D staining was localized in the apical part of glandular cells during the proliferative phase and diffused throughout the cytoplasm during the luteal phase. In contrast, in women with anovulatory cycles, cellular localization of cathepsin D remained apical in glands, regardless of the day of biopsy. In conclusion, this study shows that the cytoplasmic localization of cathepsin D might be a qualitative biological indicator of endometrial gland responses to progesterone. This could be a useful tool for evaluating cell function, which is poorly tested by histology alone.

Key words: cathepsin D/cell localization/endometrium/immuno-staining/ovulatory cycle

Introduction

Implantation of the blastocyst depends in part on the quality of the secretory endometrium. Histological analysis is currently considered to be the best method of determining endometrial quality. While many of the disadvantages of traditional histological evaluation are rectified by histometric analyses (Li et al., 1988), some forms of infertility and/or repeated in-vitro fertilization (IVF) failures remain unexplained. Biological markers might thus be more sensitive and also more informative about the aetiology of the sterility. The presence of progesterone is important for development of human endometrial receptivity and assays of progesterone-induced proteins could reflect on the functional state of the endometrium. Cathepsin D is a ubiquitous lysosomal aspartyl protease induced by progesterone in the endometrium (Moulton et al., 1983; Touitou et al., 1989). In a preliminary study (Maudelonde et al., 1990), we have shown that endometrial cathepsin D is higher during the luteal phase and that this increase is mainly explained by a higher concentration in glandular cells. Since cathepsin D is auto-activated (Rochefort, 1992), it might induce the proteolytic cascade necessary for trophoblast invasion. The aim of the present study was to assay, in situ, endometrial cathepsin D immunoreactivity throughout the menstrual cycle and to assess its potential value as a marker of endometrial quality in a population of women with anovulatory menstrual cycles.

Materials and methods

Population

A total of 32 women consulting the Department of Gynaecology and Obstetrics of the hospitals of Montpellier and Amiens (France) for primary or secondary infertility were included in this study. Of these women, 17 had normal menstrual cycles and were multiparous, and 15 had anovulatory menstrual cycles, as determined by a biological study of ovarian function prior to inclusion in this project. Anovulatory patients with amenorrhoea were excluded. Informed consent was obtained from the patients and, in both Amiens and Montpellier, the project received the approval of the Committee of protection for people agreeing to participate in biological research. Biopsy samples were obtained by aspiration from the fundus of the endometrium until the region of the isthmus with an helicoidal movement using a pipelle endometrial suction curette (Hill et al., 1989). The procedure was identical for every patient. All the sampling was performed by the same person. Each woman had two separate biopsies taken during one menstrual cycle, the first during the proliferative phase or between day 6 and day 12 for the anovulatory group, and the second 14 days later, during the luteal phase, or between day 19 and day 26. Part of the biopsy was immediately frozen in liquid nitrogen for cathepsin D immunostaining. The remaining part was fixed in 3.7% formaldehyde in 0.01 mol/l phosphate-buffered saline (PBS) to date the tissue according to the histological criteria of Noyes et al. (1950). Seven out of the 17 women with ovulatory cycles were excluded because one or two of the endometrial samples were too small to be analysed or contained fewer than six glandular structures to analyse. Six of
Table I. Clinical data for women with ovulatory cycles

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>Age (years)</th>
<th>Menstrual cycle</th>
<th>Progesterone (ng/ml)</th>
<th>Oestradiol (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Length (days)</td>
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<td></td>
<td></td>
<td></td>
<td>Histo</td>
<td>LH peak</td>
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<td>+3</td>
<td>+3</td>
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<td>+12</td>
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<tr>
<td>10</td>
<td>38</td>
<td>31</td>
<td>+12</td>
<td>+12</td>
</tr>
</tbody>
</table>

*The day in the menstrual cycle was expressed as a function of that on which the LH urinary peak was exhibited (day 0). Histo = histological evaluation according to the criteria of Noyes et al. (1950).

Table II. Clinical data for the women with anovulatory cycles

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>Age (years)</th>
<th>Menstrual cycle</th>
<th>Progesterone (ng/ml)</th>
<th>Oestradiol (pg/ml)</th>
</tr>
</thead>
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<tr>
<td></td>
<td></td>
<td>Length (days)</td>
<td>Day of biopsy*</td>
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</tr>
<tr>
<td></td>
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<td>26 (late)</td>
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<td>19</td>
<td>35</td>
<td>32</td>
<td>9 (middle)</td>
<td>23 (late)</td>
</tr>
</tbody>
</table>

*The length of the studied menstrual cycle was determined according to the first day of the next cycle.
Histological evaluations of the theoretical period in the proliferative phase are given in parentheses.

The 15 women with anovulatory menstrual cycles were excluded for similar reasons. The day in the menstrual cycle of ovulatory women was expressed as a function of the urinary luteinizing hormone (LH) concentration, with the peak being day 0. In anovulatory cycles, the proliferative phase was categorized as early, middle, and late. The length of the studied menstrual cycle was determined according to the first day of the next cycle.

LH and oestradiol were analysed using enzyme-linked fluorescent assays (Vidas; Biomerieux, Lyon, France). The sensitivities of the assays were <10 pg/ml for oestradiol and 1 mU/ml for LH. Progesterone concentrations were determined with an immunoenzyme assay (Biomerieux). The sensitivity was 0.20 ng/ml.

Immunohistochemical staining of cathepsin D

Frozen sections, 5 μm thick, of endometrial tissues were prepared on a cryocut (SLEE, London, UK). Two serial sections were collected on a gelatin-coated glass slide for specific and negative control staining using the same class of antibodies. They were immediately fixed sequentially for 10 min in 3.7% paraformaldehyde in 0.01 mol/l PBS, pH 7.3, 4 min in methanol at −20°C and 2 min in acetone at −20°C, then washed for 10 min in PBS and stored at −80°C until use.

Staining was performed using the M1G8 monoclonal antibody as described by Maudelonde et al. (1990) with slight modifications. Tissue sections were incubated for 60 min at room temperature with 100 μl of a 1 μg/ml M1G8 solution. The monoclonal antibody was then revealed with biotinylated antimouse antibody and avidin-peroxidase (ABC kit; Vectastain, Burlingame, CA, USA) using 3,3′-diaminobenzidine tetrahydrochloride as substrate. Slides were then counterstained with Harris haematoxylin, dehydrated and mounted.

The specificity of M1G8 staining was determined using a mouse monoclonal antibody of the same subclass (IgG1, MOPC 21 from Letton Bionetics Inc., Kensington, MA, USA). In each experiment, MCF7 cells were used as a positive cathepsin D control. They were cultured in Dulbecco's modified Eagle's medium with 10% fetal calf serum at 80% confluence. They were then cytopspun and stored at −20°C in 250 mmol/l sucrose glycerol, PBS (vol/sol) after fixation in 3.7% formaldehyde according to the biopsy procedure.

Quantification of cathepsin D staining by image analysis

The staining was quantified with a computerized image analyser (SAMBA 2005 TITIN; Alcatel, Grenoble, France). This system included a KY-15, 3CCD video camera (JVC, Tokyo, Japan) mounted on a BH-2 light microscope (Olympus Optical Co, London, UK) and was connected to a microcomputer. Colour image processing utilized acquisition through the red, green and blue channels (Charpin et al., 1988). Mean optical density and the percentage of stained area were quantified on a digitalized image. Background was removed automatically by deleting a digital image of the slide beside the section. The density of cathepsin D immunostaining was evaluated at high sensitivity through the blue channel. Cell surfaces manifested by haematoxylin counterstain were measured through the red channel. We separately estimated the staining intensities of glandular and stromal cells in one section. Staining intensity was calculated from integrated optical densities of the entire section at X40 final magnification. This quantification technique has been validated previously (Maudelonde et al., 1992). Cytospun MCF7 cells were used as a positive stained control, but no correction was applied since their staining was constant (SD between experiments was 10%). Staining intensity of the negative control was subtracted from the intensity obtained with the specific antibody. Results were expressed as a quantitative immunohistochemical (QIC) score = percentage of surface stained in epithelial cells×mean staining intensity×10 (Bacus et al., 1988).

Statistical methods

Statistical analyses were performed using the STAT-ITCF software package (Institut Technique des Céréales et des Fourrages, Paris, Paris 1988).
Characteristics of the population

The clinical parameters of the ovulatory and anovulatory women were similar, but values of biological parameters varied between the two groups. The ages of women with ovulatory cycles (33.2 ± 2.8 years; mean ± SD) (Table I) were not significantly different from those with anovulatory cycles (31.8 ± 3.5 years) (Table II). The menstrual cycle was longer (mean ± SD) in women with anovulatory cycles (32 ± 2 versus 29 ± 1 days; \(P < 0.01\)). In the group with normal menstrual cycles, determination of the menstrual cycle day according to the LH peak confirmed the histological evaluation, except for two samples (Table I). One of these was evaluated as day 13 postovulation, but the LH peak had occurred 10 days before and the other one was histologically evaluated at day 10 postovulation instead of day 9. The mean ± SD plasma progesterone assayed between the 3rd and the 12th day after the LH peak was 10.2 ± 4.4 ng/ml, and the mean ± SD plasma oestradiol was 140.2 ± 47.4 pg/ml (Table I). The anovulatory group had their biopsies between the 5th and the 13th day and between the 19th and the 26th day after the previous menstruation. All patients of this group had a plasma progesterone concentration <0.4 ng/ml (Table II).

Cathepsin D distribution in the endometrium

Regardless of the status of oestrogen and progesterone expression, cathepsin D was always expressed in all cells. Cathepsin D staining was cytoplasmic, granular, brown and present in both stromal cells and glandular cells (Figure 1). In women with ovulatory cycles, cathepsin D immunostaining in glandular cells was mainly apical during the proliferative phase (Figure 1A) and spread throughout the cytoplasm during the luteal phase (Figure 1B and D). Conversely, distribution of cathepsin D remained mainly apical regardless of the day of the menstrual cycle in glandular cells from women with anovulatory cycles (Figure 1C). In stromal cells, cathepsin D distribution was homogeneous regardless of the stage of the menstrual cycle in both groups of women.

Cathepsin D expression levels in the endometrium

Quantification using the image analysis system showed that cathepsin D QIC scores were highly variable in both glandular and stromal cells. They ranged from 0.6 to 213 AU in the population studied. However, in glandular cells, QIC scores for the two groups of women reached higher levels than in stromal cells (91.6 ± 8.6 versus 50.8 ± 6.1 AU). In the group of women with ovulatory cycles, there was a tendency to a positive correlation between cathepsin D staining and progesterone and oestradiol concentrations in glandular \(r = 0.59\) (\(P = 0.07\)) and 0.55 (\(P = 0.09\)) respectively] but not in stromal cells \(r = 0.16\) (\(P = 0.66\)) and 0.10 (\(P = 0.78\)) respectively. As shown in Figure 2, cathepsin D concentrations...
were significantly increased in glandular cells of women with ovulatory cycles during the luteal phase (118.2 ± 58.9 AU) as compared to the proliferative phase (51 ± 38.1 AU). Concentrations in stromal cells of these women were not significantly different between the proliferative phase (28.6 ± 26.9 AU) and the luteal phase (41.5 ± 43.1 AU). In contrast, in women with anovulatory cycles, fluctuations of cathepsin D were not seen in glandular cells between the two biopsy periods (Figure 2). The mean staining level in glandular cells, at the first biopsy, was significantly higher (P = 0.05) in the group of women with anovulatory cycles (95 ± 43 AU) than in the ovulatory group and remained high at the second biopsy (104 ± 51.3 AU). Indeed, mean values at the second biopsy in both groups of women were similar and resembled those obtained for the first biopsy in women with anovulatory cycles, suggesting an absence of protease down-regulation in these women. Staining in stromal cells was not significantly different according to the period of biopsy in either group of women. However, mean staining levels in stromal cells at the first and second biopsies were higher in women with anovulatory cycles (respectively 61.8 ± 33.8 and 75 ± 32.6 AU) than in women with ovulatory cycles (P = 0.05).

We then investigated statistically the correlation of cathepsin D concentrations with the day of biopsy during the menstrual cycle. In glandular cells of women with ovulatory cycles, we found a positive correlation, with increasing amounts of cathepsin D from day -15 to day +14 of the cycle (r = 0.71, P < 0.001; Figure 3). No correlation was found in glandular cells from women with anovulatory cycles or in stromal cells from any group.

Discussion

We had previously found that cathepsin D synthesis is regulated by progesterone in human endometrium and that cathepsin D immunostaining is higher in glandular cells during the luteal phase (Maudelonde et al., 1990). The present study confirmed these preliminary results and showed a progressive increase in cathepsin D QIC scores in endometrial glands monitored during the follicular phase and early luteal phase. This is consistent with the increase in progesterone receptors found in glandular cells (Lessey et al., 1988). However, the persistent increase in cathepsin D staining in glands during the mid- and late luteal phase contrasts with the decreased progesterone receptor activity in glandular cells described after the 22nd day of the menstrual cycle (Nisolle et al., 1994; Salat-Baroux et al., 1994). In stroma, on the contrary, both the cathepsin D QIC score in most patients and progesterone receptor concentration (Lessey et al., 1988) remained constant throughout the menstrual cycle. If cathepsin D is mainly synthesized under progesterone control, then a transfer of cathepsin D from stromal cells to the glandular epithelium could be postulated. However, the higher cathepsin D RNA concentration in glandular cells during the secretory phase (data not shown) is not in line with this hypothesis. The large amount of intracellular cathepsin D in endometrial glands following the decrease in progesterone receptor concentration could also be explained by a long half-life of the protein and/or RNA, leading to progressive accumulation during the luteal phase.
In anovulatory cycles, the high QIC score throughout the menstrual cycle suggests cathepsin D synthesis to be under the control of factors other than progesterone. The high levels of cathepsin D immunostaining associated with progestation could be caused by growth factors, since the unopposed oestrogen-induced growth of endometrium is partially mediated by the production of growth factors. For instance, epidermal growth factor is able to reproduce the oestadiol effect connected with the stimulation of mouse female genital tract growth and differentiation in vivo (Nelson et al., 1991). In humans, it stimulates the synthesis of cathepsin D in endometrium (Toitou et al., 1989). Some other growth factors (Giudice et al., 1993) have also been suggested to be involved in endometrial shedding. Another hypothesis is that, in women with anovulatory cycles, glandular cells have lost a factor that normally regulates cathepsin D synthesis during the proliferative phase, since this factor is progressively repressed by progesterone throughout ovulatory cycles.

An important finding of this study is that cellular localization of cathepsin D immunostaining in endometrial glands varied according to the phase of the menstrual cycle. Cathepsin D staining was apical and granular in endometrial glands of the follicular phase, suggesting its secretion, which is consistent with the high levels of protease activity described during the proliferative phase and the mid-cycle in endometrial fluid (Casslen and Astedt, 1981). Surprisingly, this preferential localization disappeared in the luteal phase and cathepsin D staining was dispersed throughout the cytoplasm of glandular cells. This is evidence against a progesterone effect alone on cathepsin D secretion since the cellular localization of the secreted endometrial proteins induced by progesterone (such as PP14) remained apical in glandular cells throughout the luteal phase (Cornillie et al., 1991). In humans, the presence of cathepsin D has not been studied in endometrium at the luteal phase (Comillie et al., 1991). In women with anovulatory cycles, glandular cells have lost a factor that normally regulates cathepsin D synthesis during the proliferative phase, since this factor is progressively repressed by progesterone throughout ovulatory cycles.

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Cyclic variations of endometrial cathepsin D


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