Immunohistochemical detection of cathepsin D in endometrium from long-term subdermal levonorgestrel users and during the normal menstrual cycle

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A previous report has shown that progesterone up-regulates cathepsin D expression in human endometrial cell culture. In women using the levonorgestrel-releasing implant Norplant®, the plasma levonorgestrel and immunoreactive endometrial progesterone receptor concentrations are elevated. However, the functional status of these receptors is not known. This study used endometrial cathepsin D expression both as an indirect marker for the functional status of endometrial progesterone receptors, and to identify the cell types that express cathepsin D. The results show that cathepsin D is primarily found in glandular epithelia and luminal epithelia in control and Norplant® endometria. There is no significant difference in cathepsin D expression between the control and Norplant® endometria, between the various stages of the menstrual cycle, or between Norplant users with varying degrees of breakthrough bleeding. Cathepsin D is also detected in cells scattered in the stroma in both control and Norplant endometria. The majority of these cells are macrophages. These data indicate that there is no evidence for progesterone regulation of cathepsin D in the human endometrium. Cathepsin D thus cannot be used as a marker for the functional status of progesterone receptors found in the Norplant-exposed endometrium.

Key words: cathepsin D/endometrium/human/Norplant®/progesterone

Introduction

One of the side effects associated with the use of the subdermal levonorgestrel-releasing Norplant® contraceptive implants is menstrual disturbance. The pattern of menstrual disturbance varies greatly, including frequent, prolonged and irregular bleeding, and amenorrhoea. The precise mechanisms causing these menstrual disturbances are not known. During the first year of use, release of levonorgestrel from the implants is approximately 0.05 and 0.08 mg per day (Robertson et al., 1983) and the peripheral plasma levonorgestrel levels remain steady at around 1.2 nM (Affandi et al., 1987; Olsson et al., 1987). Despite the peripheral levonorgestrel concentrations being constantly elevated, the immunoreactive progesterone receptor concentrations remain high in the endometrium of Norplant users (Critchley et al., 1993). This contradicts current beliefs that progesterone down-regulates its own receptors. In addition, the functional status of these receptors is not known. A previous report indicated that the lysosomal protease cathepsin D is up-regulated by progesterone in human endometrial cell culture (Maudeleone et al., 1990). The aim of this study is to use the expression of cathepsin D in the endometrium during the menstrual cycle and in Norplant users as an indirect marker for the functional status of endometrial progesterone receptors, and to identify the cell types that express cathepsin D.

Materials and methods

Endometrial biopsies were taken from 45 control Australian women and from 46 Indonesian Norplant users. All human subjects were recruited on the basis of fully informed consent. Ethical approval was obtained from the Medical Faculty of the University of Indonesia Ethics Commission in Research on Humans, and Monash University Standing Committee on Ethics in Research on Humans. The biopsies were collected by using either a Pipelle suction curette (Prodimed 60530, Neuilly-en-Thelle, France) or a microhysteroscope. The control biopsies were dated by an experienced histopathologist and were categorized into nine stages of the normal menstrual cycle (Rogers et al., 1993) as follows: menses (n = 6), early proliferative (n = 6), early-to-mid proliferative (n = 7), mid-proliferative (n = 6), mid-to-late proliferative (n = 4), late proliferative (n = 1), early secretory (n = 5), mid secretory (n = 6) and late secretory (n = 4). The Norplant biopsies were taken from women 3–12 months after Norplant insertion and with varying degrees of breakthrough bleeding ranging from no bleeding up to 79 days bleeding in the 90-day reference period prior to biopsy. Since it was not possible to identify the bleeding and non-bleeding areas by these biopsy techniques, endometrial samples were taken blind from Norplant users. The number of spotting/bleeding days, as defined by the World Health Organization (Rogers et al., 1993), was assessed retrospectively by means of a menstrual diary. The biopsies were routinely fixed in 10% buffered formalin in phosphate buffered saline (PBS) for 4–6 h then rinsed and stored in PBS at 4°C. The control biopsies were embedded in paraffin wax within 1–2 weeks while the Norplant biopsies were shipped to Monash University, Australia at this stage for embedding.

Staining for cathepsin D

Sections of 5 μm thickness were cut, dewaxed and washed in three changes of 100%, 100% and 75% ethanol. The sections were rehydrated and stained for cathepsin D using a commercially available mouse monoclonal antibody (NCL-CDm: Novocastra Laboratories,
Newcastle upon Tyne, UK) against human cathepsin D and a Biotin-Streptavidin/Aminoethyl Carbazole (AEC) system (Zymed Laboratory, San Francisco, USA). Endogenous peroxidase activity was blocked by incubating the sections with 3% H2O2 in 50% methanol. Non-specific binding was overcome by pre-absorbing the sections with 10% normal rabbit serum for 10 min at room temperature. Aliquots of 100 μl of the primary antibody at 1:15 dilution (i.e. 1 μg/ml) were applied to each section. The sections were incubated at 37°C for 60 min. The same volume of a biotinylated rabbit anti-mouse polyclonal antibody (Zymed Laboratory) at 1:100 dilution (6 μg/ml) was then applied and incubated for 15 min at room temperature. The horseradish peroxidase–streptavidin conjugate (Zymed Laboratory) was then applied to the sections at a dilution of 1:400 and incubated at room temperature for 15 min. H2O2 substrate and the AEC chromogen (Zymed Laboratory) mixture were applied to the sections, which were incubated at room temperature for 10 min. At the end of the incubation period the sections were rinsed with distilled water and mounted with an aqueous mount (Clearmount™; Zymed Laboratories). PBS washes were carried out in between reagents. Two negative controls were included during the development of the technique and in initial runs. One of these negative controls substituted the primary antibody with PBS, and the remaining negative control substituted the primary antibody with a non-immune mouse monoclonal antibody (Serotec, Oxford, UK) of the same subclass (IgG2b, 1 μg/ml) as the primary antibody. Since the PBS negative control was proven to be redundant, it was not included in subsequent runs, and only the non-immune mouse IgG2b negative control was used. A positive control using either an oestrogen receptor-positive breast tumour or mid-late proliferative endometrial tissue was included in every run.

**Staining for macrophages**

The immunohistochemical staining for macrophages is similar to that described for immunohistochemical staining for cathepsin D, except that a 0.1% trypsin (Sigma, porcine pancreatic trypsin, 1130 units/ml) digestion of the sections was carried out prior to the incubation with 10% normal rabbit serum. The primary antibody was a mouse monoclonal antibody against human CD68 antigen (clone KP1, Dako, Cambridge, UK). The dilution of the antibody was at 1:50 (7.6 μg/ml). The sections were incubated in primary antibody for 30 min at room temperature. Alkaline phosphatase–streptavidin conjugate (Zymed Laboratory) and bromochloro-indolyl phosphate/nitroblue tetrazolium (BCIP/NBT) substrate-chromogen were used. Endogenous phosphatase activity was blocked by incubating the sections with 5 mM levamisole (Sigma, Castle Hill, Australia) for 10 min at room temperature prior to the application of BCIP/NBT. Washes were carried out as described above. A positive and a negative control were included in every run. The negative control substituted the primary antibody with a non-immune antibody of the same subclass and concentration (IgG1, Serotec).

**Double immunostaining for cathepsin D and macrophages**

Double staining for both cathepsin D and macrophages was a combined procedure of the two individual staining protocols with the cathepsin D staining being carried out before the staining for macrophages. A double staining enhancer (Zymed Laboratory) was applied to the sections at the end of the cathepsin D staining for 30 min at room temperature. Trypsin digestion was performed prior to the normal rabbit serum block in the cathepsin D staining. Three positive controls (one for cathepsin D, one for CD68 and one for combined cathepsin D and CD68) using a mid-late proliferative endometrial tissue were included in every run. A negative control (using both non-immune mouse IgG2b and IgG1 in place of the two primary antibodies) using the same tissue was also included in every run.

**Data analysis**

Staining intensity was scored on a semi-quantitative scale from 0–4, where no staining was given a score of zero, little staining was given a score of 1, moderate staining was given a score of 2, intense staining was given a score of 3 and a very intense staining was given a score of 4. The number of individual cells in the stroma which stained positive for cathepsin D were counted for each section under light microscopy. The sections were photographed using a Sony Colour Video Camera (CCD-IRIS) and a Sony Camera Adaptor and a print out of the sections was obtained through an on-line Sony Videographic Printer (UP-811). The area of the sections was estimated by weighing the print area and comparing it to a print of known area. The number of cathepsin D-positive cells per mm2 was calculated. The data were subject to linear regression analyses and ANOVA. The correlation between cathepsin D staining intensity and the length of time between biopsy and the last spotting/bleeding episode was analysed by linear regression.

**Results**

In the control endometria, cathepsin D was detected in the majority of biopsies throughout the menstrual cycle. Staining was primarily detected in the apex of the glandular epithelia and in both the luminal and basal sides of the luminal epithelia (Figure 1). There was no significant difference in the glandular staining across the nine stages of the menstrual cycle (regression coefficient $r = 0.167, P > 0.05$, Figure 2). Cathepsin D was also detected in individual cells scattered in the stroma of the majority of biopsies (Figure 1). There were significantly more cathepsin D positive cells during the menstrual-follicular phase than the luteal phase of the menstrual cycle (regression coefficient $r = 0.442, P < 0.01$, Figure 3).

Norplant-exposed endometrial biopsies gave similar cathepsin D staining results to the control (Figure 4). There was no correlation between the staining intensity in glands or the number of cathepsin D-positive cells in the stroma and the number of spotting/bleeding days in the 90 days reference period (regression coefficient for glands was $r = 0.026, P >$
Cathepsin D in normal and Norplant endometrium

Figure 2. Regression analysis with 95% confidence limits of immunostaining for cathepsin D in the glands of control endometria, showing no variation across the control menstrual cycle ($r = 0.167, P > 0.05$).

Figure 3. Regression analysis of immunostaining for cathepsin D in individual cells scattered in the stroma of control endometria, showing significantly more positive cells in the menstrual/proliferative stages of the cycle ($r = 0.442, P < 0.01$).

Figure 4. Immunostaining for cathepsin D in a Norplant endometrium. The staining pattern was the same as in the control (Figure 1). Original magnification $\times 400$.

Figure 5. Regression analysis of immunostaining for cathepsin D in the glands of Norplant-exposed endometria and with varying degree of breakthrough bleeding, showing no correlation between cathepsin D staining and breakthrough bleeding ($r = 0.026, P > 0.05$).

Figure 6. Regression analysis of immunostaining for cathepsin D in individual cells scattered in the stroma of Norplant-exposed endometria, showing no correlation between cathepsin D staining and breakthrough bleeding ($r = 0.060, P > 0.05$).

Discussion

We have found in this study that cathepsin D is localized primarily in the glandular and luminal epithelium with relatively little staining in the stromal cells. These results are consistent with previous findings (Maudelonde, 1990). How-

0.05, Figure 5, and for cells $r = 0.060, P > 0.05$, Figure 6). When compared with the secretory phase of the normal menstrual cycle, Norplant-exposed endometria had a significantly higher number of cathepsin D-positive cells ($3.2 \pm 1.1$ cells/mm$^2$ in the secretory endometrium versus $47.9 \pm 10.4$ cells/mm$^2$ in the Norplant endometrium, $P < 0.05$). No difference was observed when the cell density in the proliferative phase ($46.1 \pm 13.9$ cells/mm$^2$) was compared with the Norplant endometria. There was no significant difference in glandular staining between the Norplant and the control groups.

Double staining for both cathepsin D and CD68 showed that the majority of the cathepsin D-positive cells in the stroma of the normal and Norplant-exposed endometria were macrophages. A small number of cells (approximately 5%) in both the normal and Norplant endometria stained positive only for cathepsin D but not CD68. There was no correlation between cathepsin D staining intensity and the length of time between biopsy and the last spotting/bleeding episode ($r = 0.12, P > 0.05$).
ever, no significant difference was observed in the immunohistochemical staining for cathepsin D between the various stages of the normal menstrual cycle. This is at variance with a previous report indicating that cathepsin D concentrations in the endometrial cytosolic extract were higher during the luteal phase than the follicular phase (Maudelonde, 1990). These discrepancies may result from the different techniques and sample sizes used in the two studies. This study also found no difference in cathepsin D staining between Norplant users and the controls, or between Norplant users with varying degrees of break-through bleeding. The lack of an increase in the expression of endometrial cathepsin D during the secretory phase (during which time progesterone is elevated) of the normal menstrual cycle and in Norplant users (in whom levonorgestrel concentrations are constantly elevated) do not provide evidence to support a role for progesterone in the up-regulation of endometrial cathepsin D. Consequently, endometrial cathepsin D cannot be used as a marker for the functional status of endometrial progesterone receptors. However, a note of caution should be made that immunohistochemical studies do not provide information on turnover of a molecular species. It is therefore possible that there may be a larger cathepsin D turnover in progesterone/progestogen-exposed endometria which immunohistochemical staining failed to detect.

It has been shown that cathepsin D is released from macrophages in culture (Levy et al., 1989; Rhodes and Andersen, 1993). To our knowledge, this is the first study in which cathepsin D has been localized in macrophages in the human endometrium using a double immunohistochemical staining technique. The precise role of cathepsin D (both endometrial and macrophage-derived) in endometrial function is not known. A role for lysosomal proteases in endometrial bleeding has been proposed by Henzl et al. (1972). However, conflicting results regarding the changes in lysosomal enzymatic activity during the menstrual cycle have been reported (Rosado et al., 1977; Cornillie et al., 1991). The lack of an increase in cathepsin D levels during premenstrual stages and the lack of correlation between bleeding and cathepsin D levels in the Norplant-exposed endometrium does not suggest a direct role for cathepsin D in endometrial bleeding. There have been a number of reports on the presence of macrophages in the human endometrium during the normal menstrual cycle (Morris et al., 1985; Kamat and Isaacman, 1987; Bonatz et al., 1992). We have consistently detected the presence of macrophages in the endometrium throughout the normal menstrual cycle using an anti-CD68 antibody. These results are in agreement with the previous studies. The role of macrophages in endometrial function is unclear. Based on their ability to release mediators such as interleukin-1 (IL-1) and tumour necrosis factor (TNF-α) and growth factors such as transforming growth factor (TGF-β), platelet-derived growth factor (PDGF), insulin-like growth factor (IGF) and platelet activating factor (PAF), a role for macrophages in endometrial haemostasis has been proposed (Clark and Daya, 1990).

It has been shown that progesterone can down-regulate its own receptors (Nardulli and Katzenellenbogen, 1988) but constant elevation of levonorgestrel resulted in high expression of immunoreactive progesterone receptor in the Norplant-exposed endometrium (Crichtley et al., 1993). Oestrogen is known to have an up-regulatory effect on progesterone receptors (Nardulli et al., 1988). It is unlikely that oestrogen is responsible for the high levels of progesterone receptors in the Norplant endometrium. In Norplant users ovarian activity is largely suppressed during the first year of use (Brache et al., 1985) and there are few oestrogen receptors in the Norplant exposed endometrium during this time (Crichtley et al., 1993). The high activity of immunoreactive progesterone receptors in Norplant-exposed endometrium (Crichtley et al., 1993) thus suggests that either the degradation of the receptors is reduced or there is an increase in progesterone receptor synthesis in these tissues. The turnover of progesterone receptors in the normal and Norplant endometria is not known. We are currently investigating the expression of endometrial progesterone receptor mRNA during the normal menstrual cycle and in Norplant users.

In summary the present study did not find a positive correlation between progesterone levels and endometrial cathepsin D expression. This indicates that endometrial cathepsin D may not be up-regulated by progesterone as suggested previously. Therefore, cathepsin D cannot be used as a marker for the functional status of endometrial progesterone receptors. The functional status of the progesterone receptor detected by immunohistochemistry in the Norplant endometrium remains unclear.

References


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