Endometrial lysosomal enzyme activity in normal cycling endometrium

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The objective of this study was to evaluate the possible role of four lysosomal enzymes in endometrial function and remodelling during the normal menstrual cycle by fluorimetric measurement (acid phosphatase, N-acetyl-β-D-glucosaminidase, α-L-fucosidase and α-D-mannosidase). A prospective study was conducted of 45 endometrial biopsies obtained from women with normal menstrual cycles. Activity of all four enzymes was identified in human endometrium. Activity of acid phosphatase and N-acetyl-β-D-glucosaminidase was relatively high, whilst that of α-L-fucosidase and α-D-mannosidase was low. There was no significant change in the activity of any of the four enzymes from the proliferative to the secretory phase of the cycle. This study suggests that the activity of these enzymes remains constant throughout a major portion of the normal cycle.

Key words: acid phosphatase/dysfunctional uterine bleeding/lysosomal enzymes/ menorrhagia

Introduction

Lysosomes are membrane-bound intracellular vacuoles containing hydrolytic enzymes (De Duve and Waitiaux, 1966). They are known to be present in the endometrium throughout the menstrual cycle. Endometrial lysosomes and their enzymes are capable of cellular digestion, tissue destruction and remodelling. The autophagic and heterophagic functions of lysosomes are responsible for the digestion and removal of excess intracellular and extracellular material. These functions are important in the regular ‘housekeeping’ of endometrial tissue (De Duve and Waitiaux, 1966; Dean, 1977). In addition, lysosomes can release their contents which contain a variety of hydrolytic enzymes which are important in glycoprotein metabolism and capable of digesting the intra and extracellular matrix. The release of enzymes may be triggered by hormonal withdrawal at the end of a menstrual cycle (Henzl et al., 1972; Wang and Fraser, 1989). Enzyme release may be complete or it may be a slow leak due to changes in lysosomal membrane permeability influenced by alterations in steroid hormone concentrations (Szego, 1974). Although the processes of bleeding and tissue shedding are under a multitude of complex control mechanisms, it has been suggested that a progressive accumulation of lysosomes and their enzymes in endometria in the secretory phase are important mechanisms contributing to later tissue shedding and menstrual bleeding (Henzl et al., 1972; Rosado et al., 1977; Tabibzadeh, 1996).

In this study, a precise, reproducible, but complex fluorimetric technique was used to examine endometrial lysosomal activity of four selected lysosomal enzymes, i.e. acid phosphatase, N-acetyl-β-D-glucosaminidase, α-L-fucosidase and α-D-mannosidase at different stages of the menstrual cycle. Contrary to traditional studies where enzyme activities were expressed in terms of wet tissue weight or protein content, the authors have evaluated enzyme activities on a cellular basis by expressing activities in terms of DNA content. The aim was to relate biochemical assessment of enzyme activity to the role of lysosomes and their enzymes in normal endometrium as they change through the proliferative and secretory phases (but not the perimenstrual phase) for later comparison with subjects with disturbances of endometrial function.

Materials and methods

Clinical recruitment

Subjects were recruited during admission to hospital if they were aged 20–45 years with a record of entirely normal, regular menstrual cycles (24–35 days), not on recent hormonal treatment, attending the operating theatre for laparoscopic sterilization or treatment of low grade cervical intraepithelial neoplasia. A careful medical history was taken and women with gynaecological disease or any condition which might influence endometrial or lysosomal function were excluded. Day of menstrual cycle was accurately documented with prospective recording of the last menstrual period and menstrual cycle history, by one investigator (IYW). The study was approved by the Ethics Review Committee of the Central Sydney Area Health Service.

In all cases, informed consent was obtained. The first strip of the endometrium was collected at dilatation and curettage with care taken to avoid contamination with vaginal fluid. The tissue was passed immediately to a research assistant for processing. A portion of the endometrium was sent for precise blinded histological dating, using the criteria of Noyes et al. (1950), but with additional precision,
taking into account the more recent morphometric observations of Li et al. (1988) and Johannisson (1990). All dating was carried out by the same experienced gynaecological specialist pathologist.

**Homogenization**
Within 5 min of collection, the sample of endometrium was divided into different portions using a sharp scalpel blade. These portions were labelled and stored in liquid nitrogen for transport. The tissue was weighed and 1 ml of EDTA/bicarbonate buffer (pH 7.6) was added to the tissue. The tissue was sonicated following homogenization with a Braun homogenizer with a Teflon grinder. The homogenate was stored in aliquots at −76°C until assayed within 3 months of collection. Lysosomal enzymes and DNA content were measured by fluorimetry.

**Enzyme assays**
The four lysosomal enzymes: acid phosphatase, N-acetyl-β-d-glucosaminidase, α-d-mannosidase and α-l-fucosidase were measured using enzyme-specific substrates containing the fluorophore, 4-methylumbelliferone (4MU). These were: 4MU-phosphate (25 mM; Sigma, St Louis MO, USA) for acid phosphatase, 4-2-acetamido-2-deoxy-β-d-glucopyranoside (3 mM; Boehringer Mannheim, Mannheim, Germany) for N-acetyl-β-d-glucosaminidase, 4MU-α-l-fucopyranoside (1 mM; Sigma) for α-l-fucosidase and 4MU-α-d-mannopyranoside (10 mM; Sigma) for α-d-mannosidase.

The homogenates and substrates were allowed to react at optimal pH using acidic buffers; pH 5.5 for acid phosphatase, pH 4.7 for N-acetyl-β-d-glucosaminidase, pH 5.5 for α-l-fucosidase and pH 4.5 for α-d-mannosidase (Cornillie et al., 1990). After incubation at 37°C for 1 h, the reaction was stopped using glycine buffers (pH 10.6). The amount of 4MU released was proportional to enzyme concentration in the homogenates. A blank was prepared under the same conditions except that no homogenates were added, i.e. a blank was prepared with the same volume of substrate at the required pH. This sample was also incubated, and glycine buffer was added at the end of the incubation period.

4MU standards were prepared by dissolving 5 mg of 4MU (Sigma) in 100 ml glycine buffer (pH 10.5). Working dilutions were then prepared with serial dilutions of glycine buffer. The 4MU released from the test sample was measured against a 4MU standard curve containing 4MU solutions in known concentrations of 0.02–0.5 µM/g.

The experiments were carried out using both standards and blanks. The amount of 4MU released was measured by fluorimetry using a Hitachi F-3000 fluorimeter (Hitachi Ltd, Tokyo, Japan) under the same wavelengths (excitation 360 nm and emission 452 nm) and bandpasses (excitation 3 mm and emission 1.5 mm). Enzyme activity was expressed as ‘activity’ (µIU) (1 µIU = 1 nmol/min) over DNA (µg) content.

**DNA determination**
Compound Hoechst 33258 (2-[2-(4-hydroxyphenyl)-6-benzoimidazolyl]-6-(1-methyl-4-piperazyl)-benzimidazol-3HCl) (American Hoechst Co, Behring Diagnostic, La Jolla, CA, USA) was the fluorophore used for DNA measurement (Labarca and Paigen, 1980).

Working solutions were prepared by diluting the dye reagent with standard sodium chloride/sodium citrate buffer (SSC). Calf thymus double-stranded DNA (Sigma) was used for standards in known concentrations of 2.5–100 µg/ml and endometrial homogenates were measured against this standard curve as follows: a standard or homogenate (100 µl) was made up to a volume of 2 ml with SSC buffer (pH 7.0). Diluted dye (1 ml) was then added to the standard or homogenate before reading the fluorescence at excitation wavelength of 360 nm, bandpass of 10 nm and emission wavelength of 450 nm, bandpass of 5 nm. All tests were performed in duplicate and standards were run in triplicate to ensure that the variation between replicates was <10%.

**Calculations and statistical analyses**
Concentrations of enzymes, and DNA were calculated firstly according to the standard curve obtained by regression analysis (fluorescence versus concentration of standards). Concentration of the unknown was obtained as follows:

$$\text{concentration of unknown} = \frac{\text{fluorescence of unknown} \times \text{concentration of standard}}{\text{fluorescence of standard}}$$

The detailed formula for the calculation is as follows:

$$\text{Enzyme activity (nmol/min)} = \frac{A \times 3.1 \times CS \times 1000}{176.16 \times A (CS) \times 0.02 \times 60}$$

where: A = fluorescence of sample; 3.1 (ml) = total volume in enzyme assay; CS (µg/ml) = concentration of standards; 1000 = conversion from µmol to nmol; 176.16 = molecular weight of 4MU; A (CS) = fluorescence of standards (regression of plot-fitted value); 0.02 (ml) = volume of homogenate; 60 (min) = time of incubation.

Traditionally, enzyme activity has been expressed in terms of protein content (µIU/µg protein). This method examines enzyme variation against a background of tissue protein content which includes both intra- and extracellular matrix. When enzyme activity is expressed in terms of DNA content (µIU/µg DNA), this should reflect enzyme activity on a cell number basis and may be a better reflection of cellular events (Sloan and Bird, 1977; Umaphartsavan and Jones, 1980; Cornillie et al., 1990).

The methods of statistical analysis used assume that the errors are normally distributed, and a logarithmic transformation was applied. Comparison of enzyme activity between groups was analysed using the F-test after logarithmic transformation, while polynomial regression was used to analyse the possible relationships between enzyme activity and day of the cycle.

**Results**
A total of 45 samples were obtained from normal cycles: three in the menstrual phase, 21 in the proliferative phase and 21 in the secretory phase. Table I provides information on actual numerical values of enzyme activity for normal subjects in the proliferative, secretory and menstrual phase.

**Endometrial lysosomal enzymes in normal endometrium**
Table I provides an overview of enzyme activity amongst proliferative, secretory and menstrual endometrium of normal subjects. Activity of acid phosphatase and N-acetyl-β-d-glucosaminidase was ~50–100-fold higher than activity of α-d-mannosidase and α-l-fucosidase in the endometrium. Median acid phosphatase activity was 2.20 µIU/µg DNA (range 0.48–6.72 µIU/µg DNA) in the proliferative phase and 2.54 µIU/µg DNA (range 0.81–17.91 µIU/µg DNA) in the secretory phase. Median N-acetyl-β-d-glucosaminidase activity was 1.65 µIU/µg DNA (range 0.38–6.72 µIU/µg DNA) in the proliferative phase and 1.04 µIU/µg DNA (range 0.015–10.53 µIU/µg DNA) in the secretory phase. Median α-l-fucosidase activity was 0.049 µIU/µg DNA (range 0.011–0.11 µIU/µg DNA) in the proliferative phase and 0.073 µIU/µg DNA (range 0.0078–0.72 µIU/µg DNA) in the secretory phase. Median α-d-mannosidase activity was 0.025 µIU/µg DNA (range 0.011–0.13 µIU/µg DNA) in the proliferative phase and 0.036 µIU/
µg DNA (range 0.0029–0.41 µIU/µg DNA) in the secretory phase (Table I). The activity of all four lysosomal enzymes showed no differences between the proliferative and secretory phases.

For menstrual phase endometrium, median acid phosphatase (3.70 µIU/µg DNA, range 1.49–8.64 µIU/mg DNA) and N-acetyl-β-d-glucosaminidase (2.18 µIU/µg DNA, range 0.93–6.07 µIU/µg DNA) showed a trend towards higher levels than the proliferative and the secretory phase, although trends were not statistically significant. However, this pattern was not seen with α-L-fucosidase or α-D-mannosidase. Only three biopsies were obtained from women in the early menstrual phase since the 1950s using colorimetry and more recently fluorimetry. Quantitative lysosomal enzyme analysis has been available for any of the four enzymes tested (Figure 1). This study examined specific changes in enzyme activity against the background variation in total tissue protein composition. However, this includes all the extracellular protein matrix and may not give a true reflection of cellular enzyme activity. Similarly, tissue weight is an unreliable parameter of cellular activity (Umamaheswaram and Jones, 1980). When enzyme activity is expressed in terms of DNA content, this reflects enzyme activity per cell which is a better reflection of activity of a substance which is a cellular product (Cornillie et al., 1991).

**Discussion**

Quantitative lysosomal enzyme analysis has been available since the 1950s using colorimetry and more recently fluorimetry (Mead et al., 1955; Rosado et al., 1977; Lombardo et al., 1984; Hansen et al., 1985; Cornillie et al., 1991). Fluorimetry, using substances such as 4-MU as fluorophores, is far more sensitive than colorimetry, but technically is a very difficult assay (Mead et al., 1955; Rosado et al., 1977; Lombardo et al., 1984; Cornillie et al., 1991). Enzyme activity can exhibit large, erratic variations if fluorimetry readings are affected by artefact due to background activity released by the various buffers or by changes in the reaction environment such as pH, temperature or ionic concentration (Urbanke, 1983). In this study, all experimental conditions were closely standardized and scrutinized and the experiments carried out with meticulous care to ensure accuracy. All four enzymes were tested confirm stability, that they could withstand storage (~76°C) and that they could withstand at least one freeze–thaw cycle without loss of activity (Faber and Glew, 1984). The experimental conditions were monitored with the assays performed at optimal pH, standardized temperature and ionic concentration for the lysosomal enzyme in question. In addition, the standards were performed in triplicate to ensure accuracy. Consistent results of test samples were accepted when the variation between replicates was <10%.

Acid phosphatase was selected in this study because it has traditionally been a marker enzyme for lysosomal activity in histochemical and biochemical studies although its function remains unknown. N-acetyl-β-d-glucosaminidase is important in glycoprotein metabolism and is present at different intracellular sites and in multiple forms. It is also an active lysosomal enzyme, often used as the ‘new’ lysosomal marker (Cornillie et al., 1990). Two other lysosomal enzymes, α-L-fucosidase and α-D-mannosidase were also selected in this study. These two enzymes are probably active in glycoprotein metabolism but detailed function in the endometrium remains unknown. Although rarely studied previously in the endometrium (Cornillie et al., 1991), it was felt to be important to examine their activity in association with two well recognized lysosomal markers for comparison.

Traditionally, enzyme activity has been expressed in terms of tissue protein content (µIU/mg protein). This approach examines specific changes in enzyme activity against the background variation in total tissue protein composition. However, this includes all the extracellular protein matrix and may not give a true reflection of cellular enzyme activity. Similarly, tissue weight is an unreliable parameter of cellular activity (Umamaheswaram and Jones, 1980). When enzyme activity is expressed in terms of DNA content, this reflects enzyme activity per cell which is a better reflection of activity of a substance which is a cellular product (Cornillie et al., 1991).

Histological and ultrastructural studies have demonstrated increased lysosomal activity in late secretory phase compared with the proliferative phase (Garcia-Bunuel and Brandes, 1966; Baron and Esterly, 1975; Ferenczy, 1976). In these studies, lysosomal activity was demonstrated by increased numbers of lysosomes as well as increased morphological changes in these

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**Table I. Enzyme activity expressed in terms of DNA (µIU/µg).**

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Time of cycle</th>
<th>Minimum</th>
<th>Median</th>
<th>Maximum</th>
<th>No. of samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acid phosphatase</td>
<td>Proliferative</td>
<td>0.48</td>
<td>2.20</td>
<td>6.72</td>
<td>21</td>
</tr>
<tr>
<td></td>
<td>Secretry</td>
<td>0.81</td>
<td>2.54</td>
<td>17.91</td>
<td>21</td>
</tr>
<tr>
<td></td>
<td>Menstrual</td>
<td>1.49</td>
<td>3.70</td>
<td>8.64</td>
<td>3</td>
</tr>
<tr>
<td>α-L-fucosidase</td>
<td>Proliferative</td>
<td>0.011</td>
<td>0.049</td>
<td>0.11</td>
<td>21</td>
</tr>
<tr>
<td></td>
<td>Secretry</td>
<td>0.0078</td>
<td>0.073</td>
<td>0.72</td>
<td>21</td>
</tr>
<tr>
<td></td>
<td>Menstrual</td>
<td>0.011</td>
<td>0.084</td>
<td>0.37</td>
<td>3</td>
</tr>
<tr>
<td>N-acetyl-β-d-glucosaminidase</td>
<td>Proliferative</td>
<td>0.377</td>
<td>1.65</td>
<td>6.72</td>
<td>21</td>
</tr>
<tr>
<td></td>
<td>Secretry</td>
<td>0.015</td>
<td>1.04</td>
<td>10.53</td>
<td>21</td>
</tr>
<tr>
<td></td>
<td>Menstrual</td>
<td>0.93</td>
<td>2.18</td>
<td>6.07</td>
<td>3</td>
</tr>
<tr>
<td>α-D-mannosidase</td>
<td>Proliferative</td>
<td>0.011</td>
<td>0.025</td>
<td>0.13</td>
<td>21</td>
</tr>
<tr>
<td></td>
<td>Secretry</td>
<td>0.0029</td>
<td>0.036</td>
<td>0.41</td>
<td>21</td>
</tr>
<tr>
<td></td>
<td>Menstrual</td>
<td>0.017</td>
<td>0.028</td>
<td>0.075</td>
<td>3</td>
</tr>
</tbody>
</table>
particles, in the form of increased autophagic, heterophagic vacuoles or secretory vesicles (Ferenczy, 1976). These morphological changes reflected changes in lysosomal morphology without reference to lysosomal enzyme activity.

In the current study, biochemical assays enabled the quantitative study of lysosomal enzymes, but did not reveal information about lysosomal numbers or morphology. The activities of acid phosphatase and \( N \)-acyetyl-\( \beta \)-D-glucosaminidase were similar to each other and appeared quite high in human endometrium. The activities of \( \alpha \)-L-fucosidase and \( \alpha \)-D-mannosidase were low, their levels being 50–100-fold less than those of acid phosphatase and \( N \)-acyetyl-\( \beta \)-D-glucosaminidase. In addition, the activity of endometrial \( \alpha \)-L-fucosidase and \( \alpha \)-D-mannosidase showed minimal fluctuations during the cycle. Past investigations have suggested the presence of different populations of lysosomes in rat myometrium (Sloan and Bird, 1977) and it can be speculated that there might be different populations of lysosomes with varying activity in different compartments within the human endometrium.

Few investigators have examined biochemical assays of lysosomal enzymes in normal endometrium (Rosado et al., 1977; Cornillie et al., 1991). Rosado et al. (1977), also using 4MU as fluorophore, showed an increase in acid phosphatase, \( N \)-acyetyl-\( \beta \)-D-glucosaminidase and \( \beta \)-glucuronidase activity from the proliferative to the secretory phase. In contrast, Cornillie et al. (1991), using a comparable technique to the current study, found a 40% drop in enzyme activity from the proliferative to the secretory phase. Although the finding from Rosado et al. (1977) was in agreement with other morphological studies (Garcia-Bunuel and Brandes, 1966; Baron and Esterly, 1975; Ferenczy, 1976), the results were expressed in terms of wet tissue weight, a parameter which did not accurately reflect cellular activity. This study also employed the process of centrifugation and separation of cell fractions making it difficult to interpret results. Whilst centrifugation can separate lysosomes of different sizes and shapes (the dense lysosomes being more resistant to shearing forces than large autophagic vacuoles), the process does not necessarily reflect the release of lysosomal enzymes (Cornillie et al., 1990). In contrast, this study and Cornillie et al. (1991) examined total tissue enzyme activity after homogenization to disrupt lysosomal membranes. Although all three studies showed quite different results, partly...
because enzyme activities were expressed in different ways; in terms of tissue weight (Rosado et al., 1997), protein content (Cornillie et al., 1991) and DNA content (this study). In addition, biochemical enzyme activity carries large intra and inter-subject variations. Furthermore, all three studies suffered from relatively small numbers because of the difficulties of recruitment and time-consuming assays. In this study, more biopsies, particularly in late secretory and the menstrual phase, would have been required to ascertain short-term lysosomal enzyme variation preceding onset of menstruation.

This study found no evidence of a progressive accumulation of lysosomal enzymes from the normal proliferative to secretory phase even with adequate numbers of samples in the mid-part of each phase. This study was not designed to rule out the possibility that enzymes contained in endometrial lysosomes may be released in the very late secretory phase to facilitate tissue shedding, menstruation and remodelling (Garcia-Bunuel and Brandes, 1966; Fraser, 1989; Henzl et al., 1972; Baron and Esterly, 1975; Ferenczy, 1976). However, it would appear that total lysosomal enzyme concentration remains relatively constant in relation to day to day activity in the endometrium. It is possible that other endometrial lysosomal enzymes which were not measured in this study may show changes during the cycle, but the four enzymes studied are all regarded as important lysosomal enzymes and could have been expected to show changes reflecting overall lysosomal activity. The relative lack of variation in concentration through most of the cycle is in agreement with the ‘remodelling’ or a ‘storage’ role in preparation for future tissue needs (De Duve and Waittaux, 1966; Henzl et al., 1972; Dean, 1977).

Hence, the results from this study demonstrated fairly conclusively that total endometrial concentrations of several important lysosomal enzymes do not change from the early proliferative to the end of the mid-secretory phase of the cycle. This does not rule out some change occurring immediately before or during menstruation. It can also be speculated that ‘pathological’ conditions may be associated with tissue damage and increased total concentration of lysosomal enzymes. This study has provided a basis for demonstrating changes in lysosomal activity under other physiological or pathological conditions.

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References


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