Oestrogen receptor in glands and stroma of normal and neoplastic human endometrium: a combined biochemical, immunohistochemical, and morphometric study

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SUMMARY Cryostat sections of unfixed human endometrial tissue from normal, hyperplastic, and carcinomatous endometrium were assayed for oestrogen receptor content using a radiolabelled oestradiol binding assay with isoelectric focusing to separate receptor proteins. The proportions of myometrium, stroma, and glandular tissue were estimated in adjacent sections by point counting. Further cryostat sections were stained for oestrogen receptor immunohistochemically with a monoclonal antibody and the relative staining intensity of the various tissue components assessed. The area proportions and relative immunohistochemical staining intensity of each tissue component in the cryostat sections were then used to adjust the biochemical results and derive separate values of oestrogen receptor content in myometrium, glands, and stroma. The greatest difference in oestrogen receptor content through the menstrual cycle and between normal, hyperplastic, and malignant endometrium occurred in the glandular element.

In common with all oestrogen responsive tissues, normal endometrial epithelium and stroma express oestrogen receptor. Values tend to be lower in the secretory than the proliferative phase of the cycle, similar to the proliferative phase in hyperplasia, and lowest of all, or zero in carcinomas.1 2 Oestrogen receptor may predict survival in carcinoma3 4 or response to endocrine treatment.5 The availability of monoclonal antibodies to oestrogen receptor6 and the recent discovery that part of the oestrogen receptor has sequence homology with the c-erb-A oncogene7 have heightened interest in measurements of oestrogen receptor.

Oestrogen receptor may be assayed biochemically by measuring the binding of radiolabelled oestrogens. Unbound and non-specifically bound steroid is removed either by incubation with dextran coated charcoal or by separating the receptor protein by isoelectric focusing. Such techniques do not usually take account of tissue heterogeneity, although in one report an attempt has been made to correct for the presence of connective tissue by measuring oestrogen receptor by isoelectric focusing on cryostat sections of breast tumour with simultaneous histological examination of adjacent tissue sections.8 This method assumed that breast connective tissue is oestrogen receptor negative, an assumption which may be incorrect even for breast tissue and is certainly not applicable to non-glandular tissue in the uterus. This study aims to deal with the problem of tissue heterogeneity by combining Underwood’s method with data from immunohistochemical demonstration of oestrogen receptor. This allows calculation of separate glandular, stromal, and myometrial oestrogen receptor values to be made.

Material and methods

Thirty five endometrial specimens were obtained from uteri removed for routine clinical indications. The table lists the diagnoses. After hysterectomy whole uteri were transported immediately to the laboratory and endometrial specimens (about 0.5 x 0.5 x 0.3 cm) were removed from the fundus and rapidly frozen by quenching in liquid nitrogen. Specimens

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obtained following curettage were similarly quenched in liquid nitrogen within 10 minutes of removal. The samples were then stored at $-80^\circ$C until assayed. The maximum storage time was two months.

**Biochemical Measurement of Oestrogen Receptor**

The method of Underwood was used. Briefly, duplicate 1 cm² 40 μm cryostat sections were cut and each transferred on a glass cover slip to a plastic tube on ice where the cover slip was crushed with a glass rod and immersed in 100 μl Edetic acid-Tris buffer (1.5 mmol disodium edetic acid, 10 mmol Tris-hydrochloric acid, 0.05% bovine serum albumin, 0.02% sodium azide; pH 7.4). One adjacent 10 μm section was then cut from the same block with minimal trimming for staining with haematoxylin and eosin. One hundred microns of tissue were then cut and discarded and repeat duplicate sections for biochemical assay and a single section for haematoxylin and eosin staining taken. Each section for oestrogen receptor assay was incubated on ice for one hour in 200 μl Edetic acid-Tris buffer containing $1 \times 10^{-9}$ mol (2,4,6,7-3H) oestradiol (specific activity 93 Ci/mmol). Edetic acid-Tris buffer (100 μl) containing $2 \times 10^{-9}$ mol oestradiol was added to each tube. The tubes were then centrifuged at 1000 g for 10 minutes at 4°C and the supernatant removed. The protein content was estimated in a 50 μl sample of the cytosol by the Coomassie blue method of Bradford. Trypsin (Sigma type 3) (1 mg/ml) was added in the proportion 5 μl per optical density 0.15 at 595 nm in the Bradford protein assay and calcium chloride was added to give a final concentration of 2 mmol. The cytosol was then incubated for a further 30 minutes at 10°C. The receptor bound oestradiol was then separated from free and non-specifically bound oestradiol by isoelectric focusing of the supernatant in a polyacrylamide gel.

**Isoelectric Focusing**

Seventy five μl trypsin treated supernatant was transferred to plastic sample application frames 1 cm from the cathode strip on the surface of a preformed thin layer polyacrylamide gel (LKB) containing Ampholine carrier ampholytes (LKB). The apparatus was maintained at 4°C in a refrigerated room. The focusing conditions were similar to those used by Gustafsson et al. The electrode strips were soaked in 1M NaOH (cathode) and 1M H₃PO₄ (anode). The gel was prefocused for 10–15 minutes at 5 mA. The samples were then added to the application frames and focusing performed for two hours at a current of 10 mA. The sample frames were removed after 90 minutes and any remaining cytosol removed with filter paper to minimise radioactive contamination. Reference isoelectric point markers with a pH range of 4.7–10.6 (BDH) were added to one sample frame. The gel was cut into 1 cm strips, one per sample track and each strip cut into 0.5 cm pieces. These were transferred to individual glass scintillation phials containing 5 ml Cocktail T scintillation fluid (BDH). The radioactivity in each phial was measured on a 2000 CA Tricarb liquid scintillation counter (United Technologies Packard) with correction for efficiency with an external standard. A graph was then plotted of counts per minute (CPM) against each slice number. Two peaks were typically present. The first within 3 cm of the cathode represented free and non-specifically bound oestradiol and was disregarded. A second peak, if present, occurred at 4.5–6 cm from the cathode and represented specifically bound oestradiol. The total specific binding for each specimen was calculated by subtracting the diagrammatic baseline from each slice value and adding the results obtained for the one, two, or three slices corresponding to the second specific peak. A typical graph from

**Table** Histopathological assessments

<table>
<thead>
<tr>
<th>Histology</th>
<th>No of specimens</th>
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</thead>
<tbody>
<tr>
<td>Menstrual endometrium</td>
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<tr>
<td>Normal proliferative</td>
<td>6</td>
</tr>
<tr>
<td>Normal secretory</td>
<td>6</td>
</tr>
<tr>
<td>Atrophic postmenopausal</td>
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</tr>
<tr>
<td>Moderately differentiated carcinoma</td>
<td>5</td>
</tr>
<tr>
<td>Poorly differentiated carcinoma</td>
<td>2</td>
</tr>
<tr>
<td>Total</td>
<td>35</td>
</tr>
</tbody>
</table>

**Fig. 1** Counts per minute for each slice of polyacrylamide gel after isoelectric focusing. Left hand peak represents non-specific binding; smaller right hand peak above baseline represents specific estradiol binding to oestrogen receptor.
Estimation of oestrogen receptor in glands and stroma of endometrium

an oestrogen receptor positive specimen is shown in fig 1. The readings were then converted to femtomoles of oestradiol per mg cytosol protein by correcting for the specific activity of the oestradiol and for the cytosol protein concentration as calculated by the Bradford assay. All determinants were done in duplicate and a mean result obtained. With experience it was found that the second specific peak was always located on the same point on the gel and therefore only pieces of gel corresponding to 3.0-7.5 cm from the cathode were counted.

IMMUNOHISTOCHEMICAL OESTROGEN RECEPTOR ASSAY
This was performed with the Abbott oestrogen receptor-ICA monoclonal antibody kit and the method is briefly described as follows. Two 7 μm frozen sections were cut from the same specimens as the sections for the biochemical assay described above, placed on slides coated with tissue adhesive, and immediately fixed for 10-15 minutes in 3-7% formaldehyde in 0.1M phosphate buffered saline (PBS) pH 7.4 and then transferred to fresh PBS for a minimum of five minutes. They were then immersed in methanol at -20°C for four minutes, acetone at -20°C for two minutes, and returned to PBS. Non-specific binding sites were blocked by incubation in normal goat serum in PBS for 15 minutes before primary rat monoclonal antibody to oestrogen receptor (0.1 μg/ml) was applied to the test slide and control normal rat antibody 0.1 μg to the control slide for 30 minutes. After washing in PBS bridging antibody (goat antirat antibody 10 μg/ml) was applied to both slides for 30 minutes followed by another wash and then rat peroxidase-antiperoxidase complex 0.5 μg/ml for 30 minutes. Slides were then coloured in diaminobenzidine solution with hydrogen peroxide and counterstained with 2% methyl green. The relative intensity of specific nuclear staining in the oestrogen receptor labelled slides was measured semiquantiatively with each tissue component (myometrium, stroma, and glands) being scored separately. A score of 0-3 was given for the maximal staining intensity (0 = no specific stain, 1 = weak, 2 = moderate, 3 = strong), and a score of 0-3 was also given for the proportion of specifically stained nuclei (0 = less than 11%, 1 = 11-30%, 2 = 31-60%, 3 = more than 60%). Thus each tissue component had a score of between 0 and 6, and each specimen had a score between 0 and 18 if it contained three tissue components. If a slide contained only one or two tissue components the total specimen score was increased by a factor of 3 or 1.5, respectively, to provide a comparable total score for analysis. An endometrial staining score was also derived for use in calculating a separate endometrial and myometrial oestrogen receptor as follows: endometrial score = (gland score × proportion of glands in a haematoxylin and eosin stained section) + (stromal score × proportion of stroma in a haematoxylin and eosin stained section).

MEASUREMENT OF TISSUE COMPONENTS IN THE BIOCHEMICAL ASSAY
The adjacent frozen sections corresponding to each paired sample analysed biochemically for oestrogen receptor by isoelectric focusing were stained with haematoxylin and eosin. The proportions of myometrium and endometrium, and, within the endometrium, the proportions of glands and stroma were estimated on each section by measuring the relative area of each by point counting. A graticule with 81 regularly distributed points was used in the focal plane of a × 10 eyepiece with a × 10 objective. The number of points coincident with each tissue component was counted over the whole section and proportions calculated. Points overlying the lumen of glands were not counted.

CALCULATION OF SEPARATE OESTROGEN RECEPTOR VALUES
This was done by solving the two halves a) and b) of a simultaneous equation containing two unknown variables. Separate myometrial [ER]m and endometrial ER [ER]e values were calculated first. [ER] is the mean value of oestrogen receptor measured by isoelectric focusing in paired sections with an adjacent section stained for haematoxylin and eosin.

Let M = the proportion of myometrium in the haematoxylin and eosin section.

Let E = the proportion of endometrium in the haematoxylin and eosin section. Equation a) is thus:

\[ M \times [ER]_m + E \times [ER]_e = [ER] \]

A second equation b) was then derived from the immunohistochemical scores, assuming that the ratio of scores is equal to the ratio of oestrogen receptor values.

\[ \frac{[ER]_m}{[ER]_e} = \frac{\text{myometrial score}}{\text{endometrial score}} \]

This equation can be rearranged as:

\[ [ER]_m = \frac{\text{myometrial score} \times [ER]_e}{\text{endometrial score}} \]

substituting this expression for [ER]m in equation a) gives:

\[ \left( \frac{M \times \text{myometrial score} + E \times \text{endometrial score}}{\text{endometrial score}} \right) \times [ER]_e = [ER] \]
This is then arranged as:

c) \[ [ER]_c = \frac{[ER]}{\left( \frac{M \times \text{myometrial score} + E}{\text{endometrial score}} \right)} \]

Having derived \([ER]_c\), \([ER]_m\) was calculated by inserting the value of \([ER]_c\) in equation a).

The process was then repeated for the second paired sections from each specimen to give duplicate myometrial and endometrial oestrogen receptor. The endometrial oestrogen receptor and the relative proportions of glands and stroma in the endometrium were then used in similar equations to derive glandular and stromal oestrogen receptor.

**Results**

The immunohistochemical oestrogen receptor assay gave specific nuclear staining (fig 2). The total oestrogen receptor score of 18 obtained from this
**Estimation of oestrogen receptor in glands and stroma of endometrium**

![Graph showing adjusted oestrogen receptor values](image)

**Discussion**

Most reports of oestrogen receptor values in endometrium have not referred to the tissue components. This may not matter if pure tumour tissue consisting only of carcinoma cells can be analysed, but usually endometrial specimens also contain variable amounts of stroma and myometrium which normally contain moderate amounts of oestrogen receptor. Such contamination may theoretically cause a falsely high or low result. Two groups of workers measured separate endometrial and stromal oestrogen receptor by using limited collagenase digestion to separate the components before assay.\(^{11}\)\(^{12}\) Such methods have two potential disadvantages. Firstly, oestrogen receptor is a highly soluble protein and it may be lost during the separation procedure, and secondly, oestrogen receptor values may vary during short term culture. There is evidence that this did indeed occur as whole tissue oestrogen receptor was consistently higher than both components' separated oestrogen receptor.\(^{12}\)

Methods of oestrogen receptor estimation using isoelectric focusing\(^{10}\) to separate receptor proteins have the advantage that small amounts of tissue can be used (5 \(\times\) 10\(^{-4}\)–10\(^{-6}\) cells). The results obtained also correlate well at high and low oestrogen receptor values with those of high affinity oestrogen receptor measured by dextran coated charcoal separation of unbound oestrogen.\(^{8}\) The correlation, however, is poor over the range 15–80 fm/mg, perhaps reflecting tissue heterogeneity. The isoelectric focusing method does not detect non-specific binding sites as the isoelectric profile of this closely approximates the diagrammatic baseline.\(^8\) An incubation with dextran coated charcoal can therefore safely be omitted. It may, however, detect the specific low affinity oestrogen binding (type II receptors) that have been reported in breast tumours.\(^{13}\) We cannot exclude the possibility that the present assay also measures these type II receptors, which have been reported in the rat uterus.\(^{14}\)

Simultaneous histological examination of near facsimile sections confirms the composition of the tissue assayed.\(^8\) This approach at least avoids the problem of measuring oestrogen receptor on specimens containing no tumour.\(^{15}\) Assuming, however, that oestrogen receptor values are proportional to the degree of immunohistochemical staining,\(^{16}\) then the calculations described in the present study will also provide more accurate estimates of stromal and epithelial receptor values. Although immunohistochemical techniques do accurately localize receptor sites, they are only semiquantitative.

The finding that oestrogen receptor is lower in the secretory phase of the cycle, similar to the proliferative phase in hyperplasia, and decreased with
decreasing differentiation in carcinoma confirms the results of most previous work. The results for separate glandular and stromal oestrogen receptor are at variance with those of Fleming et al. They reported that in normal endometrium both glandular and stromal oestrogen receptor varied equally through the cycle. Our data indicate that the major variation is in the glandular oestrogen receptor. Studies using purely immunohistochemical detection of oestrogen receptor have also shown that the variation through the normal cycle is similar in glandular and stromal tissue. There have been no previous reports of separate glandular and stromal oestrogen receptor in hyperplastic and malignant endometrium. Again, the present data indicate that most of the variation occurs in glandular oestrogen receptor. If this is confirmed it may also follow that glandular oestrogen receptor will be a better predictor of behaviour than total endometrial oestrogen receptor, but this will require prolonged follow up of more subjects.

J G Thornton is a Yorkshire Cancer Research Campaign Research Fellow.

References


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