OESTROGEN RECEPTOR STATUS OF BREAST TUMOUR BIOPSIES IN MALAYSIAN PATIENTS

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SUMMARY

This communication describes the quantitative and qualitative analysis of oestrogen receptors in breast tumour biopsies of Malaysian patients. This preliminary investigation establishes certain rigid criteria that makes possible the classification of patients most likely to respond to hormonal therapy. 20 percent of the patients had oestrogen receptor positive tumours while 60 percent were oestrogen receptor negative.

INTRODUCTION

It was recognised in 1896 by Beatson ¹ that 25 - 30 percent of patients with advanced breast cancer experience remission as a response to hormone therapy. Therefore identification procedures of potential responders and the choice of endocrine therapy is clearly important in the management of breast cancer patients. An index of response to hormone therapy for patients with breast cancer has been the identification of oestrogen receptors in biopsy specimens. ² ³ ⁴ ⁵ In most of these investigations it became evident that patients with advanced breast cancer are likely to respond to endocrine therapy only when the tumour contained oestrogen receptors in both the cytosol and nuclear fraction ⁶ of the tumour homogenate. Therefore, it was felt that similar work be conducted in this country to identify patients in our hospitals who will benefit from hormonal manipulative treatment. This paper is a report of a preliminary investigation of the oestrogen receptor status of breast tumours in Malaysian patients.

MATERIALS AND METHODS

Breast tumour biopsies were collected from 200 patients undergoing Surgery at various hospitals. Of these 175 had primary disease and 25 secondary or recurrent disease of the breast.

Biopsy specimens and tissue handling

Tissue was collected fresh from the operating theatre. Adjacent sections were sent for histopathological examination. Tissue was then transferred to the laboratory in ice or when tissue could not be processed fresh they were stored at -20°C in 0.25M sucrose, 1.5 mM MgCl₂, 10mM HEPES pH 7.4, 50% glycerol (v/v). This procedure has been found to cause minimal loss of receptor over 30 days period. Tissue at 50mg/ml was homogenised in HED buffer containing 1.5mM EDTA (ethylenediaminetetra acetic acid) 0.5mM DTT (Dithiothreitol), 10mM HEPES (N-2-hydroxy-piperazine N'-2 ethane' sulphonic acid) pH 7.4 using an ultra-Turrax homogeniser and a glass Dual Kontes homogeniser. Homogenisation was carried out at 2°C.

The homogenate was centrifuged at 5,000g for 10 mins. The supernatant was collected and this constituted the cytosol fraction and the pellet was resuspended to its original volume in 0.15M NaCl, 10mM HEPES buffer at pH 7.4. Both fractions
TABLE I
DISSOCIATION CONSTANT OF OESTROGEN RECEPTORS AND STATUS IN BREAST TUMOUR BIOPSIES

<table>
<thead>
<tr>
<th>GROUP</th>
<th>CYTOSOL FRACTION</th>
<th>NUCLEAR FRACTION</th>
<th>RECEPTOR STATUS</th>
<th>PERCENT OF PATIENTS</th>
</tr>
</thead>
<tbody>
<tr>
<td>A(12)</td>
<td>1.2 - 1.7 x 10^{-10}M</td>
<td>1.8 - 2.2 x 10^{-10}M</td>
<td>RC+/R N+</td>
<td>20%</td>
</tr>
<tr>
<td>B(16)</td>
<td>0.2 - 0.9 x 10^{-10}M</td>
<td>2.4 - 2.9 x 10^{-10}M</td>
<td>RC-/R N-</td>
<td>60%</td>
</tr>
<tr>
<td>C(12)</td>
<td>1.8 - 2.8 x 10^{-10}M</td>
<td>3.8 - 3.6 x 10^{-10}M</td>
<td>RC-/R N-</td>
<td>60%</td>
</tr>
<tr>
<td>D(120)</td>
<td>0.4 - 3 x 10^{-8}M</td>
<td>Ambiguous Scatchard</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

were retained at 0°C.

Oestrogen Receptor Assay
(i) Cytosol Receptors
The method with slight modification has been described elsewhere. Briefly a series of 9 tubes were set up each containing 50ul of tritium-labelled oestradiol (Amersham Radiochemicals) such that the final concentrations of oestradiol was 1x, 1.5x, 2.5x, 4x, 6x, 8x, 10x10^{-10}M. A parallel series of tubes was set up but this time in the presence of 100 fold excess of unlabelled steroid (Diethylstilbesterol). To both series of tubes were added 150ul cytosol. All tubes were incubated at 4°C for 18 hours, and after incubation, 0.9ml EDTA, 10mM HEPES buffer pH 7.4 and 0.5ml of dextran coated charcoal was added to each tube. Each tube was thoroughly mixed and further incubated at 0°C for 15 mins. All tubes were centrifuged at 1000g and Irnl aliquouts were removed from each supernatant into 10ml Triton-Toluene scintillant and radioactivity counted in Tricarb-Packard liquid scintillation counters at 30% efficiency.

(ii) Nuclear Receptors
A similar series of tubes as described before was set up except that 150 of nuclear suspension replaced the 150 cytosol in the cytosol assay. Incubation as before subsequent to which 100ul aliquouts were removed and each added to 5ml saline. The saline containing nuclear suspension was poured down the millipore filtration unit onto a wet Whatman GF/C glass fibre filter. The filter was washed several times with saline after which the filter was transferred into a scintillation vial and dried overnight at 60°C. Filters were countered in 10ml Toluene/PPO liquid scintillants in the Tricarb-Packard liquid scintillation counters at 35% efficiency.

(iii) DNA and Protein Assay
DNA in the nuclear fraction was assayed by the method of Burton and protein in the cytosol was assayed as described by Lowry.

(iv) Scatchard analysis of hormone-receptor interaction.
Concentration of oestrogen receptor in the cytosol and nuclear fractions and its affinity for oestrogen is measured simultaneously using a Scatchard analysis. Affinity of receptor for oestrogen is defined by the dissociation constant (Kd) of the hormone-receptor interaction activity. The Kd of the oestrogen-receptor complex from human breast tissue is in the range of 10^{-10} to 10^{-9} mol, a range similar to that of plasma oestradiol.

RESULTS
Oestrogen receptors of breast tumours
Tumour biopsies were analysed for the presence of oestrogen receptors. Tumours were classified as either oestrogen receptor 'positive' or 'negative'. For a breast tumour biopsy specimen to be considered as "positive" for oestrogen receptors it is necessary to demonstrate that the specimen contain intact and functional receptor molecules that show a very high affinity and specificity for oestrogens. Table I identifies the biopsy specimens containing both nuclear and cytosol oestrogen receptors. Binding was specific, sensitive to competition with excess diethylstilboesterol and of high affinity with a dissociation constant in the range of 1 - 3 x 10^{-10} mol/L.

Tumours biopsies also demonstrated qualitative differences in the dissociation constants (Kd) although the variation in value of Kd between groups are not significant. On the basis of this tumour biopsies in Table I are grouped as group A,
Fig. 1 The oestrogen-receptor interaction in the presence of competition with excess diethyl stibesterol is shown. The method of analysis is described in the text.

Figs. 1A, B, C demonstrate the Scatchard plot of oestrogen-receptor interaction of oestrogen-receptor positive tumour biopsies (groups A-C in Table I). Inserts show the cytosol concentration of receptors and dissociation constants. Fig. 1D demonstrates an ambiguous Scatchard plot of oestrogen-receptor interaction of oestrogen-receptor negative tumours (group D in Table I).

B and C each differing in their dissociation constants. Tumours grouped as group D although showing the presence of compatible oestrogen binding in both fraction do not show a dissociation constant (Kd) of the order of $10^{-8}$ mol/L (Fig. 1). Therefore group D tumours merely contain oestrogen binding molecules that have very low affinity for oestrogen. Similar molecules have been reported in several types of cancers not normally responsive to endocrine therapy. These results are consistent with the definition of a receptor positive sample in that it (i) should yield a Scatchard plot that produces a straight line and (ii) the Scatchard plot should have a slope or Kd in the range of $10^{-11} - 5 \times 10^{-10}$ mol/L.

Thus tumour biopsies (Groups A-C, Table I) that are both nuclear and cytosol receptor positive fitted the above criteria as positive samples in terms of oestrogen receptors. The Scatchard plot for nuclear as well as cytosol tumour fractions in biopsies of groups A, B, C and D are shown in Fig. 1. The slopes of plots for group A, B and C yielding a Kd in the range of $1-5 \times 10^{-10}$ mol/L is tabulated in Table I.

**Oestrogen receptor content of breast tumour biopsies**

All tumour biopsies identified and classified as in Table I were analysed individually for the quantitation of receptor concentration in the cytosol and nuclear fraction. Cyttoplasmic and nuclear concentrations of oestrogen receptors are reported as fmol/mg cytosol protein and fmol/mg protein.
TABLE II
OESTROGEN RECEPTOR CONCENTRATIONS IN CYTOSOL AND NUCLEAR FRACTIONS OF BREAST TUMOUR BIOPSIES

<table>
<thead>
<tr>
<th>GROUP</th>
<th>CYTOSOL FRACTION</th>
<th>NUCLEAR FRACTION</th>
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<tbody>
<tr>
<td></td>
<td>fmol /mg Cytosol protein</td>
<td>fmol /mg DNA</td>
</tr>
<tr>
<td>I (6)</td>
<td>80 - 200</td>
<td>1000 - 1300</td>
</tr>
<tr>
<td>II (10)</td>
<td>220 - 500</td>
<td>1300 - 1800</td>
</tr>
<tr>
<td>III (24)</td>
<td>500 - 700</td>
<td>1900 - 2200</td>
</tr>
</tbody>
</table>

DNA respectively. From this analysis it became evident that the tumour biopsies could be reclassified quantitatively as follows:- Group I: Breast tumour biopsies with cytoplasmic receptor concentration detectable at 80 - 200 fmol/mg protein and 1000 - 1300 fmol/mg DNA nuclear oestrogen receptor. Group II:- Breast tumour biopsies with cytoplasmic receptor concentration detectable at 250 - 500 fmole/mg protein and nuclear receptor detectable at 1300 - 1880 fmole/mg DNA. Group III: 550 - 700 fmole/mg protein of cytoplasmic receptor and 2000 - 2500 fmole/mg DNA nuclear oestrogen receptor. The above data is tabulated in Table II. From this data it is seen that in receptor - positive biopsies there is significant quantitative variation in receptor content. It should be noted that when the assay method was used on receptor rich and receptor positive quality control samples results yielded good quantitative agreement. When the group D samples (Table I), that had been classified as oestrogen receptor negative on the basis of Scatchard analysis were analysed for the content of receptors in the cytosol and nuclear fraction they yielded results that showed cytoplasmic receptor content and nuclear content in the range of 10 fmole/mg protein and 40 fmole/mg DNA respectively. There has been similar observation that demonstrated variations in receptor content between assays on different samples from the same tumour although there was little variation in the value of Kd observed. For the latter group of tumours (group D from Table I) they are classified as receptor negative for cytosol and nuclear fraction (Rc / RN ). It is worthy of note that it is misleading to emphasize relative numbers of oestrogen receptors as a criteria for the minimal response to hormone therapy. This is consistent with the findings of Leake et al. It is therefore imperative that to predict response to hormonal therapy a breast tumour must be shown to contain intact, biologically active, specific high affinity receptors for oestrogens. There is much evidence to show that very few patients with a Kd for oestrogen receptor activity outside 1 - 5x10^-10 mol/L and a receptor status of RN /Rc , respond to hormone therapy. 

It should be emphasised also that the results tabulated in Table II do not consider primary and secondary disease or differentiate between pre- and post menopausal patients or patients of different ethnic or cultural background.

DISCUSSION
Recent findings have demonstrated that oestrogen receptor status of breast tumour biopsies can be successfully used as an index of response. It should be stressed that in these investigations the presence of high affinity, saturable oestrogen - binding protein and a receptor status of RN /Rc was the main criterion used in identifying patients who may respond favourably to hormone therapy and not simply by criteria of concentrations of cytoplasmic oestrogen receptors. In this investigation it was possible to select biopsies that contain both cytoplasmic and nuclear receptor (Rc /RN ). The percentage of patients presenting with a Rc /RN receptor status in the Malaysian patients constituted only 20 percent and those presenting with a receptor status of Rc /RN constituted 60 percent (Table I). The rest of the patients were either negative for cytoplasmic receptor but positive for nuclear (Rc /RN ) or positive for cytoplasmic but negative for nuclear fraction (Rc /RN ). This category of patients is the subject of further investigation. In this investigation there is no existing therapy being administered to the patients. Evidence has shown that the presence of both cytoplasmic and nuclear receptors is indeed a good indicator of potential response to hormone therapy. The number of patients that come under this category is, however, small, but their tumour biopsies demonstrated the presence of specific, high affinity receptors in the cytosol as well as nuclear fraction, making this group of Malaysian patients very likely to respond to hormone therapy. The other group of patients, which is surprisingly high in proportion did not demonstrate the above criteria and therefore is not likely to respond to hormone therapy. However, in a recent review of the literature, Hawkins reported that, overall, the
response rate of breast cancer patients to endocrine therapy is only in the 25 - 30\% range.

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