Evaluation of an Enzyme Immunoassay for Estrogen Receptors in Human Breast Cancers

Robert I. Nicholson, Paul Colin, A. Barrie Francis, Ramesh Keshra, Pauline Finlay, Mike Williams, Chris W. Elston, Roger W. Blarney, and Keith Griffiths

Tenovus Institute for Cancer Research, University of Wales College of Medicine, Cardiff, United Kingdom CF4 4XX [R. I. N., P. C., A. B. F., R. K., P. F., K. G.], and Department of Surgery, City Hospital, Nottingham, United Kingdom NG5 1PB [M. W., C. W. E., R. W. B.]

Abstract

An estrogen receptor enzyme immunoassay kit (ER-EIA) has been evaluated in 70 human breast carcinomas against a routine cytoplasmic cytosol protein. Above this value, the ERU underestimates the concentration of receptor. The ERU gave a lower number of estrogen receptor-positive tumors (50 of 70) than did the ER-EIA assay (59 of 70). In the ERU-negative ER-EIA-positive tumors, receptor values as determined by the ER-EIA assay all fell below 50 fmol/mg of protein (mean, 19.9 ± 4.2 fmol/mg of protein). Application of an exchange procedure which estimates the total steroid binding capacity of the cytosol gave positive results in 7 of 9 ERU-negative ER-EIA-positive tumors (mean, 16.9 ± 2.95 fmol/mg of protein). Subdivision of the binding data according to the menopausal status of the patient indicates low receptor values in premenopausal women by each assay. A correlation between the ER-EIA assay and the histological grade of tumors was observed; Grade I well-differentiated tumors were all positive, while Grade II and III tumors were 86% and 75% positive, respectively. No correlation between the ER-EIA assay and tumor lymph node stage or tumor size was observed.

Introduction

Assays for estrogen receptors in breast cancer specimens have been carried out on a routine basis in the Tenovus Institute since 1974. These assays have yielded considerable amounts of valuable information relevant to the clinical management of the disease (1, 2). The method used, however, relies on the binding of radiolabeled estradiol to the receptor and is therefore only directed towards this, albeit, important site on the receptor protein. An alternative procedure is now available that utilizes rat monoclonal antibodies raised against human estrogen receptors isolated from MCF-7 breast cancer cells and detects antigenic determinants on the receptor protein (3). Clearly a combination of these techniques may provide complementary information concerning the functional integrity of the receptor and may have important clinical ramifications.

The present paper examines the recently described ER-EIA (4) in a series of 70 human breast carcinomas and contrasts the results with those determined in our routine estradiol binding assay (ERU), which measures unoccupied ER. The specificity and reliability of the ER-EIA assay for human breast tumor estrogen receptors have been evaluated (3).

Materials and Methods

Patients. Samples from 70 patients with histologically recognized breast cancer presenting to one surgeon (R. W. Blamey) were included in the study. The majority of women had primary breast cancer (57 of 70) and were under 70 yr of age at mastectomy (47 of 57). The remaining patients had recurrent loco-regional disease. Tumor size, lymph node staging, and histological grade of malignancy were assessed as previously reported (2). Briefly, lymph node stage was based on triple lymph node biopsies removed from the lower axilla, the apex of the axilla, and the internal mammary chain. Patients without tumor histologically evident in any node were classified as Stage A. Those with tumor only in the nodes from the lower axilla were Stage B and Stage C if apical axillary or internal mammary nodal involvement had occurred. Histological grade was assessed according to the method of Elston (5, 6) with Grade 1 being the most differentiated and Grade III, the least. Patients in the primary breast cancer series (47 of 70) were followed up at 3 monthly intervals and received no treatment until recurrence.

Receptor Analysis. Tumor tissue was rapidly frozen after excision, stored at -70°C, and transported on dry ice to the Breast Cancer Unit at the Tenovus Institute for receptor analysis. Tissue for assay (∼300 mg) was pulverized after immersion in liquid nitrogen and homogenized at 4°C in 10 mM Tris-HCl buffer, pH 7.4, containing 1 mM EDTA and 10% (v/v) glycerol (Buffer A) using a glass-on-glass homogenizer. All assays were carried out on a cytosol fraction prepared by ultracentrifugation (105,000 x g; 1 h) of the homogenate. The protein concentration of the cytosol was adjusted to 1–2 mg/ml with Buffer A.

Unoccupied receptor sites were measured in a 3-ml aliquot of cytosol prepared in Buffer A to which 100 µl of 30 mM monothioglycerol were added. The cytosol was incubated overnight at 4°C with a range of [2,4,6,7-3H]estradiol-17β concentrations (0.1–10 nM; specific activity, 93 Ci/mmol).

An estimate of the total number of estrogen binding sites was made using a recently developed exchange procedure. Briefly, a mercurial reagent, mersalyl acid (0.2 mM), was incorporated into Buffer A containing 0.1–10 nM [3H]estradiol to aid the dissociation of endogenous estradiol (approximately 0.2 mM) (7) from the receptor. This assay, although increasing the dissociation rate of the reaction, does not prevent the reassociation of [3H]estradiol with the receptor and at equilibrium (24 h at 4°C) gives an estimate of the total estradiol binding capacity of the cytosol.

Nonspecific binding was assessed in each binding assay in a parallel set of incubations containing a 1000-fold excess of diethylstilbestrol. At the end of the overnight incubation period, free steroid was removed by its adsorption onto charcoal [Tris-HCl buffer, pH 7.4, containing 0.5% (w/v) Norit A, 0.05% (w/v) Dextran T-70, and 0.1% (w/v) gelatin]. Estimates of binding parameters were made using Scatchard analysis (8).

The ER-EIA monoclonal kit from Abbott Laboratories (4) is an enzyme immunoassay based on the "Sandwich principle" and uses monoclonal rat antibodies to MCF-7 human breast cancer estrogen receptors (3). Cytosol (100 µl) prepared in Buffer A containing 1 mM monothioglycerol and a 1:3 dilution of the cytosol were incubated at 4°C for 18 h with an antibody (rat anti-ER; DS47) coated polystyrene bead which binds estrogen receptor proteins. A second antibody (rat anti-ER; H222) conjugated to horseradish peroxidase was then incubated with the bead at 37°C for 1 h to label the bound receptor. A further incubation (30 min at room temperature) of the bead with the enzyme substrate hydrogen peroxide and the color reagent/electron donor o-phenylenediamine-2HCl produced a phenazine polymer chromophore which was measured using a dual wavelength analyzer at 492 and 600 nm. Each incubation step was completed by washing the bead with distilled water, and the enzyme reaction was stopped by the

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2. To whom requests for reprints should be addressed.

3. The abbreviations used are: ER-EIA, estrogen receptor enzyme immunoassay; ERU, unoccupied estrogen receptor binding assay; ER, estrogen receptor.
addition of N sulfuric acid. A standard curve was obtained by plotting the ER concentration of human estrogen receptor standards (0.0 and dilutions of 2.5 pmol/ml of stock, supplied in the Abbott kit) versus their absorbance. The ER concentration of specimens was determined from this curve.

Reproducibility Evaluation. The evaluation was carried out on assays of three preparations of lyophilized human estrogen receptor protein, with mean receptor contents of 89, 173, and 364 fmol/mg of protein, respectively. On 5 separate days samples from two of the three preparations were assayed blind, in replicates of five.

Reagents. All reagents for the ER-EIA were supplied by Abbott Laboratories, Diagnostics Division, North Chicago, IL, in the Abbott ER-EIA monoclonal kit. 17β-[2,4,6,7-3H]Estradiol was supplied by Amersham International, Amersham, Bucks, United Kingdom.

Results

Methodology Studies. Monoclonal rat antibodies to MCF-7 human breast cancer estrogen receptor protein have been used to study the ER content of cytosol fractions from 70 human breast cancer specimens. Using the Abbott ER-EIA kit, values of ER ranged from <5 to over 4000 fmol/mg of protein (Fig. 1); 59 (84%) of the samples were ER positive (>5 fmol/mg of protein). Sixteen (27%) of the ER-EIA-positive samples had receptor values in excess of 1000 fmol/mg of protein. These high values were restricted to samples removed from women over 52 yr of age who were postmenopausal (Fig. 2). All premenopausal women had receptor values under 300 fmol/mg of protein. Of 23 samples from premenopausal women, 18 (78%) were positive, compared with 41 of 46 samples (89%) from postmenopausal women.

Comparison of the ER values determined using the Abbott kit and the ERU assay showed a linear correlation (Deming regression analysis) for ER-EIA values up to 400 fmol/mg of protein (Deming slope, 1.84; r = 0.85; P < 0.001) (Fig. 3). Above this value the routine procedure severely underestimates the number of binding sites. Samples were not reasayed using higher concentrations of [3H]estradiol, and therefore no further analysis of these data was possible. Using the cut-off value of 5 fmol/mg of protein for each assay the ERU method gave a lower number of ER-positive tumors. For the 9 ERU-negative ER-EIA-positive tumors, receptor levels all fell below 50 fmol/mg of protein [19.9 ± 4.2 (SD)], as determined by the ER-EIA. Application of an exchange procedure, which estimates the total steroid binding capacity of the cytosol rather than the unoccupied fraction, gave positive results in 7 of 9 of these tumors with a mean receptor value of 16.9 ± 2.95 fmol/mg of protein. Receptor values measured by the the ER-EIA on cytosols diluted 1:3 in Buffer A were in close agreement with those for undiluted cytosol fractions when the former fell within the standard range (not illustrated).

Reproducibility evaluation of the ER-EIA by multiple assay of lyophilized human receptor pools gave intraassay and inter-

Fig. 1. ER-EIA values in breast cancer specimens. Cytosol fractions of human breast cancer specimens (n = 70) were assayed for their estrogen receptor content by ER-EIA. The majority of samples gave results within the standard range for the assay (0–500 fmol of ER per ml; •). Four tumors (△), however, were outside this range. Results are expressed as fmol of ER per mg of protein.

Fig. 2. ER-EIA values in breast cancer specimens: effect of age and menopausal status. Cytosol fractions of human breast tumors (n = 66) from women of known age and menopausal status were assayed for their estrogen receptor content by ER-EIA. O, premenopausal (all values <500 fmol of ER per ml); •, postmenopausal (values <500 fmol of ER per ml); △, postmenopausal (values ≥500 fmol of ER per ml). Results are expressed as fmol of ER per mg of protein.
EVALUATION OF ER-EIA IN HUMAN BREAST CANCERS

Fig. 3. Correlation between ER values measured by ER-EIA and a routine steroid binding assay. Cytosol fractions from 70 human breast tumors were assayed for their estrogen receptor content by ER-EIA and a steroid binding assay. a, all values; b, values of ER-EIA <400 fmol/mg of protein (Deming slope of correlation, 1.84; r = 0.85; P < 0.001).

Table 1 Correlation of histological grade, lymph node stage, and size of primary breast tumor with estrogen receptor status measured by ER-EIA

<table>
<thead>
<tr>
<th>Grade</th>
<th>Stage</th>
<th>Size (cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>II</td>
<td>III</td>
</tr>
<tr>
<td>ER-EIA positive (&gt;5 fmol/mg protein)</td>
<td>15/15 (100)*</td>
<td>19/22 (86)</td>
</tr>
<tr>
<td>ER level in ER-EIA-positive samples (fmol/mg protein)</td>
<td>854 ± 266*</td>
<td>905 ± 296</td>
</tr>
<tr>
<td></td>
<td>A</td>
<td>B</td>
</tr>
<tr>
<td>6/7 (86)</td>
<td>11/12 (92)</td>
<td>26/31 (84)</td>
</tr>
</tbody>
</table>

* Numbers in parentheses, percentage.
* Mean ± SE.

Table 2 Distribution of histological grade of tumor in patients of known menopausal status

<table>
<thead>
<tr>
<th>Grade</th>
<th>I</th>
<th>II</th>
<th>III</th>
</tr>
</thead>
<tbody>
<tr>
<td>Premenopausal</td>
<td>3 (13)*</td>
<td>7 (30)</td>
<td>13 (57)</td>
</tr>
<tr>
<td>Postmenopausal</td>
<td>12 (28)</td>
<td>15 (35)</td>
<td>16 (37)</td>
</tr>
</tbody>
</table>

* Numbers in parentheses, percentage.

Clinical Correlations. In this series there was a correlation between the proportion of tumors that were ER-EIA positive and the histological grade of malignancy (χ², 4.996; P < 0.1). Thus, while all well-differentiated Grade I tumors were ER-EIA positive, the corresponding values for Grade II and III tumors were 19 of 22 (86%) and 24 of 32 (75%) ER-EIA positive, respectively (Table 1). In addition, Grade I and II tumors also had higher mean ER-EIA values than Grade III disease. This, however, may result from a disproportionate number of postmenopausal women in these groups (Table 2). No relationship between tumor ER-EIA content and lymph node staging of the disease or tumor size was evident (Table 1).

Discussion

Previous studies using the rat monoclonal antibodies D547 and H222 have established their specificity for human ER proteins (3). These antibodies now form the basis of a simple and sensitive assay for ER in cytosol fractions of breast tumors (4) with, in the present study, a low intraassay coefficient of variation of 5.4% and 16.7%, respectively. The coefficients of variation of <6% and <18%, respectively, for the ER-EIA determined in a European multicenter trial (9) indicate the reproducibility and reliability of the assay.

Parallel assays of cytosol fractions from 70 breast tumors using the ER-EIA and ERU methods revealed an excellent linear correlation between the assays at low and medium receptor levels (<400 fmol/mg of cytosol protein by ER-EIA). Unfortunately, this correlation was not maintained at high receptor values where the ERU assay severely underestimated the receptor concentration (Fig. 3). This result was, however, expected since the concentration range of [³H]estradiol used in the ERU only accurately measures receptor binding up to approximately 200 fmol/mg of protein. At higher concentrations of receptor the use of subsaturating quantities of radiolabeled estradiol introduces a major negative bias into the assay. In contrast, quality control studies have demonstrated that the ER-EIA accurately measures ER concentrations up to 500 fmol/ml of cytosol, and the inclusion of a 1:3 dilution of the cytosol in the assay gives an effective range up to 1500 fmol/ml. Thus in diluted cytosols the ER-EIA measured receptor concentrations in excess of 4000 fmol/mg of protein.

In addition to the above source of error in the ERU results, the ERU assay also gave lower receptor values than the ER-EIA for receptor concentrations below 400 fmol/mg of protein (approximately 40% lower in all cases) and a higher proportion of ER-negative tumors (20 to 70 ERU negative; 11 of 70 ER-EIA negative). Three not mutually exclusive characteristics of the assays may explain these results.

(a) Antigenic sites are less labile than steroid binding sites (10). In the routine assay thiol reagents are normally included.

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in the buffer at all stages of cytosol preparation to protect the steroid binding site from inactivation. In this study, however, since the action of the mercurial reagent used in the exchange procedure is blocked by thiol reagents, the cytosol pool was prepared in the absence of monothioglycerol. The steroid binding sites were thus unprotected for approximately 1 h after homogenization, and some loss of binding capacity is therefore to be expected. Moreover, the thiol reagent normally used in our routine assay is dithiothreitol, and in the present study this was substituted with monothioglycerol. It is our experience that this substitution introduces a small (10%) but significant decrease in the binding of [3H]estradiol to its receptor.\(^5\)

(b) The routine binding assay was carried out under conditions which measure only unoccupied receptor sites, whereas the ER-EIA measures both occupied and unoccupied receptors. Thus, under conditions of occupancy of the steroid binding sites by endogenous estrogens the ERU would underestimate the true value of ER. Indeed, a degree of receptor occupancy was observed since the application of a [3H]estradiol exchange procedure to the 9 ERU-negative ER-EIA-positive samples indicated some steroid binding capacity in 7 tumors. Similarly, Thorsen (11) using an exchange procedure based on the hydroxylapatite technique has shown significant levels of occupied receptor in ERU-negative samples. Moreover, studies from our own laboratories examining the concentration of estradiol in breast tumor homogenates by gas chromatography-mass spectrometry have shown that substantial quantities of estradiol are present in both ERU-positive (55 ± 92 pg/mg of cytosol protein) and ERU-negative (23 ± 43 pg/mg of cytosol protein) tumors. These data are therefore consistent with a degree of receptor occupancy which may be detected either by an exchange procedure or by the ER-EIA. Interestingly, the detection of receptor occupancy by the ER-EIA assay was observed in both pre- and postmenopausal women and reflects the similar levels of endogenous estradiol in tumors from these two sources (7).

(c) Finally, receptor protein fragments or precursors without steroid binding capacity may be detected by ER-EIA. This explanation is, however, unlikely, since the second antibody (H7222) detects an antigenic determinant close to the steroid binding site (3).

No data are as yet published on the prognostic significance of the ER-EIA assay with respect to breast tumor growth and progression. Its emerging relationship with the hormone sensitivity of breast tumors \(^6\) and its correlation with the histological grade of malignancy of tumors (Table 1), however, favor an improved prognosis for women with ER-EIA-positive tumors.

References


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\(^5\) R. I. Nicholson, unpublished observations.

\(^6\) R. I. Nicholson, R. A. McClelland, and C. Coombes, unpublished observations.
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