Comparison of Immunochemical and Radioligand Binding Assays for Estrogen Receptors in Human Breast Tumors

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Abstract

We have compared a new enzyme immunoassay (EIA) for estrogen receptors (ER) with our conventional radioligand binding assays (multipoint dextran-coated charcoal assay for cytoplasmic ER and hydroxylapatite exchange assay for nuclear ER). Cytoplasmic ERs were measured in 76 human breast cancer specimens by EIA and by five-point Scatchard analysis. The correlation between the two assays yielded a straight line with a slope of 0.92 (r = 0.95; P < 0.001); conversely, in 31 nuclear salt extracts, linear regression analysis of hydroxylapatite exchange assay data with EIA showed a clear correlation (r = 0.93; P < 0.001) but a slope of 1.7, demonstrating that EIA detects more ER sites. The binding of the antibody to the cytoplasmic ER molecules was investigated by sucrose density gradient analysis, which showed that EIA recognizes both cytoplasmic forms (9 and 3S), but does not distinguish between them. Advantages and drawbacks of this method are discussed with respect to its application for routine receptor determination for clinical management of breast cancer patients.

Introduction

Our present knowledge concerning the biochemical properties and the mechanism of action of ER1 has been obtained by use of methods based on the interaction of a radioactive specific ligand with the receptor protein. Despite the large number of results obtained by this method, the need has emerged for an alternative assay that will recognize receptors irrespective of their hormonal binding activity. In fact, the binding activity can be affected by many factors, such as the concentration of exogenous reduced sulphhydryl groups, temperature, pH and ionic strength of the medium, and even by endogenous hormones that occupy the binding site and do not allow the detection of the receptor under normal assay conditions (1). The limitations inherent in traditional binding assays have been recently overcome by the preparation of a series of monoclonal antibodies to ER, which provide an alternative approach for the detection, purification, and characterization of ER (2, 3). A solid-phase, sandwich type EIA for human ER has been developed by Abbott Laboratories using two antibodies that recognize different antigenic sites on the ER molecule. This assay allows the processing of human tumors for ER detection in an easier and quicker way.

The clinical usefulness of biochemical ER assays in specimens of human breast cancer has been clearly established by correlations of patient responses to endocrine therapies (4–6). There is also evidence that patients with certain ER concentrations in their tumors have a better prognosis than do ER-negative cases (7–10). Since these results have been obtained using radioligand binding assays, some caution should be used in the clinical interpretation of results obtained by immunochemical techniques. This could be the case for premenopausal breast cancer patients, for whom most investigators have estimated mainly the unoccupied fraction of ER binding sites, whereas the antibody recognizes indistinctly apo- and holoreceptors (11); moreover, irrespective of the occupancy of endogenous ligands, from a theoretical point of view monoclonal antibodies could also detect those receptors which for some obscure reason lack binding activity; therefore, for clinical purposes traditional binding methods should not be neglected until immunochemical techniques have been extensively evaluated in comparative studies performed on human tissue specimens.

In the present study we compared the new EIA with conventional assays in a series of 76 breast cancer patients using a five-point DCC assay and a HAP exchange assay for the cytosolic and nuclear fractions, respectively. We also checked the ability of the antibody to recognize either occupied or unoccupied receptors and differential molecular forms of ER recovered from the cytoplasmic fraction and identified by sucrose density gradient analysis.

Materials and Methods

Materials. 16α-[125I]Iodoestradiol (200 Ci/mmol in ethanol solution) and 17β-[2,4,6,7-3H]estradiol (85–110 CI/mmol in toluene ethanol solution) were obtained from the Radiochemical Centre (Amersham, England). Radioinert 17β-estradiol, diethylstilbestrol, Norit-A charcoal, gelatin, dextran T70, human γ-globulin, and bovine serum albumin were obtained from Sigma Chemical Co. (St. Louis, MO). Abbott ER-EIA monoclonal kits were purchased from Abbott Laboratories Diagnostic Division (North Chicago, IL). All other chemicals were of analytical grade.

Tumor Cytosol Preparation. Tissue samples obtained at surgery were immediately frozen and stored in liquid nitrogen for not more than 4 weeks. Homogenization was performed by mechanical disruption with a microdismembrator (Braun, Melsungen, West Germany), and the obtained powder was resuspended in 8 volumes of ice-cold 20 mM K2HPO4, pH 7.4 at 4°C, 1 mM EDTA, and 3 mM NaN3 buffer. After 15 min the charcoal was pelleted by centrifugation at 2000 x g for 10 min. Total and nonspecific binding data were analyzed using the formula of Eldridge and Gough (12). Where specified, an exchange assay was performed essentially as described above but incubating for 2 h at 25°C. Total and nonspecific binding data were analyzed according to the method of Scatchard (13) and expressed as fmol of...
specifically bound iodinated steroid per mg of cytosolic protein. Total protein content was measured using the Coomassie blue G250 dye (Bio-Rad, Munich, West Germany) essentially according to the method of Bradford (14) using a mixture of human albumin and human \( \gamma \)-globulin (5:3) for the calibration protein curve.

Nuclear HAP Exchange Assay. The nuclear myofibrillar pellet was quickly washed twice with 10 mM K\( _2 \)HPO\( _4 \), pH 7.4 at 4°C, 5 mM MgCl\( _2 \), 3 mM Na\( _2 \)SO\( _4 \), 250 mM sucrose, and 0.1% Triton X-100 buffer and twice with the same buffer but without Triton X-100. The washed nuclear myofibrillar pellet was extracted with a suitable volume of homogenization buffer containing 0.5 M KCl with vortexing at 20-min intervals, and after 1 h extracts were spun at 100,000 \( \times \) g for 20 min to obtain a soluble nuclear extract. HAP (DNA grade Bio-Gel HTP, Bio-Rad, Richmond, CA) was washed several times according to the method of Carola and McGuire (15). Nuclear extracts were incubated with a HAP slurry in 50 mM Tris-HCl, pH 7.2 at 4°C-10 mM K\( _2 \)HPO\( _4 \)-3 mM Na\( _2 \)SO\( _4 \) buffer for 45 min with gentle vortexing and finally centrifuged at 800 \( \times \) g for 5 min to discard the supernatant. Nuclear pellets were incubated with 5 nM 17\( \beta \)-[\( ^3 \)H]estradiol in the presence or absence of a 200-fold \( M \) excess of diethylstilbestrol for 2 h at 30°C. At the end of the incubation, pellets were washed twice with the HAP suspension buffer supplemented with 1% Tween 80 to remove unbound ligand and finally extracted overnight with ethanol at room temperatures. The alcohol extracts were counted in 5 ml of a commercial scintillation cocktail (Pico-Fluor 15; Packard, Downers Grove, IL) using a Packard 300D spectrometer with automatic quench correction on each sample. ER values were expressed in fmol/mg DNA. DNA concentrations were determined on nuclear pellets by the diphenylamine method essentially according to the method of Burton (16).

EIA. The EIA was performed on the cytosolic and nuclear fractions by use of an Abbott ER-EIA monoclonal kit. A Quantum I spectrophotometer (Abbott) was used to measure the absorbance at 492 nm and for data reduction. ER concentrations could be quantified only in the range of 0–500 fmol/mg of cytosol.

Sucrose Density Gradient Analysis. Cytosols undergoing sucrose density gradient analysis were incubated with 17\( \beta \)-[\( ^3 \)H]estradiol in the absence or presence of a 200-fold \( M \) excess of diethylstilbestrol at 0–4°C for 4 h. Unbound and weakly bound ligands were removed by treating the reaction mixtures (300 \( \mu \)l) with a DCC pellet obtained from a 500-\( \mu \)l suspension essentially as described for the saturation analysis. Clear supernatants (200 \( \mu \)l) were layered on 5–20% (w/w) linear sucrose gradients (4 ml) prepared in the homogenization buffer without thioglycerol. Gradients were spun at 370,000 \( \times \) g for 16 h at 3°C using an SW60Ti rotor in an L8-70 ultracentrifuge (Beckman, Palo Alto, CA). A mixture of \( ^3 \)H-labeled human \( \gamma \)-globulin and \( ^3 \)C-labeled bovine serum albumin, prepared by reductive alkylation according to the method of Rice and Means (17), was run as an internal marker. Fractions of seven drops each were collected from the bottoms of the tubes. Radioactivity counting was carried out as described for nuclear ER, except that appropriate \( ^3 \)H/\( ^3 \)C standard efficiency curves were used for counting of dual-labeled samples.

Results

Cytoplasmic ER determinations of 76 consecutive breast cancer specimens were carried out by EIA and multipoint Scatchard analysis, and the results were compared. Values obtained by these methods were expressed in fmol ER per mg cytosol protein and plotted (Fig. 1). A very clear correlation was noted between the 2 sets of values as demonstrated by regression curve analysis (\( r = 0.95 \); \( P < 0.001 \)). A straight line with a slope of 0.92 was obtained, meaning that on the average the EIA and our radioligand binding assay gave the same estimation of receptor concentrations. In a small subgroup of patients (three premenopausal, four postmenopausal), ER levels were determined by normal and exchange assays and the results were compared to values obtained with the EIA in order to assess the contribution of occupied sites. No differences were found since there was a good correlation between the results obtained with the first 2 assays and those obtained with the latter (\( r = 0.999 \) and 0.998, respectively), and in both cases the slope was about 1.

The influence of total protein content in cytosols processed by the EIA was studied by serial dilutions with homogenization buffer. ER levels of the undiluted cytosol and five serially diluted samples were separately determined by EIA. The third sample was chosen as a reference, since its protein concentration fell within the range recommended by the instructions for the kit. Table 1 reports the results of the EIA expressed as fmol per mg of cytosol together with the dilution factors and theoretical ER values calculated starting from the reference value. Differences between theoretical and experimental data were more evident for the less diluted samples, corresponding to a total protein concentration higher than 2 mg/ml.

The comparative study between the EIA and the radioligand binding assay was carried out also on 31 nuclear extracts. The linear regression of values expressed as fmol per mg of DNA yielded a correlation coefficient of \( r = 0.93 \) and \( P < 0.001 \). Although we used the HAP exchange assay for detection of total nuclear sites, ER concentrations obtained by the EIA were always higher than the corresponding ones determined by our nuclear assay; however, there was a significant correlation between the data obtained by these two methods (\( r = 0.93 \); \( P < 0.001 \)). Linear regression analysis of cases yielded a straight line but with a slope of 1.74, indicating the ability of the EIA to detect a greater number of ER sites. The results are reported in Fig. 2.

Some of the tumors were also characterized by sucrose density gradient analysis. For most of these cases the sedimentation profile showed the presence of both main cytoplasmic molecular forms (9 and 3S). A typical sedimentation pattern is shown in
by linear regression analysis of data. Data on abscissa and ordinate are expressed in fmol of specifically bound steroid per mg of DNA.

HAP exchange assay on 31 human breast cancer specimens. The plot was drawn conventionally with radioligand binding assays has become particular for measuring cytoplasmic ER in 76 breast cancer patients. Linear regression analysis demonstrated a clear and significant correlation between the two methods, which detected similar levels of ER as shown by the slope value of about 1; moreover, this method is not influenced by the molecular form of the ER, as demonstrated by sucrose density analysis performed in parallel on some of our samples, which yielded ER values very close to those found with the EIA despite the prevalence of one form over the other.

We did not find a better correlation with the EIA using the exchange assay than with low-temperature methods, which generally allow the detection only of unfilled sites. A possible explanation is that in our breast cancer patients the fraction of occupied sites was very low and did not contribute significantly to the total ER levels. In the case of nuclear ER, the overestimation of binding sites obtained with the EIA with respect to the HAP assay was due to the fact that in the nucleus a larger fraction of receptors are expected to be saturated by endogenous steroids. Discrepancies between the two methods could therefore depend on an incomplete exchange with endogenous hormones or on a temperature-dependent inactivation of the receptor during the radioligand binding assay. The EIA is therefore a reliable technique for measuring nuclear ER, and it allows a simplification of the experimental procedure with respect to the rather cumbersome HAP exchange assay; however, it appeared that the total cytosol protein content influenced ER-EIA results outside a certain concentration range. In fact, cytosol protein values higher than 2 mg/ml caused the EIA to overestimate ER levels, whereas at low protein concentrations the protein assay was not always reliable.

Conversely, in the case of cytoplasmic ER, we would not recommend the introduction of the EIA for routine use despite the good correlation found by Scatchard analysis. In our opinion, before switching to immunochemical methods, ER data obtained by the EIA should prove to be at least as reliable as those obtained with the radioligand binding assay for predicting response to hormone therapy and prognosis.

The routine use of the EIA does not actually represent A simpler way to obtain receptor data for management of breast cancer patients, since recent data support the need to measure also the progesterone receptor (10, 18, 19); unfortunately, the only way to measure the progesterone receptor is with radioligand binding assays (20); however, we suggest the use of the EIA for a quick and reliable estimation of nuclear ER, whereas in the case of cytoplasmic ER we think that until there are data showing a correlation between EIA results and clinical follow-up.

Discussion

In the last few years, the need for an alternative approach to conventional radioligand binding assays has become particularly evident. In fact, results obtained by these methods are influenced by slight variations in the experimental procedure and should not be used by laboratories lacking experience.
up, immunochemical assays should not replace conventional radioligand binding techniques.

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