Estrogen Receptors by Immunofluorescence: Comparison with a Dextran Coated Charcoal Technique* 

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ABSTRACT

Biopsy specimens from 21 women with primary operable breast cancer were analyzed in a double blind study designed to compare fluorescent antibody methods for detection of estrogen receptors with estrogen receptors measured biochemically with a previously reported dextran coated charcoal technique (DCC). Fluorescent antibody methods employed both direct techniques: (1) rabbit antisera to 6-O-Carboxymethyl-Oxime-17 beta Estradiol tagged with Fluorescin Isothiocyanate (FITC) and (2) indirect techniques (sheep antisera to 11-Hemisuccinate-17 beta Estradiol reacted with FITC tagged rabbit antisheep sera). Fluorescent antibody methods showed 100 percent correlation with each other but only 67 percent correlation with the DCC assay. Fluorescence was striking when present. Molecular forms were not easily predicted. Divergent results occurred largely in specimens sparsely populated with malignant cells (18 percent) though neoplasms showing no fluorescence demonstrated elevated receptor concentrations with DCC (15 percent). Indirect fluorescent techniques are the simplest of those studied. Fluorescent techniques have not replaced biochemical techniques in the authors' laboratories.

Introduction

Estrogen receptor (ER) analysis is of importance in predicting response to hormonal therapies in women with breast cancer. Biochemical assays currently employed measure the estradiol binding capacity of proteins in the cytosol fraction of tissue homogenates. Results are influenced by the actual mass of the epithelial component of the cancer in the tissue submitted for assay, the amount of cancer receptor protein extractable into the cytosol, and the proteins extractable from other noncancerous tissue components. ER is determined by extrapolation of Scatchard analysis of specific binding
data for estradiol. Cytochemical methods have been proposed to investigate estradiol binding of individual cancer cells\textsuperscript{5,9,10,11,12,13} and to serve (potentially) as an alternative or as a supplementary predictor with the biochemical assay.

Direct and indirect immunofluorescent (IF) techniques have been examined for ER in a double blind study to analyze 21 unselected biopsy specimens from patients with breast cancer. The results of ER determinations by IF study were compared to those of conventional biochemical analysis by dextran coated charcoal (DCC) assay.

**Materials and Methods**

The biopsy specimens consisted of 21 primary breast cancers (infiltrating ductal carcinoma) submitted to the laboratory for analysis. Fresh biopsy tissue was frozen in liquid nitrogen and stored at \(-80^\circ\). One portion was submitted for ER by DCC and the remainder was processed for ER by IF study.

**Dextran Coated Charcoal Assay**

Cytosol is prepared by mincing 200 to 300 mg of frozen tumor tissue in ice. Minced tissue is placed in chilled buffer of tris (hydroxymethyl) aminoethane (10 mM per L), ethylenediaminetriacetate (1.5 mM per L), and dithiothreitol (0.5 mM per L), pH 7.4. The tissue is then homogenized in an ice bath. The homogenate is placed into a cellulose nitrate centrifuge tube and centrifuged at 4\(^\circ\) for 45 min at 105,000 \(\times g\). The cytosol is removed and pipetted into chilled assay tubes, inoculated with 1.25 \(\times 10^{-9}\)M diethylstilbestrol (DES)* in absolute ethanol, and evaporated to dryness. 17 Beta-(2,4,6,7(n)-\(^3\)H) Estradiol, \(\dagger\) 90–100 Ci per mM, without further purification is evaporated to dryness, dissolved in buffer, and diluted to specific concentrations for use (160, 320, 480, 640, 800 fM). These are pipetted into tubes containing cytosol, with and without DES. Tracer is also pipetted in quadruplicate into liquid scintillation vials for total counts.

All tubes are mixed by vortex and allowed to incubate 16 hrs at 4\(^\circ\)C. An aliquot of cytosol is taken for protein determination. Cold, 1 percent DCC is added to each assay tube to end the incubation (Norit A activated charcoal, \(\dagger\) Dextran T-70\(\S\)). Tubes are vortexed and allowed to incubate for 10 min before centrifuging at 4\(^\circ\) at 2500 \(\times g\) for 15 min. Supernatant is pipetted into scintillation vials and scintillation cocktail is added. A quench curve is also prepared. Known positive and negative human breast cancer controls are employed in each run.

A BASIC program on a desktop computer\(\dagger\) is used to handle receptor data analysis. Count rates for total, assay, and blank (DES) tubes, the specific activity of tracer, and cytosol protein concentration are the raw data required. Count data is corrected for efficiency. Assay counts are blank corrected. This normalized bound data is then converted into moles bound per mg of cytosol protein and plotted on the abscissa. The ordinate is bound/free. Linear least mean squares regression is then performed to obtain slope, y-intercept, and x-intercept. Ninety-five percent confidence intervals are determined for each point. Slope variability is examined with the F-ratio because the slope is the inverse of the binding constant and is used to calculate receptor concentration (x-intercept); the variability of the slope can be used to estimate the variability of the receptor concentration. The F-ratio can be used as an aposteriori test of univalent binding and, thus, as an estimator of

\(\dagger\) Fisher Scientific, King of Prussia, PA.
\(\S\) Pharmacia Fine Chemicals, Piscataway, NJ.
\(\dagger\) Hewlett Packard 9831A.
"linearity." Assumptions of normality (and also, by extension, univalent binding) are not required to obtain valid receptor concentrations.

Protein concentrations of the cytosol approximate 2 mg per ml. Lower concentrations could result in false negative results; higher concentrations render interpretation difficult.

Results of DCC are considered positive when a receptor concentration greater than 10 fM per mg of cytosol protein is obtained and a dissociation constant on the order of 1nM is seen.

INDIRECT FLUORESCENT ANTIBODY ASSAY

Following the method of Pertschuk,12 commercially prepared polyestradiol phosphate (PEP)* was purified by gel filtration on a Sephadex G25 column, 2.5 x 100 mm, at 4°, and eluted with deionized water. Steroid content of eluates was measured by ultraviolet absorption at 279 nm. PEP from peak 1, which eluted with the void volume, was used in a concentration of 1.0 mg per ml in phosphate buffered saline (PBS), pH 7.2.

Frozen sections 4 μm thick were cut and mounted on gelatin coated glass slides. One section was stained with hematoxylin and eosin, and other sections were taken for IF studies. All steps for IF study were carried out at 4°. Slide sections were incubated for 60 min with PEP and washed for 15 min in PBS. Sections were then incubated for 30 min with a 1:10 dilution of sheep anti-11-hemisuccinate-17 beta estradiol serum † and washed with PBS for 15 min. The tissue sections were then covered with a 1:10 dilution of fluorescein conjugated rabbit anti-sheep immunoglobulin † and washed with PBS for 60 min. All dilutions were made in PBS containing 4 percent bovine serum albumin (BSA). All specimens were additionally subjected to a competitive binding study with antiestrogen by incubation of sections with tamoxifen § 1.0 mg/ml in phosphate buffered saline for 60 min following exposure to PEP.

Parallel sections of each tumor were incubated in PBS instead of PEP to allow for detection of estradiol bound in vivo. Controls included sections overlaid with non-immune sheep serum (serum control), sections treated with labelled antirabbit sera (conjugate control), sections overlaid with unlabelled rabbit anti-sheep serum prior to labelled serum (blocking control), and sections processed with estradiol antiserum previously absorbed with estradiol (absorption control). Known positive and negative human breast cancer tissue controls were also employed. Sections were mounted in buffered glycerol for examination by incident UV light using an AO Fluorostar microscope with an ABG 12 exciter filter and barrier filter unit 1100 with a 200 watt mercury arc lamp. Sections stained with hematoxylin and eosin were examined by light microscopy.

Tumors were evaluated for the presence of fluorescent cells and the pattern of fluorescence (cytoplasmic or nuclear). Positive identification of ER was accepted only when blocking, conjugate, serum, and absorption controls were negative and tamoxifen but not PBS inhibited the observed fluorescence. A decision as to positive or negative for ER was made without prior knowledge of the results of DCC assay. Decisions as to positive or negative by IF followed criteria outlined by Pertschuk.12

DIRECT FLUORESCENT ANTIBODY ASSAY

Fluorescin conjugated rabbit antiserum to 6-O-Carboxymethyl-Oxime-17 beta es-

* Ayerst Laboratories, NY.
† Calbiochem, La Jolla, CA.
‡ Wellcome Reagents, Research Triangle Park, NC.
§ Upjohn Company, Kalamazoo, MI.
tradiol was prepared. Rabbit anti-6-O-carboxymethyl-oxime-17 beta estradiol-BSA sera* and fluorescin isothiocyanate (FITC)† were obtained. The method of Riggs14 was employed. Antiserum was diluted 1:10 in 0.5 M carbonate-bicarbonate buffer, pH 9.5 FITC on celite was added to the solution and stirred for four hrs at room temperature. Celite was removed by centrifugation. The supernatant was purified through a Sephadex G25 column previously equilibrated with phosphate buffer, pH 7.8. Eluted material was dialyzed against the same buffer for 48 hrs. Molecular fluorescin per protein ratio was determined (2.0). Silica gel thin layer chromatography with a solvent system of ethyl acetate: n-hexane: ethanol: acetic acid (72:13.5:4.5:10) was employed to look for free fluorescin or hormone fragments.5 None was seen.

Frozen sections for direct IF study were hydrated with PBS for 15 min. All steps were carried out at 4°. Sections were incubated with fluorescin conjugated antisera for 30 min and washed in PBS for 60 min. Sections were mounted in buffered glycerol for examination by fluorescent microscopy. Competitive binding studies with tamoxifen and the use of controls as described with the indirect IF assay were employed.

Tumors were evaluated for the presence of fluorescent cells and the criteria of Pertschuk were employed.12 Again, a decision as to positive or negative for ER was made without prior knowledge of the results of the DCC assay.

Results

Tumor cells positive for ER fluoresced readily with incident light UV microscopy. The staining intensity of the cells was independent of topographic location. Tumor cell populations were heterogeneous for ER as has been noted by others.5,8,10,11,12,13 Distinction between positive and negative specimens, however, was readily made in that fluorescence when present in positive lesions was striking and tended to involve the entire tumor mass. Cell counts were superfluous.

The location of ER within malignant cells could be determined but with some difficulty. Cytoplasmic staining predominated in all lesions. Nuclear patterns could be seen.

Blocking, conjugate, and serum controls were negative. Absorption of specific antisera with estradiol eliminated anti-estradiol activity. Competition studies with tamoxifen were successful in abolishing specific fluorescence of viable malignant cells. Nonspecific binding of estradiol was not a problem in the IF assay.

In vivo bound estrogen was detected in one specimen, from a premenopausal woman, and was displaced by tamoxifen but not by buffer. The presence of in vivo bound endogenous hormone did not affect negative ER assay by DCC or IF study. In vivo bound estrogen was not found in other tumors.

The results of ER by both IF assays are compared in table I. Complete concordance was noted. The results of ER by the direct IF study are compared to ER by DCC in table II; by the indirect IF study, in table III. Twelve specimens (57 percent) were positive by all three assays. Two specimens (10 percent) were negative by all three assays. The results of ER by IF did not correlate with DCC in seven specimens (33 percent).

### TABLE I

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<tr>
<th>Comparison of Results of Estrogen Receptors by Direct and Indirect Immunofluorescence Study</th>
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<tr>
<td>Positive direct IF and indirect IF</td>
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<td>Negative direct IF and indirect IF</td>
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<td>Failure to correlate</td>
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IF = Immunofluorescence
Those tissues which did not correlate by IF and DCC were studied further. Four were positive by IF but negative by DCC. These four were sparsely populated by malignant cells. In these cases it is possible that DCC was not sufficiently sensitive to detect the relatively small concentration of receptor present in the cytosol. In three other specimens, negative by IF but positive by DCC, differing results could not be explained. The estradiol employed in the direct IF assay retains immunologic specificity. Sampling variation, as has been proposed by another group, was not felt to be likely in this instance.

Discussion

Current biochemical methods of ER assay present certain disadvantages. Sucrose gradient analysis (SGA) demonstrates the molecular form of ER. The technique is limited by the number of specimens which can be processed. Sensitivity is comparable to DCC. Protamine sulfate (PS) precipitation and DCC assays can be used to process large numbers of specimens with clinically valid results. PS and DCC require an ultracentrifuge and a liquid scintillation counter as does SGA. Gel filtration techniques require multiple columns for each assay, and electrophoretic methods do not separate the estrogen binding peak from contaminating proteins. Both PS and DCC fail to give information about molecular forms of ER. Recent evidence suggests tumors with the bulk of ER sedimentation at 4S (nuclear) may not respond to endocrine therapy. Techniques lacking the ability to differentiate molecular forms may have limited value, though the determination of progesterone receptor by similar techniques may provide the same information as molecular form.

IF overcomes objections of equipment availability and the need to distinguish molecular forms. The methodology is applicable to the study of small specimens unsuited for biochemical analysis. Because the technique is primarily morphologic, intact tissue may be examined and the presence, proportion, and distribution of estrogen receptor can be evaluated. Both the direct and indirect assay have been employed in our hands with acceptable results. Cell culture is not required as in another reported assay. Progesterone receptors may also be examined by IF techniques. The disadvantage of IF study is the present lack of standardization and the difficulty in quantification of results.

The conundrum of negative IF and positive DCC results has yet to be dealt with in any meaningful fashion. IF has not yet replaced or supplemented DCC in our hands.

References

1. Bloom, N., Tobin, E., and Dagenshein, G. A.: Clinical correlations of endocrine ablation with estrogen and progesterone receptors in ad-


