A Hydroxylapatite Micromethod for Measuring Estrogen Receptor in Human Breast Cancer

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ABSTRACT

The concentration of estrogen receptor (ER) in a human breast tumor is a critical variable predicting the response to endocrine therapy and the course of the disease. Since many tumor specimens are quite small, a reliable and simple ER assay requiring a minimum of tissue is desirable. We here describe a hydroxylapatite assay for ER that (a) requires only a single saturating concentration of [3H]estradiol, (b) agrees with more complex multiple-concentration assays and with the standard dextran-coated charcoal assay at normal protein concentrations, (c) is far more reliable than the latter at low protein concentrations, and (d) can be adapted to an accurate and reliable ER microassay requiring less than 50 mg of tissue.

INTRODUCTION

In recent years the ER content of human breast cancers has proven to be important for the selection of patients for endocrine therapies (13). This biochemical parameter has prognostic value as well (8). Other steroid receptors are also present in human breast cancer cells and may provide additional information. The presence of progesterone receptor may be a further indicator of hormone dependence (12). Androgen receptor has also been found in breast cancers (19), although its role is not known. The human breast cancer cell line MCF-7 (derived at the Michigan Cancer Foundation) (17) has been demonstrated to contain all of these plus glucocorticoid receptor (6). Although the significance of these additional steroid receptors is not yet clear, it is probable that simultaneous assays for all of these receptors in human breast tumors will be useful. However, the average tumor specimen is not large enough to allow all of these determinations utilizing existing methods.

In an effort to establish quicker and more accurate receptor assays requiring less tissue, this laboratory previously validated a simpler DCC assay for ER (11). We now demonstrate the use of a hydroxylapatite "batch" assay (20) to measure cytoplasmic ER in human breast cancer tissue and report a micromethod for use with even smaller tissue samples.

MATERIALS AND METHODS

Sample Preparations. Human breast cancer tissues are stored and pulverized as described in our previous work (4, 5). The pulverized tissue is homogenized in 2 ml of phosphate buffer (5 mM sodium phosphate, pH 7.4; 1 mM monothioglycerol; and 10% glycerol) per g of tissue with three 10-sec bursts of a Polytron PT-10-ST homogenizer at a setting of 3.2. Each homogenate is transferred to a polycarbonate tube for centrifugation at 105,000 x g for 30 min at 4°C to obtain the cytosol fraction. In the case of the hydroxylapatite micromethod, if a very small volume of cytosol is required (<0.3 ml) homogenization can be accomplished in a glass-glass microhomogenizer. The homogenate is then transferred instead into a 4.8- x 19.9-mm cellulose nitrate tube and centrifuged at 100,000 rpm for 10 min at 4°C in a miniature fixed-angle rotor type A-100 in a Beckman Airfuge. This is sufficient to clear particles with a sedimentation coefficient of 70S or more.

The protein concentration of both cytosol preparations is first estimated by absorbance at 260/280 nm (9) and is later confirmed by the method of Lowry et al. (10). The protein concentration is routinely diluted to between 1 and 2 mg/ml.

DCC-SSD and HAP-SSD Assays. Cytosol (0.2 ml) is incubated for 4 hr at 4°C with 0.2 pmol [3H]estradiol (approximately 100 Ci/mmol; Amersham Corp., Arlington Heights, III) delivered in a volume of 25 µl phosphate buffer. Parallel tubes contain a 100-fold excess of DES (Sigma Chemical Co., St. Louis, Mo.) in addition to [3H]estradiol. Each assay is performed in triplicate.

For the DCC-SSD assay 0.5 ml of DCC suspension (0.25% Norit A (Sigma Chemical Co.) and 0.0025% dextran (Grade C; Schwarz/Mann, Orangeburg, N. J.) in 0.01 M Tris, pH 8.0) is added to each tube, which is then further incubated for 30 min at 4°C with vigorous shaking in an Eberbach shaker (Eberbach Corp., Ann Arbor, Mich.). The tubes are centrifuged for 10 min at 4°C at 1600 x g. A 0.5-ml aliquot of each supernatant is counted for radioactivity in 5.0 ml of modified Bray's solution (125 g naphthalene, 7.5 g PPO, and 0.377 g POPOP made up to 1 liter with dioxane) with a Beckman LS 233 counter at 40% efficiency.

For the HAP-SSD assay DNA-grade Bio-Gel HTP hydroxylapatite (Bio-Rad) is gently washed with TP buffer (50 mM Tris and 10 mM K2HPO4, pH 7.2, at 4°C) until the pH of the washes remains at 7.2. The settled hydroxylapatite is then resuspended in 2 columns of TP buffer, and 0.25 ml is added to each tube containing cytosol incubated as above; the mixture is incubated for 30 min at 4°C with gentle blending on a vortex mixer every 10 min. The samples are then centrifuged at 800 x g for 2 min, and the pellets are
Hydroxylapatite Method for ER

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**RESULTS**

Hydroxylapatite-ER Adsorption and Pellet Extraction. Previous reports have demonstrated the adsorption of ER (2) as well as other proteins by hydroxylapatite in columns (18). The first question approached here was the adsorptive capacity of hydroxylapatite in batch (14, 16, 20) for cytoplasmic ER from human breast tumors. A tumor cytosol of known ER content was incubated with [3H]estradiol and treated with DCC to remove excess free hormone. It was then adsorbed with hydroxylapatite, which was washed and then extracted with ethanol. All fractions were counted. The results, shown in Table 1, demonstrate that ER adsorption by hydroxylapatite and subsequent extraction of [3H]estradiol by ethanol are very nearly quantitative. Similar results were obtained with this procedure with the use of other tissues.

**Nonspecific Binding.** The nonspecific binding of [3H]estradiol is determined by DES competition and subtracted to find the specific receptor value. It represents the binding of [3H]estradiol to low-affinity sites other than receptor, such as serum contaminants. When specific binding is exceedingly low, nonspecific binding contributes significantly (50% or more) to the total binding, thus obscuring receptor measurements. To reduce nonspecific binding without interfering with specific binding, we tried several wash buffer systems as described in Table 2. As suggested by Clark et al. (1), we also tried washing pellets with different concentrations of phosphate, but in our system this procedure does not reduce nonspecific binding (data not shown). We found that the most successful procedure is to wash the hydroxylapatite pellet twice with 2 ml 1% Tween 80 in phosphate buffer. This method diminishes the nonspecific binding by about 40% without interfering with specific binding, as was previously observed (3). These results were verified with other tumors.

**Effect of Protein Concentration.** Since many tumor tissues sent for assay are quite small (approximately 25% weighing less than 0.3 g), it is clear that low protein concentrations will sometimes be encountered. Chart 1 compares values obtained by DCC-SSD and HAP-SSD assays as a function of cytosol protein content.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Total (fmol/mg protein)</th>
<th>[3H]Estradiol (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DCC supernatant</td>
<td>280</td>
<td>100</td>
</tr>
<tr>
<td>Hydroxylapatite wash</td>
<td>20</td>
<td>7</td>
</tr>
<tr>
<td>Hydroxylapatite extract</td>
<td>250</td>
<td>89</td>
</tr>
<tr>
<td>Hydroxylapatite pellet</td>
<td>5</td>
<td>2</td>
</tr>
</tbody>
</table>

***Table 2***

**Effect of various washes on hydroxylapatite-adsorbed cytosol [3H]estradiol**

A human breast tumor cytosol was incubated with [3H]estradiol and adsorbed with hydroxylapatite as described in "Materials and Methods." The hydroxylapatite was washed twice with 2 ml of the indicated buffer and then extracted with 100% ethanol for radioactivity determination. Each value was determined in triplicate.

<table>
<thead>
<tr>
<th>Wash buffer</th>
<th>Nonspecific (cpm)</th>
<th>Specific (cpm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phosphate buffer</td>
<td>1,631</td>
<td>19,556</td>
</tr>
<tr>
<td>+ 0.1% Triton X-100</td>
<td>758</td>
<td>18,315</td>
</tr>
<tr>
<td>+ 0.3% Triton X-100</td>
<td>595</td>
<td>18,207</td>
</tr>
<tr>
<td>+ 0.5% Triton X-100</td>
<td>572</td>
<td>18,207</td>
</tr>
<tr>
<td>+ 1% Tween 80</td>
<td>916</td>
<td>19,226</td>
</tr>
<tr>
<td>+ 0.1% Triton X-100 and 10% ethanol</td>
<td>498</td>
<td>13,416</td>
</tr>
<tr>
<td>+ 10% ethanol</td>
<td>1,192</td>
<td>19,696</td>
</tr>
</tbody>
</table>

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function of protein concentration. The DCC-SSD assay underestimated receptor content when protein was lower than 1 mg/ml, and the effect was exaggerated as protein decreased. In contrast, the hydroxylapatite assay produced constant ER values with protein concentrations down to 0.6 mg/ml and a smaller error than that produced by the DCC assay at lower concentration.

Similar findings have led to suggestions that DCC adsorbs some receptor at low protein concentrations (1, 7, 16). In experiments not shown we examined the loss of receptor and total cytosol protein and found that DCC treatment reduced total cytosol protein content by an average of 32% in dilute (<1 ng/ml) cytosols. In each instance the ER values assayed by DCC were lower than the values obtained by hydroxylapatite assay at these low protein concentrations.

For further determination of the limits of protein concentration for accurate hydroxylapatite assays, 3 different tumors containing, respectively, a low (7 fmol/mg), an average (40 fmol/mg), and a relatively high (82 fmol/mg) ER value were selected for assay by the hydroxylapatite method over a range of protein concentrations. The results presented in Chart 2 suggest that, when tumor cytosols contain less than 10 fmol ER per mg protein, at least a 1-mg/ml protein concentration should be used to achieve an accurate ER value, although even at 0.5 mg/ml the hydroxylapatite procedure can still measure at least 50% of the ER present.

Hydroxylapatite Micromethod. Although the above work demonstrates that the hydroxylapatite assay is accurate even at low protein concentrations when the ER value is average or high, the problem of achieving high sensitivity at low protein concentration remained. We therefore reduced the cytosol volume from 200 to 50 µl to preserve higher concentrations with smaller tumor samples. (This micromethod was described in “Materials and Methods.”) We first determined the acceptable limit of protein concentration. Chart 3 shows the data obtained from the micromethod of 4 human breast tumor cytosols with ER values (previously determined by DCC-SSD assay) of 3, 17, 35, and 124 fmol/mg protein, respectively, with the use of a range of protein concentrations for each. Even with low ER values, good replication is obtained above 1 mg/ml, and for the microassay a sufficient quantity of 1-mg/ml cytosol may be obtained from 30 to 50 mg of tumor tissue. In the case of the cytosol with 17 fmol/mg protein, the replication was good even with a protein concentration as low as 0.41 mg/ml, and the cytosols with higher ER values were measured accurately at even lower protein concentrations.

Since the micromethod seemed to be a feasible answer to the small-tissue problem, we proceeded to validate the method by assaying a random sampling of 20 human breast tumors. Chart 4 presents the results of the assay of these 20 tumors by a 6-point binding analysis, as compared with a single-dose assay. In all cases the highest [3H]estradiol concentration, $8 \times 10^{-10}$ M, which is the dose used in the single-dose assay, showed saturation of specific binding. The chart shows an excellent correlation (correlation coefficient, 0.99) between the values obtained from the full Scatchard analysis and the single saturating dose assay. In all cases the $K_d$ of the tumors studied was lower than $5 \times 10^{-10}$ M. A representative Scatchard plot from this series is shown in Chart 5.

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**Chart 1. Comparison of hydroxylapatite (HAP) and DCC assays of a human breast cancer cytosol at different protein concentrations. Portions of the cytosol were diluted with phosphate buffer to the concentrations shown. ER values were determined simultaneously by the HAP-SSD and DCC-SSD methods. Each point is the mean of triplicate determinations.**

**Chart 2. Effect of protein concentration on ER assays by HAP-SSD. The ER values of 3 tumors were previously determined by DCC-SSD assay (protein concentration, 1 to 2 mg/ml). The tumor cytosols were diluted to various protein concentrations and assayed by HAP-SSD. One hundred % represents the ER values obtained at higher protein concentration with the use of hydroxylapatite, which are in all cases very nearly the same as those obtained by DCC. Each point is the mean of triplicate determinations.**

**Chart 3. Effect of protein concentration on ER assays by the hydroxylapatite micromethod. Otherwise the procedure is the same as that of Chart 2. The 100% value for the tumor of 3 fmol/mg was identical by DCC-SSD assay and by hydroxylapatite assay.) Each point is the mean of triplicate determinations.
HYDROXYLAPATITE METHOD FOR ER

We have demonstrated in this paper that the hydroxylapatite assay is an excellent method to measure cytoplasmic ER in biopsies of human breast cancers and is, under low protein conditions, more accurate than the widely used DCC assay. Even though the background radioactivity of the assay is somewhat high and there is some variability in replication of low ER values when protein concentration is also low, the assay is still superior to the DCC-SSD assay. We have previously shown that hydroxylapatite also permits the accurate measurement of ER from tumor cell nuclei (5), so that the same basic method can be used for both cytoplasmic and nuclear receptor.

This report also validates the use of a new hydroxylapatite micromethod that allows the assay of very small tumor specimens for ER and that should be applicable to the assay of slightly larger tumor specimens for multiple receptors. The assay is rapid, inexpensive, and reliable. It is able to measure ER accurately with the use of only 30% of the material required for the standard DCC-SSD assay; with the same amount used in the DCC-SSD assay, it can measure the ER content by full Scatchard analysis with duplicate samples. To show the advantage of this method for small tumor specimens, we describe a typical example. A biopsy specimen of 0.03 g homogenized in 0.2 ml of buffer will yield 0.2 ml of cytosol with a protein concentration of about 1.5 mg/ml. To run an interpretable single-dose DCC assay in duplicate only, at least 1 ml of cytosol would be required. To obtain this 1 ml of cytosol, we would have to dilute the 0.2 ml by a factor of 5, reducing the protein concentration to 0.3 mg/ml. We demonstrated earlier that this concentration of protein is far below the range of accuracy for the DCC assay. In the case of the hydroxylapatite micromethod, however, only 0.3 ml of cytosol would be required to perform the assay in triplicate and obtain an accurate ER value. This method therefore represents a definite advance toward the goal of obtaining maximum information from minimum tumor samples with minimum labor and expense.

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REFERENCES

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