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Estrogen Receptors in Human Breast Cancer: Comparative Features of the Hydroxylapatite- and Dextran-coated Charcoal Assay*

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Abstract—*Samples of human breast cancer tissue were analyzed for estrogen receptor content (ER) with a hydroxylapatite method (HAP) as well as a dextran-coated charcoal method (DCC). It was found that both methods revealed artificially low ER concentrations in cytosols with low protein concentration. It was also found that the DCC assay underestimated the ER activity in the tumors compared to the HAP assay. No differences in terms of low affinity binding nor K_d-values of high affinity estrogen binding were observed. A significant higher background radioactivity was obtained in the HAP assay, and in some tumors one assay would classify them as ER-negative and another as ER-positive.*

INTRODUCTION

IT HAS been shown recently [1–3] that breast cancer patients who have ER-rich primary tumors are more likely to respond to endocrine therapy when they develop advanced disease compared to those who have ER-poor and ER-negative tumors.

It is therefore important that methods used for ER analysis are not only able to distinguish between ER-positive and ER-negative tumors, but must also be able to measure the ER concentration accurately.

Tumor samples received for ER analysis are frequently small, and the widely used dextran-coated charcoal (DCC) assay is likely to reveal 'false negative' and artificially low ER results in these cases [4]. Garola and McGuire [5] have therefore proposed that small tumor samples should be analyzed by hydroxylapatite assay (HAP), because they found this method to be more sensitive.

In the present study we compared our routine DCC assay, which is comparable to the method recommended by the EORTC Breast Cancer Cooperative Group [6], with the HAP assay as published by Garola and McGuire [5], and report that we were unable to demonstrate any advantages by using the latter assay.

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MATERIALS AND METHODS

Human breast cancer tissue was stored and processed as previously described [4]. The tissue was homogenized in a Schwingmühle (Retch, F.R.G.). The homogenate was suspended in H-buffer (5 mM NaH_2PO_4, 5 mM Na_2HPO_4, 1 mM monothioglycerol, 10% v/v glycerol, pH 7.5), and centrifuged at 100,000 *g* at 4°C for 1 hr. The cytosol was adjusted to protein concentrations as detailed under Results.

DCC assay

Fifty microliters of cytosol were incubated at 2–4°C for 24 hr with tritiated 17-β-estradiol (100 Ci/mmol, The Radiochemical Centre, Amersham, U.K.) and at least seven different concentrations of estradiol ranging from 0.1 to 15 nM were used (5 μl estradiol in ethanol/H-buffer + 25 μl H-buffer; ethanol concentration less than 0.5%). All the assays were performed in duplicate. The incubation was terminated by addition of 250 μl dextran-coated charcoal suspension [0.25% charcoal (Norit A), 0.0025% dextran (T-70)]. After adsorption for 30 min at 2–4°C, the charcoal was centrifuged (800 *g*, 10 min, 4°C) and the bound radioactivity was determined by liquid scintillation counting (2.5 ml Instagel) in a Packard Tricarb 3255 spectrophotometer (efficiency for tritium approx. 45%). Quench correction was carried out by the channel ratio method.

To correct for low-affinity binding, cytosols were run in parallel with 100-fold excess of diethylstilbestrol. Buffer blanks were used to check the background radioactivity.

HAP assay

DNA-grade Bio-Gel HTP hydroxylapatite (Bio-Rad) was washed with buffer (50 mM Tris, 10 mM KH_2PO_4, pH 7.2) four times and the final HAP:buffer ratio was 0.7. Two hundred and fifty microliters were added to each tube containing cytosol incubated as above. The mixture was incubated for 30 min at 2–4°C with vortexing every 10 min. The samples were centrifuged at 800 *g* for 4 min and the pellets were washed three times with 1.0 ml phosphate buffer which contained 1% Tween 80 (Sigma Chemical Co.). The pellets were extracted with 1.0 ml absolute ethanol at room temperature for 30 min on a vortex mixer. The mixture was centrifuged at 800 *g* for 10 min and the supernatant counted for radioactivity (see above).

Data evaluation

The measured low-affinity binding was subtracted from total binding to obtain values for specific binding. These values were analyzed by the method described by Scatchard [7] (see Fig. 1). The protein determinations were done according to the method published by Lowry *et al.* [8]. The data are presented as fmol bound estrogen/mg cytosol protein.

RESULTS

In order to obtain an estimate of the intra-assay variation for the two methods, the following experiment was done: in four individual tumors, the specific estrogen binding as well as the protein content was measured separately 10 times from the same cytosol by both methods outlined in Materials and Methods. The mean value ($\bar{X}$) of the resulting data (fmol/mg protein) from each tumor was calculated as well as the standard deviation (S.D.). It was observed that this variation, expressed as $\frac{\text{S.D.} \times 100}{\bar{X}}\%$, for the DCC assay varied from 9 to 16% and for HAP assay from 7 to 18% in these four tumors (data not shown).

Figure 2 shows the influence of cytosol protein concentration on the estimation of binding capacity in seven different tumors.

It is seen when ER concentrations are low that more concentrated cytosol preparations are

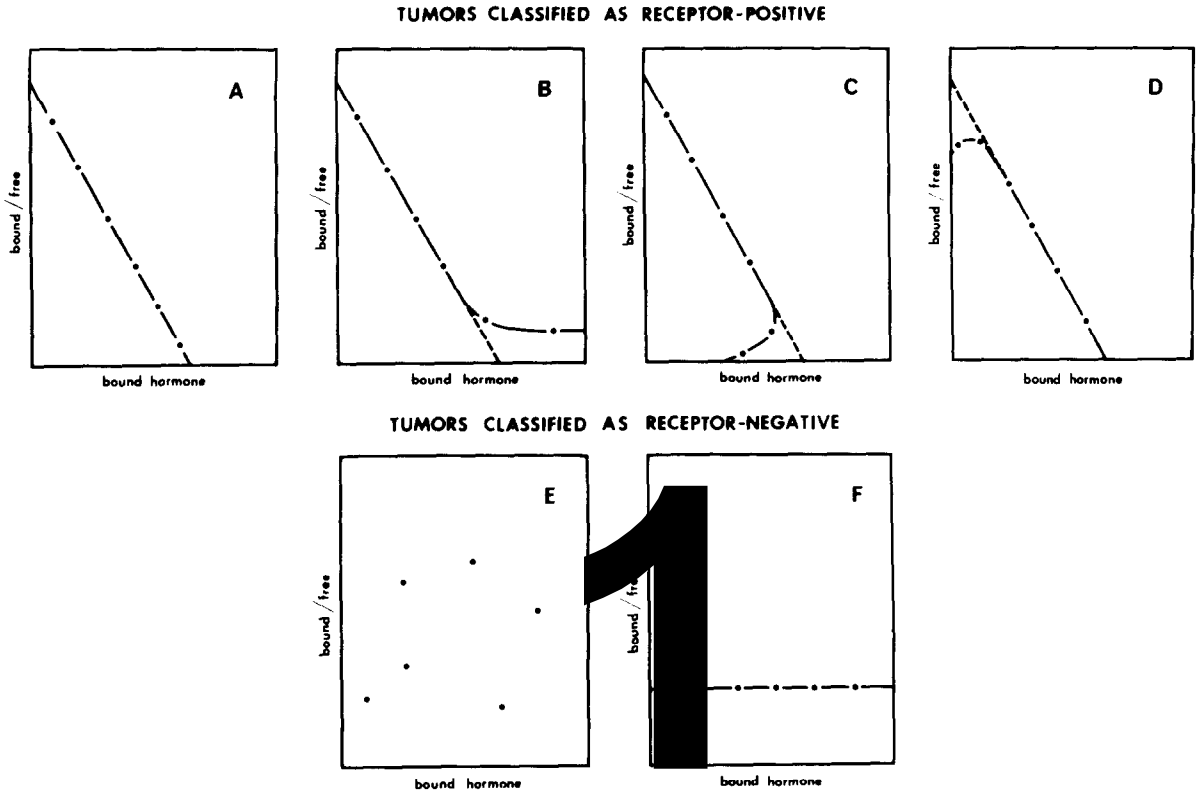

Fig. 1. Scatchard analysis of specific binding data. Regression line is calculated by the least-squares fit from the results of the steepest part of the curve. The interception of this line with the abscissa gives the binding capacity, whereas the slope of the line gives the approximate value of the dissociation constant of the estradiol receptor complex (A—D). Cytosols, in which no line could be evaluated with any confidence (E) and where the Scatchard analysis revealed a straight line with no slope (F), were defined as ER-negative.

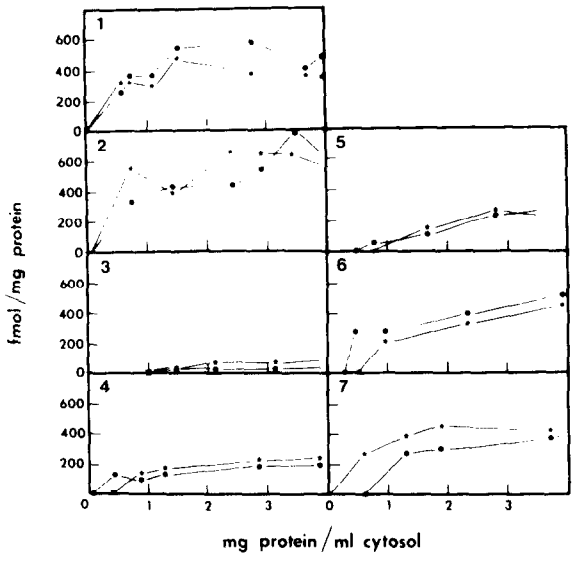

Fig. 2. Effect of protein concentration on ER assays by the hydroxylapatite (★) and the dextran-coated charcoal methods (●) in 7 different tumors. The points represent the estimated binding capacity at different cytosol protein concentrations.

needed (tumor No. 3). It is also seen that in some tumors (Nos 4–6) the DCC assay appeared to be more sensitive in terms of detecting receptors in cytosols with low protein content than the HAP assay. In another tumor (No. 7) the reverse was observed.

The two methods were compared in 50 cases. The tumor cytosol protein concentrations were adjusted to 3 mg/ml. The background activity for the DCC assay was 0.5–2.3% and for the HAP assay 1.6–4.3%, which is significantly different (see Table 1). It is also seen from Table 1 that no differences were observed in terms of low-affinity binding, K_d-values or the hormone concentration, which appeared to be able to nearly saturate the high-affinity receptor binding in the cytosols.

Idealized types of Scatchard plots obtained are shown in Fig. 1. Similar binding data were observed in 35 cases: Type A: 12; Type B: 6; Type C: 2; Type D: 7; Type E: 2; and Type F: 6. The distribution of unequally assessed types of plots were as follows: HAP, DCC: 9A, 6B and 3E; 1B, 1A; 3F, 2A and 1C; 1E, 1A; 1F, 1E.

From Fig. 3 it is seen that a good correlation was obtained of estimated data. It can be noted that 7 cases would be defined as ER-positive by one method and ER-negative by the other. The ER values from the HAP assay were significantly higher than those obtained from the DCC assay ($P < 0.001$, Wilcoxon's test for pair differences).

It was observed that for all tumors which contained receptor binding this binding approached saturation at a hormone concentration of 7.5 nM (See Table 1). We therefore used the measured specific value of bound estrogen at that concentration to compare single-point data. It can be seen from Fig. 4 that a good correlation was observed, and again the ER values from the HAP assay were significantly higher than those measured by the DCC assay ($P < 0.01$, Wilcoxon's test for pair difference). It can also be noted that discordant results were observed in terms of defining the tumor as ER-positive and ER-negative.

DISCUSSION

In this paper it has been shown that the sensitivity of the DCC assay is comparable to the HAP assay. 'False negative' results are likely to

Table 1. Comparative features of HAP- and DCC methods

Characteristics	Median	Range	Significance
Background activity			
HAP/DCC ratio ($n = 10$)	1.30	0.80–2.70	$P<0.01$, Wilcoxon's test for paired differences
Low affinity binding			
HAP/DCC-ratio ($n = 50$)	1.10	0.10–2.30	N.S.*, Wilcoxon's test for paired differences
Dissociation constants			
HAP-ASSAY, nM ($n = 37$)	0.45	0.03–4.50	N.S.*, Mann-Whitney
DCC-ASSAY, nM ($n = 38$)	0.59	0.17–5.90	U-test
Concentration of estrogen appearing to saturate ER			
HAP-assay, nM ($n = 37$)	0.75–7.50		
DCC-assay, nM ($n = 38$)	0.30–7.50		
Unequally assessed cases			
nM ($n = 7$)	0.75–3.00		

*N.S.: not significant at a P-level less than 5%.

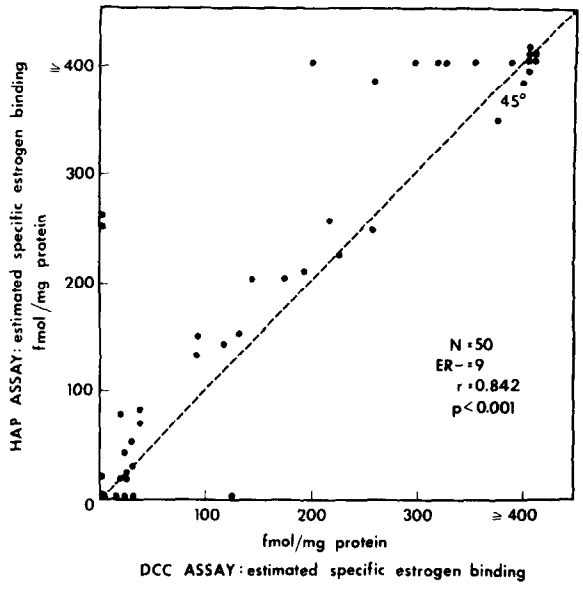

Fig. 3. Comparison of ER values by the HAP- and DCC methods of estimated data. Dashed line represents equality (Spearmann's rank-correlation test).

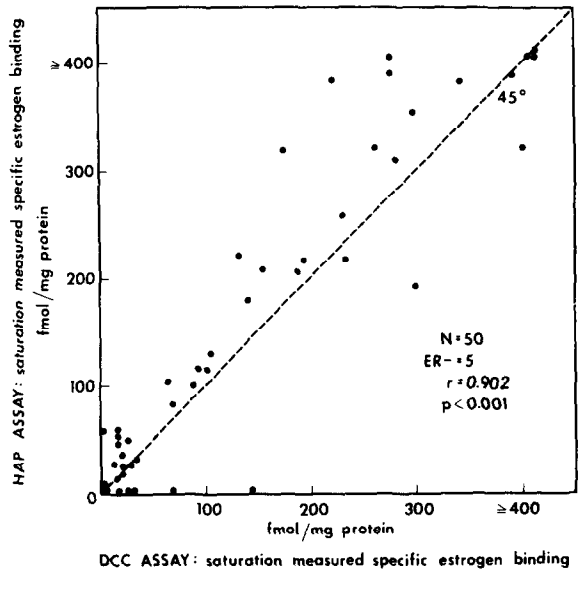

Fig. 4. Comparison of ER values by the HAP- and DCC methods of single-point data. Dashed line represents equality (Spearmann's rank-correlation test).

occur in cytosols with low protein concentration. In cytosols which contain less than 1.5–2 mg/ml protein, it is therefore not possible to evaluate whether tumors are truly ER-negative. Our data are therefore at variance with the data published by Garola and McGuire [5], who found that the HAP assay was superior to the DCC assay in cytosols with low concentrations of protein. The methods applied were comparable except for one factor, viz. the homogenizing procedure. In the study of Garola and McGuire [5], a combination of pulverization and homogenization was used, whereas in the present study a microdismembrator technique was used as recommended by the EORTC Breast Cancer Cooperative Group [6]. The yield of protein might be the same in quantity but not in quality. This could, to some extent, be a reasonable explanation for the discordant results. King [9] did not find, however, that different homogenizing procedures were of major importance when he compared results obtained from analysis of the same tumor from 5 different laboratories. In addition, Wagner and Jungblut [10] have shown that the dismembrator technique was superior to other homogenizing procedures.

It was also shown that the ER activity as measured by the HAP assay was significantly higher than that obtained from the DCC assay. The reason is probably that DCC adsorbs some receptor protein [4]. This method therefore probably underestimated the ER activity. The important feature is, however, that this underestimation seemed to be a general phenomenon. If one therefore uses one specific assay in a study, the data seem to be comparable. Difficulties, however, are introduced if alternate methods are used both in terms of interpretation differences in quantitative ER results and also in the interpretation of Scatchard plots.

It was observed that these plots were often curved or demonstrated different 'hooks'. This observation is in agreement with data published recently by Koenders [11]. This could indicate that many tumors contain more than one specific class of estrogen binding site and that cooperativity occurred [12–14]. It must be expected, however, that these features should be independent of the method used. Our data clearly demonstrate that they are not reproducible, and thus no efforts have been made to resolve and check the curvilinear plots as suggested by several investigators [14–16]. The K_d-values, however, were comparable in tumors equally assessed as ER-positive by both methods, which strongly suggests that the same kind of binding sites was measured with the two assays used in this study.

In conclusion, our data do not lend support to the recommendation made by Garola and McGuire [5] that the HAP assay should be used in ER analysis of small tumor samples. The HAP assay, as well as the DCC assay, is likely to give low ER results in tumors with low cytosol protein concentrations.

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