Measurement of Progesterone Receptor in Human Breast Cancer Biopsies

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ABSTRACT

The measurement of progesterone receptor in human breast cancer biopsies could prove to be an important addition to estrogen receptor determinations in predicting response to endocrine therapy. We therefore undertook a critical evaluation of \( ^{3} \text{H} \)R5020 progesterone receptor assays by several different methods in an effort to characterize the true nature of the competitive binding detected by sucrose density gradient centrifugation. Use of the swinging-bucket rotor caused an underestimation of 8S receptor, presumably due to the prolonged run time (16.3 hr) required. We used a vertical tube rotor which eliminated this problem. The competitive 4S component of the gradient profile was shown probably not to be progesterone receptor by the failure of 4S gradient fractions to adsorb to hydroxylapatite. Dextran-coated charcoal and hydroxylapatite Scatchard plots confirmed this finding in that values derived from these methods correlated very well with the 8S component of the gradient profile and not with the sum (8S + 4S) in a series of 27 human breast cancer specimens. In the same series, we also studied the validity of using dextran-coated charcoal or hydroxylapatite single-saturating-dose assays to solve the problem of samples too small for other methods of analysis. These assays also correlated very well with the 8S component of sucrose gradients. However, until more experience with these types of correlations is compiled from various laboratories, we feel that the 8S component of sucrose density gradient profiles as obtained from vertical tube rotor centrifugation yields the most reliable data on progesterone receptor concentrations in human breast tumors.

INTRODUCTION

It is well known that measurement of ER is very useful in predicting the response to endocrine therapy in patients with advanced breast cancer (5). At the present time, the presence of ER in a tumor promises 55 to 60% objective remission rate of ER in a tumor promises 55 to 60% objective remission rate. The measurement of progesterone receptor in human breast cancers is a reliable marker of hormone dependence in human breast cancer patients.

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We therefore undertook a critical evaluation of \( ^{3} \text{H} \)R5020 progesterone receptor assays by several different methods in an effort to characterize the true nature of the competitive binding detected by sucrose density gradient centrifugation. Use of the swinging-bucket rotor caused an underestimation of 8S receptor, presumably due to the prolonged run time (16.3 hr) required. We used a vertical tube rotor which eliminated this problem. The competitive 4S component of the gradient profile was shown probably not to be progesterone receptor by the failure of 4S gradient fractions to adsorb to hydroxylapatite. Dextran-coated charcoal and hydroxylapatite Scatchard plots confirmed this finding in that values derived from these methods correlated very well with the 8S component of the gradient profile and not with the sum (8S + 4S) in a series of 27 human breast cancer specimens. In the same series, we also studied the validity of using dextran-coated charcoal or hydroxylapatite single-saturating-dose assays to solve the problem of samples too small for other methods of analysis. These assays also correlated very well with the 8S component of sucrose gradients. However, until more experience with these types of correlations is compiled from various laboratories, we feel that the 8S component of sucrose density gradient profiles as obtained from vertical tube rotor centrifugation yields the most reliable data on progesterone receptor concentrations in human breast tumors.

MATERIALS AND METHODS

Human Breast Tumor Tissue Preparation. Tumor tissue, frozen in liquid nitrogen, was shipped by air in dry ice to San Antonio, Texas; local hospitals delivered directly to our laboratory. Upon arrival, tissues were stored at —70° in a Revco freezer. Tissues were powdered in the frozen state with a Thermovac tissue pulverizer and stored at —70° until ready for assay. They were homogenized in phosphate buffer (50 mM sodium phosphate, 10% glycerol, and 1 mM monothioglycerol, pH 7.4), 2 ml/g of tissue, with a Polytron PT-10-15 ST homogenizer at the lowest setting. All operations were performed at 4° on ice. Three 30-sec bursts were required with 1 min cooling between each burst. The homogenate was centrifuged at 40,000 rpm (102,000 x g) for 30 min at 4° to obtain the cytosol fraction.

SDG. Samples for sucrose density gradient analysis were prepared as follows. \( ^{3} \text{H} \)R5020 (\( ^{6} \text{H}-^{7} \text{H}-^{17} \text{H}-^{21} \text{H}-^{20} \text{H}-^{4} \text{H} \)) nor-4,9-pregne-3,20-dione, 55.4 Ci/mmol; a gift from J. P. Raynaud, Roussel-Uclaf, Romainville, France) was added at 2 x 10^{-8} M (final concentration) in 2 μl ethanol to 250 μl undiluted cytosol (6 to 14 mg protein per ml) and incubated for 4 hr at 4°. Parallel samples were preincubated for 10 min with a 100-fold excess of unlabeled R5020. A 10-fold excess of unlabeled DHT was added to all samples to bind any androgen receptor which might otherwise bind R5020. For separation of bound and free ligand, pellets were prepared from 1 ml DCC (0.25% Norit A and 0.0025% dextran in 10 mM Tris-HCl, pH 8.0, at 4°) by a 10-min centrifugation at 3200 rpm (2000 x g). The supernatants were discarded, and the charged cytosol was transferred to the DCC pellets with cold Pasteur pipets, shaken, and immediately centrifuged (2000 χ g) for 10 min. A 200-μl aliquot of the supernatant was applied to a linear sucrose gradient (5 to 20%) prepared in homogenization buffer. An additional 200 μl of glass distilled water was layered above the sample. Gradients were prepared manually at room temperature in 5-ml polyacrylamide tubes and allowed to diffuse overnight at 4°. 14C-Labeled bovine serum albumin was added to each cytosol before layering as an internal marker. Gradients were centrifuged in a Sorvall TV-665 vertical rotor at 65,000 rpm for 1.75 hr. Fractions (200 μl) were collected from the...
bottom of the tube by displacement with paraffin oil. Five ml of ACS (Amersham, Arlington Heights, Ill.) were added, and samples were counted in a Beckman LS 233 scintillation counter at 38% efficiency.

For comparison purposes, gradients of selected tumor cytosols were also centrifuged in a Beckman SW 60 Ti swinging-bucket rotor for 16.3 hr at 53,000 rpm. Gradients were prepared in 4.0-ml polyallomer tubes. Samples were incubated and layered as before. However, no water was layered above the sample. Collection and counting were performed as for vertical rotor centrifugation.

**HAP Precipitation of SDG Fractions.** Samples were prepared and run as for vertical rotor centrifugation, with the exception that the cytosol was not treated with DCC prior to layering and centrifugation.

HAP (DNA grade Bio-Gel HTP; Bio-Rad, Richmond, Calif.) was washed several times with Tris-phosphate buffer (50 mM Tris and 10 mM potassium phosphate, monobasic, pH 7.2, at 4°) to achieve pH 7.2 in the supernatant wash (9). The final packed HAP:buffer ratio was approximately 0.7.

Gradient fractions were collected directly into glass test tubes (12 × 75 mm) containing 250 µl of the above HAP slurry. Incubation was continued for 30 min at 4° with gentle vortexing periodically. The tubes were centrifuged at 2000 rpm (800 × g) in a Beckman TJ-6 tabletop centrifuge for 3 min. Following aspiration of the supernatants, the pellets were washed with 2 ml homogenization buffer, vortexed, and centrifuged, and the supernatant was discarded. This procedure was repeated, and the final HAP pellet containing PGR was then resuspended in 2 ml ethanol and allowed to stand overnight at room temperature. The tubes were then gently vortexed and centrifuged at 800 × g for 10 min. The supernatant ethanol containing the extracted [3H]R5020 was carefully decanted into counting vials. The ethanol was evaporated under a gentle stream of air. After addition of 200 µl homogenization buffer containing 10% sucrose (w/v) and 5 ml ACS, the vials were capped and counted as for normal gradients.

**Scatchard Analysis of PGR.** Cytosol was diluted to obtain sufficient volume for 2 Scatchard plots. Aliquots of cytosol (200 µl, 0.6 to 4.0 mg protein per ml) were incubated in duplicate with increasing concentrations of [3H]R5020 (2 × 10⁻¹⁰ to 3 × 10⁻⁸ M) in the presence of a 10-fold excess of DHT. Parallel samples, also in duplicate with DHT, were incubated in the presence of a 100-fold excess of unlabeled R5020 to allow estimation of nonspecific binding. At the end of a 4-hr incubation at 4°, bound and free ligand were separated by one of the following 2 methods.

For DCC, 0.5 ml DCC (prepared as for SDG analysis) was added to each tube, and the tubes were shaken at high speed in a reciprocating shaker for 5 min at 4°. After centrifugation at 2000 × g for 10 min, 500-µl aliquots of the supernatants were counted in 5 ml of modified Bray’s scintillation cocktail (125 g naphthalene, 7.5 g PPO, 0.377 g POPOP, and 1 liter of toluene) in a Beckman LS 233 scintillation counter at 38% efficiency.

For HAP, cytosol incubates were precipitated with 250 µl HAP slurry. The remainder of the procedure was as delineated for precipitation of sucrose density gradient fractions. However, the ethanol was not evaporated but was mixed with 5 ml of a toluene-based scintillation cocktail (4.0 g PPO, 0.05 g POPOP, and 1 liter of toluene) in a Beckman LS 233 counter at 35% efficiency.

Data obtained from the 2 methods were plotted according to the method of Scatchard (8). Protein concentrations in aliquots of cytosol were determined according to the method of Lowry et al. (6).

**RESULTS AND DISCUSSION**

Tumor specimens were chosen for study on the basis of previous routine measurements of their PGR content made in our laboratory. These PGR assays are performed routinely on incoming tumor samples by sucrose density gradient analysis in a Beckman SW 60 Ti swinging-bucket rotor. Availability of tumor samples was limited by the multiple analyses required for each tumor specimen; at least 1.0 g of pulverized tumor was needed to provide cytosol of sufficient volume and protein concentration for SDG and Scatchard analyses.

Rather than subject the receptor molecule to the prolonged 16-hr run time required for swinging-bucket centrifugation, we chose to use the vertical rotor system for routine gradient analyses. Because the sample traverses the gradient horizontally, the distance of migration is considerably shortened, and run time can be reduced to less than 2 hr. Chart 1 shows a typical sucrose density gradient profile on the Sorvall TV-865 vertical rotor (a) together with the swinging-bucket gradient profile of the same tumor cytosol (b). Certain advantages of the vertical rotor are seen. Under the described conditions, the receptor migration provides better resolution. Calculation of the specific binding in the individual regions of the gradients reveals some additional differences. Although the sum (8S + 4S) obtained from the 2 gradients is the same, the relative proportion of 8S to 4S is not. Computation of the suppressible binding from the 8S region of the gradient profile as obtained from the vertical rotor centrifugation shows considerably more receptor than the same component in the swinging-bucket gradient, suggesting that the prolonged run time required for gradient analysis in the swinging-bucket rotor may have detrimental effects on the 8S molecule. This result is not unique. Five tumors were compared in the manner described in Chart 1, and the average ratio, VTR 8S to SW60 8S, was 2.18 ± 0.45. Data from 17 tumors in which the 2 gradient methods were performed on the same tumor but with different cytosol preparations yielded a ratio of 2.33 ± 0.35. There also seems
to be less interference by unbound radioactive ligand in the 4S region of the gradient when the vertical rotor is used. These considerations lead us to prefer the vertical rotor for SDG analysis.

We further examined the nature of the 8S and 4S competitive binding by adsorbing each gradient fraction with HAP, which is known to adsorb other steroid receptors (9). Chart 2 shows one of these experiments. On the left is our standard vertical rotor gradient of DCC-stripped cytosol with each fraction counted directly. On the right, each fraction of a parallel gradient performed on untreated cytosol has been adsorbed with HAP, and the HAP was then washed and extracted for counting. It is clear that 8S material was adsorbed, while most of the competitive binding in the 4S region was not. Similar experiments on 24 tumor cytosols are summarized in Chart 3. Very little of the 4S competitive binding was ever adsorbed by HAP, suggesting that the greater part may not be PGR. When a large amount of 8S PGR was present, a small but distinct portion of the 4S peak was often adsorbed by HAP; this portion may represent true PGR subunits, while the remainder of the 4S competitive binding is probably divided between [3H]R5020 which was initially bound to relatively high-affinity plasma proteins and that which was initially bound to PGR but which dissociated during centrifugation and rebound to plasma proteins. The persistent loss of 8S binding during the HAP procedure may have resulted from a slight dissociation of [3H]-R5020 during fractionation and precipitation or from interference by high concentrations of sucrose.

Additional evidence against the 4S binding being PGR comes from Scatchard analysis of tumor cytosols which contain very little 8S binding but large amounts of 4S binding. By routine gradient analysis, such tumor cytosols could often have been considered to have more than 100 fmol PGR per mg cytosol protein if the 8S- and 4S-suppressible components of the gradient were summed. Yet, Scatchard plots, using either DCC or HAP methods, suggest that the true PGR value is in close agreement with the very small 8S component of the gradient alone.

Chart 2. Sucrose density gradient assay on vertical tube rotor of PGR in a human breast tumor biopsy. a, [3H]R5020 only; b, [3H]R5020 plus 100-fold excess unlabeled R5020; a, DCC-treated cytosol; b, untreated cytosol precipitated with HAP after centrifugation and fractionation; arrows, sedimentation of 14C-labeled bovine serum albumin.

Chart 3. Effect of HAP precipitation on 8S and 4S components of sucrose density gradient profile. Data compiled from 24 human breast tumor biopsies. Routine gradient analysis of DCC-treated cytosol was performed, and untreated cytosol was precipitated with HAP after centrifugation and fractionation.

Chart 4. Comparison of sucrose density gradient centrifugation and Scatchard assays of human breast tumor PGR. For DCC (8S + 4S), summation of 8S and 4S components was determined from routine gradient analysis of DCC-treated cytosol; the DCC plot value was determined by Scatchard analysis according to the DCC method. Both axes are log scale.

Chart 5. Comparison of the 8S component of sucrose density gradient profile and DCC Scatchard assay of human breast tumor PGR. Both axes are log scale.
Comparisons of assays for PGR in human breast cancer

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<th>HAP SSD</th>
<th>DCC Scatchard plot</th>
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* The numbers are listed as correlation coefficient/slope of regression line for each pair of PGR assay methods applied to 27 separate human breast tumor specimens.

The remaining charts show the relationship between quantitative receptor values from the 4 different methods of analysis. As expected, the DCC Scatchard analysis does not correlate well with the total competitive binding (8S + 4S) as determined by sucrose density gradient analysis of DCC-treated cytosol (Chart 4). Correlation of HAP Scatchard results with gradient 8S + 4S yields the same poor result (not shown). Values derived from these gradients are overestimations because of the large and uncertain contribution of competitive binding from the 4S component which is not true PGR.

We next correlated only the 8S portion of the gradients with the DCC Scatchard analysis (Chart 5). Even those tumor cytosols with relatively low levels of binding agree closely by these techniques, so that the possible presence of a small amount of legitimate 4S PGR does not much affect the results. The HAP Scatchard analysis agrees in much the same way with the gradient 8S value (not shown).

Because we were seeking a method for measurement of PGR which is both accurate and applicable to processing small samples, we considered the correlation of values obtained from a SSD, either DCC or HAP, with various components or combinations of the gradient profile. Experience with 27 tumors had shown us that the highest dose used in the Scatchard analysis by both methods was sufficient to saturate essentially all of the receptor present. The value of this dose ranged from $2 \times 10^{-8}$ to $3 \times 10^{-8}$ M and was the same for both DCC and HAP. Consequently, we used the data from this highest dose to generate the SSD assay receptor values. Linear regression analyses of these data suggest that the best correlations are obtained when one compares the SSD analyses, by either DCC or HAP, with SDG-8S. These 2 scattergrams are shown in Charts 6 and 7. Table 1 provides a summary of all of the correlation data relating SDG, SSD, and Scatchard plot results using HAP or DCC. Between the 8S component of SDG, the SSD assays (either by HAP precipitation or DCC treatment), and the HAP Scatchard plot, the cross-correlation is extremely good. No correlation coefficient is less than 0.92, and no slope is less than 0.90. The fact that such varied methods of analysis correlate so well with the 8S component of the vertical tube rotor SDG profile together with the high order of specificity inherent in 8S binding reinforces our belief that this is the most reliable method for analysis of PGR.

It seems to be a general rule that the higher the number of binding sites present in a tumor cytosol the greater is the variation among the methods for measurement. From a practical standpoint, this is probably of little consequence since all of these tumors would be classified as high-positive for PGR by whatever method was chosen for analysis. It is more critical to know the absolute value of PGR present in tumor cytosols which exhibit low binding. The decision to classify such tumors as positive or negative rests solely upon the reliability of the method selected for analysis. The most desirable method is the determination of the 8S competitive binding by sucrose density gradient centrifugation utilizing the vertical tube rotor. In those special cases where small amounts of tissue absolutely preclude measurement by SDG or Scatchard analysis, a SSD assay by HAP or DCC might be a substitute. Nevertheless, we would not recommend the SSD assay for routine use.

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