

Evaluation of Estrogen Receptor Assays in Human Breast Cancer Tissue¹

William L. McGuire, Minerva De La Garza, and Gary C. Chamness

Department of Medicine, University of Texas Health Science Center, San Antonio, Texas 78284

SUMMARY

Standard dextran-coated charcoal (DCC) and sucrose gradient centrifugation assays for estrogen receptor were compared in a series of human breast cancer tissues. From a quantitative standpoint the results were remarkably similar. A simplified version of the DCC assay compared to the sucrose gradient assay yielded acceptable results. We conclude that, in spite of the lack of specificity controls inherent in the sophisticated standard assays, the simplified DCC assay might be useful if the biopsy specimen is too small to provide the number of aliquots for a standard DCC assay or sufficient protein for a sucrose gradient analysis. It also might be useful in research laboratories attempting to develop assays for multiple receptors or other constituents in a single tumor biopsy specimen.

INTRODUCTION

ER² assays have been shown to be of considerable value in the selection of breast cancer patients for endocrine therapies (6), while biochemists investigating ER have been concerned with molecular conformation, nuclear translocation, and chromatin acceptor sites. These types of experiments demand precise quantitation and rigorous proof of the specificity of binding. In our own laboratory we have used both SGC and DCC assays analyzed by Scatchard plots (DCC) to determine ER in our human breast tumor specimens (7, 8). SGC is quantitative and demonstrates the 8 S molecular form of ER as a characteristic property of the receptor. DCC is also quantitative and, additionally, yields an estimate of the dissociation constant (K_d) that reveals a distinctively high affinity for steroid receptor binding. We have routinely used both assays on each specimen to ensure reliability of tumor ER values.

More recently, we have suggested that the measurement of additional steroid receptors in breast tumor tissue may provide even more information about the endocrine responsiveness of the tumor (2). This demand for multiple analyses on the same tumor specimen is often hampered by the initial amount of available biopsy material. We therefore determined whether it is actually necessary to perform both SGC and DCC assays and, furthermore, whether even sim-

pler assay procedures might suffice when biopsy material is limited or when multiple receptor analyses are required.

MATERIALS AND METHODS

Sample Preparations. Tissues are excised, stored, and pulverized as described in our previous work (7, 8). The pulverized tissue is then homogenized in 2.0 ml buffer per g tissue with a Polytron P-10-ST homogenizer at the lowest setting. The buffers used are Tris-EDTA-dithiothreitol buffer (0.01 M Tris-HCl-0.0015 M EDTA-0.5 mM dithiothreitol, pH 7.4) or phosphate buffer (5 mM sodium phosphate-10% glycerol-1 mM monothioglycerol, pH 7.5). We have determined that either Tris-EDTA-dithiothreitol or phosphate buffer gives the same ER value, but progesterone receptor assay requires phosphate buffer. The homogenate is centrifuged at 3200 rpm ($1600 \times g$) for 10 min to obtain the low-speed cytosol fraction. An aliquot of this fraction is transferred to an assay tube and stored at 4° for later assay. The remainder of the low-speed cytosol fraction is mixed with its pellet and transferred to a polycarbonate tube for centrifugation at 40,000 rpm ($104,000 \times g$) for 40 min to obtain the high-speed cytosol fraction. Protein of low- and high-speed cytosols is first estimated by absorbance at 260 and 280 nm (3) and later quantitated by the method of Lowry *et al.* (5). The estimated protein value is used to dilute each cytosol to the appropriate protein concentration, *i.e.*, 2 to 6 mg/ml for sucrose gradients and 1 to 2 mg/ml for SSD assay and DCC saturation analysis.

Sucrose Gradients. Ten to 30% sucrose gradients, 4.3 ml, are prepared in Tris-EDTA-dithiothreitol buffer and 5 to 20% sucrose gradients, 4.3 ml, are prepared in phosphate buffer with an ISCO gradient former. Samples for gradient analysis are prepared by incubating 250 μ l cytosol with 1.0 pmole [³H]E (approximately 100 Ci/mole; New England Nuclear, Boston, Mass.) in 3 μ l homogenizing buffer-10% ethanol for 4 hr at 4°. Control cytosols are preincubated with 100 pmoles nonradioactive DES in 1 μ l homogenizing buffer-2% ethanol 15 min prior to the addition of [³H]E. After incubation the nonbound estradiol is removed by mixing 250- μ l incubation mixture with 1600 $\times g$ DCC pellet derived from 1 ml DCC suspension [0.25 g % Norit A (Sigma Chemical Co., St. Louis, Mo.-0.0025 g % dextran Grade C (Schwarz/Mann, Orangeburg, N. Y.) in 0.01 Tris-HCl, pH 8.0)]. The DCC pellet is resuspended in the cytosol incubation mixture and allowed to sit at 4° for 20 min. The mixture is then centrifuged at 1,600 $\times g$ for 10 min and 200 μ l of the supernatant are applied to the gradient. A 5- μ l internal

¹ Supported in part by National Cancer Institute Grants CA11378 and CB-23862 and American Cancer Society Grant BC23.

² The abbreviations used are: ER, estrogen receptor; SGC, sucrose gradient centrifugation; DCC, dextran-coated charcoal; SSD, single saturating dose; [³H]E, tritiated estradiol; DES, diethylstilbestrol.

Received July 19, 1976; accepted October 19, 1976.

standard of [¹⁴C]bovine serum albumin is also added (9). The tubes are centrifuged in an SW56 and SW60 rotor at 54,000 and 53,000 rpm, respectively, for 16.3 hr. The bottoms of the tubes are punctured, and 4-drop fractions of the gradients are collected by a Buchler gradient collector. After each gradient is collected, the bottom of the tube is cut off and counted as Fraction 1. All fractions are counted for radioactivity (39% counting efficiency) in 5 ml of a modified Bray's scintillation cocktail (125 g naphthalene, 7.5 g PPO, and 0.377 g POPOP per liter of solution in *p*-dioxane).

DCC Saturation Analysis. Either cytosol or homogenizing buffer, 200 μ l, is incubated with increasing quantities of [³H]E (0.015, 0.025, 0.0375, 0.05, 0.075, 0.1, and 0.2 pmole) for 18 hr at 4° in duplicate. An additional set of cytosol or buffer samples with 0.2 pmole [³H]E is incubated with 40 pmoles DES at 4° for 15 min prior to the addition of [³H]E and is used to determine nonspecific binding. DCC suspension, 0.5 ml, is then added and shaken vigorously with an Eberbach shaker at the highest setting for 30 min at 4°. The mixture is then centrifuged for 10 min at 1600 \times g, and 500 μ l of the supernatant are counted for radioactivity in modified Bray's scintillation cocktail as described above. The binding data are analyzed by the method of Scatchard as previously described (1, 10).

SSD Assay. Cytosol, 200 μ l, is incubated with 0.2 pmole [³H]E in triplicate for 4 hr at 4°. Triplicate control samples of 200 μ l cytosol are incubated with 40 pmoles DES at 4° for 15 min prior to the addition of [³H]E. The method for the removal of unbound estradiol and the counting of the samples for radioactivity is identical to that described in the DCC saturation analysis procedure.

RESULTS AND DISCUSSION

We first compared the SGC and DCC assays in 29 human breast tumor specimens. Chart 1 illustrates the remarkable correlation between the 2 assays. The calculated correlation coefficient is 0.92. The only area of uncertainty is that

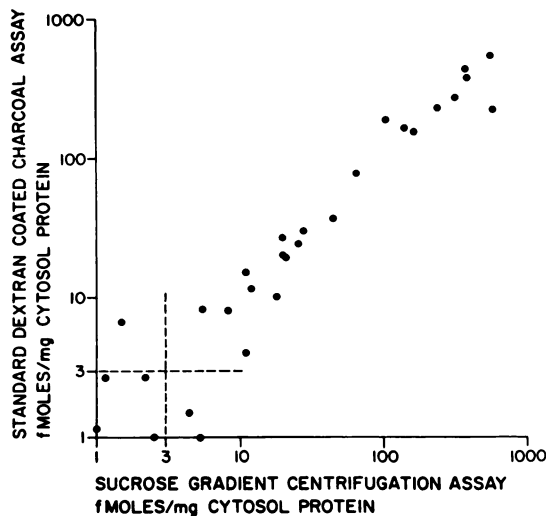


Chart 1. Comparison of SGC and standard DCC assays for human breast tumor ER. Tumors containing less than 3 fmoles/mg cytosol protein are interpreted as negative.

below 7 fmoles/mg cytosol protein, which is approaching the limits of sensitivity for the 2 assays. We conclude that, from a quantitative standpoint, the SGC and DCC assays are equally reliable. The demonstration of 8 S peaks in the SGC provides considerable additional confidence, but the limitation in the number of samples that can be processed (3/day/centrifuge) offsets this advantage. The DCC assay with Scatchard analysis permits more specimens to be assayed than the SGC does but still requires approximately 35 test tubes/assay and becomes cumbersome if many samples are involved.

We therefore proceeded to use the SGC assay as our standard to test a simplified version of the DCC assay. Leung et al. (4) had reported that the DCC assay could yield meaningful ER data with only a single concentration of [³H]E \pm 5000-fold excess of the antiestrogen Nafoxidine to determine nonspecific binding. This is in contrast to the 7 concentrations and Scatchard analysis needed for our standard DCC assay. We modified Leung's assay by increasing the [³H]E concentration to achieve receptor saturation and

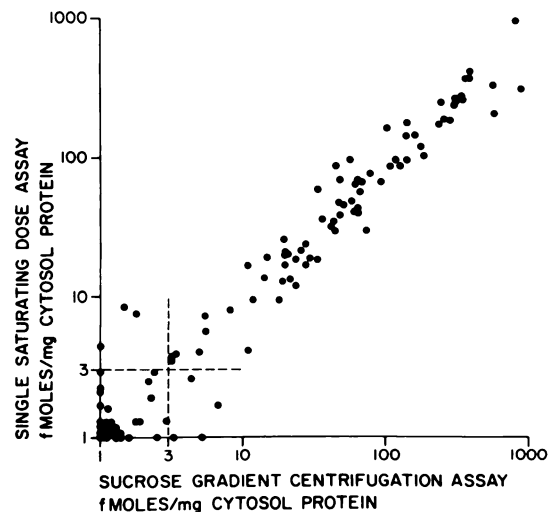


Chart 2. Comparison of SGC and SSD assays for human breast tumor ER.

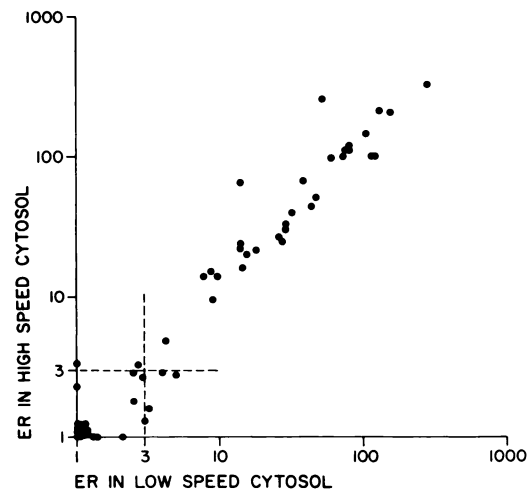


Chart 3. Comparison of ER values in low-speed (1,600 \times g) and high-speed (104,000 \times g) cytosol fractions of human breast tumors. The SSD assay was used.

by using DES as a competitor to avoid measuring [³H]E binding to contaminating plasma proteins in the sample.

Chart 2 presents 115 breast tumor cytosols analyzed both by this new SSD assay and by the SGC. Except for some uncertainty in the low range (3 to 10 fmoles/mg cytosol protein) as noted before, there was excellent correlation between the 2 analyses. The correlation coefficient was 0.95.

We also determined whether a 1,600 × *g* supernatant fraction of tumor homogeneity rather than a high-speed cytosol could be used to measure cytoplasmic ER. In Chart 3 we compare cytoplasmic ER values obtained from 1,600 × *g* and 105,000 × *g* supernatants of tumor homogenates. Both fractions gave similar values with a correlation coefficient of 0.98.

We conclude that the SSD assay described here under properly controlled conditions provides cytoplasmic ER data quantitatively similar to the SGC and standard DCC assays and that low-speed as well as high-speed cytosols may be used. Nevertheless, the SSD lacks the specificity controls such as 8 S peaks or *K_d* inherent in the other assays. For this reason we are reluctant to recommend its routine use in laboratories that are not exclusively devoted to receptor analyses. A SSD assay could generate false information if faulty reagents or techniques could not be checked by the more sophisticated procedures. On the other hand, in those special circumstances in which a biopsy specimen is too small to provide the number of aliquots for a standard 7-dose DCC assay or sufficient protein for an SGC analysis, the SSD assay may prove valuable. Another major application would be in those research labo-

ratories trying to develop assays for multiple receptors or other constituents in a single tumor biopsy.

ACKNOWLEDGMENTS

We thank Dennis Perotta for his excellent technical assistance.

REFERENCES

1. Chamness, G. C., and McGuire, W. L. Scatchard Plots: Common Errors in Correction and Interpretation. *Steroids*, 26: 538-542, 1976.
2. Horwitz, K. B., McGuire, W. L., Pearson, O. H., and Segaloff, A. Predicting Response to Endocrine Therapy in Human Breast Cancer: A Hypothesis. *Science*, 189: 726-727, 1975.
3. Layne, E. Spectrophotometric and Turbidimetric Methods for Measuring Proteins. *Methods Enzymol.*, 3: 447-454, 1957.
4. Leung, B. S., Moseley, H. S., Davenport, C. E., Kippaehne, W. W., and Fletcher, W. S. Estrogen Receptor in Prediction of Clinical Responses to Endocrine Ablation. *In*: W. L. McGuire, P. P. Carbone, and E. P. Vollmer (eds.), *Estrogen Receptors in Human Breast Cancer*, pp. 107-129. New York: Raven Press, 1975.
5. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. Protein Measurement with the Folin Phenol Reagent. *J. Biol. Chem.*, 193: 265-275, 1951.
6. McGuire, W. L. Current Status of Estrogen Receptors in Human Breast Cancer. *Cancer*, 36: 638-644, 1975.
7. McGuire, W. L., and De La Garza, M. Improved Sensitivity in the Measurement of Estrogen Receptor in Human Breast Cancer. *J. Clin. Endocrinol. Metab.*, 37: 986-989, 1973.
8. McGuire, W. L., Pearson, O. H., and Segaloff, A. Predicting Hormone Responsiveness in Human Breast Cancer. *In*: W. L. McGuire, P. P. Carbone and E. P. Vollmer (eds.), *Estrogen Receptors in Human Breast Cancer*, pp. 17-30. New York: Raven Press, 1975.
9. Rice, R. H., and Means, G. E. Radioactive Labeling of Proteins *in Vitro*. *J. Biol. Chem.*, 246: 831-832, 1971.
10. Scatchard, G. The Attraction of Proteins for Small Molecules and Ions. *Ann. N. Y. Acad. Sci.*, 51: 660-672, 1949.