Estrogen Receptor in Very Small Breast Tumor Specimens: A Modified Charcoal-Gelatin Assay

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ABSTRACT

We described recently a modified charcoal-gelatin (MCG) assay for measuring progesterone receptor activity in low-protein cytosols. We showed that pre-mixing of gelatin with sample cytosols (final gelatin concentration 0.1%) and removal of unbound steroid by a 1% charcoal suspension with 0.1% gelatin but without dextran preserves the progesterone receptor activity in dilute cytosol. For estrogen receptor (ER), as for progesterone receptor, the efficiency of the standard dextran-coated charcoal (DCC) assay drops rapidly as samples are diluted much below 1 mg protein per ml. We have therefore applied the MCG procedure to the assay of ER in breast tumor cytosols. We find that MCG is far more efficient for ER at low protein concentrations than either the DCC method or other three methods recommended previously for dilute samples, retaining at least 60% efficiency even at 0.01 mg protein per ml. The measured Ks of the receptor for estradiol is the same by MCG as by DCC. A series of breast tumor biopsies assayed by MCG at 0.1 mg protein per ml gave about the same ER values (fmol/mg) as at 1 mg/ml, while the DCC efficiency for ER at the lower concentration averaged only 32%. In combination with 125I-labeled estradiol, this MCG method should allow accurate ER assays of extremely small breast cancer specimens.

INTRODUCTION

ER is an important prognostic indicator for predicting the endocrine dependence and likelihood of recurrence of breast tumors (2, 3). More recently, the additional measurement of progesterone receptor has been shown to add further prognostic information (4, 5). Since the commonly used DCC method (6) for estimating steroid receptors is limited to samples containing at least 1 mg protein/ml (7-9), receptor analyses are difficult or impossible when the tumor specimen is limited, as in needle biopsies or biopsies of small metastatic lesions. We reported recently a MCG assay for measuring progesterone receptor activity in cytosols with low endogenous protein concentration (10). We will show here that the MCG procedure also permits accurate determinations for ER in low-protein cytosols, such as those from very small breast cancer biopsies.

MATERIALS AND METHODS

125I-labeled 16a-iodo-3,17β-estradiol (2200 Ci/mmol) and [2,4,6,7-3H(N)]estradiol (90.4 Ci/mmol) were obtained from the New England Nuclear Corporation, Boston, MA. Dextran was purchased from Schwarz/Mann, Orangeburg, NY. Diethylstilbestrol, bovine serum albumin (fraction V, A-4503), gelatin (Porcine skin, Type II, G-2625), activated charcoal (untreated, C-5260), and Sephadex LH-20-100 were obtained from Pharmacia Sweden. Nuchar PT-10-ST homogenizer at the lowest setting. All operations were performed in a Teflon-glass Potter-Elvehjem homogenizer. The homogenate was centrifuged (150,000 x g for 30 min) to obtain the cytosol fraction. Cytosol protein content was assayed immediately by the method of Lowry et al. (12). This chemical used were of analytical purity grade or the best grade available. The disposable glassware (e.g., tubes, pipets, etc.) was obtained from American Scientific Products, McGaw Park, IL. Polycarbonate centrifugation tubes were from the Nalge Co., Rochester, NY. Cytosol transfers and dilutions were done with disposable plastic pipet tips (Eppendorf Brinkmann Instruments, Inc., Westbury, NY).

Preparation of Cytosol

Human Breast Cancer Cells. Growth medium for MCF-7 human breast cancer cells consisted of minimum essential medium (Earle’s basal salts) supplemented with 2 mM L-glutamine, 1% non-essential amino acids, gentamicin (25 μg/ml), bovine insulin (6 ng/ml), 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer, pH 7.4, 0.2% NaHCO3, and 5% calf serum stripped of endogenous steroids by treatment with dextran-coated charcoal (11). The cells were allowed to grow in a 5% CO2 atmosphere at 37°C. Confluent cells were harvested by a 10-min incubation at 37°C with 1 mM EDTA in Ca2+-Mg2+-free Hanks’ balanced salt solution and pelleted by low speed centrifugation. Cell pellets were washed with Hanks’ solution and stored at −70°C until required for receptor assay. The MCF-7 cytosol was prepared by suspending the frozen cells in cold TEDGM12 buffer and homogenizing in a Teflon-glass Potter-Elvehjem homogenizer. The homogenate was then centrifuged at 150,000 x g for 30 min in a Beckman 75 Ti rotor to yield a cytosol supernatant. Protein concentration of cytosol was immediately determined by the method of Lowry et al. (12). This protein value was then used to dilute the cytosol serially to the desired concentrations with TEDGM12 buffer. For the MCG assay, a portion of the cytosol was premixed with a stock gelatin solution (1% in TEDGM12 buffer, dissolved by heating at ~50°C) to obtain a final gelatin concentration of 0.1%. It was then serially diluted to the desired endogenous cytosol protein concentrations using TEDGM12 buffer containing 0.1% gelatin.

Human Breast Tumors. Breast tumor samples were frozen in liquid nitrogen immediately after excision and stored at −70°C in a Revco freezer. Tissues were powdered in the frozen state with a Thermovac tissue pulverizer and stored at −70°C until required for receptor assay. Powdered tissues were homogenized in TEDGM12 buffer with a Polytron PT-10-ST homogenizer at the lowest setting. All operations were performed in an ice-water bath. Three 30-s bursts were given, with 1 min of cooling between each burst. The homogenate was centrifuged at 150,000 x g for 45 min in a Beckman 75 Ti rotor at 4°C to obtain the cytosol fraction. Cytosol protein content was assayed immediately by the method of Lowry et al. (12) using BSA as a standard. Procedures for dilution of cytosols and addition of gelatin (final concentration 0.1%) were essentially the same as described above for the MCF-7 cytosol.

Charcoal Assays

Single Saturating Dose Assays. Aliquots of cytosol (0.2 ml) were incubated with 2nM [3H]estradiol or 1 nM 125I-labeled estradiol for 3 h at 0-4°C. Parallel incubation in the presence of a 100-fold excess of diethylstilbestrol yielded the non-specific binding. To remove the unbound estradiol, 0.5 ml of the appropriate charcoal suspension, prepared in 10 mM Tris-HCl (pH 8.0), was added to each tube. The standard DCC suspension contained 0.25% charcoal and 0.0025% dextran, while our new MCG suspension had 1% charcoal and 0.1% gelatin. In the DCC-BSA method 0.1% BSA was added to the standard DCC suspension. A mixed DCC-gelatin formulation used by Renor et al. (14) contained 0.5% charcoal, 0.05% dextran, and 0.2% gelatin.

After addition of charcoal, the mixture was incubated for 15 min with occasional shaking. The tubes were centrifuged at 2000 x g for 10

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3 The abbreviations used are: ER, estrogen receptor; TEDGM12, buffer, 10 mM Tris-HCl:1.5 mM EDTA:0.5 mM dithiothreitol:10% (v/v) glycerol:10 mM sodium molybdate, pH 7.4 (4°C); DCC, dextran-coated charcoal; MCG, modified charcoal-gelatin; BSA, bovine serum albumin.

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min to remove the charcoal. Specific bound estradiol was estimated from the radioactivity of the supernatant after subtracting that of the parallel nonspecific binding tubes. The number of binding sites was expressed as fmol/mg of protein.

Scatchard Analysis. Aliquots of 0.2 ml of cytosol, without or with 0.1% gelatin as appropriate, were incubated with increasing concentrations of 125I-labeled estradiol (0.016–2.0 nM) for 18 h at 0.4°C. The compositions of the DCC and MCG suspensions used for removing the unbound estradiol were the same as described above. Free estradiol was calculated as the difference between the radioactivity of total and bound estradiol. The binding parameters were estimated on Scatchard plots (15, 16).

LH-20 Gel Filtration Assay

Conditions for incubation with [3H]estradiol were exactly the same as described above for the single-saturating dose charcoal assays. For the separation of bound and free [3H]estradiol, 0.2 ml of the incubation mixture was passed into Sephadex LH-20 columns (bed volume, 2.5 ml; prepared in a 5-ml pipet) pre-equilibrated in TEDGM10 buffer at 0–4°C. Then 0.6 ml of TEDGM10 buffer was applied to each column and allowed to pass in. The receptor proteins were then eluted with another 0.8 ml TEDGM10 buffer and collected. Receptor-bound [3H]-estradiol was counted as above.

Calculation of Assay Efficiency

In each case, the ER value obtained with the standard DCC assay at high protein concentration (1 mg/ml) was taken as 100%. The assay efficiency of other procedures or for other conditions was then determined as percentage observed/expected specific binding, the latter being calculated by dividing the standard ER value by the protein dilution factor.

RESULTS

We first compared the ER-measuring efficiency of our MCG assay with the standard DCC method and with three other methods recommended by other investigators for dilute cytosols. Cytosol from the MCF-7 breast cancer cell line was diluted serially to obtain protein concentrations ranging from 1.28 to 0.01 mg/ml. These cytosol dilutions were then assayed for ER using [3H]estradiol and the five procedures described in "Materials and Methods." The values of assay efficiency are plotted against the protein concentration in Fig. 1. Above 1 mg protein/ml, all methods gave about the same results, while ER content as measured by other methods at low protein concentrations (≤0.64 mg/ml) was always lower than that measured by the MCG assay. For example, at 0.04 mg protein/ml the MCG assay measured about 75% of the receptor activity, while the standard DCC assay registered only 10%. The LH-20 gel filtration assay did not even equal the DCC. Addition of 0.1% BSA or 0.2% gelatin to the charcoal suspension gave some improvement over the standard DCC procedure, but the MCG was better. At 0.01 mg protein/ml, MCG measured close to 60% of

![Fig. 1. Comparison of the efficiency of various assays (as described in “Materials and Methods”) for ER in MCF-7 cytosol at decreasing protein concentrations. The average [3H]estradiol binding obtained by the different methods in concentrated cytosol was set at 100% (15,693 cpm/tube in this experiment), and the assay efficiency at all protein concentrations was calculated as percentage observed/expected specific binding. The values shown are means of triplicate determinations.](image)

![Fig. 2. Comparison of the efficiency of the standard DCC and the MCG assay for ER in a typical human breast cytosol at decreasing protein concentrations. The average 125I-labeled estradiol binding obtained by the DCC and the MCG at 0.8 mg/ml was taken as 100% (66,632 cpm/tube in this experiment). Assay efficiency was calculated as described in Fig. 1.](image)

![Fig. 3. Scatchard analysis for ER by DCC and by MCG in a typical human breast tumor cytosol at 0.1 mg protein/ml using 125I-labeled estradiol. Experimental details are given in "Materials and Methods." Bound (B) and free (F) estradiol concentrations are given in fmol/ml incubation mixture; both Kd are about 4 × 10^−11 M.](image)

| Table 1 | Estrogen receptor content in a series of human breast tumor specimens determined by standard DCC and our MCG assay at standard (1 mg/ml) and low (0.1 mg/ml) protein concentrations using 125I-labeled estradiol. |
|---|---|---|---|---|
| No. | ER assayed at 1 mg/ml (fmol/mg protein) | ER assayed at 0.1 mg/ml (fmol/mg protein) |
| DCC | MCG | DCC | MCG |
| 1 | 41 | 46 | 16 | 48 |
| 2 | 27 | 31 | 10 | 25 |
| 3 | 51 | 49 | 17 | 49 |
| 4 | 54 | 59 | 3 | 51 |
| 5 | 73 | 69 | 28 | 58 |
| 6 | 95 | 116 | 37 | 91 |
| 7 | 240 | 221 | 76 | 232 |
| 8 | 288 | 305 | 78 | 284 |
| 9 | 482 | 463 | 168 | 459 |
| 10 | 665 | 660 | 236 | 612 |

*Mean ± SD.
These results are plotted as percentage of assay efficiencies for containing a wide range of ER levels by the DCC and the MCG by DCC, as expected.

60% of the ER activity, while standard DCC detected no difference between DCC and MCG with a cytosol directly from a typical human breast cancer biopsy using 12SI-labeled estradiol protein (see Table 1).

Subsequently, we assayed a series of breast cancer biopsies containing a wide range of ER levels by the DCC and the MCG procedure at 1 and 0.1 mg/ml. The results are given in Table 1. The standard DCC values at 1 mg/ml ranged from 41-665 fmol/mg protein. All ER values obtained by the MCG assay at 1 mg cytosol protein/ml were close to the DCC values. At 0.1 mg/ml, however, the overall mean efficiency of the standard DCC was only 32% as compared to 96% for the MCG assay. In each individual tumor in Fig. 4.

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