Stability of Estrogen- and Progesterone-Receptor Concentrations in Human Uterus Tissue

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Homogeneous control specimens for estrogen receptor (ER) and progesterone receptor (PR) assays were prepared from freshly collected human uterus. After removal of the connective tissue, the specimen was washed with isotonic saline, cut into small pieces, quickly frozen in liquid nitrogen, and stored at −70 °C until analyzed. Cytosol prepared from this specimen was lyophilized and stored at −70 °C. A single step of reconstitution, with glycerol (100 mL/L) in water, is sufficient to prepare a control. Two specimens prepared this way were found to be reasonably stable for 20 months (first specimen, mean ± SD: ER = 22.1 ± 2.9 fmol/mg, PR = 136.5 ± 26.9 fmol/mg; second specimen: ER = 107.2 ± 11.7 fmol/mg, PR = 922 ± 71.6 fmol/mg). Another specimen, prepared similarly but not frozen in liquid nitrogen soon after collection, was less stable; its ER and PR concentrations deteriorated faster.

In recent years, the presence of estrogen receptors (ER) and progesterone receptors (PR) in cancerous breast tumors has been shown to correlate well with response to endocrine therapy (1–3). As a result, these receptors in all surgically resected breast cancers are routinely measured at all major institutions (4–6).

The method commonly used for measurement of these receptors include: sucrose density-gradient ultracentrifugation, adsorption on dextran-coated charcoal, single saturation-dose analysis (7), gel filtration, electrophoresis (2), and histochemical methods (8).

We have been quantifying ER and PR in breast tumors since 1976, using successively sucrose density-gradient ultracentrifugation, dextran-coated charcoal, and single saturation-dose analysis. Although these methods have been well established, each needs a control specimen to validate the ER and PR assays. Because no commercial control was available, we looked into the possibility of preparing our own control material from freshly collected human uterus. This article describes the preparation and stability of ER and PR in human uterus tissue.

Materials and Methods

Preparation of the Control

Collection of the human uterus. Human uterus from women undergoing routine hysterectomy for uncontrolled dysfunctional bleeding was obtained at surgery. Connective tissue was removed by dissection and the freshly collected uterus was washed in cold isotonic saline, promptly cut into small pieces, and quickly frozen in liquid nitrogen. These tissues were transported on solid CO₂ to the laboratory and stored at −70 °C until analyzed.

Preparation of cytosol. The frozen pieces of uterine tissue were pulverized in a pulverizer (Thermovac Div., Island Park, NY 11558) that had been precooled in liquid nitrogen. The pulverized powder was suspended in three to five volumes of ice-cold buffer (per liter: 10 mmol of Tris HCl pH 7.4, 1 mmol of EDTA, and 0.5 mmol of dithiothreitol). The sample was then homogenized (Polytron homogenizer, Brinkmann Instruments, Westbury, NY 11590). The tubes were constantly kept in crushed ice. Four 15-s bursts at approximately 1-min intervals gave a consistent homogenate. The homogenate was transferred to 25 × 90 mm polyallomer tubes (Beckman no. 326823) and centrifuged for 45 min (105 000 × g). The temperature was maintained at 2 °C throughout the centrifugation. The clear supernate was filtered through glass wool and stored at 0 °C.

Protein determination. An aliquot of the cytosol was serially diluted in the buffer and the protein concentration was determined spectrophotometrically (9). First, the volume of cytosol was adjusted to approximately 2.5 g/L with the buffer and its protein content was determined by the method of Lowry et al. (10). After making several dilutions of the cytosol, to obtain more accurate determination of protein concentration, the cytosol volume was then adjusted to exactly 2 g/L in the buffer. A few milligrams of sodium azide was added and 2-mL fractions were aliquoted into 10-mL bottles and stored at −70 °C.

From homogenization to storage, every step in this 10-h process was carried out without interruption.

The following day, frozen cytosol fractions were lyophilized overnight. Approximately 50 samples could be lyophilized in a single batch. The bottles were then corked and stored at −70 °C, ready for use as a control material.

Reconstitution of control lyophilized powder. On the day of the experiment, the lyophilized control was reconstituted in 2 mL of glycerol/water (10/90 by vol), mixed, and left on ice until used as a control in assays of both ER and PR.

Methods of Analysis

Sucrose density-gradient method: Linear gradients of sucrose (100–300 g/L) were prepared the day before and equilibrated at 4 °C overnight. Two 250-μL samples of cytosol were incubated with 1.0 pmol of radioactive estradiol (2,4,6,7-[3H]estradiol-17β) for 4 h at 4 °C. One of the samples was incubated with 100 pmol of diethylstilbestrol (DES) for 15 min before the tritiated estradiol was added. Charcoal pellets used to separate the free from bound radiolabeled estradiol were prepared as follows: 1 mL of a suspension containing 2.5 g of charcoal and 25 mg of dextran per liter of Tris HCl buffer (10 mmol/L, pH 8.0) was centrifuged at 1600 × g for 10 min and the supernatant fluid discarded. The 250 μL of cytosol was transferred onto the pellet and the mixture was incubated at 4 °C for 20 min. After centrifugation, 200 μL of the clear supernate was layered onto the gradients. These gradients were centri-
fuged for 18 h at 207 000 × g. Then 0.2-mL fractions were collected with a gradient fractionator and their radioactivity was measured with use of modified Bray's scintillation cocktail.

Dextran-coated charcoal (DCC) assay: We incubated 200 μL of cytosol (1–2 mg/mL of protein) with increasing amounts of radiolabeled estradiol, ranging from 15 to 200 pmol. We also incubated 200 μL of cytosol with 100 pmol of DES for determination of nonspecific binding, and 200 μL of buffer blanks. All samples were incubated at 4 °C for 16 h, then 500 μL of charcoal suspension was added to all the tubes and incubated for 20 min at 4 °C. After centrifuging the tubes at 4 °C for 10 min, we then counted the radioactivity of the supernate, using Bray's scintillation cocktail. The data were analyzed by the method of Scatchard (11).

Single saturation-dose method: In this assay a single dose of tritiated estradiol was used to saturate the receptors. We incubated 200 μL of cytosol with 0.25 pmol or more of tritiated estradiol for 4 h at 4 °C. A parallel sample of 200 μL of cytosol was incubated with 100 pmol of DES 15 min before adding the tritiated estradiol. After adding 500 μL of charcoal suspension and incubating for 20 min at 4 °C, we centrifuged the samples at 1600 × g for 10 min and counted the supernate for radioactivity, using Bray's scintillation cocktail. This method is especially useful when the amount of the tumor tissue for analysis is limited and when multiple receptor determinations are needed on a single specimen.

Progesterone receptors were quantified similarly (3). R5020 (17,21-dimethyl-19-nor-pregna-4,9-diene-3,10-one) and radiolabeled R5020 obtained from New England Nuclear (Boston, MA 02118) were used for the PR assays.

Results

Stability of human uterus powder (HUP) was studied for ER and PR concentrations for several months. To analyze ER and PR, the dextran-coated charcoal assay was our routine procedure, but we used sucrose density-gradient ultracentrifugation and single saturation-dose methods periodically to check the accuracy of the system. Figure 1 shows a Scatchard plot analysis for ER in HUP #1, and

Figure 2 shows the results of sucrose density-gradient analysis for both ER and PR. The Scatchard plot confirms the presence of ER (Kd 4.9 × 10⁻¹¹ mol/L, Ks 2.03 × 10¹⁰ L/mol), and the sucrose density-gradient assay confirms the presence of both 8S and 4S fragments of estrogen receptors. To prevent loss of receptor activity, the freshly obtained samples were quickly frozen in liquid nitrogen, transported on solid CO₂ to the laboratory, and pulverized at liquid nitrogen temperatures. Homogenization was at 0 °C, ultracentrifugation between 2 and 4 °C. Scatchard plot analyses on the lyophilized powders showed no loss in receptor activity. After storage of the lyophilized samples at −70 °C freezer, HUP #1 had an average ER of 22.1 (SD 2.9) fmol/mg over a period of 20 months (Figure 3). The average PR value over the same period for HUP #1 was 136.5 (SD 26.9) fmol/mg. In another human uterus powder (HUP #2), prepared earlier, the average ER for 20 months for HUP #2

Fig. 1. Scatchard analysis of ER binding sites
Receptor content (femtomoles) is represented on the x-axis and B/F on the y-axis. Kd = 4.9 × 10⁻¹¹ mol/L, Ks = 2.03 × 10¹⁰ L/mol

Fig. 2. Sucrose density-gradient analysis for PR (A) and ER (B)

Fig. 3. Stability of ER and PR in HUP #1 and HUP #2 during 20 months

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was 107.2 (SD 11.7) and the PR was 922.1 (SD 71.6) fmol/mg (Figure 3).

HUP #3 was not frozen in liquid nitrogen soon after collection, but the remaining steps in preparing the lyophilized powder were carefully followed as with HUP #1 and 2. Although the sample appeared to be stable for four months, ER and PR activity deteriorated thereafter (Figure 4). These findings suggest the possible importance of freezing the uterus in liquid nitrogen.

Discussion

As information regarding ER and PR becomes useful in selecting patients for endocrine treatment, it is critical to set up rigid controls for ER and PR assays in the laboratory. Several approaches have been previously reported. In England, frozen human breast tissue was used; however, cellular heterogeneity and receptor instability were drawbacks (12). The EORTC group in Holland and Germany used lyophilized tissue powder from human and animal sources (13); the concentration of ER remained stable under various storage conditions. Similar studies with tissue powder have been instituted by the National Surgical Adjuvant Breast Protocol (NSABP) and the Cancer and Leukemia Group B (CALGB) in the United States. These samples require homogenization of the control specimen before use in the assay.

The method we describe for preparing a homogenous lyophilized specimen derived from human uterus requires only one simple step of reconstituting the lyophilized control specimen with water. The homogeneity of the receptors is maintained for a considerable period—as long as 20 months in quickly frozen, freshly collected human uterine tissue. Using such controls would greatly improve the quality of estrogen and progesterone receptor assays.

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References