Estradiol Receptor Analysis in Human Breast Cancer Tissue by Isoelectric Focusing in Polyacrylamide Gel[1]

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Abstract

Isoelectric focusing in polyacrylamide gel combined with limited proteolysis is a simple and specific method for quantitation of estradiol receptors in breast cancer tissue. At least eight different samples can be analyzed simultaneously on one gel, and the whole procedure, including sample preparation, takes less than 7 hr. In comparison with sucrose gradient centrifugation, isoelectric focusing is more sensitive, possibly due to the short time (1.5 to 2 hr) needed for the analysis. Furthermore, only one incubation with tritium-labeled estradiol is needed for an analysis, which means that a smaller amount of tumor tissue is needed than for most other methods. This fact allows analysis of the estrogen receptor content in tumor material obtained from fine-needle biopsy.

Introduction

Target cells for steroid hormones have been shown to contain receptor proteins for these hormones (3). The receptor proteins are recovered in the soluble fraction after homogenization and centrifugation of a target tissue. They can be demonstrated by incubating the soluble fraction with tritium-labeled hormone that is bound to the receptor protein with high affinity and low capacity. Breast cancer is a common malignant disease that sometimes (in one-third of the cases) is hormone dependent in its growth, and in these cases tumor growth may be inhibited by manipulating the endocrine environment (6). It has been demonstrated that patients bearing tumors that contain estradiol receptors have a high probability of benefiting from endocrine therapy, whereas patients with estradiol receptor-negative tumors are unlikely to benefit from such treatment (6).

Consequently, there has been a need for simple and specific methods for estradiol receptor analysis in human breast cancer tissue. This report contains a brief description of how to assay estradiol receptors in such tissues by the use of isoelectric focusing in slabs of polyacrylamide gel and how this technique may be applied. The first description of the use of isoelectric focusing in polyacrylamide gel for estradiol receptor analysis in breast cancer tissue was published recently (10).

Materials and Methods

Tissue Material. Pieces of mammary carcinomas were

cut out immediately after surgery and kept in 0.9% NaCl solution on ice. They were either analyzed within 1 to 2 hr or stored without buffer at −70°.

Preparation of Sample for Estradiol Receptor Analysis. Tumor tissue was thawed, minced, and suspended in 2 volumes of 10 mM Tris-HCl (pH 7.4):1.5 mM disodium EDTA buffer containing 7.5 nM [^{3}H]estradiol to give a final hormone concentration of 5 nM. A parallel incubation containing a 100-fold excess of unlabeled estradiol or diethylstilbestrol, together with 7.5 nM [^{3}H]estradiol, was sometimes used as a control for nonspecific binding (9).

After incubation for 30 min at 0°, the tissue was homogenized with a Polythrone (Kinematica, Lucerne, Switzerland), and then the whole homogenate was incubated for 45 min at 0° and subsequently centrifuged at 20,000 × g for 10 min. The supernatant (cytosol) was taken for estradiol receptor analysis, and the pellet containing the cell nuclei was retained for measurement of DNA according to the method of Burton (1), as a reference for quantitation of specific binding. The protein content in a portion of the tumor cytosol was estimated by measuring the difference in absorbance at 280 nm and 310 nm ($A_{280-310}$). One $A_{280-310}$ unit of cytosol was found to correspond to about 0.7 mg of protein as analyzed according to the method of Lowry et al. (5), with bovine serum albumin as standard.

Trypsin, stored at −20° as a lyophilized powder, was dissolved in water on the day of the experiment. The tumor cytosol was made 2 mM with respect to $CaCl_2$, and 5 μg of trypsin were added per $A_{280-310}$ unit of cytosol. The mixture was incubated for 30 min at 10° and then 1/3 volume of dextran-coated charcoal was added [dextran:charcoal, 1:10 (w/w)], giving a final charcoal concentration of 1% (w/v), to remove unbound estradiol. After a 10-min incubation at 0°, the charcoal was removed by centrifugation at 800 × g for 10 min, and the supernatant was subjected to isoelectric focusing as described below. All procedures for which no temperature is given were performed as close to 0° as possible.

Thin-Layer Polyacrylamide Gel Electrofocusing. Polyacrylamide gels, 245 × 110 × 2 mm, were prepared essentially according to the recommendations given by LKB-Produkter AB, Bromma, Sweden (4) and contained Ampholine at pH ranges of 3.5 to 10 and 5 to 8 at concentrations of 0.6% (w/v) and 0.4% (w/v), respectively. The polyacrylamide gel had a value of T = 5% (w/v) and C = 3% (w/v) (2), and glycerol was added to a concentration of 10% (w/v). Riboflavin, 1 μg/ml, was also included, and the gels were allowed to polymerize in light for 2 hr (4).

Ready-made gels from LKB-Produkter AB, containing 2.4% (w/v) Ampholine, pH 3.5 to 9.5, were also used for

estradiol receptor analysis and gave results identical with those obtained with the gel described above.

Isoelectric focusing was performed with a Multiphor (LKB-Produkter AB). Electrode strips soaked in 1 M sodium hydroxide (cathode) or 1 M sulfuric acid (anode) were used. The gel was prefocused for 15 to 20 min at a current of 15 ma.

Sample frames were used for sample application and were allowed a volume of 0.3 ml. The frames are made of acrylic plastic. After the gel was prefocused, the sample frames were placed on the gel surface 0.8 to 1 cm from the cathode electrode strip, where the final pH is about 8 to 8.5. [The hormone:receptor complex is dissociated or destroyed in the acid pH near the anode (10).] Ferritin and hemoglobin, dissolved in water and added to the charcoal suspension, were added to each sample as internal standards, these reference proteins having isoelectric points of 4.5 to 5 and 7.6, respectively. The standards are easily detected as a yellow band (ferritin) and as a brown band (hemoglobin).

Each sample was pipetted into a sample frame. Usually, 8 samples were analyzed simultaneously. If enough tumor material is available, it is convenient to place a sample volume containing 0.5 to 1 $A_{280-310}$ unit of tumor cytosol in each frame. This amount of cytosol protein is enough to detect low amounts of receptor (<0.01 fmol/μg DNA). In some experiments more than 4 $A_{280-310}$ units of tumor cytosol were applied without affecting the analysis of the receptors, which demonstrates that the risk of overloading the gel is small.

Isoelectric focusing was initiated at a current of 15 ma. The voltage was increased stepwise to 1000 to 1200 V. When the ferritin standard was focused near the anode and the hemoglobin standard was focused in front of the sample frame (after about 1 hr), the liquid inside the sample frames was aspirated with a Pasteur pipet, and the sample frames were removed. Any remaining sample solution on the gel surface was removed with a filter paper to minimize the risk of radioactive contamination.

The isoelectric focusing was continued until the current ceased to diminish. The total focusing time from the application of sample was about 1.5 hr. Effective cooling is of the utmost importance because of the rapid dissociation of the hormone:receptor complex when exposed to heat. We perform the analysis in a cold room at 2–4° and ice water is continuously pumped through the cooling plate of the Multiphor. pH was measured at 2–4° with a surface pH electrode (type 403–30; Ingold, Zürich, Switzerland).

Fractionation of the Gel. The 2-mm-thick gel was cut into strips, one for each sample track, and each strip was transferred to a slicing frame made of razor blades. A sheet of parafilm (American Can Co., Greenwich, Conn.) was placed on the gel, which was then pressed down between the razor blades with the help of a cork ring. The Parafilm was removed, and each 3.3-mm-broad gel slice was transferred to a plastic vial with a pair of forceps. Instagel (Packard Instrument Co., Inc., Warrenville, Downess Grove, Ill.), 5 ml, was added to each vial. The vials were shaken vigorously and kept at 50° for 1 hr and then shaken again and assayed for radioactivity in a liquid scintillation spectrometer. The incubation at 50° is sufficient to allow the

tritium-labeled steroid to diffuse out of the gel (data not shown).

Receptor Quantitation. The radioactivity in the vials was measured, and the efficiency was calculated according to the external standard technique. The peak of radioactivity with a pH maximum between 6.5 and 6.7 represented receptor-bound estradiol. For each sample a diagram was drawn, and the diagrammatic base-line radioactivity was subtracted from the peak (see also "Results"). Routinely, only a 5-cm-long gel strip between pH 8 and pH 4.7 was fractionated and assayed for radioactivity.

Results and Discussion

As illustrated in Chart 1A, the nontrypsinized tumor cytosol is focused as a double peak with maxima at pH 5.9 to 6.1 and 6.5 to 6.7; both these peaks disappear when a 100-fold excess of unlabeled diethylstilbestrol is present in the incubation medium. After trypsin digestion (Chart 1B), only one sharp peak, with a maximum at pH 6.5 to 6.7, is seen; this radioactivity peak also disappears when a 100-fold excess of unlabeled diethylstilbestrol is present in the incubation medium, which demonstrates that this peak represents saturable binding of estrogen. It was previously shown (10) that limited trypsin digestion results in the formation of a hormone-binding fragment of the estrogen receptor with a sedimentation coefficient of 4S in low-ionic-strength solutions and a pI of 6.5 to 6.7. The nontrypsinized (native) estrogen:receptor complex has a sedimentation coefficient of 8S in low-ionic-strength solutions and a pI of 5.9 to 6.1. Cytosol preparations from human breast cancer tissue usually contain a mixture of the 8S and 4S estrogen receptor complexes, and such samples are thus focused as a double peak (see Chart 1A). This may be due to endogenous protease activity in the cytosol sample preparation. In the experiment illustrated (Chart 1), 11% more receptor was
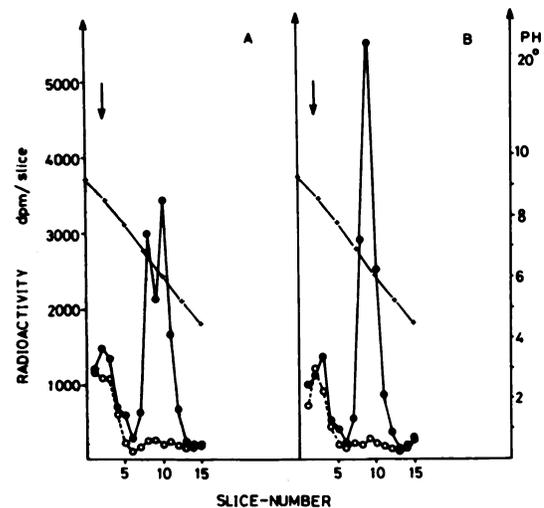

Chart 1. Isoelectric focusing in polyacrylamide gel of cytosol prepared from human mammary cancer tissue. [³H]Estradiol (5 nM) with (O) or without (●) a 100-fold excess of unlabeled diethylstilbestrol was incubated with cytosol. After 2 hr at 0°, one-half of each sample was removed (A); CaCl₂ and trypsin were added to the other half, and the mixture was incubated further for 30 min at 10° (B). The volume of the nontrypsinized controls (A) was adjusted with redistilled water, and the 4 samples were analyzed as described. Arrow, sample application point; ×, pH gradient. The gel strip between pH 4.6 and 9.3 was taken for analysis.

recovered in the trypsinized sample than in the nontrypsinized sample, as is usually the case (10); this may be due to a higher tendency of the native receptor to aggregate and stay at the application point, as compared with the trypsin-induced receptor fragment.

Human serum labeled with [³H]estradiol also gives rise to a peak of radioactivity focusing at pH 5.5 (Chart 2). The saturable estradiol-binding component in serum most probably consists of sex hormone-binding globulin. If the incubation medium also contains a 1000-fold excess of unlabeled diethylstilbestrol or estradiol, this peak contains 90 and 59%, respectively, of bound [³H]estradiol as compared with the control, demonstrating that diethylstilbestrol has little ability to compete with estradiol for binding to sex hormone-binding globulin. The pI of the radioactivity peak detected in serum does not change as a result of trypsin digestion (data not shown). Limited trypsin digestion of the tumor cytosol prior to isoelectric focusing is thus recommended for the following reasons: (a) increased specificity of the assay, due to increased separation of the trypsin-induced receptor fragment focusing at pH 6.5 to 6.7 from sex hormone-binding globulin focusing at pH 5.5 as compared with the native receptor (focusing at pH 5.9 to 6.1); and (b) increased sensitivity of the assay, due to sharper peaks and minimized risk of losses due to receptor aggregation.

As reported elsewhere (10) the loss of estrogen receptor due to proteolytic degradation during digestion with trypsin is insignificant with the recommended dose of trypsin, and the loss is 8 and 25%, respectively, when 4 or 8 times the recommended amount of trypsin is used. High resistance to further proteolytic degradation of the hormone-binding estrogen receptor fragment prepared from calf uterus was previously reported by Rat et al. (7). If too little trypsin is added to the tumor cytosol, a double peak will be formed
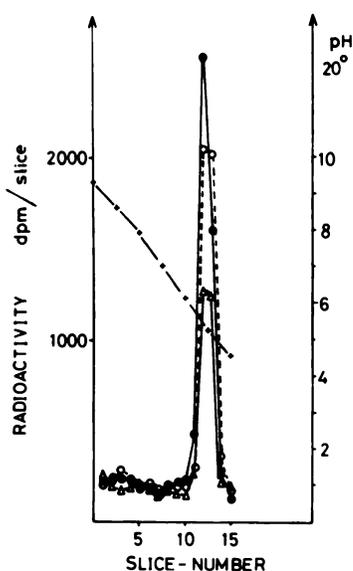
on isoelectric focusing, due to insufficient proteolysis of the native receptors focusing at 5.9 to 6.1 (See Chart 1).

The intraassay variation of the assay was 4.6% when 5 samples of the same tumor cytosol were analyzed simultaneously. The recovery of estrogen:receptor complex after limited trypsin digestion and isoelectric focusing is about 85% when compared with the amount of specifically bound [³H]estradiol determined by dextran-coated charcoal treatment in the presence and absence of a 100-fold excess of unlabeled diethylstilbestrol.

The peak focused at pH 6.6 has been found by repeated experiments to represent receptor-bound estradiol and is always absent in a control incubation containing an excess of unlabeled estradiol or diethylstilbestrol. These and other data (10) have shown that no estradiol-binding protein other than the proteolytically induced estradiol receptor fragment is focused at pH 6.6, and thus we have found that a control incubation with excess of unlabeled competitor is unnecessary in the routine assay. Thus, one incubation and one analysis per tumor are sufficient, thereby reducing the amount of tumor tissue needed for an analysis to a minimum. In fact, our method allows quantitation of estradiol receptor content in tumor material obtained by fine-needle biopsy (Chart 3). Such a biopsy normally contains about 5 to 100 μg of DNA which is equivalent to about 5×10^4 to 10^6 cells. An aliquot of the sample may be taken for cytological examination. This way of collecting tumor tissue for estrogen receptor analysis has several advantages: (a) the tumor material is obtained without an operation, which decreases the stress for the patient; (b) the tumor cells are obtained in a suspension, which means that the material taken for cytological diagnosis is qualitatively identical with the material taken for estrogen receptor analysis; (c) a new sample is easily taken if the amount of cells is insufficient as judged from cytological examination; and (d) almost exclusively tumor cells are obtained by fine-needle biopsy, probably because other cells present in the tumor tissue are more strongly fixed to the stroma than are the tumor cells.

Chart 2. Isoelectric focusing in polyacrylamide gel of serum from pregnant women. Serum (10%), v/v was incubated with 5 nM [³H]estradiol without competitor (●), in the presence of a 1000-fold excess of diethylstilbestrol (○), or in the presence of a 1000-fold excess of estradiol (△) for 2.5 hr at 0° and was then analyzed as described. The sample was applied to the gel at a place corresponding to Fractions 1 to 3; ×, pH gradient.
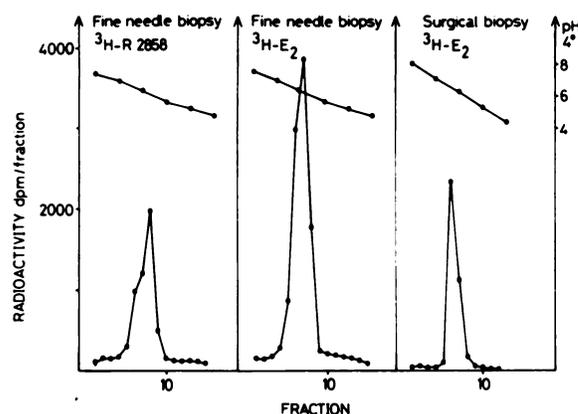

Chart 3. Tumor tissue from the same patient obtained with surgical biopsy or fine-needle biopsy analyzed with isoelectric focusing in polyacrylamide gel. For conditions of incubation of tissue obtained with surgical biopsy, see "Materials and Methods." The tissue obtained with fine-needle biopsy was incubated with 5 nM [³H]estradiol (³H-E₂; 151.3 Ci/mmol) or 20 nM [³H]R 2858 (52 Ci/mmol). These concentrations of ligand gave saturation of receptor sites under the conditions used. The following values (fmol/μg DNA) were obtained in the 3 analyses: 0.75 (surgical biopsy); 2.62 (fine-needle biopsy, [³H]estradiol); and 3.47 (fine-needle biopsy, [³H]R 2858).

One disadvantage with the fine-needle biopsy technique is the fact that, since the tumor cells are sucked into the syringe by vacuum, the samples usually are heavily contaminated with blood. This may disturb the receptor analysis also when performed with isoelectric focusing; therefore, we now use the synthetic estrogen R 2858[2] as tritiated ligand in estradiol assays of fine-needle biopsy specimens (Chart 3). R 2858 was introduced by Raynaud *et al.* (8), who demonstrated that R 2858 binds to the estrogen receptor but has no affinity for serum proteins such as sex hormone-binding globulin. We have found [³H]R 2858 to be useful for estrogen receptor assays for these reasons.[3]

At present we are carrying out comparative estradiol receptor analyses with isoelectric focusing using tissue material from the same patients obtained with both surgical biopsy and fine-needle biopsy. Although it is quite clear that the latter technique yields sufficient tissue material for estradiol receptor analysis, it is still too early to draw any conclusions concerning the correlation between the results obtained with the 2 methods.

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[2] The abbreviation used is: R 2858, 11β-methoxy-17α-ethinyl-1,3,5(10)-estratriene-3,17β-diol.

[3] S. Okret, Ö. Wrange, B. Nordenskjöld, Claes Silfoerswärd, and J-Å. Gustafsson. Estrogen Receptor Assay in Human Mammary Carcinoma with the Synthetic Estrogen 11β-methoxy-17α-ethinyl-1,3,5(10)-estratriene-3,17β-diol (R 2858). Cancer Res., *38:* 3904–3909, 1978.

References

1. Burton, K. Determination of DNA Concentration by Diphenylamine. Methods Enzymol. *12B:* 163–166, 1968.
2. Hjertén, S. Molecular Sieve Chromatography on Polyacrylamide Gels, Prepared according to a Simplified Method. Arch. Biochem. Biophys., Suppl. *1:* 147–151, 1962.
3. Jensen, E. V., and De Sombre, E. R. Mechanism of Action of the Female Sex Hormones. Ann. Rev. Biochem., *41:* 203–230, 1972.
4. Karlsson, C., Davies, H., Öhman, J., and Andersson, U.-B. LKB Application Note 75. Bromma, Sweden: LKB-Produkter AB, 1973.
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., Carbone, P. P., Sears, M. E., and Escher, G. L. Estrogen Receptors in Human Breast Cancer: An Overview. *In:* Estrogen Receptors in Human Breast Cancer (McGuire, W. L., Carbone, P. P. and Vollmer, E. P., eds.) pp. 1–7, Raven Press, New York, 1975.
7. Rat, R. L., Vallet-Strouve, C., and Erdos, T. The Properties of a Uterine Estradiol Receptor after Limited Proteolysis. Biochimie, *56:* 1387–1394, 1974.
8. Raynaud, J.-P., Bouton, M.-M., Gallet-Bourquin, D., Philibert, D., Tournemine, C., and Azadian-Boulanger, G. Comparative Study on Estrogen Action. Mol. Pharmacol., *9:* 520–533, 1973.
9. Soloff, M. S., Creange, J. E., and Plotts, G. O. Unique Estrogen-binding Properties of Rat Pregnancy Plasma. Endocrinology, *88:* 427–432, 1971.
10. Wrange, Ö., Nordenskjöld, B., and Gustafsson, J.-Å. Cytosol Estradiol Receptor in Human Mammary Carcinoma: An Assay Based on Isoelectric Focusing in Polyacrylamide Gel. Anal. Biochem., *85:* 461–475, 1978.

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