Estrogen Receptor and Proteolytic Activity in Human Breast Tumor Nuclei

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SUMMARY

We have previously found that human breast cancer cells in tissue culture contain estrogen receptor in their nuclei despite the absence of estrogen. We have now investigated solid human breast cancer biopsies and find that proteolytic activities in extracts from the nuclear pellets of these biopsies interfere with or prevent the measurement of nuclear estrogen receptor when the protamine assay is used. However, the problems of receptor degradation can be avoided by the use of a hydroxylapatite assay.

INTRODUCTION

It is now generally accepted that the presence of unoccupied cytoplasmic estrogen receptor in a human breast tumor indicates that the tumor is likely to regress following endocrine therapy (11). Recently, we found a human breast cancer cell line that contained the majority of the cellular estrogen receptor in the nucleus, even in the absence of estrogen (15). The finding of this "free" nuclear receptor (RN) was unexpected, since in normal tissues with identical assay procedures we have seen unoccupied estrogen receptor primarily in the cytoplasm (16). Nuclear estrogen receptor is usually found only in bound form (RNE) following estrogen translocation of unoccupied cytoplasmic estrogen receptor.

To see whether this unusual distribution of estrogen receptor could be found in human breast cancers in vivo or was limited to breast tumor cells in tissue culture, we studied biopsy specimens of solid human breast cancers. We now report the presence of proteolytic activity in extracts from the myofibrillar nuclear pellet of human breast tumors, which interferes with or prevents the measurement of RN and RNE by the protamine sulfate assay. We further demonstrate, however, that the proteolytic activity is avoided by the use of a hydroxylapatite assay (3, 14).

MATERIALS AND METHODS

Human Breast Tumors. Biopsies were frozen in liquid nitrogen, transported to the laboratory in dry ice, and stored at -70° in a Revco freezer (Revco, Inc., West Columbia, S. C.).

Rat Uteri. Female Sprague-Dawley rats (Holtzman Co., Madison, Wis.) weighing 200 to 250 g were ovariectomized and adrenalectomized under ether anesthesia 48 hr prior to use. Rats were sacrificed by cervical dislocation 30 min after i.p. injection of 10 µg 17β-estradiol given in 10% ethanol and 0.15 M NaCl. Uteri were removed, cleaned, and frozen in liquid nitrogen.

Preparation of Cytoplasmic and Nuclear Extracts. Frozen tissues were pulverized with a Thermovac tissue pulverizer (Thermovac, Inc., Copiaque, N. Y.), weighed, and thawed to 4°. The powder was homogenized in 4 volumes of phosphate buffer prepared immediately before use [5 mM sodium phosphate (pH 7.4), 1 mM monothioglycerol, 10% (v/v) glycerol] with three 10-sec bursts of a Polytron PT-10-ST homogenizer set at 3.2. The homogenate was centrifuged at 800 x g and twice resuspended in phosphate buffer. All the supernatants were combined and centrifuged at 105,000 x g for 30 min, and supernatant cytosol was obtained. The washed 800 x g nuclear myofibrillar pellet was extracted for 60 min with 4 volumes of TK buffer (10 mM Tris-HCl (pH 8.5), 0.6 M potassium chloride, 1 mM monothioglycerol, and 10% (v/v) glycerol). Tubes were vortexed at 10-min intervals during the extraction and then centrifuged at 105,000 x g for 30 min to obtain the supernatant nuclear extract.

Nuclear Protamine Receptor Preparation. The protamine receptor assay was performed as previously described (16). Cytosol (200 µl) diluted with phosphate buffer to a final protein concentration of 0.75 to 1.25 mg/ml or nuclear extract (500 µl) diluted 6- to 10-fold to achieve a final KCI concentration of less than 0.1 M was incubated 5 min with 250 µl protamine sulfate (U.S.P. injection, without phenol preservative; Eli Lilly & Co., Indianapolis, Ind.), 1.0 mg/ml in phosphate buffer. Tubes were centrifuged at 800 x g for 10 min, the supernatant was discarded, and the precipitated receptor was assayed as described below.

Nuclear Hydroxylapatite Receptor Assay. Following the approach used in the protamine assay, unfilled and filled estrogen receptors were preprecipitated from nuclear extract by hydroxylapatite (Bio-Rad DNA grade). Nuclear extract (200 µl) previously diluted with phosphate buffer to produce a final protein concentration between 0.4 and 1.0 mg/ml was added to bovine serum albumin-washed tubes (2). A hydroxylapatite slurry (250 µl), prepared as described by Williams and Gorski (14) in a packed/liquid volume ratio of approximately 0.7, was added to the tubes. The mixture was incubated on ice for 30 min, vortexed every 10 min, and
centrifuged at 800 x g for 2 min, and the supernatant was discarded. Hydroxylapatite-bound receptor was then assayed as described below.

**Nuclear Binding Site Determination.** The number of specific estrogen receptor sites precipitated by protamine or hydroxylapatite was determined by adding 5 nM 17β-[^3H]estradiol, 107 Ci/m mole (in triplicate), with or without 500 nM diethylstilbestrol to measure nonspecific binding. Times and temperatures are indicated in the chart legends. Following incubation the pellets were washed twice with phosphate buffer for the protamine assay or with phosphate buffer plus 1% Tween 80 (Sigma Chemical Co., St. Louis, Mo.) for the hydroxylapatite assay and then were extracted overnight at room temperature with 1 and 2 ml ethanol, respectively. The extracts were counted in 5 ml scintillation fluid (4.0 g PPO, 0.05 g POPOP, 1 liter toluene). Protein content was initially determined by A<sub>260</sub>/280 (8) and later was confirmed by the method of Lowry et al. (9). DNA was determined according to the method of Burton (1).

**RESULTS**

**Temperature-Time Course for the Binding of[^3H]estradiol to Breast Tumor Nuclear Extract.** Our initial approach for measuring RN was to add a saturating amount of [^3H]estradiol to a protamine-precipitated nuclear extract at 4°. We have previously shown that no exchange of [^3H]estradiol for nonradioactive estradiol bound to receptor (RNE) occurs at this temperature in rat uterine cells or breast cells in culture, (15). To measure RNE, we added saturating amounts of [^3H]estradiol to the protamine-precipitated nuclear extract as before but incubated the mixture at elevated temperature to effect an exchange of labeled ligand for unlabeled ligand. In our previous studies of rat uterine cells and breast cells in culture, we found that RNE was very stable during these incubations at elevated temperatures (15, 16). Chart 1 illustrates our experience with nuclear extracts from solid human tumor biopsies. This representative experiment demonstrates that RN can be quantitated at 4° and supports our previous findings in cultured breast cancer cells (15). However, under the conditions of elevated temperature required to effect an exchange (RNE assay), RN is rapidly degraded, suggesting the possibility of a temperature-dependent degradative process in nuclear extracts of solid human tumor biopsies.

**Effect of a Breast Tumor Nuclear Extract on Uterine Nuclear-bound Estrogen Receptor.** To determine whether a factor exists in nuclear extracts of solid human breast tumors that degrades estrogen receptor, we incubated equal parts of a nuclear extract containing RNE from rat uterus with a nuclear extract from a solid human breast tumor. Each nuclear extract was diluted to the same protein concentration prior to incubation. Chart 2 shows that the nuclear extract of the breast tumor markedly reduced our ability to measure RNE in the rat uterine nuclear extract. We examined this effect in more detail by mixing the uterine RNE-containing nuclear extract with increasing quantities of breast tumor nuclear extract. For control purposes, a parallel incubation was performed with a breast tumor nuclear extract that had been heated to 70° to destroy any proteolytic enzyme activity. Chart 3 demonstrates an inverse relationship between the amount of breast tumor nuclear extract added and the amount of RNE measurable in the uterine nuclear extract. The inhibition was completely prevented by the 70° heat treatment of the breast tumor nuclear extract. This suggests that the breast tumor nuclear extract might have contained a protein with proteolytic activity, which degraded the uterine nuclear estrogen receptor.

**Protease Inhibitor Study.** There are several reports of proteases in transformed cells (7, 10, 13). If, indeed, breast tumor nuclear extracts contain a protease that degrades estrogen receptor or in some way interferes in the assay of receptor, this might be revealed with the use of protease inhibitors. Chart 4 demonstrates that Trasylol, a potent protease inhibitor, can prevent the loss of RNE from uterine nuclear extracts incubated with breast tumor nuclear extracts. This supports our suggestion that the loss of RNE is due to a nuclear protease present in human breast tumor biopsies.

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**Chart 1.**[^3H]estradiol binding to protamine-precipitated human tumor nuclear extract as a function of time and temperature. See "Materials and Methods" for details.
Estrogen Receptor and Protease Activity

DISCUSSION

We have successfully used the protamine exchange assay to measure cytoplasmic and nuclear estrogen receptor in normal reproductive tissue (16) and in human breast cancer cells in tissue culture (15). This paper demonstrates that in solid human breast tumor biopsies the protamine exchange assay is unable to successfully quantitate RNE because of a temperature-dependent protease in the nuclear extracts from these tumors. This appears to be a general finding, since we have detected protease activity in the majority of 30 tumors examined.

We find that when the protamine receptor pellet is warmed above 15°, \(^{3}H\)estradiol binding is quickly lost. The results of our mixing experiments with an exogenous source of RNE from rat uteri suggest that the loss is due to proteolytic activity in the breast tumor nuclear extracts, since either heat inactivation of these nuclear extracts or addition of protease inhibitors prevents the loss of RNE.

There is considerable precedent for the presence of proteases in cell extracts. They have been described in transformed cell lines (7, 10), rat uteri (6), and human mammary and uterine tumors (5). From our data we are unable to determine how much of the \(^{3}H\)estradiol binding loss is due to proteolytic activity directed against the receptor and how much is due to activity directed against the protamine to which the receptor is bound. The latter possibility is suggested by data from other laboratories, in which protamine has been used as a substrate for the assay of proteolytic enzymes (6, 12).

In any case, the problem of nuclear protease activity can be avoided by using a modification of the hydroxylapatite assay (3, 14), in which the receptor is preprecipitated by hydroxylapatite and then incubated with the hormone. It would seem that the proteolytic activity is not bound by hydroxylapatite, and therefore the assay of nuclear estrogen receptor is unaffected.

We have now begun to examine systematically human breast tumor biopsies using the hydroxylapatite assay. We find that certain tumors do indeed contain RN and RNE.

Protamine versus Hydroxylapatite Assays of RNE. Since protamine is a basic polypeptide often used as a substrate for measuring certain proteases, such as plasminogen activator, trypsin, etc. (6, 12), we wondered whether part of the receptor loss seen when using the protamine assay might be due to proteolytic degradation of the protamine itself. Therefore, we compared the protamine assay and the hydroxylapatite assay under the same experimental conditions described earlier. An RNE-containing nuclear extract from rat uteri was incubated with a nuclear extract from a breast tumor biopsy and assayed by the 2 methods. With the protamine assay the usual receptor loss was seen when increasing quantities of breast tumor extract were added (Chart 5). In contrast, the hydroxylapatite assay was able to measure the RNE in spite of the presence of the breast tumor nuclear extract. At this point we have not determined whether the loss of RNE is due to an effect on RNE or on the protamine, or both. In any case, it appears that the hydroxylapatite assay avoids the problem entirely.

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supporting the relevance of similar data obtained from human breast cells in tissue culture (15). These data are the subject of the accompanying paper (4).

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REFERENCES

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