Nuclear Estradiol-binding Sites in Human Breast Cancer

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INTRODUCTION

Following the demonstration by Jensen et al. (16) that the presence of estrogen receptors in human breast cancer correlated with response to endocrine therapy, determinations of ERC\(^*\) have been used extensively in many laboratories. However, the presence of ERC in a tumor results in only a 55 to 60% chance of remission in response to endocrine-ablative or -additive therapy (17). From these observations, it is obvious that the presence of ERG alone is not a sufficient marker of hormonal sensitivity.

Estrogen receptors may recognize estrogens but may not be able to initiate events beyond the binding step; thus, a product of estrogen action might be a more accurate indicator of the estrogen receptor by itself. PGR, the synthesis of which is known to be controlled by estrogens (15, 32), are thought to be valuable additional criteria of estrogen sensitivity (2). According to the multicenter study of Osbourne and McGuire (29), 77% of patients with tumors containing both ERC and PGRC will respond to endocrine manipulations. It remains unclear why the remaining 23% do not respond to such treatments, despite the presence of PGR suggesting that the estradiol system is operating. Several explanations can be offered to account for this. (a) It may be due to the heterogeneity of the tumoral tissue; tumors might contain a heterogeneous population of hormone-dependent and autonomous cell types (23). (b) PGR may reflect only a part of estrogen action; this could explain the dissociation between the effects of estrogen on growth and PGR induction (24). (c) It has been demonstrated that the presence of PGR may occur in some cells unresponsive to estradiol (14, 18, 26).

This consideration prompted different authors to investigate other criteria which may be more closely linked with mammary tumor growth such as prolactin (3, 33, 35, 37), estradiol-induced protein (7, 47), and estradiol nuclear receptors (1, 10, 12, 13, 28, 40, 42, 43).

In the present report, we have investigated nuclear binding sites in human breast tumors in order to improve the selection of patients who would benefit from hormone therapy.

MATERIALS AND METHODS

Tumor Specimens

Mammary tumor specimens were exclusively adenocarcinomas. At the time of collection, fat was removed, and samples were divided into 2 pieces. One was submitted for histological studies, and the other was frozen and stored in liquid nitrogen until assays were performed. For some experiments, tissue from several ERC-, PGR-positive tumors were pooled in order to provide sufficient material.

Reagents and Buffers

\[^{[\text{H}]\text{Estradiol}}, 160 \text{ Ci/mmol}; ^{[\text{H}]\text{estradiol}}, 1000 \text{ Ci/mmol}; ^{[\text{H}]\text{R 5020}}, 90 \text{ Ci/mmol}; \text{ and unlabeled R 5020 were purchased from New England Nuclear, Boston, Mass. DES, cortisot, dihydrotestosterone, and progesterone were from Steraloids, Inc. (Pawling, N. J.). Sephadex LH 20, Sephadex G-200, and the standard protein calibration kit were purchased from Pharmacia, Uppsala, Sweden; the Bio-Rad protein kit assay was from Bio-Rad Laboratories, Richmond, Calif.}\]

The buffers used were P buffer, TK buffer, TP buffer, N\(_4\) buffer, and N\(_2\) buffer. Bio-Gel HT HAP (Bio-Rad) was washed extensively at 4\(^{\circ}\) with TP buffer until the wash buffer reached pH 7.2. The HAP was resuspended in TP buffer (70% HAP-30% buffer).

Tissue Processing

All procedures were carried out at 4\(^{\circ}\) unless otherwise stated. The frozen tissues were weighed and then pulverized with a Thermovac tissue pulverizer (Thermovac Industries, Inc., Copaque, N. Y.). The resulting fine powder was homogenized in P buffer using a motor-driven homogenizer (Interbraun, Melsungen, Germany). The crude nuclear fraction was prepared by centrifuging the homogenate at 800 \(\times\) g for 20 min; the nuclear pellet was then washed 3 times in P buffer.
supernatants were ultracentrifuged in a Beckman L5-50 ultracentrifuge at 105,000 x g for 30 min to yield the cytosol, from which a thin layer of fat was aspirated. The washed nuclear pellet was extracted with 4 volumes of TK buffer under agitation for 60 min and then ultracentrifuged at 105,000 x g for 30 min in order to obtain the supernatant nuclear extract.

Preparation of Purified Nuclei

Tissues were homogenized in N2 buffer. Purified nuclear pellets were prepared from these homogenates according to the method of McEven and Zigmond (21); this involves 3 additional washes in N2 buffer without Triton X-100, followed by centrifugation at 60,000 x g for 1 hr in 2.2 M sucrose (N2 buffer).

Characteristics of [3H]Estradiol Binding to Nuclear Receptors

Association Studies. Samples of the HAP-precipitated nuclear extracts were incubated for various times at 4° or at 30° with 5 nM [3H]estradiol in the presence or absence of a 300-fold excess of DES.

Dissociation Studies. The HAP-precipitated nuclear extracts were equilibrated with [3H]estradiol in the presence or in the absence of a 300-fold molar excess of DES. Free steroids were removed by centrifugation and 4 washes with P buffer. Dilutions of the pellet were performed in either the absence or the presence of unlabeled estradiol. Dissociation of the complexes was monitored for 6 hr under conditions in which the rebinding of dissociating radioactive hormone to the receptor was not possible. Amounts of specific binding remaining on HAP were compared to the amounts at T = 0 hr.

Analysis of Nuclear Extracts on Sephadex G-200 Columns. Sephadex G-200 (5 g, bead form) was suspended in TP buffer and allowed to swell at 4° for 3 weeks. Gel was packed into column (100 x 1.6 cm) and equilibrated in TP buffer. Samples of each nuclear extract incubation product (300 µl) were first applied to a column (6 x 0.5 cm) of Sephadex LH-20 equilibrated in TP buffer in order to separate macromolecular bound and free estradiol. The macromolecular bound estradiol was placed directly on the column of Sephadex G-200. Elution was conducted at 4° with TP buffer at a rate of 7.5 ml/hr, and 1.6-ml fractions were collected. Elution profiles were monitored by absorption of the column effluent at 280 nm for standard proteins of known molecular weight.

Receptor Assays

ERC and PGRC Levels. [3H]Estradiol and [3H]R 5020 bindings were measured using dextran-coated charcoal assays (8). Increasing masses of [3H]estradiol (0.25 to 10 nM final concentrations) or [3H]R 5020 (0.5 to 20 nM final concentrations) were incubated in duplicate at 4° for 16 hr with cytosol aliquots. Nonspecific binding was accounted for by preparing the same incubation series with the addition of a 200-fold excess of DES as estrogen competitor and a 200-fold excess of R 5020 as progesterone competitor. After adsorption of free hormones on dextran-coated charcoal, the number of binding sites and the dissociation constant (Kd) were calculated by Scatchard analysis of specific bound hormones (36). The results were expressed in fmol/mg protein; ERC and PGRC were considered as positive when greater than 3 and 25 fmol/mg protein, respectively.

Nuclear Estrogen Receptor Levels. The procedure is a modification of the methods described by Erdos et al. (9) and Garola and McGuire (12); 200 µl of the nuclear extract were first adsorbed on 250 µl of HAP slurry for 30 min and centrifuged at 1500 x g for 2 min; then incubations of the pellet with 50 µl of [3H]estradiol with or without a 300-fold excess of DES were performed for 4 hr at 30° and for 16 hr at 4°. In each experiment, the nonspecific binding of [3H]estradiol to HAP or plastic tubes was estimated when bovine serum albumin standard solution replaced nuclear extracts. In order to confirm specificity, Scatchard analyses were also carried out with unlabeled progesterone and with unlabeled testosterone present in buffer. After incubation and centrifugation, free hormones were discarded, and the pellets were washed 4 times with P buffer containing 1% Tween 80 and extracted overnight with 2 ml ethanol. Binding parameters were analyzed by the method of Scatchard; the concentration of binding sites and the equilibrium dissociation constant of each component of the binding system were determined by the method of Rosenthal (34). Specific binding sites that were measured at 30° were termed TNR, while those measured at 4° were termed BNR. The difference, TNR-BNR, was designated as ANR and was considered as positive when higher than 10 fmol/mg protein.

When large samples were available, binding studies were performed with a 0.1 to 100 nM concentration range. Routinely, single-dose assays were performed in 134 breast tumors with 5 nM [3H]estradiol for 4 hr at 4° and 30°.

Other Analytical Methods

Protein concentrations were determined in cytosol by the method of Lowry et al. (20) and in nuclear extract by the Bio-Rad protein assay. [3H]Estradiol and [125I]estradiol were counted using a Beckman LS 6800 liquid scintillation spectrometer and a Beckman gamma 5500 counter, respectively. Counting efficiencies were 70% for [125I]estradiol and 30% to 50% for [3H]estradiol.

The significance of differences between results was determined using Spearman’s test. All analyses were carried out using a programmable CBM Model 8032 Commodo calculator.

RESULTS

Validity of the Method

To eliminate the possibility of cytosolic contamination in the nuclear preparations, the nuclear receptor content before and after purification of the nuclei was determined in 10 experiments. In the method involving purified nuclei, the inclusion of 0.25% Triton X-100 in the homogenization buffer strips away the outer membrane, reducing the possibility of contamination by cytoplasmic receptor; then a partial purification of nuclear preparation is achieved by suspension in 2.2 M sucrose and centrifugation at 60,000 x g for 1 hr. The mean values obtained per mg protein from the 2 methods were not significantly different (p < 0.001; n = 10), showing that nuclear receptors could not be attributed to cytosolic contamination.

In order to ascertain that estrogen cannot bind to HAP and mimic specific binding at any estradiol concentration used, a bovine serum albumin standard solution in TK buffer was precipitated on HAP, and [3H]estradiol, with and without a 300-fold excess of unlabeled DES, was added over a range of 0.1 to 100 nM estradiol; incubations were performed at 30° for 4 hr and at 4° for 16 hr. Results are plotted in Chart 1; no “specific binding” was observed at any estradiol concentration used.

Association Studies. The effect of incubation time of the binding of [3H]estradiol at 4° and 30° to binding sites in the nuclear extracts is shown in Chart 2. At 30°, the binding was complete after incubation for 3 hr, but values dropped after 5 hr, this presumably being due to a temperature-dependent destruction of the nuclear receptor by the proteolytic enzyme described by Garola and MacGuire (11). At 4°, the binding was nearly complete after incubation for 4 hr, and the estradiol-binding material remained stable after incubation for up to 24 hr.

Saturation Analysis. The results of saturation analysis over a wide range of [3H]estradiol concentrations (0.1 to 100 nM) in HAP-precipitated nuclear extracts are shown in Chart 3. When incubations were performed at 30°, a biphasic curve consisting of 2 apparently linear parts was obtained. The concentration of
Nuclear Receptors in Human Breast Cancer

Chart 1. Analysis of [³H]estradiol ([³H]E₂) binding to HAP. Bindings were measured on HAP-precipitated bovine serum albumin solution (1 mg/ml TK buffer). Incubations were performed at 30° using [³H]estradiol (C) and [³H]estradiol with a 300-fold excess of DES (D) and at 4° using [³H]estradiol (E) and [³H]estradiol with a 300-fold excess of DES (F).

binding sites and the equilibrium dissociation constant of each component of the binding system were determined by the method of Rosenthal: the first part, corresponding to low estradiol concentrations, is a steep curve, indicating high affinity (Kₐ = 0.45 ± 0.5 nM) and a limited number of sites (ANR sites); the second part, corresponding to higher estradiol concentration, is less steep, indicating low affinity and a very high number of sites (BNR sites). In order to confirm that the lower-affinity binding component was not due to binding of [³H]estradiol to progesterone or androgen receptors, the Scatchard analyses were repeated in the presence of excess of unlabeled progesterone and dihydrotestosterone. The results were identical to those obtained in the absence of such unlabeled hormones. Contrary to these results, the Scatchard plots of experiments carried out at 4° for 16 hr show only the more horizontal curve with apparent positive cooperativity, and only one type of binding site with high capacity and low affinity (Kₐ = 30 nM) was exhibited. They were similar to the BNR sites with high capacity and low affinity observed on the plotting obtained from the 30° analysis (Chart 4).

Specificity of Nuclear Binding Sites

The specificity of [³H]estradiol binding to nuclear binding sites was determined by measuring the ability of various hormones to compete for binding with [³H]estradiol. Competition studies for binding of 5 nM [³H]estradiol on ANR sites were carried out at 30° for 3 hr, i.e., on tumors that did not bind estradiol at 4° so that only type A was present, while competition studies for binding of 50 nM [³H]estradiol or 5 nM [³H]estradiol on BNR sites only were carried out at 4° for 16 hr, i.e., on tumors that bound the same amount of [³H]estradiol at 4° as at 30°; therefore, only

Chart 2. Binding of [³H]estradiol ([³H]E₂) to HAP-precipitated nuclear extract as a function of time and temperature. Binding sites were measured at 4° (C) and at 30° (F) by single-dose assays as described under "Materials and Methods." Values have been corrected for nonspecific binding.

Chart 3. Scatchard analysis of binding data from HAP assay. The HAP-precipitated nuclear extracts were incubated at 30° for 4 hr with a 0.1 to 100 nM [³H]-estradiol concentration range. The plot is the average of 7 experiments, 4 pooled extracts and 3 individual tumors. Kₐ = 0.45 ± 0.5 (S.D.) nM; Kₐ = 24 ± 8 nM. The Kₐ of each component was established by using the graphic solution of the biphasic curves (---) as described by Rosenthal.

Chart 4. A, saturation analysis of [³H]estradiol binding to HAP-precipitated nuclear fraction. The HAP assays were performed at 4° for 16 hr with a 0.1 to 100 nM [³H]estradiol concentration range. The plot is the average of 9 experiments, 4 pooled extracts, and 5 individual tumors, 2 of which contained only type B receptors. B, Scatchard analysis of the data in A; Kₐ = 30 ± 10 nM.
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Table 1
Hormonal specificity of nuclear estrogen binding sites

<table>
<thead>
<tr>
<th>Competitor concentration (300-fold molar excess)</th>
<th>ANR: 5 nM [3H]estradiol</th>
<th>5 nM [3H]estradiol</th>
<th>50 nM [3H]estradiol</th>
</tr>
</thead>
<tbody>
<tr>
<td>DES</td>
<td>9</td>
<td>9.1</td>
<td>19</td>
</tr>
<tr>
<td>Estradiol</td>
<td>8.5</td>
<td>5.7</td>
<td>7.3</td>
</tr>
<tr>
<td>Dihydrotestosterone</td>
<td>91</td>
<td>94.5</td>
<td>93</td>
</tr>
<tr>
<td>Progestrone</td>
<td>93</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Cortisol</td>
<td>99.7</td>
<td>96.5</td>
<td>100</td>
</tr>
<tr>
<td>Estradiol</td>
<td>28</td>
<td>12.4</td>
<td>14.7</td>
</tr>
<tr>
<td>Estriol</td>
<td>45.5</td>
<td>12.3</td>
<td>29.5</td>
</tr>
<tr>
<td>2-Hydroxyestrone</td>
<td>76.5</td>
<td>23.1</td>
<td>30.8</td>
</tr>
</tbody>
</table>

* Mean of 3 separate experiments.

The results in Table 2 show that, after 4 hr, approximately 50% of bound [3H]estradiol had dissociated from BNR sites in Tumor 1, which contained only type B binding sites. In Tumors 2 and 3, which contained type A and type B binding sites, the amounts of [3H]estradiol that had dissociated from binding material were the same, either after incubations at 30° where ANR and BNR were labeled or after incubation at 4° where only BNR were labeled. Thus, only [3H]estradiol bound to type B binding sites had dissociated after 4 hr.

In some cases, when material was sufficient, the dissociation study was monitored for 6 hr. Chart 6 shows that, following incubation with 5 nM [3H]estradiol at 4°, the rate of dissociation of binding material was higher (Kd 1.16 10⁻³ min⁻¹; t½, 269 min) than the rate of dissociation of binding material following incubations at 30° (Kd 5.44 10⁻⁴ min⁻¹; t½, 553 min). In the first case, only one type of binding site with low affinity for estradiol (BNR) was present; in the second case, 2 binding sites were present with high (ANR) and low (BNR) affinity for estradiol.

Single-Dose Assays

Both types of nuclear receptors have been measured by single-dose assays using 5 nM [3H]estradiol in 134 tumors. ANR with high affinity were saturated at this concentration, BNR were measured only in a small part since much higher concentrations of hormones are needed for these sites to be saturated; the nonspecific binding represented 20 to 70% of total binding according to the tumor studied.

ANR were found to be positive in 47 of 134 tumors (35.1%
Table 2

Dissociation studies of labeled nuclear binding sites

HAP-precipitated nuclear extracts were equilibrated with various concentrations of \[^{3}H\]estradiol; the macromolecular bound material was then diluted, and dissociation experiments were carried out for 4 hr at 4°.

<table>
<thead>
<tr>
<th>Tumor</th>
<th>[^{3}H]Estradiol bound (pm) at following dissociation times</th>
<th>% of dissociated material</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 hr</td>
<td>4 hr</td>
</tr>
<tr>
<td>1(d)</td>
<td>80.4(b)</td>
<td>41.8(b)</td>
</tr>
<tr>
<td>2(c)</td>
<td>12.4(c)</td>
<td>9.6(c)</td>
</tr>
<tr>
<td>3(b)</td>
<td>8.1(b)</td>
<td>5.4(b)</td>
</tr>
</tbody>
</table>

---

Analysis of the different hormone binding parameters showed that ANR and TNR values were significantly higher (\(p < 0.001\)) in ERC-positive, PGR-positive tumors when compared to values from other positive cytosolic classes (Table 4).
DISCUSSION

We measured 2 types of binding sites in the nucleus of human mammary tumors. Our experiments on purified nuclear preparations have discounted the possibility that nuclear receptors arise from cytosolic contaminations. Secondary binding sites for estrogen have been frequently described in many tissues such as the rat uterus (4), guinea pig uterus (41), rabbit corpus luteum (48), chick oviduct (39), and mouse mammary cancer (46).

Recently, in human mammary cancer, 2 binding sites have been shown in the cytosol (31) and in the nucleus (42) of tumor cells. In the nucleus of human mammary tumors, we also measured at 3° the total nuclear content TNR, which is composed of 2 types of binding sites (Kd = 1 nm and Kd = 30 nm). However, only the low-affinity type of binding site (BNR, Kd = 30 nm) was obtained at 4°. Subtraction of BNR from TNR yielded the contribution made by the solely high-affinity type of binding site (ANR, Kd = 1 nm).

Dissociation constants of ANR sites were always below 1.5 nm and corresponded to usual values encountered with estrogen receptors. As found in mouse mammary cancer (46), they probably represent active nuclear receptors, which result from nuclear translocation from cytoplasm. At 5 nm [3H]estradiol, these sites are saturated. In some cases, BNR alone were encountered; the nature and the significance of these types of sites are unknown. When performing assays at 4°, with a low estradiol concentration range, many authors have related unoccupied receptors in the nucleus of human mammary tumors (13, 30, 40, 43, 49). However, we have shown that at 4° an exchange takes place on these sites. Like ANR sites, the BNR sites have a molecular weight of about 37,000; they are moreover estrogen specific. It is worth noting that competition by weak estrogens such as estrone, estriol, and 2-hydroxyestrone for BNR binding sites is more important than competition for ANR binding sites. We have shown that estradiol complexed to the BNR sites dissociates rapidly at 4°, whereas estradiol complexed to ANR sites is stable at 4° at least during 6 hr. Thus, the dissociation constant of BNR sites obtained by dissociation studies as well as the Scatchard plot is high. The possibility that these sites are complexed to estrogens with low affinity and rapid rates of dissociation was discounted by Levy et al. (19), at least in human uterine tissues. In human mammary tumors, they could represent in vivo a lower-affinity ligand such as catechol estrogens, which may be present at high cellular concentrations, but which dissociate rapidly from the receptor binding sites. The levels of these receptors might be regulated directly or indirectly by the intranuclear content of receptors with high affinity for estradiol. However, these nuclear sites with low affinity for estradiol are thought to reflect the estrogen-responsive state of tissues in mouse mammary carcinoma (46). Thus, they may represent an additional indicator for the assessment of estrogen dependence.

From a practical point of view, the tumor size was often too small to perform Scatchard analysis; then single-dose assays were performed at 5 nm [3H]estradiol, and TNR and ANR were measured routinely; they appeared to be linked in almost all cases (only 2 dissociations: TNR positive consisting of ANR negative plus BNR positive) (Table 3).

We found 63% ANR positives in ERC-positive PGRC-positive tumors and 25, 28, and 26% ANR positives in ERC-positive PGRC-negative, ERC-negative PGRC-positive, and ERC-negative PGRC-positive tumors, respectively. It is of interest to note that these percentages are close to the likelihood of success with hormone therapy reported in a consensus report as a function of cytosolic status (5). Although the likelihood of finding ANR positive sites is higher in PGRC-positive tumors, 27.6% of our tumors lacking ANR were PGRC positive. It can be argued that our assays could yield false-negative ANR results since they do not take into account the nonextractable nuclear binding sites; we are, in addition, attempting to perform more complete extraction using NaSCN. Although this possibility cannot be ruled out, other factors should be considered such as the action of hormones other than estradiol which could determine the presence of PGRC. In the chick oviduct, it has been demonstrated that the coefficient of sedimentation of the PGR is 4 to 5S in cytosol from unstimulated glands but is 6 to 7S after estrogen stimulation (45). These findings imply that 2 species of PGR may be encountered and that molecular aggregates constitute a physiologically significant occurrence depending upon estrogen stimulation. Thus, we could suggest as found in cancer cell lines that, among PGR receptors, some are estrogen independent (26) and differ from other receptors by their molecular size (44).

Other factors that could be involved in growth regulation have thus to be selected. Among them, prolactin is of particular interest because of its key position in controlling the growth of hormone-dependent mammary tumors (6, 25). Interplay between prolactin and estradiol has recently been studied in normal mammary tissue (27). In this model, prolactin dramatically enhances nuclear translocation of ERC induced by estradiol. Therefore, nuclear receptors are believed to reflect the final step of the action of all hormones involved in the promotion of tumor growth. We are now establishing clinical correlations between the presence of nuclear receptor binding sites and response to

<table>
<thead>
<tr>
<th>ERC</th>
<th>PGRC</th>
<th>TNR</th>
<th>ANR</th>
</tr>
</thead>
<tbody>
<tr>
<td>fmoi/mg cytosol protein</td>
<td>Kd (nm)</td>
<td>fmoi/mg cytosol protein</td>
<td>Kd (nm)</td>
</tr>
<tr>
<td>ERC positive (n = 89)</td>
<td>208 ± 34*</td>
<td>0.66 ± 0.03</td>
<td>299 ± 58</td>
</tr>
<tr>
<td>PGRC positive (n = 64)</td>
<td>226 ± 44</td>
<td>0.63 ± 0.084</td>
<td>337 ± 68</td>
</tr>
<tr>
<td>ERC positive PGRC positive (n = 57)</td>
<td>170 ± 53</td>
<td>0.71 ± 0.17</td>
<td>18.1 ± 4.5</td>
</tr>
<tr>
<td>ERC negative PGRC positive (n = 7)</td>
<td>78 ± 60</td>
<td>1.37 ± 0.39</td>
<td>21 ± 15</td>
</tr>
<tr>
<td>ERC negative PGRC negative (n = 38)</td>
<td>3.5 ± 1.3</td>
<td>2.9 ± 1.1</td>
<td></td>
</tr>
</tbody>
</table>

* Mean ± S.E.
endocrine therapy with the aim of verifying the value of these receptor measurements in predicting tumor responsiveness.

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REFERENCES


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