Uncharged Nuclear Receptors for Estrogen in Breast Cancers

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ABSTRACT

This study was undertaken to define the incidence and concentration of uncharged estrogen receptors (RN) in human breast cancer. The concentrations of RN and cytoplasmic uncharged receptor were determined on sucrose gradients following a 4-hr incubation at 4°C with 1.6 nM 17β-[3H]estradiol in 139 tumor specimens from 137 patients. RN was extracted from washed nuclear pellets in buffer containing 0.4 M KCl. The receptor molecule extracted had a high affinity for 17β-[3H]estradiol (Kd = 0.9 to 7.6 nM) and was specific for estrogen. The possibility of artifact due to cytoplasmic contamination of the nuclear fraction or high-ionic-strength-induced exchange of charged nuclear receptors was rendered unlikely by validation experiments performed with pooled tumor tissue. Significant amounts of cytoplasmic uncharged receptor (greater than 7 fmol/mg protein) were found in 63.3% of the tumors. Similar significant amounts of RN were found in 29.5% of the tumors. Significant amounts of RN in the presence of undetectable cytoplasmic uncharged receptor were found in 2.3% of the tumors. The percentage of tumors that contain significant amounts of RN is approximately the same percentage of estrogen receptor-positive tumors that do not respond to ablative therapy.

INTRODUCTION

Early studies by Jensen et al. (10) and Gorski et al. (6) led to the current hypothesis of estrogen-receptor interaction in hormone-dependent tissue (9). This hypothesis states that estrogenic steroids bind to specific cytoplasmic receptors (RC), followed by a temperature-dependent translocation of hormone-receptor complex to the nucleus. Nuclear hormone-receptor complex (RNE), which is derived from the transformation of cytoplasmic hormone receptor complex, regulates cell function by controlling the expression of various mRNA's (14). Jensen et al. (8) were the first to show that the presence of estrogen receptors (RC) in human breast cancer could be correlated with response to endocrine therapy. This finding, indicating that the estrogen receptor mechanism is intact in many breast cancers, has been verified in a carefully controlled study involving several groups (12), yet almost 45% of those patients whose tumors contain estrogen receptors (RC) do not respond to endocrine manipulation (12). McGuire et al. (13) have summarized several possible explanations for these treatment failures. The most provocative is that breast tumors contain estrogen receptors that exist and function in the nucleus in the absence of estrogen. Studies of tumor cell lines in vitro (21) tend to support this hypothesis. Recently, Garola and McGuire (5) reported the presence of significant amounts of RN in several human breast cancers. This study was undertaken to define the incidence and concentration of uncharged nuclear receptors for estrogen (RN) in human breast cancer. Our data reveal that almost 30% of the human breast cancers examined contain amounts of uncharged nuclear estrogen receptor (RN) greater than or equal to 7 fmol/mg cytoplasmic protein. This study is directed not at RNE but at RN, a species of receptors not thought to be active normally.

MATERIALS AND METHODS

Tumor Specimens. A total of 139 pathologically confirmed mammary tumor specimens was obtained from 137 patients, including 2 males. The specimens obtained from local hospitals were promptly stored in liquid nitrogen. Tumor specimens obtained from distant hospitals were stored at less than −40°C until shipment on dry ice. In addition to the University of Virginia Hospital, 1 local and 10 out-of-town hospitals contributed material for this study.

Estrogen Receptor Assays. The method used was a modification of the methods of Jensen et al. (8) and Wittliff et al. (20). The tumor specimen was shattered while being maintained at a temperature of less than −60°C with a Thermovac tissue pulverizer (Glass Seal Division, Thermovac Industries, Inc., Copiague, N. Y.). The resulting fine powder was suspended in 5 volumes of TMG buffer. All remaining steps were carried out at 4°C unless otherwise indicated. The suspension was homogenized 3 times for 10 sec with a Tissuemizer SDT-100 (Tekmar Industries, Cincinnati, Ohio) at a setting of 35. Pauses of 30 sec were observed between homogenization steps. The homogenate was centrifuged for 10 min at 800 × g, and the supernatant was reserved. The pellet was washed twice by resuspending in an equal volume of the TMG buffer and centrifuging at 800 × g for 10 min. Both the crude homogenate and the washed pellet of 12 tumors were examined for whole cells with phase-contrast microscopy and light microscopy following staining. No whole cells were seen. The washed pellet was resuspended in an equal volume of TMGK buffer and allowed to incubate for 1 hr. At the end of the hr, the original supernatant, containing the RC, and the resuspended nuclear pellet, containing the RN, were centrifuged for 30 min at 105,000 × g in a type 50 rotor...
in a Beckman L5-50 ultracentrifuge (Beckman Instruments, Inc., Spincov Division, Palo Alto, Calif.). Aliquots were set aside for protein determination by the method of Waddell (18). The protein concentration of the TMGK extracts (RN fraction) ranged from 1 to 1.7 mg/ml.

Cytoplasmic (RC) and nuclear (RN) receptor sites for estrogen were determined in the following manner. Aliquots (0.15 ml) of the fractions were incubated for 4 hr in a total volume of 0.25 ml with either 1.6 nM 17β-[^3H]estradiol (specific activity, 56.4 Ci/mmol; New England Nuclear, Boston, Mass.) or 1.6 nM 17β-[^3H]estradiol plus 100 nM DES. Following incubation the reaction mixtures were transferred with a 0.1-ml wash to test tubes containing a sedimented pellet derived from 0.5 ml of a DCC suspension (0.025% dextran:0.25% Norit A in TMG buffer). After transfer the tubes were vortexed, incubated for 5 min, and centrifuged at 2000 x g for 10 min. Aliquots (0.2 ml) of the supernatant were layered on 5-ml 10 to 30% sucrose gradients (in 1 mM EDTA and 0.01 M Tris-HCl, pH 7.4, at 4°C) and collected in 0.2-ml fractions into Bio-vials (Beckman Instruments, Inc.) The fractions were dissolved in 3 ml of Formula 947 scintillation counting fluid (New England Nuclear, Boston, Mass.) or 1.6 nM 17ß-[3H]estradiol plus 100 nM DES. Following incubation the reaction mixtures were transferred with a 0.1-ml wash to test tubes containing a sedimented pellet derived from 0.5 ml of a DCC suspension (0.025% dextran:0.25% Norit A in TMG buffer). After transfer the tubes were vortexed, incubated for 5 min, and centrifuged at 2000 x g for 10 min. Aliquots (0.2 ml) of the supernatant were layered on 5-ml 10 to 30% sucrose gradients (in 1 mM EDTA and 0.01 M Tris-HCl, pH 7.4, at 4°C) and collected in 0.2-ml fractions into Bio-vials (Beckman Instruments, Inc.) The fractions were dissolved in 3 ml of Formula 947 scintillation counting fluid (New England Nuclear) and counted for 5 min in a Packard Tri-Carb scintillation spectrophotometer (Packard Instrument Corp., Downers Grove, Ill.). Counting efficiency was 26%.

Specific binding of estrogen to uncharged nuclear (RN) sites was obtained by subtracting the SS-region nonspecific binding in the presence of DES from the SS-region total binding tubes. Results were expressed as fmol/mg cytoplasmic protein.

In the validation studies RN was determined by the DCC method (19). The DCC method was also used to determine the Kd of the RN as well as its steroid specificity. For 17 tumors the Kd of the RN was determined and ranged from 0.9 to 7.6 nM with a mean of 1.1 nM. For 37 tumors [3H]estradiol was incubated with crude nuclear extract in the presence of 100-fold excess of unlabeled progesterone, testosterone, or estradiol. Only estradiol competed with [3H]estradiol for binding sites.

**Triton Treatment of Nuclei.** The crude nuclear pellet was stripped of cytoplasm by the Triton: glycerol method of Schibler and Weber (17). Nuclear preparations were examined under the light microscope and were found to be free of cytoplasmic remnants. DNA was determined by the method of Burton (2).

**Prelabeling of RN.** The suspension of the crude nuclear pellet was incubated in TMG buffer with 1.6 nM 17β-[^3H]estradiol or 1.6 nM 17β-[^3H]estradiol plus 100 nM DES in a total volume of 0.25 ml. Following incubation for 4 hr at 4°C, the nuclei were sedimented at 2,000 x g for 10 min. The nuclear pellet was washed in 0.25 ml of TMG buffer. The nuclei were then extracted in 0.25 ml of the TMGK buffer for 1 hr and then centrifuged at 105,000 x g for 1 hr. RN was determined in the resulting supernatant by the DCC method.

**RESULTS**

**Analysis of Individual Tumors.** The concentrations of both uncharged cytoplasmic estrogen receptor (RC) and nuclear uncharged estrogen receptor (RN) were determined in 139 tumors. A statistical description of the results of these determinations is shown in Table 1. An estimate of total uncharged estrogen receptors was obtained by adding the RC and RN values. A RN:RC ratio of ≥1 was found in 17 tumors. Also included in Table 1 is a statistical description of the age of the patient population from which the tumors were obtained. It is clear from Table 1 that the parameters RN, RC, RN + RC, or RN:RC are not normally distributed. The log-transformed parameters present a more nearly normal distribution. The tumors were classified as to whether they were positive for RC, RN, or both. Based on results from the literature (8) and on our own experience, tumors containing 7 fmol or more of estrogen receptor per mg cytoplasmic protein are considered positive. The results of this classification are shown in Table 2. An examination of Table 2 suggests that the presence of significant amounts of RC is associated with significant amounts of RN since RN is 7 fmol/mg protein or more in 37 of 86 (42%) of the tumors in which RC was 7 fmol/mg protein or more while RN was 7 fmol/mg protein or more in only 4 of 51 (7.8%) of the tumors in which RC was less than 7 fmol/mg protein. Regression analysis indicates that RN is correlated with RC as is log RN with log RC. The appropriate equations are RN = RC (0.289) - 0.021 (r = 0.81) and log RN = log RC (0.671) - 0.529 (r = 0.67). The age of the patient could not be correlated with RN, RC, RC + RN, or RN:RC.

**Validation Studies.** The correlation of RN with RC raises the possibility that the RN detected could be an artifact, representing RC contamination of the RN fraction. For exclusion of this possibility, experiments were performed with pooled breast tumor tissue from 7 different tumors. An aliquot of the crude nuclear pellet from the pooled breast

**Table 1**

<table>
<thead>
<tr>
<th>Mean ± S.E.</th>
<th>Range</th>
<th>Median</th>
<th>0.25</th>
<th>0.75</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (yr)</td>
<td>57.3 ± 1*</td>
<td>27-84</td>
<td>56.5</td>
<td>49</td>
</tr>
<tr>
<td>RC*</td>
<td>35.9 ± 4.6</td>
<td>0.1-255</td>
<td>14.3</td>
<td>3</td>
</tr>
<tr>
<td>RN</td>
<td>10.3 ± 1.6</td>
<td>0.1-118</td>
<td>3.4</td>
<td>1.3</td>
</tr>
<tr>
<td>RC + RN</td>
<td>46.2 ± 6</td>
<td>0.2-329</td>
<td>18</td>
<td>5.9</td>
</tr>
<tr>
<td>RN:RC</td>
<td>0.48 ± 0.05</td>
<td>0.01-4.3</td>
<td>0.26</td>
<td>0.13</td>
</tr>
</tbody>
</table>

* fmol/mg cytoplasmic protein.

**Table 2**

<table>
<thead>
<tr>
<th>Estrogen receptor content*</th>
<th>Cytoplasmic</th>
<th>Nuclear</th>
<th>No. of tumors</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative</td>
<td>Negative</td>
<td>47</td>
<td>33.8</td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>Positive</td>
<td>4</td>
<td>2.9</td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>Negative</td>
<td>51</td>
<td>36.7</td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>Positive</td>
<td>37</td>
<td>26.6</td>
<td></td>
</tr>
</tbody>
</table>

* Estrogen receptor concentrations of less than 7 fmol/mg cytoplasmic protein are considered negative. Estrogen receptor concentrations larger than or equal to 7 fmol/mg cytoplasmic protein are considered positive.
tumor tissue was stripped of cytoplasm by the Triton:glycerol method. RN was then determined in both the crude and purified nuclear fractions. The results (Table 3, Experiment 1) indicate that comparable amounts of RN are found in both the crude and purified nuclear fractions.

Another possible source of error in the RN determination could occur if the high-ionic-strength extraction buffer, TMGK, induced stripping of estrogen from RNE. For exclusion of this possibility, 2 additional experiments were performed with pooled breast tumor tissue. RN was labeled and quantitated before and after extraction with TMGK buffer. The results of this experiment (Table 3, Experiment 2) indicate that comparable amounts of RN are found whether the labeling step occurs before or after extraction with the TMGK buffer.

In the second experiment, the rate of dissociation of 17β-[3H]estradiol from RN extracted from the crude nuclear pellet by the TMGK buffer was measured. The results shown in Chart 1 indicate minimal dissociation of [3H]estradiol from the 0.4 M KCl-extracted RN after 21 hr. In addition, the rate of [3H]estradiol dissociation from RN extracted from the crude nuclear pellets and partially purified on 0.4 M KCl-containing sucrose gradients was determined. The results, also shown in Chart 1B, show no detectable dissociation of 17β-[3H]estradiol after 6 hr.

**DISCUSSION**

The nuclear receptors described herein are uncharged; i.e., they are not complexed with estrogen. This type of nuclear receptor has not been thought to be involved directly in the action of estrogen in target tissue until recently (21).

The results presented in Tables 1 and 2 indicate that almost 30% of human breast cancers contain significant amounts of uncharged nuclear receptors. At least with respect to age of the patient and the concentration and incidence of RC, this tumor population is similar to those reported by other groups (12). The presence of significant amounts of RN is most often found in those tumors that contain significant amounts of RC, although 8% of RC-deficient tumors also contain RN.

Regression analysis was performed with log-transformed parameters since the untransformed parameters were not normally distributed (7). Despite the observed correlation no appreciable amount of RN could be attributed to contaminating RC. In addition, the finding that the RN:RC ratio was ≈1 in 17 cases makes the possibility of significant cytoplasmic contamination less likely. High ionic strength did not alter the rate of dissociation of estradiol from RNE. Therefore, the RN measured could not result from the exchange of RNE. Because it has been shown (3) that 0.4 M KCl will not extract 100% of the RNE from rat uterine nuclei, the possibility that the RN values determined are underestimated cannot be excluded.

The presence of significant amounts of RN in normal target tissue has not been reported previously (1, 19). While this paper was in preparation, 2 reports on nuclear receptors in human breast cancer appeared. Laing et al. (11) reported that RNE from human breast cancer showed complete exchange of all filled sites when incubated for 18 hr at 4°C. The results from pooled human breast cancer presented in Chart 1 are at variance with their observation. The exchange conditions reported by Zava and McGuire (22) for the RNE of the MCF-7 cell line and by Garola and McGuire (4, 5) with individual human breast cancers also contradict the findings of Laing et al. (11). A partial explanation of this disparity between the results may relate to the proteolytic activity at temperatures of 15°C or greater found in pooled breast cancer tissue (W. B. Panko, unpublished observations) and in individual tumors (4). Such proteolytic activity could account for the failure to demonstrate increased binding of 17β-[3H]estradiol at 20 or 37°C when compared to the binding at 4°C, as reported by Laing et al. (11). If exchange of RNE does not occur during an 18-hr incubation at 4°C, the methods of Laing et al. should measure RN instead of RNE. In fact, the results of Laing et al. could be interpreted as demonstrating RN in 39% of human breast cancers, a figure that corresponds roughly with our report of 29%. Since their report does not include a definition of
the level of nuclear receptor for a tumor to be classified as
nuclear receptor positive, a more critical comparison is not
possible.

Garola and McGuire (5) reported the presence of signifi-
cant amounts of uncharged nuclear receptor (RN) in ap-
proximately 40% of the 28 human breast tumors assayed.
The disparity between our results and those of Garola and
McGuire (5) might be explained on the basis of our under-
estimation of RN in human breast cancer due to loss of RN
during sucrose gradient centrifugation. Since the sucrose
gradient method used in this work for the estimation of RN
involves a 16-hr period during which the receptors are
exposed to nonequilibrium conditions for [3H]estradiol, it is
possible that RN sites are lost during this period, par-
icularly since the prior DCC treatment might strip 17β-
[3H]estradiol from the RN (16). Two factors may work to
minimize this effect: (a) the time of exposure to charcoal
and the percentage of charcoal in the suspension was
considerably below the optimal for stripping; (b) the KCl
concentration of the reaction mixture during DCC exposure
was reduced to 0.24 M, an amount also below the optimal.
The experiments of Peck and Clark (16) used cytoplasmic,
not nuclear, receptors.

Two recent papers (21, 22) reported that, in MCF-7 cells
(derived from a human breast cancer) grown in estrogen-
stripped media, 75% of the total estrogen receptors exist in
the RN form. The RN were not incapable of binding estradi-
ol since cells grown in the presence of estradiol contained
exclusively RNE. Most importantly, while estrogen was not
particularly effective in stimulating thymidine incorporation
by this cell line, treatment of these cells with the antiestro-
genase, mafenoxidine resulted in a marked reduction of thymidine
incorporation by these cells. Simultaneous treatment with
estradiol could block the mafenoxidine effect.

The findings reported within this paper and the work of
Garola and McGuire (22) suggest that the observations with
the MCF-7 cell line do not represent a unique aberration of
a cell line derived from malignant tissue. In fact an appreci-
able number of human breast cancers contained signifi-
cant amounts of RN. The implication is that human breast
cancer frequently contains an estrogen receptor mecha-
nism that is at the very least different from that found in
normal target tissue and that may represent the defective
expression of the normal estrogen receptor mechanism.

Zava et al. (21) suggested that a number of human breast
cancers may fail to respond to therapeutic approaches that
deprive them of estrogen because they contain RN, which
are capable of forming effective complexes with the nuclear
acceptor site of the tumor genome. Our results, which do
not indicate the function of RN in human breast cancer, at
least imply that the hypothesis warrants further examina-
tion. The 30% of human breast cancers that contain RN
may represent a subclass in which antiestrogen therapy
would be a better mode of attack than would endocrine
ablation, as suggested by Zava et al. (21). An insufficient
number of these patients have undergone endocrine ther-
apy for recurrent disease to indicate whether any therapeu-
tic significance can be attached to the presence of RN in
a breast cancer. However, the percentage of tumors that
contain significant amounts of RC and also contain signifi-
cant amounts of RN is approximately the same as the
percentage of estrogen-receptor-positive tumors that do
not respond to ablative therapy (12).

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