Immunological Quantitation of Nuclear Receptors in Human Breast Cancer: Relation to Cytosolic Estrogen and Progesterone Receptors

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ABSTRACT

Nuclear estrogen receptors (ERn) can now be reliably analyzed using the monoclonal estrogen receptor enzyme immunoassay. In a consecutive series of 135 breast cancer biopsies, ERn as well as cytosolic estrogen receptor (ERc) and progesterone receptor (PgR) concentrations were determined to evaluate whether ERn assays provide additional valuable information for the clinical management of the disease. Furthermore, by performing analyses on this relatively large number of patients, we sought explanations for the occurrence of the receptor profiles of ERc negative PgR positive and ERc positive PgR negative, which are found in a significant proportion of tumor biopsies.

Eight-four percent of all tumors are classified as ERn positive (≥10 fmol/mg nuclear extract protein) using the monoclonal assay technique. Two trends are evident: (a) ERn positivity was found to be associated with ERn positivity (≥10 fmol/mg cytosol protein) in 98% of the cases investigated; and (b) PgR positivity (≥10 fmol/mg cytosol protein) was found to be associated with ERn positivity in 95% of the cases investigated. However, a major proportion (~28%) of ERn positive tumors are either ERc negative or PgR negative. The pattern of ERc negative ERn positive occurs almost exclusively among younger women, most of whom also had detectable amounts of PgR in their tumor tissues, while the pattern of ERn positive PgR negative occurs primarily among older women.

ERn concentration was found to be significantly correlated to the concentration of both PgR and ERc. While the correlation between ERn and PgR was found to be strongest among women younger than 50 years of age, the correlation between ERn and ERc was strongest among women older than 50 years. Young women were found to have a significantly higher proportion of total tissue estrogen receptor present as ERn than older women (27% versus 14%).

The information obtained by performing ERn analyses concurrently with or in place of ERc and PgR analyses does not appear to be valuable for the clinical management of the disease. However, this new method for determination of ERn is a significant advance in receptor technology that permits reevaluation of established enigmas concerning the biology and natural history of breast cancer.

INTRODUCTION

ERn is now accepted as being localized in the nucleus in living cells. The fact that ER is detected in the cytosol of tissue homogenates is assumed to be an artifact resulting from the preparation of the tissue for analysis (1-4). In undisturbed cells both "free" and "filled" ER may be expected to be found within the nucleus. The free form is not bound to endogenous hormone, is easily extracted from tissue homogenates with buffers of low ionic strength, and is the form of ER routinely determined that has been called ERc. Filled ER is bound to endogenous estradiol, is extracted only with buffers of high ionic strengths, and is the form of receptor that is referred to as ERn. The relative amounts found of each form of receptor can be expected to vary according both to the level of receptor occupancy by hormone and to the methods used to fractionate the cells (5). In the field of breast cancer research, almost all data relating ER concentrations to either response to endocrine therapy or prognosis of the disease are based upon determinations of ERc (6-10).

Although ERc determinations are useful in evaluating which form of treatment may be best suited for the individual patient, other factors believed to be of predictive value have been investigated. With only one known exception (11) PgR synthesis occurs exclusively in the presence of both estradiol and a normally functioning ER (12). In accord with this, simultaneous determination of PgR with ER in biopsies has been demonstrated to provide a better foundation for patient evaluation than ER analyses alone (13, 14). Alternatively, knowledge of ERn concentrations has been suggested to serve the same purpose (15). One of the basic premises for this suggestion is that nuclear ER assays yield information that is different from that which is obtained by performing ERc and PgR assays. Reports of primary breast tumors that are ERc positive but ERn negative range in frequency from 17 to 58% in the literature (16-20), and such reports have supported the foundation for this premise. Meanwhile, demonstration of the presence of either ERn or PgR could, a priori, be expected to provide evidence that ER functions normally in the given tissue.

Nuclear ER assays have been performed in few previous investigations. The most reliable techniques previously available for quantitating nuclear ER in breast cancer biopsies (hydroxyapatite, nuclear exchange) have not been suitable for routine use. These methods demand relatively large amounts of tissue, they are time consuming, and they are sensitive to procedural details. However, since the recent development of monoclonal antibodies that recognize epitopes on the human estrogen receptor, a new method for detection of ER has become available. The ER-EIA kit (Abbott Laboratories) that is designed for detection of ER in the cytosol of human biopsies has been demonstrated to yield reproducible, quantitative results under high salt conditions (21) as well as low salt conditions (22, 23). Furthermore, the assay method is simple, is rapid, and requires only small amounts of tissue. The kit is, therefore, well-suited for routine determination of ERn in high salt extracts of nuclear myofibrillar pellets. The purpose of the present investigation was to determine ERn, ERc, and PgR in a consecutive series of biopsies from breast cancer patients in order to evaluate whether determination of ERn provides additional information that is valuable for selection of patient treatment.

MATERIALS AND METHODS

Chemicals. All reagents were of analytical grade. [2,4,6,7-3H]Estradiol (TRK.322, 85-110 Ci/mmol) and 16-ethyl-21-hydroxy-19-nor[6,7-
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3Hpregn-4-en-3,20-dione (3H-ORG 2058, TRK 629, 40–60 Ci/mmol) were purchased from Amersham.

The Abbott ER-EIA monoclonal kit (Abbott Laboratories) was used.

Preparation of Tumor Tissue for Analysis. Human breast cancer biopsies routinely sent to the laboratory for receptor determination were kept at −80°C for a maximum of 14 days until further handling. Tissue precooled in liquid nitrogen was pulverized in a Micro-Dismembrator (Braun, Melsungen, Germany). Tissue powder was suspended in ice-cold PB in a tissue:buffer ratio of 1:4, and samples were centrifuged at 800 × g for 10 min. The supernatant from this first wash of tissue powder was ultracentrifuged at 105,000 × g for 1 h (4°C) and used for routine determination of ERc and PgR. The pellet from the 800 × g centrifugation was resuspended using a Vortex mixer with the same volume of PBS as that used for the first wash and repelleted at 800 × g twice more. The supernatants from these washes are denoted supernatants 2 and 3. Prior to the protein determination they were also ultracentrifuged at 105,000 × g for 1 h (4°C). After the third wash, the crude nuclear pellet (nuclear myofibrillar pellet) was resuspended in 0.6 M KCl:Tris buffer (1:5; w/v). High salt extraction was performed by vortexing every 10 min for 1 h. Extracts were centrifuged at 105,000 × g for 1 h (4°C), and the supernatant following this centrifugation is termed the nuclear extract.

A total of 135 biopsies histologically verified as being malignant cancer mammae were investigated. Data from ERn analyses are available for all 135 patients, while ERc and PgR data are available for 127 and 118 patients, respectively.

DCC Assay. The ERc-DCC and PgR assays have been performed in accord with the recommendations of the EORTC Receptor Group (24). Cytosols were diluted to concentrations of approximately 2–3 mg cytosol protein/ml, and 50-µl aliquots were used for the assays.

Table 1 Percentage of total cytosol protein present in subsequent washes of tissue with buffer of low ionic strength

<table>
<thead>
<tr>
<th>Supernatant</th>
<th>N</th>
<th>% of total cytosol protein</th>
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<tr>
<td>First</td>
<td>107</td>
<td>67 ± 8.7*</td>
</tr>
<tr>
<td>Second</td>
<td>27</td>
<td>25 ± 7</td>
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<tr>
<td>Third</td>
<td>27</td>
<td>8 ± 4</td>
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* Mean ± SD.

Statistical Methods. Conventional statistical tests have been applied to the data. The particular test used in the individual circumstance is indicated in the text or in the relevant table or legend. A value of P < 0.05 was accepted as being significant.

RESULTS

Nature of Nuclear Estrogen Receptor. Before examining data regarding ERn, it is relevant to consider whether the ER extracted from the crude nuclear pellet could be a remnant of ERc that has not been removed by proper washing. To address this question, quantitative analyses were performed to determine the protein content of the supernatants derived from the three washes of the crude nuclear pellet (Table 1). For the majority of the samples, the second and third supernatants were pooled before protein analysis. As can be seen in Table 1, the yield of each wash is approximately one-third of that of the previous one. Since the concentration of ERc is constant in consecutive washes with buffer of low ionic strength, approximately 3% of the total cytosolic ER would be expected to be included in the high salt extract of the nuclear myofibrillar pellet that, in effect, constitutes a fourth wash. Such receptors would incorrectly be regarded as nuclear ER.

Both ERc and ERn concentrations exceed the detection level in 83% (106 of 127) of the cases investigated. Of these 106 samples, quantitative analyses have been performed to establish the total cytosolic protein content for 71 biopsies. In only 2 of the 71 biopsies has the number of ERn been so low as to be less than 3% of the total cytosolic ER. Therefore, ERn can be considered to be different from ERc in at least 96% of the cases reported here.

The overall fraction of total tissue ER that occurs as ERn in these biopsies is 18% (median value; first and last quartiles are 9 and 27%, respectively). However, the proportion of ER found as nuclear ER is higher among the younger than the older women, as can be seen in Fig. 1. This difference in distribution of the fraction of total tissue ER present as ERn among women younger and older than 50 years of age is highly significant (Student's t test for unpaired data, P = 0.0016).

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All of ERc positive biopsies are also ERn positive (87 of 89); women. Most notable, however, are the two trends: (a) almost women have a high occurrence of ERc negative PgR positive according to ERc and PgR status (Table 3), the frequency of conventional way showing the four possible combinations ac according to age group in Table 2. Approximately 85% of all activity is significantly higher among the older women than tumors are ERn positive. The frequencies of receptor positivi- among younger women. When the data are examined in a Table 4 Results from analyses of patients found to be ERc negative PgR negative but ERn positive
Individual results (given in fmol/mg protein) from analysis of tissue of the four biopsies classified as ERc positive that belong to the receptor profile ERc negative PgR negative are shown. The method of analysis used for determination of ER is indicated.

Occurrence of ERn in Biopsies. The frequencies of receptor positivity found here for ERc, ERn, and PgR for biopsies within each age group and overall. The correlation between the results of ERc determinations using the DCC and the EIA assay methods was excellent (linear regression equation, ER-DCC = -12.1 + 1.1 ER-EIA, N = 113, r = 0.97). If the result from either the ER-DCC assay or the ER-EIA assay was ≥10, the sample has been classified as being ERc positive.

The frequency of receptor positivity (using a cutoff level of 10 fmol/mg protein) is shown for ERc, ERn, and PgR for biopsies within each age group and overall. The four exceptions to the second trend (PgR associated with ERn positivity) all had low PgR values (<23 fmol/mg cytosol protein) and ERn values ranging from 5 to 8 fmol/mg nuclear extract protein.

If the two above trends are consistent, namely that ERn occurs simultaneously with ERc and PgR, then ERc negative PgR negative tumors might also be expected to be ERn negative. This is found to be the case in 15 of 19 patients here. The results from the 4 patients who are exceptions are shown in Table 4. Measurable but low values of either ERc or PgR are found in all four cases. Thus, the few “misclassifications” that we observe in relation to the postulated trends could appear to be a consequence of the arbitrary choice of a cutoff level of 10 fmol/mg protein.

Correlations between ERc, PgR, and ERn Concentrations. The concentration of PgR is significantly correlated to concentrations of both ERn and ERc among those patients with detectable levels of both receptors (Fig. 2). This correlation is found to be stronger among younger than among older women. ERc concentrations are also significantly correlated to ERn concentrations (Fig. 3). Consequently, there is a weak but insignificant correlation between PgR and ERc concentrations (Pearson correlation coefficient of the log transformed values, N = 68, r = 0.18). The direct proportionality between ERn and ERc that is observed in Fig. 4 occurs mainly in the older group of women. Younger women have higher concentrations of ERn than ERc and a weaker correlation between the two receptor concentrations.

Table 3 Classification of patients according to receptor profile
Receptor profiles among younger and older women are shown together with the frequency of ERn positivity for each profile.

Table 4 Results from analyses of patients found to be ERc negative PgR negative but ERn positive

respectively) that exceed the mean value of ERn negative tumors [3.3 ± 2.7 (SD) fmol/mg nuclear extract protein, N = 22]. The four exceptions to the second trend (PgR associated with ERn positivity) all had low PgR values (<23 fmol/mg cytosol protein) and ERn values ranging from 5 to 8 fmol/mg nuclear extract protein.

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Individual results (given in fmol/mg protein) from analysis of tissue of the four biopsies classified as ERc positive that belong to the receptor profile ERc negative PgR negative are shown. The method of analysis used for determination of ER is indicated.

Occurrence of ERn in Biopsies. The frequencies of receptor positivity found here for ERc, ERn, and PgR are shown according to age group in Table 2. Approximately 85% of all tumors are ERn positive. The frequencies of receptor positivi- are approximately the same within the older and younger age groups for ERn and PgR while the frequency of ERc positivity is significantly higher among the older women than among younger women. When the data are examined in a conventional way following the four possible combinations according to ERc and PgR status (Table 3), the frequency of profile patterns found is in accord with that found earlier in our laboratory among a larger group of patients (27); young women have a high occurrence of ERc negative PgR positive tumors in relation to older women, and the frequency of ERc positive PgR negative tumors is highest among the older women. Most notable, however, are the two trends: (a) almost all of ERc positive biopsies are also ERn positive (87 of 89); and (b) almost all PgR positive tumors are ERn positive (71 of 75). The two patients who are exceptions to the first trend (ERc positive being associated with ERn positive) had low ERc values (23 and 10 fmol/mg cytosol protein) and measurable concentrations of ERn (8 and 6 fmol/mg nuclear extract protein,
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**Fig. 2.** Relationship between ERn and PgR concentrations for all 83 patients with detectable ERn and PgR (note log scale). △, women younger than 50 years; □, women 50 years or older. The Pearson correlation coefficients of the log transformed values are as follows: all patients, \( r = 0.54 \); those <50 years, \( N = 23 \), \( r = 0.80 \); and those 50 years or older, \( N = 63 \), \( r = 0.38 \).

**Fig. 3.** Relationship between ERn and ERc concentrations determined using the ER-EIA method. △, women younger than 50 years; □, women 50 years or older. The regression analysis equation for the total population (\( N = 116 \)) is ERc = -15.8 + 1.0 ERn, \( r = 0.80 \). When divided into the two age groups, the following regression analysis equations and coefficients of correlation are found: patients <50 years, ERc = 9.43 + 0.2 ERn, \( r = 0.65 \); and patients ≥50 years, ERc = -4.4 + 1.0 ERn, \( r = 0.81 \).

ERn in women younger than 50 years while only 14% is found as ERn among the older women (Fig. 1).

**DISCUSSION**

Quantitative determinations demonstrate that the amounts of ER being extracted from the crude nuclear pellet exceed by far those that could be expected had it been possible to extract more ER under low salt conditions. Thus, ER extracted from the crude nuclear pellet under high salt conditions does not appear to be merely a contamination of the nuclear extract with ERc. Moreover, biologically meaningful results are observed. A greater fraction of total tissue ER is found more tightly bound to the chromatin among younger women than among older women. This observation is consistent with the fact that endogenous estradiol concentrations are significantly higher among premenopausal than among postmenopausal women.

The premise that ERn may be a better measure for biologically functional ER than is ERc is central to the present investigation. Several lines of evidence indicate that the mere presence of ERc in tissue does not necessarily confer the capacity to respond to estrogen upon the tissues. A major proportion of ERc positive tumors do not respond to endocrine therapy (28-30), which has led to the speculation that some tumors may have dysfunctional ER. Furthermore, two experimental cell lines have been described that contain ERc but are not capable of synthesizing PgR (31, 32). In the biopsies analyzed in the present study, ERc positivity was found to be associated with ERn positivity in 98% of the cases. These results contrast with those reported by others where the reported frequency of ERn positivity among ERc positive tumors ranges from 42 to 82% (16-20).

One of the major differences between previous studies and the present one is the difference in methods used to detect nuclear ER. Most earlier studies have either estimated binding of radioactive estradiol to nuclear preparations themselves (16, 20, 33, 34) or quantitated ER in high salt extracts from nuclear preparations using the hydroxyapatite technique (17, 18, 35). Both of these methods probably yield a number of false negative results due to either a high level of nonspecific binding of...
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[3H]estradiol or a temperature-dependent degradation of ER during the assay incubation (21).

Not only was ERc always found to occur simultaneously with ERn but a significant relationship was also found between the two receptor concentrations. Such a correlation has been reported earlier by another group using the hydroxyapatite technique (17). Here, higher concentrations of ER were found in the nuclear than in the cytosolic fractions among younger women (<50 years), while a 1:1 ratio was found between ERc and ERn among the older group of women. Thus it might appear that the ratio of ERn to ERc may reflect the endogenous estrogen concentration in the tumor tissue. A varying, high ratio is found in the younger women who experience cyclical fluctuations of endogenous estrogen concentrations, while there appears to be a lower but more constant ratio between ERc and ERn in the tissues of older women who have a more constant endogenous estrogen concentration. PgR positivity was associated with ERn positivity in 95% of the cases investigated here, and a significant correlation was found between PgR and ERn concentrations among tumors with detectable levels of both receptors. Since PgR is synthesized consequent to estrogen stimulation of target tissue, this result is consistent with our perception of the mechanism of ER action and it confirms an earlier report regarding a correlation between ERn and PgR concentrations (36).

Although ERc and PgR are always found to occur together with ERn, the converse is not true. A considerable number of ERn positive biopsies are ERc positive (7 of 25). These occur almost exclusively among the younger women, most of whom also have measurable concentrations of PgR. Thus, the fact that ERn is found in such biopsies explains the previous enigma that a significant number of especially premenopausal patients are found with a receptor profile of ERc positive and PgR negative. Our inability to detect ER in the cytosols from these patients has not been because the patients’ tissues have been devoid of ER but because the ER has been so tightly bound to the chromatin that it has not been extracted under low salt conditions. This result may call for a revision of our perception of the biological significance of the traditional receptor profiles. Thus there may be three rather than two or four biologically relevant profiles: ERc positive PgR positive and ERc negative PgR positive tumors may constitute a single class; ERc positive PgR negative tumors, a second class; and ERc negative PgR negative tumors, a third class.

Approximately 60% of all PgR negative tumors are ERn positive (23 of 39). Thus, in some tumors it might appear that there is a defect in the mechanism of action of ER after the binding of the hormone-receptor complex to the chromatin. Alternatively, there may be a form of ER in some tissues that becomes tightly associated with the chromatin without being bound to estradiol. It may be noted that the majority of the patients who are ERn positive PgR negative are postmenopausal. Clarification of this phenomenon requires further studies.

From the literature, it might appear that ERn determinations might add to the predictive and prognostic information that can be obtained from ERc and PgR analyses (16, 20, 37, 38). However, the data presented here do not support this viewpoint because nearly all ERn positive tumors are either ERc positive or PgR positive. In fact, only 1 of the 135 patients may inappropriately have been classified as being ER negative PgR negative on the basis of the routine receptor determinations (Patient 4 in Table 3).

It would have been fortuitous if ERn determinations could have been substituted for PgR determinations since the latter analyses continue to be more difficult to perform reproducibly than ERc determinations (39) or ERn analyses using the ER-EIA method (21). However, since not all ERn positive tumors contain PgR, valuable information for the classification of the patients’ receptor status would be lost were this approach to be followed. ERn determinations could reasonably replace ERc determinations; such an approach would reduce the frequency of occurrence of ERc negative PgR positive biopsies. However, since tissue preparation is more time consuming for the ERn assay than for the ERc assay, such a choice would not be reasonable based upon our present knowledge.

The application of the monoclonal ER-EIA assay method to determine ERn represents a major advance in receptor technology. However, the major value of ERn determinations may remain in the realm of experimental situations in which questions concerning the biology of breast cancer are addressed. The method yields reliable results that present a reasonable explanation for the curious phenomenon of the occurrence of ERc negative PgR positive tumors in some patients. While this issue can, perhaps, be put to rest, another remains to be answered. A large fraction of ERc positive PgR negative tumors have ERn.

The nature of this abnormality in the mechanism of ER action in these tumor tissues as well as whether this abnormality is of clinical consequence remains to be elucidated.

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