Enzyme Immunoassay of Estrogen Receptors in Fine Needle Aspirates of Breast Tumors

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Abstract

Enzyme immunoassay of estrogen receptors (ER-EIA) was compared to radioligand assay (ER-RLA) in fine needle aspirates of breast tumors. Fine needle aspiration is a relatively atraumatic means of harvesting malignant cells from breast tumors. Fine needle aspiration provides a homogeneous suspension (about 90% malignant cells) with a sufficient amount of cellular material (10 to 50 µg DNA per sample) for single point radioligand assays of extractable estrogen (ER) and/or progesterone receptor (PR) in about 85% of primary adenocarcinomas at the time of diagnosis.

Sixty-one different samples of malignant mammary cells were obtained by fine needle aspiration from 43 adenocarcinomas, 11 metastatic axillary nodes, and 7 cutaneous nodules. Thirteen patients were under antiestrogen therapy (tamoxifen). ER-EIA was performed with Abbott’s reagents, following the manufacturer’s protocol. ER-RLA was a single saturation (5 nM) dextran-charcoal assay with [3H]R2858 as the labeled estrogen. The sensitivity of ER-EIA allowed dilution of the sample up to 10 times (according to sample cellularity and ER level) with less than 20% deviation from undiluted samples. Three levels of dilution of the samples (1/1, 1/2, and 1/10) allowed them to fall at least once into the range of the ER-EIA standard curve. Quantitative correlation between ER-EIA and ER-RLA was high (r = 0.86), and highest (r = 0.97) when samples from patients undergoing tamoxifen treatment were excluded. Major discrepancies between ER-EIA and ER-RLA appeared in those patients undergoing tamoxifen therapy; much higher values were obtained by ER-EIA. Eight of 13 of these patients were ER negative by ER-RLA but ER positive by ER-EIA. This preliminary observation indicates that in vivo ER modulation by hormones and antihormones should be reevaluated.

Introduction

FNA is a relatively atraumatic means of collecting cell samples from pathological tissues. It offers many advantages, especially when the therapy does not involve surgery. Sampling is atraumatic and anesthesia is not necessary. Even deep organs can be punctured, under echographic control (2). Repetition of sampling during the course of treatment is well accepted by the patient, which allows sequential studies and follow-up of the disease.

The scarcity of contaminating cells (less than 1% stromal cells, less than 10% peripheral blood nucleated cells) makes FNA very specific for the collection of tumor cells. Being multidirectional within the tumor, the sample is representative of cellular heterogeneity of the tumor. Fine needle aspiration has been applied to breast pathology for diagnosis (3–5). More recently it has been used to obtain specimens for the determination of ER and PR by radioligand assays in tumors for which therapy does not involve surgery (6–9). The availability of monoclonal antibodies to ER, in the form of ready to use reagents (Abbott Laboratories, North Chicago, IL), prompted us to evaluate the practicability of this new assay on fine needle aspirates from patients with primary or advanced breast cancer and to compare it to a classical dextran coated charcoal radioligand assay.

Materials and Methods

Sixty-one samples were obtained by aspiration of 43 breast adenocarcinomas, 11 axillary nodes, and 7 cutaneous nodules metastatic from breast carcinoma. Thirteen of these samples were from patients undergoing long term antiestrogen therapy with tamoxifen. However, since the present study was aimed only at methodological evaluation, samples were not selected for sequential study of receptor modulation by hormonal therapy.

Fine needle aspiration was performed by trained cytologists as described by Franzen and Zajicek (10). The needle (22-gauge; 0.6 mm in diameter) was fitted to a 20-ml disposable syringe mounted in a syringe holder (Camco, Sweden) to facilitate the aspiration of cellular material from the solid tumor. The cellular material collected in the shaft of the needle was rinsed out with 0.5 ml of buffer (10 mM phosphate-10% glycerol-0.4 M KCl-2 mM dithiothreitol, pH 7.6, 0°C) and immediately frozen in liquid nitrogen. The whole operation from puncture to liquid nitrogen freezing took generally less than 30 s. The cellularity of the sample was assessed on a smear of a minute aliquot of the cell suspension.

Estrogen Receptor Assay. All operations, except the ER-EIA itself, were performed at 0°C on crushed ice. Homogenization was performed by sonication of the thawed sample for 6 to 8 s at minimal power setting with a 3-mm microprobe (Branson Sonifier 15B; Branson, Danbury, CT). The homogenate was centrifuged for 1 h at 105,000 × g. The entire supernatant was collected by aspiration with a pipet, and its volume was made up to 700 µl with homogenization buffer. The pellet was assayed for DNA by fluorimetry (11). ER-RLA was performed by a standard dextran coated charcoal assay on 100-µl aliquots of supernatant (2 aliquots for total binding, 2 for nonspecific binding) at one single saturation concentration, 5 nM, of the estrogen tag [3H]R2858, with or without a 100-fold excess of unlabeled R-2858. [3H]R2858 and R2858 were purchased from New England Nuclear (Boston, MA). Conditions for ER-RLA with [3H]R2858 were those described previously (9).

ER-EIA was performed either in duplicate or on 1/1, 1/2, and 1/10 dilutions of the supernatant. The assay was done with the Abbott ER-EIA kit, following the manufacturer’s instructions. ER values were not statistically different from zero when below 100 fmol/mg DNA and were given this latter value when logarithmic values of ER were used in correlation analysis.

Results

The cytosols from the 61 aspirates were classified in three groups (Table 1) according to the ratio of DNA (µg DNA) measured in the 105,000 × g pellet, which reflects the cellularity of the sample, to the volume of supernatant (ml) obtained for ER assay. Twenty-three % (14 of 61) of the samples had what is considered as “very low” cellularity in radioligand assay (<10 µg DNA/ml), 16% (10 of 61) had “low cellularity” (10 to 20 µg DNA/ml), and 61% (37 of 61) had “high” cellularity (>20 µg DNA/ml). Because the standard curve of ER-EIA is limited to 500 fmol/ml, several dilutions of the samples were assayed whenever possible in order to fall into this range. Samples with very low cellularity were assayed without further dilution (1/1). Samples with low cellularity were assayed undiluted (1/1) and...
diluted to one-half (1/2). Samples with high cellularity were assayed at dilutions 1/1, 1/2, and 1/10.

Fig. 1 shows the precision of ER-EIA between dilutions of a given sample, expressed as the percentage of deviation of two given dilutions from the mean ER value calculated from each dilution. Variation between dilutions very seldom exceeded 15% and only in the case of low ER levels. Above 10 fmol/mg DNA, the variation is below 10%, except in one sample (see legend to Fig. 1). Thus, ER-EIA appears reliably sensitive, allowing up to 10-fold dilution of FNA samples without loss of accuracy.

Correlation between ER-EIA and ER-RLA. The regression analysis between ER-EIA and ER-RLA (log values) on 61 unselected fine needle aspirates is shown in Fig. 2A. ER values which were underestimated by ER-EIA because of insufficient dilution were included in the regression analysis as the maximum value found at the highest dilution (Fig. 2, arrows). The correlation is highly significant (r = 0.86; P < 10⁻⁴) despite the fact that it does not hold for samples from patients undergoing tamoxifen therapy (circled cases). In those cases, the ER-RLA value is much lower (and often zero) than the ER-EIA value. In terms of positivity, i.e., defining ER negative as below 500 fmol/mg DNA and ER positive as above 600 fmol/mg DNA, the correlation was absolute when samples “under tamoxifen” were excluded. In the series of 13 samples under tamoxifen, 2 cases were ER negative by both assays; 3 cases were ER positive by both assays, with much higher values by ER-EIA; and 8 cases were strictly ER negative by ER-RLA while definitely ER positive by ER-EIA. ER-EIA and ER-RLA are not, therefore, equivalent when patients are undergoing tamoxifen therapy. When these patients were excluded from the regression analysis, the correlation coefficient for the 48 remaining cases was 0.97 and log(ER-EIA) = 1.02 log(ER-RLA) + 0.06. Finally, if we further excluded the 8 cases with very low cellularity (<10 μg DNA/ml) for which the validity of both assays may be suspect (especially the negative results), the regression analysis of the 40 remaining samples gave a correlation coefficient of 0.97 with log(ER-EIA) = log(ER-RLA) + 0.10 as shown in Fig. 2B. The correlation in terms of positive or negative values was absolute for these 40 cases as shown in Table 2.

Discussion

The present work was aimed at testing the suitability of the Abbott ER-EIA for the assay of estrogen receptors in small samples of breast tumor tissue, such as those obtained by fine needle aspiration. Estrogen and progesterone receptors are routinely assayed in our laboratory by radioligand methods, which are either classical (single saturation dose, dextran-charcoal assay (7–9) or include high pressure liquid chromatography for the simultaneous determination of ER and PR (12). Although the limited amount of ER-EIA reagents available precluded further investigations on clinically selected patients, at least two important conclusions could be drawn.

1. ER-RIA is a highly sensitive assay which allows ER determination on fine needle aspirates. ER-EIA appears superior to ER-RLA as regards sensitivity. The sensitivity of the test has been clearly demonstrated for surgical samples (13); the assay is reliable at cytosol protein concentrations as low as 0.2 mg/ml (1.5 mg/ml for routine RLA). This sensitivity was confirmed for FNA, since up to 10-fold dilutions of samples containing more than 10 μg DNA gave consistent results. Since reliability of ER-RLA must be strongly questioned when cellularity is low (<20 μg DNA/ml cytosol) (9), EIA appears reliable for samples with much lower cellularity (down to 10 μg DNA/ml and even below). The accuracy of EIA is apparent from its high degree of linearity upon dilution, so that ER samples can be assayed at two or three dilutions with less than 20% deviation. Due to the limited range of the standard curve for EIA, several dilutions should be assayed for any sample, whenever possible. A strategy of dilution is proposed in Table 3, according to the cellularity of the samples, which can be assayed by fluorimetry prior to ER assay (see “Materials and Methods”).

The reproducibility of ER-EIA could not be determined as rigorously as for surgical samples (13) due to the limited amount of cellular material available in FNA samples. However, when samples were assayed in duplicate, the coefficient of variation never exceeded 5% for ER values above 10 fmol/ml. The stability of the sample as measured by ER-EIA has not been tested in the present study, but it is reported as superior to that for site binding (ER-RLA). The presence of blood contamination in some FNA samples introduced a high protein concentration which slightly (up to 20%) interferes in the assay (data not shown) whereas ER-RLA with [³H]J2858 as estrogen tag was not susceptible to this interference (data not shown). However, the slight interference of blood with ER-EIA becomes negligible as soon as the sample is diluted. As a result of its intrinsic qualities, the ER-EIA results agreed favorably with the ER-RLA, despite our lack of previous experience with the ER-EIA technique. Therefore, ER-EIA is a very promising assay for samples containing minute amounts of tumor cells.

2. Taking advantage of the fact that fine needle aspiration, being relatively atraumatic, allows the sampling of tumor tissue during the course of nonsurgical treatment, in particular during

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Table 1 Cellularity of FNA samples

<table>
<thead>
<tr>
<th>DNA (μg/ml cytosol)</th>
<th>2.3–10 (very low)</th>
<th>10–20 (low)</th>
<th>20–200 (high)</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>14</td>
<td>10</td>
<td>16</td>
<td>61</td>
</tr>
<tr>
<td>%</td>
<td>23</td>
<td>16</td>
<td>61</td>
<td>100</td>
</tr>
</tbody>
</table>

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Fig. 1. Variation between dilutions of a sample according to the extent of dilutions and to the level of ER.

% of variation = \( \frac{1}{2} \) (Difference between ER values at 2 dilutions) / Mean ER value of the 2 dilutions

Thirty-nine pairs of dilutions were analyzed. Curves are estimates of maximum deviations for each set of dilutions. Points above curves at upper right are from dilutions of the same sample.

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4 P. M. Martin, unpublished data.
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The course of antiestrogen therapy by tamoxifen, we were able to show a clear difference between ER-RLA and ER-EIA. The majority of the samples under tamoxifen had much lower ER values by ER-RLA than by ER-EIA, even though ER-RLA assay was performed under exchange conditions (16 h incubation at 0-2°C). Low values of ER are generally reported under tamoxifen therapy (14). Since our results are very preliminary, a working hypothesis can only be proposed as an explanation. The epitope for the monoclonal antibody of ER-EIA is not affected by the state of the estrogen binding site (bound or free), whereas in ER-RLA bound tamoxifen is not displaced by [3H]R2858. Thus, these preliminary results could lead to a reevaluation of the in vivo modulation of estrogen receptors by hormones and antihormones according to the definition of the entity studied, i.e., the ER molecule (ER-EIA) or the estrogen binding site (ER-RLA). Sequential FNA samples from patients treated by various protocols of hormone therapy are currently being investigated by both methods in our laboratory to bring new insight to this problem.

Table 2 Correlations between ER-EIA and ER-RLA

Patients under tamoxifen therapy and samples with very low cellularity (<10 µg DNA/ml) are excluded. n = 40.

<table>
<thead>
<tr>
<th>ER-EIA</th>
<th>ER-RLA</th>
</tr>
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<tbody>
<tr>
<td>+</td>
<td>27</td>
</tr>
<tr>
<td>±</td>
<td>0</td>
</tr>
<tr>
<td>-</td>
<td>0</td>
</tr>
</tbody>
</table>

Table 3 Proposed schedule of dilutions of the original FNA sample for ER-EIA, according to its cellularity

<table>
<thead>
<tr>
<th>DNA (µg/ml cytosol)</th>
<th>Dilutions</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;10</td>
<td>1/1</td>
</tr>
<tr>
<td>10-20</td>
<td>1/1</td>
</tr>
<tr>
<td>&gt;20</td>
<td>1/2</td>
</tr>
</tbody>
</table>

Acknowledgments

ER-EIA reagents were kindly provided by Abbott Laboratories. We are grateful to Dr. M. Lonsdorfer (Abbott Laboratories, Frankurt, Germany) for helpful discussion on ER-EIA methodology. This study could not have been performed without the expert technical help of M. Thioux (ER-EIA) and C. Lainé-Bidron and E. Boudou (ER-RLA).

References

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