
Colette Charpin, Pierre-Marie Martin, Jocelyne Jacquemier, Marie Noelle Lavaut, Natalie Pourreau-Schneider, and Maurice Toga

Departments of Pathology [C. C., M. N. L., M. T.] and Experimental Oncology [P. M. M., N. P. S.], University of Marseille, and the Department of Pathology, Institut Paoli Calmettes [J. J.], Marseille, France

Abstract

An estrogen receptor (ER) immunocytochemical assay (ER-ICA) was applied to 115 malignant breast carcinomas and the results were compared to those of steroid binding assays performed on cytosol extracts of the same tumors.

Immunoperoxidase (peroxidase-antiperoxidase) staining was performed on frozen sections using rat monoclonal antibody to estrogen receptor H222SPγ. A preembedding method was used for the immunoelectron microscopy study. A semiquantitative analysis and a computerized image analysis system were used to evaluate the positive ER immunostaining. Positive immunostaining (81 of 115) was always located in the nucleus of tumor cells and of normal cells in adjacent breast tissue. The immunostaining pattern differed from one tumor to another, due to variations in either the intensity or the percentage of positive cells.

When immunohistochemical staining was correlated to biochemical assay, (a) there was an 88% correlation, and (b) staining intensity and percentage of positive cells significantly increased (P < 0.01) with cytosolic ER levels and were independent of cellularity.

These results indicated that (a) ER-ICA is to date the most reliable histochemical method for ER detection and correlated in 88% of the cases with ER biochemical assay; (b) ER-ICA constitutes a method particularly valuable to screen ER negative tumors on condition that tumor fragment quality (sampling and storage) is perfectly controlled; (c) ER-ICA provides additional information for heterogeneous ER distribution within tumors; (d) ER-ICA as a qualitative method is unable to replace the quantitative ER determination obtained with biochemical assay although the computerized system (SAMBA 200) for image analysis of microscopic preparations constitutes a valuable improvement of immunostaining analysis; and (e) ER-ICA based on ER antigenic site detection is complementary to biochemical assay based on ER functional site determination.

Introduction

The importance of estrogen receptor measurements for making therapeutic decisions is widely accepted for breast cancer. Therefore, the development of receptor investigation methods has drawn considerable interest.

ER hallmarks consist of their specificity, their high affinity binding capacity, and their antigenic properties. ER are included in cell compartments in which occupied and unoccupied receptors are in equilibrium (1). ER positive cells are heterogeneously distributed in tissues, including tumors. Also stromal epithelial cell interactions may account for possible regulation of ER expression. Consequently, ER determination methods for detection and quantification in human tissue must face three major problems: (a) the specificity; (b) the receptor accessibility; and (c) the heterogeneous distribution of receptors in tissues. Estrogen receptor determination methods may be classified in two categories: biochemical assays using radioligands performed on cell homogenates and measuring mean receptor levels; and morphological methods performed on tissue sections or intact cells and providing information about ER distribution within cells and tissues. Most of these methods rely on steroid binding properties of receptors. Recently, specific monoclonal antibodies against ER have been developed (2–4) allowing either ER enzyme immunoassays or ER-ICA based on ER antigenic site detection.

Since currently available histochemical or immunohistochemical methods (5–9) have been shown to be unreliable methods for ER detection (10), we undertook a prospective study in a large series of breast carcinomas using the monoclonal anti-ER antibody H222SPγ in conjunction with an immunoperoxidase method; our objective was (a) to compare ER immunocytochemical assay results with current biochemical assays, (b) to study variations of ER distribution in tumors, and (c) to define as accurately as possible ER intracellular distribution of immunoreactive ER using immunoelectron microscopy.

Materials and Methods

Materials

Tissue samples from 115 surgically removed breast carcinomas were collected (1983–1984) in the pathology departments of Timone Hospital and the Institut Paoli Calmettes. Carcinomas were histologically typed according to the WHO classification (11) and the Scarff-Bloom-Richardson grading system (12) routinely used in pathology. Tumor histological types and grades were distributed in our material as reported previously (13). In all cases two tissue blocks were sampled, one for ER-ICA and one for binding assays in perfectly controlled conditions as reported previously (14–16). In three cases two fragments from each tumor quarter were sampled for ER-ICA and for estradiol binding assay to determine the sampling variability of the tissue used in both assays.

Light Microscopy Immunostaining Procedure

Tissue Preparation. The specimens were rapidly frozen (within 15 min in the operating room) in liquid nitrogen and stored embedded in OCT (Tissue-Tek; Miles) at −80°C. Tumor blocks were cut into 8-mm slices and then mounted on glass slides coated with the tissue adhesive provided in the ER-ICA kit. The sections were immediately fixed for 10 min in 3.7% formaldehyde in 0.1 M PBS (pH 7.3), washed for 10 min in PBS, subsequently fixed for 4 min in methanol at −25°C and 2 min in acetone at −25°C, and then washed for 5 min in PBS. For each tumor, one section was processed. In 5 cases, 10 sections from the same tissue block were tested.

Immunostaining Procedure. The immunostaining was performed according to the method of King et al. (17) using an Abbott ER-ICA kit (Abbott Laboratories Diagnostics Division, North Chicago, IL). Briefly,
the sections were incubated for 15 min with the blocking reagent to suppress nonspecific binding and then for 30 min with monoclonal anti-ER H222SPγ, rinsed for 2 × 5 min in PBS and then incubated for 30 min with the bridging antibody, rinsed for 2 × 5 min in PBS and incubated 30 min with the peroxidase-antiperoxidase complex, rinsed in PBS, and finally incubated for 6 min in chromogen (diaminobenzidine and 0.06% hydrogen peroxide in PBS) and counterstained with Harris hematoxylin.

Negative controls were assessed using PBS and ER-ICA kit control serum (normal rat IgG) instead of specific monoclonal anti-ER. MCF-7 cells served as positive controls.

Immunostaining Quantitative Analysis. In each section a semiquantitative method was used to evaluate the immunocytochemical staining with a double grading system: (a) the SI (0 to 3+); and (b) the PC (1 = 5 to 100%; 2 = 30% to 70%; 3 = 71 to 100%). Also a global score referred to as SIC was established by adding both SI and PC grades (0 to 6).

Also, to improve and control this easily routinely performable method of immunostaining evaluation, a computerized system of image analysis was used as a comparison to the semiquantitative analysis in 6 tumors and in MCF-7 cytospins. This system, previously referred to as SAMBA 200 (TITTN) (18, 19), provides the accurate percentages of positive cells and the histograms of the staining intensity distribution. In this analysis, several fields (X40) on the same slide were examined. A variable number of cells (from 350 to 600) per field were counted using the nuclear staining obtained with the hematoxylin counterstain. For both the counterstaining and the immunostaining evaluation, the detection thresholds were first determined on the microscope and on the computer screen (X40); then, the cell count, the immunostaining detection, and the staining intensity analysis were automatically performed. The results were represented on diagrams and histograms.

Immunoelectron Microscopic Study

For electron microscopy tumor fragments removed in the operating room were immediately cut into 5- × 5- × 2-mm slices and fixed as described above. Thick sections of 100-μm-thick sections were cut with a Vibratome (Lancer 1000). The preembedding method was also applied on vibratome sections obtained from frozen tissue blocks. The immunostaining was performed on free floating sections in Petri dishes, using the same procedure as above described for light microscopy. Then the sections were postfixed in 1% osmium tetroxide solution (30 min), dehydrated, and embedded in Araldite. Ultrathin sections were obtained using a diamond knife, then collected on 300 mesh copper grids, and examined with a Jeol 1200 EX electron microscope.

Biochemical Determination of ER

Cytosolic ER determination was measured by dextran coated charcoal assay as previously described (14-16). Tumors with less than 10 fmol/mg protein were considered negative, and those with >10 fmol/mg protein were considered positive.

Statistical Analysis

For correlation of the semiquantitative analysis of immunocytochemical results and binding assays, statistical analysis was performed using the χ² and Fisher’s exact tests. As reported previously (18) the Fourier transform was applied to analyze the objective SAMBA 200 parameters.

Results

Semiquantitative Analysis

Positive Immunostaining Distribution. In positive tumors the staining was located in the nucleus of carcinomatous cells or of epithelial cells of nontumorous adjacent breast tissue (Fig. 1). Positive cells were heterogeneously distributed in the tissue sections in all cases (Fig. 1). The staining intensity varied within a given tumor and the percentage of immunostained cells varied among the positive cases (Fig. 2).

Controls. Multiple sections from one tissue block displayed the same immunostaining pattern. No positive immunostaining was observed in negative controls, ruling out nonspecific reactions due to the kit reagents. The immunostaining intensity was unchanged when tumors were stored for more than 1 year (1 to 3 years). In the cases in which four fragments were sampled, the intensity but not the percentage of positive cells varied slightly as did the binding assay in the adjacent area (ER = 159 to 271 fmol/mg protein). In the negative case, however, all four fragments were negative.

Semiquantitative Evaluation. Among the 115 carcinomas tested, 81 (70%) were ER-ICA positive and 34 (30%) were ER-ICA negative. The staining intensity in positive cases increased with the cytosolic ER levels. For those tumors with SI = 1 (31.5%) the ER mean value was 63 fmol/mg protein; for SI = 2 (33%) the ER mean was 145 fmol/mg protein; for SI = 3 (35.5%) the ER mean was 246 fmol/mg protein; correlation SI/ER, P < 0.001. Similarly the percentage of positive cells increased significantly (P < 0.01) with ER levels. For those tumors with PC = 1 (19%) the ER mean was 33 fmol/mg protein; for PC = 3 (42%) the ER mean was 215 fmol/mg protein.

Although the cellularity was related to the cytosolic ER levels, it was not related to the intensity or to the proportion of tumor cells stained.

When ER-ICA positive staining was evaluated according to the immunocytochemical score, SIC, established by adding both SI and PC grades, high SICs, i.e., SIC = 6 and SIC = 5 (71 and 87%, respectively), were correlated with an 80% probability of positive response to hormone therapy defined on the age adjusted ER level curve (P < 0.01) (20).

ER Immunostaining and ER Biochemical Assay. Cytosolic ER levels were ≥10 fmol/mg protein in 74% (85 of 115) of the tumors included in our series. This rate of positive ER tumors correlates with that in previous reports (14, 16).

In 101 of 115 of the cases (88%) ER-ICA results were correlated with binding assays and a discrepancy was observed in 14 cases (12%). Among the 81 ER-ICA positive cases, 5 had ER levels <10 fmol/mg protein which constitutes 32% (5 of 14) of the discrepant cases. In these 5 cases, tumors had SI = 2 and PC = 1 in two cases and SI and PC = 2 in 3 cases. Also 3 cases were progesterone receptor positive (progesterone receptor = 23, 35, and 1360 fmol/mg protein). These data suggest that at least in these 3 cases negative binding assay values most probably result from unsuitable tissue sampling. Among the 34 ER-ICA negative cases, 9 had ER levels from 20 to 451 fmol/mg protein by steroid binding assay (mean range, 75 fmol). In 2 cases the tumors measured 10 mm. In 3 cases the tissue fragment used for ER-ICA contained few tumor cells and was not representative of the whole tumor tissue. These results suggest that in at least 6 cases the discrepancies between both methods can be attributed to the fact that tumor fragments sampled for ER-ICA and biochemical assays were different. Also, the correlation with the binding assay was better in the cases with positive ER-ICA results (97%) than in ER-ICA negative samples.

ER-ICA Computerized (SAMBA 200) Quantitative Analysis

MCF-7 Cytospins. MCF-7 cytospins, which served as positive controls, were first evaluated by this method. The SAMBA 200 analysis showed that the percentage of positive cells varied from one slide to another in the range of 51 to 66%. These variations were not detected by simple subjective semiquantitative analysis. Also, immunostaining intensity differences which were not
accurately evaluated by semiquantitative analysis, were clearly shown by the histograms (Fig. 3).

**Tissue Sections.** In each tumor, the histogram of the staining intensity in positive cell nuclear surfaces was established as shown in Fig. 4. Also, the mean of the percentage of positive surfaces of each field was compared to the semiquantitative analysis (Table 1). Some discrepancies could be observed, in particular, the percentage of positive cells evaluated by semiquantitative analysis being overscored when the staining intensity was marked and vice versa (Table 1).

**Immunoelectron Microscopy**

The immunoelectron microscopy provided more accurate data than did the light microscopy for intracellular immunoreactive ER distribution. This method showed a consistent intranuclear positive immunostaining. The positive staining, consisting of osmiophilic black dots, was variable and irregularly distributed in nuclei (Fig. 5). However, it appeared most often diffusely spread in the nucleoplasm and did not involve the nucleolus. Positive cells were observed adjacent to completely negative cells, but usually positive cells were gathered in clumps or sheets (Fig. 5).

**Discussion**

Until very recently, the information about subcellular estrogen receptor distribution and quantitative assays for ER was based on the binding of radioactively labeled steroids. The recent development of a number of monoclonal (2-4, 17) antibodies against ER provides a new approach to the determination of ER based on the detection of antigenic sites instead of the binding of the labeled hormone. Monoclonal antibodies allow the detection of occupied and unoccupied sites (4) and are suitable for light and electron microscopy immunocytochemical assays. This is particularly useful to assess ER intra-
ER IN BREAST CARCINOMAS

Fig. 2. Immunoperoxidase (peroxidase-antiperoxidase), monoclonal anti-estrogen receptor antibody, Harris hematoxylin counterstaining. Variations of positive staining in carcinomas. The staining intensity is variable: (a) some tumors enclose a majority of positive cells lightly stained (SI = 1+) and (b) others, a majority, strongly stained cells (SI = 3+) (x 750). Also, (c) some tumors exhibit a low percentage of positive cells (PC = 1+) in contrast to others (d) with a high percentage of positive cells (PC = 3+) (x 750).

cellular localization and ER positive cell distribution in tissues. In accordance with previous reports in which the anti-ER antibody H222SPγ and a similar immunostaining procedure peroxidase-antiperoxidase were used (2–4, 17), we observed in our study positive staining only in cell nuclei in frozen sections. Positive nuclear immunostaining was also consistently obtained.
with different monoclonal anti-ER antibodies, as recently reported by others (2, 21, 22). Nevertheless, questions may arise about the accuracy and reliability of light microscopy, even on high power magnification, to study the intracellular antigen distribution, particularly when the immunostaining procedure is performed on shortly fixed frozen sections. In our experience, however, immunoelectron microscopy correlated with light microscopic findings and confirmed the nuclear localization of immunoreactive ER in morphologically well preserved carcinomatous cells. This nuclear localization of immunoreactive ER confirms some previous studies (1, 23, 24) which suggested the nuclear ER localization. Recent reports using well documented monoclonal anti-ER antibodies also supported the view that ER, traditionally referred to as cytosolic, and nuclear receptor depending on preparation are located in the nucleus (2-4, 17). According to this hypothesis, a nonactivated receptor is loosely associated with the nucleus and can easily be extracted from the cytosol by low salt buffers. In contrast, hormone-receptor complexes are tightly associated with nuclear components, requiring the use of high salt buffers for receptor extraction from the nuclear compartment (1, 17). However, since autoradiographic studies revealed some cytoplasmic staining in addition to nuclear staining (1, 23), it can be suggested that the absence of immunoreactive ER in the cytoplasm may result from the fact that the antigenic determinants are unavailable to the anti-ER antibody in the cytosol. Indeed, when immunoas-
ER IN BREAST CARCINOMAS

says were performed in MCF-7 cytospins or tissue sections preincubated with various concentrations of estradiol or tamoxifen at variable temperatures, we observed (results not shown).

Table 1 ER-ICA in tissue sections from 6 breast carcinomas: comparison of the semiquantitative analysis and the quantitative analysis performed with the computerized image analysis system (SAMBA 200, TITN)

<table>
<thead>
<tr>
<th>Case</th>
<th>SI*</th>
<th>PC*</th>
<th>SAMBA 200 analysis (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>++</td>
<td>+</td>
<td>57</td>
</tr>
<tr>
<td>2</td>
<td>+++</td>
<td>+</td>
<td>51</td>
</tr>
<tr>
<td>3</td>
<td>+</td>
<td>++</td>
<td>66</td>
</tr>
<tr>
<td>4</td>
<td>+</td>
<td>+++</td>
<td>56</td>
</tr>
<tr>
<td>5</td>
<td>+++</td>
<td>+</td>
<td>74</td>
</tr>
<tr>
<td>6</td>
<td>+++</td>
<td>+++</td>
<td>51</td>
</tr>
</tbody>
</table>

* SI graded from SI = 1 to SI = 3+.
* PC graded from PC = 1+ (5 to 30%), PC = 2+ (31 to 70%), to PC = 3+ (71 to 100%).

Hormone therapy response rates are related to the tumor estrogen receptor content and there is an increasing response rate with increasing receptor content of the tumor (4, 25). Indeed, about 70% of patients with receptor rich carcinomas respond to endocrine therapy (4), indicating that the quantitative information from estrogen receptor assay improves the predictability of response to hormone therapy. However, quantitative ER analysis with current biochemical assays requires substantial amounts of tissue and is expensive and time consuming to perform. Also lability of receptors requires rigorous technical quality controls for interlaboratory reproducibility and adequate use of quantitative receptor results in national and international clinical trials. In contrast, ER antigenic sites have been shown to be more stable than steroid binding activity, and immunoassays are reproducible, are simpler to perform, and can be applied to small biopsies (4).

Also, ER-ICA allows assessment of ER heterogeneity among cells or regions within a tissue or lesion, in contrast to binding assays which only measure an average receptor level in cell homogenates.

In fact, breast carcinomas are heterogeneous within their histological types and their degree of differentiation. Moreover, tumors enclose heterogeneous cell populations as demonstrated particularly by a variety of epithelial cell associated antigens heterogeneously distributed. Some clones enclose so-called differentiation antigens such as casein (26, 27), lactalbumin (28, 29), and gross cystic disease fluid protein (31); other clones contain carcinoembryonic antigen (32, 33) or lactoferrin (33). Also, breast carcinomas are composed of tumor cells exhibiting a variable amount of basement membrane component synthesis and protease release such as type IV collagenase (34-36). Similarly immunocytochemical assays showing that each tumor is a pool of ER positive and ER negative cells should permit a deeper insight into hormone responsiveness. Indeed, it may be suggested that hormone therapy failure in patients with ER positive tumors results from this ER heterogeneity, which is not evaluated by quantitative ligand binding assays. However, whether the heterogeneity of immunostaining intensity and distribution results from the polyclonal origin of tumor cells or from a variable expression of the receptor phenotype needs further investigation.

Most of the histochemical or immunohistochemical techniques which have been used for ER detection in tissue sections (5-9) have been shown previously to be inconsistent with the detection of estrogen receptor (10). In contrast, ER-ICA is well correlated with binding assays, suggesting its potential value for anti-estrogen response prediction. In our study ER-ICA was correlated in 88% of the cases to binding assay which is close to the findings of King et al. (17) and Pertshuck et al. (22) (84% and 86%, respectively). In our material there was strong evi-
dence that differences between both assays could have resulted from inadequate tissue sampling in 6 cases, indicating that ER-ICA reliability could be improved by a rigorous quality control of tissue sampling.

Although ER immunocytochemical assay constitutes basically a qualitative ER analysis in tissues, complementary to quantitative evaluation of binding assays, it seems promising to improve this qualitative analysis using immunocytochemical grades and scores which can serve as an index to allow a correlation with biochemical assays and clinical data. This type of semiquantitative analysis is currently used for analysis of immunocytochemical results and has recently been shown to be useful in correlating ER content in tumors and the response to hormonal therapy (22). Also, it can be suggested that the semiquantitative analysis of ER immunostaining could replace quantitative biochemical ER analysis of specimens of small size such as biopsies. However, attention must be focused on the fact that the immunostaining quantification, even performed by trained pathologists, is a method involving mainly a subjective appreciation that may lead to erroneous and nonreproducible results. In this respect, the use of a computerized system of image analysis provides more accurate and reliable data as shown by our preliminary results from the SAMBA 200 analysis of ER immunostained MCF-7 cytopsins. In tissue sections, this computerized method of analysis has to be improved by double staining methods using epithelial cell markers such as keratins or cell membrane antigens in addition to monoclonal anti-ER; these methods, presently under investigation, will permit accurate determination of the percentage of ER positive cells included in the epithelial compartment of tumors.

However, whatever the degree of the improvement of the immunocytochemical assay analysis by such methods may be, it is too early to suggest that ER-ICA could replace ER biochemical quantitative assays since no accurate data for hormone therapy decision and prognosis evaluation are thus far available for ER-ICA. Patient follow-up during a long period of time is required to judge the value of ER-ICA in terms of therapy decision and prognostics. Combined ER analysis in tumors by both binding assays and ER-ICA could improve the predictability of response to hormone therapy.

Acknowledgments

The authors thank W. J. King and M. Lonsdorfer for providing ER-ICA kits and F. Bres, N. Bianco, and R. Gochgargagn for the manuscript preparation.

References


Cancer Res 1986;46:4271s-4277s.