Quantitative Enzyme-linked Immunosorbent Assay for the Estrogen-regulated M, 24,000 Protein in Human Breast Tumors: Correlation with Estrogen and Progesterone Receptors

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ABSTRACT

A competitive enzyme-linked immunoassay has been developed to quantitate the M, 24,000 estrogen-regulated protein (p24) in human breast tumors and tumor cell lines. The concentration of monoclonal antibody used to coat polystyrene microtitrater assay plates was critical in optimizing assay sensitivity versus precision and demonstrated marked differences between plate types. Analysis of 114 human breast tumor cytosols revealed a 4-log range in p24 concentration with a median value of 1 μg/mg cytosol protein. The abundance of p24 in breast tumors in vivo is therefore similar to that observed previously in hormone-responsive breast tumor cells in vitro. Elevated expression of p24 in these specimens was found to correlate well with presence of estrogen and progesterone receptors (P = 0.0002 by Pearson χ² analysis) with the correlation coming primarily through estrogen receptor. This result was confirmed by immunoblot analysis of p24 in 76 additional tumor cytosols and suggests that p24 may be a valuable marker for differentiation in human breast cancer.

INTRODUCTION

In the search for additional markers of hormone sensitivity in human breast cancer, our laboratory has discovered (13) and characterized (12) a p24 cytoplasmic protein (termed 24K in previous publications) that is regulated by estrogen in the MCF-7 human breast cancer cell line. Unlike other proven or proposed markers, e.g., ER (19), PGR (10, 14) or the M, 52,000 secretory glycoprotein (27, 29, 30), p24 is an abundant cytosol protein which permitted rapid development of highly specific monoclonal antibodies, e.g., ER (19), PGR (10, 14) or the M, 52,000 secretory glycoprotein (27, 29, 30), p24 is an abundant cytosol protein which permitted rapid development of highly specific monoclonal antibodies. However, p24 was subsequently used these antibodies to demonstrate by immunohistochemistry selective p24 staining in breast tumor cell lines which retain ER and PGR. The protein is not commonly expressed in normal or lactating breast, in receptor-negative breast tumor cells, or in nonendocrine tissues and tumors (6). However, p24 is expressed in tissues of the normal human female reproductive tract (6), an expression that in endometrium appears to be hormone-regulated during the menstrual cycle (7). This evidence implies that p24 may have a role in the normal reproductive process that is subverted by hormone-sensitive breast tumors. To address this question and others regarding the mechanism of hormone effects on p24 and the expression of this protein in human breast tumors, we have developed a quantitative competitive ELISA. In this report, we confirm our preliminary finding (3) that p24 is present in significant amounts in certain human breast tumors, namely those that retain functional levels of ER and PGR.

MATERIALS AND METHODS

Materials. BSA (Cohn Fraction V), NSB, o-phenylenediamine, 30% hydrogen peroxide, and polyoxyethylene sorbitan monolaurate (Tween 20) were from Sigma Chemical Co., St. Louis, MO. ABC (ABC Reagent Kit PK4000) was obtained from Vector Laboratories, Inc., Burlingame, CA. Immulon I (Lot CR401) and Immulon II (Lot CR252) flat-bottomed polystyrene 96-well microtiter plates came from Dynatech Laboratories, Inc., Alexandria, VA.

Methods. Steroid hormone receptors were assayed in tumor cytosols prepared in buffer (10 mM Tris, pH 7.4 at 4°C:1.5 mM Na₂EDTA:0.5 mM diithiothreitol:5 mM NaMoO₄) at protein concentrations of 2 to 4 mg/ml. ERs were measured by the dextran-coated charcoal technique (20) and PGRs by the sucrose density gradient method (23). Immunoblot analysis of p24 protein in tumor cytosols was done as reported (3) except that the p24 band was excised from the aminophenylthioether paper and counted in a gamma counter (Nuclear-Chicago).

Preparation and Conjugation of Purified p24 Protein. The p24 protein was purified by monoclonal antibody affinity chromatography as described previously (2) and stored in aliquots at −20°C. The purified protein was biotinylated by a modification of the method of Clark and McGuire (9). Following overnight dialysis against 2 changes of 500 ml of PBS at 4°C, 1 ml of protein (0.168 mg/ml) was added to 1 ml of NSB solution (4.0 mg NSB first dissolved in 0.5 ml dimethyl sulfoxide and then diluted with 1.5 ml water) and incubated for 2 h at room temperature. The biotinylated p24 was dialyzed extensively at 4°C against PBS and then titrated against monoclonal antibody G3.1 using ELISA as described below. A dilution of 1:32,000 gave 1 absorbance unit at 490 nm; consequently, a 1000X stock solution was made by dilution in storage buffer (0.35M NaCl:1% BSA in PBS, pH 7.4) and 50-μl aliquots were stored at −20°C.

Solid-Phase ELISA for p24. Derivation of monoclonal antibody G3.1 against p24 protein has been described previously (3). The antibody was purified from mouse ascites fluid by ammonium sulfate precipitation and anion-exchange and gel-permeation chromatographies (2). Immunol I or II microtiter plates were coated overnight at 4°C with 150 μl of either BSA (Wells A1 to A6) or with antibody G3.1 (remaining wells) diluted in PBS to 6.67 μg/ml (1 μg/well). Coating solutions were removed, and the remaining protein-binding sites were blocked by addition of block buffer (1% BSA in PBS, pH 7.4), 300 μl/well, followed by incubation for at least 1 h at room temperature. The blocking solution was removed, and the plates were washed 3 times with PBST. Plates that were not used...
calculated as:

\[ \text{Input concentration} \times \text{Oil 50} \]

and which makes removal of nonadsorbed enzyme label difficult (8). With this problem solved, we proceeded to characterize the assay, finding the optimal conditions described under "Methods." We then began analyzing breast tumor cytosols. After assaying 69 samples, we observed a significant and highly variable increase in the nonspecific binding even though we continued to use plates from the same lot. This problem was not affected by either the concentration or choice of blocking agent (i.e., BSA, ovalbumin, or gelatin). Consequently, we switched to Immulon I plates (in which the nonspecific binding is negligible) and assayed the remaining 45 samples included in this work. Our results indicate distinct differences between these plate types, illustrating the importance of plate selection.

Chart 1 shows that p24 ELISA sensitivity (defined as that concentration of p24 standard giving 10% inhibition) increases inversely with coating-antibody concentration to a maximum of 5 ng/ml or 0.75 ng/well (Immulon I) and 10 ng/ml or 0.15 ng/well (Immulon II). However, there is a concomitant loss in the total binding level that is reflected in a decrease in the slope of the standard curve. Thus, a compromise coating dose must be chosen to optimize assay sensitivity while maintaining assay precision. In the case of Immulon I plates, this dose is 1 µg/well, whereas for Immulon II it is 0.3 µg/well. For consistency, a coating dose of 1 µg/well was used on both plate types throughout this work, yielding sensitivities of 3 and 8 ng for Immulon I and II, respectively.

Table 1 summarizes the variation observed in standard curves

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Immulon I (n = 23)</th>
<th>Immulon II (n = 36)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Interassay variation</td>
<td>0.608</td>
<td>0.933</td>
</tr>
<tr>
<td>Non-specific binding (mg/ml)</td>
<td>0.009</td>
<td>0.007</td>
</tr>
<tr>
<td>Standard concentration at 50% inhibition (ng/ml)</td>
<td>132.1</td>
<td>169.3</td>
</tr>
<tr>
<td>Slope of standard curve</td>
<td>0.812</td>
<td>1.220</td>
</tr>
<tr>
<td>Correlation coefficient</td>
<td>0.986</td>
<td>0.987</td>
</tr>
<tr>
<td>Intrassay variation (CV%)</td>
<td>5.0</td>
<td>4.0</td>
</tr>
</tbody>
</table>

\*Response in the absence of competitor corrected for non-specific binding. The CV of the replicates at each dose on the standard curve; an average of the range given in "Results."
for both plate types. The intraassay CV was similar, ranging from 2.59 to 7.31 on Immulon I and 1.81 to 8.02 on Immulon II at the 7 doses of standard. The interassay variation indicates that standard curves run on Immulon II plates give better precision and less variation than do those on Immulon I. However, the non-specific binding is higher and the sensitivity is lower (as expected from Chart 1). The correlation coefficient of the weighted logit-log regression line was equally good with either plate type. Assay reproducibility was also assessed in crude samples by reassaying cytosol from estrogen-treated MCF-7 cells in the experiment shown in Chart 1. For the same sample assayed at different doses of coating antibody (n = 4), Immulon I gave 4.75 ± 1.26 (SD) ng/mg (CV = 28.5%) and Immulon II gave 9.95 ± 1.67 ng/mg (CV = 16.8%). Thus, plate differences in p24 ELISA variation seen with a crude sample reflect the variation seen with purified protein. This experiment also shows that the absolute p24 value was significantly different between plate types. Consequently, our statistical treatment of the data considered these data groups separately (see below). Furthermore, because the interassay variation of the 50% inhibition point used for computing p24 values was significantly higher on Immulon I plates than the variation seen on Immulon II, standard curves were routinely run on each plate regardless of plate type.

A concern in developing any quantitative assay is the stability of the purified standard. This is particularly true for this work, since the same preparation of p24 standard, both unconjugated and biotinylated, was used throughout. Chart 2 shows that the antigenic site on purified, biotinylated p24 recognized by monoclonal G3.1 is stable to storage at −20°C for long periods. The slight negative slope of the regression line (−0.002 Amax/day) is not significant given the degree of variation in the data. These data do not reflect the stability of p24 in crude tumor cytosols, which we were unable to assess due to the limited amount of sample available. However, ELISA analysis of p24 in a set of samples stored at −70°C for as long as 5 years gave similar correlations with receptor status as samples assayed fresh, and in fact, one of these samples gave the highest level of p24 of any tumor sample tested. Furthermore, immunoblot analysis of stored tumor cytosols did not reveal any extensive degradative fragments (data not shown).

Analysis of p24 in Human Breast Tumor Biopsies. A total of 114 human breast tumor cytosols were assayed for p24 (90 fresh and 24 frozen samples) of which 102 had valid ER and PGR assays. The frequency distribution of the p24 data is presented in Chart 3 classified according to receptor status. Table 2 summarizes the same data as the p24 ELISA results were divided on Immulon I and II plates. Neither the p24 nor the receptor data fit a normal distribution, showing a small but significant positive skewness and kurtosis. In addition, the median value for p24 in samples assayed on Immulon I plates was about twice that in samples assayed on Immulon II. Although this difference is due partly to a plate effect, it also reflects more receptor positive tumors in the Immulon I group (compare median ER and PGR values). The overall median p24 value of about 1 μg/mg cytosol protein suggests that p24 is an abundant protein in human breast tumors (extending over a 4-log concentration range) as well as in the MCF-7 human breast tumor cell line (where it represents 1 to 2% of cytosol protein).

Since the data is not normally distributed, p24 was correlated with ER and PGR as continuous variables by using the Spearman coefficient of rank correlation analysis. Table 3 shows that p24 measured by the quantitative ELISA on Immulon II plates is significantly correlated to ER, less so to PGR, while neither correlation is as high as that between the receptors themselves. The p24 correlation observed with a smaller number of samples assayed on Immulon I plates was lower as was the correlation between ER and PGR. To obtain an independent confirmation of the p24 correlation with receptor status, we analyzed p24 in a separate set of tumors using a Western blot technique in which iodinated antibody bound to p24 was quantified. This technique has the added advantage that only p24 of the correct molecular weight is analyzed. Correlations identical to those seen with the p24 ELISA on Immulon II plates were found (Table 2, Column 4).

The p24 protein was also correlated with ER and PGR status after stratifying the data (Table 4). Since we have no correlation of p24 levels with response to endocrine therapy as yet, p24 values were divided into low and high using as cut points median values for the individual plate types found in Table 2. Receptor cut points were set at 10 fmol/mg, which most clinical studies...
agree is a functional level of receptor. The Pearson $\chi^2$ statistic on this data indicates a significant positive correlation between $p24$ and the presence of ER and PGR in human breast tumors ($P = 0.0002$). A Mantel-Haenzel test was then used to determine whether the correlation of $p24$ and receptor is due primarily to ER even after adjustment for PGR status ($P = 0.0032$); the correlation was less significant between $p24$ and PGR adjusted with ER even after adjustment for PGR status ($P = 0.0002$). The correlation is not quantitative and is lower than the correlation between the receptors themselves. This is not surprising.

**DISCUSSION**

In our design of a quantitative immunoassay for $p24$, we chose to avoid the inherent disadvantages of radiommunoassay (short isotope half-lives, degradation of standard during labeling and storage, health hazards during handling and disposal, greater instrumentation costs) in favor of an ELISA approach. ELISA protocols are now nearing the sensitivity of radioimmunoassay, particularly with the advent of amplification steps either through antibody sandwiching or as we have used through bridging with avidin-biotin. This method, first developed by Guesdon et al. (15) and modified by Hsu et al. (17) is up to 40 times more sensitive than other immunoperoxidase methods (16). Even further amplification can be achieved by using both these methods with enzyme-coupled reactions (21). Although there are a variety of solid phases for ELISA, polystyrene microtiter plates are the most convenient and most easily adapted to automated detectors. The tradeoff for these advantages is the variability within and between plates. We found Immulon plates exhibit significant variation within and between each lot, with plates from some lots showing essentially no binding of antibody at all. Recently, microtiter plates have been produced that have a certified binding capacity and variability (Nunc Immunoplates). We have tested these plates with the $p24$ ELISA and found them to be as sensitive as Immulon II but with higher precision and reduced plate-to-plate variation (Lot 4283).

Aside from plate type, purity and stability of the $p24$ standard and the anti-$p24$ monoclonal are other factors that we found to be important. The $p24$ standard, purified to homogeneity by ion-exchange and gel filtration chromatographies that is at least 90% pure by gel electrophoresis. However, even using purified antibody, we observed significant and highly variable nonspecific binding on Immulon II plates in contrast to Immulon I (Table 1). These results suggest that albumin contamination of coating binding sites. In a detailed survey of plate effects in ELISA, Kenny and Dunsmoor (18) classified plates by their high or low adsorption of albumin. Immunoglobulin adsorbed well on all plate types, but those plates binding albumin (e.g., Immulon II) gave a higher level of nonspecific binding of the detecting conjugate. A major factor in this reaction is the presence of detergent (0.1% Tween 20) in the diluent buffer reduces the nonspecific binding on Immulon II plates in contrast to Immulon I (Table 1). We have since found that inclusion of nonionic detergent (0.1% Tween 20) in the diluent buffer reduces the nonspecific binding to $p24$ levels making Immulon II or similar plates the solid phase of choice with a coating antibody concentration of 0.67 to 1.33 $\mu$g/ml (0.1 to 0.2 $\mu$g/well).

Results from 2 different immunoassays (ELISA and Western blot) on individual tumor sets indicate that presence of $p24$ is correlated with that of ER and PGR in breast tumor cytosols. The correlation is not quantitative and is lower than the correlation between the receptors themselves. This is not surprising.

### Table 2

**Frequency distributions of $p24$ and steroid receptors in human breast tumor biopsies**

<table>
<thead>
<tr>
<th>p24</th>
<th>ER</th>
<th>PGR</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of samples</td>
<td>Median ng/mg</td>
<td>Range</td>
</tr>
<tr>
<td>Immunon I</td>
<td>45</td>
<td>1771</td>
</tr>
<tr>
<td>Immunon II</td>
<td>69</td>
<td>843</td>
</tr>
<tr>
<td>Combined</td>
<td>114</td>
<td>1041</td>
</tr>
</tbody>
</table>

*ER assayed by dextran-coated charcoal method.  
PGR assayed by sucrose density gradient method.  
Maximal range.

### Table 3

**Correlation of $p24$ with receptor status spearman rank correlation coefficient analysis**

<table>
<thead>
<tr>
<th>p24 vs. ER</th>
<th>p24 vs. PGR</th>
<th>ER vs. PGR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coefficient</td>
<td>P</td>
<td>Coefficient</td>
</tr>
<tr>
<td>p24 ELISA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Immulon I</td>
<td>0.2630</td>
<td>0.0811</td>
</tr>
<tr>
<td>Immulon II</td>
<td>0.3588</td>
<td>0.0031</td>
</tr>
<tr>
<td>p24 Immunoblot</td>
<td>0.4130</td>
<td>0.0003</td>
</tr>
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</table>

### Table 4

**Correlation of $p24$ with receptor status Pearson $\chi^2$ analysis**

<table>
<thead>
<tr>
<th>ER</th>
<th>PGR</th>
<th>Low</th>
<th>High</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>$-$</td>
<td>$-$</td>
<td>26</td>
<td>6</td>
<td>32</td>
</tr>
<tr>
<td>$+$</td>
<td>4</td>
<td>0</td>
<td>0</td>
<td>4</td>
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<tr>
<td>+</td>
<td>11</td>
<td>7</td>
<td>18</td>
<td></td>
</tr>
<tr>
<td>+</td>
<td>17</td>
<td>31</td>
<td>48</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>58</td>
<td>44</td>
<td>102</td>
<td></td>
</tr>
</tbody>
</table>

$\chi^2 = 19.93$
since we are correlating different protein properties, antigenicity and hormone binding. Other factors may also affect the correlation of p24 and receptors. Of 114 tumors tested, 50 gave an incomplete cross-reaction in the p24 ELISA, i.e., the slope of the sample dose response was more than 2 SDs from the standard slopes given in Table 1. Since we did not observe nonparallelism in cytosols from tumor cell lines, this effect may due to sample heterogeneity such as variable contamination of tumor biopsy cytosols with serum. If tumors with nonparallel slopes are excluded from the analysis, the correlation of p24 with receptor status is in fact higher.

Although a number of hormone-regulated proteins have now been described in human breast cancer (1), few of these proteins have been evaluated as markers for hormone sensitivity in breast tumor biopsies. Aside from PGR, clinical data are available only on PAA, an enzyme that is secreted by MCF-7 cells within 8 h of estrogen treatment independent of any mitogenic effect of the hormone (4, 5). Sutherland (25) has measured PAA in 119 human breast tumor cytosols and found a significant positive correlation between PAA and steroid hormone receptors, particularly PGR. Using median values for all 3 parameters as cut points, 80% of the tumors positive for both ER and PGR also had elevated PAA, whereas in ER- and PGR-negative specimens, only 37% were positive for PAA. (The comparable values for p24 using the cut points in Table 4 are 65 and 19%, respectively.) In a small patient population, Sutherland also found that elevated PAA was moderately correlated with response to hormone treatment (P = 0.0549). Thorsen (26) has likewise reported that PAA sharply discriminates PGR-positive from PGR-negative tumors, irrespective of ER content. Thus, PAA appears to identify the same tumor subgroup as does PGR. The p24 protein, on the other hand, is correlated more with ER than with PGR and may define a slightly different tumor population. This apparent association of PAA and p24 with different levels of breast tumor differentiation is also reflected in the expression of these proteins in normal breast, since PAA is present during involution of the normal lactating breast (22) while p24 is absent (6). The p24 protein could therefore be a valuable additional parameter in classifying differentiated human breast tumors.

ACKNOWLEDGMENTS

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