Relation of Estrogen Receptor Expression to Clonal Growth and Antiestrogen Effects on Human Breast Cancer Cells

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ABSTRACT

Expression of estrogen receptor (ER) was studied in the ER-positive human breast cancer cell line MCF-7 using immunoperoxidase staining with monoclonal antibodies to ER. Using a soft agar colony assay and liquid culture, effects of growth and the antiestrogen tamoxifen were examined. Heterogeneity in expression of ER was observed between different clones in the agar cultures as well as among cells within the same clone. Clonal expression of ER increased progressively with increasing cell number within a clone. At pharmacological doses, tamoxifen significantly reduced clonal growth but also markedly reduced the expression of ER within clones that grew despite the presence of the antiestrogen. These findings are consistent with the hypothesis that ER-positive colonies arise from ER-negative progenitors and that ER expression occurs along with differentiation of cells within clones. Furthermore, the findings are consistent with tamoxifen exerting its antineoplastic action beyond the level of the tumor stem cell. Such therapy would therefore be capable of suppression but not eradication of breast cancer clonal progenitors.

INTRODUCTION

Human breast cancer has long been known to be influenced by hormonal factors. Identification of the ER in selected normal and neoplastic tissues by Jensen and coworkers (1, 2) and subsequent studies by a number of investigators have provided rationale for understanding effects of hormonal therapy in breast cancer.

In clinical studies, ER has been detected in significant quantities in the primary tumors of at least 60% of women with breast cancer (3). Patients whose tumors are ER positive have been documented to have a longer relapse-free survival after surgery than those that are ER negative (4–6). Furthermore, additive or ablative endocrine therapy or use of antiestrogens appears to be of benefit to only 5% or less of patients with ER-negative tumors, whereas such therapy provides objective benefit in about 70% of women with ER-positive tumors, suggesting ER-mediated effects of hormonal therapy. Despite the beneficial effects of endocrine therapy in ER-positive patients, the effect appears to be primarily a palliative one, as tumor recurrence eventually occurs even in women whose cancers are strongly ER positive. Quite recently, MoAb have been raised against the ER and applied to its immunoquantitation with good correlation to biochemical methods (7). Such MoAb have also been applied immunohistochemically and have provided evidence for cellular heterogeneity in staining even in breast cancers known to be biochemically positive for ER (8).

Acquisition of drug or hormone resistance in cancers is often thought to be related to clonal heterogeneity in receptor expression and/or spontaneous mutation to drug resistance. In an attempt to better identify endocrine-sensitive malignant cells in human breast cancer, we have applied MoAb to ER and immunohistochemical techniques in an in vitro soft agar clonal assay system with the stable ER-positive human breast cancer cell line MCF-7 (9). Use of an assay for clonogenic cells was selected, as cells which exhibit clonogenicity in vitro are thought to be closely related to tumor stem cells in vivo (10, 11). Tumor stem cells are capable of numerous cycles of self-renewal and are responsible for recurrence after subcurative therapy in vivo (11). A major objective of our studies was to determine whether cellular expression of ER occurred on a clonal basis (with some colonies positive while others are negative) or as a function of the differentiation process, wherein cells within the same clone might be either negative or positive.

MATERIALS AND METHODS

The clonogenic assay system used was based on the 2-layer semisolid agar system (in 35-mm plastic Petri dishes) as described by Hamburger and Salmon (10). However, a special modification was required in order to optimize immunohistochemical staining for ER in the clonogenic assay for MCF-7 cells (12). For these studies, fetal calf serum was "stripped" of endogenous estrogenic steroids by adsorption with dextran-coated charcoal (FCS-S). E-McCoy was used as the main constituent of the culture medium and contained 94.5% (v/v) M-McCoy (Grand Island Biological Co.), 2.0% (v/v) 20 mg/ml glutamine (Grand Island Biological Co.), 1.0% (v/v) 100X concentrated nonessential amino acids (Flow Laboratories), 1.0% (v/v) 200 mg/ml sodium pyruvate (Grand Island Biological Co.), 0.5% (v/v) insulin (200 µg/ml; Sigma Chemical Co.), and 1.0% (v/v) penicillin/streptomycin (10,000 units/ml; Grand Island Biological Co.). FCS-S, an appropriate volume of 3.0% (w/v) Bacto agar, and the same volume of double-strength M-McCoy were added to the E-McCoy so that the underlayer contained 0.5% agar and 20% FCS-S, and the overlayer contained 0.3% (w/v) and 20% (v/v) FCS-S. The liquid medium added above the agar consisted of 80% E-McCoy and 20% FCS-S. A 1.0-ml aliquot of medium for the underlayer was transferred by pipet into a 35-mm plastic Petri dish (Nunc) and solidified. Prior to cell inoculation, special moist conditions were maintained to prevent the inoculated cells on the GFF from being damaged by drying. Under sterile conditions, a 7-cm-diameter conventional filter paper was placed in a 9-cm Petri dish, and a 5-mm-diameter circular cutout of thick GFF (Gelman Sciences) was placed on the filter paper. Liquid medium (1.2 ml) was transferred by pipet onto the filter paper. The medium permeated the thick filter pad,
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after which a 7-mm GFF was placed on the pad with the smooth side down. To support the agar for washing and staining, 7-mm-diameter circular cutouts of GFF (Whatman GF/A) were used as a rigid but clear down. To support the agar for washing and staining, 7-mm-diameter circular cutouts of GFF (Whatman GF/A) were used as a rigid but clear support for clonal growth in soft agar with minor modifications of the original description of Kodama et al. (12). As the filter discs can be readily plucked from the cultures, clusters and colonies can be obtained at any time point and washed free of culture medium. This permits optimal fixation and immunohistochemical staining for ER. MCF-7 cells (>95% viable) maintained in estrogen-free medium with estrogen-depleted serum for over 3 months were harvested at subconfluence as a single cell suspension with trypsin/EDTA solution and used for these experiments. Five thousand viable cells suspended in 20 μl of medium were inoculated onto each premoistened GFF with a micropipet, and the GFF were immediately transferred to the surface of the 0.5% agar underlayer for the cultures. One-half ml of medium containing molten 0.3% agar was gently transferred by pipet onto the surface of the underlayer (avoiding direct transfer onto the GFF). The overlayer of soft agar rapidly permeates the GFF prior to gelation. After gelling, 1.0 ml of liquid medium was poured onto the surface of the agar. Triplicate cultures were then incubated in a fully humidified 37°C incubator with a controlled low-oxygen atmosphere (6% O2/8% CO2/86% N2) and harvested on Days 0, 5, 7, 9, and 11. In additional experiments, Day 11 harvest was used to assess the effects of the antiestrogen tamoxifen, on clonal growth and ER expression. In order to establish a concentration of tamoxifen which would suppress clonal growth to 30 to 50% of control, it was initially tested at a wide range of concentrations. Subsequent to this titration, tamoxifen was used at the concentration of 1.5 μM (by continuous exposure in culture) in all quantitative comparisons. All experiments were repeated to assure reproducibility of results. After harvesting, each GFF containing clusters and colonies was covered with 30 μl of molten 0.5% agar medium to reduce the background. After gelling of the agar, the GFF were transferred into a buffered picric acid/paraformaldehyde fixative solution as described by Stefani et al. (13) for 2.5 min at room temperature, then transferred into a Buchner funnel containing prewetted filter paper, and washed with dropwise addition of 0.01 M PBS (pH 7.4) from a 10-ml pipet. Washing was continued until the filtrate was cleared of any tinge of yellow picric fixative coloring. GFF were covered with 10% (w/v) ovalbumin in PBS and incubated for 15 min. After an additional wash, the GFF were overlayed for 30 min with an equal mixture of 2 different rat MoAb to ER from MCF-7 cells (clones D547 and D75) (protein concentration, 50 μg/ml for each MoAb) (14). This combination of MoAb yields better histochemical staining with the PAP staining technique than is obtained with either MoAb alone. Control incubations were obtained by parallel incubation of additional GFF with the same concentration of normal rat IgG. Subsequent to an additional washing on the filter (40 drops of PBS per filter), GFF were incubated with a 1/500 dilution of the secondary antiserum (goat anti-rat IgG) for 30 min, washed again, incubated in McCoy’s Medium 5A with 10% serum for 30 min at 37°C (to reduce background staining), and again washed. GFF were then incubated for 30 min with a 1/80 dilution of PAP reagent (15), washed with PBS, stained with a mixed reagent comprised of 3,3’-diaminobenzidine (0.5 mg/ml; Sigma) and 0.06% H2O2, and again washed. Counterstaining was done subsequently with 0.1% methylene blue for 10 min, followed by washing and dehydration in an ethanoyleneo series which renders the filters transparent, after which they were mounted permanently on glass slides. At defined points in agar culture, permanent mounts of MoAb-stained slides were prepared. Each cell in clusters and colonies was counted by light microscopy (×400) and distinguished as to the positive reaction color (brown) of the PAP stain. The size of clonal growth units was classified as follows: small cluster (4 to 20 cells); large cluster (21 to 39 cells); and colony (40 or more cells) (Fig. 1). The plating efficiency of MCF-7 cells in the GFF system approximated 10% for small clusters, 3% for large clusters, and 3% for colonies. Tritiated thymidine autoradiography and monoclonal antibody staining were carried out on MCF-7 cells in a few preliminary experiments. In these experiments, MCF-7 cells from liquid-culture flasks maintained in E-McCoy/FCS-S were adhered to coverslips in Lab Tek tissue culture chambers/slides (No. 4838; Miles Laboratories, Naperville, IL) and exposed to high-specific-activity tritiated thymidine (10 μCi, 40 Ci/mM) for 1 h, followed by washing and monoclonal antibody staining for ER. The slides were then processed autoradiographically using the high-speed scintillation technique (16).

RESULTS

Intracellular localization of ER was restricted to the nuclei in almost all of the cells. Such localization would not have been predicted from the usual "cytosol" assays (17), but it is consistent with the results of biochemical assay of ER in MCF-7 cells and in enucleation studies with rat GH3 cells (18, 19) and also with recent findings with immunohistochemical staining with MoAb to ER by King and Greene (8). In order to classify the extent of ER staining within clones, the following grades of positivity were defined: 0, no staining; 1, 1 to 50%; and II, >50% of cells staining (Fig. 1). In most instances, more than 100 clones in each of the 3 clonal size classes were differentially counted per GFF as to the ER positivity grade (Table 1). Two hundred cells in the single cell preparation on Day 0 and 1000 cells in the clonal units present on Days 5 to 11 were also differentially counted as to ER positivity (Table 2). Many clusters and colonies generated contained both ER-positive and -negative cells within them, and only a minority of clones contained either all positive or negative cells. The percentage of ER-positive cells in individual clones was quite variable, particularly within the smaller clonal growth units. Among clusters, there were always some which were ER negative (Grade 0), as well as many which were ER positive (Grades I and II). However, as the clones grew larger, the ER positivity grade rose and converged at Grade II, with none of the growth units of colony size being ER negative (Table 1; Chart 1).

In comparing the 3 size classes of clones for any given harvest day, we consistently recognized that, the larger the clonal size class was, the higher the average proportion of ER-positive cells (Table 2). The difference in the average proportion of ER-positive cells between the small clusters and colonies on the same days of culture was statistically significant (P < 0.05). These data indicate that ER expression increases as the breast cancer

### Table 1

<table>
<thead>
<tr>
<th>Clonal growth stage</th>
<th>ER positivity grade</th>
<th>% of ER positivity grade distribution of the following days of culture</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>5</td>
</tr>
<tr>
<td>Small cluster</td>
<td></td>
<td>10.0 ± 2.0</td>
</tr>
<tr>
<td>Large cluster</td>
<td></td>
<td>24.3 ± 2.3</td>
</tr>
<tr>
<td>Colony</td>
<td></td>
<td>65.7 ± 4.2</td>
</tr>
</tbody>
</table>

*Mean ± SE.*

b^{±}, minimal growth.
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Table 2

Proportion of ER-positive cells in single cells and clones

<table>
<thead>
<tr>
<th>Cell growth stage</th>
<th>% of ER-positive cells in single cells and clones at the following days of culture</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Single cell</td>
<td>54.8 ± 4.8&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Small cluster</td>
<td>66.1 ± 0.4&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Large cluster</td>
<td>±</td>
</tr>
<tr>
<td>Colony</td>
<td>±</td>
</tr>
</tbody>
</table>
<sup>a</sup> Mean ± SE.
<sup>b</sup> NC, not counted.
<sup>c</sup> ±, no clonal growth; ±, minimal clonal growth.

Table 3

Effect of tamoxifen on ER expression in breast cancer colonies

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of colonies/ GFF</th>
<th>% of ER-positive cells in colonies</th>
<th>% Distribution of ER positivity grades&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>125.3 ± 24.0&lt;sup&gt;b&lt;/sup&gt;</td>
<td>53.9 ± 3.5&lt;sup&gt;c&lt;/sup&gt;</td>
<td>P &lt; 0.05&lt;sup&gt;c&lt;/sup&gt;  0  25.0 ± 1.7  75.0 ± 1.7  11.8 ± 10.8</td>
</tr>
<tr>
<td>Tamoxifen&lt;sup&gt;d&lt;/sup&gt;</td>
<td>40.7 ± 9.8</td>
<td>29.7 ± 6.8</td>
<td>2.9 ± 2.9  85.3 ± 10.2  11.8 ± 10.8</td>
</tr>
</tbody>
</table>
<sup>a</sup> The ER positivity grade of colonies was defined according to the proportion of ER-positive cells in a colony (see text).
<sup>b</sup> Mean ± SE.
<sup>c</sup> The Student t test was used to determine the difference between the control and tamoxifen-treated groups as to the mean percentage of ER-positive cells in clones.
<sup>d</sup> Tamoxifen was added at a final concentration of 1.5 × 10<sup>−6</sup> M and present in continuous exposure throughout the culture period.

Chart 1. ER positivity of breast cancer clones on Day 9 of agar culture. Clonal growth units [small clusters (■), large clusters (△), and colonies (○)] were differentially counted as to their average percentage of ER positivity. Colonies expressed the highest percentage of positivity.

Clones grow larger. The percentage of ER-positive cells in clones was the highest on Day 7, and with increasing culture duration, ER staining declined.

In preliminary qualitative studies, we examined the relationship of ER positivity of MCF-7 cells to cell proliferation by studying the occurrence of scheduled DNA synthesis. In these experiments, we found that most of the breast cancer cells remained positively for ER. In contrast, most cells in DNA synthesis (as determined by significant nuclear [3H]thymidine labeling) were ER negative. However, a lesser proportion of cells exhibited unequivocal ER positivity as well as significant thymidine labeling.

Finally, using the immunohistochemical system as described, we studied the effects of tamoxifen on MCF-7 colony growth and ER staining. The results of these studies are summarized in Table 3. In the in vitro studies with tamoxifen, we found that tamoxifen simultaneously suppressed both clonal growth and the expression of ER in surviving clones. In fact, some entirely ER-negative colonies developed, a phenomenon which was never found in the untreated controls (Table 3). Thus, the antiestrogen induced an increased proportion of ER-negative cells in clones resistant to the antiproliferative effects of 1.5 μM tamoxifen.

DISCUSSION

These studies combining clonal growth studies with immunohistochemical staining for the ER help clarify the relationship between clonal growth, ER expression, and the effects of antiestrogens in breast cancer. In the unperturbed state, ER-negative colonies are not observed, but rather there is a gradual decrease in ER positivity until clonal growth units reach colony size. The findings demonstrate that there is heterogeneity in expression of ER between different clones as well as within the same clones of MCF-7 cells, and that the degree of heterogeneity is greater with small clones than large ones (Tables 1 and 2). These findings are consistent with the hypothesis that ER-positive colonies can arise from ER-negative progenitors in ER-positive human breast cancer. After Day 7, ER positivity decreased in mature colonies, suggesting that there was an increase in ER degradation or an increasing dead cell population within clones with prolonged cultivation and decline of nutrients within the medium. Protein degradation on deprivation of nutrients in cultured mammalian cells has been reported (20). Our observations suggest that, in ER-positive breast cancer, ER expression does not occur as a clonal phenomenon (with some colonies negative and others positive), but rather that ER expression depends upon cell differentiation associated with cell proliferation and is seen in all developing colonies. Analogous obser-
vations have been made in hematological cancers, wherein differen-
tiation markers are expressed with clonal expansion (21, 22). Despite an imperfect association between ER status and histological grade of differentiation of breast cancer, it is widely accepted that ER expression is associated with increasing morphological differentiation (23–26). In a recent cytokine study of MCF-7 cells in monolayer culture, ER expression (as assayed radiometrically) was reported to be inversely related to the cellular proliferation rate (27). These observations could also be interpreted as indicating that ER expression occurred with differentiation and a declining proliferative rate of breast cancer cells. Our data are thus complementary and suggest that ER is a possible differentiation marker for clonal growth in breast cancer. Our experimental results are also compatible with the concept of a proliferation-differentiation hierarchy for human breast cancer, wherein expression of cell differentiation is inversely related to the cellular proliferation potential (28).

It is well known that tamoxifen inhibits growth of ER-positive breast cancer both experimentally and in the clinic (29–34).

Our findings (Table 3) suggest that antiestrogen therapy for breast cancer acts beyond the level of the tumor stem cell and might, therefore, suppress but not eradicate human breast cancer clones. Current clinical trials, using tamoxifen as an adjuvant agent for a defined time period (e.g., 2 years). Our findings, as well as the in vivo murine studies of Jordan et al. (35), suggest that the antiestrogen should be administered for as long as evidence of recurrence is suppressed. Further in vitro cloning studies on fresh tumor biopsies from the time of initial surgery and on recurrence after adjuvant antiestrogen therapy would be of interest.

ACKNOWLEDGMENTS

We thank Dr. C. M. McGrath of the Michigan Cancer Foundation for providing the MCF-7 cells, Dr. W. J. King for helpful suggestions, and Barbara Soehnlen for technical assistance.

REFERENCES

Fig. 1. Immunoperoxidase staining for ER of breast cancer clones in agar culture using MoAb. Glass fibers in the supporting filter can be seen in the background. A, small cluster in a control culture (no staining present); B, small cluster exhibiting Grade I ER positivity. Dark areas in the nuclei of cells (arrow) represent positive staining; C, colony with Grade II ER positivity. The thickness of the GFF prevents some cells from being in focus. Cellular localization of ER is restricted to the nuclei.
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