Correlation of Lactogenic Receptor Concentration in Human Breast Cancer with Estrogen Receptor Concentration

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ABSTRACT

The presence of receptors for lactogenic hormones in human breast cancer tissue has been documented previously, but the relationship between the expression of these receptors and estrogen receptor (ER) status has not been adequately studied. In this report, the specificity of $^{125}$I-human growth hormone (HGH) binding in both cultured human breast cancer cell lines and tumor biopsies was studied to establish that HGH was a suitable ligand for investigating lactogenic receptor concentration in these tissues. In addition, the relationship between specific binding of $^{125}$I-HGH and ER concentration in human breast cancer was investigated. Specific $^{125}$I-HGH binding to 14 breast cancer cell lines in long term culture and to membrane preparations (microsomal and plasma membrane fractions) from 31 breast cancer biopsy specimens was examined.

Human prolactin and HGH were approximately equipotent in inhibiting binding of $^{125}$I-HGH to both cultured breast cancer cell lines and to membrane preparations from breast cancer biopsy specimens. Competitive inhibition experiments using lactogenic and somatogenic hormones established that the specificity of $^{125}$I-HGH binding to breast cancer biopsy material was similar to that of cultured breast cancer cell lines and similar to that reported for subprimate lactogenic receptors.

Saturable, high-affinity ($K_a = 0.53$ to $2.33 \, \text{nm}^{-1}$), low-capacity ($330$ to $6560 \, \text{sites/cell}$) growth hormone binding sites were found on each of the ER-positive cell lines, whereas no specific $^{125}$I-HGH binding to ER-negative cell monolayers was detected. When all cell lines were considered, a significant linear correlation ($r = 0.745, p < 0.001$) between ER and lactogenic receptor concentrations was found. Significant specific $^{125}$I-HGH binding, greater than 1% of the total radioactivity added, was detected in 20 of 31 (65%) breast tumor biopsy specimens. The mean affinity and capacity of the lactogenic receptor as measured in 8 separate membrane preparations were $K_a = 0.52 \pm 0.09 \, \text{S.E.} \, \text{nm}^{-1}$ and $255 \pm 85 \, \text{fmol/mg protein}$. Membrane preparations from ER-negative tumors (<3 fmol ER/mg cytosol protein) bound significantly less $^{125}$I-HGH than did membrane preparations from ER-positive tumor biopsies ($1.22 \pm 0.44 \, \text{versus} \, 3.21 \pm 0.56 \, \text{fmol/mg}$, $p < 0.05$). A significant linear correlation between specifically bound $^{125}$I-HGH and ER concentration ($r = 0.412, p < 0.02$) was demonstrated in the 31 breast cancer biopsy specimens studied.

This report provides the first clear demonstration of a correlation between ER and lactogenic receptor in cultured breast cancer cell lines and suggests that a similar relationship exists in breast cancer biopsy specimens.

INTRODUCTION

In contrast to the majority of experimental rodent mammary tumors, human breast cancer is only partially hormone responsive (31). The predictive value of the ER$^3$ status of human breast cancer biopsy material in determining those patients likely to respond to endocrine therapies has been clearly established in many clinical trials (9, 11) and has encouraged the search for other hormone receptors in human breast tissue. Progesterone (13), insulin (7), and lactogenic receptors (1, 2, 4, 7, 14, 19, 20, 22, 27, 30) have been demonstrated in human breast cancer biopsies. While the progesterone receptor concentration has been shown to correlate with ER status (13), the relationship of insulin and lactogenic receptors in breast cancer biopsies to ER status has not been established.

Iodinated preparations of OPRL, HPRL, and HGH have been used to measure lactogenic receptors (2, 20, 30) in breast cancer biopsies. However, the specificity of the lactogenic receptor in this tissue has not been adequately documented and as a consequence the most appropriate iodinated ligand for assaying this receptor remains undefined. In this report we have investigated the specificity of $^{125}$I-HGH binding sites in cultured human breast cancer cell lines and in membrane preparations from breast cancer biopsies and have established that HGH is a suitable ligand for determining lactogenic receptor concentration in these tissues. In addition, we have demonstrated a significant correlation between ER and lactogenic receptor concentrations in both cultured breast cancer cells and breast cancer biopsy material.

MATERIALS AND METHODS

HGH was a generous gift from the Human Pituitary Advisory Committee (Canberra, Australia). A highly purified preparation (2 IU/mg) was iodinated by the method of Greenwood et al. (6) as described in Ref. 16. A less pure HGH preparation (0.9 IU/mg) was used for displacement studies. The source and purity of other hormones used were as stated previously (16, 17).

Cell Cultures. A total of 14 cell lines was studied. In addition, $^{125}$I-HGH binding to MCF-7 cells from 3 different sources and T-47D cells from 2 sources were examined. The source of each of these cell lines is listed in Table 1. Mycoplasma-free cells were routinely grown as monolayer cultures in RPMI 1640 medium supplemented with 20 mm 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 14 mm sodium bicarbonate, 6 mm L-glutamine, gentamicin (20 \mu g/ml), porcine insulin (10 \mu g/ml), and 10% (v/v) fetal bovine calf serum (Flow Laboratories, Sydney, Australia) as described previously (29).

Estrogen Receptor Analyses. Confluent cell monolayers were harvested with 1 \text{mM} EDTA in Dulbecco's phosphate-buffered saline (1.5

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1Recipient of a Garvan Research Foundation Fellowship. To whom requests for reprints should be addressed.

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3The abbreviations used are: ER, estrogen receptor; HGH, human growth hormone; HPRL, human prolactin; OPRL, ovine prolactin; GH, growth hormone; TES, (10 \text{mM} \text{Tris-HCl,} 1.5 \text{mM} \text{EDTA,} 0.25 \text{mM} \text{su} \text{crose buffer, pH} 7.4).
mm KH₂PO₄-8.1 mm NaH₂PO₄-2.7 mm KCl-140 mm NaCl) and washed with TES. This and all further steps were carried out at 4°C. The cell pellet was then homogenized in TES (3 × 10⁷ cells/ml) using a Teflon-glass homogenizer, and a crude nuclear pellet was prepared by centrifugation (800 × g for 10 min). The resulting supernatant was centrifuged further at 135,000 × g for 1 hr to give the soluble fraction containing cytosol ER. The crude pellet was washed once with 1% (w/v) Triton X-100 in TES and 3 times with TES. Nuclear ER was extracted from the washed crude nuclear pellet with 0.5 μl NaCl in TES (equivalent of 3 × 10⁷ cells/ml) as described previously (28). In some experiments, a whole cell extract was prepared by homogenizing cells (3 × 10⁷ cells/ml) in 10 mm Tris-HCl-1.5 mm EDTA buffer, pH 7.4, containing 1 μl NaCl and centrifuging the homogenate at 135,000 × g for 1 hr. This extract contained the total extractable ER, i.e., cytoplasmic ER plus salt-extractable nuclear ER. Both methods of preparation yielded similar results for estimates of total extractable ER concentration, and thus the data were pooled.

Endogenous estrogens were removed from the extracts by a 30-min incubation with 5% charcoal-0.5% dextran solution (100 μl/ml). ER concentration was measured in charcoal-dextran-treated cell extracts by saturation analysis as described previously (23), and data were analyzed by the method of Scatchard (24) following correction for nonspecific binding.

Breast cancer biopsies were obtained from 31 female patients and stored in liquid nitrogen. The frozen tissue specimens were pulverized using a Braun Mikro-Dismembrator II, added to 5 volumes of ice-cold buffer (20 mm Tris-3.0 mm disodium EDTA, 10 mm KH₂PO₄, pH 7.4), and centrifuged for 1 hr at 100,000 × g. ER concentration was measured in this supernatant as described above, and data were expressed as fmol/mg protein following measurement of the protein concentration by the method of Lowry et al. (10) using bovine serum albumin as a standard. Cytosol preparations with an ER concentration of less than 3 fmol/mg protein were considered negative (13).

It is important to note that these techniques measure predominantly unoccupied ER and little exchange of ER occupied with estrogens would be expected to occur under these assay conditions. Binding of 125I-HGH to cell monolayers was measured by incubating 0.1 ng (30,000 cpm) of 125I-HGH with 1 to 5 × 10⁶ cells in the presence or absence of increasing concentrations of unlabeled HGH for 4 hr at 30°C, as described for GH receptors in mouse and human fibroblasts (16-18). Receptor affinity and concentration were calculated from Scatchard transformations (24) following correction for nonspecific binding. Each cell line was assayed on at least 2 separate occasions, and data have been expressed as the mean of 2 or more determinations ± S.E. where appropriate. In assays where different somatogenic and lactogenic hormones were compared, parallel incubations with and without excess unlabeled HGH were performed. Specific binding was calculated as the difference between binding in the presence or absence of unlabeled HGH (10⁶ ng/ml). Since different batches of 125I-HGH varied in specific activity and binding ability, data have been expressed as a fraction of the maximal specific binding (B/Bₒ), where B is the number of each batch of 125I-HGH bound in the presence of excess receptor, i.e., T-47D cell monolayers.

For studies on the binding of 125I-HGH to membranes from human cancer biopsies, a crude membrane fraction was prepared as follows. The 100,000 × g-for 1-hr pellet obtained from preparation of the cytosol for ER assays was homogenized using a Teflon-glass homogenizer in ice-cold 0.25% sucrose and centrifuged at 10,000 × g for 30 min at 4°C. The resultant supernatant was centrifuged at 40,000 × g for 40 min at 4°C, and the pellet was resuspended in 0.05 μl Tris-HCl buffer, pH 7.4, and washed by recentrifugation. This membrane preparation contained both microsomal and plasma membrane fractions. Binding of 125I-HGH was determined by incubating 75 to 100 μl of membrane protein with 0.1 ng of 125I-HGH (approximately 30,000 cpm) in 400 μl of binding buffer (12.5 mm Tris-HCl-10 mm CaCl₂-0.5% bovine serum albumin, pH 7.4) for 20 hr at room temperature (18-22°C). The incubation was terminated by the addition of 2 ml of ice-cold binding buffer followed by centrifugation at 1500 × g for 1 hr. The supernatant was decanted, and the pellet was counted in an NE 1600 gamma counter. All assays were performed in duplicate. As insufficient membrane preparation was available from individual tumor biopsies for full displacement curves in many cases, 4 membrane pools from 30 to 40 breast cancer biopsies were prepared for the study of specificity of 125I-HGH binding to breast cancer membranes. Specific binding of 125I-HGH to membranes was expressed as the difference in the percentage of added 125I-HGH bound in the absence and presence of an excess of HGH (10⁴ ng/ml) per 100 μg of membrane protein.

RESULTS

Breast Cancer Cell Lines. Binding of 125I-HGH to 13 breast cancer cell lines and one cell line derived from human colostrum (HBL-100) was examined. The specificity of 125I-HGH binding to the T-47D cell line is shown in Chart 1. HPRL was approximately equipotent with HGH in inhibiting 125I-HGH binding to its saturable binding sites in T-47D cells while human placental lactogen and rat prolactin were 3.5 and 1.5%, respectively, as potent as HGH. Bovine and rabbit GH did not inhibit 125I-HGH binding to these high-affinity sites. The specificity of 125I-HGH binding to MCF-7 and BT-474 cells was also studied, and in these cell lines, HGH and HPRL were again approximately equipotent in inhibiting the saturable binding of 125I-HGH. Thus, the specificity of 125I-HGH binding to breast cancer cells in this study is similar to that reported by Shiu (25) who used 125I-HGH as the labeled ligand and demonstrated that HPRL and HGH were equipotent in inhibiting binding of the iodinated ligand to T-47D cells. Such data are also compatible with our previous observation that HGH and HPRL are mutually competitive for binding to the lactogenic receptor in cultured breast cancer cell lines (15). The affinity and concentration of the lactogenic receptor in each of the cell lines studied are shown in Table 1. High-affinity (Kₛ = 0.53 to 2.33 nm⁻¹), low-capacity (330 to 6560 sites/cell) lactogenic receptors were expected to occur under these assay conditions.
Table 1

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Source</th>
<th>Lactogenic receptor concentration (sites/cell)</th>
<th>ER concentration (sites/cell)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCF-7</td>
<td>Dr. C. McGrath, Michigan Cancer Foundation</td>
<td>0.94 ± 0.15a 4140 ± 2300</td>
<td>9114 ± 1693b</td>
</tr>
<tr>
<td>MCF-7LC</td>
<td>Dr. M. Lippman, NCI</td>
<td>0.95 4135</td>
<td>6828 ± 2541</td>
</tr>
<tr>
<td>R27</td>
<td>Dr. M. Lippman, NCI</td>
<td>0.53 3810</td>
<td>2219 ± 494</td>
</tr>
<tr>
<td>R98</td>
<td>Dr. M. Lippman, NCI</td>
<td>1.44 2340</td>
<td>5229 ± 1411</td>
</tr>
<tr>
<td>T-47D</td>
<td>Dr. R. Whiteshead, Ludwig Institute,</td>
<td>1.68 2212</td>
<td>3803 ± 548</td>
</tr>
<tr>
<td></td>
<td>Melbourne</td>
<td></td>
<td></td>
</tr>
<tr>
<td>T-47Dw</td>
<td>E. G. and G. Mason Research Institute</td>
<td>1.70 ± 0.38 6560 ± 1930</td>
<td>4345 ± 1089</td>
</tr>
<tr>
<td>MCF-7L</td>
<td>E. G. and G. Mason Research Institute</td>
<td>1.16 2120</td>
<td>4585</td>
</tr>
<tr>
<td>ZR 75-1</td>
<td>E. G. and G. Mason Research Institute</td>
<td>0.72 4135</td>
<td>2933 ± 897</td>
</tr>
<tr>
<td>BT-474</td>
<td>E. G. and G. Mason Research Institute</td>
<td>0.70 330</td>
<td>1885 ± 698</td>
</tr>
<tr>
<td>MDA-MB-361</td>
<td>E. G. and G. Mason Research Institute</td>
<td>2.33 435</td>
<td>2505 ± 958</td>
</tr>
<tr>
<td>MDA-MB-134</td>
<td>E. G. and G. Mason Research Institute</td>
<td>?a</td>
<td>2659</td>
</tr>
<tr>
<td>MDA-MB-157</td>
<td>E. G. and G. Mason Research Institute</td>
<td>Not detected</td>
<td>Not detected</td>
</tr>
<tr>
<td>MDA-MB-330</td>
<td>E. G. and G. Mason Research Institute</td>
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<td>Not detected</td>
</tr>
<tr>
<td>MDA-MB-231</td>
<td>E. G. and G. Mason Research Institute</td>
<td>Not detected</td>
<td>Not detected</td>
</tr>
<tr>
<td>BT-20</td>
<td>E. G. and G. Mason Research Institute</td>
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<td>Not detected</td>
</tr>
<tr>
<td>Hs0578T</td>
<td>E. G. and G. Mason Research Institute</td>
<td>Not detected</td>
<td>Not detected</td>
</tr>
<tr>
<td>HBL-100</td>
<td>E. G. and G. Mason Research Institute</td>
<td>Not detected</td>
<td>Not detected</td>
</tr>
</tbody>
</table>

a Mean ± S.E.; n = 9 for MCF-7 cells and n = 8 for T-47D cells, or the mean of duplicates for the other cell lines.

b Mean ± S.E., n = 3 to 8 and are taken from a separate study to be published elsewhere (Footnote 4).

Subscripts have been used to denote different sources of the same cell line.

NCI, National Cancer Institute.

Specific binding detected but too low to determine receptor affinity and concentration.


d Specific binding detected but too low to determine receptor affinity and concentration.

were found in all ER-positive cell lines while no specific 125I-HGH binding was detected in the 6 cell lines where no saturable [3H] estradiol binding to cytosols and nuclear extracts was measured. These latter cell lines have been described as ER-negative cell lines in Chart 2. In addition, no specific 125I-HPRL binding to either the BT-20, Hs0578T, or HBL-100 cell monolayers was detectable. When considered as a group, there was a significant correlation (r = 0.745, p < 0.001) between ER and lactogenic receptor concentrations in the breast cell lines (Chart 2).

The ER concentration data presented in Table 1 and used to establish this correlation were from a separate study in which the relationship between ER concentration and sensitivity to the growth inhibitory effects of tamoxifen was investigated in human cancer cell lines. Detailed data on the sources of these cell lines, their growth behavior under our assay conditions, the validity and limitations of the ER assays, and the relationship of measured ER values to those reported previously for these cell lines will be presented in a separate manuscript{\textsuperscript{4}} and will not be discussed further here.

Breast Cancer Biopsies. The specificity of binding of 125I-HGH to the high-affinity binding site in pooled breast cancer membranes is shown in Chart 3. HGH, HPRL, and OPRL readily inhibited binding of 125I-HGH to saturable sites in this tissue. PRL and OPRL were marginally less potent, whereas human placental...
lactogen was significantly less potent than was HGH in inhibiting 125I-HGH binding to breast cancer membranes. Bovine GH did not compete with HGH for binding to this receptor. A similar specificity was observed when 125I-HPRL was used as the labeled ligand (Chart 4) indicating that HPRL and HGH are mutually competitive ligands for the lactogenic receptor in human breast cancer tissue.

The affinity and concentration of lactogenic receptors in breast cancer tissue were derived from Scatchard plots. Representative Scatchard plots for membrane preparations from individual breast cancer biopsies and for a pooled membrane preparation are shown in Chart 5. The mean receptor affinity and concentration as determined from 8 separate membrane preparations (2 pools and 6 individual biopsies) were $K = 0.52 \pm 0.09$ (S.E.) nm$^{-1}$ and 255 \pm 85 fmol/mg protein, respectively.

Under the assay conditions described in "Materials and Methods" for estimation of lactogenic receptors in breast membranes, the percentage of 125I-HGH specifically bound was proportional to the amount of membrane protein added to the incubation over the range 0 to 100 \mu g/tube ($r^2 = 0.964, p < 0.001$). In most assays, 100 \mu g of membrane protein was used per incubation, but because of the small size of some biopsy specimens smaller quantities of membrane protein had to be assayed. In these situations, specific binding of 125I-HGH was corrected for the reduced membrane protein concentration and expressed as a fraction of the maximal specific binding, which varied from 1.6 to 7.5% of the total radioactivity added.

Membrane preparations from ER-negative tumors (<3 fmol ER/mg cytosol protein) bound significantly less 125I-HGH than did membrane preparations from ER-positive tumor biopsies (1.22 \pm 0.44 versus 3.21 \pm 0.56%, $p < 0.05$); and membrane preparations from breast cancer biopsies with moderately high (101 to 150 fmol/mg protein) or very high (>150 fmol/mg protein) ER concentrations demonstrated significantly more specific binding of 125I-HGH than did ER-negative tumors or tumors with low ER concentrations. A significant correlation between ER concent-
turation and specific ¹²⁵I-HGH bound was also found with this tumor biopsy material (Chart 6; \( r = 0.412, p < 0.02 \)).

**DISCUSSION**

We have demonstrated a significant correlation between ER concentration and specific ¹²⁵I-HGH binding in both cultured breast cell lines and breast cancer biopsies. Since HGH and HPRL bind to the lactogenic receptor in breast cancer cells with similar affinity (15, 18, 25), ¹²⁵I-HGH is an acceptable ligand for determining lactogenic receptor concentration and affinity. In addition to binding to the lactogenic receptor in breast cancer tissue, HGH has been shown to exert significant effects on this tissue at physiological concentration (11, 15). The data reported here for receptor affinity and concentration are in general agreement with those reported by Shiu (25) who used ¹²³I-HPRL as the ligand. In addition, he also demonstrated low but detectable levels of specific ¹²³I-HPRL binding to the ER-negative cell lines MDA-MB-231, Hs0578T, and HBL-100 (25). Using either ¹²³I-HGH or ¹²³I-HPRL, we have been unable to confirm saturable binding in these cell lines. If lactogenic receptors are present in the HBL-100 cell line, it appears that they are inactive, since HPRL is not degraded by these cells (26); and in contrast to the MCF 7 cell line, prolactin does not stimulate protein synthesis in these cells (3). The lactogenic receptor concentration reported by Shiu (25) in the ER-negative cells was approximately one-half the receptor concentration in the BT-474 cell line, which has low ER levels and approximately 5% of the lactogenic receptor concentration of T-47D cells (Table 1). If low concentrations of lactogenic receptor are present in ER-negative cell lines, this would not negate the significant correlation between ER levels and lactogenic receptor concentration demonstrated in this report. The ER status of each of the cell lines is in general agreement with other published data (5); however, the ER status of the T-47D and MDA-MB-231 cell lines differs from that reported by Horwitz et al. (8).

Since most of these cell lines are thought not to be clonally derived, it is not surprising that cells which have been maintained for prolonged periods in different laboratories have different ER and lactogenic receptor concentrations.

The specificity of the lactogenic receptor in breast cancer is controversial. While Holdaway and Friesen (7) reported that HGH is equipotent with HPRL in displacing ¹²⁵I-HPRL, Morgan et al. (14) found that HGH did not compete for binding of OPRL. In addition, the percentage of tumor biopsies with significant specific binding of iodinated HGH, HPRL, or OPRL varied considerably even within the one laboratory (1, 2, 4, 14, 19, 20, 22, 27, 30). In this report, we have demonstrated that specificity of ¹²⁵I-HGH binding to membranes from breast cancer biopsies and breast cancer cell lines is similar. HGH and HPRL are mutually competitive at this receptor. In this study, OPRL was less potent than was either HGH or HPRL in inhibiting binding of either ¹²⁵I-HGH or ¹²⁵I-HPRL to breast cancer membranes. In contrast, HGH and HPRL have been found to be less potent than is OPRL in inhibiting binding of ¹²⁵I-OPRL to breast cancer membranes (20). Significant specific binding of ¹²⁵I-HGH (greater than 1% of the added radioactivity) was found in 20 of 31 (65%) tumors. This is consistent with data which have been reported by Bonneterre et al. (2) and Stagner et al. (27) where 72 and 70% of tumors, respectively, demonstrated significant specific ¹²⁵I-HGH binding. In contrast, Turcot-Lemay and Kelly (30) reported that only 12% of tumor biopsies demonstrated significant binding of ¹²⁵I-HGH while 58 and 30% of tumors bound significant amounts of ¹²⁵I-HPRL and ¹²⁵I-OPRL, respectively. Since HGH, HPRL, and OPRL all bind to the lactogenic receptor in human breast cancer tissue with similar affinities, the observations of Turcot-Lemay and Kelly are difficult to explain. The differences in the percentage of tumor biopsies considered lactogenic receptor positive in the published reports may relate to the different assay conditions used, different methods of membrane preparation, the use of MgCl₂ to desaturate occupied receptor, and the quality of the radiolabeled hormone used for the binding studies. In addition, considerable cell type heterogeneity has been recognized in breast cancer biopsies (22).

A correlation between ER and lactogenic receptor concentration in breast cancer biopsies has been reported previously in abstract form by Stagner et al. (27) and more recently by Bonneterre et al. (2). In both studies, ¹²⁵I-HGH was used as the labeled ligand. In a series of 55 human breast cancer biopsy specimens reported by Rae-Ventor et al. (22), 58% demonstrated specific binding of iodinated prolactin. Furthermore, they found that tumors with ER concentrations of 6 to 100 fmol/mg of protein had a significantly higher mean concentration of lactogenic receptor than did ER-negative tumors. The failure of these investigators to demonstrate a correlation between ER and lactogenic receptor concentration was probably due to the low prolactin binding observed in tumors with very high ER concentrations (>250 fmol/mg of protein). In other studies in which ¹²⁵I-OPRL or ¹²³I-HPRL have been used, no significant correlation between ER and lactogenic receptor concentration was found (7, 20, 31).

The significant correlation between ER and lactogenic receptors in both cultured breast cancer cell lines and breast cancer biopsy tissue suggests that expression of these 2 receptors is coupled in mammary tissue. Although the most likely explanation for this observation is that ER-positive tumors arise from hor-
monally sensitive cell types which also possess lactogenic receptors, the possibility that estrogens have a direct or indirect effect on lactogenic receptor expression in human breast cancer tissue, as has been reported in rodent hepatic tissue (21), cannot be excluded. The therapeutic benefits of endocrine therapies in patients with ER-positive tumors have been assumed to result from their estrogen-lowering effects and inhibition of ER-mediated events; however, ovariectomy, hypophysectomy, antiestrogen, and progestin therapy may have other hormonal effects which lower the concentration of hormones acting through the lactogenic receptor. Further studies are needed to evaluate the usefulness of lactogenic receptor determinations in the management of human breast cancer and the role of lactogenic hormones in the control of proliferation and function of human breast cancer cells.

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