Decrease in Estradiol-stimulated Progesterone Receptor Production in MCF-7 Cells by Epidermal Growth Factor and Possible Clinical Implication for Paracrine-regulated Breast Cancer Growth

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ABSTRACT

These studies have evaluated the modulation by epidermal growth factor (EGF) of estrogen receptor (ER) levels and estradiol-stimulated progesterone receptor (PgR) synthesis. Short-term culture of MCF-7 cells in an "estrogen (phenol red indicator)-free" environment caused a rise in ER concentration that is inhibited by EGF at 10−8 M and 10−7 M. Estradiol at 10−10 M induced a 5-fold increase of PgR over a 5-day assay period. However, the rise in PgR was diminished or prevented by increasing concentrations of EGF (10−8 M to 10−5 M). Similarly, the concentration-related rise in PgR caused by estradiol (10−10 M to 10−6 M) was abolished after a 7-day pretreatment with EGF (10−7 M). For both the ER and PgR receptor, EGF treatment caused a decrease in receptor number without an apparent change in receptor affinity. Thus, EGF appears to down regulate the ER by approximately 50% and to diminish the ability of estradiol to induce PgR. In addition, a survey of ER+PgR+ and ER+PgR− values of primary breast tumors from women between the ages of 55 and 70 demonstrated significantly less (50%) (85 to 39 fmol/mg of cytosol protein) ER in ER+ PgR− tumors (P = 0.0005). The median PgR values for the PgR-positive tumors were 139 fmol/mg of cytosol protein. We propose that ER+ breast cancer that has changed to a paracrine growth factor-driven system (from stromal cells or ER− breast cancer cells) is less responsive to gonadal steroids. The loss of PgR in these ER+ carcinomas may be an indicator of this type of hormone independence.

INTRODUCTION

The heterogeneous nature of breast cancer has provided a challenging dilemma for the treatment of the disease. Much effort has been placed in designing protocols with different treatment strategies which address the diversity of the cancer. For this reason the identification of physical, genetic, or protein markers has been eagerly sought to predict the response to a particular treatment (1–8).

ER+ and PgR levels in breast tumor samples are highly predictive of a patient’s response to endocrine therapy. ER+ PgR− patients respond well to treatment, with an objective response of approximately 80% (9). Women who are ER+ but PgR− have only a 30% chance for objective response (9, 10). Therefore, the estradiol-stimulated production of PgR is important for clinical diagnosis of hormone dependency. A lack of either ER or PgR is associated with a poor prognosis and usually indicates a decrease in a disease-free interval and a decreased survival (10).

There has not been a systematic investigation to address the relationship of steroid receptors and growth factors in breast cancer. Some in vitro studies imply that steroid hormone receptor levels may be modulated by EGF (11–15). Because of the clinical importance of these receptors, this relationship bears investigation.

Estradiol treatment enhances the secretion of EGF-like peptides and insulin-like growth factor I from MCF-7 breast cancer cells (16). These two factors are mitogenic to MCF-7 cells and other breast cancer cell lines (16–18). Estradiol also stimulates growth and PgR synthesis in MCF-7 cells (19, 20). Therefore, we evaluated the dose-response effects of EGF in the MCF-7 breast cancer cell line to determine the impact on receptor levels. We noted a down regulation of ER and a decrease in estradiol-stimulated PgR synthesis. Based upon these data we considered that perhaps excessive growth factor production by ER− or stromal cells in breast tumors may cause a down regulation of ER in the positive cells and prevent PgR production (by an unknown mechanism). A survey of primary tumors from women between the ages of 55 and 70 demonstrated that ER+ PgR− samples had only 50% as much ER as did the ER+ PgR+ tissues. We hypothesize that the lack of PgR in an ER+ system may be an indication that the tumor as a whole is no longer hormone responsive and depends upon the paracrine influences of growth factors from the receptor-negative cells for growth.

MATERIALS AND METHODS

Materials. EGF was obtained from Collaborative Research (Bedford, MA). Radioinert estradiol and dexamethasone were obtained from Sigma (St. Louis, MO). Radioactive estradiol, R5020, and unlabeled R5020 were obtained from New England Nuclear (Wilmington, DE).

Cell Culture. MCF-7 cells were obtained from Dr. Dean Edwards (San Antonio, TX) who obtained the stock from the Michigan Cancer Foundation. These cells were karyotyped as MCF-7 by Dr. Lorraine Meisner of the University of Wisconsin. MCF-7 cells were grown in minimal essential medium with 0.29 mg of l-glutamine/ml, 100 units of penicillin/streptomycin/ml, 6 ng of insulin/ml, 0.35 g of NaHCO3/liter, 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, and 5% charcoal-stripped calf serum. Medium components were obtained from Gibco (Grand Island, NY). Cells were harvested by an initial wash with a CMFH-1 mM EDTA solution followed by a 5-min incubation at 37°C with 2 to 5 ml of CMFH-1 mM EDTA. MCF-7 cells were plated at a density of 14,000 cells/well in 24-well culture plates in phenol red-free medium (Sigma) unless otherwise indicated. Media were changed each day for 2 days after plating, and compounds were added 4 days after plating.

Receptor Assays. Estrogen receptor was measured by whole cell uptake of [3H]estradiol (95.4 Ci/mmol), 5 nM, in CMFH. For PgR, 2 nM [3H]R5020 (86.2 Ci/mmol) was used; 25 nM dexamethasone was added to reduce binding of R5020 to the glucocorticoid receptor. One hundred-fold excess of nonradioactive ligand was added in separate wells to measure nonspecific binding. After incubation with 0.5 ml of the tritiated ligand for 45 min at 37°C, the wells were washed 3 times with 1 ml of CMFH containing 1 mg/ml of bovine serum albumin followed by two extra washes with just CMFH. The washed cells were sonicated in 1 ml of the wash buffer without bovine serum albumin.

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4The abbreviations used are: ER, estrogen receptor; EGF, epidermal growth factor; PgR, progesterone receptor; CMFH, calcium- and magnesium-free Hanks’ balanced salt solution; BSA, bovine serum albumin; DES, diethylstilbestrol.
diluted 1:9 with water, and samples were taken to determine the radioactivity and DNA. DNA was measured fluorometrically using Hoechst 33258 (Calbiochem-Behring) according to LaBarca and Paigen (21).

Statistics for clinical data. Scatchard analyses done in this laboratory of human breast tumor samples were surveyed from a database of assays done from 1985 to 1987. Only information from postmenopausal patients between the ages of 55 and 70 who had primary tumors was used. This was to avoid the effects of circulating steroids on receptor levels in premenopausal women. Two groups of patients were sorted, those who were ER+PgR+ or ER+PgR−. Positive values were those greater than 10 fmol/mg of protein. Scatchard analyses with greater than 4 points were used. Kd values were in the order of 10−10 M or less.

The estrogen receptor data were analyzed using a two-way analysis of variance where the two independent variables are age of the patient and the presence or absence of PgR. The response variable, ER, was transformed to the log scale to satisfy the assumptions of normality and constant variance. The estimates for the mean of the ER for each group are a least-square mean to take into account the unequal sample sizes.

**ER Analyses for clinical samples.** Human breast tumors, received on dry ice in storage buffer (10 mM Tris:0.25 mM sucrose:1 mM EDTA), were stored at −70°C until assayed. The tumors were homogenized (two 10-s bursts with ice water cooling) with a Polytron tissue homogenizer (Brinkman Instruments) at 1:10 (w/v) in 5 mM sodium molybdate:10 mM Tris-HCl (pH 7.4):1.5 mM EDTA:5 mM monothioglycerol:10% (v/v) glycerol. The homogenate was centrifuged for 30 min (4°C) at 100,000 × g using a TFE 45.6 rotor in an OTTD-8 Sorvall ultracentrifuge. The supernatant (cytosol) was removed, taking care not to disturb the fat layer on the surface, and kept in an ice water bath before use. Aliquots of cytosol (0.1 ml) were incubated overnight (16 to 20 h) at 2°C with six different concentrations of [3H]estradiol (3.0 to 0.294 nM) in 0.05 ml of 5 mM sodium molybdate:10 mM Tris-HCl (pH 7.4):1.5 mM EDTA:5 mM monothioglycerol:10% (v/v) glycerol. Parallel incubations of cytosol with a 200-fold excess of unlabeled DES were used to determine the nonspecific binding. Bound and free hormones were separated by adding 0.3 ml of dextran-coated charcoal (0.25% charcoal:0.025% dextran in 5 mM sodium molybdate, 10 mM Tris-HCl, and 1.5 mM EDTA). After incubation for 15 min at 0°C, the tubes were vortexed and centrifuged at 2000 × g for 15 min at 4°C. The supernatants were decanted into vials containing scintillation fluid and analyzed for radioactivity. All total binding (i.e., those without excess cold DES) tubes in the assay were done in duplicate (provided that enough cytosol was available). The nonspecific binding tubes (i.e., those with excess cold DES) were done singly. The binding parameters were calculated with linear regression of both Scatchard and Woolf plots analyses. Actual binding capacity (in fmol/ml) and Kd values (in nM) were taken from the Scatchard plot data with the Woolf plot used to lend confidence to the results. A calf uterine cytosol (=50 fmol/mg of cytosol protein) prepared from a powder was used along with tumor samples as a positive control.

**PgR analysis for clinical samples.** Progesterone receptor analysis was run simultaneously on the above cytosol in an analogous fashion. The ligand was used [3H]R5020 in a concentration range of 8.0 to 0.25 nM. A 200-fold excess of cold R5020 was used as the competitor. Due to the instability of this ligand, the ligand was kept in ethanol and added as 10-μl aliquots to the appropriate tube already containing 50 μl of buffer. The remainder of the procedure was identical to the ER analysis.

**Quality control for clinical samples.** A calf uterine cytosol prepared from lyophilized calf uterus (ER =50 fmol/mg of cytosol protein, PgR =100 fmol/mg of cytosol protein) was run simultaneously with the human tumor samples to provide daily quality control for all assays. ER and PgR values for the control are highly consistent and typically fall in the range of 50 ± 10 and 100 ± 20 fmol/mg of cytosol protein, respectively. The steroid receptor laboratory is externally quality controlled by Dr. J. L. Wittliff (Louisville, KY) in cooperation with the North Central Cancer Treatment Group program and the Southwest Oncology Group. The laboratory routinely reports reference sample results within target ranges specified by these two groups.

**RESULTS**

**EGF effects on ER.** Phenol red is an estrogen agonist and as such can down regulate ER (22). The culture of MCF-7 cells in phenol red-free medium causes an increase in ER (20). Therefore, to determine the effect of EGF on ER levels, MCF-7 cells were grown for specified times out of phenol red-containing medium. Experimental groups were treated with EGF (10−8 M or 10−7 M). ER levels were measured for up to 7 days. The up regulation of the ER was maximum at 5 to 7 days out of phenol red (Fig. 1). However, EGF treatment prevented a full increase in ER. Some reduction of ER is seen with 10−8 M EGF, but an approximately 50% decrease in ER from Days 3 to 7 is obtained if cells are treated with 10−7 M EGF.

Binding assays were performed to determine whether the lower ER resulted in a decrease in receptor number or a change in receptor affinity. MCF-7 cells were grown for a total of 10 days out of phenol red; on the last 7 days an experimental group of cells was treated with EGF. At the end of treatment, binding assays were done and analyzed by the method of Woolf (23). Only those cells receiving EGF (10−7 M) gave a significant difference in maximum bound ER versus control values (Fig. 2, A to C). ER, in this case, was approximately 43% that of control. This is very similar to the 50% decrease seen at this concentration in Fig. 1. There were no apparent differences in the Kd values among the groups (see figure insets). Therefore, the decreased binding of ER after EGF treatment appears to be due to a reduction in receptor number.

**EGF effect on PgR.** Because estradiol stimulates PgR synthesis in MCF-7 cells, we wished to determine the effect of an EGF-induced decrease in ER on this action of estradiol. Maximum PgR induction is attained after 2 days of estradiol (10−10 M) treatment (Fig. 3) and remains constant for up to 5 days. We used either a 3- or 4-day estradiol treatment, as indicated, in our experiments before measuring PgR. If cells were simultaneously treated with estradiol (10−10 M) and EGF (10−9 M to 10−7 M) for 4 days, there was an EGF concentration-related reduction in PgR synthesis (Fig. 4). EGF at 10−7 M was the least effective in inhibiting PgR synthesis, while EGF at 10−7 M was the most effective. EGF alone, at all the concentrations tested, had no noticeable effects on PgR levels compared with control. Estradiol alone, on the other hand, increased PgR 5-fold higher than control. Binding analyses also reflected these differences in receptor levels without an apparent effect on receptor affinity. Maximum binding with estradiol (10−10 M) alone was 9.63 ± 1.86 fmol/μg of DNA, with a Kd of 0.79 nm. When EGF (10−7 M) was added, PgR was reduced to 4.49 ± 0.23 fmol/μg of DNA but displayed a similar Kd of 0.28 nm.

We wanted to determine if EGF could cause MCF-7 cells to become refractory to estradiol treatment. We used the induction

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**Fig. 1.** Effects of phenol red on ER. MCF-7 cells were grown for at most 7 days out of phenol red-containing medium. ER levels were determined on experimental groups treated with EGF (10−8 M) (V), EGF (10−7 M) ( ), and compared with control ( ) ER levels. Points, mean; bars, SEM.
EGF REDUCTION OF ER AND PgR LEVELS

Fig. 2. ER binding analyses of MCF-7 cells treated with EGF. MCF-7 cells were grown without EGF (A) or in the presence of EGF, $10^{-8}$ M (B), or EGF, $10^{-7}$ M (C), for 7 days. ER was measured by saturation binding assays, and ER levels and ER binding affinity were determined by Woolf plot analysis. F/B, free/bound.

of PgR synthesis as an end point measurement on the effects of estradiol in this system. Pretreatment of MCF-7 cells for 7 days with $10^{-7}$ M EGF, followed by 3 days of simultaneous addition of increasing concentrations of estradiol ($10^{-13}$ M to $10^{-9}$ M), resulted in no stimulation of PgR synthesis (Fig. 5). However, estradiol treatment alone, at these concentrations, was stimulatory. EGF modulation of ER under these conditions can cause MCF-7 cells to lose their responsiveness to estradiol stimulation of PgR synthesis.

Clinical Observations. We surveyed our clinical steroid receptor laboratory database to determine if differences in ER levels could be found in ER-positive tumor samples of postmenopausal women who were either PgR+ or PgR−. In the ER+PgR+ group, data from 115 samples were analyzed, and in the ER+PgR− group, 47 samples were analyzed (Figs. 6 and 7). The ER status between the two groups was analyzed using a two-
**DISCUSSION**

The evaluation of steroid hormone receptor status in breast cancer has provided a powerful tool for the prediction of prognosis and responsiveness to therapy for nearly two decades (24, 25). Because of the importance of steroid receptors in the management of breast cancer and the reported effects of EGF on steroid receptor levels, we wanted to assess the action of EGF on ER and PgR.

Simultaneous treatment of MCF-7 cells with EGF and estradiol can inhibit estradiol-stimulated PgR synthesis by at least 50% of control value in a 4-day assay. Moreover, pretreatment for 7 days with EGF completely abolishes estradiol stimulation of PgR. ER, in this case, is decreased by approximately 50%.

This suggests that the induction of PgR synthesis may be regulated by a specific population of ER which can be modulated by both estradiol and EGF. We have previously described such a model for the regulation of breast cancer cell growth (26). Alternatively, the PgR response may require a minimum threshold level of ER. It is worthy of note that the PgR was not consistently increased by EGF treatment alone in these studies, which concurs with an earlier report by Iacobelli and Natoli (27). In contrast, it has recently been shown that uterine cells from fetal guinea pig increase PgR with EGF in culture (28). The explanation for these different findings is unclear.

Treatment of MCF-7 cells with estradiol also causes between a 40 and 60% decrease in ER within 6 h (29). Down regulation of ER corresponds to an increase in PgR synthesis. Since PgR stimulation is rate limited with maximum stimulation occurring at Day 2 (see Fig. 3), then the decrease in ER may serve as a regulating mechanism to inhibit further induction of PgR. By similar reasoning, down regulation of ER by EGF, prior to estradiol treatment, should prevent PgR synthesis. Both treatments, however, still result in the growth of the cell (30), suggesting that ER-stimulated growth and PgR synthesis are disassociated events.

Increased PgR primes breast cells for differentiation by progesterone (31). By preventing differentiation, EGF regulation of PgR may then aid the progression of the cancer to a more malignant state. EGF also inhibits or delays the terminal differentiation of cultured mouse mammary epithelial cells, rat ovarian granulosa and testicular cells, and human epidermal keratinocytes (32-34).

The presence of PgR in breast cancer biopsies is thought to indicate that the ER pathway is intact and functional (35). In support of this hypothesis is the fact that objective response to hormonal therapy is improved from 60% to 80% when the cancer is both ER+ and PgR+ (9). Studies looking at PgR measurements from sequential biopsies from the same patient showed that 44% of the patients who were initially PgR+ were later assayed as receptor negative (36). Loss of PgR correlated with positive axillary lymph nodes and decreased survival.

Our in vitro studies indicate that PgR can be diminished by EGF while the cells remain ER+, although ER levels are significantly reduced. It is interesting to note that postmenopausal breast cancer patients who are PgR negative also showed an ER mean value which is approximately 50% lower than the ER mean value for ER+PgR+ women.

The cellular environment of the tumor during growth and progression may be important to change the steroid receptor status. Therefore, as a hormone-dependent breast cancer becomes less responsive to estradiol, the PgR levels seem to be one of the first estrogen-regulated responses to be affected. Indeed, there is a decrease of PgR with increasing primary breast tumor size (37). With larger tumors the chances of greater numbers of hormone-independent cells are increased. This increase may lead to sufficient production of growth factors which may then reduce PgR and ER levels. We hypothesize that tumors which are ER+PgR− may have the ability to become autonomous, primed by high growth factor concentrations, a decrease in ER, and a decrease in the ability of estradiol to stimulate PgR. These tumors are known to be less estrogen responsive although they are ER+. In an earlier paper we have also demonstrated that antiestrogens do not function effectively in ER+ cells in a growth factor-rich environment (30). Therefore, the poor response to endocrine therapy by ER+PgR−...
tumors may be due to paracrine growth factor stimulation which subverts the normal regulation of replication through the ER.

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