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REGULATION OF EPIDERMAL GROWTH FACTOR-RECEPTOR BY ESTROGEN AND ANTIESTROGEN IN THE HUMAN BREAST CANCER CELL LINE MCF-7

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Summary: Regulation of breast tumor proliferation depends in a large part on a variety of hormones and growth factors. In this report we show that estrogen and antiestrogen modulate epidermal growth factor-receptor (EGF-R) level in the human breast cancer MCF-7 cells with opposite mechanisms. Although a short-term treatment (24h to 48h) with estradiol leads to a decrease in EGF-R number, the addition of hormone in cell culture for 5 days increases EGF-R level with a maximal effect observed at 10^{-10} M estradiol. In contrast, when cells are treated with the antiestrogen hydroxytamoxifen, a dose-dependent decrease in EGF-R level occurs. We also report that EGF is able to induce estrogen receptors and, to a lesser extent, progesterone receptors when added to MCF-7 cell cultures. These results demonstrate an interaction between both estrogen receptor and EGF receptor growth promoting systems in target cells. The implications of such an interaction in the understanding of human breast cancer hormone responsiveness and, in the development of therapies, are discussed.

A large variety of hormones and growth factors may be involved in the regulation of breast tumor proliferation and contribute to a complex series of hormonal interactions in the target tissue (1). Estrogens are known to play an important role in breast cancer cell biology (2, 3). The mechanism by which estrogens induce cell proliferation has been extensively investigated. Although it is generally accepted that estradiol effects on breast cancer cell proliferation are mediated largely through the estrogen receptor (4, 5), the post-receptor events leading to the growth stimulation need further investigation. Recently, it was shown that human breast cancer cell lines release in vitro growth promoting agents such as epidermal growth factor (EGF) and EGF-related polypeptides (6, 7) whose the secretion is regulated by estrogens (8, 9). These growth factors, particularly EGF, modulate the growth of the mammary gland as well as the proliferation of hormone-dependent human breast cancer cells in vitro (10, 11). Its mammary tumor-promoting role has also been demonstrated in several models (12). Presently it is accepted that EGF specifically interacts with the surface binding domain of a transmembrane receptor (EGF-R) leading to activation of its intrinsic tyrosine kinase activity (13). In human breast cancer biopsies, quantitation of EGF-R revealed that estrogen-receptors (ER) and progesterone-receptors (PR) correlated inversely with EGF-R levels, and a high EGF-R content in mammary carcinoma metastases and tumors was associated with poor prognosis (14-16). Since growth factors influence the growth and function of reproductive tracts, some steroids may elicit their effect through regulation of growth factors, their receptors, or both. For instance, progesterone has been shown to induce an increase in EGF-R in the human breast cancer cell line T47D (17, 18). In the same cells, Koga and Sutherland (19) recently showed that EGF may reverse the inhibitory effect of antiestrogens on cell proliferation. Inversely, an antagonistic effect of antiestrogens on the EGF-growth stimulation was recently

Abbreviations: DMEM, dulbecco minimum essential medium; F12, Ham F12; CDCS, dextran-coated charcoal-treated calf serum; EGF-R, epidermal growth factor-receptor; E_2, 17-β estradiol; OH-TAM, [trans-1-(4-β-dimethylamino-ethoxyphenyl)-1-4-hydroxyphenol)-2-phenybut-1- ene]; ORG 2058, (16α-ethyl-21-hydroxy-19-nor [6,7-^{3}H] pregn-4-en-3,20 dione); ER, estrogen-receptor; PR, progesterone-receptor.

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reported in the MCF-7 cell line (20). All these data prompted us to investigate the effects of estrogens and antiestrogens on the regulation of EGF-R in MCF-7 cells.

MATERIALS AND METHODS

Materials

4-hydroxytamoxifen [trans-1-(4-β-dimethylamino-ethoxyphenyl)-1- (4-hydroxyphenyl)-2-phenybut-1-ene] was supplied by ICI Pharmaceuticals Division. 17β-estradiol was obtained from Sigma. Steroids were prepared as 1,000-fold concentrated stock solutions in ethanol and stored at –20°C. [^{3}H]E_2 (SA = 90 Ci/mmole), and [^{3}H]ORG.2058 (16 α-ethyl-21-hydroxy-19-nor [6, 7-^{3}H] pregn-4- en-3,20 dione) (SA = 51 Ci/mmole) were obtained from Amersham. Murine EGF, tissue culture or receptor grade, was supplied by Oncogene Science (Birmingham, AL). EGF was prepared as 100 µg/ml in PBS/0,1 % BSA and stored in aliquots at –20°C.

Cell cultures

The MCF-7 cell line was obtained from Dr. Marc Lippman's Laboratory (NIH, Bethesda). The cells in passages 15-50, were grown in 50 % Dulbecco Minimum Essential Medium / 50 % Ham F12 (DMEM/F12, Gibco) containing 16 ng/ml insulin, 2 mM glutamine, 1% non-essential amino acids, 0.06 % Hepes buffer, 50 UI/ml penicillin, 50 µg/ml streptomycin and 10 % heat-inactivated calf serum. The cells were cultured at 37°C in a humid atmosphere of 5 % CO_2 in air. They were grown in the presence of 10 % dextran-coated charcoal-treated calf serum (21) for 2 weeks before use for experiments. All treatments were done in phenol red-free culture medium (22).

Receptor determinations

ER and PR were determined on cytosol and nuclear extract. Briefly, cells were removed by a 10 min incubation at 37°C with 1 mM EDTA in Hank's balanced salt solution, washed with Hank's solution, then with phosphate salin buffer. Cells were resuspended in phosphate buffer, homogenized in a Dounce homogenizer using the B pestle, then cytosol and nuclear extracts were prepared as previously described (23). Aliquots were added to 10 nM [^{3}H]E_2 for ER assay or 10 nM [^{3}H]ORG for PR measurement, and nonspecific binding was determined in the presence of a 100-fold excess of unlabeled ligand. The mixture was incubated for 18 h at 4°C, then free ligand was removed by a 10 min incubation at 4°C with dextran-coated charcoal slurry. After centrifugation, an aliquot of the supernatant was removed and the radioactivity was determined. DNA measurements were performed on nuclear pellet by DABA assay (24).

EGF-R was measured by [^{125}I]EGF binding on cell monolayers. Receptor-grade murine EGF was iodinated by the chloramine-T method. [^{125}I]EGF was purified on Sephadex G25 (Pharmacia) in PBS pH 7.4/0.1 % BSA and stored at –20°C. [^{125}I]EGF had a specific activity of 200,000-350,000 cpm/ng. Binding studies were performed on cells grown in 24-wells culture plates. Preconfluent monolayers were washed in Hepes-buffered DMEM containing 0.1 % BSA, then incubated in DMEM/BSA in presence of 5 nM [^{125}I]EGF for 1 h at 37°C. For some experiments, Scatchard analysis were performed by incubating cells with various concentrations of radiolabeled-EGF for 4h at 4°C. In the all cases, nonspecific binding was determined in the presence of a 100-fold excess of unlabeled EGF. Incubations were stopped by washing cells 5 times with cold PBS/BSA, and cells were suspended in 1N NH_4OH/0.1 % TX 100. DNA was then measured after determination of cell-associated radioactivity.

RESULTS

The effect of estrogens and antiestrogens on EGF-R level, was measured on cell monolayers treated with E_2 and OH-TAM for various periods of time.

Measurements were first performed by incubating cells for 1h at 37°C with 5nM [^{125}I]EGF. The time-course experiments are reported in figure 1. Cell treatment with 10^{-7} M OH-TAM led to a slight decrease in EGF-binding observable 3 days following the antiestrogen addition. EGF-R number was then reduced to 25% of control when treatment was extended to 5 days. A 40% decrease in EGF-binding was also detected when MCF-7 cells were submitted to 10^{-9} M estradiol for 24h to 48h. However, by 3 days of hormone-treatment control EGF-R level was restored and a significant increase was observed by 4 day-incubation with estradiol. The maximal enhancement in EGF-cell binding (200% of control) was attained when estradiol was added for 5 days in MCF-7 cell culture.

Dose-response effects of estrogens and antiestrogens on EGF-R level were then investigated. Figure 2 shows that cell treatment with 10^{-10} M to 10^{-7} M OH-TAM led to a dose-dependant decrease in EGF-R number which attained a minimal level (25% of control) in presence of 10^{-7} M antiestrogen. In contrast, the increase in EGF-binding was significantly induced by 10^{-11} M to 10^{-8} M estradiol. The maximal effect was observed at 10^{-10} M which increased EGF-R number to 230% of control. However, 10^{-8} M estradiol displayed a lower stimulatory activity on EGF-binding, although maintaining its level to 160% of control. In order to verify wether modifications of affinity and/or internalization could account for variations in EGF-R number observed in presence of estrogen or antiestrogen, Scatchard analysis were performed at 4°C. As previously reported (25), 2 types of binding sites with respective Kd of 0.30 nM and 1.4 nM were observed (table 1). Estradiol and

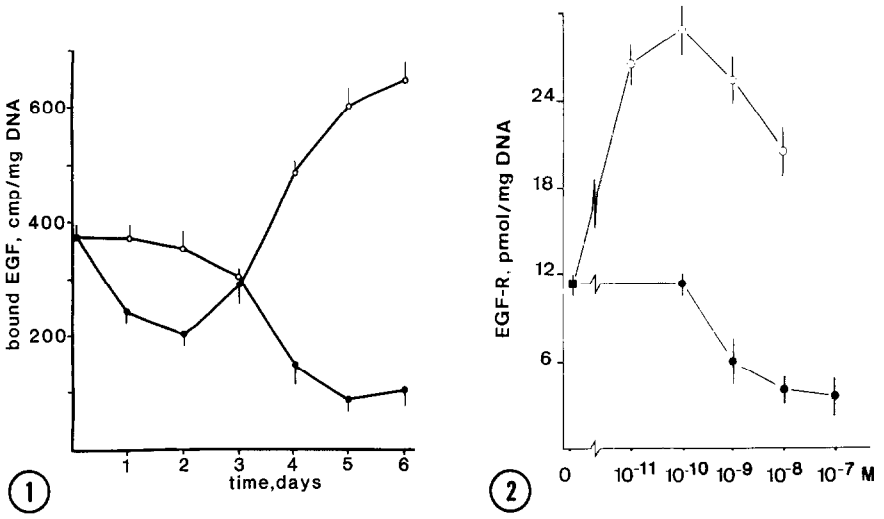

Fig. 1 : Time-course analysis of estradiol and hydroxytamoxifen effect on EGF-R levels in MCF-7 cells. Cells were grown in 12-well plates in DMEM/F12, 10 % CDCS. Medium was supplemented with 10^{-9} M estradiol (o) or 10^{-7} M hydroxytamoxifen (●) for the periods of time indicated . Control cells (■) received 0.1 % ethanol vehicle and media and hormones were changed every days. At the end ofthe hormone treatment cells were washed and EGF-R levels were determined using [^{125}I]EGF in the absence and presence of a 100-fold excess of unlabeled EGF. Data points represents the mean ± SEM of 3 wells and are representative of tree experiments.

Fig. 2 : Dose-dependant effect of estradiol and hydroxytamoxifen on EGF-R levels in MCF-7 cells. Cells were processed as mentionned in fig.1, except that cell treatment was performed for 5 days with the indicated concentrations of estradiol (○) and hydroxytamoxifen (●).
Data points are the mean SEM of 3 wells and are representative of two experiments.

hydroxytamoxifen treatment did not modify their affinity but modulation of EGF-R number by these compounds was confirmed.

In parallel the effect of estradiol on ER-level was also determined. As previously described (26), a 5 day cell treatment with 10^{-11} M to 10^{-8} M estradiol induced a dose-dependent loss in ER level (Fig. 3), decreasing receptor number from 4000 fmol/mg DNA in control cells to 1200 fmol/mg DNA in 10^{-8} M estradiol-treated cells.

The effect of EGF on estrogen and progesterone receptor cell content was then investigated and is reported in figure 4. Cells treated with 10^{-10} M to 10^{-8} M EGF exhibited a higher ER level compared to control cells. The magnitude of the EGF induction of ER was 200 % of control. Since progesterone receptor is an estrogen-

Table 1. Scatchard analysis of EGF-R in MCF-7 cells submitted to estrogen or antiestrogen treatment

		type I		type II	
		n	Kd,nM	n	Kd,nM
Control		5.1 ± 0.5	0.33	6.3 ± 1.1	1.33
E2	10^{-11}M	10.5 ± 1.2	0.30	15.4 ± 2.1	1.11
	10^{-10}M	12.0 ± 0.8	0.41	17.0 ± 2.9	1.80
	10^{-9} M	10.8 ± 1.1	0.20	14.2 ± 0.5	1.50
	10^{-8} M	9.3 ± 0.3	0.35	10.0 ± 1.3	1.75
OH-TAM	10^{-9} M	4.2 ± 0.3	0.45	3.1 ± 0.6	1.30
	10^{-8} M	2.3 ± 0.5	0.28	3.0 ± 0.2	1.66
	10^{-7} M	2.0 ± 0.6	0.29	2.6 ± 0.4	1.80

MCF-7 cells were processed as mentionned in fig.2, except that EGF-R number was determined by incubating cells for 4h at 4°C with 0.1 nM to 5 nM (^{125}I)EGF. Nonspecific binding was determined in presence of a 100 fold excess of unlabeled ligand. The results are representative of two separate experiments. n: EGF-R number are expressed as pmol/mg DNA.

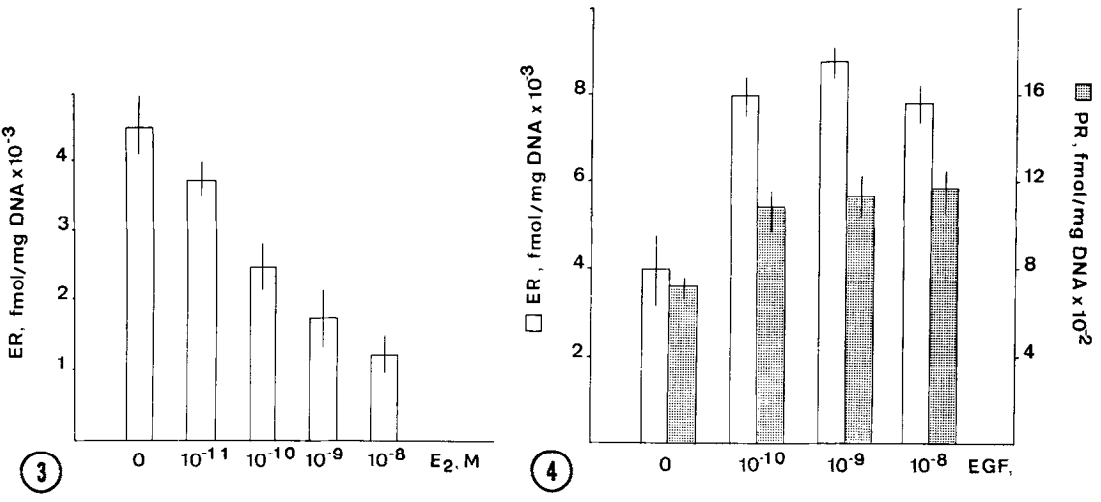

Fig. 3 : Effect of estradiol on ER number in MCF-7 cells. MCF-7 cells were seeded in T-150 flasks in DMEM/F12, 10 % CDCS. The following day, 10^{-11} M to 10^{-8} M estradiol was added. Control cells received 0.1 % ethanol vehicle. Fresh medium and hormone were added daily. On day 5, cells were harvested and processed for ER measurement as described in "Materials and Methods".

Fig. 4 : Effect of EGF on estrogen and progesterone receptors levels in MCF-7 cells. MCF-7 cells were seeded in T-150 flasks in DMEM/F12, 10 % CDCS. The following day, medium was supplemented with the indicated concentrations of EGF. Control cells received 0.1 % PBS/BSA vehicle. Medium and growth factor were renewed every two days, and on day 5, cells were processed for ER and PR as described in "Materials and Methods". Each value represents data obtained from duplicate flasks and is representative of two experiments.

stimulated protein (27), we examined the effect of EGF on progesterone receptor level in MCF-7 cells. At a lesser extent, EGF was also able to stimulate progesterone receptor level at the all concentrations used, increasing progesterone receptor number to 150 % of control.

DISCUSSION

Recently, human breast cancer cell lines have been reported to secrete into their conditioned medium a number of growth factors which may display growth promoting activity (6, 28, 29). The production of one such factor, α-transforming growth factor (α-TGF), has been shown to be stimulated by estrogens, and to be inhibited by antiestrogen cell treatment (8, 9). Although the effects of estrogens on breast cancer cell proliferation undoubtedly involve the estrogen receptor, these data suggest that estrogen stimulation of cell growth may be in part mediated, indirectly by increasing the production of autocrine growth promoting peptides. Given that α-TGF mediates its effects through interaction with the EGF-receptor (30, 31), and that both estrogens and EGF are involved in growth stimulation, regulation of their receptors seemed to us to be of fundamental interest.

The results reported herein demonstrate an interaction between steroid and growth factor cell responses. Our data indicate that estrogens and antiestrogens are able to modulate EGF-R level in the hormono-sensitive MCF-7 cells. It has been previously reported a slight decrease of the expression of EGF-R mRNA in MCF-7 cells submitted to estradiol for 24h (32). In accordance with these data, we show that a short-term estradiol treatment (24h to 48h) leads to a decrease in EGF-R number. However, when extending the hormone treatment to a few days, estradiol leads to an increase in EGF-R level with a maximal activity at 10^{-10} M. Although a high EGF-R content is maintained, a return toward basal level is observed with 10^{-9} M to 10^{-8} M estradiol. Our results agree with those of Mukku and Stancel who described a estradiol-stimulation of EGF-R level in the rat uterus (33). In our study, we show that this increase in EGF-R level by estradiol-treatment is associated with a loss of ER content, previously described as processing (26). In vivo, such an inverse correlation between ER and EGF-R has been reported on human breast cancer biopsies (14-16). Since estradiol stimulates the production of α-TGF, we suggest that the E_2-induction of EGF-R is a mechanism permitting the cells to

optimally respond to growth factors and could explain the bad prognosis associated with high EGF-R containing breast cancers (34).

Whereas estrogen treatment leads to an increase in EGF-R number, by 3 days addition in cell culture, the antiestrogen OH-TAM displays a dose-dependent inhibitory effect. Previous reports have suggested (22, 35) that antiestrogens would not affect cell growth in the absence of estrogen. In fact, our data and others, indicate that these drugs may be effective on cells deprived of estrogen but submitted to the action of other mitogens. Thus, inhibition of EGF-stimulated proliferation by antiestrogens in MCF-7 cells has recently been reported (20). This inhibition could be due to the decrease in EGF-R level observed when OH-TAM is added in cell culture, leading to a lower EGF responsiveness. Nevertheless, the relative low concentrations of estrogen and antiestrogen able to modulate EGF-R level in MCF-7 cells as well as the absence of any effects on the estrogen receptor-negative MDA-MB 231 cell line (not shown) suggest an ER-mediated effect.

It is known that EGF down-regulates its receptor (36, 37). Given the effects of estrogen and antiestrogen on EGF-R regulation, we wondered whether EGF was also able to interact with the estrogen-receptor system. Although estradiol down-regulates ER and increases EGF-R number, it is interesting to point out that EGF displays totally opposite effects. Indeed, we show that EGF-treatment leads to an induction of ER and PR in MCF-7 cells, concomitent with a decrease in EGF-R level (data not shown). Thus, similarly to observations described in vivo (14-16), these data demonstrate that in MCF-7 cells, high ER and PR content can be associated with a decrease in EGF-R number. Presently, it is not known whether these observations reflect the in vivo behavior of breast cancer cells containing high ER and PR and low EGF-R levels. This merits further investigation, since the efficacy of antiestrogens in the treatment of breast cancers depends in large part on the presence of free ER sites.

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