Endogenous Growth Factor Expression in T-47D, Human Breast Cancer Cells, Associated with Reduced Sensitivity to Antiproliferative Effects of Progestins and Antiestrogens

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ABSTRACT

To determine the functional significance of the endogenous expression of the epidermal growth factor (EGF) and transforming growth factor-α genes in T-47D, human breast cancer cells, we have examined the effects of two types of antiproliferative agents, progestins and antiestrogens, on the expression of these growth factors and the effects of exogenous EGF on the antiproliferative action of these agents. Using Northern blot analysis, the regulation of expression of these two genes by the antiproliferative agents, tamoxifen and monohydroxytamoxifen, was examined. In T-47D cells the two antiestrogens did not affect the accumulation of EGF mRNA and decreased the accumulation of TGFα mRNA. As we have shown before, the progesterin, medroxyprogesterone acetate, increased the level of both EGF mRNA and TGFα mRNA in this cell line. The regulation of expression of these endogenous growth factor genes was unrelated to the proliferative behavior of T-47D cells since both antiprogestins and progestins were antiproliferative under the conditions of the experiments. The variant cell line, T-47D-5, had no detectable EGF mRNA and contained about 1/10th the level of TGFα mRNA expressed by “wild-type” T-47D cells. T-47D-5 cells were 2.5 times more sensitive to the antiproliferative effects of both progestins and antiprogestins when compared to the growth factor expressing T-47D cells. Exogenously added murine EGF was able to decrease slightly the sensitivity of both cell lines to the antiproliferative effects of both progestins and antiprogestins as well as increase the proliferation of T-47D but not T-47D-5 cells. These data suggest that endogenous expression of growth factors may be associated with decreased sensitivity of the cells to growth inhibitory agents.

INTRODUCTION

We have previously shown that progestins regulate the expression of both the EGF3 (1) and TGFα genes4 in the human breast cancer cell line T-47D. Furthermore, in this cell line progestins also up-regulate the expression of the EGF-receptor (2). However, we and others (1, 3, 4) have shown that progestin treatment of these cells results in growth inhibition rather than enhanced proliferation. Furthermore, other growth inhibitory agents such as the anti-progestin RU 486 or growth stimulators such as estradiol had little if any effect on the expression of EGF, TGFα, and EGF-receptor genes. To further investigate the significance of endogenous EGF and TGFα gene expression in T-47D human breast cancer cells we have examined the effect of nonsteroidal antiprogestins, and the progesterin MPA on cellular proliferation of T-47D cells and a variant T-47D cell line, which does not express EGF and expresses TGFα at very low levels compared to “wild-type” T-47D cells. Here we report that the sensitivity of the nongrowth factor gene-expressing variant to antiprogestins and progestins was markedly enhanced compared to the growth factor EGF and TGFα expressing T-47D “wild-type” cells.

MATERIALS AND METHODS

Materials. MPA, hydrocortisone, estradiol-17β, and dihydrotestosterone were purchased from Sigma. R2050, [3H]R5020 (87 Ci/mmol), [3H]estradiol-17β (37.9 Ci/mmol) and [32P]dCTP were purchased from NEN (Lachine, Quebec). Tamoxifen and 4-hydroxytamoxifen were gifts from ICI (Macclesfield, Cheshire). RU 486 was a gift from Roussel Uclaf (Romainville, France). Murine epidermal growth factor was purchased from Collaborative Research (Bedford, MA). Dulbecco’s minimal essential medium powder was purchased from Gibco/BRL (Burlington, Ontario). All other cell culture medium ingredients were purchased from Flow Laboratories (Mississauga, Ontario).

Cells. The T-47D human breast cancer cell line was obtained from Dr. R. P. C. Shiu (Department of Physiology, University of Manitoba, Winnipeg, Canada) (5). The T-47D-5 variant line was kindly provided by Dr. R. L. Sutherland (Garvan Institute for Medical Research, Sydney, Australia) in whose laboratory it was originally isolated (6). The cells were grown in Dulbecco’s minimal essential medium supplemented with 5% fetal bovine serum, glucose, sodium pyruvate, penicillin-streptomycin (5). Cells were harvested by scraping the cells off the monolayer with a rubber policeman. After centrifugation the cell pellet was snap frozen and stored at −70°C until RNA or DNA isolation.

For experiments in which cells were treated with various compounds, the cells were grown in the above medium. While the cells were still subconfluent, the medium was replaced with fresh medium and the test compounds were added directly from 1000X stock solutions in ethanol to achieve the concentrations indicated.

For cell growth experiments, cells were plated at approximately 10⁴ cells/30-mm dish. 2 days later the medium was replaced with fresh medium containing varying concentrations of the drugs to be tested. Cells were harvested, in duplicate or triplicate, at the times indicated and cell numbers were counted using an electronic cell counter.

RNA Extraction and Northern Blot Analysis. RNA was isolated by the guanidinium thiocyanate/cesium chloride method (7). Poly(A⁺) RNA was isolated by one cycle of oligo(dT) cellulose chromatography (8), 10−15 µg of poly(A⁺) RNA was denatured in 50% (v/v) formamide and 2.2 M formaldehyde, size separated by electrophoresis on 1% (w/v) agarose gels containing 2.2 M formaldehyde and then blotted onto nitrocellulose (9). Filters were baked for 2 h at 80°C under vacuum and then prehybridized in hybridization solution for at least 3 h. The filters were then hybridized with the 1.9 kilobase pair human EGF cDNA insert from X EGF15(c) (10). After the signal from the previous hybridization had decayed the same filters were hybridized with a 0.90 base pair human TGFα cDNA insert. Hybridizations, usually for 48 h, were performed at 42°C in the presence of 50% (v/v) deionized formamide, 5× Denhardt’s solution (1× Denhardt’s = 0.02% w/v each of bovine serum albumin, Ficol, and polyvinylpyrrolidone), 5× SSPE (1× SSPE = 1.15 M NaCl, 0.01 M NaH₂PO₄, 1 mM EDTA), 250 µg/ml

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3 The abbreviations used are: EGF, epidermal growth factor; TGFα, transforming growth factor-alpha; MPA, medroxyprogesterone acetate; R5020, 17,21-dimethyl-19-nor-4,9-pregnadiene-3,20-dione; RU 486, 17β-hydroxy-11β-(4-di-methylaminophenyl)-17α-(1-propynyl)estra-4,9-dien-3-one; OH-Tam, 4-hydroxytamoxifen; PR, progesterone receptor; SDS, sodium dodecyl sulfate; SSC, standard saline citrate (0.15 M sodium chloride:0.015 M sodium citrate, pH 7.4).

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denatured salmon sperm DNA and 0.1% SDS. At the end of the hybridization period the blots were washed twice in 2xSSC, 0.1% SDS for 15-30 min at room temperature, followed by one wash in 0.1xSSC, 0.1% SDS for 45-60 min at 65°C. Filters were also hybridized with chicken beta-actin cDNA (11) as a control for differences in the amount of RNA loaded on the gel. Filters were exposed to Kodak XAR film at -70°C with an intensifying screen.

DNA Extraction and Southern Blot Analysis. Genomic DNA was isolated, digested with EcoRI, fractionated on a 0.8% alkaline-agarose gel and transferred to nitrocellulose according to the protocol of Davis et al. (12). Hybridization and washing procedures were as previously described (1).

Whole-Cell Estrogen and Progesterone Receptor Assays. Cells were plated in charcoal-stripped fetal calf serum containing medium into 6- and 24-multiwell plates, respectively. 3-4 days later whole-cell estrogen and progesterone-receptor assays were performed as previously described (13). [3H]estradiol-17β and excess unlabeled estradiol-17β, [3H]R5020 and excess unlabeled R5020 was used to determine estrogen and progesterone-receptor binding and nonspecific binding, respectively. The progesterone-receptor assay was also done in the presence of 100 nM dexamethasone and 100 nM dihydrotestosterone to minimize binding of R5020 to the glucocorticoid and androgen receptor in these cells.

Statistical Analysis. The paired Wilcoxon test was used to assess the statistical significance of the results obtained.

RESULTS

Effect of Growth Inhibitory Agents on EGF and TGFα mRNA Accumulation. Since the antiestrogens, tamoxifen and 4-hydroxytamoxifen, are growth-inhibitory agents in the estrogen-receptor positive T-47D cell line, we investigated the effect of these compounds on the accumulation of EGF mRNA and TGFα mRNA in these cells. Consistent with our previous findings the progestin, medroxyprogesterone acetate, increased the accumulation of the EGF mRNA but neither tamoxifen nor its more potent metabolite 4-hydroxytamoxifen had any significant effect (Fig. 1, a and b). Interestingly, the antiestrogens inhibited the accumulation of TGFα mRNA while the progestin MPA increased the accumulation of TGFα mRNA (Fig. 2, a and b). However, under the conditions of these experiments MPA (1), 4-hydroxytamoxifen (Fig. 3), and tamoxifen (data not shown) inhibited the growth of T-47D cells. The antiprogestin RU 486 alone caused a small increase in TGFα mRNA but inhibited the MPA-induced increase. RU 486 also inhibited the growth of T-47D cell (Reference 3, data not shown). These data suggest that the alteration of expression of these endogenous growth factor genes in T-47D cells is not directly related to growth inhibition per se.

In the process of our studies, it was observed that one T-47D subline, T-47D-5, was found not to express the EGF gene (Fig. 4a) and only expressed TGFα at about one-tenth the level found in our wild-type T-47D cells (Fig. 4b). While the level of TGFα mRNA in T-47D-5 cells was also increased slightly by 1 and 10 nM MPA (data not shown), this treatment did not reveal any detectable EGF mRNA in T-47D-5 cells. Any possible inhibitory effect of antiestrogens on TGFα mRNA in these cells could not be measured due to the very low to undetectable levels of this transcript in control cells.

Furthermore, Southern analysis of the EGF and TGFα genes in both cell lines (data not shown) showed that there was no amplification or gross rearrangement of these genes in either cell line.

Comparison of the Sensitivity of T-47D and T-47D-5 Cells to the Antiproliferative Action of Antiestrogens and Progestins. In order to study the possible role of growth factor expression in these cells, we have investigated the sensitivity of the growth factor expressing (T-47D) and the growth factor nonexpressing (T-47D-5) cell lines to the antiproliferative action of antiestrogens and progestins. The effects of varying concentrations of MPA and 4-hydroxytamoxifen on the proliferative rates of the two cell lines are shown in Fig. 5A and 5B, respectively. Under control conditions the growth factor-nonexpressing, T-47D-5...
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TGF-alpha

Actin

A B C D E F

TGF-alpha

Actin

A B C D E

Fig. 2. The effects of medroxyprogesterone acetate and tamoxifen on TGFα mRNA accumulation in T-47D human breast cancer cells. a, Northern blot analysis of poly(A*) RNA isolated from T-47D cells which had been treated for 64 h with A, vehicle alone; B, 10 nM MPA; C, 100 nM RU 486; D, 1000 nM OH-Tam; E, 10 nM MPA plus 100 nM RU 486; F, 10 nM MPA plus 1000 nM OH-Tam. TGF-alpha, pattern of hybridization obtained with TGFα cDNA; Actin, pattern of hybridization obtained with chicken β-actin cDNA. b, Northern blot analysis of poly(A*) RNA isolated from T-47D cells which had been treated for 24 h with A, vehicle alone; B, 10 nM MPA; C, 1 μM tamoxifen; D, 5 μM tamoxifen; E, 10 μM tamoxifen. TGF-alpha and Actin, same as in a. Time course studies have shown that TGFα mRNA remains elevated due to progestin treatment from 12 h to at least 64 h after treatment.

cell line grows 1.5 times faster than the wild-type, growth factor-expressing T-47D cell line (doubling time of 1.33 versus 2.09 days, respectively). Furthermore, the T-47D-5 cell line is 2.5 times more sensitive to the growth inhibitory effects of both MPA and OH-Tam when compared to the T-47D cell line. A similar result was obtained when the antiprogestin and antiproliferative agent, RU 486, was tested (data not shown).

Estrogen- and Progesterone-receptor Levels in T-47D and T-47D-5 Cells. Estrogen- and progesterone-receptor levels in the two cell lines were measured to determine if differences in the relative levels could account for the sensitivity difference. Both cell lines specifically bound estradiol with a Kd value of 1.2 nM and contained approximately equal amounts of ER, 113491 sites/cell and 85008 sites/cell for T-47D and T-47D-5, respectively. T-47D cells contained approximately 10 times (3 x 10^6 sites/cell, Kd = 2.2 nM) the amount of PgR present in the T-47D-5 line (0.4 x 10^6 sites/cell, Kd = 2.2 nM).

Effect of Exogenous EGF on Sensitivity of Cells to the Antiproliferative Action of Antiestrogens and Progestins. Since both cell lines contained EGF-receptor as determined by binding assay and the presence of EGF-receptor mRNA (data not shown, Ref. 2) we determined the effect of exogenously added murine EGF on the sensitivity of these cells to the antiproliferative action of MPA and OH-Tam. The results of addition of 10 ng murine EGF/dish/day on cellular proliferation in the presence or absence of 10 nM MPA and 10 nM OH-Tam, are shown in Fig. 6. A rank-paired Wilcoxon test was used to assess the statistical significance of the results obtained. The T-47D cells were inhibited by 10 nM OH-Tam and MPA and demonstrated a 9% and 12% reduction in growth inhibition, respectively, in the presence of exogenously added murine EGF. The T-47D-5 cells were inhibited by 10 nM OH-Tam and MPA and demonstrated a 5% reduction in growth inhibition in the presence of exogenously added murine EGF. Although the observed EGF-induced reductions in sensitivity in both cell lines were of small magnitude, the differences were statistically significant. The difference between MPA treatment alone and MPA treatment plus EGF was significant at P < 0.025 and P < 0.05 for T-47D and T-47D-5, respectively, N = 6, and the difference between OH-Tam treatment alone and OH-Tam treatment plus EGF was significant at P < 0.05 and P < 0.05 for T-47D and T-47D-5, respectively, N = 5. Increasing the amount of EGF added to 50 ng/dish/day did not further reverse growth inhibition (data not shown). Interestingly, EGF alone was able to stimulate the growth of T-47D cells (the difference between control T-47D cells and control cells plus EGF treatment in the MPA series of experiments was P < 0.025 and in the OH-Tam series of experiments was P < 0.05) but not the T-47D-5 cells. However, even after correction for the growth stimulatory effect of EGF on T-47D cells (i.e., the data presented as
percentage of control cell numbers), EGF was still found to decrease the sensitivity of T-47D cells to the growth inhibitory effects of antiestrogens and progestins in this cell line, at the same level of statistical significance as when the data were expressed as cell numbers.

Another series of experiments was then carried out to assess the effect of exogenously added EGF on the growth inhibitory effects of various concentrations of MPA and OH-Tam in T-47D and T-47D-5 cells. EGF was able to reduce the sensitivity of both cell lines to growth inhibition by MPA and OH-Tam at all concentrations tested (Fig. 7A and B). Again it should be emphasized that the observed reductions in sensitivity were all of small magnitude and there was little difference between cell lines in the magnitude of the effect of EGF on OH-Tam growth inhibition (Fig. 7A). However, exogenously added EGF appeared to reduce the sensitivity of T-47D to MPA markedly more than that seen in T-47D-5 cells (Fig. 7B).
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DISCUSSION

The demonstration that human breast cancer cells express a number of possible autocrine/paracrine growth factors (14) set the stage for an investigation of their relationship to both hormonally dependent and independent breast cancer cell proliferation. Lippman’s group have presented data that estrogens increase while antiestrogens decrease the level of TGFα-like peptides in the medium of MCF 7 human breast cancer cells (15). Furthermore, estrogens decreased and antiestrogens increased the level of the growth inhibitory peptide, TGFβ in these cells (16). Recently it has been demonstrated that EGF can partially reverse the growth inhibitory effects of antiestrogens (17, 18) in T-47D human breast cancer cells. These data are consistent with the hypothesis that the proliferative effects of estrogens and the antiproliferative effects of antiestrogens are mediated at least in part directly by modulation of the expression of autocrine/paracrine growth stimulatory and inhibitory factors. However, our own published data (1), that of others (19), and the data presented in this manuscript are not consistent with the regulation of endogenous expression of TGFα or EGF being directly responsible for inhibition of growth by antiestrogens in T-47D cells and possibly other human breast cancer cells. Progestins, anti-progestins, and antiestrogens are all growth inhibitory but differentially effect the regulation of the genes encoding TGFα and EGF. The data presented in this paper are, however, consistent with the hypothesis that the endogenous expression of these genes indirectly affects the sensitivity of the cells to growth inhibitory agents. That is, those cells which express endogenous growth factors (T-47D) are less sensitive or alternatively more resistant to antiestrogens and progestins. One possible explanation for this may be that the expression of these growth factors provides the cells with an alternative proliferation pathway in the presence of growth inhibitors. The T-47D-5 variant which contains approximately the same number of ER but one-tenth less PgR compared to our wild-type T-47D cells is more sensitive (2.5 times) to the growth inhibitory effects of both antiestrogens and progestins. This cell line, although it proliferates 1.5 times faster than T-47D cells, does not express the EGF gene and has a markedly reduced expression of TGFα compared to the wild-type cells. Indirectly we have demonstrated that growth factors can modulate the sensitivity of T-47D cells to antiestrogens and progestins by adding exogenous murine EGF to the medium. This manipulation reduced the sensitivity of both cell lines to the antiproliferative effects of progestins and antiestrogens. However, it should be noted that the effects of exogenous EGF were overall of small magnitude and EGF could not completely eliminate the growth inhibitory effects of antiestrogens and progestins in either cell line. Increasing the amount of EGF added to the medium did not further reduce the sensitivity of either cell line. However, we have previously shown (1) that T-47D cells secrete into the medium a major M, 40,000 and a minor M, 18,000 EGF-immunoreactive peptide and it is possible that their biological activity and/or potency may be different to that of the M, 6000 EGF which we added exogenously to the medium. Experiments which address this possibility, await the purification and biological assay of these EGF-immunoreactive peptides produced by the T-47D cells.

Interestingly, reduction by exogenous EGF of sensitivity of T-47D cells to MPA-induced growth inhibition, was the most marked response. MPA has been shown previously (2, 20) to increase EGF-receptors in T-47D cells. This may be the explanation for the increased sensitivity of these cells to EGF under these circumstances. Furthermore, it has recently been shown that exogenously added EGF reduces the level of progestosterone receptors in T-47D cells (21) which will in turn decrease the sensitivity of the cells to progestins. Our data concerning antiestrogens are consistent with that obtained by others (17, 18) although in Koga and Sutherland’s hands EGF alone does not stimulate the growth of T-47D cells. One explanation may be that we added EGF every day to the cells as opposed to one treatment only in Koga and Sutherland’s experiments (17). Consistent with our observations other groups have found EGF to be growth stimulatory for T-47D cells (22). Although both the antiproliferative effects of MPA and OH-Tam can be slightly reversed by exogenous EGF in T-47D-5, overall these effects are less pronounced than in T-47D cells and in particular T-47D-5 cells do not proliferate any faster in response to EGF under baseline growth conditions. Consistent with the view that the EGF/TGFα proliferative pathway is relatively inactive in T-47D-5 cells. The effect of exogenous EGF in T-47D cells is both to increase the growth rate of these cells as well as to partially reverse the growth inhibitory effects of MPA and OH-Tam. It has been shown previously that MPA increased the expression of EGF mRNA (1) and TGFα mRNA in T-47D cells which may be a compensatory response to the growth inhibition caused by the progestin. However, the increased expression of these growth factor genes is not a general phenomenon in response to growth inhibitory agents by T-47D cells which may be a compensatory response to the growth inhibition caused by the progestin. However, the increased expression of these growth factor genes is not a general phenomenon in response to growth inhibitory agents by T-47D cells since RU 486 (1) and antiestrogens (Fig. 2) do not significantly increase the expression of these genes but do inhibit the growth of these cells (Fig. 3) under the conditions described in this paper. It must be remembered however, that in these experiments cells are grown in 5% fetal calf serum and phenol red-containing medium, i.e., in the presence of estrogen. Antiestrogen inhibition of cellular growth and TGFα production may be due to loss of a stimulatory response rather than direct inhibition. A similar argument can, however, be made for the effect of progestins on growth inhibition. Progestin-induced growth inhibition has been reported to be an antiestrogenic response (4). Furthermore, when estrogen-responsive human breast cancer cells are grown in the absence of all estrogens, i.e., charcoal-stripped fetal calf serum plus phenol red-free medium, both
antiestrogens and progestins act to increase the proliferation of these cells (23, 24). It is therefore possible that the growth inhibition by both progestins and antiestrogens is due to loss of a stimulatory agent, i.e., estrogen. Irrespective of whether growth inhibition is a direct or indirect effect of these agents both progestins and antiestrogens differentially regulate the expression of growth factor genes in T-47D cells.

In conclusion, our data are not consistent with the antiproliferative effects of antiestrogens and progestins in human breast cancer cells being mediated directly by a decrease in the expression of endogenous growth factors. However our data do suggest that endogenous expression of growth factors may be associated with decreased sensitivity of the cells to growth-inhibitory agents. Direct testing of this hypothesis is of course necessary but if correct this may be one mechanism by which human breast cancer develops resistance to hormone-manipulative therapies.

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