Epidermal Growth Factor Gene Expression in Human Breast Cancer Cells: Regulation of Expression by Progestins

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ABSTRACT

Epidermal growth factor (EGF) is thought to be important in normal mammary development. The presence of EGF receptors in breast cancer cells suggests that it may also have a role in regulating growth of tumors of the human breast. Using a complementary DNA probe for the human EGF precursor we have examined expression of this gene in a series of human breast cancer cells in long term culture. The T-47D cell line demonstrated the highest level of EGF mRNA. EGF expression was not detectable in the MCF-7, BT 20, or HBL 100 cell lines. Surprisingly, in both T-47D and ZR 75 cells, pretreatment with progestins which exert antiproliferative effects under the conditions used increased EGF mRNA levels approximately 6-fold above untreated controls. This effect, demonstrable with as little as 0.1 nm of medroxyprogesterone acetate, was apparent as early as 12 h after addition of progestin and was reversed with the antiprogestin RU 486. Dexamethasone, estradiol, and dihydrotestosterone had no effect on EGF expression in T-47D cells. There was no evidence that the increased levels of EGF mRNA were due to gene amplification. Immunoprecipitation of biosynthetically labeled T-47D conditioned medium with antibodies to human EGF and EGF-precursor revealed the presence of both Mr 40,000 and 18,000 products. Fully processed Mr 6,000 EGF was not detectable in either conditioned medium or cell lysates. These data provide unequivocal evidence for the expression of the EGF gene in some human breast cell lines.

INTRODUCTION

The factors controlling growth and differentiation of the normal mammary gland are poorly understood. However, it has been appreciated for some time that these phenomena depend on the complex interaction of multiple hormones including estrogens, progestins, glucocorticoids, prolactin, and probably other known (e.g., EGF) and unknown factors (1). The relative roles and importance of these hormones and growth factors in breast development have not been established. Many of the same hormones and growth factors have been implicated in the pathogenesis of breast cancer (2).

EGF has potent growth promoting effects in many tissues including mammary epithelium of both rodent and human origin (3). While the precise role of EGF in controlling mammary gland growth and differentiation is not clear, many studies have implicated EGF as a normal physiological growth factor for the mammary gland (4). Recently, EGF has been shown to be present in human breast cyst fluid at higher concentrations than in colostrum or milk (5) and a role for EGF has been proposed in mouse mammary tumorigenesis (6). Receptors for EGF have been described on a number of human breast cancer cell lines (7) and in membranes prepared from breast cancer biopsies (8). Furthermore, in some but not all human breast cancer cell lines, EGF stimulates DNA synthesis and cellular proliferation (7). Taken together these observations suggest that EGF may have some role in human mammary cancer.

It has recently been appreciated that an important component of normal growth and neoplasia may involve the autocrine or paracrine action of locally synthesized growth factors. For example, in human breast cancer an autocrine function for TGF-α has been suggested (9, 10). However, the expression of EGF by human breast cancers has not been investigated. This paper presents unequivocal evidence that the EGF gene is expressed by some human breast cancer cell lines and that EGF mRNA levels are increased by progestins.

MATERIALS AND METHODS

Materials. MPA, dexamethasone, estradiol-17β, and dihydrotestosterone were purchased from Sigma. R 5020 and Org 2058 were purchased from Amersham. RU 486 was a gift from Roussel Uclaf (Romainville, France). Trans [35S]-label was from ICN Radiochemicals.

Cells. Human breast cancer cell lines, obtained from sources as previously described were grown in Dulbecco’s minimal essential medium supplemented with 10% fetal bovine serum, glutamine, glucose, and penicillin-streptomycin (7). 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 preparation.

Experiments in which cells were treated with various steroid hormones the cells were grown to confluence in the above medium, the medium was replaced with fresh medium, and the hormones were added directly from 1000× stock solutions in ethanol to achieve the concentrations indicated.

RNA Extraction and Northern Blot Analysis. RNA was isolated by the guanidinium thiocyanate/cesium chloride method (11). Poly(A)+ RNA was isolated by one cycle of oligodeoxynucleotidic acid cellulose chromatography (12). Ten−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 (13). 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 EGF15(c) (14) labeled with 32P by nick translation (Amersham, Oakville, Ontario Canada) to a specific activity of 106 cpm/μg (3× 107 cpm/hybridization were used). 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, Ficoll, and polyvinylpyrrolidine), 5× standard saline phosphate EDTA (1× standard saline phosphate EDTA = 1.15 M NaCl-0.01 M NaH2PO4-1 mM EDTA), 250 μg/ml denatured salmon sperm DNA, and 0.1% SDS. At the end of the hybridization period the blots were washed twice in 2× SSC, 0.1% SDS (1× SSC = 0.15 M NaCl-0.015 M sodium citrate) for
15–30 min at room temperature, followed by one wash in 0.1x SSC-0.1% SDS for 45–60 min at 65°C. Filters were also hybridized with chicken β-actin cDNA (15) 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. Hybridization signals were quantitated by densitometric scanning of multiple autoradiograms of various exposures and were expressed relative to the control arbitrarily assigned a value of 1.

DNA Extraction and Southern Blot Analysis. Genomic DNA, isolated from various human breast cancer cells was digested with PstI, fractionated on an 0.8% alkaline-agarose gel and transferred to nitrocellulose according to the protocol of Davis et al. (16). Hybridization and washing procedures were essentially as described above.

EGF Radioreceptor and Radioimmunoassay. Conditioned medium was obtained by incubating confluent T-47D monolayers with DMEM medium containing 0.1% bovine serum albumin for 24 h. The medium (2.5 liters) was clarified by centrifugation, dialyzed extensively against deionized water, lyophilized, and reconstituted with 5 ml of 0.01 M sodium phosphate buffer, pH 7.5. Samples were analyzed for their ability to inhibit murine 125I-labeled EGF (Collaborative Research, Inc., Bedford, MA) binding to T-47D cells (17). An EGF radioimmunoassay was established using reagents supplied by Biotopto Inc. (Seattle, WA). Rabbit anti-human EGF antisera, PA-135-R, was used at a final dilution of 1 in 1000 and hEGF (TR-130-U) was used as a standard and tracer. Under the conditions used the sensitivity of the assay was 0.5 ng/tube. Both human and mEGF were iodinated using the chloramine-T method was previously described (17).

Immunoprecipitation and SDS/Polyacrylamide Gel Electrophoresis. Rabbit polyclonal antiserum to EGF from 4 sources were used for immunoprecipitation studies: anti-mEGF (40011; Collaborative Research, Inc.); anti-h-EGF, raised against a synthetic peptide, residues 1–53 (PA-135-R; Biotopto, Inc.); anti-human EGF (C2); and anti-preproEGF-II. The latter two antisera were generously provided by Dr. L. Rall (Chiron Corp., Emeryville, CA) and were raised against synthetic human EGF and a synthetic peptide corresponding to the amino acid residues 348-691 of the mouse preproEGF-II protein (18), respectively.

MPA treated T-47D monolayers were incubated for 2.5 h with cysteine and methionine free DMEM containing 0.1 mg/ml soybean trypsin inhibitor at 37°C. This medium was removed and replaced with fresh cysteine and methionine free DMEM plus soybean trypsin inhibitor and 100 μCi/ml Tran35S-label (containing 70% [35Smethionine and 20% [35S]cysteine). The incubation was continued at 37°C for 4 h. At the end of this period the medium was collected, clarified by centrifugation, dialyzed extensively against distilled water at 4°C using dialysis membrane with a cut off value of M, 3500, lyophilized, reconstituted in immunoprecipitation buffer, and immunoprecipitation was carried out as previously described (19). Cell lysates were prepared by addition of immunoprecipitation buffer directly to the dishes. The lysate was collected, clarified by centrifugation, and the supernatant subjected to immunoprecipitation as previously described (19). Immunoprecipitated proteins were analyzed by SDS/polyacrylamide gel electrophoresis. The gels were fixed in 30% methanol/7% acetic acid followed by treatment with Amplify (Amersham), dried, and autoradiographed.

RESULTS

Presence of EGF mRNA in Some Human Breast Cancer Cells. In order to unequivocally ascertain the expression of the EGF gene in human breast cancer cells, poly(A)+ RNA was isolated from a variety of human breast cancer cell lines and analyzed by Northern blotting. Under stringent hybridization and washing conditions, a single mRNA species of approximately 5 kb was identified in some but not all human breast cancer cell lines (Fig. 1A). As well as a second EGF transcript of approximately 7 kb was apparent in the MDA 468 human breast cancer cells. RNA extracted from the male mouse submandibular gland was used as a positive control (Fig. 1B); the mouse EGF mRNA and 28S ribosomal RNA are both apparently smaller in size.

The highest levels of EGF mRNA were present in T-47D and ZR 75 human breast cancer cell lines. Furthermore, the cloned sublines of T-47D, clones 8 and 11 also contain high levels of EGF mRNA. Little if any EGF mRNA is present in the MDA 468, MDA MB 231 and HBL 100 cell lines. In order to unequivocally ascertain the expression of this gene may be under steroid hormone regulation. This possibility was tested by treating the "wild-type" T-47D cells for 24 h with 10 nM 17β-estradiol, dexamethasone, dihydrotestosterone, and various progestins (MPA, R5020, ORG 2058) (Fig. 2). Of the compounds tested, only the progestins resulted in a significant increase in EGF mRNA accumulation. This increase was not specific to the T-47D cells, because MPA but not estradiol increased EGF mRNA accumulation in ZR 75 cells (Fig. 3).

Interestingly, estradiol, if anything, caused a slight decrease in the accumulation of EGF mRNA in breast cancer cells. These hormones had little or no effect on β-actin mRNA levels (Fig. 3).

Dose-response curves indicated that a significant increase in EGF mRNA levels in T-47D cells was obtained with as little as 0.1 nM MPA while 10 nM MPA was maximal (Fig. 4A). Using the maximal concentration of 10 nM MPA, the time...
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Fig. 2. Effect of steroid hormones on EGF expression in T-47D human breast cancer cells. The relative abundance of EGF mRNA was determined by densitometry of Northern blots of RNA extracted from T-47D cells treated for 24 h with the various hormones at a final concentration of 10 nM. EGF mRNA abundance in untreated T-47D cells was arbitrarily attributed a value of 1. DHT, dihydrotestosterone; E2, 17β-estradiol; Dex, dexamethasone.

course of progestin-induced EGF mRNA accumulation was examined. The first detectable increase in EGF mRNA levels occurred 12 h after hormone treatment (Fig. 4B) and then continued to increase for up to at least 48 h after hormone treatment. Longer time course experiments revealed that EGF mRNA accumulation plateaued at around 48 h after MPA treatment and thereafter began to decline (data not shown). After prolonged exposure to MPA a second larger EGF transcript of about 7 kb was also apparent. The significance of this larger transcript is as yet unknown. These experimental manipulations had no effect on β-actin mRNA levels (Fig. 4B). In a series of six separate experiments, treatment of T-47D cells for 24 h with 10 nm MPA resulted in a 6 ± 1-fold (SEM) increase in EGF mRNA. At maximal concentrations, progestins increased the accumulation of EGF mRNA in exponentially growing as well as confluent T-47D cells. T-47D cells, which had been grown for two passages in medium containing charcoal-stripped fetal bovine serum, responded to progestin treatment with a more marked increase (approximately, 18-fold) in EGF mRNA accumulation (data not shown).

Since under certain conditions progestins have been shown to be growth stimulatory for human breast cancer cells (20) we examined the effects of MPA on T-47D cell growth under the conditions used here, where MPA stimulated the accumulation of EGF mRNA. Under these conditions a dose-dependent inhibition of cell proliferation was seen (Fig. 5A). Furthermore when cells were grown in medium containing 10% charcoal-stripped fetal bovine serum the antiproliferative effect was even more pronounced (Fig. 5B).

We also examined the effect of the anti-progestin RU 486 on EGF expression in T-47D cells. The results of this experiment are presented in Fig. 6. RU 486, an antiproliferative agent in these cells (21) under the culture conditions used in these experiments (data not shown), had no effect alone on the accumulation of EGF or β-actin mRNA (Fig. 6, lanes 3 and 4). However, RU 486 did inhibit the effect of MPA on EGF mRNA accumulation (Fig. 6, lanes 5 and 6) but it did not alter β-actin mRNA levels.

EGF Immunoreactivity in T-47D Conditioned Medium. To investigate the nature of the translation product(s) of the EGF gene in T-47D cells we initially isolated conditioned medium from T-47D cells and examined its ability to inhibit the binding of 125I-labeled EGF to its receptor (Fig. 7). Radioimmuneactive material was present in the conditioned medium of these cells. However, it has been demonstrated previously (22) that T-47D cells express and secrete immunoreactive-TGF-α-like peptides which interact with the EGF-receptor. We next used a specific EGF radioimmunoassay in an attempt to measure EGF-activity specifically in conditioned medium. Although some displacement (15%) of 125I-labeled hEGF was detected, the absence of parallel displacement with authentic hEGF suggested that it was unlikely to be due to the mature M, 6,000 EGF peptide.
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Fig. 5. A, dose-dependent effect of medroxyprogesterone acetate on growth of T-47D cells. Cells were plated at $10^4$ cells/dish in medium containing 5% fetal bovine serum; 3 days later fresh medium containing the indicated concentrations of MPA was added. Duplicate dishes of cells were harvested at the indicated times and cell numbers counted on a Coulter Counter. A representative experiment is shown; however, the mean ± SEM (bars) of 5 separate experiments after 6 days of MPA treatment were vehicle alone, $33.9 ± 5 \times 10^4$ cells/dish; 1 nM MPA, $21.2 ± 4 \times 10^4$ cells/dish; 10 nM MPA, $18.2 ± 1 \times 10^4$ cells/dish; 100 nM MPA, $17.4 ± 1 \times 10^4$ cells/dish; 1000 nM MPA, $14.8 ± 1 \times 10^4$ cells/dish. All groups except 1 nM MPA were significantly different from the vehicle only group with at least $P < 0.01$, using Student's t test. B, T-47D cells which had been grown in medium containing 1% charcoal-stripped fetal bovine serum were treated as in A except that all treatments were performed in the presence of medium containing 10% charcoal-stripped fetal bovine serum. Mean doubling times were calculated from the initial ($n_i$) and final ($n_f$) cell numbers from the equation, doubling time = $t \log 2/\log(n_f/n_i)$, where $t$ is time between $n_i$ and $n_f$. From this equation the proliferation rate could be calculated as the percentage of control

$$\text{Proliferation rate (control)} = \frac{\text{doubling time (control)} \times 100}{\text{doubling time}}$$

Results (plotted as the mean ± SEM from three experiments) obtained with cells grown in medium containing 5% fetal bovine serum are shown by $\bullet$ and results obtained with cells grown in medium containing 10% charcoal-stripped fetal bovine serum are shown by $\oplus$. The difference between growth conditions was significant, at $P < 0.05$, for all points on the curve except the 1000 nM point, using Student's t test.

Fig. 6. Effect of RU 468 on EGF mRNA abundance in T-47D human breast cancer cells. Cells were treated for 24 h with vehicle alone (lane 1), 10 nM medroxyprogesterone acetate (lane 2), 10 nM RU 486 (lane 3), 100 nM RU 468 (lane 4), 10 nM medroxyprogesterone acetate plus 10 nM RU 486 (lane 5), and 10 nM medroxyprogesterone acetate plus 100 nM RU 486 (lane 6). Bottom, pattern of hybridization obtained with chicken &-actin cDNA. $\ast$, position of the 28 and 18S ribosomal markers, top and bottom, respectively. Relative exposure time of blots to X-ray film for EGF versus actin hybridization was 3 to 1.

Fig. 7. EGF-radioreceptor assay of conditioned media obtained from T-47D cells. Media were conditioned by T-47D cell monolayers, dialyzed, lyophilized, and reconstituted as described in "Materials and Methods." $\oplus$, displacement of $^{125}$I-labeled EGF from T-47D EGF receptors by murine EGF.

(data not shown). Subsequently we have used immunoprecipitation and SDS/polyacrylamide gel electrophoresis to analysis [S]$^{35}$methionine/cysteine biosynthetically labeled material in both the conditioned medium and cell lysates of T-47D cells. Using three different anti-EGF antisera (Biotope anti-human EGF, anti-human EGF-C2, anti-preproEGF-II), one major protein band of approximately $M_r$, 40,000 has been specifically immunoprecipitated from the conditioned medium of T-47D cells (Fig. 8). An additional less abundant protein of $M_r$, 18,000 was specifically immunoprecipitated with two (Biotope anti-human EGF, anti-human EGF-C2, anti-preproEGF-II), one major protein band of approximately $M_r$, 40,000 has been specifically immunoprecipitated from the conditioned medium of T-47D cells (Fig. 8). An additional less abundant protein of $M_r$, 18,000 was specifically immunoprecipitated with two (Biotope anti-human EGF, anti-human EGF-C2) of the three antisera. However the $M_r$, 33,000 EGF precursor (14, 18) or the fully processed $M_r$, 6,000 EGF peptide was not detected under these conditions. We were unable to specifically immunoprecipitate any proteins in T-47D cell lysates, suggesting that the translation product of the EGF gene in these cells is rapidly synthesized and secreted from the cells.

Nonamplification of EGF Gene in Human Breast Cancer Cells Having High Levels of EGF mRNA. The possibility that expression of the EGF gene in some human breast cancer cells was due to amplification or rearrangement of the gene was investigated by Southern analysis of DNA isolated from a number of
human breast cancer cell lines and comparison to that of normal human placental DNA. The results, presented in Fig. 9 show that there was no amplification of the EGF gene in any of the human breast cell lines tested. In two of the cell lines, HBL 100 and BT 20, there were additional PstI fragments (Fig. 9). It is unknown at this time if these effects indicate a subtle rearrangement of the EGF gene in these cells or are due to undescribed restriction fragment length polymorphism. The latter result seems more likely.

DISCUSSION

There is a considerable amount of circumstantial evidence which supports a role for EGF or EGF-like proteins in normal mammary gland development. In addition, EGF or other growth factors that bind the EGF receptor could contribute to the unregulated proliferation of breast tumor cells. TGF-α, a growth factor that is structurally related to EGF and is a ligand for the EGF receptor, has been identified in human breast cancers (9, 22) and Dickson et al. (22) have suggested that TGF-α may function in an autocrine fashion to mediate estrogen induced proliferation in MCF-7 cells. In this study we have demonstrated that EGF mRNA is expressed at relatively high levels in both T-47D and ZR 75 human breast cancer cell lines. Since a number of investigators have presented data that exogenous EGF stimulates human breast cancer cell DNA synthesis and cell proliferation (7, 19, 23) it is surprising that progestins which are antiproliferative under these conditions (Ref. 24; Fig. 5A) increase the expression of the endogenous EGF gene. Since the control of cellular proliferation is now thought to involve a complex interaction of mitogens and growth inhibitory factors (25), one possible explanation is that expression of the EGF gene, encoding a putative growth-stimulatory factor, could represent a compensatory response to growth inhibition by progestins. Although this explanation would be consistent with our observation that an EGF nonexpressing variant of T-47D cells is much more sensitive to the antiproliferative effects of progestins than the EGF expressing cell line, an increase in endogenous EGF expression in T-47D cells cannot be a general response to inhibition of proliferation since nonsteroidal antiestrogens and the antiprogestin RU 486 do not effect EGF expression in T-47D cells. Furthermore, the antiproliferative effects of both progestins and antiestrogens in T-47D cells can be partially reversed by the exogenous addition of the M, 6,000 EGF peptide.7 Alternatively the progestin effect on T-47D cells may be due to a growth inhibitory EGF effect. Although considerable emphasis has been placed on the mitogenic actions of EGF it is of interest that in certain circumstances EGF is growth inhibitory. For example, the proliferation of certain cell lines such as A-431 and MDA-468 is actually inhibited by EGF (26, 27). However, this is not the case for T-47D cells where the exogenous addition of M, 6,000 EGF increases cellular proliferation and partially reverses progestin-induced growth inhibition.7 Since T-47D cells appear to synthesize and secrete EGF-immunoreactive proteins of M, 40,000 and 18,000 but not the M, 6,000 EGF peptide per se, it may be that these larger proteins have different biological activities from those described for the M, 6,000 EGF peptide. Different processed forms of EGF proteins have been reported previously to be present in the kidney (14, 18), human urine (28), and platelets (29). The biological activity of these different EGF-immunoreactive proteins as well as those now identified in conditioned medium from T-47D cells is as yet unclear. Further speculation as to the significance of EGF expression in T-47D cells has been presented previously (9, 22).

Fig. 8. Autoradiograph of [35S]methionine and cysteine biosynthetically labeled medium from T-47D cells immunoprecipitated with preimmune rabbit serum (B) and Biotope anti-human EGF antiserum (C) and analyzed on a 12% polyacrylamide/SDS gel. Molecular mass markers (A) are 125I bovine serum albumin (M, 70,000), ovalbumin (M, 44,000), carbonic anhydrase (M, 30,000), and cytochrome C (M, 12,300). kDa, kilodaltons, expressed as molecular weight throughout; ←, protein specifically immunoprecipitated using EGF antisera, as described in text.

Fig. 9. Southern blot analysis of DNA from human breast cancer cells. Ten μg of genomic DNA were digested with PstI, fractionated, and subjected to Southern blot analysis and hybridization with 32P-labeled EGF cDNA as described under “Materials and Methods.” A, MCF 7; B, T-47D; C, HBL 100; D, BT 20; E, MDA MB 231; F, human placental DNA.
cells awaits the characterization of the biological activities of the EGF-immunoreactive proteins produced by T-47D cells.

The observations reported here provide the first unequivocal evidence that the EGF gene is expressed and hormonally regulated in human breast cancer cells. While the biological role of this expression is as yet unclear our observations raise the possibility that EGF gene expression may have a biological role in human breast cancer. The T-47D model will be useful in addressing this question.

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