Epidermal Growth Factor Stimulation of Human Breast Cancer Cells in Culture

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ABSTRACT

Epidermal growth factor (EGF), a polypeptide found in human and animal blood and secretions, has been found to stimulate a variety of tissues in vitro including normal and malignant rodent mammary epithelium and human breast epithelial cells and fibroadenoma. We have studied the influence of EGF on malignant human breast tissue with a model system comprising human breast carcinoma cells growing in tissue culture. EGF stimulated growth of MCF-7 cells in serum-free medium. After 7 days in culture, a 2-fold increase in cell number and DNA content and a 3-fold increase in total protein were observed in cells incubated with EGF (10 ng/ml). As little as 0.01 ng/ml of EGF stimulated growth; 10 ng/ml was maximal. EGF effects on growth were noted for cells plated at a high as well as sparse (cloning) density. EGF also stimulated the rates of thymidine, uridine, and leucine incorporation into macromolecules in a dose- and time-dependent fashion. Stimulation of uridine and leucine incorporation was evident by 3 hr, whereas EGF stimulation of thymidine incorporation was delayed until 12 to 18 hr. EGF increased the proportion of cells active in DNA synthesis by nearly 2-fold. The combination of optimal concentrations of insulin (also a growth factor for these cells) and EGF did not stimulate growth above that seen with either hormone alone, suggesting a common step in their mechanism of action. The EGF effect was not dependent on the presence of serum and was not enhanced by dexamethasone as reported for other types of cells. EGF had no effect on another human breast cancer cell line, the MDA-231. These studies suggest that growth of some human breast cancers may be influenced by EGF.

INTRODUCTION

EGF is a single-chain polypeptide isolated first from mouse submaxillary glands (7) and later from human urine (8). Although mouse EGF and human EGF differ slightly with regard to molecular weight, amino acid composition, and immunological properties, they both compete for the same cell membrane receptor and demonstrate similar biological activities (6). The tissue site of EGF production and the regulation of its secretion are not understood. However, it is known that daily urinary excretion of EGF in humans exceeds 50 μg/day, is significantly higher in females, and is 50% higher in women who are taking oral contraceptives (9). In addition, EGF has been detected in human plasma, human milk, and other secretions in measurable concentrations (ng/ml) (9, 22). EGF appears to be closely related if not identical to the gastric antisecretory hormone urogastrone (12).

Although the relevance of EGF to normal human physiology and disease has not been clarified, data suggest that this polypeptide may be an important regulator of cell growth. EGF is mitogenic for a panoply of ectoderm-, mesoderm-, and endoderm-derived cells in tissue culture (11). However, not all cells in culture respond to EGF, which suggests some target tissue specificity and supports the notion that it may have a physiological role in vivo.

Several studies indicate that EGF may regulate growth of mammary epithelium: (a) EGF promotes growth of normal rodent mammary tissue and rodent breast cancer (25); (b) it enhances growth of normal human mammary epithelial cells in short-term culture (24); and (c) it stimulates mitosis of cells derived from benign human breast fibroadenomas (23). The effect of EGF on malignant human breast tissue has not been reported in detail, but one might speculate that certain breast cancers could retain the sensitivity to EGF observed with nonmalignant mammary cells. In the present studies, we have examined the effects of EGF on human breast cancer cells maintained in long-term tissue culture, a system well suited for biochemical studies of hormone action since the environmental milieu of the cells can be carefully defined. This model system has been characterized in detail and is used by several laboratories for studies of steroid and peptide hormones (18). We find that EGF stimulates proliferation of a human breast cancer cell line, suggesting that it may be an additional hormonal factor regulating breast cancer growth in certain patients with this disease.

MATERIALS AND METHODS

Materials. Mouse EGF was purchased from Collaborative Research, Inc. (Waltham, Mass.). Porcine insulin (Lot 615-D63-10, 25.4 units/mg) was the gift of Dr. M. Root, Lilly Research Laboratories (Indianapolis, Ind.). Crystalline TCA was purchased from Baker Chemical Co. (Phillipsburg, N. J.), and [3H]thymidine (15 Ci/mmol), [5-3H]uridine (29 Ci/mmol), and L-[U-14C]leucine (342 mCi/mmol) were obtained from the Radiochemical Centre, Amersham, England.

Cells and Tissue Culture Techniques. Both human breast cancer cell lines were initially derived from malignant effusions of women with metastatic breast cancer and have been in continuous tissue culture for at least 4 years. Characterization of the human and mammary origin of these cells has been summarized previously (10, 13, 18). The MCF-7 cell line was
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provided by Dr. H. D. Soule at the Michigan Cancer Foundation (21). These cells are maintained in monolayer culture in IMEM (Grand Island Biological Co., Grand Island, N. Y.) supplemented with glutamine (0.6 g/liter), penicillin (62 mg/liter), streptomycin (130 mg/liter), insulin (1.0 nm), and 5% calf serum. The MDA-231 cell line was initially isolated by Dr. R. Cailleau at M. D. Anderson Hospital and Tumor Institute (4) and supplied to us by Dr. M. Lippman of the National Cancer Institute. These cells are maintained in the same medium as the MCF-7 cells but with 10% fetal bovine serum and no insulin supplementation. Both cell lines are grown in a humidified incubator in 5% CO2 at 37°, and both are free of Mycoplasma contamination. The cells are subcultured weekly with the use of 0.05% trypsin:0.02% EDTA in 150 mm NaCl to remove cells from the culture dish. In all experiments, cells were harvested by suspending them with this same solution.

Precursor Incorporation and Growth Studies. Methods for the measurement of precursor incorporation into macromolecules and cell growth have been summarized previously (17, 20). Briefly, incorporation studies were performed by placing cells in replicate to a subconfluent density (about 7 x 105 cells/well) in 4 ml of IMEM supplemented with the appropriate serum in multiwell culture dishes (FB-4-TC; Linbro Chemical Co., New Haven, Conn.). After the cells had attached to the dish (24 hr), the medium was exchanged for serum-free IMEM. After a further 24 hr, hormones (or hormone-free carrier for controls) were added directly to the incubation medium, and the cells were incubated for specified times. During the last 2 hr of incubation, cells were pulsed with labeled thymidine (0.5 μCi/ml), uridine (1.0 to 2.0 μCi/ml), or leucine (0.5 μCi/ml) and then were harvested. An aliquot of the cell suspension was taken for DNA determination (3), and the remaining cells were disrupted by sonicating for 3 sec in a Heat-Systems Ultrasonic sonicator (Plainview, N. Y.) with a microtip at the lowest setting. Precursor incorporation was determined by measuring the radioactivity precipitated in 10% TCA after collection on Millipore filters (0.45 μm, type HA). The rates of precursor incorporation were linear under the conditions used.

Cell growth studies were performed by determining the total DNA (3) or protein (15) content of cells plated in replicate on Petri dishes (Falcon Plastics, Oxnard, Calif.) after incubation in serum-free medium (unless stated otherwise) with or without EGF or other factors. Cell number was determined by visual counts in a hemocytometer. Changes in DNA content closely paralleled changes in population size.

TLI. The TLI was determined by a modification of previously published methods (14). Cells were plated in replicate and incubated with hormones, as described for the incorporation studies. At the end of the incubation period, they were pulsed with 3H-thymidine (5 μCi/ml), washed twice, harvested, and centrifuged (600 rpm for 2 min). The supernatant containing the harvesting medium was discarded. The cells were resuspended in chilled (4°) Dulbecco’s phosphate-buffered saline (pH 7.4, without Ca2+ or Mg2+) at a concentration of about 2.5 x 105 cells/ml. Two-tenths ml of the dilute cell suspension was added to a cytcentrifuge cup and centrifuged for 5 min at 1000 rpm (Shandon Elliott, Southern Instruments, Inc., Sewickley, Pa.). After a drying time of 4 hr, the cytcentrifuge-prepared slides were dipped 10 times in each of 2 staining dishes containing 5% cold TCA and then dipped 5 times in a dish containing methyl alcohol. After drying, the slides were dipped in Kodak NTB-2 emulsion, exposed for 24 hr in a light-tight box, developed with D19 developer, and fixed with Kodak general purpose fixer. The slides were dried and then stained with May-Grünwald-Giemsa. A total of 500 cells were counted for each of 4 slides representing one experimental group (2000 cells total). The labeling index (labeled/unlabeled cells) was calculated by counting the fraction of intact cells containing 8 or more grains over the nucleus. Background counts for the autoradiographs were always less than 5 grains/cell nucleus.

Statistical Analysis. When applicable, the means of experimental groups were compared by using Student’s t test.

RESULTS

Effects of EGF on Growth. EGF significantly stimulates growth of the MCF-7 human breast cancer cells (Chart 1 A and B). Cell proliferation as determined by measuring the total DNA and protein content of dishes plated in replicate was augmented by the addition of EGF (10 ng/ml) to cells growing in serum-free medium. Saturation density of the cells was reached by 7 days in both control and EGF-treated cultures. At this time, DNA content was increased nearly 2-fold and total protein was increased 3-fold in cultures incubated with the polypeptide. The increased DNA content in this experiment paralleled a 2-fold increase in actual cell number after 5 days [control = 7.2 ± 0.2 x 105 (S.D.) cells/dish; EGF = 13.3 ± 0.9 x 105 cells/dish], indicating that EGF stimulates cell division and not just synthesis of DNA and protein. The observation that EGF stimulates protein synthesis to a greater extent than DNA synthesis and mitosis (a hypertrophic effect) was reproducible in multiple experiments. In a separate but similar experiment (Chart 1C), the effect of EGF on actual cell number was determined. The initial doubling time was reduced from about 36 to 24 hr by the hormone. It should be emphasized that control cells in serum-free medium alone were not dying under these conditions but continued to synthesize macromolecules and to divide. Thus, the EGF effect is not simply to delay or prevent cell death but to increase multiplication of these cells.

Growth of MCF-7 cells is extremely sensitive to EGF (Chart 2). Stimulation of DNA and protein synthesis was observed with as little as 0.01 ng of EGF per ml (p < 0.05 and 0.005, respectively). Half-maximal stimulation was noted with concentrations between 0.1 and 1.0 ng/ml, and maximal stimulation was noted at 10 ng/ml. These EGF concentrations are similar to those required for a mitogenic effect in other tissues (5, 11). Furthermore, these concentrations are within the range of concentrations reported for EGF in human secretions and plasma by radioimmunoassay (9, 22). A modest but consistent reduction in the stimulatory effect of EGF was noted at concentrations of 50 ng/ml or greater.

The effect of EGF on growth is evident even when MCF-7 cells are plated at a very low ("cloning") density (Table 1). In this experiment, cells were plated at a density of 500 cells/60-mm Petri dish and incubated for 15 days in serum-free medium with and without EGF or 5% calf serum. Surprisingly, cells maintained in serum-free medium alone slowly divided to reach a saturation density of 11.4 x 104 cells. The addition of EGF resulted in a 2-fold increase in saturation density. However, the effect of EGF is only about 10% of that observed with calf
again, no EGF effect was observed (Table 2). Similar experiments using higher serum concentrations (1%) or serum stripped of steroid hormones and probably many proteins by treatment with dextran-coated charcoal failed to produce EGF stimulation of the MDA-231 cells (data not shown). Of interest is our previous observation that these cells are also insensitive to insulin (20). The factors in serum responsible for growth stimulation of this cell line remain unknown.

**Effects of EGF on Precursor Incorporation.** We also examined the effects of EGF on the rates of incorporation of radioactive precursors into macromolecules in MCF-7 cells. The sensitivity of these cells to physiological concentrations of EGF was confirmed by the increased incorporation of \(^{3}H\)thymidine, \(^{3}H\)uridine, and \(^{14}C\)leucine into TCA-precipitable material (Chart 3). Slight stimulation was observed with 0.10 ng of EGF per ml, and maximal stimulation was observed with 10 ng/ml, in parallel with the dose-response curve of growth stimulation (Chart 2). The magnitude of the maximal EGF stimulation of precursor incorporation above control values (30

### Table 1

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>EGF</th>
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<tbody>
<tr>
<td>No. of cells (x 10^5)</td>
<td>11.4 ± 0.6</td>
<td>13.5 ± 0.3</td>
</tr>
<tr>
<td>DNA (μg)</td>
<td>9.1 ± 0.2</td>
<td>14.3 ± 0.3</td>
</tr>
<tr>
<td>Protein (μg)</td>
<td>55.9 ± 4.0</td>
<td>109.4 ± 6.1</td>
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</table>

*Mean ± S.E. of quadruplicate determinations.

### Table 2

**Effect of EGF on growth of the MDA-231 cell line**

Cells were plated as described in Chart 1 (4 x 10^5 cells/100-mm Petri dish) and EGF was added at the indicated concentrations. The cells were fed fresh medium on Day 3 and harvested on Day 5 for DNA and protein determination. Results are the means of quadruplicates. Bars, S.E.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>EGF</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA (μg)</td>
<td>1561 ± 34</td>
<td>2016 ± 68</td>
</tr>
<tr>
<td>Protein (μg)</td>
<td>1355 ± 34</td>
<td>2016 ± 68</td>
</tr>
</tbody>
</table>

*Mean ± S.E. of triplicate determinations.*
to 100% depending on the experiment) was similar to the maximal stimulation induced by insulin in these cells (17).

The time course of the effects of EGF on thymidine, uridine, and leucine incorporation into macromolecules is also similar to that observed with insulin (17). Stimulation of leucine and uridine incorporation occurred shortly after EGF addition (by 3 hr) and was maximal from 12 to 18 hr (Chart 4). On the other hand, EGF stimulation of thymidine incorporation was evident only after a lag period of about 12 hr; maximal stimulation occurred at about 24 hr incubation with EGF. This ordered, sequential stimulation of precursor incorporation by EGF suggests that it is not simply due to altered precursor uptake into the cell (usually an immediate effect) with subsequent changes in the specific activity of the precursor pool. The fact that EGF also stimulates the thymidine labeling index (see below) supports that contention and suggests that an effect of EGF may be to increase the proportion of cells actively synthesizing DNA.

**TLI.** The TLI provides an estimate of the fraction of cells in a population actively engaged in DNA synthesis at a given time (14). EGF significantly augmented the TLI of MCF-7 cells (Table 3). Nearly a 2-fold increase in TLI above that seen in cells maintained in control medium was observed for cells incubated with EGF for 24 hr. This effect on the TLI was identical to that seen with insulin (10 nM). Interestingly, no additive effect on TLI was observed in cells incubated with optimal concentrations of the 2 hormones together. Thus, a major effect of EGF and insulin in these cells may be to increase the fraction of cells in the population that synthesize DNA. Additional studies will be required to more accurately define the effects of these hormones on other parameters such as cell cycle transit times.

Because the effects of EGF and insulin on the TLI were not additive, we wondered whether cell proliferation was influenced in a similar fashion by these 2 growth factors. When MCF-7 cells were incubated with optimal concentrations of EGF or insulin for 6 days, the effect on cell growth as measured by total DNA or protein content of dishes plated in replicate was strikingly similar (Table 4). A 50% increase in DNA content and a 75 to 100% increase in total protein were observed. However, an additive effect on DNA and protein content was not observed when the cells were incubated with both hormones. On the contrary, a slight decrease in DNA was noted for cells exposed to both EGF and insulin together, and only a minimal increase (not additive) in total protein was noted in this group. These data suggest the possibility that the stimulation of MCF-7 cells by EGF and insulin may involve a common step in the mechanism of action of these hormones. Furthermore, they suggest that the pathways of regulation of DNA and protein synthesis by these hormones may be different, since changes in protein content do not always parallel changes in DNA or cell number.

**Influence of Glucocorticoids and Serum on the EGF Response.** Several reports have indicated that the presence of either serum or the glucocorticoid dexamethasone enhances the mitogenic response of human fibroblasts in culture to EGF (1, 5, 11, 23). Presumably, serum provides a cofactor necessary for a maximum EGF response, whereas dexamethasone may increase the binding of EGF to its receptor. In contrast, the response of MCF-7 cells to EGF is not enhanced by dexamethasone or by low concentrations of calf serum (Tables 5 and 6, respectively). Dexamethasone (1.0 or 100 nM) for 6 days resulted in a minimal increase in total cell protein or DNA per dish (Table 5). However, when dexamethasone was added with EGF, a decrease in protein and DNA compared to EGF alone was observed (p < 0.025). This antagonism of the EGF response by dexamethasone is similar to antagonism by dexamethasone of the mitogenic response to insulin in MCF-7 (17) and ZR 75-1 (19) human breast carcinoma cells.

Similarly, calf serum did not synergistically enhance the effect of EGF in MCF-7 cells (Table 6). In serum-free medium EGF caused a 3-fold rise in total protein per dish and a 70% increase in DNA over untreated cells. Calf serum stimulated DNA and protein accumulation in a dose-dependent manner.
but did not augment the EGF effect. In fact, in the presence of 0.5% calf serum EGF increased protein accumulation 30% above that seen with the calf serum alone, but no additional stimulation of DNA content was observed. Thus, serum factors are not required for, nor do they synergistically enhance, the mitogenic response to EGF of MCF-7 cells.

**DISCUSSION**

The importance of EGF for cellular growth regulation in vivo has not been defined. *In vitro* studies, however, suggest that a variety of tissues may be targets for EGF action. Mammary epithelium is a potential target for this hormone. It is of interest that levels of EGF in humans are higher in females and are increased further by pregnancy or exogenous sex steroid hormone administration (9), conditions which drastically alter mammary gland growth and development. Furthermore, cell culture studies suggest that EGF regulates growth of rodent and human mammary epithelial cells (23-25). The present studies demonstrate that EGF is also a potent growth stimulus for human breast cancer cells in culture, thus confirming a preliminary report that EGF is a necessary ingredient for growth maintenance of these cells in a defined serum-free medium (2). Physiological concentrations of EGF enhance MCF-7 cell multiplication and increase the rates of thymidine, uridine, and leucine incorporation into macromolecules in these cells. A major effect of this hormone appears to be an increase in the number of S-phase cells in the population. Whether this is due to recruitment of nonproliferating cells into the cell cycle or changes in cell cycle transit times will require further study. The observation that another breast cancer cell line, the MDA-231, is not sensitive to EGF suggests that only certain breast tumors retain the biomolecular machinery necessary for an EGF effect and that the stimulatory effect of EGF on the MCF-7 cells is not an artifact of tissue culture conditions.

Unlike effects on certain other types of cells in culture, the effect of EGF on MCF-7 cells is evident in serum-free medium and is not exaggerated in the presence of calf serum. In addition, dexamethasone does not augment the EGF response in these cells, but, in fact, slightly antagonizes the response. These disparate results among different cells in culture may be due to culture conditions, or may reflect genuine differences among tissues in their hormonal responsiveness. We have also observed that the mitogenic action of insulin is antagonized by glucocorticoids in human breast cancer cells in tissue culture (17, 19). One possible explanation for these effects is that dexamethasone could cause a nonspecific alteration of the cell membrane, thereby affecting both EGF and insulin receptors. Another possibility is that dexamethasone opposes the action of EGF and insulin at a common site distal to receptor binding.

The effects of EGF on the MCF-7 cell line are strikingly similar to the effects we have reported previously for insulin (17, 19). (a) The stimulation of growth and precursor incorporation into macromolecules is quantitatively equivalent with both hormones. (b) Both hormones stimulate cell proliferation and the TLI, and the effects are not additive when the cells are exposed to optimal concentrations of both hormones. (c) The time course of stimulation of thymidine, uridine, and leucine incorporation by insulin and EGF are nearly identical. (d) Glucocorticoids modulate the effects of both hormones in a similar manner. (e) The MDA-231 cell line, which is unresponsive to insulin, also fails to respond to EGF. These observations suggest that EGF and insulin may stimulate a common biochemical pathway in MCF-7 cells leading to enhanced growth. Since specific receptors for each of these polypeptides have been detected in target cells and since neither hormone competes for the receptor of the other in MCF-7 cells (20), it is unlikely that a "common pathway" involves a common receptor binding site at the cell membrane. However, after initial binding of these hormones to receptors dispersed on the cell surface, it has been shown by fluorescent techniques that bound insulin and EGF rapidly migrate on the membrane to form patches where both are internalized within the same vesicle by a common pathway (16). Whether patching and internalization are related to the growth effects of these hormones is not known, but it is interesting to speculate that the similar responses to EGF and insulin observed in MCF-7 cells are related to this event.

In summary, we have shown that EGF stimulates growth of human breast cancer cells in tissue culture. These results, in concert with other studies of rodent and human mammary epithelium, suggest the possibility that EGF has a regulatory role in the growth and development of the breast and perhaps of breast cancer.

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* C. K. Osborne and B. Hamilton, unpublished data.

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**Table 4**

| Effect of EGF and insulin on MCF-7 cell growth |
| DNA/dish (µg) | Protein/dish (µg) |
| Control | 33.5 ± 2.4* | 406 ± 24 |
| EGF | 49.6 ± 0.7 | 806 ± 27 |
| Insulin | 50.4 ± 2.5 | 728 ± 20 |
| Insulin + EGF | 44.5 ± 1.4 | 731 ± 56 |

* Mean ± S.E.

**Table 5**

| Effect of dexamethasone on the MCF-7 response to EGF |
| DNA/dish (µg) | Protein/dish (µg) |
| Control | 115 ± 5d | 1709 ± 113 |
| EGF | 216 ± 4 | 4233 ± 125 |
| Dexamethasone (1.0 nm) | 133 ± 4 | 2321 ± 176 |
| Dexamethasone (1.0 nm) + EGF | 185 ± 6 | 3403 ± 180 |
| Dexamethasone (100 nm) | 137 ± 8 | 2405 ± 24 |
| Dexamethasone (100 nm) + EGF | 164 ± 4 | 3518 ± 280 |

* Mean ± S.E.

**Table 6**

| Effect of serum on the MCF-7 response to EGF |
| DNA/dish (µg) | Protein/dish (µg) |
| Control | 19.6 ± 1.5 | 234 ± 43 |
| EGF (10 ng/ml) | 33.6 ± 0.5 | 676 ± 30 |
| Calf serum (0.1%) | 30.4 ± 0.4 | 513 ± 17 |
| Calf serum (0.1%) + EGF | 46.2 ± 0.8 | 965 ± 23 |
| Calf serum (0.5%) | 62.7 ± 1.6 | 1062 ± 117 |
| Calf serum (0.5%) + EGF | 56.1 ± 1.6 | 1326 ± 87 |

* Mean ± S.E.
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