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Biological action of epidermal growth factor and its functional receptors in normal mammary epithelial cells

(mammary gland/epidermal growth factor receptor/tumor promoter/cell proliferation/differentiation)

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ABSTRACT **Normal murine mammary epithelial cells possessed the capacity to bind epidermal growth factor (EGF) in a specific and saturable manner. Scatchard plot analysis showed a curvilinear pattern. Assuming that this is not due to negative cooperativity of EGF binding, the data suggest the presence of two classes of receptors with a high and a low affinity: K_d values = 1×10^{-10} M and 3.6×10^{-9} M, respectively. The number of high- and low-affinity receptors was estimated to be 800 per cell and 8,600 per cell, respectively. The occupancy of EGF receptors for a half-maximal stimulation of DNA synthesis or inhibition of casein synthesis was about 10% and 6% of total receptors, respectively. A potent tumor promoter, 12-*O*-tetradecanoylphorbol 13-acetate, acted synergistically with EGF in terms of stimulation of DNA synthesis but not in terms of inhibition of casein synthesis when the two agents were added at a suboptimal concentration. The presence of the tumor promoter increased the amount of EGF bound to mammary cells in culture and also decreased a loss in the amount of EGF in the culture medium. These results indicate that mammary epithelial cells possess functional receptors for EGF, which are modulated by the tumor promoter.**

Epidermal growth factor (EGF) is a single-chain polypeptide hormone produced and secreted by the salivary gland (1), but its physiological function has not been fully established. It has been shown (2) that the level of an EGF-like substance in serum is elevated markedly during pregnancy when the mammary gland undergoes extensive cell proliferation and its ability to synthesize milk proteins is suppressed. Recently, we have found that EGF stimulates mammary cell proliferation and inhibits the synthesis of milk proteins casein and α-lactalbumin as well as the accumulation of milk protein mRNAs in a primary mouse mammary epithelial cell culture system (3). Because EGF elicits its actions at physiological concentrations, we have postulated that EGF serves as a regulator for the growth and differentiation of mammary epithelial cells (3). Because biological actions of EGF are thought to be mediated through specific high-affinity receptors on the plasma membrane (4), it is important to demonstrate the presence of EGF receptors and to examine their properties in this system.

Earlier studies have indicated that some of the biological actions of EGF can be mimicked by 12-*O*-tetradecanoylphorbol 13-acetate (TPA), a potent tumor promoter (5, 6). Recently, we have shown that TPA, like EGF, stimulates mammary cell proliferation and inhibits differentiation in the cell culture system (7). These findings prompted us to study the interaction between EGF and TPA in terms of the EGF receptors and biological actions on mammary epithelium.

In this paper, we describe the functional receptors specific for EGF in normal mammary epithelial cells and show that TPA synergizes with EGF to stimulate DNA synthesis by affecting the EGF receptor system.

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MATERIALS AND METHODS

Materials were obtained as follows: ^{3}H-labeled L amino acid mixture (1 mCi/ml; 1 Ci = 3.7×10^{10} Bq), [*methyl*-^{3}H]dThd (77 Ci/mmol), and ^{125}I (carrier-free), from New England Nuclear; medium 199 (Hanks' salts) and fetal bovine serum, from GIBCO; TPA and its analogs, from P-L Biochemical; mouse EGF (receptor grade) and anti-EGF antibody, from Collaborative Research (Waltham, MA); collagenase (CLS III, 118–120 units/mg), from Millipore; DNase I (bovine pancreas, chromatographically purified, 2,000 units/mg), from Sigma; crystalline porcine zinc insulin, from Eli Lilly; bovine prolactin (lot no. B5), from the Hormone Distribution Program, National Institute of Arthritis, Diabetes, and Digestive and Kidney Diseases.

Iodination of EGF. ^{125}I-Labeled EGF (^{125}I-EGF) was prepared according to the method of Hunter and Greenwood (8). The labeled hormone was separated from unreactive Na^{125}I by passage through a Sephadex G-50 column. Iodinated EGF had a specific activity of $\approx$200 μCi/μg and retained its biological activity with potency equal to native EGF in mammary epithelial cells in culture.

^{125}I-EGF Binding Assay. About 5 g of thoracic and abdominal mammary glands from C3H/HeN lactating mice were freed of muscle and lymph nodes, chopped finely, and transferred into a tissue culture flask containing 50 ml of medium 199 with 1% bovine serum albumin and 0.1% collagenase. The tissues were incubated for 80 min at 37°C in a shaking water bath. DNase at a final concentration of 0.0001% was added during the last 10 min of incubation. The incubation mixture was passed through double layers of nylon mesh (149 μm). The filtered cells were washed three times with medium 199 containing 0.25% bovine serum albumin by centrifugation. When examined under a microscope, cells thus obtained formed small aggregates of relatively equal size consisting of four to six cells. The EGF binding assay was carried out by incubating dissociated cells (2×10^6) in 0.5 ml of medium 199 containing 0.25% bovine serum albumin and ^{125}I-EGF ($\approx$120,000 cpm) with or without 1 μg of unlabeled EGF at 37°C for 2 hr with constant shaking. After incubation, the cells were washed four times with ice-cold phosphate-buffered saline containing 0.25% bovine serum albumin, and the cell-associated radioactivity was counted in a gamma scintillation counter. Specific ^{125}I-EGF binding was determined by subtracting the amount of radioactivity bound nonspecifically in the presence of excess unlabeled hormone. Nonspecific binding represented about 1–2% of the total ^{125}I-

Abbreviations: EGF, epidermal growth factor; TPA, 12-*O*-tetradecanoylphorbol 13-acetate.

EGF added. The binding assay was conducted in triplicate or quadruplicate.

Measurement of Immunoreactive ^{125}I-EGF in Medium. The amount of ^{125}I-EGF in the medium was determined by indirect immunoprecipitation by using rabbit anti-mouse EGF antibody and goat anti-rabbit IgG antibody (2).

Primary Mammary Epithelial Cell Culture. C3H/HeN lactating mice, 3 to 5 days postpartum, were obtained from the Animal Breeding Facility, National Institutes of Health. Primary culture of mammary epithelium was performed by using attached collagen gels as described (3). Briefly, epithelial cell-enriched fractions obtained by collagenase digestion of mammary tissue were plated onto collagen gel-coated multi-well tissue culture plates (16 mm, diameter; Falcon) at a density of 1.0×10^5 cells per cm^2. Each well contained 0.5 ml of medium 199 supplemented with 10% fetal bovine serum. Cells were incubated at 37°C in humidified air with 5% CO_2. After 24 hr, culture medium was replenished and cell culture was continued in medium containing 5% fetal bovine serum and indicated combinations of hormones. Cell culture was usually carried out for 4 days with a daily change of medium. The concentrations of hormones used were as follows: insulin, 5 μg/ml; prolactin [free of contaminating vasopressin and oxytocin (9)], 5 μg/ml; and cortisol, 3 μM.

DNA Synthesis. The extent of DNA synthesis was determined by allowing cells to incorporate [^{3}H]dThd (0.5 μCi/ml) into trichloroacetic acid-insoluble materials for 24 hr as described (3).

Casein Synthesis. Casein synthesis was determined by allowing cells to incorporate radioactivity from the ^{3}H-labeled L amino acid mixture (10 μCi/ml) for 24 hr at the indicated times. The amount of [^{3}H]casein was measured by an indirect immunoprecipitation method (3).

RESULTS

Fig. 1 shows the time course of cell-associated binding of ^{125}I-EGF to dissociated mammary epithelial cells. Apparent equilibrium was reached at 120 min at 37°C, although the binding increased gradually up to 180 min at 25°C. The cell-associated binding of ^{125}I-EGF at 4°C was about 25% of that at 37°C at 180 min of incubation. In subsequent assays, cells were incubated for 2 hr at 37°C.

The cell-associated ^{125}I-EGF binding to mammary cells was specific in the sense that unlabeled EGF at 1 ng/ml caused about 50% suppression of binding and uptake of ^{125}I-EGF (Fig. 2), whereas this process was inhibited by not greater than 10% by the following agents: fibroblast growth factor, multiplication-stimulating activity, and nerve growth factor (each at 1 μg/ml); platelet-derived growth factor (100 μg/ml); bovine prolactin and porcine insulin (each at 5 μg/ml); equine luteinizing hormone and porcine follicle-stimulating hormone (each at 1 international unit/ml).

Scatchard analysis of the cell-associated ^{125}I-EGF binding showed an apparent curvilinear pattern (Fig. 2 *Inset*). Assuming that this is not due to negative cooperativity of EGF binding, the data suggest the presence of two classes of EGF receptors with different affinities. By the use of a graphic analytical method for resolution of Scatchard plots (10), high- (K_d values = 1×10^{-10} M) and low- (K_d values = 3.6×10^{-9} M) affinity classes of receptors were detected. The number of high- and low-affinity receptors was estimated to be 800 per cell and 8,600 per cell, respectively.

Fig. 3 shows the cell-associated EGF binding and biological activities in mammary epithelial cells as a function of EGF concentration. The cell population used was the same as that in the experiments in Fig. 2. As reported previously (3), EGF stimulated DNA synthesis and inhibited casein synthesis at a concentration as low as 0.1 ng/ml, and its effect was maximal at 50–100 ng/ml. The ED_{50} values for stimulation of DNA synthesis and inhibition of casein synthesis were about 3 ng/ml and 1 ng/ml, respectively. From the data in Fig. 3, it was estimated that EGF exerted its biological actions maximally when 78% of the total EGF receptor sites were occupied. However, only about 6–10% of occupancy was required for EGF to elicit a half-maximal response in terms of DNA synthesis or casein synthesis.

Previously, we have shown that TPA, like EGF, stimulates

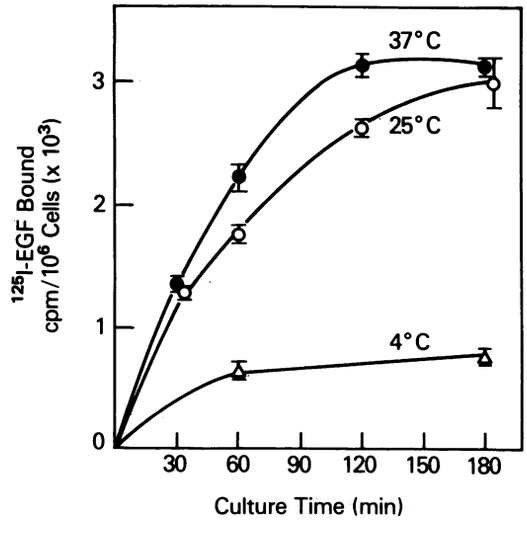

FIG. 1. Time course of cell-associated ^{125}I-EGF binding to mouse mammary epithelium at different temperatures. Mammary cells were incubated at the indicated temperature in 0.5 ml of medium containing 0.25% bovine serum albumin and ^{125}I-EGF (≈1.2×10^5 cpm per tube). At indicated time points, the amount of ^{125}I-EGF binding to mammary cells was determined. All values were corrected for nonspecific binding. Each value represents the mean ± SEM of triplicate determinations.

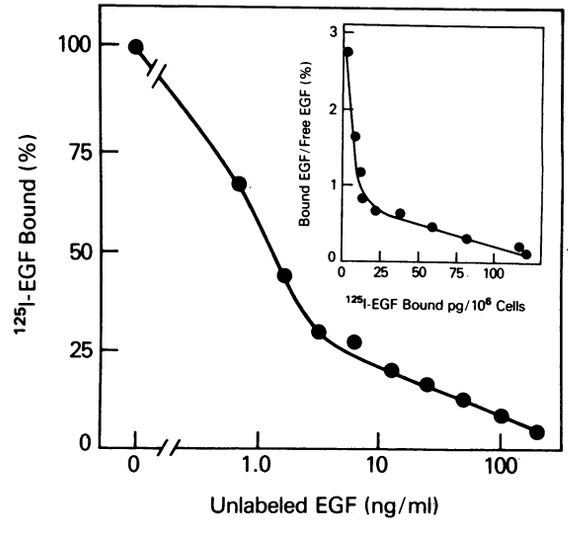

FIG. 2. Competition of unlabeled EGF with ^{125}I-EGF for binding to mouse mammary epithelium. Mammary cells were incubated with ^{125}I-EGF (1.2×10^5 cpm per tube) and different concentrations of unlabeled EGF. The amount of ^{125}I-EGF binding was determined by incubation at 37°C for 2 hr. The amount of specific ^{125}I-EGF binding was expressed as the percentage of that occurring in the absence of the unlabeled EGF. Each value represents the mean of triplicate determinations. (*Inset*) Scatchard analysis of the ^{125}I-EGF binding data.

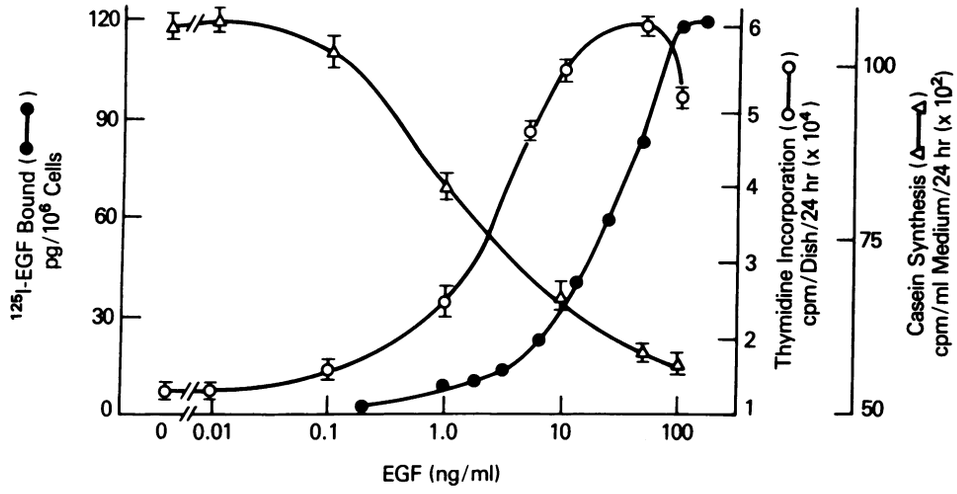

FIG. 3. Effect of EGF concentration on cell-associated ^{125}I-EGF binding, DNA synthesis, and casein synthesis in mouse mammary epithelium in culture. The amount of ^{125}I-EGF binding to mammary cells was determined at zero time before plating. The synthesis of DNA and of casein was measured in mammary cells cultured for 4 days in medium containing insulin, cortisol, prolactin, and the indicated concentrations of EGF (see *Materials and Methods*). Each value represents the mean ± SEM of five to eight separate determinations.

cell proliferation and inhibits milk protein synthesis in cultured mammary epithelial cells (7). The effect of TPA on DNA synthesis and casein synthesis was apparent at concentrations of 0.1 ng/ml and 0.01 ng/ml, respectively, during a 4-day culture (7). As shown in Table 1, addition of TPA at 0.01 ng/ml caused about 15% reduction in casein synthesis and had no effect on DNA synthesis in mammary cells cultured for 4 days. EGF at a concentration of 0.01 ng/ml neither was able to stimulate DNA synthesis nor to inhibit casein synthesis. However, EGF at a concentration of 0.01 ng/ml in combination with TPA (0.01 ng/ml) stimulated DNA synthesis in a synergistic manner. When EGF was added at a maximal concentration—i.e., 10 ng/ml—no synergism between EGF and TPA was observed. The data also indicate that synergism between EGF and TPA was not apparent in terms of casein synthesis regardless of the concentration of the two agents.

To gain some insight into the mechanism of the synergism between EGF and TPA, the effect of TPA on both cell-associated binding and degradation of ^{125}I-EGF was examined in cultured cells. The data in Table 2 indicate that the presence of TPA increased the amount of ^{125}I-EGF binding by 2.7-fold over that in the absence of TPA in 48 hr. TPA also increased the amount of ^{125}I-EGF remaining in the culture medium, which was decreased to about 28% of the initial level in 48 hr in the absence of TPA. No proteolytic activity to degrade ^{125}I-EGF was detected in conditioned media obtained from the two culture systems. When DNA synthesis was examined in parallel cultures, TPA enhanced the effect of EGF on DNA synthesis synergistically by about 50% in 48 hr.

Table 1. Effect of EGF and TPA on the synthesis of DNA and of casein in cultured mouse mammary epithelial cells

Culture condition	Thymidine incorporation, cpm/dish/24 hr	Casein synthesis, cpm/ml of medium/24 hr
Insulin/cortisol/prolactin	21,380 ± 730	9,260 ± 200
+ TPA	21,320 ± 870	7,850 ± 20
+ EGF (0.01 ng/ml)	22,480 ± 750	9,260 ± 710
+ EGF (0.01 ng/ml) + TPA	30,650 ± 203	8,090 ± 160
+ EGF (10 ng/ml)	128,500 ± 5,990	4,280 ± 330
+ EGF (10 ng/ml) + TPA	126,310 ± 4,980	3,890 ± 240

Mammary cells were cultured for 4 days in medium containing insulin, cortisol and prolactin and TPA (0.01 ng/ml) or the indicated concentrations of EGF, or both. The synthesis of DNA and of casein was determined on day 4. Each value represents the mean ± SEM of five to eight determinations.

DISCUSSION

Previously, we showed that EGF at physiological concentrations stimulates mammary cell proliferation and suppresses milk protein synthesis in cultured mammary cells (3). The stimulatory effect of EGF on mammary cell proliferation has also been reported by other investigators (11, 12). The present study has demonstrated the presence of functional receptors for EGF in mammary epithelial cells. Scatchard plot of ^{125}I-EGF binding to mammary cells displays a curvilinear pattern, suggesting the presence of a high- and a low-affinity receptor with K_d values $= 1.0 \times 10^{-10}$ M and 3.6×10^{-9} M, respectively. The present findings provide strong support for our proposal that EGF plays a vital physiological role in regulation of the growth and differentiation of mammary epithelium.

Graphic analysis of the curvilinear Scatchard plot also indicates that the low-affinity receptor was about 10 times as abundant as the high-affinity receptors. A similar pattern was also observed in a human epithelial cell line (13) and fibroblasts (14). On the other hand, both murine fibroblasts (15) and embryonal carcinoma cells (16) exhibited a rectilinear pattern of Scatchard plots, suggesting a single class of high-affinity receptors.

Table 2. Effect of TPA on the cell-associated ^{125}I-EGF binding and the level of EGF in medium in cultured mouse mammary epithelial cells

Culture condition	^{125}I-EGF binding, cpm/10^6 cells	^{125}I-EGF in medium, cpm/0.5 ml
Without TPA	510 ± 12	2,780 ± 70
With TPA	1,390 ± 186	5,360 ± 30

Mammary cells were cultured for 4 days in medium containing insulin, cortisol, and prolactin. On day 2, ^{125}I-EGF (about 10,000 cpm/0.5 ml of medium) was added into the culture medium with or without TPA (1 ng/ml). On day 4, ^{125}I-EGF bound to cells and the amount remaining in the medium were determined. Each value represents the mean ± SEM of four separate determinations.

In our previous studies (3), it has been shown that the inhibitory effect of EGF on the synthesis of milk proteins is dissociable from its mitogenic action. This suggests the presence of different intracellular pathways of EGF actions leading to these two distinct biological events. The present findings, that synergism between EGF and TPA is only observed in terms of DNA synthesis but not of casein synthesis, are consistent with our previous interpretation. However, the question remains unanswered as to why EGF does not synergize with TPA to inhibit casein synthesis.

The data in Table 2 indicate that the presence of TPA increased the amount of ^{125}I-EGF binding to mammary cells in culture by almost 3-fold. This increase can be accounted for, at least in part, by the elevated level of EGF in the medium containing TPA. At present, it is not known whether TPA also increases the number and affinity of EGF receptors.

According to recent studies (17, 18), EGF, after binding to its receptors, is internalized by the process of adsorptive endocytosis and delivered to lysosomes where it is degraded. This results in the inability of cells to rebind fresh EGF because of fewer exposed receptors, the event termed "down regulation." One possible explanation for the increase in EGF binding in the presence of TPA is that TPA prevented the down regulation of EGF receptors. However, our preliminary experiments indicate that down regulation of EGF receptors did not occur appreciably in the presence or absence of TPA (unpublished data).

The observed loss of EGF from the culture medium during culture is apparently mediated by intracellular degradation of EGF because no such a loss occurred in the absence of cells and no proteolytic activity was found in conditioned media. EGF degradation has been shown to be intimately coupled with binding and internalization (17, 18). Accordingly, the change in EGF degradation may involve altered binding or internalization of EGF, or both. In short-term cultures, TPA was found to decrease EGF binding in mammary cells without affecting the ratio of ^{125}I-EGF internalized to that bound to cell surface (unpublished data). Earlier studies by Lee and Weinstein (19) also showed that TPA exerts similar effects on various types of cells. Thus, it is possible that TPA decreases the degradation of EGF by decreasing EGF binding. This may be an important aspect of the ability of TPA to elicit synergistic action with EGF in this system as well. Such an action would spare the degradation of the growth factor in the cellular milieu and would be more significant when levels of growth factors are limiting. However, the possibility that TPA itself exerts some mitogenic activity by acting directly on pivotal processes associated with DNA synthesis to elicit synergistic action with EGF cannot be ruled out presently.

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