Possible physiological role of epidermal growth factor in the development of the mouse mammary gland during pregnancy

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Epidermal growth factor stimulated cell proliferation in a primary mammary epithelial cell culture derived from mice at different stages of pregnancy. Moreover, the peptide hormone inhibited casein production induced by the synergistic actions of insulin, cortisol and prolactin. The inhibitory effect of epidermal growth factor was influenced by the gestational stages of the mammary gland. These effects of epidermal growth factor were exerted at physiological concentrations. The dual actions of epidermal growth factor on mammary cells implicate its participation in regulation of the growth and differentiation of the mammary gland during pregnancy.

Epidermal growth factor *Mammary gland* *Growth* *Differentiation* *Milk protein synthesis* *Pregnancy*

1. INTRODUCTION

Epidermal growth factor (EGF) is a polypeptide hormone synthesized and secreted by the salivary gland [1]. A number of workers have shown that EGF acts as a mitogen to various types of cells [1]. However, the physiological function of EGF is unknown at present.

The concentration of circulating EGF has been shown to increase during pregnancy [2] when the growth of the mammary gland is markedly stimulated. During this period, the mammary gland is unable to produce milk proteins despite the presence of high levels of lactogenic hormones such as glucocorticoid and prolactin [3]. For this reason several factors such as progesterone [4] and cyclic AMP [5] have been postulated to play a negative regulatory role to suppress lactogenesis during pregnancy. However, these factors are unable to stimulate mammary cell proliferation, and thus the growth factor for mammary cell proliferation during pregnancy remains to be identified.

Recently, we have developed a primary mammary epithelial cell culture system in which the synergistic actions of insulin, cortisol and prolactin induce milk protein synthesis [6]. Using this system, we examined the effect of EGF on both cell proliferation and milk protein synthesis in order to assess its possible role in the regulation of the growth and differentiation of mammary epithelium during pregnancy. The data demonstrate that EGF at physiological concentrations stimulates mammary cell growth and inhibits milk protein synthesis in cultured mammary epithelial cells derived from mice at various stages of pregnancy. These dual actions of EGF implicate its participation in the development of the mammary gland during gestation.

2. MATERIALS AND METHODS

C3H/HeN mice at various stages of the first pregnancy were obtained from the Animal Breeding Facility, National Institutes of Health. Bovine prolactin (NIH B5) was obtained from the Hormone Distribution Program, NIADDK, National Institutes of Health. Crystalline porcine zinc insulin was a generous gift from Lilly Research Laboratories. Cortisol was purchased from Calbiochem-

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Behring. L-^{3}H-labeled amino acid mixture (1 mCi/ml), [*methyl*-^{3}H]thymidine (77 Ci/mmol), and Protosol (tissue solubilizer) were purchased from New England Nuclear. Mouse EGF (receptor grade), fibroblast growth factor, multiplication stimulating activity, 2.5 S-nerve growth factor, and platelet-derived growth factor were purchased from Collaborative Research and Medium 199 (Hanks' salts) and fetal bovine serum were obtained from GIBCO. Collagenase (CLS III 120 U/mg) was purchased from Millipore Corporation and DNase from Sigma Chemical Co. Purified collagen was purchased from Flow Laboratories.

Cell culture – Thoracic and abdominal mammary glands were removed, freed of muscle tissues, lymph nodes and connective tissue. The glands were finely chopped and incubated in M 199 containing 1% bovine serum albumin and 0.1% collagenase at 37°C for 80 min to dissociate mammary epithelial cells from fat cells. The resultant cell suspension was passed through a double layered nylon mesh, and the filtrate was centrifuged at 800 rev./min for 3 min at 25°C. After removal of the supernatant, the cell pellet was suspended in M 199 containing 10% fetal bovine serum and plated evenly onto a collagen-coated well which was prepared as described [7]. Cells were incubated at 37°C in humidified air with 5% CO_2. After 24 h, culture medium was replenished and cell culture was continued in M 199 containing 5% fetal bovine serum and indicated combinations of hormones for 4 days with a daily medium change.

DNA synthesis – The extent of DNA synthesis was determined by allowing cells to incorporate [^{3}H]thymidine (0.3 μCi/ml) into trichloroacetic acid (TCA) insoluble materials for 24 h at the indicated times. At the end of labeling, cultured cells on collagen gels were harvested and gels were digested by collagenase. Cells were then treated successively with ice-cold 10% and 5% trichloroacetic acid for 15 min each, and finally washed with 95% ethanol. The final residues were air-dried, digested in 0.8 ml of Protosol and counted for radioactivity. Over 95% of [^{3}H]thymidine incorporated into TCA insoluble materials was sensitive to DNase treatment.

Casein synthesis – Casein synthesis was determined by allowing cells to incorporate ^{3}H-labeled amino acid mixture (10 μCi/ml) for 24 h during day 4 and 5. At the end of labeling, the culture medium was harvested and centrifuged at 3000 rev./min for 10 min to remove cell debris. The resultant supernatant was used for the assay of casein synthesis. The amount of ^{3}H-labeled casein was determined by the indirect immunoprecipitation using rabbit anti-mouse casein antiserum and goat anti-rabbit IgG antiserum as previously described [8].

3. RESULTS

The addition of EGF stimulated mammary cell proliferation in a dose-dependent manner during a 4-day culture of mammary epithelial cells derived from mice at various stages of pregnancy (fig.1). The stimulatory effect of EGF was manifested at concentrations as low as 0.1 ng/ml and was maximal at 10 ng/ml. The ED_{50} value was about 0.5–0.8 ng/ml. The maximal extent of increase in the cell number was about 3-fold over that in the absence of EGF regardless of the stage of pregnancy of mice used for culture experiments.

The time course study indicated that the stimulatory effect of EGF on DNA synthesis was apparent

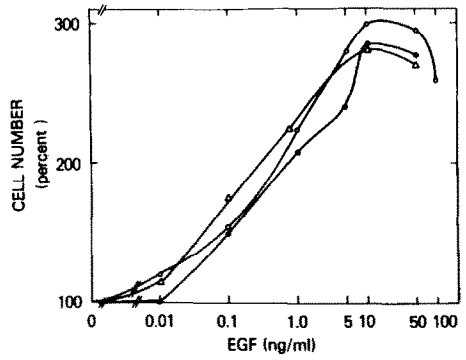

Fig.1. The effect of EGF concentrations on cell growth in cultured mammary epithelial cells from mice at different gestational stages. Mammary epithelial cells derived from mice at 8–10 days (△—△), or 11–14 days (○—○) or 15–20 days (●—●) of pregnancy were cultured for 4 days in medium containing insulin (5 μg/ml), cortisol (3 μM), prolactin (5 μg/ml) and the indicated concentrations of EGF. At the end of culture, the number of cells were counted in a hemocytometer. Each point represents the mean of closely-agreeing (SE < 3%) of triplicate determinations. Results are expressed as viable cell count compared with controls, i.e., no EGF addition.

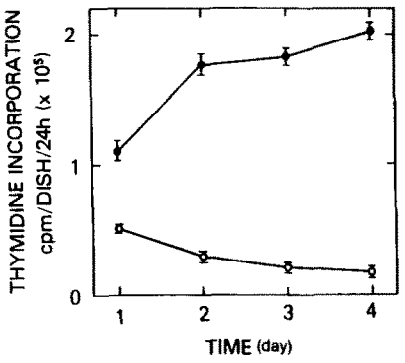

Fig.2. The effect of EGF on DNA synthesis in cultured mammary epithelial cells from pregnant mice. Mammary epithelial cells from 11–14 days pregnant mice were cultured for 4 days in medium containing insulin, cortisol, prolactin with (●—●) or without (○—○) EGF (10 ng/ml). Cells were labeled with [^{3}H]thymidine for 24 h prior to the indicated times. Other details are given in section 2 as well as in the fig.1 legend. Each point represents the mean ± SE of 5 separate determinations.

on day 1 and thereafter maintained at higher levels (fig.2). In the absence of EGF, DNA synthesis decreased precipitously after day 1.

As previously observed in organ culture of the mammary gland [8], the synergistic actions of insulin, cortisol and prolactin stimulated casein production in primary cultures of mammary epithelium derived from mice at various stages of pregnancy (fig.3). During a 4-day culture, the extent of increase in casein production induced by the three hormones was about 8–10-fold over that in culture with insulin and cortisol in cultured mammary cells derived from mice at different stages of gestation (data not shown). The addition of increasing amounts of EGF up to 50 ng/ml to the medium containing the three hormones caused progressive inhibition of casein production in mammary cells derived from 15–20 day pregnant mice. The inhibitory effect of EGF was apparent at concentrations as low as 0.1 ng/ml and was maximal at about 10 ng/ml, under which condition casein production was inhibited as much as 80%. The ED_{50} value was about 0.5 ng/ml. However, the inhibitory ef-

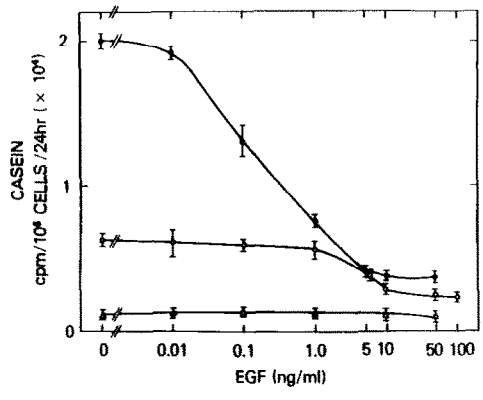

Fig.3. The effect of EGF concentrations on casein production in cultured mammary epithelial cells from mice at different gestational stages. Mammary epithelial cells derived from mice at 8–10 days (Δ—Δ), or 11–14 days (○—○), or 15–20 days (●—●) of pregnancy were cultured for 4 days in medium containing insulin (5 μg/ml), cortisol (3 μM), prolactin (5 μg/ml) and the indicated concentrations of EGF. On day 3, cells were labeled with ^{3}H-labeled amino acid mixture for 24 h, and casein production was determined as described in section 2. Each point represents the mean ± SE of 8 separate determinations.

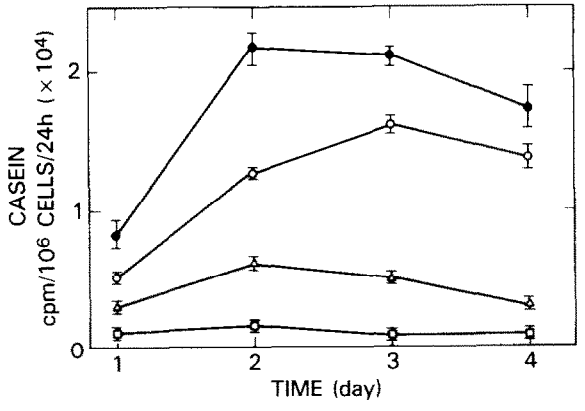

Fig.4. The effect of EGF on the time course of casein production in cultured mammary epithelial cells from pregnant mice. Mammary epithelial cells derived from 15–20 days pregnant mice were cultured for 4 days in medium containing insulin and cortisol (□—□) or insulin, cortisol and prolactin (●—●) or the combination of the three hormones plus either 0.1 ng/ml (○—○) or 50 ng/ml of EGF (Δ—Δ). Cells were labeled with ^{3}H-labeled amino acid mixture throughout the culture period, and casein production was determined at the indicated times as described in section 2. Each value represents the mean ± SE of 8 separate determinations.

Table 1
Effect of various growth factors on DNA synthesis and casein production in cultured mammary epithelial cells

Culture condition	Thymidine incorporation cpm. $dish^{-1}$. 24 h^{-1}	Casein production cpm. 10^6 $cells^{-1}$. 24 h^{-1}
I + F	18980 ± 1500	1680 ± 65
I + F + P	20720 ± 800	20010 ± 720
I + F + P + EGF	182370 ± 6960	3830 ± 208
I + F + P + Fibroblast growth factor	21720 ± 2530	19590 ± 313
I + F + P + Multiplication stimulating activity	19540 ± 1545	18465 ± 1560
I + F + P + Nerve growth factor	20020 ± 465	19696 ± 1150
I + F + P + Platelet-derived growth factor	19334 ± 1502	19025 ± 245

Mammary epithelial cells derived from 15–20 days pregnant mice were cultured for 4 days in medium containing insulin (I), cortisol (F), prolactin (P) and the indicated growth factors. Each growth factor was used at a final concentration of 50 ng/ml, except platelet-derived growth factor which was used at a concentration of 1 μg/ml. Other details are given in the legend to fig.2 and fig.3. DNA synthesis was measured on day 3. Each value represents the mean ± SE of 5 to 8 separate determinations

fect of EGF on casein production was much less pronounced in cultured mammary cells derived from mice at less advanced stages of pregnancy. The results indicate that the inhibitory effect of EGF on casein production is influenced by the gestational stage of the mammary tissue.

As shown in fig.4, the inhibitory effect of EGF (50 ng/ml) on casein synthesis was manifested as early as day 1 and continued throughout a 4-day culture. A lower concentration of EGF (0.1 ng/ml) exerted a smaller inhibition of casein production during this period.

The data in table 1 indicate that the effect of EGF to stimulate mammary cell proliferation and inhibit casein production is specific in the sense that other growth factors such as fibroblast growth factor, multiplication stimulating activity, nerve growth factor and platelet-derived growth factor were ineffective in this system.

4. DISCUSSION

In the present study, we have shown that EGF stimulates mammary cell proliferation and exerts an inhibitory action on casein production in cultured mammary epithelial cells derived from pregnant mice. While the mitogenic action of EGF was manifested equally on mammary cells derived from different stages of pregnancy, its inhibitory effect on casein production was found to be influenced by the gestational state of mammary cells. The ED_{50} value for these effects was about 0.5 ng/ml in the case of mammary cells from 15–20 days pregnant mice, which is in the range of physiological concentrations of circulating EGF [2]. The serum concentration of EGF increases to about 1.5 ng/ml during pregnancy, whereas its level in the non-pregnant state is often below the level of sensitivity of assay [2]. The mitogenic action of EGF on the mammary tissue in vitro has been reported by other workers [9–11]. Based on these findings, it is possible that EGF participates in the regulation of the development of the mammary gland by enhancing cell growth and preventing premature production of milk protein during pregnancy.

The present data indicate that the sensitivity of mammary cells to the mitogenic action of EGF dif-

fers from that to the inhibitory effect of EGF on casein production as a function of gestational stage, suggesting that the two effects of EGF are not causally related. Our recent studies of a tumor promotor, 12-*O*-tetradecanoylphorbol 13-acetate (TPA) in this system also indicated that the two events are dissociable in terms of TPA action [12]. One possible explanation for these phenomena is that the population of mammary epithelial cells is heterogeneous, consisting of cells destined to proliferate and those committed to differentiate and the two types of cells respond differently to EGF. The relative abundance of these types of cells may vary during different stages of pregnancy as a result of changes in hormonal environments. Alternatively, the two actions of EGF are exerted on the same population of cells, but cellular pathway leading to cell proliferation and differentiation involves different mechanisms that are affected differently by EGF.

The mechanism by which EGF inhibits casein production is not known at present. Our preliminary data indicate that EGF inhibits the accumulation of casein mRNA in this system and also reduces the binding capacity of prolactin, an essential lactogenic hormone. Since the receptors for EGF and prolactin are present on plasma cell membranes [13,14], it is possible that the binding of EGF to its receptors alters the properties of cell membranes and thus inhibits the action of prolactin. Studies are in progress to assess this possibility.

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