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Epidermal growth factor replaces estrogen in the stimulation of female genital-tract growth and differentiation

(uterus/vagina/DNA synthesis)

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ABSTRACT **The *in vivo* studies presented here demonstrate that epidermal growth factor (EGF) is an important autocrine and/or paracrine mediator of estrogen-induced growth and differentiation in mouse uterus and vagina. An antibody specific for EGF significantly inhibited estrogen-induced uterine and vaginal growth, thereby implicating EGF involvement in estrogen action. Furthermore, EGF administered via slow-release pellets in ovariectomized mice acted as a potent uterine and vaginal mitogen as well as an inducer of vaginal keratinization. Experiments with ovariectomized, adrenalectomized, hypophysectomized mice indicated that EGF mitogenesis does not require pituitary or adrenal hormones. Treatment with EGF also mimicked estrogen in the induction of uterine lactoferrin (a major estrogen-inducible secretory protein) mRNA and protein. These data suggest that EGF has estrogen-like effects in the promotion of cell growth in the reproductive tract and that EGF may serve as an important mediator of estrogen action *in vivo*.**

Estrogen-induced growth of various target tissues is mediated, in part, by the production of polypeptide growth factors, which may act in an autocrine or paracrine fashion to stimulate proliferation (1, 2). Two polypeptide growth factors, insulin-like growth factor I and epidermal growth factor (EGF), have been associated with the induction of uterine growth by estrogen (2, 3). Our laboratory and others have demonstrated that immunoreactive EGF precursor is present in the mouse uterus (3, 4), that estrogen treatment enhances the expression of EGF (3, 4) and EGF receptor (5) in the uterus, and that EGF is a potent mitogen in mouse uterine cell cultures (6). These studies provide evidence suggesting that EGF plays an important role in estrogen-induced uterine growth.

Previous studies (3, 6) suggesting that EGF plays a role in uterine growth led us to further evaluate the role of EGF as a potential regulator of reproductive-tract growth *in vivo*. In this study, we demonstrate that estrogen action is inhibited by an EGF-specific antibody and that *in vivo* exposure to EGF alone mimics estrogen in the induction of uterine and vaginal growth and differentiation. Our results strongly suggest that EGF acts as an estrogen-inducible physiological mediator of mouse reproductive-tract growth *in vivo*.

MATERIALS AND METHODS

Quantitation of Uterine and Vaginal Growth Following Exposure to an EGF-Specific Antibody. To determine whether an antibody specific for EGF could inhibit estrogen-induced growth, slow-release cholesterol-based pellets containing an antibody against EGF (whole antiserum; 2.5 mg per pellet; Collaborative Research) were prepared by Innovative Research of America. These pellets were divided into fourths and one quarter was implanted under the kidney capsule of 8-week-old female CD-1 mice that had been ovariectomized for at least 3 weeks. Appropriate control pellets containing normal whole serum (rabbit; 2.9 mg per pellet; Calbiochem) were also implanted. Three days following pellet implantation, the animals were injected (s.c.) with 17β-estradiol (10 μg/kg) or vehicle (corn oil) and DNA synthesis was measured by incorporation of [^{3}H]thymidine (2 μCi per gram of body weight, injected 1 hr before sacrifice) into nuclei 16 hr after estrogen administration (peak of estrogen-induced DNA synthesis). The tissues were prepared for autoradiographic analysis by Bouin's fixation, embedded in paraffin, sectioned (4–5 μm), and dipped in photographic emulsion (NTB2, Kodak). After 4–6 weeks of incubation, the slides were developed and stained with hematoxylin and eosin. For quantitation, tissue from at least four animals in each treatment group was evaluated. Counts of [^{3}H]thymidine-labeled cells were made on at least 400 cells per uterus or vagina; data are presented as a percentage of the mean plus or minus the standard error of the mean.

For analysis of the effect of exogenously administered EGF on DNA synthesis in mouse uterus and vagina, pellets were prepared (Innovative Research of America) containing HPLC-purified mouse α-EGF (Biomedical Technologies, Stoughton, MA). Whole pellets containing EGF (3 μg per pellet) or placebo were divided into fourths (750 ng of EGF per quarter pellet per mouse) and implanted under the kidney capsule of adult ovariectomized CD-1 mice. At various times following pellet implantation, the animals were injected with [^{3}H]thymidine and the tissues were processed for autoradiography as described above. Radioimmunoassay of EGF remaining in the implants revealed that >90% of the EGF was released within 96 hr following implantation (data not shown). In addition, radioimmunoassay for estradiol in the sera of the ovariectomized mice exposed to EGF was performed to ensure that there was not a significant amount of estrogen present that could influence the growth analyses (data not shown).

Immunohistochemical, Immunoblot, and Polymerase Chain Reaction (PCR) Analyses of Uterine Lactoferrin. For lactoferrin immunohistochemistry, uteri were placed in Bouin's fixative, embedded in paraffin, and incubated first with an affinity-purified rabbit antibody specific for mouse lactoferrin (compliments of Y. Chen, Institute of Biochemical Sciences, National Taiwan University, Taipei, Taiwan) and then with an alkaline phosphatase-conjugated secondary antibody (Vector Laboratories).

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Abbreviations: EGF, epidermal growth factor; PCR, polymerase chain reaction; TGF, transforming growth factor.

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For immunoblotting, uteri and vaginas were homogenized (0.5 g/ml) in 5% SDS/10 mM Tris, pH 7.5, centrifuged at 100,000 × *g* for 45 min, and stored at −70°C until analysis. Aliquots (200 μg) of each tissue sample and 500 ng of purified mouse lactoferrin were fractionated by SDS/8% PAGE and transferred to nitrocellulose. Lactoferrin was detected by sequential incubations with the affinity-purified antibody against mouse lactoferrin and an ^{125}I-labeled donkey anti-rabbit antibody (Amersham).

For PCR, total RNA was isolated (7) from uteri of each treatment group. RNA was also isolated from lactating mouse mammary glands to serve as a positive control, since lactoferrin is expressed at a high level in this tissue during lactation. Total RNA (1 μg) was reverse-transcribed with Superscript reverse transcriptase (Life Technologies, Grand Island, NY) and the cDNA was amplified with a GeneAmp kit (Perkin–Elmer/Cetus) using the oligodeoxynucleotides TACAAGGGAGTGCCACCTGGCC and ACACCATGTACCCGGGCCTT as 5′ and 3′ primers for lactoferrin (8) and GTGGGCCGCTCTACGGACCA and CGGTTGGCCTTAGGGTTCAGCAGGGGGG as 5′ and 3′ primers for actin (9). The reaction proceeded for 30 cycles in a Perkin–Elmer/Cetus thermal cycler; each cycle consisted of denaturing at 94°C (45 sec) annealing at 55°C (45 sec), and extension at 72°C (2 min). Excess primers were removed by repeated water washes and centrifugation in Centricon 100 microconcentrators (Amicon). The samples were then electrophoresed through a 4% NuSieve (FMC) agarose gel and stained with ethidium bromide. The size of the resulting DNA fragment following PCR amplification was 321 base pairs (bp) for lactoferrin and 245 bp for actin.

RESULTS

Estrogen Action Is Inhibited by an Antibody Specific for EGF. Evidence to further substantiate that EGF may be an important component of an autocrine/paracrine mechanism regulating estrogen action was obtained by investigating the effects of an antibody specific for EGF on estrogen-stimulated uterine and vaginal growth *in vivo*. Slow-release pellets containing an EGF-specific antibody were implanted under the kidney capsule of ovariectomized adult female mice 3 days prior to treatment with estrogen (17β-estradiol, 10 μg/kg), and DNA synthesis was measured by [^{3}H]thymidine incorporation into nuclei 16 hr after estrogen administration, a time at which maximal uterine DNA synthesis is induced. Exposure to the EGF-specific antibody resulted in a 60–70% inhibition of estrogen-induced epithelial cell proliferation in mouse uterus and vagina (Fig. 1).

***In Vivo* Administration of EGF Induces Uterine and Vaginal Growth.** Direct *in vivo* studies examining the putative role of EGF as a physiological regulator of reproductive tract growth were performed next. Slow-release pellets containing HPLC-purified EGF (750 ng per mouse) were implanted under the kidney capsule of ovariectomized mice. EGF significantly increased DNA synthesis in mouse uterine luminal epithelial cells, with maximal synthesis occurring 24–48 hr after treatment (Figs. 2 and 3*A*). At all time points examined, EGF exposure resulted in significantly increased levels of luminal epithelial cell DNA synthesis compared with placebo-treated controls (Fig. 3*A*). Within 72 hr of EGF pellet implantation, the uterine luminal epithelium exhibited morphology characteristic of classic estrogen stimulation (10–12), with a significant increase both in the number of luminal epithelial cells per unit basement membrane and in epithelial cell height (Fig. 2). This stimulated morphology persisted for 14 days, even though most of the EGF was released from the pellets by 96 hr postimplantation. Uterine stromal and muscle cells also responded to exogenous EGF, with maximal DNA synthesis occurring 48–72 hr after treatment (Fig. 3*B*). Interestingly, as with estrogen, the induction of DNA synthesis by EGF in uterine stromal and muscle cells occurred 12–24 hr after DNA synthesis was noted in uterine luminal epithelial cells (Fig. 3*A*). DNA synthesis was also observed in the endothelial cells of uterine blood vessels. The response of multiple uterine cell types to EGF is consistent with studies demonstrating that all uterine cell types contain EGF binding sites, thereby suggesting that all of the cell types may be potential targets of EGF action (13). However, EGF at the dose used here did not significantly stimulate DNA synthesis in liver, kidney, duodenum, lung, or mammary gland.

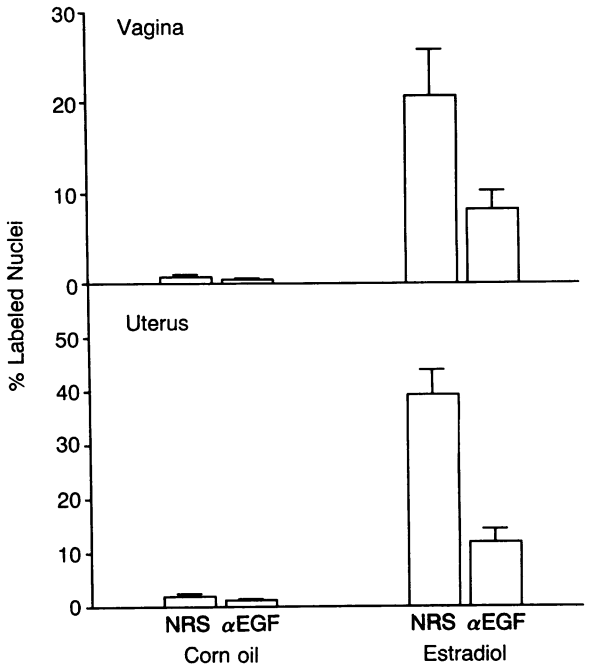

FIG. 1. Inhibition of estrogen-induced epithelial proliferation in ovariectomized CD-1 (8-week-old) mouse uterus and vagina by an antibody specific for EGF. Slow-release pellets containing anti-EGF (αEGF) or control nonimmune immunoglobulin (normal rabbit serum, NRS) were implanted under mouse kidney capsules. Three days after pellet implantation, animals were treated with 17β-estradiol or vehicle (corn oil) and DNA synthesis was measured by quantitation of [^{3}H]thymidine incorporation into nuclei. Bars represent means + SEM.

Significant changes in growth and differentiation were also induced in mouse vagina following *in vivo* exposure to EGF (Figs. 2 and 3*A*). Increased nuclear labeling of vaginal epithelial cells was apparent within 12 hr of EGF treatment, reached a peak by 24 hr, and remained significantly elevated over the controls for at least 4 days (Fig. 3*A*). In addition, mouse vaginal epithelium showed pronounced keratinization within 72 hr of EGF exposure (Fig. 2), which is remarkably similar to the keratinization of vaginal epithelium seen under the influence of estrogen during estrus in the mouse (12, 14).

Pituitary and Adrenal Hormones Are Not Necessary for EGF Growth Effects. To determine whether the effects of EGF on mouse reproductive-tract growth were possibly mediated indirectly via pituitary or adrenal hormones, we investigated the influence of EGF on uterine and vaginal DNA synthesis in ovariectomized, hypophysectomized, adrenalectomized mice. Stimulation of nuclear labeling and the induction of characteristic morphological changes were seen in the uterus and vagina of these mice in response to EGF (Fig. 4); in fact, the uterine response to EGF was significantly greater than the response observed in the sham-operated animals. Thus, these data suggest that neither pituitary nor adrenal hormones were necessary for EGF-induced uterine and vaginal mitogenesis.

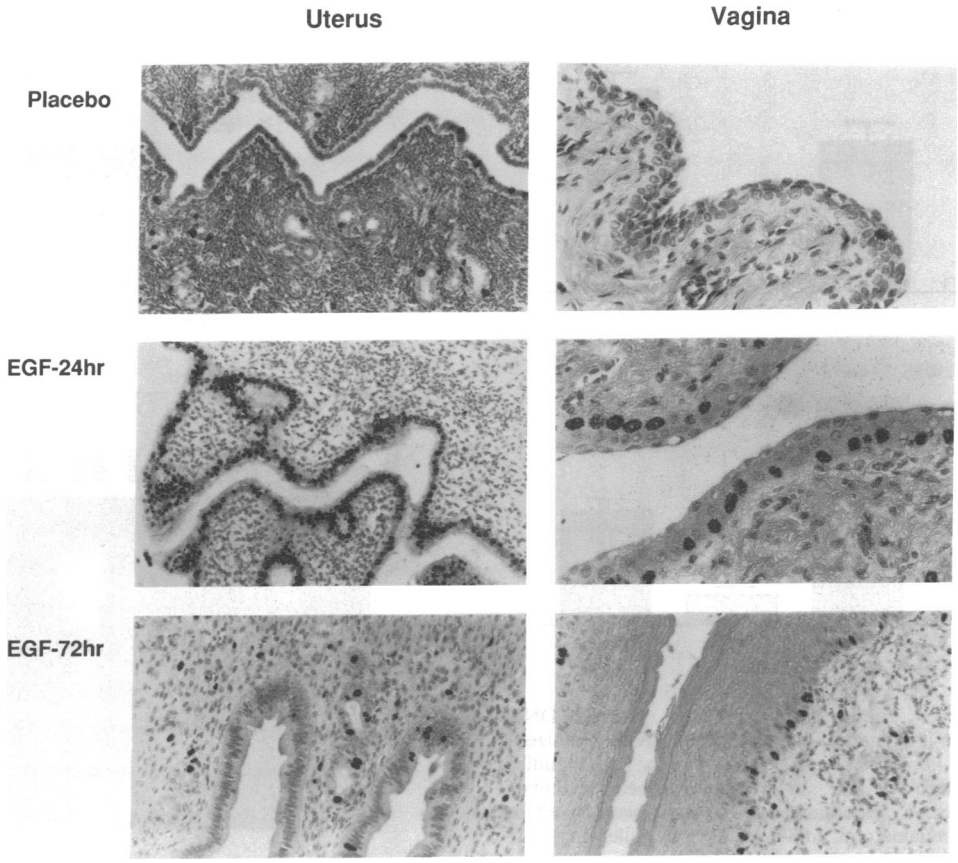

FIG. 2. Autoradiographs demonstrating DNA synthesis in ovariectomized mouse uterus and vagina 24 hr and 72 hr following kidney-capsule implantation of EGF (750 ng)-containing cholesterol-based pellets or 24 hr after implantation of placebo.

EGF Induces an Estrogen-Regulated Gene in Mouse Uterus. Further evidence that EGF and estrogen induce similar changes in the mouse reproductive tract was obtained by examining the induction of lactoferrin by immunohistochemistry, immunoblot, and RNA analysis. Lactoferrin is an estrogen-inducible, iron-binding protein found associated with the epithelium of the mouse uterus and vagina (8, 15). Like estrogen, EGF induced uterine lactoferrin mRNA (Fig. 5*B*) and a M_r 70,000 protein (Fig. 5*A*), which was localized by immunohistochemistry to the uterine epithelium (Fig. 6).

DISCUSSION

Locally derived, paracrine/autocrine-acting peptide growth factors may play crucial roles in the control and maintenance of growth and differentiation of the male and female repro-

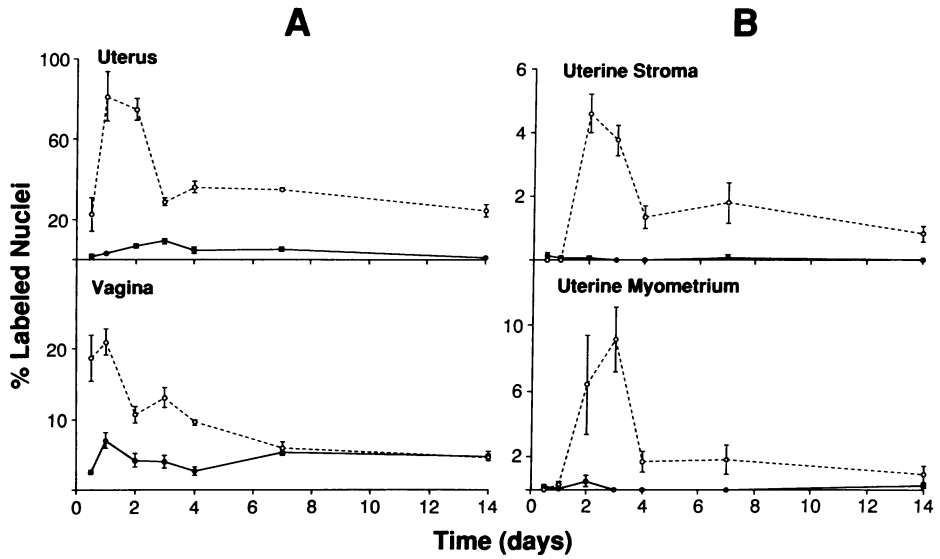

FIG. 3. Time-course study of DNA synthesis in uterine luminal epithelial and vaginal epithelial cells (*A*) and in uterine stroma and myometrium (*B*) following *in vivo* exposure of ovariectomized CD-1 mice to EGF (○) or placebo (●). DNA synthesis was quantitated by autoradiographic analysis of [^{3}H]thymidine-labeled nuclei following kidney-capsule implantation of EGF-containing or placebo pellets.

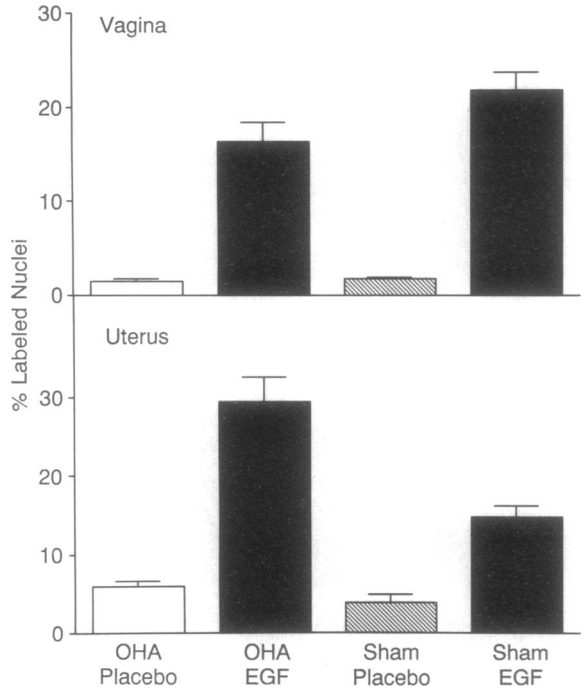

FIG. 4. EGF effects on uterine and vaginal epithelial DNA synthesis 48 hr after implantation of pellets into ovariectomized, hypophysectomized, adrenalectomized (OHA) mice. Pellet studies [EGF (750 ng) or placebo] were performed in 8-week-old female CD-1 mice that had been ovariectomized and hypophysectomized for 4 weeks and then adrenalectomized 3 days prior to pellet implantation. Other mice underwent sham operations.

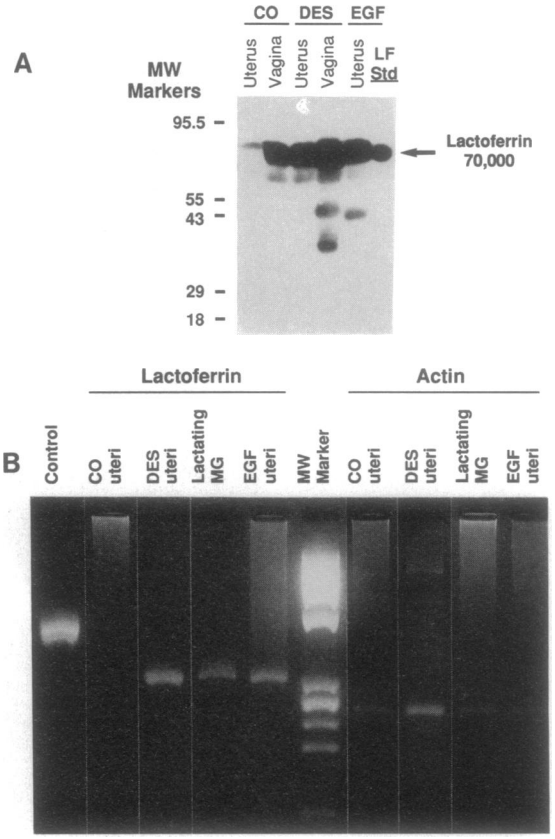

FIG. 5. Western blot (*A*) and PCR (*B*) analysis of lactoferrin 48 hr following exposure of ovariectomized mice (8 weeks old) to EGF (750 ng) pellets, diethylstilbestrol (DES; 10 μg/kg in corn oil, s.c.), or corn oil (CO) vehicle control. (*A*) EGF, like the synthetic estrogen DES, stimulates the appearance of uterine lactoferrin, M_r 70,000 (arrow). Lactoferrin standard (LF Std, 500 ng) is shown in lane at right. Molecular weight markers ($M_r \times 10^{-3}$) are at left. (*B*) Lactoferrin mRNA (321 bp) detected by PCR is present in mouse uterus following EGF or estrogen (DES) treatment. Lactating mammary glands (MG) express high levels of lactoferrin and were used as a positive control for the PCR. In addition, the control lane represents a positive control for the PCR in which bacteriophage λ DNA was used as a template and primer to define a 500-bp fragment. "Molecular weight" (MW) marker was *Hae* III-digested replicative-form DNA of phage ϕX174.

ductive tract. Expression of transforming growth factor α (TGFα)/EGF in Sertoli and peritubular cells of the seminiferous tubule is speculated to be involved in the maintenance of testicular function (16). Androgen regulation of EGF and the EGF receptor in rat prostate also suggests that EGF may have an important physiological role (17). Gonadotropin induction of various growth factors (EGF, TGFα, TGFβ, insulin-like growth factor I) in the ovary has been proposed to provide a self-amplification mechanism that perpetuates the gonadotropin signal and thereby allows for fine control of thecal and granulosa cell growth and differentiation (18).

The uterus and vagina are dynamic tissues undergoing cyclic changes in growth and differentiation under the influence of steroid hormones (10–14). Despite intensive studies of estrogen-induced growth, the factors that may induce DNA replication in the uterus and vagina remain largely unknown. The present investigation provides insight into the estrogen regulation of mouse uterine and vaginal growth, suggesting that this process is at least partially dependent on endogenously produced growth factors. In our study, the role of EGF in the female mouse reproductive tract was examined, and the results suggest that this factor may be a physiological inducer of uterine and vaginal DNA synthesis. An antibody specific for EGF was shown to reduce estrogen-mediated growth, which supports the hypothesis that EGF acts as a mitogen in the reproductive tract. The partial inhibition of DNA synthesis may be due to the existence of privileged sites where the antibody does not have access to the growth factor. Alternatively, it is possible that the antibody does not recognize all the active forms of EGF that are produced during synthesis and processing. Finally, EGF is probably only one of multiple growth factors involved in uterine growth, each of which may act independently of EGF; therefore, blocking EGF action may not completely interfere with estrogen mitogenesis. Under the experimental conditions of our *in vivo* pellet studies, EGF alone appeared to substitute for systemic estrogen in the reproductive tract in terms of stimulating DNA synthesis, inducing uterine lactoferrin, and modifying cell morphology. Furthermore, EGF stimulation of growth in the reproductive tract does not appear to be species-specific, since we have also found that EGF significantly increases DNA synthesis in the uterus and vagina of ovariectomized rats (data not shown).

Our observation that EGF promotes vaginal growth and keratinization is quite similar to a number of reports suggesting that EGF/TGFα may function as autocrine/paracrine growth/differentiation factors for keratinocytes (19–22). *In vivo* administration of EGF/TGFα has been demonstrated to stimulate basal cell proliferation and to induce epidermal hyperplasia that results in an enhancement of wound healing in adult mice (19, 21, 22) and in precocious development in neonatal mice (accelerated eyelid opening and incisor eruption) (20). Furthermore, administration of EGF to 14- to 18-day-old mice has been shown to induce precocious vaginal opening (23), which provides additional support for a critical role for EGF in vaginal development.

Similar to our *in vivo* growth factor studies of the reproductive tract, exogenous EGF has been shown to be able to replace estrogen in the stimulation of growth and morpho-

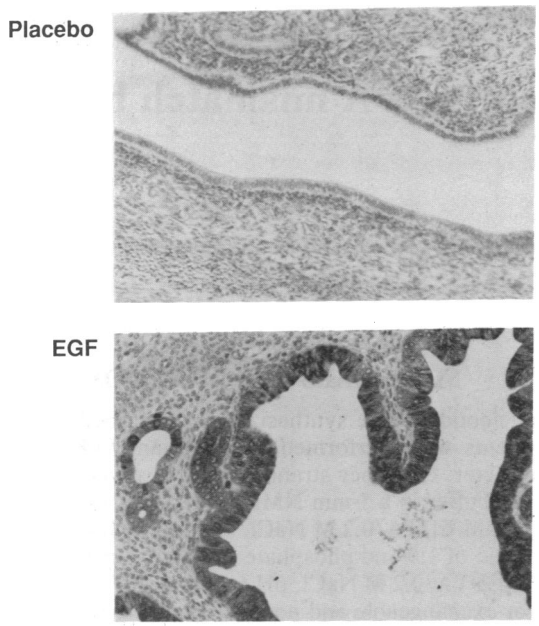

FIG. 6. Photomicrograph demonstrating that EGF (750 ng) treatment for 48 hr stimulates the appearance of immunodetectable uterine lactoferrin, an estrogen-inducible protein. Note the intense staining of the uterine epithelium following EGF treatment.

genesis of the immature mammary gland (24). In fact, EGF effects are not limited to estrogen target tissues, in that exogenously administered EGF has been shown to influence the growth and differentiation of several tissues including the eyelid (20), tooth (21), skin (22, 25), lung (26), pancreas and gastrointestinal tract (27, 28), hair follicle (29), ear canal (30), and parotid gland (31). We have attempted to address the contribution of EGF in mediating the mitogenic effects of estrogen, and our data provide supportive evidence that EGF may be a component of the normal pathway of cell growth that occurs in the reproductive tract in response to estrogen.

Certainly, the results presented in this communication demonstrate that responses usually associated with estrogen (uterine and vaginal cell proliferation, morphological differentiation, and selected gene expression) can be mimicked *in vivo* by certain peptide growth factors in the absence of estrogens. This opens a new approach to elucidating the mechanisms for steroid hormone action in target tissues.

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