Proc. Natl. Acad. Sci. USA
Vol. 78, No. 1, pp. 382–386, January 1981
Cell Biology

Differential response to growth factor by rat mammary epithelium plated on different collagen substrata in serum-free medium

(epidermal growth factor/glucocorticoids/type IV collagen/basement membrane)

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Communicated by Renato Dulbecco, October 15, 1980

ABSTRACT **Primary cultures of rat mammary epithelial cells proliferate and synthesize basement membrane collagen (type IV collagen) in a serum-free medium supplemented with epidermal growth factor (EGF), hydrocortisone or dexamethasone, insulin, transferrin, and Pedersen fetuin. The growth response of the cells to EGF and glucocorticoids but not to insulin or transferrin varies depending on the substratum on which the cells are plated. Cell growth is 4 times more sensitive to omission of EGF or glucocorticoid on type I collagen or plastic substratum than on type IV collagen substratum. The mechanism by which these two growth factors differentially affect cell growth appears to be linked to an increase in type IV collagen synthesis and a stabilization of secreted type IV collagen in the extracellular matrix. Glucocorticoids suppress the elaboration of type IV collagenolytic activity by the cells whereas EGF stimulates amino acid incorporation into type IV collagen. The results suggest that EGF and glucocorticoids affect mammary epithelial cell growth by facilitating the accumulation of the appropriate cell substratum.**

The basement membrane scaffolding contains type IV collagen, glycosaminoglycans, and glycoproteins such as laminin (1–5). This extracellular matrix functions as an important determinant during development by controlling morphogenetic movements and tissue–tissue interactions such as those that occur between adjacent epithelial and mesenchymal cells during differentiation (1, 4). In the male embryonic mammary gland rudiment and in the adult female gland, glandular involution is associated with and probably preceded by degradation of the basement membrane (6, 7). Based on studies on *in vivo* and *in vitro*, we have postulated that degradation of the basement membrane may in fact be the trigger of the involution process (7, 8).

Our experiments have suggested that contact with the basement membrane substratum is important for the growth and survival of rat mammary epithelium (7, 9). For example, *in vivo* an involution-like degeneration of the epithelium is produced when rats with proliferating mammary glands are treated with *cis*-hydroxyproline, a proline analogue that selectively inhibits collagen deposition, thus blocking basement membrane deposition (7). Additionally, isolated rat mammary epithelial (RME) cells are killed by *cis*-hydroxyproline when the cells are plated on type I collagen-coated dishes whereas growth, though reduced, is still appreciable when the treated cells are given a substratum of type IV collagen (9), the collagen that these cells normally synthesize and deposit (8).

Other studies *in vitro* have indicated that synthesis and turnover of basement membrane collagen (type IV) by RME cells are influenced by the hormones added to the cultures (8). Because a serum supplement was present in the culture medium during these studies, there was some uncertainty about the identity of factors that modulated collagen turnover. In the present experiments, a more defined growth medium is used (10). We now show that the growth regulation by epidermal growth factor (EGF) and hydrocortisone of rat mammary cells is mediated by their effect upon the synthesis of type IV collagen.

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MATERIALS AND METHODS

Cell Cultures. Mammary ducts and alveoli were isolated from perphenazine-stimulated rats by limited collagenase digestion, differential filtration, and velocity sedimentation through Ficoll gradients (11). Minced mammary tissue was digested with bacterial collagenase (Worthington CLS ii), 600 units/ml in Earle's balanced salt solution/2% (vol/vol) fetal calf serum with a reaction volume of 1 g of tissue per 10 ml, at 37°C for 1 hr. After the collagenase digestion, 1000 units of DNase (Worthington) was added to each 10 ml, and the mixtures were incubated for 10 min at 37°C. The digested tissue was filtered through Nitex cloth, washed by centrifugation, layered onto a gradient of 0–20% Ficoll in 100 ml of balanced salt solution, and centrifuged at 500 rpm for 5 min (Sorval RC3, HL8 rotor). The first 60 ml of the gradient contained single cells; the last 40 ml contained the organoid structures.

The organoids were plated without dissociation into single cells because in the organized state viability was close to 100% whereas it was less than 10% after dissociation. The organoids were plated in improved minimal Eagle's medium-zinc option (ref. 12) supplemented with fetuin (type III, Sigma; 1 mg/ml), insulin (crystalline, bovine pancreas; 5 μg/ml), transferrin (Sigma, human; 5 μg/ml), fibroblast growth factor (FGF) (Collaborative Research, Waltham, MA; 100 ng/ml), EGF (Collaborative Research; 10 ng/ml), and hydrocortisone (0.2 μM) or dexamethasone (5 nM). Indirect immunofluorescence assays for types I and III collagens were negative, indicating an absence of contaminating stromal fibroblasts (11). Type IV collagen staining was intense, especially so for spindle-shaped cells believed to be myoepithelial cells. These cells formed a basal layer upon which the epithelial cells rested (13).

Collagen Substrata. Type I collagen was purified from lathyritic rat skin (14), and type IV collagen was prepared in homogeneous form from an EHS murine sarcoma as described (15). Both types of collagen were applied to the culture dishes at 10 μg/cm^2 by air evaporation of the 0.1 M acetic acid solvent. For assay of collagenase production by the cultures, cells were grown on labeled type IV collagen that had been biosynthetically prepared by incubating dimethylbenzanthracene rat mammary tumor minces with [^{14}C]proline (50 μCi/ml for 6 hr; 1 Ci = 3.7×10^{10} becquerels) in proline- and glutamine-free growth medium. The labeled collagen was then purified by acetic acid extraction, salt precipitation, and DEAE-cellulose chromatog-

Abbreviations: EGF, epidermal growth factor; FGF, fibroblast growth factor; RME cells, rat mammary epithelial cells.

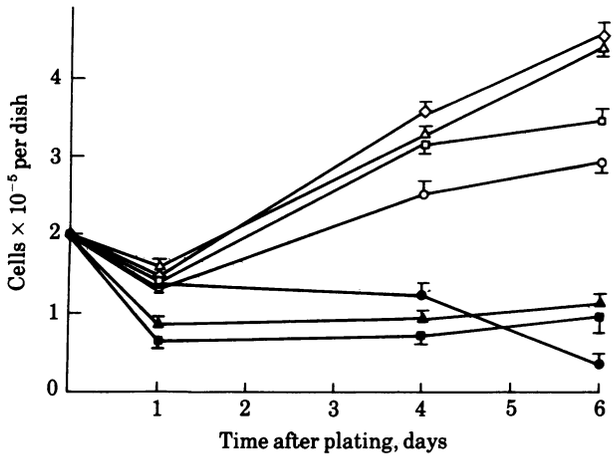

FIG. 1. Growth of RME cells in serum-free medium. Cells (2×10^5) were plated as organized structures in medium with all factors present or with individual factors omitted. The number of cells plated was determined after dissociation into single cells with trypsin/EDTA. ◇, Medium containing EGF, dexamethasone, insulin, transferrin, FGF, and fetuin; △, FGF omitted; □, transferrin omitted; ○, insulin omitted; ●, fetuin omitted; ▲, EGF omitted; ■, dexamethasone omitted.

raphy (8). The purified protein was insensitive to plasmin or type I collagenase, but was cleaved by a collagenase from a fibrosarcoma that is specific for type IV collagen (16). Plasminogen activator activity in the medium was assayed as described with [^{3}H]fibrin (17).

Collagen Synthesis. The accumulation of labeled collagen in the cultures was assessed by measuring [^{14}C]proline-labeled protein rendered acid soluble after digestion with protease-free bacterial collagenase (18) or, alternatively, by quantitating the amount of cell-associated, labeled 4-hydroxyproline. In the latter assay, the total cell protein was acid hydrolyzed (8) and the amino acids were converted to their phenylthiohydantoin derivatives (19) and separated by high-pressure liquid chromatography (20). The peaks corresponding to 4-hydroxyproline and proline were collected and radioactivity was measured.

RESULTS

Effect of Substratum on Cell Growth in Serum-Free Medium. As shown in Fig. 1, primary cultures of RME cells attach and proliferate on plastic dishes in improved minimal Eagle's medium (12) supplemented with 10 ng of EGF per ml, 100 ng of FGF per ml, 5 nM dexamethasone, 5 μg of insulin per ml, 5 μg of transferrin per ml, and 1 mg of Pedersen fetuin per ml. To determine which of the components were obligatory for cell attachment or growth (or both), we seeded cells in the absence of individual growth factors (Fig. 1). In the absence of either EGF or dexamethasone, attachment of the cells to the substratum within the first 24 hr of culture was reduced by 50% compared to cells seeded in complete medium. Furthermore, the growth of attached cells was minimal in the absence of these two factors. Although removal of fetuin did not affect cell attachment, the cells failed to proliferate and eventually died after 6 days in culture. In the absence of transferrin, the cells attached and grew normally for the first 4 days. However, after this time, cell growth ceased. Omission of insulin did not affect cell attachment, but reduced cell growth by 50%. Removal of FGF from the serum-free medium had no appreciable effect on either attachment or growth. Therefore, FGF was not included in the medium in subsequent experiments.

Qualitatively and quantitatively similar responses for attachment and cell growth were observed for these components when RME cells were seeded on type I collagen in place of plastic dishes (Fig. 2). Furthermore, the concentration of each of these growth factors used in the serum-free medium was optimized on plastic dishes for stimulating cell growth as determined from dose–response curves (data not shown).

Compared to the growth of cells on plastic or type I collagen, cells seeded on type IV collagen-coated dishes exhibited a completely different set of responses for cell attachment and growth upon omission of EGF or dexamethasone from the serum-free medium (Fig. 2). Growth of the cells on plastic, type I collagen, or type IV collagen was monitored 3 days after cells were plated in culture in medium lacking one of the constituent factors (Fig. 2). Reduction of growth by omission of insulin or transferrin was the same for type I and type IV collagens and for plastic. Omission of EGF or dexamethasone resulted in a 45–60% decrease in cell growth on plastic or type I collagen but barely affected cell growth on a type IV collagen substratum. The requirement for fetuin was absolute on any of the substrata. The data illustrate that EGF or dexamethasone removal produced a 3- to 4-fold greater reduction in cell growth on either plastic or type I collagen in comparison with growth on type IV collagen (14% reduction on type IV collagen compared to 45–55% reduction on plastic or type I collagen.

Effect of Substratum on EGF and Glucocorticoid Receptors in RME Cells. Biological responses produced by either EGF or glucocorticoids in a variety of cell types are mediated by the presence of specific cell-surface or intracellular receptors for these two classes of hormones (21, 22). The differential growth response of RME cells to either EGF or dexamethasone on type I compared to type IV collagen was not due to the modulation of receptors for either hormone on these different substrata. Fig. 3 illustrates that RME cells possessed high-affinity, saturable cell-surface receptors for ^{125}I-labeled EGF. Moreover, cells cultured on either type I or type IV collagen possessed an equal number of receptors (78,000 receptor sites per cell) with an equivalent affinity (K_d = 0.34 nM). Likewise, intracellular receptors for [^{3}H]dexamethasone were identified in RME cells grown on either type of collagen. As with EGF receptors, no differences in either receptor concentration (66,400 receptor sites per cell) or affinity (K_d = 5.4 nM) for dexamethasone were found for cells cultured on either substratum.

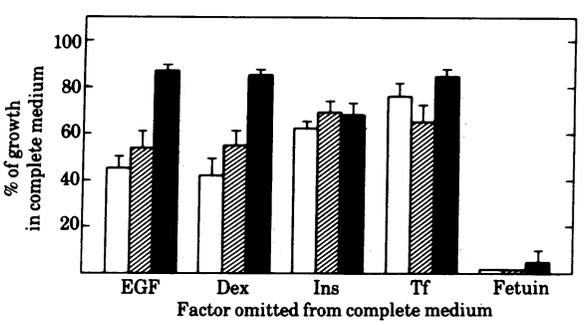

FIG. 2. Substratum affects responsiveness of RME cells to growth factors. Cells (1×10^5) were plated in 1.5 ml of serum-free improved minimal Eagle's medium with EGF, dexamethasone (Dex), insulin (Ins), transferrin (Tf), and fetuin present (complete medium) or in medium with individual factors omitted on plastic (□), type I collagen (▨), or type IV collagen (■). After 24 hr to permit cell attachment, the medium was removed along with any unattached cells and fresh medium of the same composition was added. Values represent the percent of cell growth after 3 days in culture in the absence of individual factors determined in comparison to the growth in complete serum-free medium (100%) and represent the average from duplicate experiments ±SD.

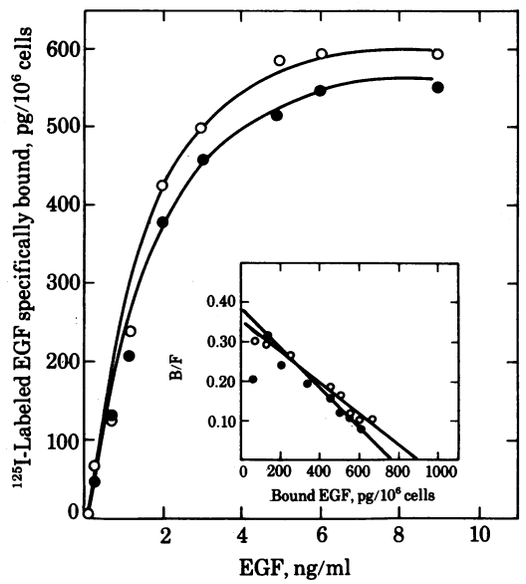

FIG. 3. ^{125}I-Labeled EGF binding to RME cells. Cells (2×10^5) were plated as described in the legend for Fig. 1 in serum-free medium containing all factors except EGF on type I (○) or type IV (●) collagen-coated dishes for 3 days. Specific binding of different concentrations of ^{125}I-labeled EGF to the monolayers was determined as described (10). (*Inset*) Scatchard plot of the binding data. B, bound; F, free.

Effect of Growth Factors on Collagen Synthesis and Turnover *in Vitro*. Previous studies have demonstrated that collagen from mammary epithelial cells pulse-labeled in the presence of a complement of hormones (insulin, hydrocortisone, estradiol-17β, progesterone, and prolactin; the hormone concentrations optimal for *in vitro* growth of the rat epithelium had been previously determined) in 5% fetal calf serum was completely degraded in the subsequent 24-hr chase period if all hormones were omitted from the culture medium but not if the hormones were present (8). Therefore, individual hormones were added during the chase period to define which factor(s) modulated turnover. As shown in Fig. 4, pulse–chase experiments revealed that the stabilization of newly formed collagen against turnover was affected primarily by a single medium supplement, hydrocortisone. Marginal inhibition of turnover was observed when estradiol-17β, insulin, prolactin, or progesterone alone was individually present during the chase period.

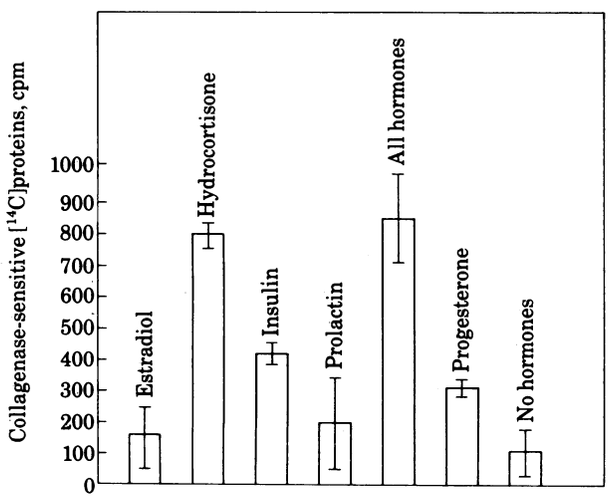

FIG. 4. Effect of various hormones on the stabilization of newly synthesized collagen. Cells (10^5) were plated in medium containing 5% fetal calf serum and 1 ng each of progesterone and estradiol-17β, 500 ng of hydrocortisone, 0.1 μg of insulin, and 200 ng of prolactin per ml. Cells were allowed to attach for 24 hr; then [^{14}C]proline (5 μCi/ml, Amersham; 250 μCi/mol) and ascorbic acid (4 μM) were added. Cells were incubated for 24 hr. Then this medium was replaced with medium lacking [^{14}C]proline and with individual factors omitted as indicated. Twenty-four hours later, the amount of collagenase-sensitive protein (cpm/0.5 $\times$ 10^5 cells) in the cell layer was determined (18).

The turnover of newly formed collagen appears to be mediated by a collagenolytic activity that is elaborated by the epithelium in the absence of hydrocortisone. In serum-free medium the cultured epithelium supported by EGF, insulin, hydrocortisone, transferrin, fetuin, and estradiol-17β degraded little of the [^{14}C]proline-labeled type IV collagen substratum on which the cells were plated (Fig. 5). This was also the case if estradiol-17β, insulin, or EGF was individually omitted from the medium. In contrast, there was a 3-fold increase in degradation of the labeled collagen substratum when hydrocortisone was omitted and a 9- to 10-fold increase in the degradation of the labeled substratum when plasminogen was included in the cultures maintained without hydrocortisone. The potentiating effect of plasminogen occurs because plasminogen is converted to plasmin by plasminogen activator produced by the cells. Plasmin, like trypsin, then converts collagenase from an inactive to an active form (16). The overall process of degrading the labeled collagen substratum by the cultured cells is not influenced by the effects of hormone or growth factor on plasminogen activator production by the cells because this activity is comparable regardless of which of these factors is omitted from the culture medium (Table 1).

Even when cells are plated on a type IV collagen substratum, production of collagenase can lead to a decreased cell growth rate if the collagen substratum supplied is extensively degraded. In the experiment presented in Table 1, 10 μg of type I collagen was first plated and then coated with 0.1 μg of [^{14}C]proline-labeled type IV collagen (1% of the amount used in the experiment of Fig. 2). After 3 days, about 90% of the labeled collagen was degraded in cultures without hydrocortisone but containing plasminogen. Associated with type IV collagen degradation there was a decrease in the growth of the cells. Consistent with the results given in Fig. 2, there was also a reduction in the rate of cell proliferation when insulin was omitted from the growth medium. This reduction in cell division was seen re-

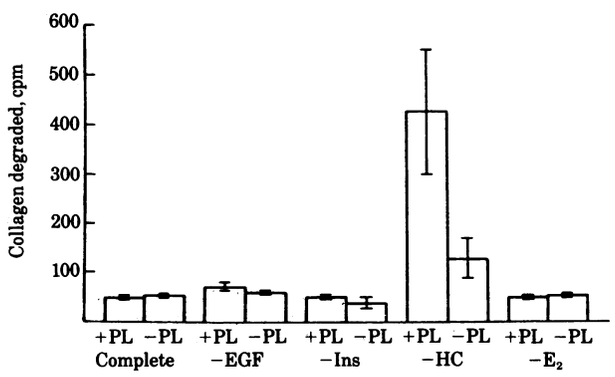

FIG. 5. Effect of various hormones on type IV collagenase activity. Cells were plated as described in the legend to Table 1. Upon changing to medium with individual factors omitted, half the dishes also received 25 μg of plasminogen (PL) per ml and the other half did not. The bars represent the range of duplicate assays. The release of labeled type IV collagen into an aliquot of the medium was measured 3 days after the medium was changed. Ins, insulin; HC, hydrocortisone; E_2, estradiol-17β. Maximum cpm = 900.

Table 1. Effect of growth factors on plasminogen activator and collagenase activity in RME cells

Factor omitted	Plasminogen activator production*	Type IV [^{14}C]collagen substratum degraded, dpm	Cells × 10^{-5}
None	710	457	1.28
EGF	680	427	1.21
Insulin	670	838	0.59
Hydrocortisone	675	3260	0.61

Cells (10^4) were plated in Linbro plates that had been coated with 10 μg of type I collagen per cm^2 and overlaid with 0.1 μg of [^{14}C]proline-labeled type IV collagen per cm^2. In addition to the growth factors, plasminogen (25 μg/ml) was included in the medium. To avoid selective cell attachment, we first plated cells in complete serum-free medium with all factors present except plasminogen. After 24 hr, when ≈80% of plated cells had attached, the medium was changed to medium without individual growth factors but with plasminogen. Three days after the medium was changed, the culture medium was harvested. Radioactivity released from the substratum was measured and the no. of cells per dish was determined. Plasminogen activator activity in the medium from parallel samples containing plasminogen (25 μg/ml) was assayed at this time by measuring the release of [^{3}H]fibrinopeptides from [^{3}H]fibrin-coated plates as described (17). Less than 100 dpm [^{14}C]collagen per ml was released during the cell attachment period. All values represent the average of duplicate experiments, with individual samples varying ±10%.
*cpm [^{3}H]fibrin solubilized per 10^4 cells per 2 hr.

gardless of the presence or absence of plasminogen. It was also not associated with an appreciable degradation of the labeled type IV collagen substratum. Omitting estradiol-17β or EGF did not affect either cell growth or collagenolytic activity in the presence or absence of plasminogen.

Hydrocortisone (or dexamethasone) thus apparently facilitates cell growth by blocking the turnover of newly synthesized collagen via inhibition of the elaboration of a collagenase. Consequently, when type IV collagen substrata are supplied in sufficient thickness (Fig. 2), there is a reduced dependency of the cells on hydrocortisone or dexamethasone. The reduced EGF dependency of the cells plated on type IV collagen suggested that this growth factor facilitates collagen deposition. That this was the case was shown by the fact that the incorporation of [^{3}H]proline into collagen was reduced when EGF was omitted from the growth medium (Fig. 6). Reduced accumulation of newly formed collagen was also observed if hydrocortisone was omitted, presumably as a consequence of an increase in collagenase activity. Omission of insulin had no effect on the relative amount of collagen synthesized by cultured ducts and alveoli.

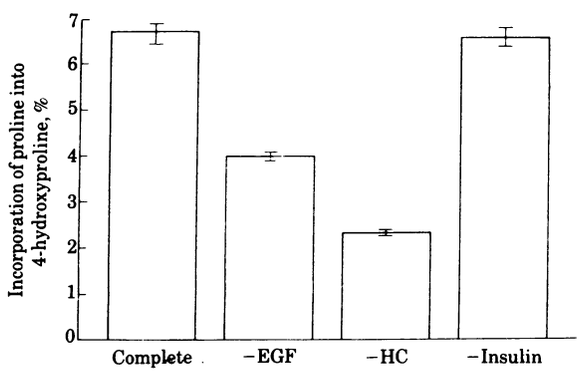

FIG. 6. Effect of hormones on collagen synthesis. Cells (10^5, undissociated ducts and alveoli) were plated onto bacterial plastic dishes in complete serum-free medium (see Fig. 1) or with complete medium minus EGF, hydrocortisone (HC), or insulin. The organoids that did not attach or divide on these dishes were then labeled with 5 μCi of [^{14}C]proline per ml for 16 hr. The organoids were recovered by pelleting, and the media and cell pellet were analyzed for proline and 4-hydroxyproline precipitable by 20% (wt/vol) trichloroacetic acid. The radioactivity in these amino acids was quantitated after acid hydrolysis.

DISCUSSION

The present studies demonstrate that RME cells can be grown in primary culture in a hormone-defined, serum-free medium. Growth rates of mammary epithelial cells are comparable on plastic, type I collagen, or type IV collagen provided that the full hormonal complement is present, but they vary on the different substrata in response to omission of individual growth factors. On all substrata, mammary epithelial cells depend upon insulin, transferrin, and Pedersen fetuin for growth. Insulin and transferrin appear to be obligatory requirements for the growth of several cell types in the absence of serum (23). The growth-stimulating or survival activity of Pedersen fetuin is apparently due to a contaminant in this preparation; the more homogeneous Deutsch and Spiro fetuin preparations cannot substitute for Pedersen fetuin (refs. 10 and 23 and unpublished observations). The growth response to EGF or glucocorticoids depends upon the type of substratum provided. On plastic or type I collagen, mammary epithelial cells show a requirement for EGF and dexamethasone for attachment and growth. However, on type IV collagen, this dependence is reduced or absent. The mitogenic effect of EGF is not unique to RME cells; human mammary epithelial cells are also stimulated to grow by EGF (24). Furthermore, bovine corneal epithelial cells become responsive to the growth-promoting effects of EGF when they are maintained on type I collagen, whereas on plastic, they are more responsive to FGF (25). In contrast, FGF is a mitogen for vascular smooth muscle cells when these cells are cultured on plastic surfaces but not when the cells are maintained on an extracellular matrix (26). From these studies it was suggested that the proliferative capacity of cells in response to specific mitogens could be determined by the cellular structure as influenced by the composition of the substratum upon which the cells rest (27, 28). If such changes in cell shape are important in determining qualitative or quantitative growth responses to particular mitogens, this is not a result of changes in receptor number or affinity for these growth factors, at least in RME cells. On type I or type IV collagen, mammary epithelial cells have an equivalent number of receptors for both EGF and glucocorticoids.

On type I and III stromal collagens, mammary epithelial cell spreading and flattening are delayed compared to these processes on type IV collagen (9); this delay is due to a requirement for *de novo* synthesis of type IV collagen (9). On type I collagen, both EGF and glucocorticoids are required by mammary epithelial cells for the deposition of type IV collagen; when an exogenous type IV collagen substratum of sufficient thickness is provided for cell attachment and spreading, mammary epithelial cells become refractory to the growth-promoting effects of these two hormones. A similar correlation between the stimulation of collagen production and growth has been described for EGF in human foreskin fibroblasts (29). Likewise, glucocorticoids increase the attachment of rat hepatoma cells by modulating the synthesis of surface glycoproteins (30).

Degradation of type IV collagen as well as its synthesis is also under hormonal control in RME cells. In the absence of glucocorticoids, a collagenolytic activity that is able to degrade type IV collagen can be detected in the culture medium. The existence of this activity is significant because previously described

mammalian collagenases fail to degrade type IV collagen (16). This enzyme is apparently secreted largely in a latent form by mammary epithelial cells because activity is enhanced by a limited trypsin or plasmin proteolytic cleavage. By gel electrophoresis we have found that physiologic concentrations of highly purified plasmin alone will not induce a significant cleavage of native type IV collagen. The mammary epithelial enzyme is similar to a neutral type IV collagen-degrading metalloprotease isolated from a murine sarcoma (16, 31). Plasminogen activator activity can be detected in the culture medium from mammary epithelial cell cultures, and this enzyme may play a role through plasmin in activating collagenase. In organ cultures of lactating mouse mammary explants, plasminogen activator has been detected and found to be under hormonal control in the lactating mammary gland (32). The lack of hormonal dependency for plasminogen activator elaboration in the present studies may be due to the fact that mammary epithelial cells used in these experiments were obtained from proliferating rather than lactating mammary glands. The hormonal responsiveness of the proliferative epithelium probably differs from that of lactating epithelial cells. Glucocorticoids therefore inhibit type IV collagen turnover in cultured RME cells by suppressing the appearance of a type IV collagenase. EGF, however, stimulates collagen synthesis without affecting collagen degradation. The dual effect of these two growth factors is to enhance the accumulation of a type IV collagen substratum and probably other basement membrane constituents such as laminin as well.

We thank Mr. Kenneth Burdette and Ms. Elise Kohn for their technical assistance, which made certain portions of this research possible.

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