Involvement of Collagen Formation in the Hormonally Induced Functional Differentiation of Mouse Mammary Gland in Organ Culture*

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In mammary gland organ culture from midpregnant mice, synergistic actions of insulin, cortisol, and prolactin stimulate differentiation of mammary epithelium and induce the synthesis of the milk proteins casein and α-lactalbumin. In the present study we examined the production of collagen and its function in the hormone-dependent development of mammary gland in vitro. The measurement of collagen production by the hydroxyproline assay and by sodium dodecyl sulfate-polyacrylamide gel electrophoresis revealed that the accumulation of collagen type I and type III in cultured mammary explants increased with the addition of insulin, cortisol, and prolactin to a chemically defined medium. When an analog of proline, L-azetidine-2-carboxylic acid (LACA), was added at a concentration of 80 μg/ml with insulin, cortisol, and prolactin at the beginning of culture, collagen production in cultured tissue was inhibited by 75% during a 3-day incubation period. This agent also inhibited the synthesis of casein and α-lactalbumin by about 77%, respectively. The inhibitory effect of LACA could be prevented by L-proline; concomitant addition of L-proline (80 μg/ml) with LACA (80 μg/ml) resulted in complete restoration of milk protein synthesis to normal levels. Measurement of the amount of milk protein mRNAs in mammary explants by a cell-free translation assay demonstrated that LACA reduced the hormone-stimulated accumulation of casein mRNA and α-lactalbumin mRNA by 79 and 76%, respectively. LACA, however, produced little inhibition of DNA synthesis in cultured tissue. These results suggest that collagen production may be involved in the phenotypic expression of milk protein genes during hormonal induction of mammary epithelial differentiation in vitro.

Collagenous proteins are widely distributed in mammalian tissues, constituting one of the major components of the extracellular matrix (1). It is well recognized (1, 2) that collagen plays an important role in the support and framework of tissues. Also, collagen has been shown to be involved in the morphological development of embryonic organs such as the lung, the salivary gland, and the rudimentary mammary gland (1-5). During the past several years, it has been shown (1, 2) that collagen is a group of heterogeneous protein molecules with common chemical and physical properties. Furthermore, individual tissues have been shown to contain different types of collagen that may arise during developmental processes (1, 2). Moreover, recent studies have revealed that collagen(s) is involved in cell attachment and differentiation and also acts as an agent responsible for chemotaxis of macrophages and fibroblasts (1). Thus, it appears that collagen potentially has various developmental and physiological functions in addition to its structural role.

Previous studies have established that the synergistic actions of insulin, cortisol, and prolactin stimulate the functional differentiation of the mouse mammary gland in organ culture and induce the synthesis of the milk proteins casein and α-lactalbumin (6). More recently, it has been shown (7) that the triple hormone treatment can stimulate the growth and differentiation of mouse mammary epithelium in a primary cell culture system, provided that collagen gels are used as a substrate. In the absence of collagen gels, cultured cells are unable to accumulate casein mRNA and synthesize milk proteins. These results suggest that collagen may be essential for the hormonal induction of mammary epithelial differentiation.

The purpose of this study is to investigate the accumulation and functional role of collagen in the hormone-dependent differentiation of the mouse mammary gland in organ culture. In order to assess this, we have used two drugs, LACA, which is a proline analog and an inhibitor of the formation and secretion of collagen (8), and β-APN, which blocks cross-linking of collagen by inhibiting lysyl oxidase (9, 10). The results show that types I and III collagen are the major species that accumulate during the induction of differentiation of the mammary gland in culture. The data also suggest that collagen(s) has a pivotal function for the phenotypic expression of milk protein genes in mammary tissue.

**EXPERIMENTAL PROCEDURES**

**Materials—**C57/H/HeN mice in the 10th to 12th day of their first pregnancy were obtained from the Animal Breeding Facility, National Institutes of Health. Chemicals were purchased as follows: Medium 199 (Hanks' salts) and Dulbecco's phosphate-buffered saline (Ca²⁺- and Mg²⁺-free) from Gibco, Grand Island, New York; L-proline, chloramine-T, silicic acid, hydroxyproline, P-APN, UDP-Gal and bovine milk galactosyl transferase (lot 29C-8015), and penicillin G from Sigma; LACA, 2-mercaptoethanol, spermidine, and cortisol from Calbiochem-Behring; reagents for polyacrylamide gel electrophoresis, including SDS and AG1-X2 ion exchange resin, from Bio-Rad. Crystalline porcine zinc insulin was a gift from Lilly, and bovine prolactin (lot NIH/B5) was obtained from the Hormone Distribution Program, National Institute of Arthritis, Diabetes, and Digestive and Kidney Diseases. Each preparation of prolactin was further purified to remove contaminating vasoactive intestinal polypeptide and oxytocin by ultrafiltration with ultrafiltration cell model 12 and PM-10 membrane from Amicon, Lexington, MA. Phenol was obtained from Mallinckrodt, Paris, Kentucky; wheat germ from General Mills, Minneapolis, MN; ATP disodium

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* A preliminary account of this work was presented at the meeting of the American Society of Cell Biology, November 12, 1981, Anaheim, California. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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1 The abbreviations used are: LACA, L-azetidine-2-carboxylic acid; β-APN, β-aminopropionitrile; I, insulin; F, cortisol; P, prolactin.
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salt, GTP, trisodium salt, creatine phosphate and creatine kinase from \text{Boshringer Mannheim}; 1,6-H-aminolevulinic acid, \text{I}^{[7]}\text{H}]-lucine (84 Ci/mmol or 60 Ci/mmol), \text{I}^{[1]}\text{H}]-proline (160 Ci/mmol), \text{[H]}\text{hydroxyproline (5.4 Ci/mmol)}], \text{[methyl-3H]}\text{thymidine (56 Ci/mmol)}, Triton X-100, and Protosol from New England Nuclear; oligo(dt)-cellulose T, from Collaborative Research, Lexington, MA; rabbit globin mRNA and \text{Triton X-100 from Miles, Elkhart, IN; type I and type III standard collagen from human placenta was a gift from Dr. Stephen I. Rennard, National Heart, Lung, and Blood Institute, National Institutes of Health.

**Organ Culture—**Mice were killed by decapitation. Mammary explants were prepared from abdominal glands under sterile conditions and cultured in Medium 199 containing 35 pg/ml of penicillin G and the indicated agents at 37 °C in humidified air with 5% CO₂ (6). **Measurement of \text{[H]}\text{Hydroxyproline Content—**Mammary explants (10-15 mg) cultured in 10 pg/ml of \text{HH}-g for 30 min at 4 °C, and the supernatant was dialyzed against 10 ml 3776 Collagen Accumulation in Mammary Cell Differentiation hydroxyproline were assayed at the same time. The same amount of unlabeled explants and a known amount of \text{[3H]}\text{hydroxyproline (5.4 Ci/mmol), \text{[methyZ-3H]}\text{thymidine (50 Ci/mmol}, Triton X-100, and homogenized in 5 ml of 0.5 M acetic acid with a Poltron tissue disintegrator (Brinkmann Instruments (dial setting 12.2 min)). The homogenates were dialyzed at 4 °C against 10 liters of 0.5 M acetic acid to remove free amino acids and small peptide fragments. The volume of dialysates was brought to a final volume of 6 ml with 0.5 M acetic acid. Each three ml aliquots of each dialysate were hydrolyzed at 150 °C in 6 N HCl for 15 h and dried in vacuo. \text{[H]}\text{Hydroxyproline in the hydrolysates was quantitated by the modified method of Juva and Prockop (11), using a silicic acid column. Each column was eluted with 55 ml of toluene, and the eluate was collected in counting vials and counted for radioactivity in a Beckman LS-200 liquid scintillation counter following the addition of scintillation fluid and a concentrated liquid scintillator (Amersham Corp.). In order to check the recovery of \text{[3H]}\text{hydroxyproline in each sample, a mixture of the same amount of unlabeled explants and a known amount of \text{[H]}\text{hydroxyproline were assayed at the same time.}

**Purification of Collagens in Cultured Mammary Gland—**The cultured explants (200-300 mg) were homogenized in 5 ml of 0.5 M acetic acid containing 1 mg of carrier collagen and 0.5 mg/ml of peptin in the Polytron. Collagen was prepared from mouse mammary gland as described below. The homogenates were left at 4 °C for 24 h with continuous stirring and then centrifuged at 10,000 × g for 30 min. The supernatants were saved, but the precipitates were again digested with 0.8 ml of 2% Triton X-100, and Protosol and counted in a toluene-based liquid scintillation fluid.

**Measurement of Casein Synthesis in Cultured Mammary Explants—**Mammary explants (10-15 mg) cultured in 10 µg/ml of \text{H}-amino acid mixture were harvested at the indicated times, weighed, and homogenized in 50 volumes (w/v) of phosphate-buffered saline containing 2% Triton X-100. The homogenate was centrifuged at 105,000 × g for 60 min. The amount of casein in the supernatant was measured using the immunoprecipitation method, which has been previously described (13). The amount of total protein synthesized in cultured explants was determined by the incorporation of \text{H}-amino acid into trichloroacetic acid-insoluble materials (15).

**Isolation of Poly(A) mRNA and the Activity of mRNA for Milk Proteins—**Total mRNA was extracted with phenol-chloroform from cultured explants (10 mg). Poly(A) mRNA was isolated, utilizing two cycles of adsorption on oligo(dt)-cellulose columns. The activity of mRNA for milk proteins was measured by the wheat germ cell-free protein-synthesizing system, as described earlier (16). Under the conditions employed the extent of translation was linear with the amount of mRNA added. The amounts of casein and α-lactalbumin in the released polypeptides were measured by immunoprecipitation of the supernatant obtained after centrifugation of the reaction mixture at 150,000 × g for 60 min (16).

**Measurement of \text{[3H]}\text{Thymidine Incorporation into DNA—**The incorporation of \text{[H]}\text{thymidine into cultured mammary explants was determined by a method described previously (17).}

**RESULTS**

As shown in Fig. 1, the accumulation of collagen in mammary gland explants cultured in the presence of insulin, cortisol, and prolactin increased progressively up to 72 h with the largest increase occurring between 24 and 48 h. At 72 h, collagen constituted about 0.7% of the total protein synthesized by explant explants (data not shown). In the absence of hormones, the extent of collagen accumulation at 72 h was about 6% of that which was cultured with the three hormones.

In order to determine what types of collagen were synthesized by mammary explants cultured with insulin, cortisol and prolactin, collagen was extracted from explants cultured in the presence of \text{[H]}\text{proline, the three hormones, and \beta-APN for 72 h. \beta-APN was included to maximize the extent of collagen extraction but had no effect on the accumulation of collagen in cultured tissue. Analysis by sodium dodecyl sulfate-gel electrophoresis indicated the presence of three peaks, which were identified as type III collagen and the α₁ and α₂ chains of type I collagen, according to their migration patterns with authentic standard collagen. The amount of radioactivity in α₁(I) chain was 2 times greater than that of the α₂(I) chain (Fig. 2). These results demonstrate that cultured mammary tissue synthesizes type I \{α₁(1)I\}α₂ and type III \{α₁(III)I\} collagen.

The addition of 80 µg/ml of LACA caused a substantial inhibition of hydroxyproline formation in explants cultured with insulin, cortisol, and prolactin for 24 h, and thereafter blocked it completely (Fig. 1). Sodium dodecyl sulfate-gel electrophoretic analysis indicated that LACA virtually abolished the formation of type I and type III collagen in cultured explants (Fig. 2).

Fig. 3 shows the effect of various concentrations of LACA on the accumulation of collagen in mammary explants cultured with the three hormones for 72 h. LACA inhibited collagen accumulation in a concentration-dependent manner. The maximal extent of inhibition attained with a LACA concentration at 80 µg/ml was about 80%.

**FIG. 1.** Time course of collagen accumulation in cultured mammary gland. Mammary explants from midpregnant mice were incubated with 10 µCi/ml of \text{[H]}\text{proline, 5 µg/ml of insulin, 1 µg/ml of cortisol, and 5 µg/ml of prolactin with (IFP) or without (IFP) 80 µg/ml of LACA for the indicated times. \text{[H]}\text{Hydroxyproline content was measured as described under "Experimental Procedures."}
concentrations of L-proline on LACA-inhibited accumulation of casein and α-lactalbumin, respectively. In both cases, almost complete restoration of the accumulation of the two milk proteins was attained in the presence of 80 μg/ml of proline. The "reversal" effect of L-proline was specific in the sense that D-proline (80 μg/ml) as well as other amino acids, L-leucine and L-lysine, each at 80 μg/ml, were ineffective in this regard.

To extend our studies on the possible relationship between collagen formation and milk-protein gene expression, the effect of LACA on the level of mRNA for casein, α-lactalbumin, and total protein in cultured mammary tissue was examined. As shown in Table I, LACA inhibited the hormone-stimulated accumulation of mRNA for casein and α-lactalbumin by 79 and 76%, respectively, whereas the amount of total mRNA was inhibited by 52%. In experiments not shown here, the extent of inhibition of the accumulation of casein mRNA and total mRNA by LACA was found to be influenced by the stage of pregnancy and the cortisol concentration in culture.

In order to assess possible causal relationships between collagen accumulation and milk protein synthesis, the effects of LACA on the accumulation of the milk proteins, casein, and α-lactalbumin, and total protein in mammary explants cultured with insulin, cortisol, and prolactin were examined. As shown in Fig. 4, casein comprised about 19% of total protein synthesized in the absence of LACA. The addition of increasing concentrations of LACA caused progressive inhibition of casein accumulation, and it was reduced by 67% in the presence of 80 μg/ml of LACA. LACA also inhibited α-lactalbumin accumulation in a concentration-dependent manner, and it was reduced by 40% in the presence of 80 μg/ml of LACA. In contrast, total protein synthesis was less sensitive to LACA and was only inhibited by 23% in the presence of 80 μg/ml of LACA. These results indicate that LACA caused a relatively selective inhibition of milk protein accumulation as compared to general protein synthesis in cultured mammary tissue.

Since LACA is an analog of proline, it was of interest to examine whether its inhibitory effect on milk protein accumulation can be reversed by the addition of a larger amount of proline. The time-course study depicted in Fig. 5 demonstrates that the addition of 80 μg/ml of proline completely overcomes the inhibitory effect of LACA on casein accumulation in cultured mammary tissue.

Fig. 6, A and B, shows the reversal effects of various concentrations of L-proline on LACA-inhibited accumulation of casein and α-lactalbumin, respectively. In both cases, almost complete restoration of the accumulation of the two milk proteins was attained in the presence of 80 μg/ml of proline. The "reversal" effect of L-proline was specific in the sense that D-proline (80 μg/ml) as well as other amino acids, L-leucine and L-lysine, each at 80 μg/ml, were ineffective in this regard.

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cells growing (data not shown). This can account for the

FIG. 6. The effect of various concentrations of proline on
LACA-inhibited accumulation of (A) casein and (B) α-lacta-
bumin in cultured mouse mammary gland. Mammary explants
from midpregnant mice were cultured in medium containing insulin,
cortisol, and prolactin (IFP) and the indicated amount of proline in
the presence (●) or absence (○) of 80 μg/ml of LACA.
Insulin and prolactin were used at a concentration of 5 pg/ml, whereas
cortisol was used at 0.01 μg/ml for α-lactalbumin accumulation and 1
μg/ml for casein.

TABLE I
Effect of LACA on the accumulation of mRNA for total protein,
casein, and α-lactalbumin in mouse mammary explants in culture

Mammary explants from midpregnant mice were cultured for 72 h
in medium containing insulin (5 μg/ml), prolactin (5 μg/ml), and
cortisol (0.01 μg/ml) with or without LACA (80 μg/ml). The amount
of mRNAs was determined as described under “Experimental Pro-
cedures.” Each value represents the average of two culture experi-
ments. The number in parentheses is the percentage of inhibition
relative to the IFP values.

<table>
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<th>Culture conditions</th>
<th>Total protein</th>
<th>Casein</th>
<th>α-Lactalbumin</th>
</tr>
</thead>
<tbody>
<tr>
<td>IFP</td>
<td>7500</td>
<td>3900</td>
<td>4 (76)</td>
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<tr>
<td>IFP + LACA</td>
<td>3600 (52)</td>
<td>820 (79)</td>
<td>16</td>
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inhibition was smaller in tissue explants from late pregnant
mice and was greater in explants cultured in the presence of
a high concentration of cortisol (1 μg/ml).

β-APN, an inhibitor of collagen cross-linking, was used to
maximize the extraction of collagen from cultured explants
(Fig. 2). This agent, however, had little effect on the accumulation
of casein, α-lactalbumin, and total protein in cultured tissue (Fig. 7). β-APN also did not interfere with the inhibitory effect of LACA on milk protein synthesis in mammary explants. These results indicate that collagen cross-linking is not required for the hormonal stimulation of milk protein accumulation in culture.

In order to assess possible cytotoxicity of LACA on cultured
tissue, its effect on [3H]thymidine incorporation into DNA
was examined. As shown in Table II, LACA at concentrations ranging from 20–80 μg/ml did not affect the incorporation of
[3H]thymidine in cultured tissue. β-APN also had no effects.

We recently demonstrated that milk protein synthesis can be
induced by insulin, cortisol, and prolactin in primary mammary
explants in culture (18) and this system was used to examine further side effects of LACA on mammary cells. The data shown in Table III indicate that LACA caused little inhibition of casein synthesis in cultured mammary epithelial cells when normalized on the basis of total protein synthesis. In the presence of LACA, however, there were less cells growing (data not shown). This can account for the

Fig. 7. The effect of various concentrations of β-APN on the
accumulation of total protein, casein, and α-lactalbumin in
cultured mouse mammary gland. Mammary explants from mid-
pregnant mice were incubated with insulin (5 pg/ml), prolactin (5 pg/
ml), cortisol (1 μg/ml or 0.01 μg/ml), and the indicated amount of
β-APN. The concentration of cortisol was used at 1 μg/ml for casein
accumulation and 0.01 μg/ml for α-lactalbumin accumulation. The
amount of total protein (●), casein (○), and α-lactalbumin (△) was measured as described under “Experimental Procedures.”

TABLE II
The effect of LACA and β-APN on [3H]thymidine incorporation in
mouse mammary explants in culture

Mammary explants from midpregnant mice were cultured for 3 days in medium containing insulin, prolactin, and cortisol. The indicated
concentrations of LACA or β-APN. [3H]Thymidine incorporation into trichloroacetic acid-insoluble materials was determined as described under “Experimental Procedures.” Each value represents the average of 2–3 determinations.

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<td>cpm/mg tissue ∙ 10^-4</td>
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<tr>
<td>IFP</td>
<td>1.50</td>
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<td>IFP + LACA, 20 μg/ml</td>
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<tr>
<td>IFP + LACA, 40 μg/ml</td>
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<td>IFP + LACA, 80 μg/ml</td>
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<tr>
<td>IFP + β-APN, 20 μg/ml</td>
<td>1.40</td>
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<tr>
<td>IFP + β-APN, 40 μg/ml</td>
<td>1.38</td>
</tr>
<tr>
<td>IFP + β-APN, 80 μg/ml</td>
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</table>

TABLE III
Effect of LACA on casein synthesis in primary mammary cell
culture

Lactating mammary epithelial cells were cultured for 5 days in
medium containing insulin, prolactin, and cortisol in the presence or
absence of LACA (80 μg/ml). Casein synthesis was determined as
described previously (13). The number in parentheses is the per-
centage of casein synthesis.

<table>
<thead>
<tr>
<th>Culture conditions</th>
<th>Total protein</th>
<th>Casein</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>cpm/ml/24 h</td>
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<td>IFP</td>
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<td>IFP + LACA</td>
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decrease in the synthesis of total protein and casein in this
system. The results thus imply that the ability of mammary
epithelium to synthesize casein in a primary cell culture is not
dependent on the formation of collagen when exogenous col-
lagen is provided.
Collagen Accumulation in Mammary Cell Differentiation

The present study has shown that accumulation of type I and type III collagen increases during hormonally induced differentiation of the mammary gland in organ culture and that LACA causes a substantial inhibition of collagen accumulation. The effect of LACA is relatively selective in the sense that LACA inhibits total protein synthesis by only 20%, whereas collagen formation is inhibited more than 80%. The observed inhibitory effect of LACA on collagen formation agrees with the results of earlier studies of LACA (3, 8, 20–24) which indicated that LACA exerted a relatively specific inhibitory effect on the synthesis and secretion of collagen without appreciable effect on the synthesis of total proteins (25, 26).

Previously, it was shown (8) that LACA, a proline analog, is incorporated in place of proline into collagen polypeptides which have a high proline-hydroxyproline content, i.e. 20% of total amino acids (19). α-Chain collagen, which contains LACA, does not undergo hydroxylation and fails to transform into a triple helical structure. Some of these peptides are degraded intracellularly (8, 20–22, 24), and other portions are secreted (27, 28). LACA has been used to explore the possible involvement of collagen in embryonic development of several organs such as the salivary gland (3), the lung (3, 29) and the teeth (30).

In the present study, LACA has been shown to inhibit the hormonal stimulation of the accumulation of the milk proteins casein and α-lactalbumin in cultured mammary tissue to a greater extent than that of total protein synthesis. It is possible that the inhibition of casein accumulation by LACA is due to direct insertion of LACA into casein, since LACA is a proline analog and casein is relatively rich in proline (31). However, this appears unlikely in the case of α-lactalbumin, whose proline content is only 3% of total amino acids (15). Moreover, LACA inhibited the accumulation of milk protein mRNAs to a greater extent than that of total mRNA. These results indicate that LACA caused a relatively selective inhibition of the phenotypic expression of milk protein genes. It is also to be noted that LACA did not inhibit DNA synthesis in cultured mammary explants.

Recently, it was shown (7, 32, 33) that collagen gels prepared from rabbit tendon are essential for the hormonal induction of differentiation of mammary epithelium in primary culture. This collagen consists of approximately 95% of type I collagen and 5% of type III collagen. The present study has shown that mammary cells cultured on collagen gels are insensitive to LACA in terms of milk protein synthesis. The observed difference in the action of LACA between organ culture and cell culture systems can be explained by the hypothesis that mammary epithelial cells cultured on a collagen gel matrix can differentiate without de novo synthesis of collagen, whereas in an organ culture system, collagen must be formed to support mammary differentiation.

Previous studies showed (34) that a type IV collagen gel matrix enhances plating efficiency and the growth rate of mammary epithelium in culture and is more effective than type I or III collagen in these processes. It was also reported (34) that the presence of cis-hydroxyproline, another proline analog, reduced plating efficiency of mammary cells in culture containing type I and III collagen gels, but not in a type IV collagen matrix. These results suggest that type IV collagen may play a role in the growth of mammary cells. Liotta et al. (35) showed that the formation and deposition of type IV collagen in cultured mammary cells are under the control of hormones. In this study, the amount of type IV collagen was too small to determine accurately and thus, the role of type IV collagen in differentiation of mammary epithelium is unknown.

The major types of collagen in cultured mammary tissue were shown to be type I and type III. Gay et al. reported previously that these types of collagen are produced by fibroblasts and thus designated them as stromal collagen (36). Although the present study has not identified the cell type in the mammary gland that produces collagen type I and type III, it is possible that they are made by stromal cells. If so, stromal cells can contribute to the differentiation of mammary epithelium through the production of collagens.

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