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 a-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 a-lactalbumin by about 77 and 70%, 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 a-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 of tissue structure. Also, collagen has been shown to be involved as 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 a-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/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, chlarnine-T, silicic acid, hydroxyproline, β-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 vasopressin 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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Collagen Accumulation in Mammary Cell Differentiation

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 mammary explants (data not shown). In the absence of hormones, the extent of collagen accumulation at 72 h was about 65% 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 [3H]proline, the three hormones, and β-APN for 72 h. β-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 radioactive activity 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(I),α2\} and type III \{α1(III),α2\} 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 III collagen in cultured explants (November 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%.

**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 mammary explants (data not shown). In the absence of hormones, the extent of collagen accumulation at 72 h was about 65% of that which was cultured with the three hormones.

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In order to assess possible relationships between collagen accumulation and milk protein synthesis, the effects of LACA on the accumulation of the milk proteins, casein, and $\alpha$-lactalbumin, and total protein in mammary explants 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 $\mu$g/ml of LACA. LACA also inhibited $\alpha$-lactalbumin accumulation in a concentration-dependent manner, and it was reduced by 40% in the presence of 80 $\mu$g/ml of LACA. In contrast, total protein synthesis was less sensitive to LACA and was only inhibited by 23% in the presence of 80 $\mu$g/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 $\mu$g/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 $\alpha$-lactalbumin, respectively. In both cases, almost complete restoration of the accumulation of the two milk proteins was attained in the presence of 80 $\mu$g/ml of proline. The "reversal" effect of L-proline was specific in the sense that D-proline (80 $\mu$g/ml) as well as other amino acids, L-leucine and L-lysine, each at 80 $\mu$g/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, $\alpha$-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 $\alpha$-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;
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 (see 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 cells cultured on collagen gels (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 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 collagen is provided.

**Table I**

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

<table>
<thead>
<tr>
<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>16</td>
</tr>
<tr>
<td>IFP + LACA</td>
<td>3600 (52)</td>
<td>820 (79)</td>
<td>4 (76)</td>
</tr>
</tbody>
</table>

**Table II**

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

<table>
<thead>
<tr>
<th>Culture conditions</th>
<th>[3H]Thymidine incorporation (cpm/mg tissue × 10⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IFP</td>
<td>1.50</td>
</tr>
<tr>
<td>IFP + LACA, 20 μg/ml</td>
<td>1.51</td>
</tr>
<tr>
<td>IFP + LACA, 40 μg/ml</td>
<td>1.35</td>
</tr>
<tr>
<td>IFP + LACA, 80 μg/ml</td>
<td>1.27</td>
</tr>
<tr>
<td>IFP + β-APN, 20 μg/ml</td>
<td>1.40</td>
</tr>
<tr>
<td>IFP + β-APN, 40 μg/ml</td>
<td>1.38</td>
</tr>
<tr>
<td>IFP + β-APN, 80 μg/ml</td>
<td>1.27</td>
</tr>
</tbody>
</table>

**Table III**

Effect of LACA on casein synthesis in primary mammary cell culture

<table>
<thead>
<tr>
<th>Culture conditions</th>
<th>Total protein</th>
<th>Casein</th>
</tr>
</thead>
<tbody>
<tr>
<td>IFP</td>
<td>198,360</td>
<td>11,305 (6.8%)</td>
</tr>
<tr>
<td>IFP + LACA</td>
<td>123,700</td>
<td>8,852 (7.1%)</td>
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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 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 rat tail 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 α2-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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