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Cell Biology

Hormonal effects on intracellular and secreted casein in cultures of mouse mammary epithelial cells on floating collagen membranes

(cell differentiation)

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ABSTRACT **Cultured on floating collagen membranes in the presence of lactogenic hormones, dissociated normal mammary epithelial cells from prelactating mice acquire the ultrastructural and biochemical characteristics of differentiated mammary secretory cells *in vivo*. The cells on floating collagen membranes in medium containing insulin alone have sparse secretory organelles, and a small amount of casein can be detected in these cells with a sensitive radioimmunoassay. These cells resemble counterpart cells in early-pregnant mice. When the cells are exposed to insulin, cortisol, and prolactin, the secretory apparatus is elaborated and significant increases in intracellular and extracellular casein are observed. In this environment, the intracellular casein content is generally four to eight times greater than in freshly dissociated cells or cells cultured in insulin alone. The amount of casein secreted into the medium by floating-collagen-membrane cultures in the three hormones is from 25 to 200 times greater than that secreted by cultures in insulin alone. Cells cultured on plastic substrates in either hormone combination fail to show any increase in intracellular or extracellular casein. On floating collagen membranes, the cells differentiate in response to hormones as they do *in vivo* and in organ culture. This cell-culture system provides an opportunity to study direct effects of environmental factors on mammary differentiation at the cellular level.**

Differentiation of mammary epithelial cells is influenced by several hormones (1). In the early- to mid-pregnant mouse, the cells of the mammary gland have few secretory organelles, and little casein synthesis is detected. The secretory cells of the late-pregnant and lactating mouse are characterized by the presence of extensive rough endoplasmic reticulum and a well-developed Golgi apparatus. At this time, there is much casein synthesis, and casein micelles are seen in secretory vesicles (2). The maintenance or induction of these characteristics can be achieved in organ culture (3). Using this system, it has been demonstrated that insulin and a glucocorticoid stimulate the development and deployment of secretory organelles. The addition of prolactin to the medium initiates casein synthesis.

Primary cultures of mammary epithelial cells dissociated from glands of pregnant mice and maintained on plastic or glass substrates rapidly lose their differentiated characteristics despite the presence of hormones. Attempts to maintain or induce biochemical differentiation have met with only limited success (4–6). Ultrastructure studies indicate that these cells lack the secretory apparatus characteristic of their counterparts *in vivo* (7).

Recently, substrates other than glass or plastic have been employed for the growth of dissociated mammary cells. The results suggest that conditions in cell culture can be modified to encourage differentiation of these cells. Cells of the human mammary tumor cell line MCF-7, grown on collagen-coated cellulose sponges, organize to assume the histologic pattern of a mammary adenocarcinoma (8). This cell line also displays a tissue-like organization when grown in an artificial capillary system; ultrastructure studies identify the presence of cell abnormalities characteristic of human breast carcinoma cells (9).

We have reported maintenance and induction of cytodifferentiation in primary cultures of dissociated normal mouse mammary epithelial cells maintained on floating collagen membranes in an appropriate hormonal environment (7). This communication describes the biochemical differentiation identified in these cells. Increases in both intracellular and extracellular casein, identified by radioimmunoassay (10), are observed in mammary cells grown on floating collagen membranes in medium containing insulin, cortisol, and prolactin.

MATERIALS AND METHODS

Dissociation and Culture Procedures. Mammary glands from 8- to 10-day pregnant C3H/Crgl or BALB/cCrgl mice were dissociated in Hanks' balanced salt solution containing 0.12% collagenase (CLS III, 125–170 units/mg, Worthington Biochemical Corp.) as previously described (7). The cells were collected by centrifugation and washed three times in Waymouth's medium MB 752/1 (Grand Island Biological Co.) containing penicillin at 100 units/ml and streptomycin at 100 μg/ml (Nutritional Biochemicals). Viable cells as indicated by trypan blue exclusion were counted in a hemacytometer. The cells were divided into three groups: (*i*) cells tested at time 0 for casein and DNA content; (*ii*) cells plated in Waymouth's medium MB 752/1 containing penicillin, streptomycin, 5% horse serum (Grand Island Biological Co.), and insulin at 5 μg/ml (bovine pancreas, Sigma); and (*iii*) cells plated in the above medium containing, in addition, cortisol at 1 μg/ml (Sigma) and prolactin at 1 μg/ml (ovine, NIH-P.S. 10). The cells were seeded at 5×10^5 cells per cm^2 directly onto 16-mm wells in Falcon plastic multiwell tissue culture plates or onto collagen-gel-coated wells. Cultures were incubated at 37° in 95% air/5% CO_2. On day 1, the horse serum was removed from the medium, and thereafter the cultures remained serum-free. The medium was changed daily. On day 4, cortisol and prolactin were added to some of the cultures from group 2.

Preparation of Floating Collagen Membranes. The collagen solution and collagen gels were prepared according to the method of Michalopoulos and Pitot (11) as modified by us (7). Eighteen–twenty hours after the cells were seeded onto collagen-gel-coated dishes, the gels were removed from the plastic substrate by rimming the gels with a scalpel blade and gently

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Abbreviations: PBS, phosphate-buffered saline; I, insulin; F, cortisol; Prl, prolactin.

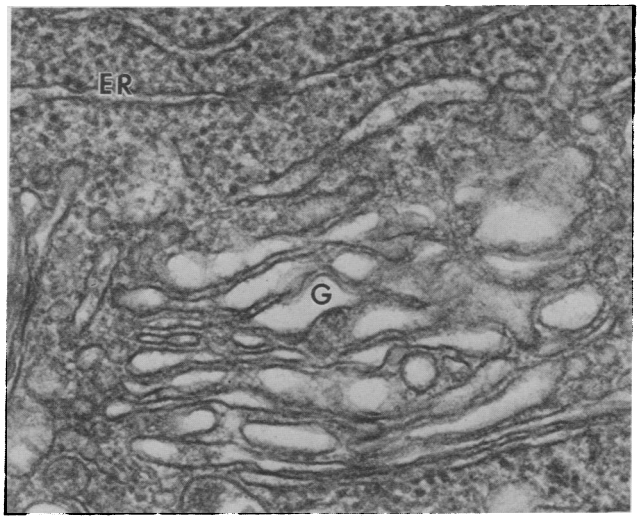

FIG. 1. Three-day culture of mammary epithelial cells on a floating gel. Cells were seeded at 5×10^5 cells per cm^2 in Waymouth's medium + 5% horse serum + insulin (I) at 5 μg/ml. On day 1, the horse serum was removed from the medium. The cell contains rough endoplasmic reticulum (ER) and a Golgi apparatus (G); however, it is void of protein granules. (×60,000.)

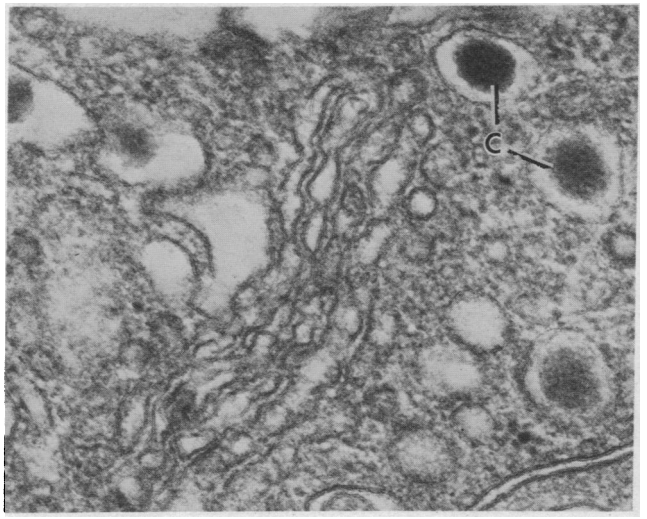

FIG. 2. Three-day culture of mammary epithelial cells on a floating gel. Cells were seeded at $5 \times 10^5/cm^2$ in Waymouth's medium + 5% horse serum + I at 5 μg/ml + cortisol (F) at 1 μg/ml + prolactin (Prl) at 1 μg/ml. The horse serum was removed on day 1. The Golgi vesicles contain casein micelles (C). (×75,000.)

shaking the dish. The gels (membranes) float beneath the medium surface.

Electron Microscopy. The cultures were prepared for transmission electron microscopy as previously described (7, 12).

Cell Processing for Determination of Intracellular Casein. The suspension of freshly dissociated cells was centrifuged at 1000 rpm for 7 min (Sorvall RC-2 centrifuge, SM24 rotor). The pellet was quick-frozen in ethanol and dry ice and stored at −20° until the conclusion of the experiment. The frozen pellet was homogenized in 0.8 ml of phosphate-buffered saline (PBS) containing 0.5% (wt/vol) Triton X-100; 0.2 ml of this was used for DNA content measurement, and the remainder was centrifuged at 10,000 rpm for 5 min (Sorvall). Aliquots (50 μl) of the supernatant were used for the radioimmunoassay. Floating collagen membranes containing cells were removed from the medium and quick-frozen. The frozen cultures were homogenized in 0.8 ml of PBS/Triton X-100 and divided as described above. The medium was removed from cultures grown on plastic substrates and PBS/triton X-100 was added to the dishes. The cells were removed with a rubber policeman and quick-frozen. The frozen cell suspension was treated like the other samples.

Medium Processing for Determination of Extracellular Casein. The medium was collected from the cultures and centrifuged at 8000 rpm for 5 min (Sorvall) to pellet the debris. The supernatant was quick-frozen and stored at −20° until the experiment was terminated. Aliquots (25 μl) of the supernatant were then mixed with 25 μl of PBS/Triton X-100 and used for the radioimmunoassay.

Radioimmunoassay. Iodination of purified mouse casein (molecular weight 22,000) and preparation of anti-casein serum were done as described previously (10). The radioimmunoassay based on competitive protein binding is a modification of the method described by Cardiff (13). Aliquots of the samples were incubated with anti-casein serum for 2 hr at 37°, ^{125}I-labeled casein was added, and incubation was continued for 2 hr. Finally, goat anti-rabbit γ-globulin (P4, Antibodies, Inc., Davis, CA) was added for 1 hr at 37° and overnight at 4°. The immune complexes were pelleted by centrifugation for 1 min (Beckman Microfuge 152) and solubilized in 2M NaOH. The solution was placed in a scintillation vial containing 0.5 ml of water and mixed with 5 ml of toluene/Triton X-100 scintillator fluid, and its radioactivity was measured in a Packard scintillation counter. The casein standard was prepared as described by enami and Nandi (10), dissolved, and stored in 4 M urea/PBS and diluted in PBS/Triton X-100 before use.

DNA Measurements. The amount of DNA in each sample was measured by a fluorometric assay modified from Hinegardner (14). The cell homogenate was treated with cold 10% trichloroacetic acid for 30 min and the pellet was collected by centrifugation at 11,000 rpm for 5 min at 4° (Beckman Microfuge B). The pellet was washed in cold 5% trichloroacetic acid, then cold 100% ethanol, and dried in a 60° oven for 2 hr. An 0.2-ml portion of a 40% (wt/vol) aqueous solution of diaminobenzoic acid dihydrochloride (Aldrich Chemicals) was added to each tube, and the samples were heated in a 60° water bath for 45 min. After 1.5 ml of 1 M HCl was added to each sample, fluorescence was measured on a Turner model III fluorometer. Calf thymus DNA (Sigma) was used as the standard.

RESULTS

Cells seeded at 5×10^5 cells per cm^2 directly onto plastic substrates form confluent epithelial pavements after 2–3 days in culture. They are identified as epithelial cells in the electron microscope by the presence of tight junctions joining adjacent cells at their apical surfaces (7, 12). However, intracellularly, they lack morphological properties that would identify them as mammary secretory cells.

Cells seeded onto collagen-gel-coated dishes form almost confluent epithelial pavements within 1 day. At this time, the gels are released from the plastic to float in the medium. In addition to having tight junctions, the cells maintained in medium containing insulin alone often have moderately developed rough endoplasmic reticulum and Golgi complexes, although they are void of secretory product (Fig. 1). Cells maintained in medium containing insulin, cortisol, and prolactin have a highly developed secretory apparatus, and their secretory vesicles contain material morphologically identical to casein micelles (15) (Fig. 2).

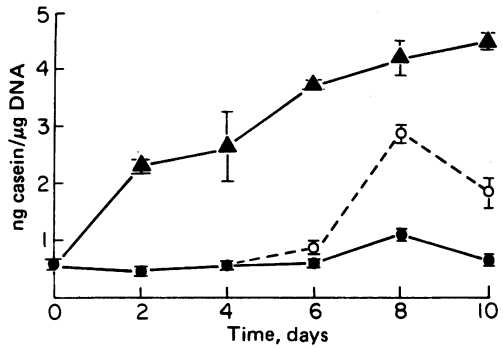

FIG. 3. Time course of casein accumulation within cultured mammary epithelial cells. Cells were dissociated and maintained on floating collagen gels in the presence of I at 5 μg/ml (●—●), or I at 5 μg/ml + F at 1 μg/ml + Prl at 1 μg/ml (▲—▲). On day 4, F and Prl were added to some cultures maintained in I alone (O- - -O). On alternate days, cultures were frozen and stored at −20°. At the conclusion of the experiment, the frozen tissue was homogenized in 0.8 ml of PBS/Triton X-100. An aliquot was removed for determination of DNA content by fluorometric assay; the remainder was processed for determination of casein content by radioimmunoassay. Freshly dissociated cells, indicated as 0 days in culture, were stored frozen and processed with the other samples. Each point and vertical bar represent mean and range from duplicate cultures.

Intracellular Casein. The casein content of cells on floating collagen gels in medium containing the three hormones is significantly greater than that of freshly dissociated cells, cells on floating collagen gels in medium containing insulin alone, or cells grown on plastic in medium containing either insulin or all three hormones.

Cells given insulin alone maintain the basal level of casein synthesis detected in freshly dissociated cells (Fig. 3). In cells maintained on floating gels in all three hormones, the casein content increases 4- to 8-fold over that in freshly dissociated cells, and this level remains constant through 10 days in culture (Fig. 3). We have detected amounts of intracellular casein as great as 30 times the amount in freshly dissociated cells (Fig. 4). Possible explanations for this variation in intracellular casein are discussed below.

When cortisol and prolactin are added to floating-gel cultures maintained for 4 days in insulin alone, there is a 3- to 4-fold increase in intracellular casein (Fig. 3).

Cells seeded on plastic show a small increase in casein content

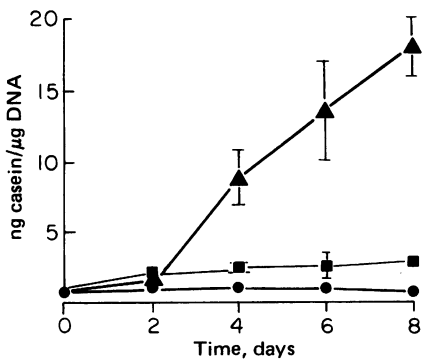

FIG. 4. Time course of casein accumulation within cultured mammary epithelial cells. Cells were dissociated and maintained on floating collagen gels in the presence of I at 5 μg/ml + F at 1 μg/ml + Prl at 1 μg/ml (▲) or maintained on Falcon plastic multiwell plates in the presence of I at 5 μg/ml (●) or I at 5 μg/ml + F at 1 μg/ml + Prl at 1 μg/ml (■). On alternate days, cultures were sacrificed and processed as described in Fig. 3. Each point and vertical bar represent mean and range from duplicate cultures.

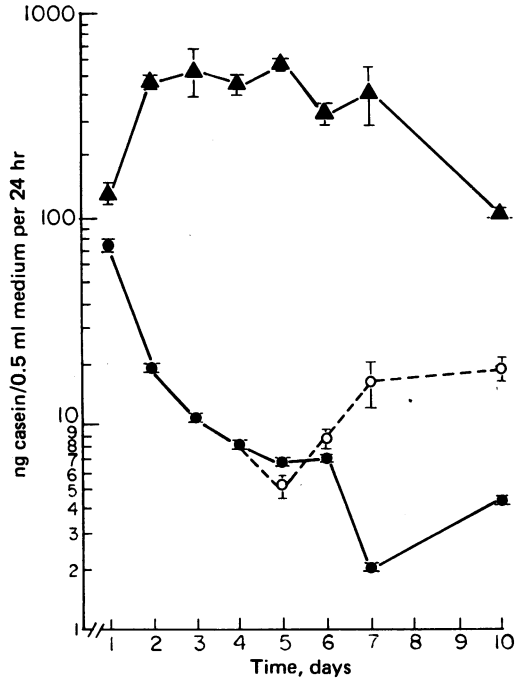

FIG. 5. Time course of casein secretion in cultured mammary epithelial cells. Cells were dissociated and maintained on floating collagen gels in the presence of I at 5 μg/ml (●—●) or I at 5 μg/ml + F at 1 μg/ml + Prl at 1 μg/ml (▲—▲). On day 4, F and Prl were added to some cultures maintained in I alone (O- - -O). Every 24 hr, the medium was collected and stored at −20°. At the conclusion of the experiment, 25-μl aliquots of the media were mixed with 25 μl of PBS/Triton X-100 and used for determination of casein content by radioimmunoassay. The gels were released to float on day 1; the value for the initial 24 hr was obtained from cells on gels still attached to the plastic dish. Each point and vertical bar represent mean and range from duplicate cultures.

in response to hormones, but this is insignificant when compared to the response of cells on floating gels in the same experiments (Fig. 4).

Extracellular Casein. The amount of casein secreted into the medium is 25 to 200 times greater in cultures grown on floating collagen membranes in medium containing the three hormones than in any other cultures (Figs. 5 and 6). Although there is a wide variation in the magnitude of response, the response is significant and is consistently reproducible.

When cortisol and prolactin are added to floating-collagen-membrane cultures maintained for 4 days in insulin only, casein secretion increases, but it remains low compared to that in cultures maintained in the three hormones from the time of cell seeding (Fig. 5).

DISCUSSION

Our results indicate that dissociated mouse mammary epithelial cells grown on floating collagen membranes respond to lactogenic hormones similarly to mammary cells *in vivo*. Cells exposed to insulin, cortisol, and prolactin contain well-developed secretory organelles and accumulate and secrete casein. Cells initially maintained in insulin alone and later exposed to all three hormones respond with a moderate increase in intracellular casein content. They respond with a similar increase in casein secretion, but the absolute amount is small relative to the amount of casein secreted by cultures initially exposed to the three hormones. The reason for this is not yet clear, but the data suggest that intracellular casein and its secretion may not be coupled. Consistent with this view is the observation that the

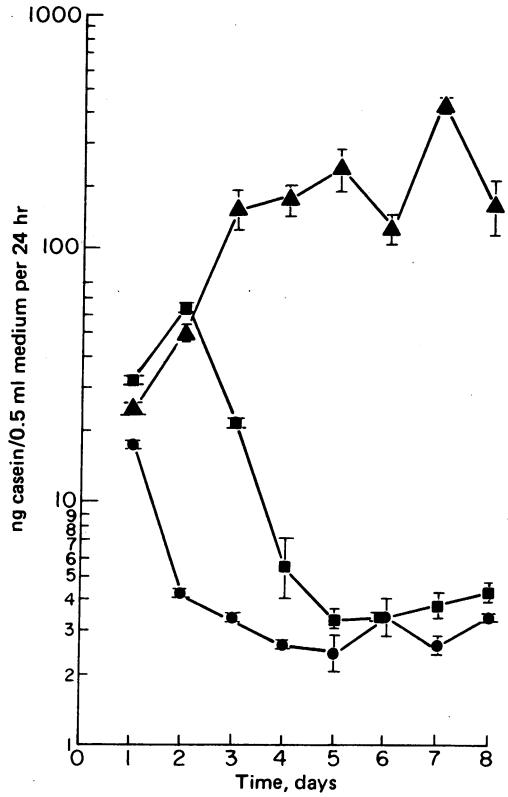

FIG. 6. Time course of casein secretion in cultured mammary epithelial cells. Cells were dissociated and maintained on floating collagen gels in the presence of I at 5 μg/ml + F at 1 μg/ml + Prl at 1 μg/ml (▲), or maintained on Falcon plastic multiwell plates in the presence of I at 5 μg/ml (●), or I at 5 μg/ml + F at 1 μg/ml + Prl at 1 μg/ml (■). Every 24 hr, the medium was collected and processed as described in Fig. 5. Each point and vertical bar represent mean and range from duplicate cultures.

intracellular casein content remains constant or increases with time in culture while extracellular casein often decreases with time in culture. It has been suggested by others (16, 17) that synthesis and secretion may be independent events in the mammary gland.

Although the amounts of intracellular and extracellular casein are consistently greater in floating-collagen-membrane cultures exposed to all three hormones than in other culture conditions, the magnitude of casein accumulation is variable. The representative experiments illustrated in this paper emphasize this point. We suggest two possibilities for this discrepancy: (*i*) The physiological state of the mammary glands may vary between experiments, although we have attempted to minimize this source of error by pooling the glands from a minimum of 10 female mice per experiment. However, at any given time, even the secretory cells of lactating glands are not all committed to producing milk components (2). Topper *et al.* (18) have demonstrated in organ culture experiments that mammary cells at different stages of development have different sensitivities to hormones. (*ii*) Differentiation of the mammary cells may be influenced by the flexibility of the collagen substrate. After the gels are released from the plastic substrate, they begin to contract (7). The degree of contraction is variable, depending in part on the hormones in the medium and cell seeding density (J. T. Emerman, unpublished data). Even under what appear to be similar conditions, the contracted gel varies from ¼ to ⅛ its original diameter. We have demonstrated that the flexibility of the floating collagen membrane allows the cells to change shape, presumably reflected by the contracting gel, and that there is a correlation between cell shape and the degree of differentiation (19).

It is difficult to assess to what degree these cells are behaving like mammary cells *in vivo* in terms of casein accumulation and secretion, because the amount of casein per unit of DNA in stimulated or unstimulated glands is not known. *In vivo* experiments have only measured casein in a given volume of milk (20).

Organ culture studies have measured cpm of [^{32}P]orthophosphate and ^{3}H-labeled amino acids incorporated into casein-like material per mg of mammary gland tissue (21, 22) and cpm per epithelial DNA content (18) to monitor the rate of casein synthesis. However, these studies have suffered from the lack of accurate methods to measure actual quantity of casein produced. Only recently have radioimmunoassay methods quantitating casein production been explored (23–25). The assay used in this study is based on purified casein preparations and can detect as little as 0.1 ng of casein (10).

Organ culture has its limitation, because the tissue is not able to secrete its casein into the bulk medium. The ducts of explants seal their cut ends (26), producing a closed system, which may limit the amount of casein that can be synthesized. It has been demonstrated that unrelieved engorgement of the glands *in vivo* leads to cessation of casein synthesis and involution of the glands (27). This problem is overcome in cell culture, because casein can be continuously secreted into the medium.

We have been able to maintain the secretory characteristics of mouse mammary epithelial cells grown on floating collagen membranes in the appropriate hormonal milieu. Other experiments done in our laboratory (J. Enami, J. Yang, and S. Nandi, unpublished data) show that casein accumulation and mammary tumor virus production follow a similar time course, and the production of mammary tumor virus is also higher in cells grown on floating collagen gels than on the plastic tissue culture dish. It has been reported that lactogenic hormones influence the production and maturation of mammary tumor virus in the mammary gland (28, 29). With the availability of culture conditions that maintain morphological and biochemical differentiation, a direct approach to the influence of environmental factors on many mammary-specific cell processes becomes possible.

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