Cell-Cell Interactions Promote Mammary Epithelial Cell Differentiation

JEDD F. LEVINE and FRANK E. STOCKDALE
Department of Medicine, Stanford University School of Medicine, Stanford, California 94305

ABSTRACT Mammary epithelium differentiates in a stromal milieu of adipocytes and fibroblasts. To investigate cell-cell interactions that may influence mammary epithelial cell differentiation, we developed a co-culture system of murine mammary epithelium and adipocytes and other fibroblasts. Insofar as caseins are specific molecular markers of mammary epithelial differentiation, rat anti-mouse casein monoclonal antibodies were raised against the three major mouse casein components to study this interaction. Mammary epithelium from mid-pregnant mice was plated on confluent irradiated monolayers of 3T3-L1 cells, a subclone of the Swiss 3T3 cell line that differentiates into adipocytes in monolayer culture and other cell monolayers (3T3-C2 cells, Swiss 3T3 cells, and human foreskin fibroblasts). Casein was synthesized by mammary epithelium only in the presence of co-cultured cells and the lactogenic hormone combination of insulin, hydrocortisone, and prolactin. Synthesis and accumulation of α-, β-, and γ-mouse casein within the epithelium was shown by immunohistochemical staining of cultured cells with anti-casein monoclonal antibodies, and the specificity of the immunohistochemical reaction was demonstrated using immunoblots. A competitive immunoassay was used to measure the amount of casein secreted into the culture medium. In a 24-h period, mammary epithelium co-cultured with 3T3-L1 cells secreted 12–20 μg β-casein per culture dish. There was evidence of specificity in the cell-cell interaction that mediates hormone-dependent casein synthesis. Swiss 3T3 cells, newborn foreskin fibroblasts, substrate-attached material ("extracellular matrix"), and tissue culture plastic did not support casein synthesis, whereas monolayers of 3T3-L1 and 3T3-C2 cells, a subclone of Swiss 3T3 cells that does not undergo adipocyte differentiation, did. We conclude that interaction between mammary epithelium and other specific nonepithelial cells markedly influences the acquisition of hormone sensitivity of the epithelium and hormone-dependent differentiation.

The mammary gland provides an excellent system to investigate the hormonal regulation of specific gene expression and differentiation. The milk proteins, particularly caseins and α-lactalbumin, represent specific molecular markers of mammary epithelial differentiation. There is substantial evidence that milk protein synthesis is under complex hormonal control by peptide and steroid hormones (1–5). Insulin, glucocorticoid, and prolactin act synergistically to induce casein and α-lactalbumin in organ culture (1–5).

Primary cultures of mammary epithelium maintained on a plastic substrate rapidly lose their differentiated characteristics even in the presence of lactogenic hormones (6, 7). Several investigators have demonstrated that nonplastic tissue culture substrates will support milk protein synthesis. Emerman et al. (8), Suard et al. (9), and Lee et al. (10) have shown that mammary epithelium cultured on floating collagen gels in the presence of insulin, hydrocortisone, and prolactin will accumulate and secrete casein. Wicha and colleagues have demonstrated increased synthesis of α-lactalbumin by rat mammary epithelium grown on "mammary gland extracellular matrix" (11). With the exception of the studies of Wicha (11), none of the previously described primary cell culture systems consistently supports both growth and differentiation.

These studies do not account for cell-cell interactions that may occur within the differentiating mammary gland in vivo (12, 13). The predominant cell-cell interactions in the mammary gland are those between epithelial cells and adipocytes and fibroblasts. These interactions are retained in organ culture of the mammary gland, an approach that has been used extensively to study the hormonal control of milk protein

synthesis (1–5). However, it has not been feasible to study regulatory interactions between mammary epithelium and adipocytes because of the difficulty in manipulating adipocytes in cell culture. Whereas epithelial cells attach to various cell culture substrates, adipocytes float and do not attach. To investigate cell interactions between mammary epithelium and adipocytes that may influence mammary cell growth and differentiation, we have used the 3T3-L1 cell line, a subclone of Swiss 3T3 cells that will differentiate as a confluent monolayer of adipocytes (14), as a cellular substrate for culture of mammary epithelium.

We have recently demonstrated that a co-culture of mammary epithelium and 3T3-L1 adipocytes has potent growth-promoting activity for mammary epithelium (15). These observations suggest that cell-cell interactions in the mammary gland may play an important role in the regulation of epithelial cell function. Insofar as mammary epithelium synthesizes milk proteins in a stromal milieu of adipocytes and fibroblasts, we have used this in vitro system to determine whether mammary epithelium exhibits hormone-dependent casein synthesis when co-cultured with 3T3-L1 adipocytes and other fibroblasts. Casein is a family of acidic phosphoproteins which comprises >50% of the mouse milk protein and consists of three major proteins of 44,000, 26,000, and 22,000 mol wt (16). Monoclonal antibodies that are specific for each of these casein proteins were generated, and these were used to analyze hormone-dependent casein synthesis in this system. We find that when mammary epithelium is cultured in the presence of 3T3-L1 adipocytes and 3T3-C2 cells, it acquires sensitivity to lactogenic hormones and synthesizes all three major casein components.

MATERIALS AND METHODS

Mammary Cell Dissociation and Culture Technique: Mammary glands from midpregnant (12–14 d) Balb/c mice were removed, minced, and dissociated with collagenase (5 U/mg tissue, 250 U/ml) (Type III, Worthington Biochemical Corp., Freehold, NJ) in Dulbecco's modified Eagle's medium (DME) for 90 min at 37°C with continuous stirring (17). Dissociated cells were centrifuged at 1,000 *g* for 5 min. The cells were then washed and pelleted three times in DME. After the final wash, the cells were resuspended in DME + 5% fetal calf serum (FCS) + 1% antibiotic/antimycotic (basal medium). This method of dissociation yields a mixture of single cells and small clusters of cells. The cell clusters were counted with a Coulter counter (Coulter Electronics, Inc., Hialeah, FL) and plated at a denisty of 2.5–5.0 × 10^4 clusters/cm^2 in 60-mm tissue culture dishes (Falcon Labware, Oxnard, CA) on confluent irradiated monolayers of 3T3-L1, 3T3-C2, Swiss 3T3 cells, human newborn foreskin fibroblasts (NFF),[1] substrate-attached material (SAM) (15), or tissue culture plastic. The cultures were incubated in a 5% CO_2 atmosphere at 37°C. The culture medium (3 ml/60-mm dish) was changed on day 2 after plating. At this time, the mammary epithelium that grew on the various substrates or plastic was readily apparent under phase optics as discrete plaques or islands of polygonal closely bunched cells. On day 3, the cultures were divided into two groups and the medium was changed to either basal medium + 5 μg/ml insulin (Sigma Chemical Co., St. Louis, MO) or basal medium + 5 μg/ml insulin + 5 μg/ml hydrocortisone (Sigma Chemical Co.) + 5 μg/ml ovine prolactin. Ovine prolactin was obtained from the National Institute of Arthritis, Metabolism, and Digestive Disease, Bethesda, MD. The medium was then changed daily for an additional 72 h.

Cell Lines: The 3T3-L1 and 3T3-C2 cell lines, which are both derived from the same 3T3 cell line, were generous gifts of Dr. Howard Green (Harvard Medical School) (14, 18). In a 4-wk incubation, 3T3-L1 cells form adipocytes, whereas 3T3-C2 cells do not. Swiss 3T3 cells were obtained from the American Type Culture Collection (Rockville, MD). Dr. Branimir Sikic (Stanford University School of Medicine) kindly provided human NFF. Stock cultures of 3T3-L1, 3T3-C2, and Swiss 3T3 cells were maintained in DME + 10% calf serum + penicillin (100 μg/ml) and streptomycin (100 μg/ml) (P/S) (Gibco Laboratories, Grand Island, NY) at 37°C in a 5% CO_2 atmosphere. NFF were maintained in DME + 10% FCS + P/S. The medium was changed every 48–72 h, and the cells were subcultured weekly. To achieve adipocyte conversion of 3T3-L1 cells, cells were plated at 10^3 cells/cm^2 in DME + 10% calf serum + P/S. When the cells were confluent, the medium was changed to DME + 10% FCS + 10 μg/ml insulin + 0.5 mM methyl-isobutyl-xanthine (Sigma Chemical Co.) + 0.25 μM dexamethasone (Sigma Chemical Co.) + P/S for 48 h (19). After 48 h, the medium was changed to DME + 10% FCS + 10 μg/ml insulin + P/S. In 1 wk, there was ~75% adipocyte conversion.

Preparation of Cellular Substrates and SAM: Confluent monolayer cultures of 3T3-L1 adipocytes, 3T3-C2 and Swiss 3T3 cells, and NFF used as cellular substrates were irradiated with 6,000 rad by using a ^{137}cesium irradiator. Dishes coated with SAM derived from monolayer cultures were prepared as previously described (15). In brief, SAM substrates were prepared from either confluent converted or unconverted 3T3-L1 cell cultures by a brief exposure to 0.02% EDTA. After gentle pipetting, the cell layer detached from the dish, which was then washed. By visual inspection, we found that these dishes contained no attached cells, but to prevent regrowth if any cells remained attached, each dish was irradiated with 6,000 rad. The dishes were used for mammary epithelial cell plating within 2–3 h of cell removal. The response of mammary epithelium was the same if the SAM dishes were prepared from converted or unconverted 3T3-L1 cells.

Preparation of Casein: Milk was obtained from 8–10-d-old lactating Balb/c mice by use of a suction apparatus 15–30 min after intraperitoneal injection of 2.5 U oxytocin (Pitocin, Parke, Davis and Co., Detroit, MI). Caseins were recovered by acid precipitation (pH 4.6) of skim milk according to the method of Enami and Nandi (20).

Monoclonal Antibody Production: Monoclonal antibodies to mouse caseins were produced by hybridoma formation between the spleen cells of a Sprague-Dawley rat immunized with acid-precipitated mouse caseins and a nonsecreting mouse myeloma cell line (AX63AG8653). Immunization, cell fusion, and selection of hybridomas were performed according to a modification of the procedures of Oi and Herzenberg (21). Initial screening of hybridoma supernatants was performed in a plate-binding assay after passive adsorption of mouse casein to polyvinyl chloride microtiter plates. Positive hybridoma supernatants were then screened on electrophoretic nitrocellulose transfers of mouse milk or caseins fractionated by SDS PAGE (see below). Hybridomas with positive supernatants were subcloned by limiting dilution on a thymocyte feeder layer (22). Supernatants from the subcloned hybridomas were used as the source of monoclonal antibodies. The antibodies used in this study were derived from subclones IC4 (anti-α casein), IVC9 (anti-β casein), and IIID10 (anti-γ casein).

Immunocytochemistry: The presence of casein or keratin in mammary epithelium was identified immunocytochemically by use of rat anti-mouse casein monoclonal antibodies or mouse monoclonal antibodies to human keratin (a gift from Dr. A. Vogel). For immunoperoxidase staining, cells in culture dishes were washed with Hanks' balanced salt solution and fixed with methanol for 2 h at 4°C. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide in methanol (30 min), and nonspecific antibody binding was blocked with 2% bovine serum albumin (BSA) (Sigma Chemical Co.) in phosphate-buffered saline (PBS), pH 7.4 (1 h). For casein detection, monoclonal antibody (1/10 to 1/100 dilution in 2% BSA/PBS) was applied to the cells overnight at 4°C. After the cells were washed with PBS, a rabbit anti-rat horseradish peroxidase (HPO)-conjugated secondary antibody (1/200 dilution, DAKO, Accurate Chemical and Scientific Corp., Westbury, NY), was applied for 4 h at room temperature. After another PBS washing, peroxidase activity was identified by incubation with a 0.04% solution of 3, 3′-diaminobenzidine (Sigma Chemical Co.) and 0.015% hydrogen peroxide in 50 mM Tris buffer, pH 7.6. Keratin was detected similarly, except that the secondary antibody used was rabbit anti-mouse HPO-conjugated antibody (1/200 dilution).

Gel Electrophoresis and Electrophoretic Transfer of Protein to Nitrocellulose (Immunoblots): SDS PAGE was performed as described by Laemmli on 10% gels (23). Caseins were transferred from SDS gels to nitrocellulose overnight at 8–9 V/cm as described by Burnette in a buffer that consisted of 25 mM Tris, 192 mM glycine, and 20% methanol (24). The transferred caseins were detected with anti-casein monoclonal antibodies according to the procedure of Burnette, except that the secondary rabbit anti-rat antibody was conjugated to HPO (23). Peroxidase activity was identified by the reaction of nitrocellulose with a solution of diaminobenzidine and hydrogen peroxide (see above).

Extracts of cultured mammary epithelial cells for PAGE and immunoblotting of casein were prepared by scraping cells from culture dishes with a rubber policeman in PBS, 1% Triton X-100, 1% deoxycholate, pH 7.4. The cell

[1] *Abbreviations used in this paper:* basal medium, consisting of DME, 5% FCS, and 1% antibiotic/antimycotic; DME, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; HPO, horseradish peroxidase; NFF, newborn foreskin fibroblasts; P/S, penicillin + streptomycin; SAM, substrate-attached material.

suspension was quick frozen in ethanol and dry ice, thawed, and homogenized with a glass homogenizer. Cellular debris was pelleted in a microfuge, and the supernatant was collected and diluted in 2× SDS sample buffer. To detect casein secreted by mammary epithelium, we centrifuged medium derived from cultures of mammary epithelium at 1,000 *g* to pellet cellular debris. The supernatant was diluted in 2× SDS sample buffer for subsequent SDS PAGE and immunoblotting of casein.

Competitive Enzyme-linked Immunoassay of Secreted Casein: To quantitate the amount of casein in the culture medium, a competitive immunoassay using monoclonal anti-β-casein antibody was developed based on a modification of the method of Crow et al. (25). Acid-precipitated mouse casein (2.5 μg/ml PBS) was passively adsorbed to polyvinyl chloride flat-bottom microtiter plates for 2 h at room temperature (50 μl/well). After adsorption, the wells were incubated with a solution of 2% BSA in PBS for 1 h at room temperature to block nonspecific protein binding. In separate glass tubes, the antibody (1/400 dilution) was mixed with serial twofold dilutions of purified β-casein or medium from cultures of mammary epithelium in PBS that contained 1% BSA, 1% Triton X-100, 1% deoxycholate, pH 7.4, and incubated for 1 h at 37°C. After incubation, 50 μl from each tube was transferred to the precoated microtiter plates and incubated overnight at 4°C. The plates were then washed and reacted with a rabbit anti-rat HPO-conjugated secondary antibody diluted in 2% BSA/PBS (1/200 dilution) for 4 h at room temperature. After additional washing, the plates were reacted with *o*-phenylenediamine (Sigma Chemical Co.), 1 mg/ml in 0.1 M citrate, pH 4.5, and 0.012% hydrogen peroxide. The reaction was stopped with an equal volume of 4 N sulfuric acid and read at 490 nm on an automatic plate scanner (Dynatech Laboratories, Alexandria, VA).

Purification of Mouse β-Casein: Acid-precipitated caseins were dansylated according to the procedure of Talbot and Yphantis (26) and were monitored during fractionation by SDS PAGE. The band corresponding to β-casein was cut from the gel, and then the protein was eluted from the polyacrylamide and concentrated in an ISCO electrophoretic concentrator (Instrument Specialties Co., Sunnyvale, CA) in the same buffer used for SDS PAGE. Protein content was determined as described by Wang and Smith (27).

RESULTS

Specificity of Anti-Casein Monoclonal Antibodies

The specificity of the rat anti-mouse casein monoclonal antibodies to the 44,000 (α), 26,000 (β), and 22,000 (γ) caseins was determined by immunoblotting. Fig. 1 *a* shows mouse casein (lane *1*), mouse milk (lane *2*), a detergent extract of lactating mammary gland (lane *3*), and bovine casein (lane *4*) stained with amido black after PAGE and electrophoretic transfer to nitrocellulose. Replicas of these blots were reacted with anti-α-casein (Fig. 1 *b*), anti-β-casein (Fig. 1 *c*), and anti-γ-casein (Fig. 1 *d*) monoclonal antibodies, then with a rabbit anti-rat HPO-conjugated secondary antibody. Fig. 1, *b–d* demonstrates that each antibody reacted with a unique casein component in acid-precipitated casein, milk, and lactating mammary gland extract corresponding to α-, β-, and γ-casein, respectively. None of the antibodies cross-reacted with bovine casein.

Immunohistochemical Staining of Mammary Epithelium with Anti-Casein Monoclonal Antibodies

Mammary epithelium was plated and incubated on confluent irradiated monolayers of 3T3-L1 adipocytes, SAM derived from 3T3-L1 adipocytes ("extracellular matrix"), or tissue culture plastic in a basal medium of DME and 5% FCS. At 72 h, the medium was changed to contain either insulin or the lactogenic hormone combination of insulin, hydrocortisone, and prolactin for an additional 72 h. Figs. 2 and 3 show these cultures after fixation and immunoperoxidase staining with monoclonal antibodies to casein. After the first 72 h of incubation in co-cultures with 3T3-L1 cells and before the addition of lactogenic hormones, no casein was detected in the mammary epithelial cells (Fig. 2 *e*). During the second 72 h of incubation on the 3T3-L1 monolayer, mammary epithelium acquired lactogenic hormone sensitivity and synthesized α-, β-, and γ-casein (Figs. 2 *a* and 3). Casein did not accumulate in cells cultured on SAM (Fig. 2 *b*) or tissue culture

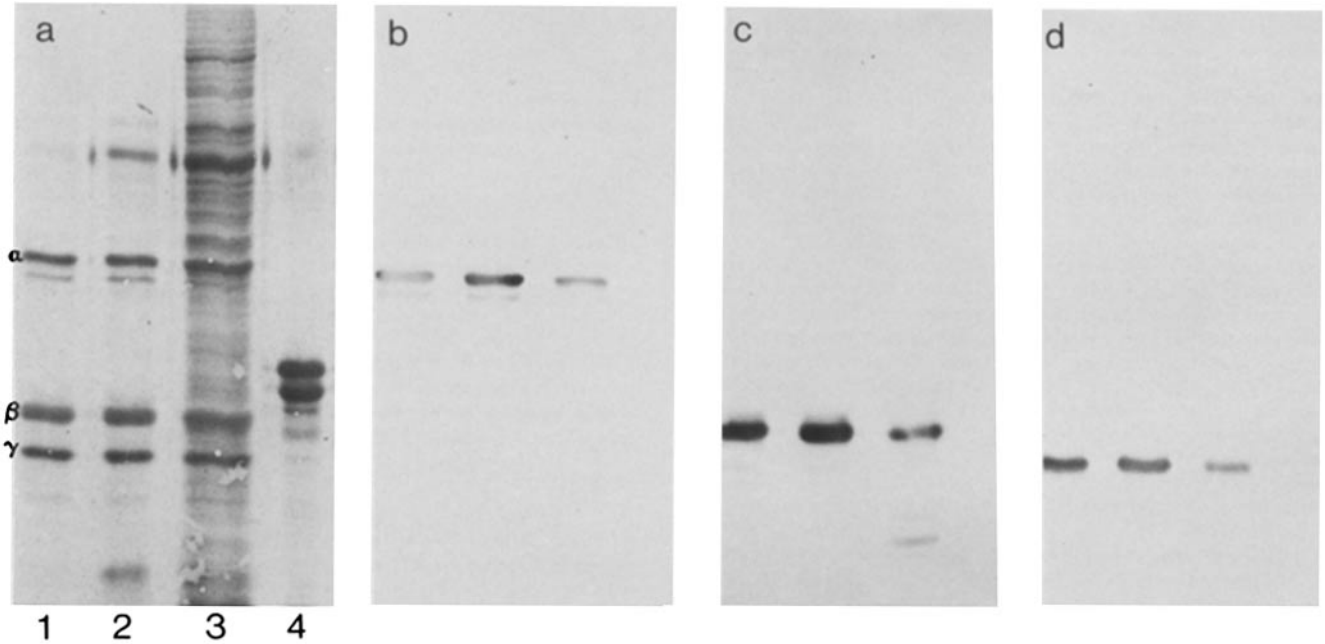

FIGURE 1 Immunoblots of caseins detected with anti-casein monoclonal antibodies. Acid-precipitated mouse casein (lane *1*), whole mouse milk (lane *2*), extract of lactating mouse mammary gland (lane *3*), and bovine casein (lane *4*) were electrophoresed on a 10% polyacrylamide gel, transferred to nitrocellulose paper, and stained with amido black (*a*). Replicas of *a* were reacted with rat anti-mouse α-casein (*b*), anti-β-casein (*c*), and anti-γ-casein (*d*), then by a rabbit anti-rat HPO-conjugated secondary antibody. Peroxidase activity was identified by addition of diaminobenzidine and hydrogen peroxide. The three major casein components are indicated on the left by symbols.

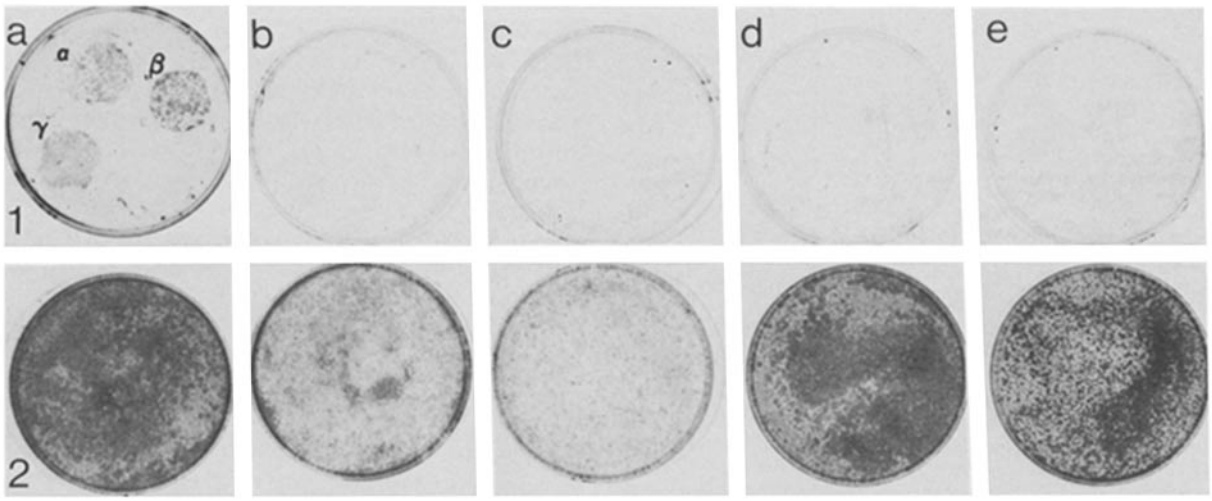

FIGURE 2 Immunoperoxidase staining of mammary epithelium with anti-casein antibodies. Mammary epithelium from midpregnant mice was plated on either a confluent layer of 3T3-L1, SAM from 3T3-L1 cells, or plastic tissue culture dishes. The epithelium was added to each dish and incubated for 3 d in the presence of DME and 5% FCS. Each dish in row *1*, *a–d*, was stained with monoclonal antibodies to α-, β-, and γ-casein after an additional 3 d of incubation in medium to which either insulin alone or insulin, hydrocortisone, and prolactin were added. Replicate plates (row *2*) were stained with hematoxylin to visualize the amount of mammary epithelium on the different substrates and hormone additions. Plates *1 e* and *2 e* were stained with the antibodies or hematoxylin before addition of any hormones. The substrates and medium conditions were: (a) 3T3-L1 cells and insulin, hydrocortisone, and prolactin; (*b*) SAM from 3T3-L1 cells and insulin, hydrocortisone, and prolactin; (c) tissue culture plastic and insulin, hydrocortisone, and prolactin; (*d*) 3T3-L1 cells and insulin; (e) 3T3-L1 cells and mammary epithelium before the addition of any hormones to the medium.

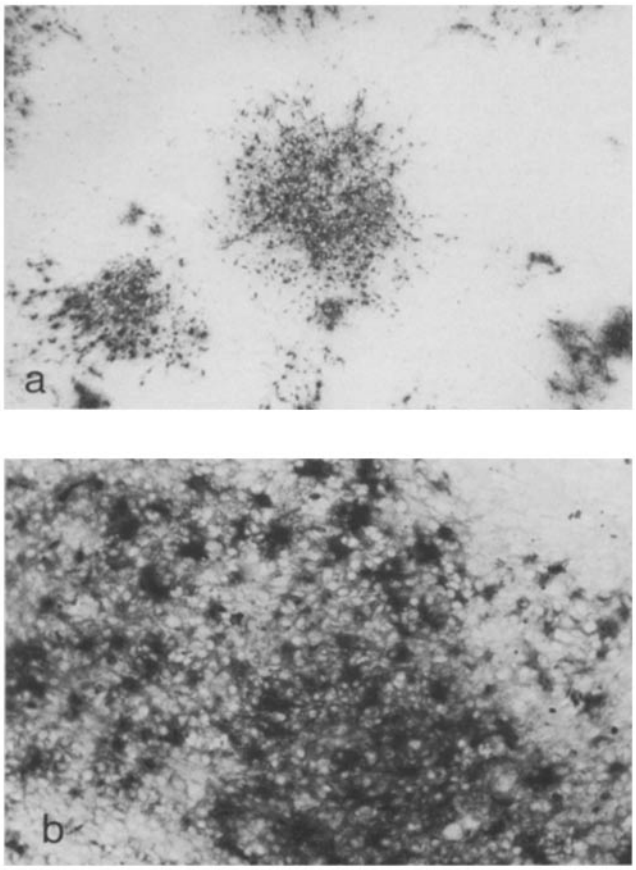

FIGURE 3 Immunoperoxidase staining of mammary epithelium with anti-casein antibody. Mammary epithelium was plated on 3T3-L1 cells in DME and 5% FCS and incubated for 72 h. At 72 h, the medium was changed to DME + 5% FCS + insulin + hydrocortisone + prolactin for an additional 72-h incubation. Photomicrographs of the cells stained with anti-α-casein antibody show that there is heterogeneity in the staining of the mammary epithelium. (*a*) × 40, (*b*) × 200.

plastic (Fig. 2*c*) even in the presence of lactogenic hormones. There was no evidence of casein synthesis in mammary epithelium cultured on 3T3-L1 monolayers in the presence of insulin alone (Fig. 2*d*). Fig. 3 shows that the positive staining of mammary epithelium with anti-casein antibodies tended to occur in discrete foci, which suggests that not all of the mammary epithelial cell population becomes responsive to lactogenic hormones when co-cultured with adipocytes.

To determine the specificity of the effect of 3T3-L1 adipocytes on mammary epithelial differentiation, we grew mammary epithelium under identical culture conditions in the presence of lactogenic hormones on monolayers of the following cells: 3T3-C2 cells (18) (a subclone of the Swiss 3T3 cells that gave rise to the 3T3-L1 line, but one which does not differentiate into adipocytes under conditions that will induce adipocyte differentiation in 3T3-L1 cells); parental Swiss 3T3 cells; or NFF. A large proportion of the mammary epithelial population was stained with anti-casein antibodies when the epithelium was plated on 3T3-L1 cells or 3T3-C2 cells, whereas only occasional mammary epithelial cells were stained when plated on Swiss 3T3 cells or NFF. The anti-casein staining of mammary epithelium on 3T3-C2 cellular monolayers appeared comparable to that detected when 3T3-L1 cells were used as the cellular substrate.

Immunoblots of Mammary Epithelial Cell Extracts and Culture Medium

To demonstrate that immunohistochemical staining of mammary epithelium in culture reflects specific reactivity of the antibodies with casein, we performed immunoblots on extracts of co-cultured mammary epithelium. Fig. 4 shows that mammary epithelium co-cultured with 3T3-L1 cells in

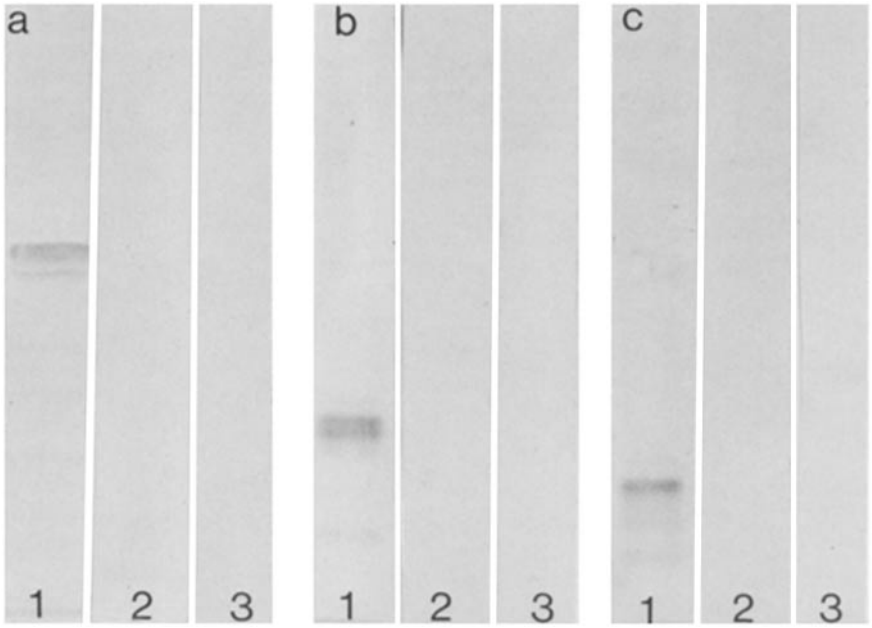

FIGURE 4 Immunoblots of extracts of mammary epithelial cell cultures. Mammary epithelial extracts derived from cells cultured on 3T3-L1 cells in the presence of DME, 5% FCS, insulin, hydrocortisone, and prolactin for 72 h (lane *1*), insulin alone for 72 h (lane *2*), and before the addition of hormones (lane *3*) were electrophoresed on a 10% polyacrylamide gel, transferred to nitrocellulose, and reacted with anti-α-casein (*a*), anti-β-casein (*b*), and anti-γ-casein (*c*), then by an HPO-conjugated secondary antibody. Peroxidase activity was identified by addition of diaminobenzidine and hydrogen peroxide. Before hormone addition, no casein is synthesized; only those extracts from cells exposed to insulin, hydrocortisone, and prolactin synthesize each casein component.

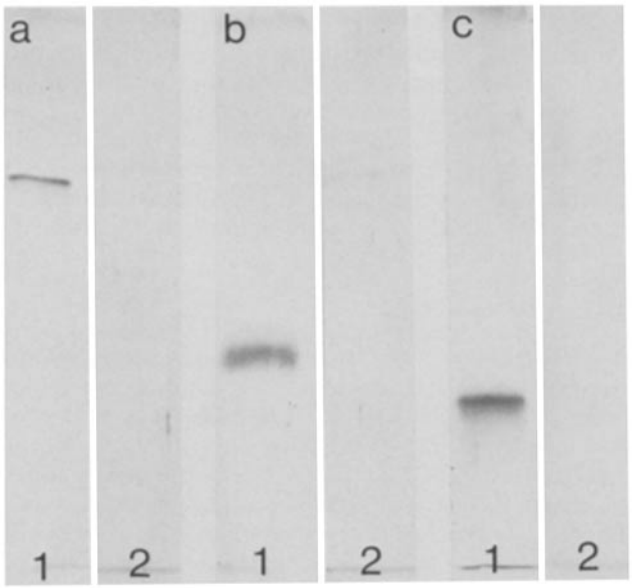

FIGURE 5 Immunoblots of culture medium of mammary epithelial cell cultures. Medium derived from mammary epithelium cultured on 3T3-L1 cells in the presence of insulin, prolactin, and hydrocortisone (lane *1*) and insulin alone (lane *2*) for 72 h were electrophoresed on a 10% polyacrylamide gel, transferred to nitrocellulose, and reacted with anti-α-casein (*a*), anti-β-casein (*b*), and anti-γ-casein (*c*), then by an HPO-conjugated secondary antibody. Peroxidase activity was identified by addition of diaminobenzidine and hydrogen peroxide. All casein components are secreted into the medium from cells exposed to the three lactogenic hormones.

the presence of lactogenic hormones for 72 h synthesized α-casein (Fig. 4*a*, lane *1*), β-casein (Fig. 4*b*, lane *1*), and γ-casein (Fig. 4*c*, lane *1*). There was no casein detected in extracts of cell cultures exposed to insulin alone (Fig. 4, *a–c*; lane *2*). After the first 72 h of incubation in basal medium, there was no casein detected by immunoblotting of mammary epithelium co-cultured with 3T3-L1 cells (Fig. 4, *a–c*; lane *3*).

Culture medium was transferred to nitrocellulose after SDS PAGE to determine whether casein was secreted into the medium when mammary epithelium was co-cultured with

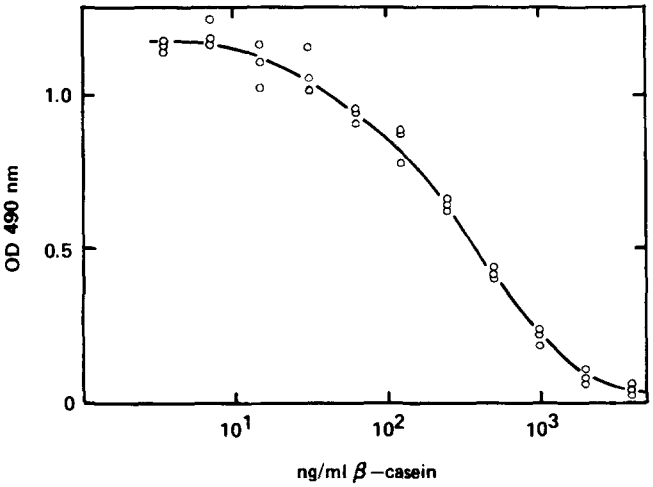

FIGURE 6 Standard curve for competitive enzyme-linked immunoassay of casein. After passive adsorption of acid-precipitated mouse casein to the bottom of a polyvinylchloride microtiter plate and blocking with BSA, serial twofold dilutions of purified mouse β-casein were mixed with anti-β-casein antibody. Aliquots of these mixtures were added to the casein-coated microtiter plate, followed by an HPO-conjugated secondary antibody. Peroxidase activity was identified by reaction with *o*-phenylenediamine and hydrogen peroxide and quantitated by determination of optical density at 490 nm.

3T3-L1 cells. Fig. 5 demonstrates that in the presence of lactogenic hormones, all three major casein components were secreted into the culture medium; no casein was found in the medium when insulin was the only hormone added.

Quantitation of Casein Secretion by Competitive Enzyme-linked Immunoassay

To quantitate the amount of casein secreted into the medium, we developed a competitive enzyme-linked immunoassay which uses monoclonal anti-β-casein antibody. A standard curve is depicted in Fig. 6 using purified β-casein as a competitor. The assay is sensitive in the range of 60–120 ng/ml β-casein. Table I demonstrates that there was measurable

TABLE I

β-Casein Accumulation in the Culture Medium

Substrate	Hormone addition	μg β-Casein/ 60-mm dish per 24 h*
3T3-L1	I, F, P‡	12.7 ± 0.29§
3T3-L1	I	< 0.18‖
3T3-C2	I, F, P	7.5 ± 0.71
Swiss 3T3	I, F, P	< 0.18
NFF	I, F, P	< 0.18
SAM	I, F, P	< 0.18
Tissue culture plastic	I, F, P	< 0.18

* Assay performed 72 h after hormone additions to the culture.
‡ Insulin (I), hydrocortisone (F), prolactin (P).
§ ± SEM.
‖ Limit of detection of the assay.

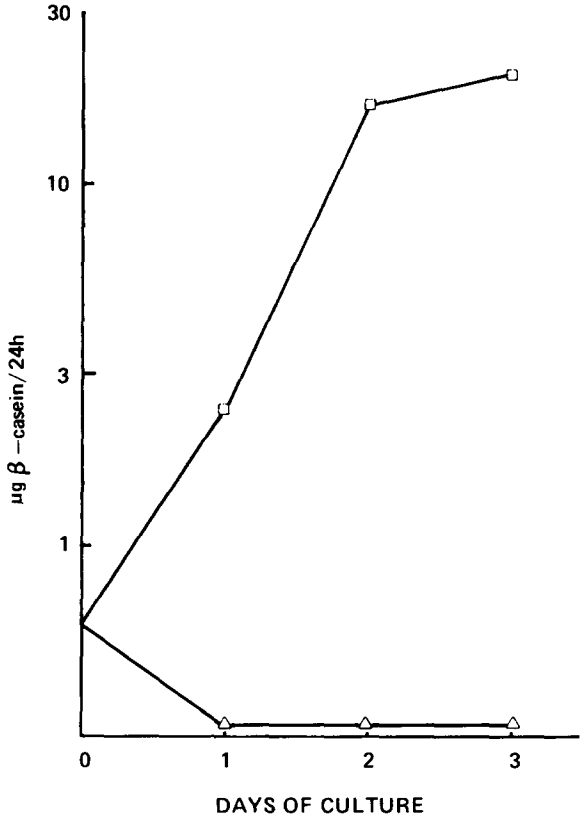

FIGURE 7 Time course of accumulation of β-casein in culture medium. Mammary epithelium was plated on 3T3-L1 cells in DME and 5% FCS (3 ml/60-mm dish). At time 0 (72 h after plating), the culture medium was changed to DME and 5% FCS and insulin, hydrocortisone, prolactin (□), or DME and 5% FCS and insulin (△). The medium was changed daily and assayed by competitive immunoassay for the quantity of β-casein every 24 h. Each point represents the mean of duplicate determinations that differed by <10%. Casein production is detected by 24 h after addition of lactogenic hormones.

casein only in medium derived from mammary epithelium cultured on 3T3-L1 or 3T3-C2 cells in the presence of insulin, hydrocortisone, and prolactin. There was no casein detected in the medium derived from mammary epithelium cultured on Swiss 3T3 cells, NFF, SAM, or plastic even in the presence of lactogenic hormones.

Fig. 7 shows the daily accumulation of β-casein in the culture medium of mammary epithelium co-cultured with 3T3-L1 cells. Each day over the course of 3 d of incubation, there was increasing accumulation of β-casein when mammary epithelium was co-cultured with 3T3-L1 cells in the presence of insulin, hydrocortisone, and prolactin. A response to lactogenic hormones is seen within 24 h of hormone addition. Between the second and third day after addition of lactogenic hormones, there was accumulation of >20 μg β-casein in the medium of a single 60-mm tissue culture dish.

Immunohistochemical Staining of Mammary Epithelium with Monoclonal Antibody to Keratin

It is important to demonstrate that when epithelium is plated, the epithelial cells attach to the various substrates. The dishes that contained either the substrates alone or those to which epithelium was added were stained with a monoclonal antibody to keratin 72 h after addition of lactogenic hormones (Fig. 8). The cellular substrates alone contain no cells that stain with the antibody to keratin (Fig. 4, row 1, *a–d*). Under these conditions, the culture dishes to which mammary epithelium was added show comparable numbers and sizes of islands of epithelial cells on 3T3-L1, 3T3-C2, and Swiss 3T3 cell substrates (Fig. 4, row 2, *a–c*). The size of the islands is smaller on the NFF and the tissue culture plastic substrates (row 2, *d* and *e*), but it is clear that there is abundant epithelium in all cases for analysis of casein production by immunohistochemistry as illustrated in Fig. 2. Thus, the difference in casein synthesis demonstrated for Swiss 3T3 cells versus 3T3-L1 or 3T3-cells in Table I is due to differences in hormone responsiveness of the mammary epithelium, not to selective overgrowth of mammary gland fibroblasts or solely to differences in the amount of epithelium attachment or growth.

DISCUSSION

The data reported in this study show that in isolated murine mammary epithelium, the acquisition of hormone sensitivity to lactogenic hormones and the synthesis of casein is dependent upon interaction with a cellular substrate. Attachment to a substrate of 3T3-L1 or 3T3-C2 cells permits mammary epithelium to undergo hormone dependent differentiation after only a brief period of co-culture. This is not a nonspecific response since not all cell substrates during this period of co-culture will elicit a significant casein response to lactogenic hormones, nor will tissue culture plastic substrates produce this response. The cellular interaction results in a significant change in epithelial cell function in that caseins accumulate intracellularly and are secreted in large quantity into the culture medium.

The magnitude of differences in numbers of cells stained by use of anti-casein antibodies and in quantity of casein production on different cellular substrates is the important point. Analysis of casein production at the individual cell level by use of immunostaining with monoclonal antibodies to different caseins indicates marked differences in the production of casein dependent upon substrate. Very few cells on Swiss 3T3 or NFF cellular substrates produce casein at the single cell level as seen under the microscope, whereas casein production is grossly apparent even to the unaided eye in epithelial cells grown on 3T3-L1 or 3T3-C2 cells. Differences solely in numbers of cells attached or the amount of growth do not account for the differences in casein synthesized. That there are large differences in hormone-dependent casein production and secretion on different substrates is confirmed by

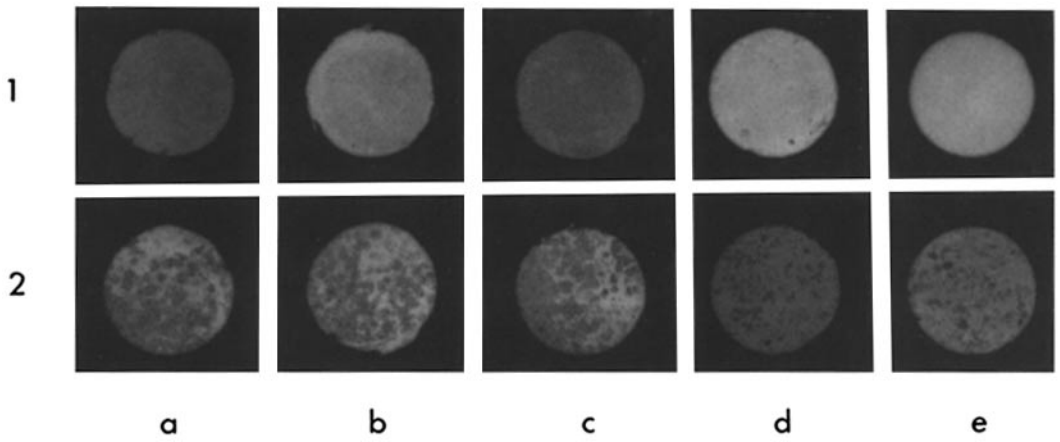

FIGURE 8 Immunoperoxidase staining of mammary epithelium with anti-keratin antibody. Cellular substrates of 3T3-L1 cells (*a*); 3T3-C2 cells (*b*); Swiss 3T3 cells (*c*); NFF (*d*); or tissue culture plastic (e) were established. Row *1*: no mammary epithelium was added. Row *2*: mammary epithelium (5×10^4 cells/cm^2) was added. All dishes were incubated for 72 h in DME and 5% FCS followed by an additional 72-h incubation in insulin, hydrocortisone, and prolactin at which time all dishes were stained with monoclonal antibody to keratin as described in Materials and Methods.

quantitation of casein in the culture medium. Whether the casein synthesis in this interaction is regulated at the transcriptional or translational level of gene expression is not known. Previous work using midpregnant mouse mammary gland in organ culture has demonstrated that new synthesis of casein is associated with new mRNA synthesis (1, 2).

The synthesis of casein in response to lactogenic hormones by epithelium from midpregnant mice growing on 3T3-L1 or 3T3-C2 cells is not simply due to retention or maintenance of this function. As shown in Figs. 2 and 4, casein synthesis in our experimental system is not detectable before the addition of lactogenic hormones. Although it is possible that the midpregnant state may enhance the magnitude of the response of mammary epithelium to lactogenic hormones, the midpregnant state is not a requirement, since in preliminary experiments epithelium from nonpregnant mice also synthesizes casein when plated on 3T3-L1 cells in the presence of lactogenic hormones (unpublished observations).

Previous work that examined in vitro milk protein synthesis in primary mammary epithelial cell culture, particularly on plastic substrates, has met with limited success (6, 7, 28). Emerman et al. (8), Suard et al. (9), and Lee et al. (10) have demonstrated that casein is synthesized by mammary epithelium cultured on floating collagen gels but not on attached gels. They suggest that the flexibility of the floating collagen gel allows the mammary cells to assume a more columnar-cuboidal shape, and that this change in morphologic orientation and organization is an important prerequisite for hormonal responsiveness and differentiation.

Our data would indicate that a more appropriate interpretation of their results is that a change in cell shape and orientation, and synthesis of a specific cell product such as casein, are associated features of mammary epithelial differentiation, rather than being causally related. That mammary epithelium can synthesize casein when cultured on an appropriate monolayer of cells indicates that a mechanically induced change in cell shape, such as that caused by collagen gel contraction, is not a requirement for synthesis of a specific cellular product. Although 3T3-L1 (and 3T3-C2) cells very likely permit a change in the three-dimensional organization and cell-cell relationships of the mammary cell population in the presence of lactogenic hormones, this should be regarded as a concomitant of the differentiative response and not a cause for it.

These data focus attention on the role of cell-cell and cell-stromal interactions in mammary gland differentiation. It has been well established that during embryonic development, normal growth and differentiation are dependent on epithelial-mesenchymal interactions (29). Epithelium and mesenchyme interact reciprocally either to regulate or amplify previously determined gene expression (28) or to induce selectively *de novo* production of specific gene products (30). The work of Kratochwil and Sakakura has demonstrated the importance of epithelial-mesenchymal interactions in embryonic mammary growth and morphogenesis (12, 13). Green et al. have suggested that similar interactions may also be a feature of adult epithelial differentiation, since they have been able to serially culture human epidermal keratinocytes with evidence of squamous differentiation only in the presence of 3T3 fibroblasts (31). Shannon and Pitelka have demonstrated morphological and biochemical differentiation of murine mammary epithelium in association with mammary stroma after injection into cleared mammary fat pads (32). Indeed, the potency of the organ culture technique in promoting milk protein synthesis is likely because important cell-cell and cell-stromal relationships are maintained.

These observations indicate that cell-cell and/or cell-stromal interactions are required for hormone-dependent differentiation of mammary epithelium. The finding that co-culture of mammary epithelium with 3T3-L1 or 3T3-C2 cells will support massive casein production, whereas co-culture with the parental Swiss 3T3 cell line or NFF will not, indicates that the role of the cellular substrate is not entirely passive or nonspecific. That 3T3-C2 cells support casein production indicates that the appearance of a fat vacuole per se is not a prerequisite for these lines to support casein production. In fact it may be fortuitous that the 3T3-L1 line is so permissive for mammary epithelial differentiation. Whereas living cell layers establish hormone responsiveness, SAM alone does not promote hormone-dependent differentiation (Table I), which suggests that other factors are required than those remaining after removal of cells with EDTA. However, matrix prepared in this way has a pronounced effect on mammary epithelial growth (15), which indicates that SAM has biological activity

on mammary epithelium. These observations do not necessarily mean that the focus of interaction is other than through matrix components since preparation of matrix by other methods (11) or longer durations of exposure to the matrix may permit differentiation to be seen. The common element in the process of mammary epithelial differentiation on cellular substrates, floating collagen gels (8, 10), and on mammary gland matrix (11) may be the stroma. Since the presence of a basal lamina is so important for normal epithelial response and function, we postulate that some cellular substrates foster more rapid basal lamina assembly than others. Those cell lines that permit mammary epithelium to differentiate may mediate this effect through elaboration of specific extracellular matrix materials and/or soluble factor(s) that facilitate basal lamina formation by the epithelium. Time parameters may also be important for the interpretation of interactions between cells, cell stroma, or matrix components. In the system reported here, casein production in response to lactogenic hormones develops within a day of adding hormones to the appropriate cellular substrates, and very large amounts of casein accumulate and are secreted. Interactions with cellular or other substrates may require more time for crucial components to be synthesized or for concentration of components to reach levels at which differentiation is frankly manifested.

Because some cell types are permissive and others nonpermissive in mediating hormone-dependent casein synthesis by mammary epithelium, these different cell types can be used to further define cell-cell and cell-stromal interactions that influence hormone-dependent mammary epithelial differentiation. Furthermore, the ability to detect different casein components with monoclonal antibodies will facilitate future study of differential regulatory effects on casein gene expression.

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