Effect of Cell Shape Change on the Function and Differentiation of Rabbit Mammary Cells in Culture

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ABSTRACT   We examined the role of cell shape, cytodifferentiation, and tissue topography on
the induction and maintenance of functional differentiation in rabbit mammary cells grown as
primary cultures on two-dimensional collagen surfaces or in three-dimensional collagen
matrices. Mammary glands from mid-pregnant rabbits were dissociated into single cells, and
epithelial cells were enriched by isopycnic centrifugation. Small spheroids of epithelial cells
(approximately 50 cells) that formed on a rotary shaker were plated on or embedded in
collagen gels. The cells were cultured for 1 d in serum-containing medium and then for up to
25 d in chemically defined medium. In some experiments, epithelial monolayers on gels were
mechanically freed from the dishes on day 2 or 5. These gels retracted and formed floating
collagen gels. On attached collagen gels, flat monolayers of a single cell type developed within
a few days. The cells synthesized DNA until the achievement of confluence but did not
accumulate milk proteins. No morphological changes were induced by prolactin (PRL). On
floating gels, two cell types appeared in the absence of cell proliferation. The cells in direct
contact with the medium became cuboidal and developed intracellular organelles typical of
secretory cells. PRL-induced lipogenesis, resulting in large fat droplets filling the apical cyto-
plasm and accumulation of casein and α-lactalbumin in vesicles surrounding the fat droplets.
We detected transferrin in the presence or absence of PRL intracellularly in small vesicles but
also in the collagen matrix in contact with the cell layer. The second cell type, rich in
microfilaments and reminiscent of the myoepithelial cells, was situated between the secretory
cell layer and the collagen matrix. In embedding gels, the cells formed hollow ductlike
structures, which grew continuously in size. Secretory cells formed typical lumina distended by
secretory products. We found few microfilament-rich cells in contact with the collagen gels.
Storage and secretion of fat, caseins and α-lactalbumin required the presence of PRL, whereas
the accumulation and vectorial discharge of transferrin was prolactin independent. There was
no differentiation gradient between the tip and the center of the outgrowth, since DNA
synthesis and milk protein storage were random along the tubular structures.

These results indicate that establishment of functional polarity and induction of cytodiffer-
entiation are influenced by the nature of the interaction of the cells with the collagen structure.
The morphological differentiation in turn plays an important role in the synthesis, storage, and
secretion of fat and milk proteins.

Onset of gestation is reflected in the mammary gland by the resumption of epithelial cell proliferation with a characteristic lobulo-alveolar outgrowth into fat tissue previously free of ductal penetration. The cell growth of the mammary gland is

accompanied by drastic morphological changes and the onset of synthesis and secretion by alveolar cells of specific milk components (for reviews, see references 1, 37). Growth and differentiation of the mammary gland during gestation are entwined in a manner that makes it difficult to dissociate and analyze them experimentally at the molecular level. Classical in vivo studies, consisting in endocrinectomy followed by hormonal supplementation, often led to conflicting results. Development of organ culture systems and more recently of new primary cell culture conditions that allow sustained growth and the induction and maintenance of a differentiated state (14, 30, 42) provided an important step forward in the interpretation of experimental data.

From the latter studies, it became evident that the expression of two mammary cell functions, i.e., cell proliferation and milk protein gene expression, was influenced by culture conditions. Thus, mammary epithelial cells isolated from midpregnant mice produced considerably more of the milk protein casein when cultured on floating gels of stromal collagen than when placed on attached collagen gel or directly on plastic dishes (14, 15). When the same cells were embedded within, rather than plated on, the surface of stromal collagen gels, there was extensive cell growth, but little differentiation (42). Recently, in studies that employed cells grown on a complex extracellular matrix isolated from pregnant rat mammary gland (28), both cell growth and functional differentiation were maintained, and cells remained viable for several months (41). These studies, however, correlated only to a limited extent the morphological features of mammary cells cultured under various conditions with gene expression.

Our purpose was to analyze in detail the molecular events that accompany the morphological differentiation of mammary cells in culture. We chose the rabbit, which offers several experimental advantages over other animal models which have been studied, for the following reasons. First, several milk proteins regulated or not by lactogenic hormones have been isolated (11). Second, the hormonal regulation of casein gene expression has already been investigated in vivo (12, 19, 20) and in vitro (13, 35). In addition, probes in the form of specific antibodies (11) or complementary DNA (32) are available, and the mammary lactogenic hormone binding site has been isolated and immunochemically characterized (18). Finally, the rabbit mammary secretory immune system has been extensively investigated (24–26).

The present work defines culture conditions that allow induction and maintenance of functional differentiation that is similar to what takes place in vivo during pregnancy. Here we also examine, at the cellular level by morphological means, the hormonal environment necessary for proliferation and expression of milk protein genes. In the accompanying paper (33), the molecular events leading to functional differentiation are assessed biochemically. Part of this work has been presented in an abstract form (31) and in a symposium (23).

MATERIALS AND METHODS

Reagents: The following enzymes and inhibitors were obtained from Worthington Biochemical Corp. (Freehold, NJ): crude collagenase type III (*Clostridium histolyticum*), which was further purified according to Schultz et al. (29); crude bovine testis hyaluronidase, 400 USP/mg (free of proteolytic activity as tested against azocoll); purified swine pancreas elastase, 60 U/mg; and purified soybean trypsin inhibitor (STI). From Armour Pharmaceutical Co. (Chicago, IL) we obtained bovine serum albumin (BSA), powder fraction V. Fetal calf serum (FCS), horse serum (HS), fungizone, trypsin-EDTA mixture, penicillin, and streptomycin were purchased from Grand Island Biological Co. (Grand Island, NY); 6-[^{3}H]thymidine, [^{3}H]leucine (200–300 Ci/mmol), [α]-dGT^{32}P (400 Ci/

mmol), [^{35}S]methionine (1,200 Ci/mmol), and Na^{125}I (carrier-free) were obtained from Amersham (Buckinghamshire, England). Phenylmethylsulfonyl fluoride (PMSF) and gentamicin were obtained from Serva (Heidelberg, Federal Republic of Germany); pepstatin, leupeptin, antipain, and aprotinin, from Sigma Chemical Co. (St. Louis, MO); lissamine rhodamine B-sulfonyl chloride was from Polysciences, Inc. (Warrington, PA); fluorescein isothiocyanate (FITC) from Miles Laboratories (Kankakee, IL); Ilford L$_4$ emulsion was purchased from Ilford Ltd. (Ilford, England). Streptavidin was obtained from BRL (Bethesda Research Laboratories GmBH, Neu Isenburg, FRG). •

Hormones: Progesterone, 17-β-estradiol, 3,5,3'-triiodo-L-thyronine (T$_3$), and dexamethasone were obtained from Fluka (Buchs, Switzerland). Prostaglandin F2α and epidermal growth factor (EGF) were purchased from Inotech (Lausanne, Switzerland). Porcine insulin was purchased from Sigma Chemical Co. Prolactin (PRL) (NIH-PS 11 and 12) was obtained through the hormone distribution program of the National Institute of Arthritis and Metabolic Diseases (Bethesda, MD). Bromocryptin (CB 154, 2-Br-ergocryptin) was a gift from Dr. E. Del Poco, Sandoz Ltd. (Basel, Switzerland).

Animals: Outbred virgin 4- to 5-mo-old New Zealand rabbits were used in all experiments. Females bred for the first time were killed on day 15 of gestation. All animals were subcutaneously injected with 2 mg of bromocryptin 36, 24, and 12 h before killing. Following induction of anesthesia (pentobarbital: 50 mg/kg wt), mammary glands were removed under sterile conditions, and the animals were then killed by an overdose of pentobarbital.

Dissociation of Rabbit Mammary Glands: Mammary glands were dissociated by collagenase, elastase, and hyaluronidase digestion, divalent cation chelation, and mechanical disruption according to a published procedure (22) with the following modifications. Mammary glands, freed of connective and fat tissue, were minced with scissors prior to the first 90-min enzyme digestion. All incubation steps were performed on a rotary shaker (180 rpm) in an incubator equilibrated with 5% CO$_2$ and 95% air at 37°C. After mechanical disruption of lobules, we filtered the cell suspension through a 20-μm Nytex filter mounted on a Millipore Sterifil filtration device under slight vacuum. The epithelial cells were separated from interstitial cells by isopycnic centrifugation, washed five times with F12/M199 medium, and finally were suspended in the same medium containing 20% HS, 5% FCS, gentamicin (100 μg/ml), and fungizone (2.5 μg/ml). On the average, the yield obtained per rabbit was 5 × 10^8 cells. To constitute a large pool of cells that would then allow comparative studies using the same starting material, freshly dispersed mammary cells were suspended at 10^7 cells/ml in F12/M199 medium containing 20% HS, 5% FCS, and 10% dimethyl sulfoxide, frozen overnight at −80°C, and then were stored in liquid nitrogen. Upon thawing, the viability of the cells, as judged by Trypan blue exclusion, was in the range of 60%. All the experiments were conducted with such cells.

Collagen Gels: We prepared rat tail collagen type I as described (5) and used at a concentration of 5 g/liter of 0.1% acetic acid. The collagen solutions were filtered sequentially through 3.0, 1.2, 0.65, and 0.45-μm Millipore filters and were stored at 4°C under sterile conditions. Gelation was achieved by mixing 0.2 ml of a 2 to 1 solution of M199 (10 times concentrated) and 0.34 N NaOH with 1 ml of collagen solution (14). For cells grown on attached or floating collagen gels, collagen was spread evenly in Costar microplates (0.15 ml/cm^2) with wells having a diameter of 35 or 16 mm (Costar, Cambridge, MA). For embedded gels, a first layer of collagen was formed under the conditions described. The collagen solution was allowed to polymerize at 37°C and the gels were then equilibrated with F12/M199 medium containing 20% HS and 5% FCS. For embedding gels, small aggregates of mammary cells (see culture conditions) were mixed at a density of 10^5 to 10^6 cells/ml with the collagen mixture at 4°C, and aliquots (0.25 ml/cm^2) were immediately overlaid on collagen wells and were allowed to polymerize by incubation at 37°C.

Culture Conditions: The standard medium for plating, growing and labeling cells was a one to one mixture of M199 and F12 media supplemented with gentamicin (100 μg/ml). Fungizone at 2.5 μg/ml was added for the first 5 d of culture. HS and FCS were heat-inactivated at 60°C before use. Before plating, freshly dispersed or thawed cells were allowed to aggregate by incubating 3 × 10^7 cells in 15 ml F12/M199 medium containing 20% HS and 5% FCS in a bacteriologic dish (10 cm in diameter) for 16 h at 37°C on a rotary shaker (37 rpm). The spheroids were recovered by low speed centrifugation (2 min at 25 × g), suspended in F12/M199 medium containing 20% HS and 5% FCS, plated on attached collagen gels at a density of 0.5 or 5 × 10^5 cells/cm^2, and were embedded at 10^6 cells/ml of collagen mixture. The cells were cultured for 24 h in the same serum-containing medium and then in serum-free medium complemented with 0.25% BSA, 10^{-10} M dexamethasone, 10^{-10} M 17-β-estradiol, 10^{-9} M triiodothyronine, 10 ng/ml EGF, and prostaglandin F2α, 10^{-8} M progesterone, 5 μg/ml insulin, and 5 × 10^{-9} M ovine prolactin (PRL). After 3 d, progesterone was removed and the cells were further cultured in the absence (controls) or presence of 5 × 10^{-9} PRL. The medium was changed each day. Floating gels were obtained 2 or 5 d after plating by mechanically detaching the attached collagen gels with a Pasteur pipet.

Immunofluorescence and [³H]Thymidine Incorporation: Mammary cell cultures were incubated for 1 h in the presence of 2 μCi/ml of [³H]thymidine, were washed in PBS, were fixed in 3% formaldehyde, and were processed for immunocytochemistry according to Tokuyasu (36). Affinity-purified anticasein, anti-α-lactalbumin, and antitransferrin antibodies were directly biotinylated and then were visualized with fluorescein or lissamine-labeled streptavidin (R. Rodewald, D. S. Papermaster, and J. P. Kraehenbuhl, manuscript in preparation). The slides were processed for autoradiography with liquid Ilford emulsion with 3 wk exposure before development. Since all biochemical measurements reported in the accompanying paper (33) were performed after digestion of the collagen gels, we have compared the immunocytochemical and autoradiographic results of cells fixed on intact collagen gels with those in which the collagen gels were first digested with purified collagenase (29) for 2 h at 37°C. We observed no difference in the labeling pattern.

Microscopy: We observed living cells with phase-contrast optics in a Zeiss inverted microscope. Cells on or in collagen gels were fixed for light or electron microscopy in 0.5% glutaraldehyde, 5% sucrose, 0.1 M cacodylate buffer, pH 7.4, for 2 h at room temperature, were postfixed in 1% osmium tetroxide, and were embedded in Epon.

RESULTS

Topographical Organization of Mammary Cells in Culture

SPHEROID FORMATION: Freshly dispersed mammary cells or thawed cells that were allowed to aggregate by overnight shaking formed small spheroids averaging 50 cells. Such spheroids were constituted by epithelial cells, which after 16 h had reestablished junctional complexes. Before plating or embedding, the spheroids were separated from dead cells and contaminating fibroblasts by low speed centrifugation (2 min at 25 × g). The spheroids were recovered in the pellet. Thus, enrichment of viable epithelial cells was achieved by this simple procedure.

ATTACHED COLLAGEN GELS: Cells organized in spheroids were plated at low (5 × 10⁴ cells/cm²) and high density (5 × 10⁵ cells/cm²). The cells were cultured for 1 d in serum-containing medium which permitted rapid adhesion of the aggregated cells to the surface of the collagen gels. The cells rapidly spread out, flattened, and achieved confluency within 5 d when plated at low density and within 24 h at high density. After 24 h, the serum-containing medium was replaced by chemically defined medium and the cells were cultured for up to 25 d. The cells remained epithelial in appearance and there was no apparent increase in the fibroblast population with time. At confluency, the mammary cells formed a flat monolayer (Figs. 1a and 2a) with microvilli present on the cell surface facing the medium and a thin dense layer separating the monolayer from the collagen gel. We observed junctional complexes in the form of tight junctions that sealed the apical end of the cells, zonula adherens, and desmosomes. We detected no morphological changes when PRL was added to the medium. However, when grown in the absence of PRL for longer periods, the cells became vacuolated and the monolayers showed signs of disorganization.

FLOATING COLLAGEN GELS: On day 2 or 5, depending on the cell density, the collagen gels were mechanically detached from the plastic wells. The gels retracted rapidly with a 50% reduction in diameter within ~6 h. We observed no retraction in the absence of cells, and the rate of retraction was proportional to the cell density. Concomitant to this retraction, the cells changed their shape and became cuboidal or columnar with many infoldings of their lateral membrane (Fig. 1b and d, Fig. 2b). The number and length of the apical microvilli increased. In the absence of progesterone and PRL, survival of these cells was poor over longer incubation times. In the

presence of these two hormones, however, the preservation of fine structure was similar to that of cells grown in serum-containing medium. The addition of PRL (5 × 10⁻⁹ M) in the absence of progesterone resulted in an accumulation of rough endoplasmic reticulum and Golgi elements and in the appearance of secretory vesicles filled with electron-dense material typical of the casein granules observed in glands from late pregnant and lactating rabbits (Fig. 2c). Large fat droplets similar to those seen in mammary cells at midpregnancy filled the apical cytoplasm (Figs. 1d and 2b). At high PRL concentrations (10⁻⁷–10⁻⁶ M), the droplets appeared rapidly after 1 d, but then disappeared within 3–5 d after which the cell cultures became morphologically indistinguishable from controls. At lower hormone concentrations (10⁻¹⁰–10⁻⁹ M), the number of fat droplets increased progressively in the cells during the first 5 d and then remained constant. Since these fat droplets could be visualized in living cells under the inverted microscope, they served as indicator of prolactin response.

In contrast to cells grown on a rigid substrate, a second cell type appeared in the cultures. These cells occupied a basal position between the secretory cells and the collagen matrix (Fig. 1b and d). Bundles of intermediate filaments filled their cytoplasm, and a rim of thin filaments was present around the cell membrane. We observed desmosomes between these microfilament-rich cells and the secretory cells, and hemidesmosomes formed with the basal lamina-like structures separating the cells from the collagen (Fig. 2d).

EMBEDDING COLLAGEN GELS: Spheroids embedded in collagen gels grew out as ductlike structures, radiating in three dimensions into the surrounding collagen matrix, and were frequently terminated by small alveoli (Figs. 1c, e, and f). Cell proliferation occurred over the 25-d culture period, provided progesterone and PRL were present in the serum-free medium. Under the electron microscope, the outgrowths appeared initially as cell aggregates that with time formed ducts and alveoli with typical lumina outlined by apical microvilli (Figs. 1f and 3a). The cells were sealed at their apex by tight junctions and separated from the surrounding matrix by a thick basal lamina-like structure (Fig. 3c). Translucent fat droplets (Fig. 1e) developed in response to PRL hormone stimulation, with the same hormone-dependency as that observed in cells grown on floating gels. The droplets appeared in the more proximal cells of the ductlike outgrowths and with time filled the cytoplasm of more distal cells. The fat droplets were frequently located in the basal cytoplasm of the secretory cells, instead of the apical cytoplasm as was the case in cells grown on floating gels. We also found some fat droplets in the collagen matrix. In the presence of PRL, the secretory apparatus filled most of the cytoplasm of the cells and the lumina of the ductlike and alveolar structures were distended with dense secretory material or fat droplets (Figs. 1f and 3a) not seen in control experiments.

Topographical Distribution of Mammary Cells Involved in DNA Synthesis and Milk Protein Storage

DNA synthesis in mammary cells grown on rigid or flexible collagen gels or within embedding gels was determined by autoradiography of cultures pulsed for 1 h with 2 μCi/ml of [³H]thymidine. The accumulation of milk protein was assessed by the immunocytochemical detection of casein, α-lactalbumin, and a milk transferrin. The autoradiographic and immunocytochemical labeling pattern of cultures grown in the presence
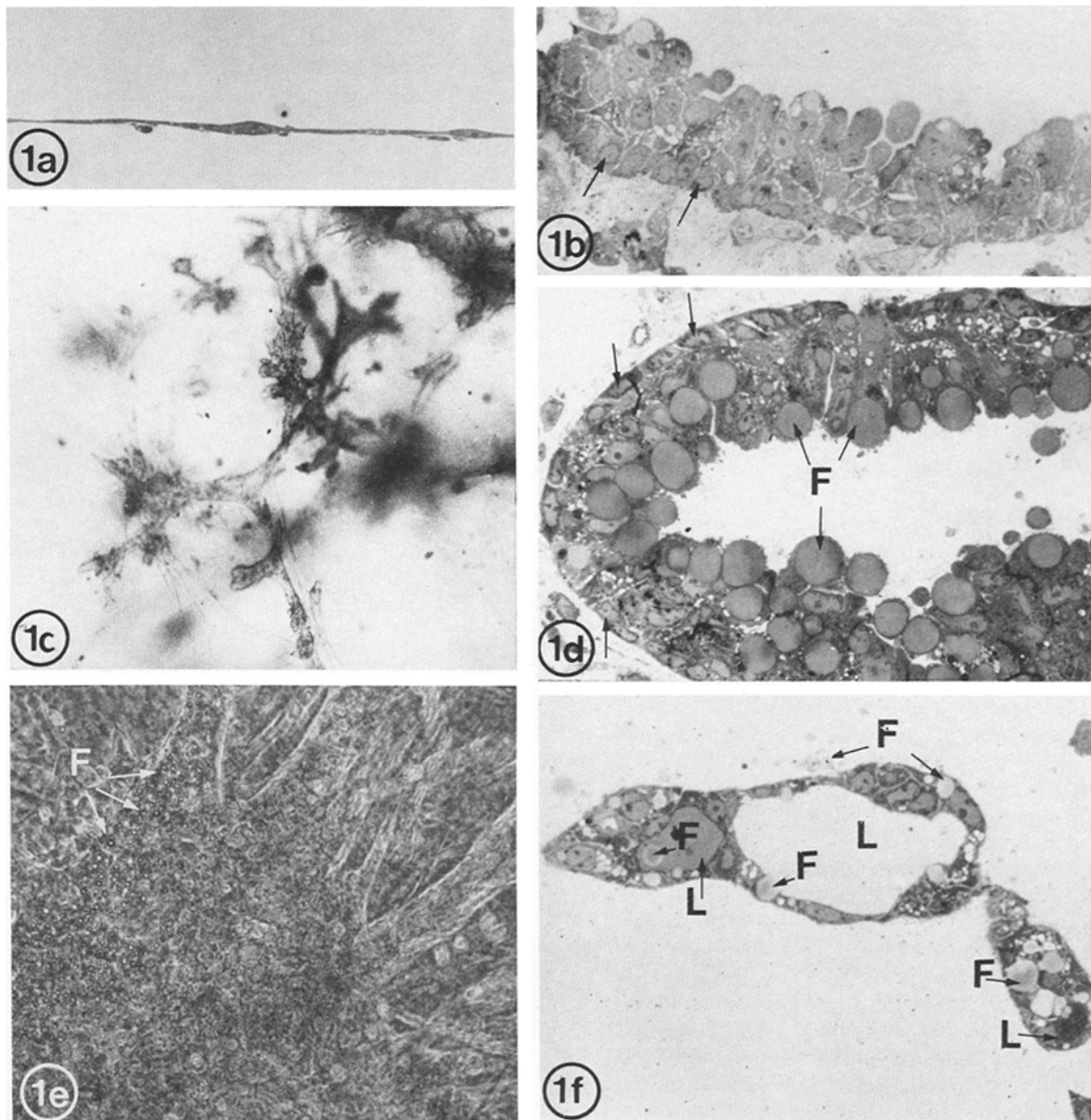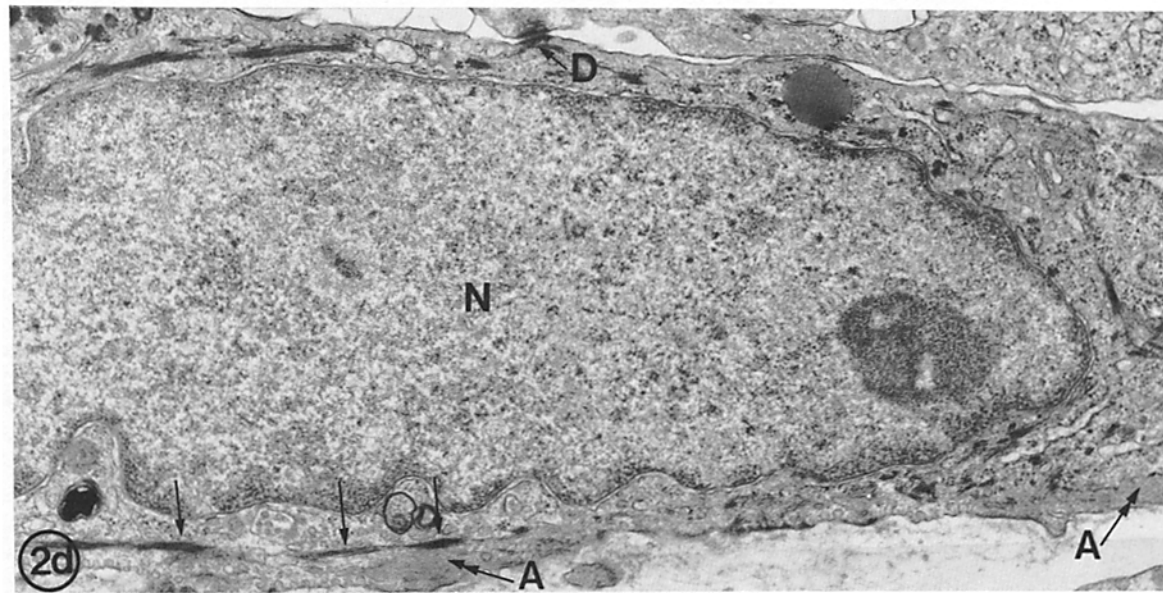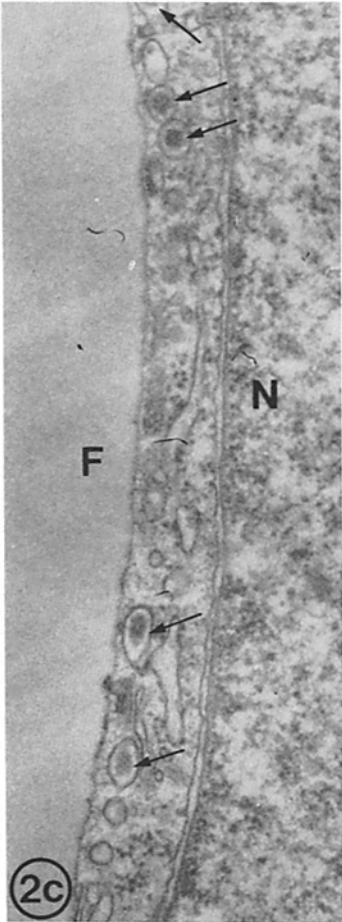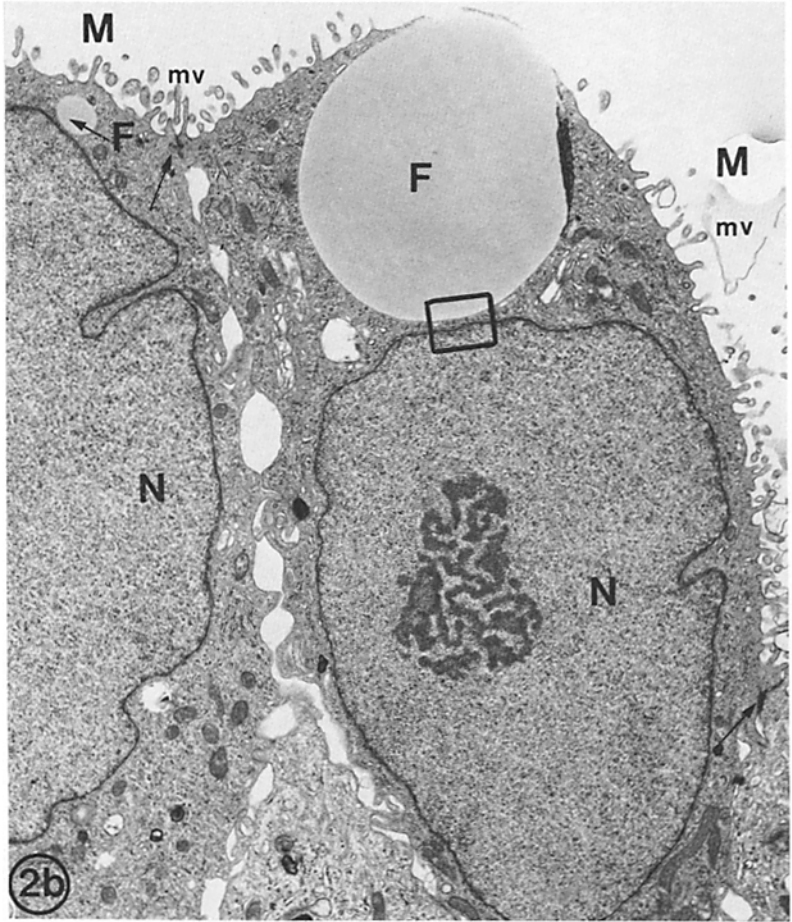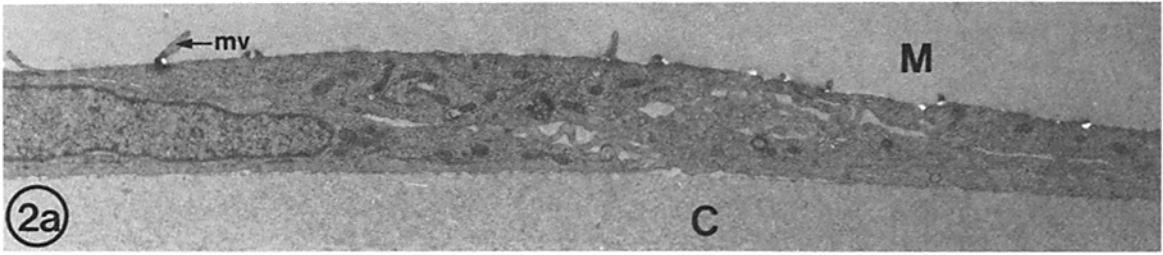
FIGURE 1   Light micrographs of rabbit mammary cells grown on attached (a), floating (b and d), and embedded in (c, e, and f) collagen gels in chemically defined medium. The cells were plated at 0.5×10^5 cells/cm^2 on the collagen gels, embedded at 10^6 cells/ml of collagen mixture, and cultured in the presence or absence of PRL (5×10^{-9} M). For the first 3 d progesterone was added to the medium. (a) 0.5-μm thick section through mammary cells grown for 10 d on attached collagen gels in the presence of 5×10^{-9} M PRL. The collagen matrix is below the cell monolayer. $\times$ 400. (b and d) 0.5-μm thick section through mammary cells grown for 10 d on floating collagen gels in the absence (b) or presence of 5×10^{-9} M PRL (d). The gels were mechanically freed on day 5. A layer of small cells (arrows; see Fig. 2 d) is situated between the secretory cells directly in contact with the medium and the collagen gel. Lipogenesis with the appearance of large fat droplets (F) in the secretory cells is induced in the presence of PRL. $\times$ 400. (d) Mammary cells grown in embedding collagen gels for 6 d (c, $\times$ 25) and 10 d (e, $\times$ 60) in the presence of 5×10^{-9} M PRL and observed by phase contrast with the invertoscope. Fat droplets (F) can be visualized over the ductlike structures. (f) 0.5-μm thick section through mammary cells grown for 10 d in the presence of 5×10^{-9} M PRL in embedding collagen gels. The cells are organized in ductlike structures with typical lumina (L), which can be filled with dark secretory products and fat droplets (F). Fat droplets are also present in the cells and in the collagen matrix. $\times$ 400.

FIGURE 2   Electron micrographs of rabbit mammary cells grown for 10 d as described in Fig. 1 containing 5×10^{-9} M PRL on attached (a) and floating collagen gels (b-d). The sections were stained with uranyl acetate and lead citrate. (a) Section perpendicular to the plane of the monolayer. Microvilli (mv) are present on the cell surface facing the culture medium (M). A thin basal lamina-like structure separates the cell monolayer from the underlying collagen gel (C). $\times$ 6,500. (b) Section through a secretory cell which has accumulated fat droplets (F). The apical membrane, which contains numerous microvilli (mv) facing the medium (M), is sealed by tight junctions (arrows). (N) Nucleus. $\times$ 7,000. (c) Higher magnification of the rectangle seen in Fig. 2 b. The cytoplasm surrounding the fat droplets (F) is filled with secretory vesicles containing retracted dense material (arrows). $\times$ 30,000. (d) Section through a basal cell situated between the secretory cells (direct contact with the medium) and the collagen gel. The cytoplasm of the myoepithelial-like cells contains intermediate (arrows) and thin filaments (A). Desmosomes (D) are present between secretory and myoepithelial-like cells. $\times$ 20,000.

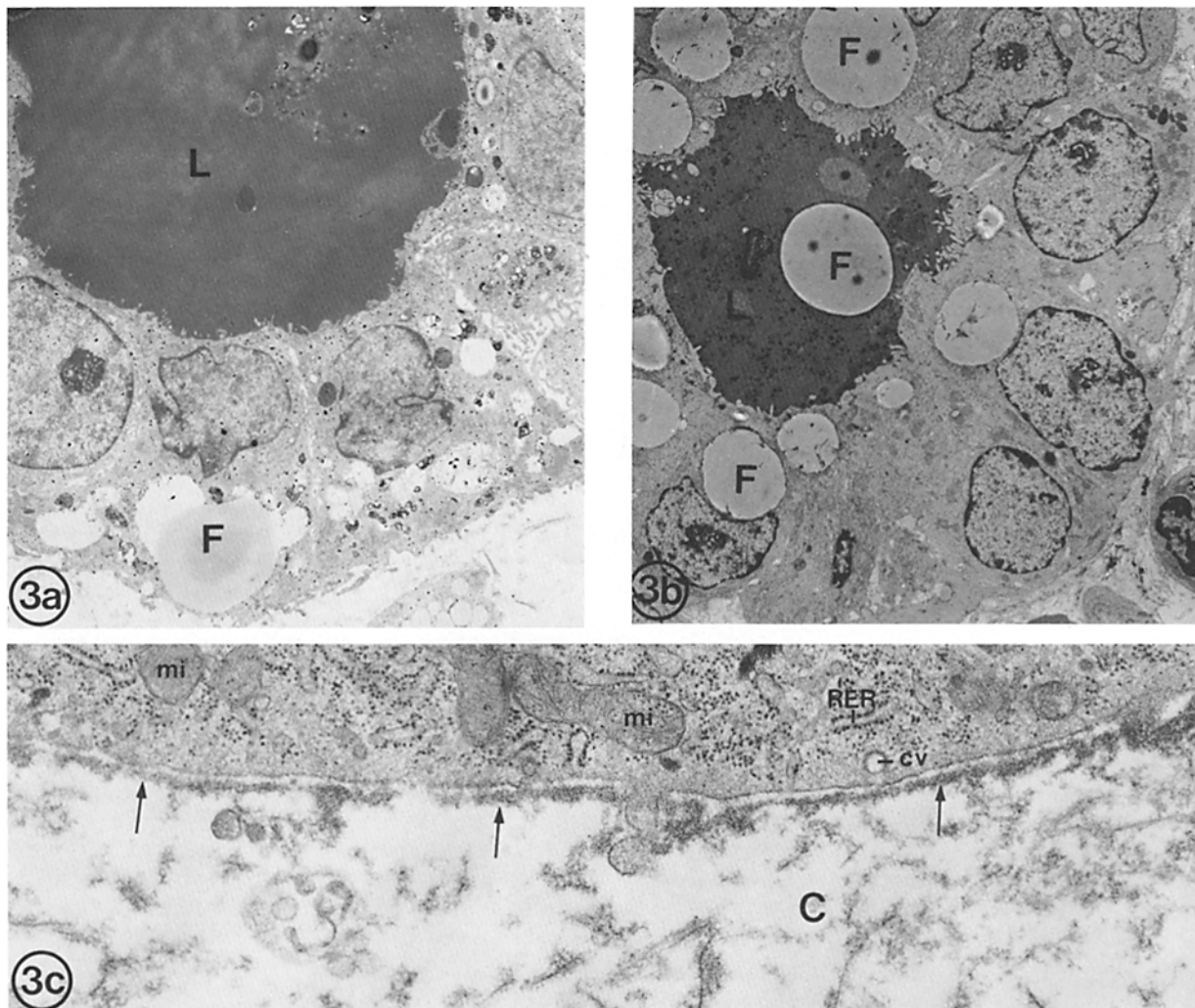1428

FIGURE 3   Electron micrographs of rabbit mammary cells grown for 10 d as described in Fig. 1 in the presence of 5×10^{-9} M PRL in embedding collagen gels (a and c). For comparison, the structure of the mammary gland of a midpregnant rabbit is shown in (b). Stained with uranyl acetate and lead citrate. (a) The mammary cells form a ductlike structure with their apical membrane facing a lumen (L) filled with dense secretory products. Fat droplets (F) are present in the cytoplasm: their orientation with respect to the nucleus is different from that in cells from the intact gland where the droplets are always in a supranuclear location. $\times$ 4,000. (b) Thin section through the mammary gland of a midpregnant rabbit. Fat droplets are present in the supranuclear region of the secretory cells and within the lumen. $\times$ 4,000. (c) Higher magnification of mammary cells grown in embedding gels. The cells contain mitochondria (mi) and rough endoplasmic reticulum (RER). Coated vesicles (cv) are seen near the basal membrane, separated from the collagen matrix (CM) by a basal lamina-like structure (arrows). $\times$ 25,000.

of PRL were determined on days 10–12 and compared to those of control experiments.

On attached collagen gels, ~10% of the cells incorporated thymidine (Fig. 4a). This synthetic activity almost completely stopped when the cultures became confluent. The labeled cells were randomly distributed along the forming monolayers. No milk proteins were detected in the mammary cells despite the presence of PRL (Fig. 4b). This could result from the establishment of an impermeable monolayer of cells that could prevent transport systems and hormone receptors on the basolateral cell surface from interacting with nutrients or hormones in the medium. To rule out the possibility that such a diffusion barrier might play a role, cells were plated and cultured on collagen-coated Millipore filters. At confluency, the filters were released from the plastic wells and allowed to float. We observed no retraction of the collagen gels and no cell shape changes. The basolateral cell surface, however, was

accessible to hormones, as reflected by the specific binding of radio-iodinated PRL (data not shown). Under these conditions, we detected no storage of milk proteins immunocytochemically.

On floating collagen gels, the secretory and the basal microfilament-rich cells were not involved in DNA synthesis, since none of the cells were labeled even after a pulse of as long as 2 h (Fig. 4c). In contrast, the secretory cells accumulated milk proteins. In the absence of PRL, we detected only transferrin in secretory cells, and it accumulated in small vesicles scattered throughout the cytoplasm. Interestingly, we also detected large amounts of this protein between the mammary cells and the collagen gels, suggesting that transferrin was secreted into the collagen matrix (Fig. 4d). Upon addition of PRL, fewer secretory cells stored casein and α-lactalbumin in vesicles surrounding the fat droplets (Fig. 4e). We detected no casein in the space between the collagen gels and the cells. In double-label-

ing experiments, the cells that contain casein also stored α-lactalbumin, but fewer cells accumulated both casein and transferrin.

In embedding collagen matrices, numerous secretory cells incorporated [³H]thymidine. The cells involved in DNA synthesis were randomly distributed along the ductlike structures and did not accumulate at the tip of outgrowths (Fig. 4f). Their number was estimated on autoradiograms of dispersed and cytocentrifuged cells (see Table I of [33]). The milk protein synthetic activity was high as reflected by the accumulation of transferrin, casein, and α-lactalbumin in the lumen of the ductlike structures (Fig. 4g–i). Whereas, transferrin was detected in the absence of PRL, this hormone was required for the accumulation of casein and α-lactalbumin in the lumina. In double-staining experiments on cultures grown in the presence of PRL, several cells stored both transferrin (Fig. 4h) and casein (Fig. 4i), but in different intracellular organelles. A few cells which incorporated [³H]thymidine also accumulated milk proteins (Figs. 4f and g) (see Table I from [33]). No milk proteins were detected in the collagen matrix.

DISCUSSION

Our results indicate that dispersed nontransformed mammary epithelial cells from midpregnant rabbits can organize themselves into polarized epithelial cell layers. Depending on culture conditions, the cells can maintain the morphological differentiation characteristic of midpregnancy. This confirms and extends previous reports (6, 14, 21, 30, 42). Furthermore, functional differentiation can be induced in these multicellular structures depending both on the substrate and the hormonal environment.

Maintenance of Morphological Differentiation

During pregnancy, the mammary tubular system further grows and infiltrates the fat pad as a result of hormone-induced cell proliferation. The end buds found at the extremity of the ducts form globular alveoli when lactogenesis is induced before parturition and during lactation. Several cell types are distinguishable in the mammary epithelium, including ductal and alveolar secretory cells and myoepithelial cells. The former cells express milk protein genes and produce milk, whereas the latter cells rich in myofilaments and prekeratin intermediate filaments (17) are situated between the ductal and alveolar cells and the basal lamina, and contract in response to oxytocin stimulation. The secretory cells represent an heterogenous cell population, as reflected by differential labeling by monoclonal antibodies directed against constituents of fat globule membrane (16). Little is known about the role of each cell type in mammary morphogenesis or about the importance of cell-cell interactions in the induction of functional differentiation which takes place during pregnancy, parturition, and lactation. The description by Yang et al. (42) of three-dimensional ductlike outgrowths from clumps of mammary cells cultured in collagen matrices contributed to the understanding of mammary morphogenesis. In such a system it is not possible, however, to define the role of each cell type in morphogenesis, due to the heterogeneity of the starting cell population. More recently, Bennet and co-workers (3, 4) report morphogenesis of branching tubules obtained in cultures of cloned epithelial cells from rat mammary gland that are transformed with chemical carcinogens. From these studies, it appears that two cell types—cuboidal secretory and elongated myoepithelial-like cells—are required for the formation of glandular outgrowths.

In the present study, the establishment of functional polarity and cytodifferentiation is analyzed in serum-free, chemically defined medium under three different culture conditions.

The morphological cell polarity, defined by a microvillar apical surface separated by junctional complexes from the basolateral cell surface, is reestablished under all culture conditions. On two-dimensional collagen surfaces, both the apical and the basolateral membranes of the cells are in contact with the medium that contains nutrients and hormones, whereas in three-dimensional collagen matrixes the apical plasma membrane is separated from the medium by the junctional complexes of the cell layers. As will be discussed later, in contrast to morphological polarity, functional polarity is greatly influenced by the various culture conditions.

The same enriched secretory cell starting material forms quite different multicellular structures under the three culture conditions. On attached gels, a single cell type produces a flat monolayer separated from the collagen gel by a basal lamina-like structure. In contrast, detachment of gels releases a constraint on the monolayer and allows the differentiation of a multilayer composed of two cell types: a secretory cell in direct contact with the medium, and a myoepithelial-like cell situated between the secretory cells and the collagen gel, but separated from the substrate by a basal lamina-like structure. Since there is no DNA synthesis nor cell division (see accompanying paper [33]) on floating gels, one can postulate that myoepithelial-like cells differentiate from the secretory cells that constitute the starting material. The origin of myoepithelial cells in vivo remains unclear. It has been proposed that these cells arise by differentiation either from a stem cell or from epithelial cells located towards the stromal side of the ducts adjacent to the basal lamina (7, 27). Our observations are consistent with the latter interpretation and contrary to recent reports indicating that myoepithelial-like cells differentiate spontaneously from a stem cell (Rama 25) derived from a dimethylbenzanthracene induced rat adeno-carcinoma (2, 38). Such cells, although considered stem cells, are poorly characterized, and could represent secretory cells, whose function is altered by changes in gene structure (e.g., methylation, rearrangement) subsequent to chemical carcinogenesis (34).

In three-dimensional collagen matrices, myoepithelial-like cells appear to a lesser degree than in floating collagen gels. We observe no difference in morphological or functional differentiation when spheroids are directly embedded in a collagen matrix or when they were first plated on a collagen gel and then overlaid 1 d later by a collagen gel layer (unpublished data). When using such a system, the orientation of cell polarity could not be modulated, as is the case for thyroid cells (8, 9).

Induction of Functional Differentiation

Establishment of cell polarity is not sufficient for the expression of mammary functions, as is the case for thyroid functions in thyroid cell cultures (9). On a rigid two-dimensional substrate, mammary cells synthesize DNA and proliferate at least until achievement of confluency. These cells, however, are unable to express their specialized genetic program, as reflected by the absence of milk protein and fat accumulation and secretion. Accessibility of the basolateral surface to nutrients and stimulators is not sufficient for morphological and functional differentiation to occur, since cells grown on Millipore filters with a fully accessible basolateral surface are indistinguishable from cells grown on attached collagen gels.

On a flexible two-dimensional surface (floating gels), change
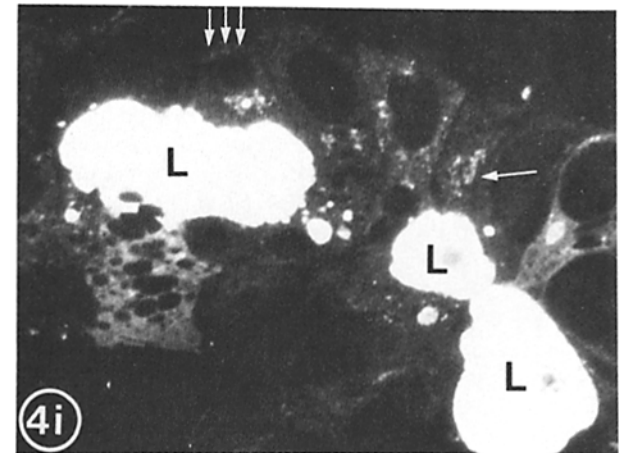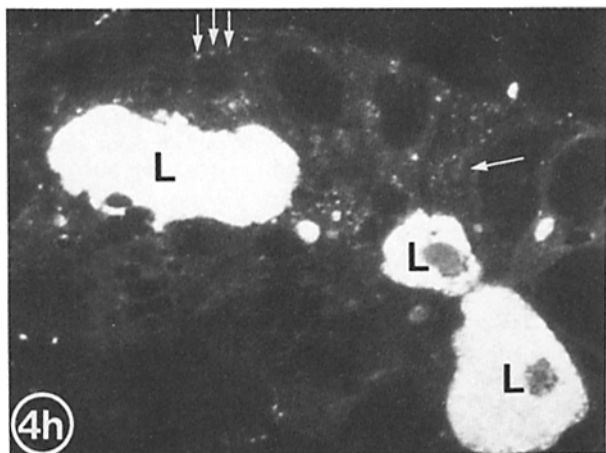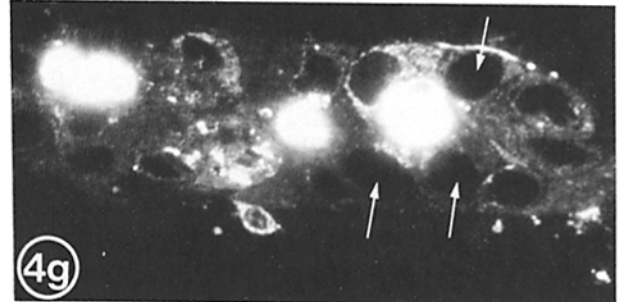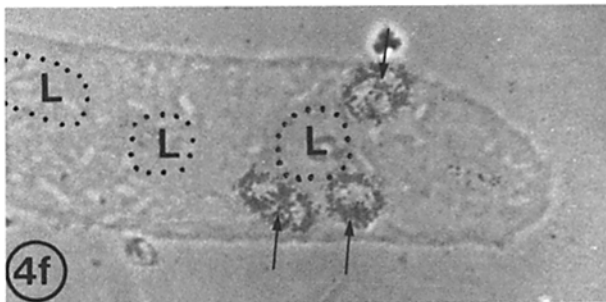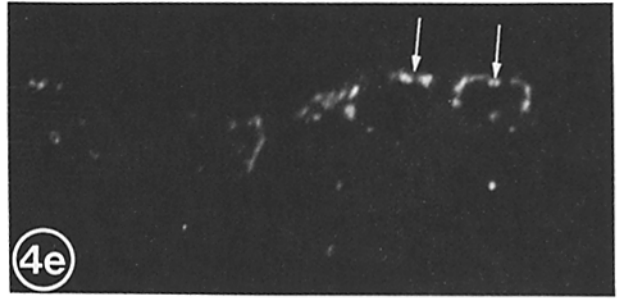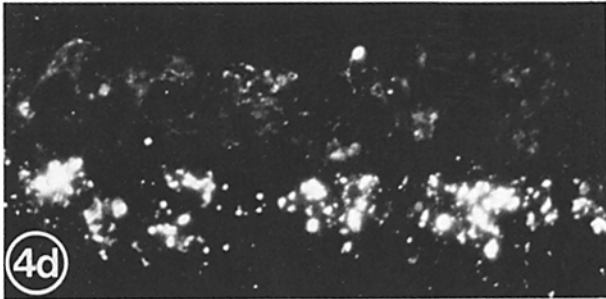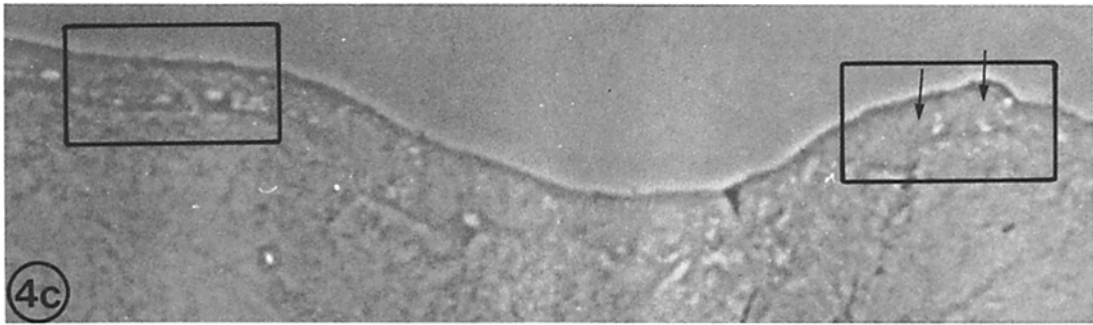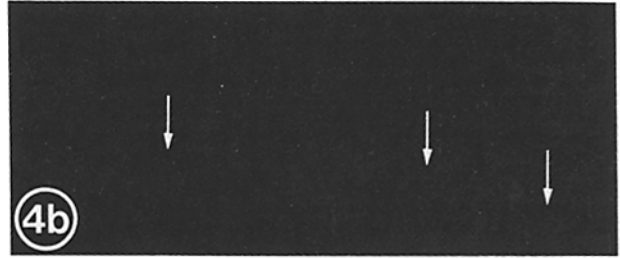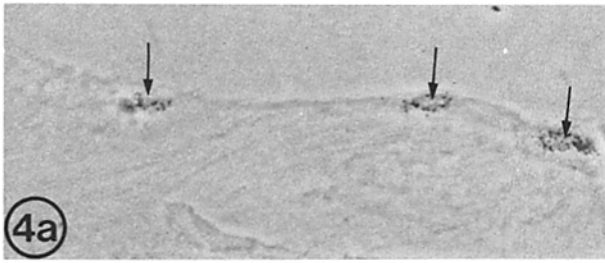
in cell shape results in the termination of DNA synthesis and of induction of milk protein gene expression. PRL stimulation induces lipogenesis and large fat droplets form and are released vectorially into the medium. Caseins and α-lactalbumin that accumulate only in the presence of PRL are also discharged vectorially into the medium. In contrast, the milk iron-binding protein, identical to serum transferrin (11) appears both in the medium and in the collagen gel, suggesting secretion from both cell surfaces.

Cell proliferation and milk production, the two mammary functions characteristic of pregnancy and lactation, are simultaneously expressed when mammary cells are grown in three dimensions. Fat droplets and milk proteins accumulate in the lumina of ductlike structures. No milk proteins appear in the collagen matrix, indicating that milk protein secretion is vectorial. Functional polarity is however, only partially reconstituted, since fat droplets that are seen in the basal cytoplasm of the secretory cells also appear in the collagen matrix.

Since both DNA synthesis and milk protein gene expression occur in cells grown in three-dimensional collagen matrices, we examined the topographical distribution of the cells involved in these functions by combined immunocytochemical and autoradiographical procedures. DNA synthesis is random along the tubular structure and is not concentrated at the tip of the ductlike structures, a site where contact inhibition is expected to be minimal. We observed a similar random distribution for cells involved in milk protein synthesis and storage. Consequently there is no differentiation gradient between the tip and the center of the outgrowths.

Fewer cells store casein and α-lactalbumin than transferrin, suggesting that two different secretory cells are involved in hormone-dependent and hormone-independent synthesis of milk proteins. However, double-labeling experiments revealed that transferrin and casein are produced in the same secretory cells, although some cells accumulate only one type of milk protein.

These in vitro studies, performed in serum-free, chemically defined media, indicate that morphological and functional differentiation can be maintained in mammary cells grown as primary cultures. It is tempting to correlate the expression of a differentiated state, i.e., milk protein gene expression, with the presence of two cell types: the secretory and the myoepithelial-like cells. The role of myoepithelial cells in functional differentiation is not known. The differentiation of rat mammary tumor cells into myoepithelial-like cells is accompanied by an enhanced synthesis of basal lamina constituents (39) and the metabolism of the basal lamina apparently plays an important role in mammary morphogenesis (10, 39, 40). Myoepithelial-like cells could therefore contribute to functional differentiation by providing basal lamina constituents. An answer

to these questions will require well characterized and fully differentiated cloned mammary cells which are still not available. However, our studies using primary cell cultures suggest that cytodifferentiation plays a role in the expression of mammary functions.

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FIGURE 4 Light and immunofluorescence micrographs of 0.5-μm-thick frozen sections of mammary cell cultures. The cells were grown for 10 d on attached (a and b), floating (c–e), and in embedding collagen gels (f–i) in chemically defined medium containing 5 × 10⁻⁹ M PRL, as described in Fig. 1. The cultures were pulsed for 1 h with 2 μCi/ml of [³H]thymidine and were fixed with 3% formaldehyde. Frozen sections were prepared and incubated sequentially with biotinylated antimilk protein antibodies and streptavidin coupled to fluorochrome and then were processed for autoradiography (see Material and Methods). (a and b) Autoradiographic grains (arrows) overlying three nuclei (a) indicate that the cells on attached collagen gels synthesize DNA. However, no casein is detected in the cells (b). (c–e) No DNA synthetic activity is detected in the cell layers from floating gels (c). The cells, however, accumulated transferrin in vesicles scattered throughout the cytoplasm; transferrin is also detected in the collagen matrix (d). Casein is present in vesicles surrounding fat droplets (arrows) (e). c, × 650, d and e, × 1,000. (f–i) Cells grown in embedding gels synthesize DNA (f) as reflected by the presence of three labeled nuclei (arrows). The same cells accumulate casein in vesicles which are visible in the apical cytoplasm close to the lumina of the ductlike structures (L). The lumina are filled with casein. × 900. In h and i, the sections were double-labeled with antitransferrin antibodies and fluoresceinated streptavidin, and then with anticasein antibodies and lissaminated streptavidin. Both proteins are detected in the same cells (arrows), but in different cell organelles. The lumina (L) contain both milk proteins. × 1000.

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