Characterization of a Myoepithelial Cell Line
Derived from a Neonatal Rat Mammary Gland

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ABSTRACT A clonal, myoepithelial-like cell line has been obtained from a primary culture established from the mammary gland of a 7-d-old rat. In a number of respects, this cell line, termed Rama 401, resembles the myoepithelial cells of the mammary gland, especially when grown on floating collagen gels. The cells grow as multilayers on the gel surface and form branching structures that do not appear to contain a lumen. They are rather elongated, with irregular-shaped, flattened nuclei that contain large amounts of peripheral chromatin. Elongated processes project from the cell surface and numerous membrane pinocytotic vesicles can be seen. The cytoplasm is filled with linear arrays of 5- to 7-nm filaments with occasional dense foci. Cell junctions with associated 8- to 11-nm tonofilaments are also observed. Immunofluorescence techniques reveal actin and myosin filaments and also intermediate filaments of both prekeratin and vimentin types. Rama 401 cells secrete an amorphous material that, when an immunoperoxidase technique is used, stains with antibodies to basement membrane-specific type IV collagen. Localized densities of the cell membrane, which resemble hemidesmosomes, are located adjacent to these extracellular deposits. Immunofluorescence staining and immunoprecipitation techniques reveal that the cells also synthesize two other basement membrane proteins, laminin and fibronectin. The type IV collagen consists of two chains with molecular weights of 195,000 and 185,000.

Myoepithelial cells are found in a number of exocrine glands, e.g., mammary (38–41, 47, 50), salivary (43), sweat (14), and lacrimal glands (30). Myoepithelial cells are located between the epithelium and the basement membrane with which they form attachment areas or hemidesmosomes. These cells are arranged with their long axis perpendicular to the long axis of the overlying epithelial cells in lactating glands (38, 39, 50, 52). In the rat mammary gland, the myoepithelial cells completely surround the ducts and also form a "basket" around the secretory alveoli (52). In response to oxytocin, mammary myoepithelial cells contract, expelling the secretory products from the gland (10). Although they are believed to be true epithelial cells, myoepithelial cells share many properties with smooth muscle cells. The cytoplasm of both cell types is filled with actin and myosin filaments with irregularly-spaced electron-dense foci, and the plasma membrane is characterized by the presence of clusters of pinocytotic vesicles (6, 40, 41). Smooth muscle cells may be distinguished from myoepithelial cells in vivo on the basis of their intermediate filament content, the former containing vimentin and desmin, the latter containing only prekeratin intermediate filaments (19–21, 36).

The origin of myoepithelial cells is obscure. On the basis of ultrastructural studies of salivary and mammary glands, it has been suggested either that myoepithelial cells develop from epithelial cells (32, 42, 43) or that a stem cell gives rise to both epithelial and myoepithelial cells (40). In either case, the formation of a fully differentiated myoepithelial cell occurs only when the cell attaches or is in close proximity to the basement membrane. In fibroblastic cells, an ordered cytoskeleton is believed to be dependent on the presence of an extracellular matrix containing fibronectin, which, through a transmembrane linkage protein, provides anchorage points for the attachment of microfilaments (9, 28). A similar mechanism may be operative when a myoepithelial or epithelial stem cell attaches to the basement membrane. We have previously shown that when a mammary epithelial stem cell line isolated from a tumor, Rama 25, differentiates into a myoepithelial-like cell, it acquires an ordered cytoskeleton and the ability to...
synthesize an extracellular matrix that contains fibronectin (55). However, myoepithelial cells from normal glands have been poorly characterized in culture. "Baskets" of myoepithelial cells have been isolated from lactating mammary glands (47) and myoepithelial cell lines have been established from apparently normal human breast tissue from an area adjacent to a tumor (25) and from a salivary gland tumor (46). In addition, evidence has been presented for the outgrowth of myoepithelial cells from normal mammary gland explants (13, 44, 48). We now report the isolation of a clonal myoepithelial-like cell line from a neonatal rat mammary gland and its ultrastructural and biochemical characterization with particular reference to microfilaments and basement membrane components.

MATERIALS AND METHODS

Isolation and Growth of Mammary Cells

Mammary glands from 7-d-old female ICRF-Wistar rats were excised, digested with 150 U/ml collagenase and 230 U/ml hyaluronidase in Dulbecco's modified Eagle's medium (DEM) and 10% fetal calf serum (FCS) for 1 h and then separated into a stromal fraction and an epithelial fraction by differential rates of attachment to plastic petri dishes (26, 44). Epithelial cells were grown in 75% Ham's F12 medium + 5% FCS adjusted to 3.7 ng/ml Na/ECO, previously exposed for 1 d to near-confluent cultures of Rama 29 (8) (conditioned medium, CM: 2 × 106 Rama 29 cells in 10 ml of medium) plus 25% DEM, 10-20 ng/ml epidermal growth factor, 50 ng/ml hydrocortisone, 50 ng/ml insulin, and 10% fresh FCS at 37°C in 10% vol/vol CO2 atmosphere. This is the optimal medium for epithelial cell growth (26). In the early stages, epithelial cells were grown with 1-2 × 105 cells/cm2 petri dish. Rama 29 feeder cells previously exposed for 12-16 h to 0.5 μg/ml mitomycin C in DEM and 10% FCS. Contaminating stromal cells were removed by two washes of the cell monolayers with phosphate-buffered saline (PBS) containing 30 μM Ca2+ followed by a 1-min digestion with 0.1 mg/ml pronase in the same buffer (8). Rama 29 feeder cells were then added back. Epithelial cells were transferred initially by detaching them with EDTA/trypsin solutions containing solutions alone, but by the third passage EDTA/trypsin solutions could be employed (8). After the 4th passage the epithelial cells were plated sparsely as separated single cells. These single cells developed into separate cell colonies and several apparently pure epithelial cell colonies were picked by means of ring cloning, i.e., isolating the colony with a steel ring attached to the petri dish by vacuum grease and detaching the entire colony within the ring with trypsin/EDTA solution. One such ring clone was termed NM7D-5 (normal myoepithelial-derived, was obtained from an apparently pure epithelial colony and consequently in NM7D-5. However, when single-cell epithelial colonies from NM7D-5 were recloned, again by picking individual cells, both epithelial (22%) and elongated cells (78%) were obtained. This suggests that NM7D-5 is a type of stem cell producing both epithelial and elongated (myoepithelial-like) cells.

In sparse cultures, Rama 401 are rather elongated, irregularly shaped cells that do not grow in colonies (Fig. 1). Confluent
the cells have a polygonal appearance produced by overlapping adjacent cells. Bar, 125 μm. × 80.

cultures appear to consist of polygonal cells, an effect apparently produced by overlapping of adjacent cells (Fig. 2). Rama 401 cells grow as multilayers up to seven cells thick. The cells have an average doubling time of ~21 h that is unaffected by the addition of insulin (50 ng/ml), hydrocortisone (50 ng/ml), or prolactin (500 ng/ml).

When grown on plastic, the cells are somewhat flattened, averaging 12–15 μm in diameter and 3.5 μm in depth, with no morphological differences between cells attached to the substrate and those on the surface (Fig. 2). The cell surface is characterized by a large number of elongated processes. Desmosomes are not found; however, the large intercellular spaces contain a weakly electron-dense amorphous material. Occasional densities, which resemble hemidesmosomes, are present on the cell membrane adjacent to these extracellular deposits. The nuclei are flattened, often indented, with large amounts of peripheral heterochromatin and a prominent nucleolus.

Growth on Collagen Gels

Rama 401 cells also grow as multilayers on top of collagen gels. However, if the gels are allowed to float, cells begin to penetrate into the collagen matrix within 2–4 d. Initially, irregular cell processes protrude below the multilayer of cells on the surface. After 5–15 d, multicellular branching structures are formed. These are composed of solid cords of cells that are joined to the surface multilayer by a narrow stalk of cells (Fig. 3). Cords branch up to maximum of four times.

The gross ultrastructure of cells grown on collagen gels is similar to that of cells grown on plastic (Fig. 4). Additional features include a more distended rough endoplasmic reticulum that is filled with an amorphous material. Mitochondria are elongated and Golgi can be observed, although these organelles occupy a very limited portion of the cell if cytoplasmic filaments are abundant. Areas of undulating extracellular electron-dense material follow the contours of the cell surface along the interface of the collagen matrix and also between the layers of the cells. Arrays of pinocytotic vesicles many of which appear to be coated, are usually observed in areas of localized densities of the plasma membrane. Although these clusters of vesicles resemble those found in smooth muscle and endothelial cells, the possibility arises that some may be secretory vesicles and that the concentration of extracellular material found in regions where these vesicles are common represents newly secreted material. The densities of the plasma membrane resemble hemidesmosomes and are often associated with tonofilaments. Cell junctions with associated tonofilaments are common between adjacent cells. Many of these junctions appear similar to intermediate junctions or zonula adhaerens (16), with parallel adjacent membranes and a weakly electron-dense material occupying the intercellular space. Filaments of ~4- to 6-nm diameter commonly run longitudinally through the cytoplasm. Some regions of abundant microfilaments contain focal dense areas that resemble myofilaments (Fig. 4 b), although these dense foci are not always seen (Fig. 5 a). In the perinuclear region, arrays of filaments with diameters of 10–12 nm are often observed (Fig. 5 b). Microtubules are sparsely distributed at random throughout the cytoplasm.

Synthesis of Basement Membrane Proteins

The amorphous extracellular material secreted by Rama 401 cells is reminiscent of basement membrane. We have, therefore, investigated the synthesis of three basement membrane proteins, laminin, fibronectin, and type IV collagen, using immunofluorescence, immunoperoxidase, and immunoprecipitation techniques. Immunofluorescence staining of Rama 401 cultures with antibodies raised against these three basement membrane proteins reveals, in the case of fibronectin and type IV collagen, intense staining of a fibrillar extracellular matrix located between, but not apparently on the surface of the cells (Fig. 6 a and b). Antiserum to laminin also stains a fibrillar extracellular material (Fig. 6 c) but with less intensity than antisera to the other two proteins, except in certain areas where accumulations of laminin-containing fibrils are observed. Immunoperoxidase staining of Rama 401 cells, at the ultrastructural level, with antiserum to type IV collagen, demonstrates strong staining of the extracellular matrix secreted beneath and between the cells (Fig. 7). Staining was also absent from the top surface of the top layer of cells. Immunoperoxidase staining with antisera to laminin and fibronectin also results in staining of the extracellular material. Absorption of the antisera with the corresponding antigen abolished staining in each case (not shown).

Analysis of the proteins secreted by Rama 401 cells labeled with [3H]proline in the presence of β-aminopropionitrile and ascorbic acid reveals five major proteins with molecular weights of approximately 220,000, 195,000, 185,000, 165,000, and 155,000 (Fig. 8). Minor proteins with molecular weights above 220,000 are also present. The 195,000 and 185,000 mol wt proteins are completely hydrolyzed by bacterial collagenase.
All five major secreted proteins are susceptible to digestion with pepsin, which results in the appearance of a pepsin-resistant fragment with a molecular weight of 126,000. The nature of this fragment and the 165,000 and 155,000 mol wt proteins is under investigation. The 220,000 mol wt protein has been identified as fibronectin and the 195,000 and 185,000 mol wt proteins as the two chains of type IV collagen by immunoprecipitation with specific antisera (Fig. 9). These molecular weights are in approximate agreement with those of type IV collagen synthesized by mouse teratocarcinoma cells (1), am-
FIGURE 4 Ultrastructure of Rama 401 cells growing on floating collagen gels. (a) An overall view of a Rama 401 cell. Note the irregular-shaped nucleus with dispersed heterochromatin; microfilaments (MF) are abundant especially in the perinuclear region. An amorphous extracellular matrix (EM) can be observed between adjacent cells and between cells and the collagen matrix. (b) A cell junction (J) with associated tonofilaments; microfilaments (MF) are again abundant with occasional focal densities (arrows). (c) Pinocytotic vesicles (PV) are particularly common in areas of membrane adjacent to extracellular matrix deposits. (d) Thickenings of the cell membrane (arrow) are seen at areas where the cell and the extracellular matrix are in close proximity. Note the abundant distended rough endoplasmic reticulum (RER). Bars: (a) 1.26, (b) 0.47, (c) 0.38, and (d) 0.46 μm. (a) X 8,375, (b) X 23,490, (c) X 29,250, and (d) X 23,760.

niotic fluid cells (11), and HT-1080 sarcoma cells (4). Type IV collagen differs from other collagens in that it can be partially digested by pepsin (45). The three subunits of laminin with molecular weights of about 400,000, 220,000, and 200,000 can
also be immunoprecipitated from a cell extract (Fig. 9). The hydroxyproline content of proteins synthesized by Rama 401 cells and retained within the cell layer is $1.07 \pm 0.02\%$ of the total hydroxyproline + proline and $4.11 \pm 0.34\%$ in the culture medium. The respective values for cells growing on floating collagen gels are $1.98 \pm 0.03\%$ in the cell layer and $3.87 \pm 0.17\%$ in the culture medium.

Rama 401 cells do not synthesize casein measured by a radioimmunoassay procedure (54) with a limit of detection of 0.2 ng of casein, and do not stain with an antibody raised against rat milk fat globule membrane that specifically stains the epithelial cells of the rat mammary gland (unpublished observations). Rama 401 cells are thus unlikely to be rat mammary epithelial cells. Rama 401 cells do not synthesize factor VIII, as judged by immunofluorescence staining with an antiserum to factor VIII–related antigen (Hoechst Pharmaceuticals, Hounslow, U. K.) and are, therefore, unlikely to be endothelial cells.

Identification of Microfilaments

We have investigated the identity of microfilament proteins found in Rama 401 cells by immunofluorescence techniques. In well-spread cells, parallel arrays of microfilament cables...
FIGURE 7 Immunoperoxidase localization of type IV collagen. Note the staining of the extracellular material between the cell layers and the absence of staining in the upper surface adjacent to the culture medium. Lack of staining of the extracellular material further down the cell layer is probably attributable to lack of antibody penetration. Bar, 0.75 μm. × 14,110.

FIGURE 8 Proteins secreted by Rama 401 cells. Confluent cultures of Rama 401 cells were labeled with [3H]proline (10 μCi/ml) in the presence of ascorbic acid (50 μg/ml) and β-aminopropionitrile fumarate (100 μg/ml) for 24 h. Secreted proteins were analyzed on a 7% polyacrylamide gel. (A) Proteins secreted after reduction with β-mercaptoethanol, (B) secreted proteins treated with collagenase (10 μg) for 30 min at 37°C, and (C) secreted proteins treated with pepsin (10 μg) for 30 min at 37°C. Note the two collagenase-sensitive proteins with molecular weights of 195,000 and 185,000, which are probably the two chains of type IV collagen, and the pepsin-resistant fragment with a molecular weight of 126,000.

FIGURE 9 Immunoprecipitation of basement membrane proteins from Rama 401 cells. Cells were labeled with [3H]proline (10 μCi/ml) for 24 h in the presence of ascorbic acid (100 μg/ml). A cell extract was incubated with antiserum to either (a) type IV collagen, (b) laminin, (c) nonimmune rabbit serum, or (d) fibronectin. Immune complexes were absorbed on protein A-Sepharose and analyzed on a 6% SDS polyacrylamide gel.

that contain actin and myosin are observed (Fig. 10 a and b). Increased staining is also seen in the perinuclear region. In many cells, “wavy” filaments radiate out from the perinuclear filament bundles. In the majority of cells, microfilaments appear to be so dense that individual filaments cannot be distinguished. These actin and myosin filaments probably correspond to the 4- to 6-nm filaments observed in the ultrastructural studies. Intermediate filaments of both the vimentin and pre-keratin type can be demonstrated (Fig. 10 c and d). Staining for both proteins is particularly strong in the perinuclear region.
and these proteins are probably constituents of the 10- to 12-nm filaments commonly seen surrounding the nucleus of Rama 401 cells. Both proteins have complex distributions throughout the cytoplasm although the prekeratin filaments are often, but not always, observed in ruffling areas. Preliminary analysis of Rama 401 cytoskeletons on two dimensional gels has failed to reveal a spot corresponding to rat desmin (pl 5.60, molecular weight 50,000).

DISCUSSION

In this paper, we have described the isolation and characterization of a clonal myoepithelial-like cell line from neonatal rat mammary glands. Ultrastructurally, this cell line, especially when grown on collagen gels, resembles the myoepithelial cells of the mammary gland in a number of respects. The cells have flattened nuclei, which in the majority of cells are irregularly shaped and contain abundant peripheral chromatin. Adjacent cells are often linked by cell junctions from which tonofilaments radiate. True desmosomes are not present in Rama 401 cells, although they are observed in vivo between adjacent myoepithelial cells, their formation possibly being inhibited by the rapid growth rate of these cells (41). The cells appear to secrete an amorphous material with which they form attachment areas. Filaments are sometimes seen in association with these attachment sites in a manner resembling the association of tonofilaments with hemidesmosomes (12). The cytoplasm contains abundant parallel arrays of 5- to 7-nm filaments that apparently contain actin and myosin and in which electron dense foci can sometimes be seen. Rama 401 cells contain both prekeratin and vimentin intermediate filament proteins. The presence of prekeratin establishes Rama 401 as a cell of epithelial rather than mesenchymal origin. Mesenchymal cells such as fibroblast, smooth muscle (23), or endothelial cells do not contain prekeratin in vivo or in vitro. In the case of vimentin, however, mammary epithelial and myoepithelial cells in vivo do not contain levels sufficiently high to be detectable by immunofluorescence techniques (19). However, Rama 401 cells contain abundant intermediate filaments of the vimentin type. Many epithelial cells in culture contain vimentin filaments (22). We have also found vimentin in a number of rat mammary epithelial cell lines and can detect low levels of vimentin in both rat mammary epithelial and myoepithelial cells using alkaline phosphatase–conjugated antibodies (unpublished observations). The perinuclear concentration of intermediate filaments observed in Rama 401 cells are similar to those observed in other cell lines and may be involved in anchorage of the nucleus (31) and the maintenance of the mitotic spindle (58).

Some of these features are apparently lacking when Rama 401 cells are grown on plastic, leading to a relatively undifferentiated appearance. During the fetal development of the mammary gland and in newborn rats, relatively undifferentiated myoepithelial cells can also be observed (5, 40). Epithelial cells grown in vitro on collagen gels, rather than on plastic, generally assume a more differentiated state, e.g., mammary epithelial cells can form duct-like structures that contain a lumen (7, 8, 56), and are more responsive to lactogenic hormones when grown on floating collagen gels (15). Similarly,
for myoepithelial cells, expression of the fully differentiated phenotype may be dependent on the nature of the substratum on which they grow. The branching structures formed by Rama 401 cells growing on floating collagen gels are clearly different from those formed by mammary epithelial cells in that they do not contain a lumen, are thinner, and have tapered rather than bulbous ends. Mammary fibroblastic cells appear to be capable of penetrating collagen gels but do not form multicellular structures (unpublished observations). Rama 401 cells retain approximately double the amount of newly synthesized collagen within the cell layer when grown on floating collagen gels. These results suggest that growth within a collagen matrix is necessary not only for the development of three-dimensional structures but also for the maximum expression of both intracellular and extracellular differentiated characteristics. We have previously described the isolation of a myoepithelial-like cell line, Rama 29, from a dimethylbenzanthracene-induced rat mammary tumor (8). Our preliminary observations suggest that Rama 29 cells represent a myoepithelial cell type that is less differentiated than Rama 401 in that its filamentous system is less well developed and the synthesis of type IV collagen and laminin is much reduced.

Myoepithelial cells in vivo are located between the basement membrane and the ductal or alveolar epithelial cells. It is not clear which cell type synthesizes mammary gland basement membrane. A number of mammary epithelial cell lines fail to synthesize an extracellular matrix containing fibronectin (57), which is a component of mammary gland basement membrane (49). However, elongated myoepithelial-like cell lines isolated from dimethylbenzanthracene-induced mammary tumor (8) synthesize an extracellular matrix that contains fibronectin (55) and two other basement membrane proteins laminin and type IV collagen (unpublished observations). Two types of cells have been observed to grow out of mammary explants: cuboidal epithelial and elongated cells (13, 33, 48). The elongated cells resemble myoepithelial cells ultrastructurally and have also been demonstrated, by immunofluorescence techniques, to synthesize type IV collagen (29). Rama 401 cells synthesize three basement membrane proteins, fibronectin (49), laminin (17), and type IV collagen (33), which are deposited in an extracellular matrix beneath the cells. A number of other cell lines synthesize basement membrane proteins in vitro, including neuroblastoma cells (3), endothelial cells (24), teratocarcinoma cells (1), amniotic fluid cells (2), and liver epithelial cells (18). The ability of elongated myoepithelial cells to synthesize basement membrane components should clearly delineate them from other elongated cells found in the mammary gland when their relative spatial positions are lost during tissue culture. Furthermore, the retention of the ability to synthesize basement membrane proteins, myofibrillar proteins, and hemidesmosome-like structures should prove useful in elucidating the role these structures may play during the development of myoepithelial cells from their precursor stem cells.

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