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

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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 16 h and then separated into a stromal fraction and an epithelial fraction by differential rates of attachment to plastic petri dishes (26, 44). Epithelial cultures were grown in 75% Ham's F12 medium + 5% FCS adjusted to 3.7 mg/ml $NaHCO_3$ previously exposed for 1 d to near-confluent cultures of Rama 29 (8) (conditioned medium, CM: 2×10^6 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 CO_2 atmosphere. This is the optimal medium for epithelial cell growth (26). In the early stages, epithelial cells were grown with $1–2 \times 10^5$ cells/5-cm petri dish of 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 Ca^{2+} 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-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 mammary gland from 7-d rat, clone number 5). These cells were subsequently grown in 50% CM rather than 75% CM, but with the other added ingredients. At the 9th passage these cells were single-cell cloned by picking individual cells with a drawn-out Pasteur pipette and plating them in separate 6-mm wells (8). The cloning efficiency was 17%. The elongated cells (differing in morphology from their parents), which grew up in one well and were termed Rama 401, were recloned with an efficiency of 78%.

Collagen gels were prepared as described by Michalopoulas and Pitot (35). Rama 401 cells were seeded at 10^6 cells/gel. When the cells became confluent, gels were removed from the plastic substrate by rimming around the edge and were allowed to float beneath the surface of the medium. Cultures were fed with fresh medium every 3 d.

Electron Microscopy

Cells grown on Thermanox cover slips (Lux Scientific Corp., Newbury Park, Calif.) or on collagen gels were washed with PBS, fixed in 2% glutaraldehyde for 1 h and postfixed in 1% osmium tetroxide for 2 h. Both fixatives were phosphate buffered (pH 7.2–7.4), and the osmotic pressure was adjusted to 350 mosmol by addition of sucrose. Samples were dehydrated in ethanol and embedded in Epon/Araldite via propylene oxide. Semithin sections (1 μm) for light microscopy were stained with toluidine blue, and regions were selected for electron microscopy. Ultrathin sections were cut on a Reichert OMU4 ultratome, stained with uranyl acetate and lead citrate, and examined in a Philips EM400 electron microscope.

For immunoperoxidase cytochemistry, cells cultured on Thermanox cover slips were washed with PBS and incubated with 200 μl of 1:150 dilution of antiserum containing 0.1% bovine serum albumin. After a 1-h incubation at room temperature, the cells were washed twice with PBS, incubated with sheep anti-rabbit peroxidase–conjugated IgG, diluted 1:75 in PBS, for 1 h, and then washed twice with PBS. The reaction was developed in 0.05% diaminobenzidine and 0.05% hydrogen peroxide, and the cultures were washed twice in PBS and processed for electron microscopy as described above.

Immunofluorescence Staining

Antibodies to fibronectin (34), type IV collagen (1, 33), laminin (53), myosin and actin (37) and vimentin (27) have been described previously. Human epidermal prekeratin was purified as described by Sun and Green (51), and antibodies were raised in rabbits. The prekeratin antibodies formed a single precipitin band in immunodiffusion tests.

To stain extracellular proteins, cells grown on plastic cover slips (Lux Scientific Corp.) were washed with PBS and fixed in 3.7% (wt/vol) formaldehyde at room temperature for 15 min. The cells were washed with PBS and incubated with antiserum (1:50 dilution) at room temperature for 40 min followed by seven washes with PBS. Incubation was then continued with fluorescein-labeled goat anti-rabbit IgG (1:10 dilution) (Miles Laboratories Ltd., Slough, U. K.). The coverslips were then washed seven times in PBS and mounted in glycerol/PBS (9:1). To visualize intracellular filaments, cultures on plastic cover slips were incubated in 0.1% Triton X-100/3.7% HCHO/PBS for 5 min at room temperature. Immunofluorescence staining was then carried out as described above. Fluorescence was observed with a Reichert Polyvar fluorescence microscope and photographs were taken on Kodak Plus-X Pan film.

Immunoprecipitation of Extracellular Matrix Components

Cultures of confluent cells were incubated in medium supplemented with β-aminopropionitrile fumarate (50 μg/ml), ascorbic acid (100 μg/ml), and L[5-^{3}H]proline (10 μCi/ml) for 24 h. A cell extract was prepared by homogenizing the cells in 1% Triton X-100/0.6 M KCl/PBS and removing insoluble material by centrifugation at 10,000 *g* for 5 min. Rabbit anti–bovine fibronectin serum, rabbit anti–mouse type IV collagen antiserum, rabbit anti–mouse laminin antiserum, or preimmune rabbit serum (5 μl) was added to the cell extract (500 μl), and the mixture was incubated at room temperature for 1 h. Protein A–Sepharose (10 μl; Pharmacia Fine Chemicals, Uppsala, Sweden) was then added and the incubation was continued for a further hour with constant mixing. The protein A–Sepharose was collected by centrifugation and washed three times by centrifuging through PBS. Immune complexes were eluted from the protein A–Sepharose by boiling for 5 min with 100 μl of "solubilizing buffer" (1% SDS, 0.1% 2-mercaptoethanol, 10% sucrose, and 10 mM sodium phosphate, pH 7.0). Aliquots were applied to polyacrylamide slab gels consisting of a 4.2% stacking gel and a 7% polyacrylamide running gel, both containing 0.1% SDS. After staining, the gels were impregnated with 2,5-diphenyloxazole and radioactivity was detected by fluorography on Kodak X-Omat H film.

Collagen Synthesis

To investigate the synthesis of collagen, confluent cultures of Rama 401 cells were incubated with [^{3}H]proline (10 μCi/ml) in the presence of ascorbic acid (50 μg/ml) for 24 h. Cell and culture medium were dialyzed extensively against 0.1 M acetic acid, and lyophilized and hydrolyzed with 6 M HCl at 110°C for 24 h. Proline and hydroxyproline were separated on a Biotronik LC 2000 amino acid analyzer equipped with a stream splitter, and the radioactivity in each was determined.

RESULTS

Isolation, Growth Characteristics, and Ultrastructure

The epithelial cell line, NM7D-5, from which Rama 401 are derived, was obtained from an apparently pure epithelial colony by ring cloning. Single-cell cloning of NM7D-5 then gave rise to cell lines with either epithelial (70%) or elongated (30%) morphology. One of these elongated clones, Rama 401, was selected for detailed study. Thus, Rama 401 may derive from a contaminant in the original epithelial colony and consequently in NM7D-5. However, when single-cell epithelial clones 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

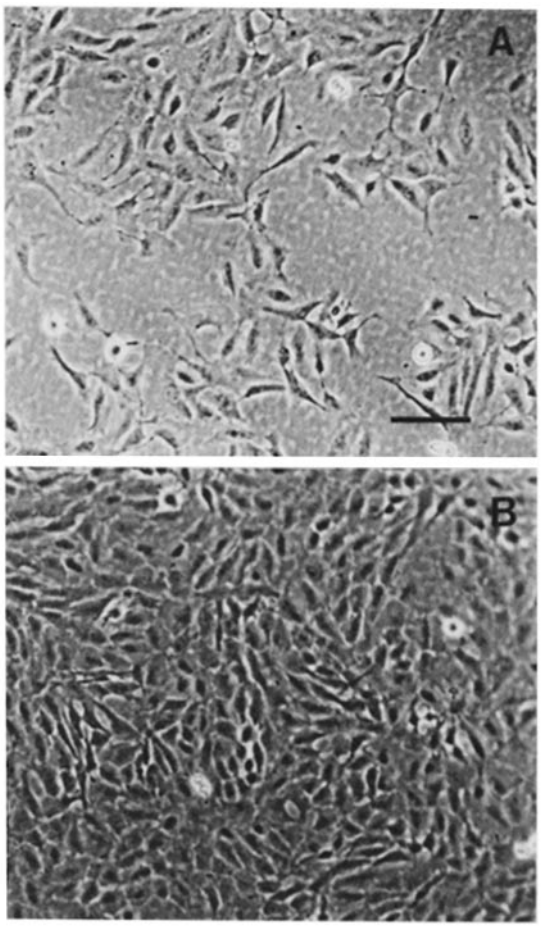

FIGURE 1 Phase-contrast microscopy of Rama 401 cells, (*A*) sparse and (*B*) confluent cultures. In sparse cultures, the cells have an irregular morphology and do not grow in colonies. In dense cultures, the cells have a polygonal appearance produced by overlapping adjacent cells. Bar, 125 μm. × 80.

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 material 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 [^{3}H]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.

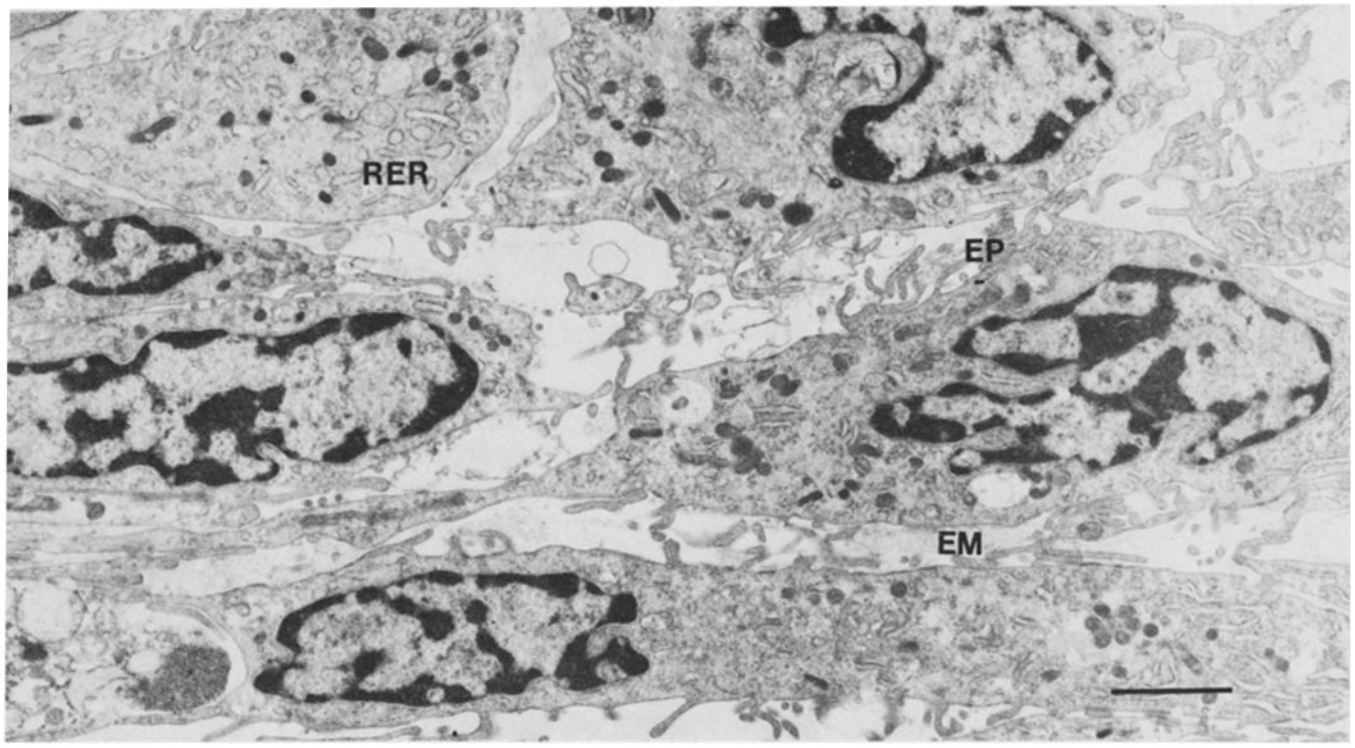

FIGURE 2 Electron microscopy of Rama 401 cells growing on plastic. Note the irregular-shaped nuclei with peripheral heterochromatin. Elongated processes (*EP*) project from the cell surface. Note the distended rough endoplasmic reticulum (*RER*) and the amorphous extracellular matrix (*EM*) filling the spaces between the cells. Bar, 1.5 μm. × 9,400.

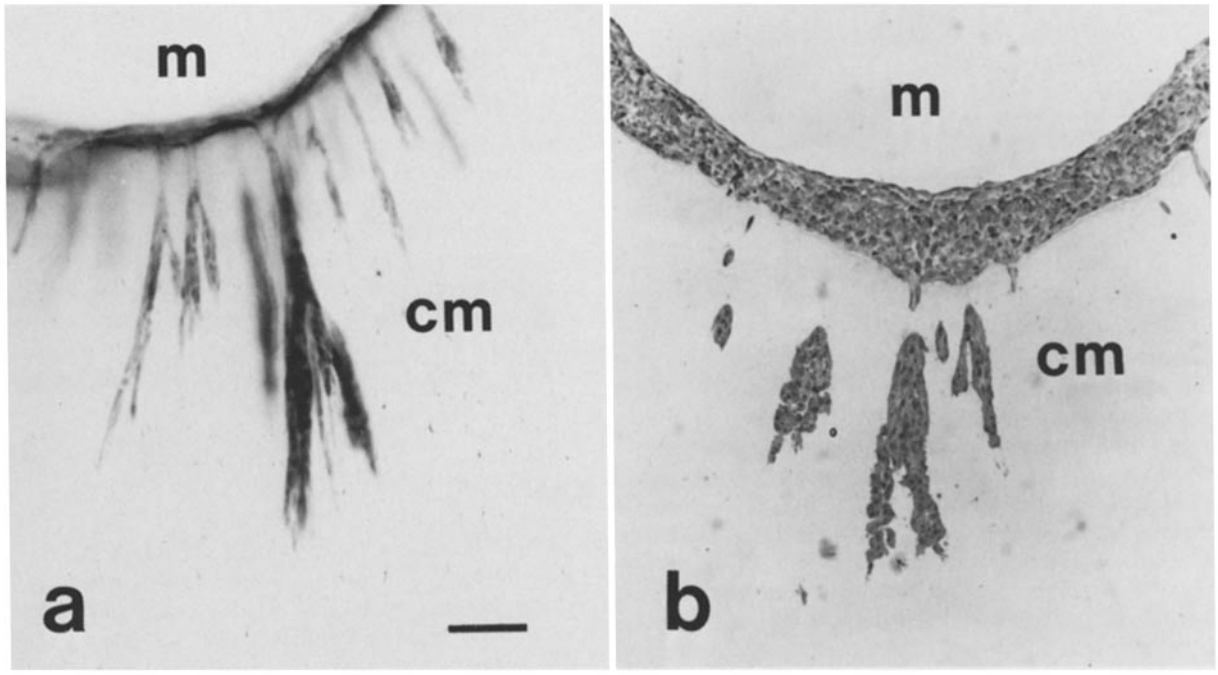

FIGURE 3 Structure formed by Rama 401 cells growing on floating collagen gels. (*a*) Phase-contrast micrograph; (*b*) 1 μm section stained with toluidine blue. After the cells reach confluency, the gel is floated and within 5–15 d the cells migrate into the collagen matrix forming multicellular branching structures composed of solid cords of cells joined to the surface by a narrow stalk of cells. (*m*) Medium, (*cm*) collagen matrix. Bar, 108 μm. × 93.

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-

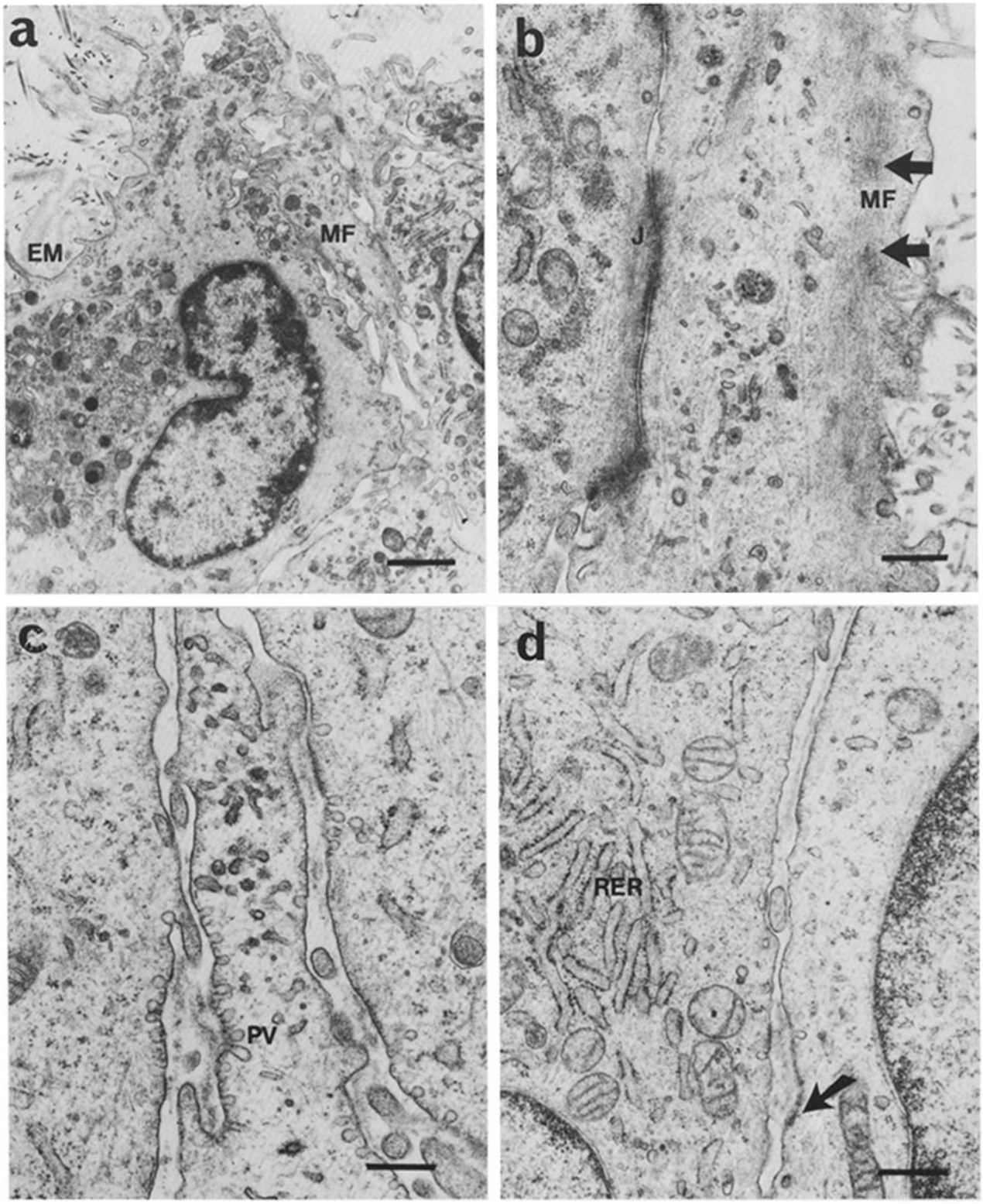

FIGURE 4 Ultrastructure of Rama 401 cells growing on floating collagen gels. (*a*) An overall view of a Rama 401 cell. Note the irregular-shaped nucleus with dispersed heterochromatin; microfilaments (*MF*) are abundant especially in the perinuclear region. An amorphous extracellular matrix (*EM*) can 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*) × 8,375, (*b*) × 23,490, (*c*) × 29,250, and (*d*) × 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

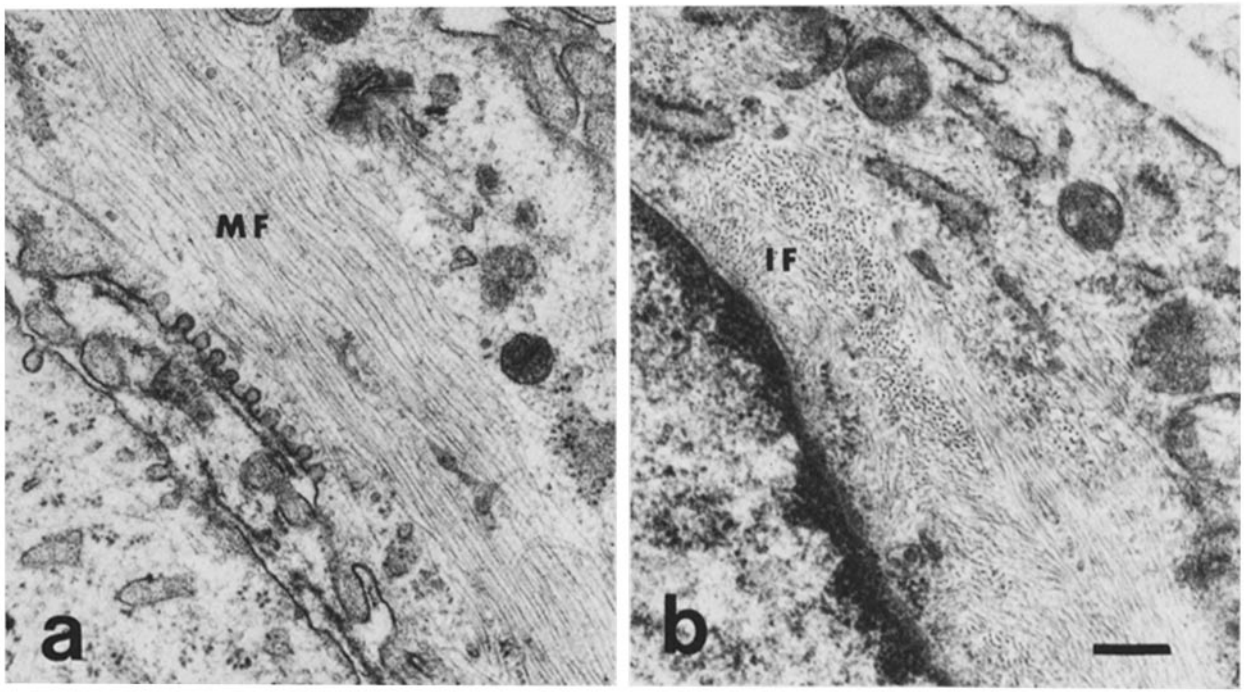

FIGURE 5 Distribution of microfilaments and intermediate filaments in Rama 401 cells. (*a*) Parallel arrays of microfilaments (*MF*) with diameters of 4–6 nm commonly occupy much of the cytoplasm. Note the absence of the focal dense bodies. (*b*) Intermediate filaments (*IF*) with diameters of 10–12 nm are located in the perinuclear region. Filaments appear to be distributed both in the plane of and perpendicular to the plane of the section. Bar, 0.25 μm. × 36,480.

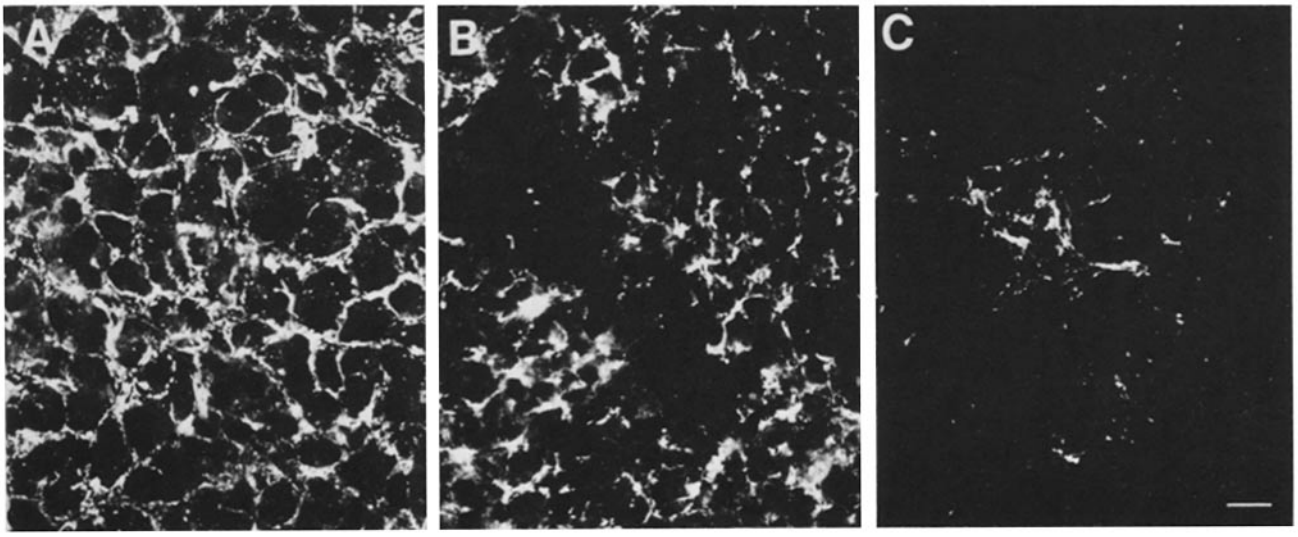

FIGURE 6 Immunofluorescence localization of basement membrane proteins, (*A*) type IV collagen, (*B*) fibronectin, and (*C*) laminin. Note the fibrillar staining around the periphery of the cells and the absence of staining over the surface of the cells. Accumulation of laminin-containing fibrils was often observed, whereas other areas were relatively devoid of staining. Bar, 20 μm. × 300.

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 ± 0.02% of the total hydroxyproline + proline and 4.11 ± 0.34% in the culture medium. The respective values for cells growing on floating collagen gels are 1.98 ± 0.03% in the cell layer and 3.87 ± 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

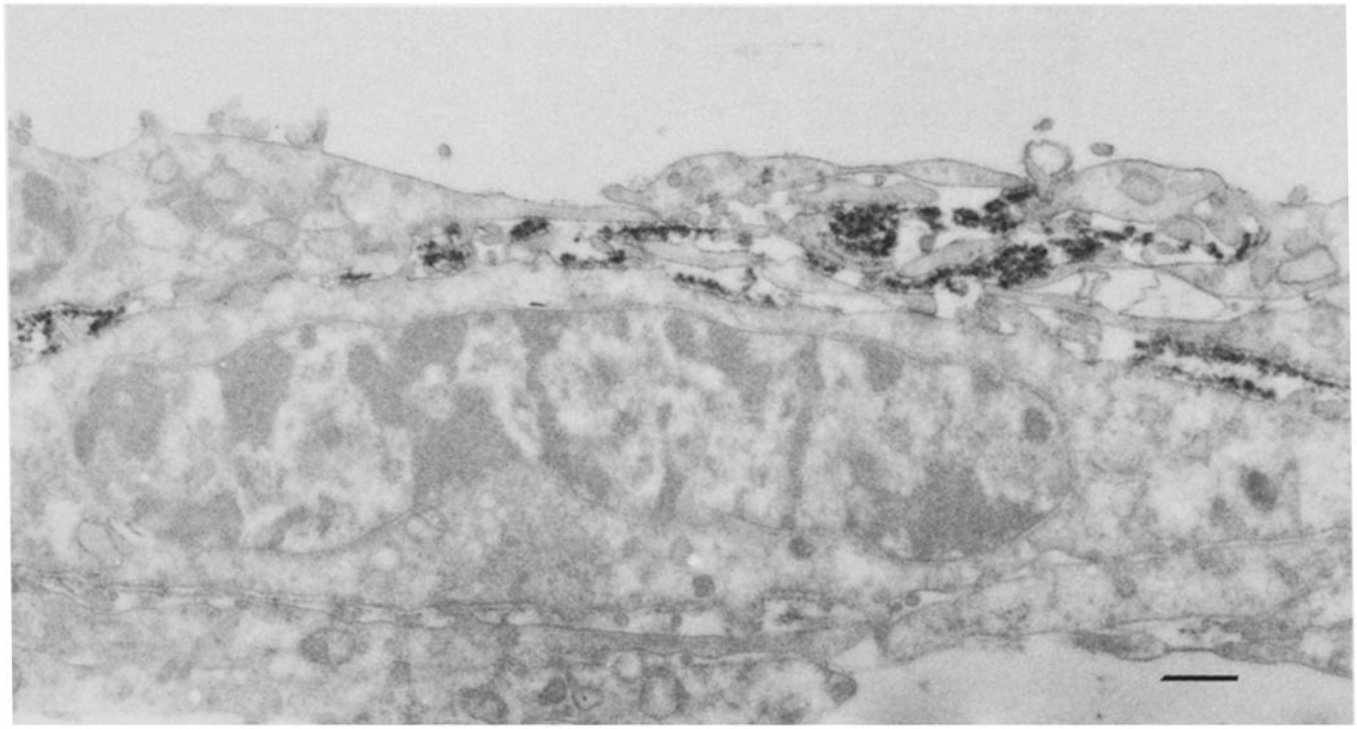

FIGURE 7 Immunoperoxidase localization of type IV collagen. Note the staining of the extracellular material between the cell layers and the absence of staining in the upper surface adjacent to the culture medium. Lack of staining of the extracellular material further down the cell layer is probably attributable to lack of antibody penetration. Bar, 0.75 μm. × 14,110.

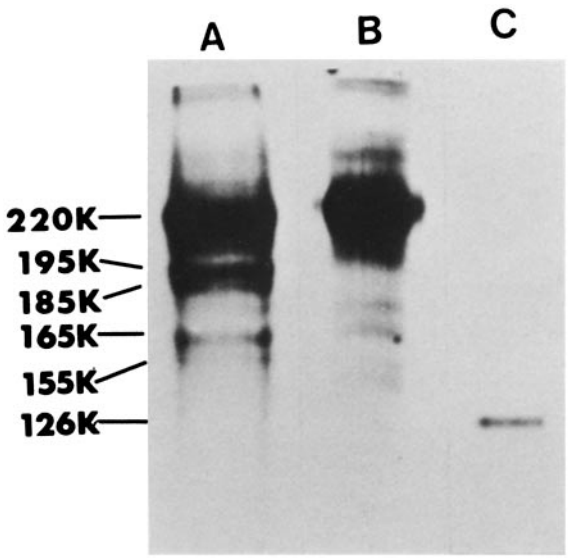

FIGURE 8 Proteins secreted by Rama 401 cells. Confluent cultures of Rama 401 cells were labeled with [^{3}H]proline (10 μCi/ml) in the presence of ascorbic acid (50 μg/ml) and β-aminopropionitrile fumarate (100 μg/ml) for 24 h. Secreted porteins were analyzed on a 7% polyacrylamide gel. (*A*) Proteins 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.

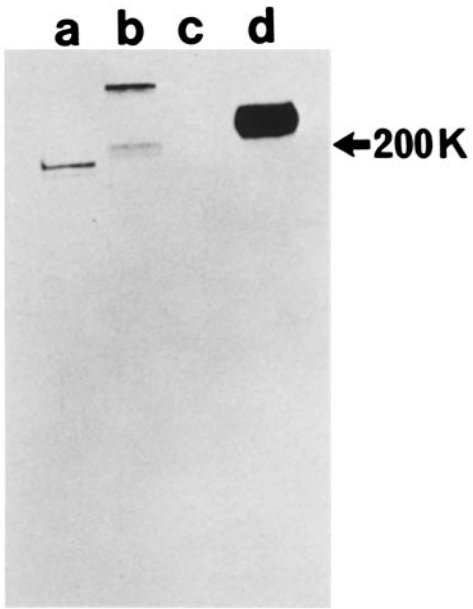

FIGURE 9 Immunoprecipitation of basement membrane proteins from Rama 401 cells. Cells were labeled with [^{3}H]proline (10 μCi/ml) for 24 h in the presence of ascorbic acid (100 μg/ml). A cell extract was incubated with antiserum to either (*a*) type IV collagen, (*b*) laminin, (*c*) nonimmune rabbit 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 prekeratin type can be demonstrated (Fig. 10 *c* and *d*). Staining for both proteins is particularly strong in the perinuclear region

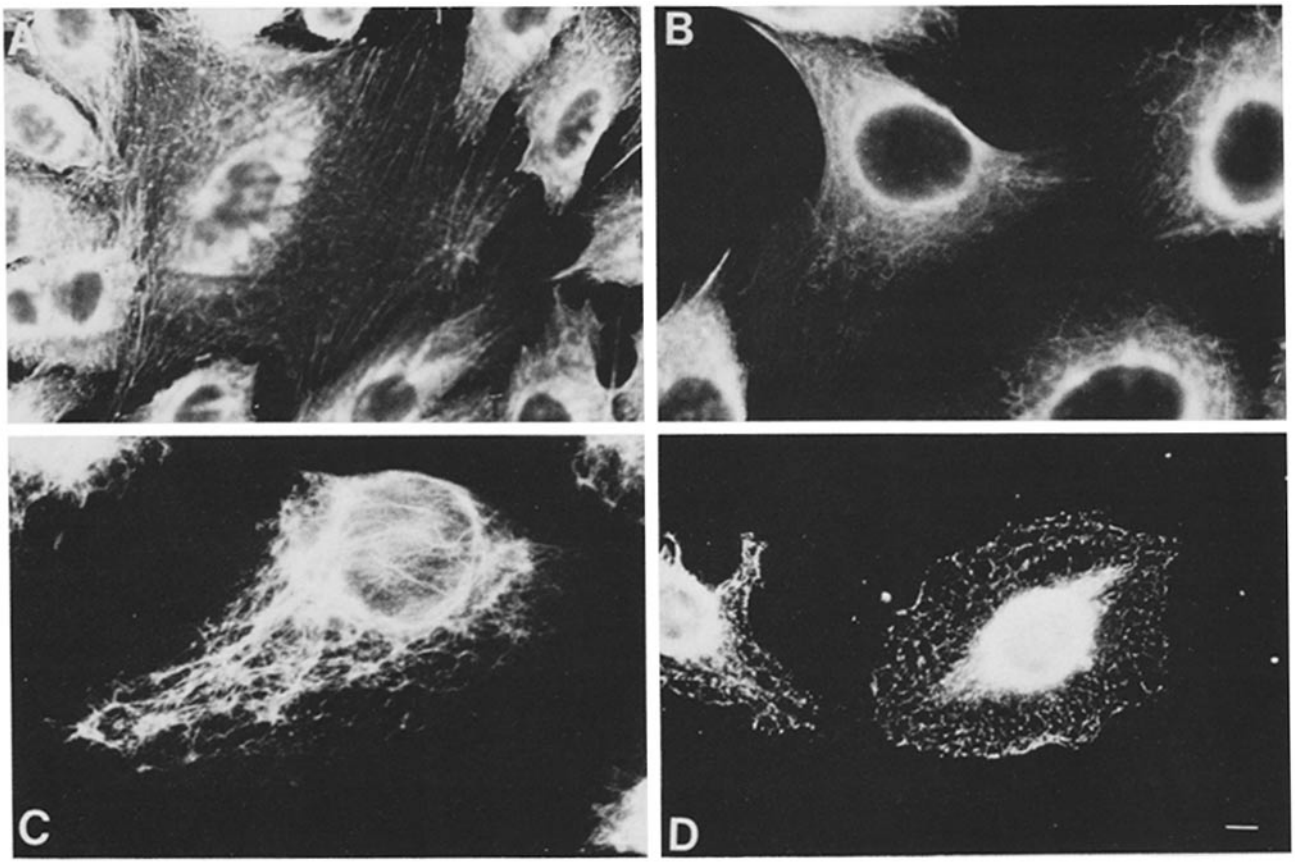

FIGURE 10 Immunofluorescence localization of microfilament and intermediate filament proteins. (*A*) myosin, (*B*) actin, (*C*) vimentin, and (*D*) prekeratin. Note the parallel arrays of actin and myosin filaments. Both vimentin and prekeratin stain intensely in the perinuclear region. Vimentin is localized in "wavy" filaments throughout the cytoplasm, whereas prekeratin containing filaments are arranged in a netlike pattern. Bar, 4 μm. × 1,100.

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 (pI 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 filamental system is less well developed and the synthesis of type IV collagen and laminin are 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 cell 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, myofilaments, 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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