

Extracellular matrix promotes mammary epithelial growth and differentiation *in vitro*

(development/basement membranes/substratum/primary culture)

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ABSTRACT The study of growth and differentiation of mammary epithelium has been hampered by the difficulty of maintaining these functions *in vitro*. We describe a system for the primary culture of rat mammary epithelium on an acellular matrix derived from whole rat mammary glands that maintains growth and differentiation for months. Cultures plated on this complex substratum produce 50 times the α -lactalbumin of those on tissue culture dishes and 5 times the α -lactalbumin of those on floating collagen gels as determined by radioimmunoassay. Unlike cultures grown on floating collagen gels, which rapidly lose the ability to secrete the milk sugar lactose, mammary cells on this matrix retain this ability for over 30 days in culture. The organ specificity of this mammary extracellular material is shown by the failure of extracellular matrix prepared from rat liver to support mammary differentiation. Within a given culture dish, cells on the surface of mammary extracellular matrix are more differentiated than those on the adjacent plastic. This is demonstrated by their increased α -lactalbumin content as shown by indirect immunofluorescence, and by their increased ability to bind fluorescein-conjugated peanut lectin. Cells on the surface of the matrix continue to synthesize DNA as determined by [³H]thymidine incorporation and autoradiography. Even when mammary epithelial cells are plated at low density, cell division continues until the matrix is covered with a confluent layer. We propose that the limited growth, differentiation, and survival of mammary cells in previously described *in vitro* systems may have been due to substrata that were inadequate to support these functions.

The study of mammary growth and differentiation has been hampered by the lack of a suitable system that is capable of maintaining these functions *in vitro*. When normal mammary epithelial cells from rodents or humans are cultured on tissue culture plastic surfaces they undergo only a few rounds of cell division and rapidly lose differentiated function (1-3). Sometimes, continuous cell lines that are easy to manipulate *in vitro* can be established from these cultures (4, 5). However, because these cells are highly selected to proliferate under artificial conditions, their control mechanisms may have little relevance to those of mammary cells *in vivo*. Organ culture has the advantage of maintaining more normal tissue orientations. However, these systems have limited viability and the presence of stromal cells makes quantitation of epithelial growth difficult (6, 7).

It has been appreciated for some time that cell behavior *in vitro* may be influenced by placing cells on matrices of stromal collagen (8, 9). More recently, Emerman and Pitelka described a system for the culture of mouse mammary cells on floating gels of stromal collagen. Mammary epithelial cells isolated from midpregnant mice produced considerably more of the milk protein casein when plated on these floating collagen gels than

when plated on attached collagen gels or tissue culture plastic dishes (10, 11). However, differentiation was limited in that these cultures failed to produce the milk sugar lactose (12). Cells that were already fully differentiated, obtained from lactating mice, lost the ability to secrete lactose within several days. Neither these cells nor those obtained from pregnant mice proliferated on the floating collagen gels (10, 12). Yang *et al.* (13, 14) reported that when mammary epithelial cells were embedded within rather than placed on top of a stromal collagen gel there was extensive cell growth but little differentiation. Thus, although the use of stromal collagen gel substrata represents a significant advance in the culture of mammary epithelial cells, no single system using this matrix has been described that can support both mammary growth and differentiation.

In an attempt to more closely simulate the *in vivo* environment, we have previously cultured rat mammary epithelial cells on a substratum of basement membrane (type IV) collagen. We have shown that these cells preferentially attach to and proliferate on this substratum compared to stromal collagen types I and III and require this matrix for normal growth *in vitro* (15) and *in vivo* (16). This matrix can reduce the cell requirements for hydrocortisone and epidermal growth factor (17). However, it is clear that the collagenous component of basement membrane alone is insufficient to support normal mammary function. *In vitro*, although mammary epithelial cells attach to and proliferate on a matrix of basement membrane collagen, this substratum does not promote cell differentiation or increase culture longevity, compared to tissue culture plastic.

The present studies were initiated to examine the effects of a more complete extracellular matrix on mammary function. Using a modification of the technique described by Rojkind *et al.* (18) for the isolation of liver "biomatrix," we have prepared a complex extracellular matrix from pregnant rat mammary glands that can be used as a substratum for primary rat mammary cultures. We now report that, unlike previously described systems, this matrix permits both cell growth and differentiation. These cultures, which remain viable for many months, should prove valuable in studying the factors that control the growth and differentiation of mammary epithelial cells.

METHODS

Cell Culture. Primary cultures of rat mammary epithelium were prepared from perphenazine-stimulated Sprague-Dawley rats by partial collagenase digestion as we have previously described (19). The resulting mammary "organoids" consist of ductal and alveolar fragments containing epithelial and myoepithelial cells. These structures were separated from stromal fibroblasts by collecting them on 26- μ m-pore nylon filters (Martin Supply, Baltimore, MD). Cultures containing 2×10^5

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Abbreviation: MGEM, mammary gland extracellular matrix.

cells per 35-mm dish were plated in medium 199 (KC Biological, Lenexa, KS) supplemented with 5% fetal calf serum, insulin at 0.1 $\mu\text{g}/\text{ml}$, ovine prolactin (National Institutes of Health) at 0.3 $\mu\text{g}/\text{ml}$, hydrocortisone at 0.5 $\mu\text{g}/\text{ml}$, progesterone at 1 ng/ml, estradiol-17 β at 1 ng/ml, and gentamycin at 50 $\mu\text{g}/\text{ml}$ and incubated at 37°C in a humidified atmosphere with 5% CO₂.

Preparation of Substrata. Mammary gland extracellular matrix (MGEM) was prepared from 14- to 16-day pregnant rat mammary glands by a modification of the technique of Rojkind *et al.* (18). Briefly, mammary glands from 20 rats were homogenized in 10 vol of water with leupeptin (Sigma) at 1 $\mu\text{g}/\text{ml}$ and soybean trypsin inhibitor (Sigma) at 10 $\mu\text{g}/\text{ml}$ at 3°C in a Brinkmann Polytron homogenizer. The insoluble material was collected on a 160- μm -pore nylon filter (Martin Supply) and washed extensively with water with the same protease inhibitors. This material was washed with 1 M NaCl with protease inhibitors for 20 hr with frequent changes of NaCl solution. Lipid was removed by layering butanol/diisopropyl ether, 2:3 (vol/vol), over an equal volume of water containing the matrix and shaking for 1 hr at room temperature. Due to the high lipid content of the mammary tissue it was necessary to repeat the delipidation procedure at least two times until the lipid was removed as determined by oil-O-red staining (19). The matrix

material was washed overnight in 1 M NaCl at 3°C and then treated with DNase at 25 $\mu\text{g}/\text{ml}$ and RNase at 100 $\mu\text{g}/\text{ml}$ in 5 vol of medium at 37°C for 1 hr with stirring. After an overnight wash in phosphate-buffered saline the matrix was frozen in medium 199 with 10% (vol/vol) glycerol and stored at -20°C. The resulting material consists of large tubular and sacular membranes as well as amorphous material. This probably represents basement membrane material from ducts, alveoli, and blood vessels as well as stromal matrix components. For attached MGEM, the matrix was frozen in Tissue-Tek II O.C.T. compound (Lab-Tek, Naperville, IL), and 10- μm sections were cut on a cryostat and deposited on the bottom of 35-mm Falcon bacteriologic plastic dishes. These were washed four times for 1 hr each with pH 7.4 phosphate-buffered saline and overnight in medium 199 prior to use. For floating MGEM, matrix frozen in medium 199 with glycerol was washed with water and weighed, and 25 mg was placed directly in a Petri dish. All matrices were sterilized with 1-3 $\times 10^5$ rads (1 rad = 0.01 gray) of cobalt-60 radiation prior to use. Cultures were fixed and processed for electron microscopy as we have described (19). Liver-derived organ matrix was prepared from rat liver by the same procedure.

Collagen gels were prepared from rat tail tendon collagen as

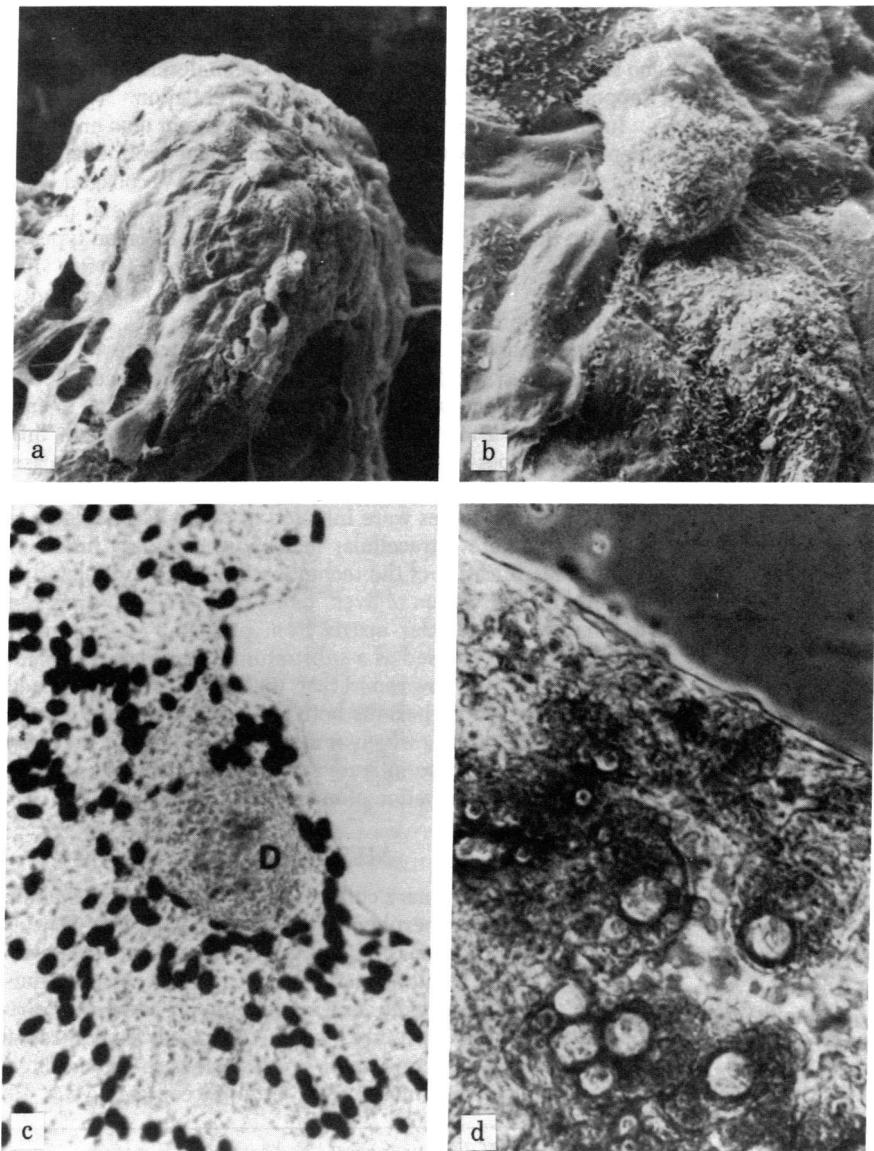


FIG. 1. Scanning electron microscopy of primary cultures on MGEM. Three-week cultures on floating MGEM. Note close cell-cell and cell-matrix contacts. ($\times 500$.) (b) Enlargement of area in a. Note numerous microvilli and rounded cell in mitosis. ($\times 2,200$.) (c) Autoradiograph of culture on attached MGEM. Cells were labeled for 22 hr at 14 days in culture with [³H]thymidine. Note dome formation (D). ($\times 120$.) (d) Phase-contrast micrograph of primary culture on attached MGEM. One month in culture. Note lipid vacuoles. ($\times 200$.)

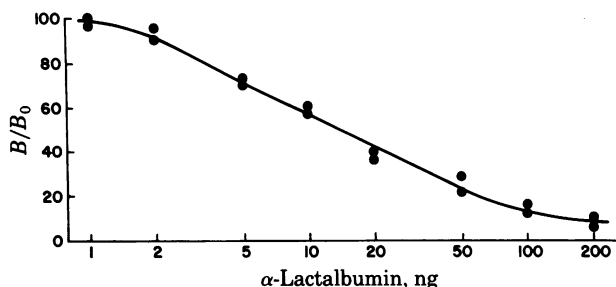


FIG. 2. Competition radioimmunoassay for rat α -lactalbumin. ^{125}I -Labeled α -lactalbumin (50,000 cpm) was used for binding, with 100% binding representing approximately 20,000 cpm precipitated. B , labeled α -lactalbumin bound; B_0 , labeled α -lactalbumin bound in the absence of competitor.

described (9). Twenty-four hours after cells were plated on these gels they were released to float as described by Emerman and Pitelka (10, 11). Autoradiography and ^3H thymidine incorporation into DNA were performed as we have described (19). DNA measurements were done by fluorometric assay as described by Setaro and Morley (20). This could be done for cultures on MGEM because DNA had been removed from the matrix with DNase treatment.

Cell Differentiation. Rat α -lactalbumin and rabbit antibody to rat α -lactalbumin were a generous gift of the National Institutes of Health Breast Cancer Task Force. Indirect immunofluorescence localization of α -lactalbumin was performed on air-dried cultures as we have described (19). Radioimmunoassay for α -lactalbumin was carried out with α -lactalbumin iodinated by the lactoperoxidase method as described by Quasba and Gullino (21) (see Fig. 2 for standard binding curve). Lactose was assayed by the colorimetric assay of Coffey and Reithel (22) as described by Burwen and Pitelka (12). Binding of fluorescein-conjugated peanut lectin from *Arachis hypogaea* (Sigma) was studied on air-dried or fixed dishes either with or without neuraminidase pretreatment (23, 24).

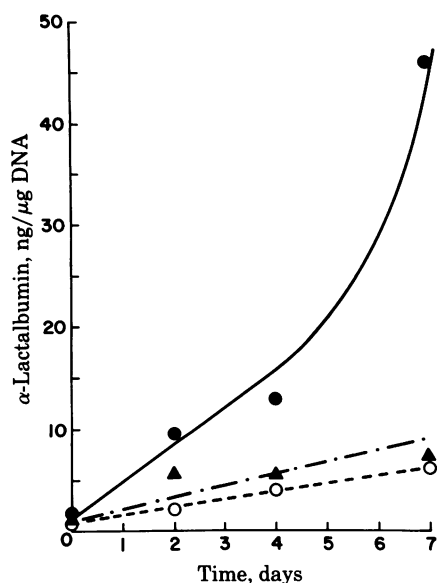


FIG. 3. α -Lactalbumin content of cells on attached MGEM (●), liver-derived organ matrix (▲), or tissue culture plastic (○). Cultures were plated on the indicated matrix, and at indicated times they were harvested in 1% Triton X-100/1% deoxycholate and an aliquot was assayed for α -lactalbumin and for DNA. Results shown are mean of duplicates repeated two times with range less than 10% of mean.

RESULTS

Morphologic Observations. We have previously shown that primary cultures of rat mammary epithelium, consisting of ductal and alveolar organoids, attach to tissue culture dishes over 10–18 hr and rapidly spread and flatten (19). Autoradiography reveals that these cultures undergo several rounds of DNA synthesis in the first week. After that time epithelial cells are gradually replaced by fibroblasts and myoepithelial cells. In contrast, rat mammary organoids plated on MGEM attach to this matrix within 1–2 hr even at cell densities below 10^4 per 35-mm dish. The cells spread over the surface of the substratum and in some areas flatten while in other areas they remain more rounded. Scanning electron microscopy demonstrates the close cell-cell and cell-substratum contacts and numerous microvilli on the epithelial cell surfaces (Fig. 1 a and b). Autoradiography reveals that DNA synthesis continues until the entire matrix surface is covered with cells (Fig. 1c). ^3H Thymidine incorporation is comparable on MGEM attached to the dish or floating in the medium. The identification of labeled cells as mammary epithelial is confirmed by peanut lectin binding (see below). Within 1–2 weeks large “domes” form to a much greater extent than on tissue culture plastic (Fig. 1c). Some cells acquire large lipid-filled vacuoles (Fig. 1d). The cultures remain viable for more than 4 months.

Differentiated Function. α -Lactalbumin is a subunit of the cytosolic lactose synthase enzyme, which is produced by differentiated mammary epithelial cells (21). The synthesis and secretion of this enzyme was quantitated by using a radioimmunoassay that can detect as little as 1 ng (see Fig. 2 for binding

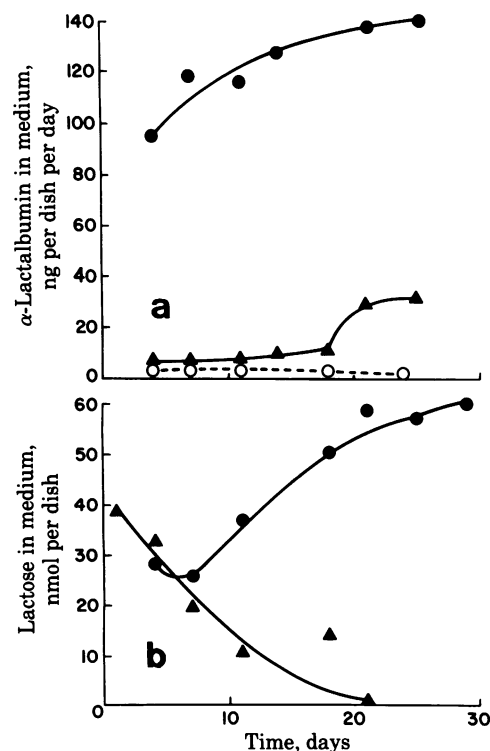


FIG. 4. Comparison of MGEM and floating collagen gels for ability to support α -lactalbumin and lactose secretion. Cultures were plated on 25 mg of floating MGEM or on floating collagen gels. After 24 hr, the collagen gels were released to float. At indicated times medium was removed and assayed for α -lactalbumin by radioimmunoassay and lactose by colorimetric assay. Results are mean of duplicates with range less than 10% of mean. (a) α -Lactalbumin: ●, MGEM; ▲, floating collagen gel; ○, tissue culture plastic. (b) Lactose: ●, MGEM; ▲, floating collagen gel.

curve). We assayed α -lactalbumin in both the cell layer and the medium for cultures plated on MGEM either attached to dishes or floating in the medium. We compared this protein to that in cells grown on tissue culture dishes or floating collagen gels. Fig. 3 shows that there is approximately 10-fold more α -lactalbumin in the cell layer for cells on attached MGEM than in cells on tissue culture plastic after 7 days. This difference was not due to cell growth alone because these values are normalized for total DNA. The organ specificity of MGEM is demonstrated by the fact that matrix prepared from rat liver failed to support differentiation (Fig. 3). Cultures plated on floating MGEM secreted even more α -lactalbumin than those plated on MGEM sections attached to the dish. α -Lactalbumin secretion into the medium was compared for cells plated on floating MGEM, floating collagen gels, or tissue culture plastic. Fig. 4a shows that at day 24, cultures on floating MGEM secreted approximately 5 times more α -lactalbumin than did cultures on floating collagen gels and 50 times more than did cultures on tissue culture plastic.

Lactose Secretion. We compared the secretion of the milk sugar lactose by primary mammary cultures plated on floating collagen gels or MGEM. As was reported by Burwen and Pitelka (12), mammary cells cultured on floating collagen gels rapidly lose the ability to secrete lactose into the medium (Fig. 4b). In contrast, the amount of lactose secreted into the medium of cells on MGEM increases with time. After 3 weeks in culture there is no detectable lactose secreted into the medium of cells on floating collagen gels, but 60 nmol per dish per day was secreted by cells on MGEM (Fig. 4b).

Histochemical Localization of Differentiated Function. In order to confirm that cells on the surface of the MGEM matrix expressed more differentiated function than did those on plastic, intracellular α -lactalbumin was localized by indirect immunofluorescence. Within a given dish there is more α -lactalbumin in cells on the surface of MGEM than on the surrounding plastic surface (Fig. 5a). Fluorescence is especially intense in areas of dome formation (Fig. 5b), which is consistent with the concept that these structures contain differentiated cells (25). These cells also show increased binding of fluorescein-conjugated peanut lectin compared to cells on plastic. It has been shown that the peanut lectin binds to oligosaccharides containing the terminal sequence β -D-galactose-(1,3)-N-acetyl-D-galactosamine (23). This lectin specifically binds to epithelial cells within the mammary gland. In the undifferentiated gland, this disaccharide is masked by sialic acid; the sialic acid is removed during differentiation. Thus, in the absence of neuraminidase pretreatment, binding of this lectin is a marker of mammary epithelial differentiation (23, 24). Using a combination of [3 H]thymidine autoradiography and peanut lectin binding, we have been able to follow both cell growth and differentiation. Within a given culture dish there is DNA synthesis in cells on and off the edge of MGEM sections, but differentiation occurs to a much greater extent in cells on the surface of MGEM matrix (Fig. 5c and d).

DISCUSSION

We have developed a system for the primary culture of rat mammary epithelium on an extracellular matrix prepared from

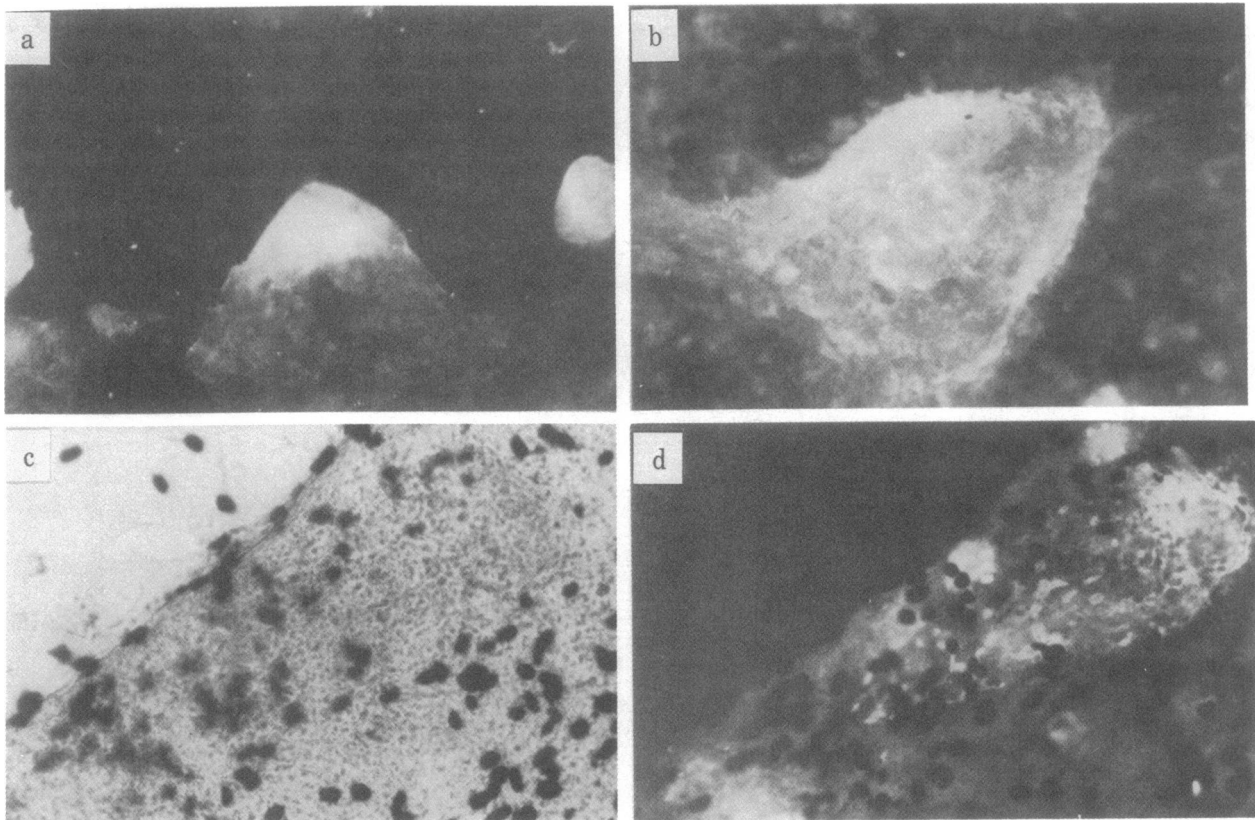


FIG. 5. Histochemical localization of differentiated function. (a and b) Indirect immunofluorescence with rabbit antibody to rat α -lactalbumin. Three-week-old cultures. ($\times 120$.) (a) Note increased fluorescence in cells on MGEM surface. Dark area, top, is off MGEM surface. (b) Note intense fluorescence in dome. (c and d) Fluorescein-conjugated peanut lectin binding and autoradiography of mammary cells on MGEM. Cells were labeled with [3 H]thymidine for 22 hr after 3 weeks in culture and processed for autoradiography and peanut lectin binding. ($\times 73$.) (c) Autoradiography, brightfield. Note labeled cells on (lower right) and off (upper left) the surface of MGEM. (d) Fluorescence of field in c. Only cells on surface of MGEM show fluorescence.

midpregnant rat mammary glands. Unlike previously described culture systems utilizing tissue culture plastic or collagenous matrices as substrata, this more complex extracellular matrix promotes both long-term growth and differentiation of normal rat mammary epithelium. As shown by α -lactalbumin and lactose secretion, cells on MGEM express substantially more differentiation than those on floating collagen gels or tissue culture plastic. Furthermore, there is extensive DNA synthesis in cells on MGEM, as shown by autoradiography, which continues until the entire matrix is covered with cells. These cells are identified as mammary epithelial by α -lactalbumin localization and peanut lectin binding.

There is increasing evidence in other systems that the behavior of cells *in vitro* may be influenced by extracellular matrix components. Gospodarowicz *et al.* (26, 27) have cultured endothelial cells on matrix material previously deposited by other endothelial cells. They found that this matrix affected the cell response to growth factors present in the serum. Overton (28) demonstrated that epithelial and mesenchymal cells cultured on the basement lamella of tadpole skin mimicked aspects of normal tissue organization. Rojkind *et al.* (18) described a process for the extraction of an extracellular matrix from rat liver. They found that this material promoted the differentiation and long-term survival of rat hepatocytes. Cell growth was not measured. Our studies with the rat mammary system extend these earlier observations and reinforce the importance of extracellular matrix components in epithelial growth and differentiation.

Mammary epithelial cells *in vivo* are anchored to a basement membrane that is composed of type IV collagen, laminin, glycosaminoglycans, and glycoproteins (29–32). Preliminary studies using immunofluorescence reveal that MGEM contains type IV collagen and laminin as well as fibronectin. Because none of these purified components can substitute for MGEM in the support of mammary differentiation (unpublished observation), it may be that the orientation of these components or other unidentified elements in MGEM are important. Furthermore, the support of differentiation by MGEM appears to be organ specific. Matrix prepared from rat liver failed to support mammary differentiation. Organ specificity in stromal–mesenchymal interactions has been described in embryonic systems and appears to be important in organ formation *in vivo* (33). Our experiments indicate that there is organ specificity in the extracellular matrix that does not require the stromal cellular component.

The configuration of MGEM as well as its composition appears to influence the expression of differentiated function. Mammary cells on floating MGEM secrete more α -lactalbumin and lactose than those on MGEM that is sectioned and attached to the bottom of Petri dishes. This may indicate the importance of cell shape or geometry in the differentiation process, as has been proposed by others (10, 11, 26).

We also have preliminary evidence that there is enhanced mitogenic responsiveness to estrogen of mammary cells grown on MGEM compared to those on tissue culture plastic. This is consistent with the observation of Gospodarowicz *et al.* regarding the altered response of endothelial cells to growth factors when the cells are cultured on a preformed extracellular matrix (26, 27).

The studies described in this report support the concept that the extracellular matrix is important in normal mammary function (16). The limited growth, differentiation, and survival of mammary epithelial cells in previously described *in vitro* systems may have been due to substrata that were inadequate to support these functions. Partial differentiation of cells on floating collagen gels may be due to the ability of this substratum to promote the cellular deposition of some basement membrane

components by these cells. David and Bernfield (34) have recently shown that stromal collagen may facilitate the deposition of glycosaminoglycans by mammary epithelial cells into an extracellular matrix. This cell-generated matrix still may not be sufficient to maintain both mammary growth and differentiation *in vitro*. MGEM may provide an extracellular matrix closer to that found *in vivo*, which is capable of sustaining normal mammary function. This system should facilitate the study of mammary growth and differentiation and the role that extracellular matrix components play in these processes.

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