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Influence of a reconstituted basement membrane and its components on casein gene expression and secretion in mouse mammary epithelial cells

(extracellular matrix/heparan sulfate proteoglycan/cell shape/messenger RNA/milk proteins)

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ABSTRACT **When primary mouse mammary epithelial cells are cultured on plastic, they rapidly lose their ability to synthesize and secrete most milk proteins even in the presence of lactogenic hormones, whereas cells cultured on released type I collagen gels show greatly enhanced mRNA levels and secretion rates of β-casein and of some other milk proteins. We show here that culture on a reconstituted basement membrane from Engelbreth–Holm–Swarm tumor (EHS) allows >90% of cells to produce high levels of β-casein. By comparison, 30–40% of cells on released type 1 gels and only 2–10% of cells on plastic express β-casein after 6 days in culture. Because only 40% of cells from late pregnant gland produced β-casein before culture, the EHS matrix can both induce and maintain an increased level of casein gene expression. Individual basal lamina components were also evaluated. Type IV collagen and fibronectin had little effect on morphology and β-casein mRNA levels. In contrast, both laminin and heparan sulfate proteoglycan increased β-casein mRNA levels (1.5- to 4-fold and 2- to 8-fold, respectively). However, for heparan sulfate proteoglycan, increased message was not accompanied by increased secretion of β-casein. Profound morphological differences were evident between cells cultured on plastic and on EHS matrix, the latter cells forming ducts, ductules, and lumina and resembling secretory alveoli. These results emphasize the vital role of the extracellular matrix in receiving and integrating structural and functional signals that can direct specific gene expression in differentiated tissues.**

Study of the development and regulation of the mammary gland poses a challenging opportunity for understanding tissue-specific gene expression because the gland changes continuously in its spatial organization and functional differentiation from embryo to aging adult. A primary regulator of these changes is the complex interaction of hormone and gland during different stages of life (for reviews, see refs. 1–3).

Until a decade ago, most investigators cultured cells on plastic surfaces, which led to drastic alterations of morphology and function from the parent tissue (4). Following the example of Emerman and Pitelka (5), we and others have shown that several aspects of functional epithelium can be maintained when primary mouse mammary epithelial cells (PMME) are cultured on "released" (RG or "floating") collagen type I gels instead of plastic. These include polarization of organelles, appearance of apical microvilli, formation of a basal lamina (5, 6), changes in glucose metabolite pattern (7), lumina formation (8), altered synthesis and compartmentalization of extracellular matrix (ECM) components (9, 10), enhancement of most milk protein synthesis and secretion (11), and increases in β-casein (12) and transferrin (13, 14) mRNA levels. These results signify the importance of cell–substratum interactions in regulating tissue-specific functions and point to a possible regulatory role for ECM *in vivo*.

Cells on released gels (and not on plastic or flat gels) were shown to synthesize an intact basement membrane (5) containing high levels of heparan sulfate and other sulfated glycosaminoglycans (9), type IV collagen, and laminin (10). We reasoned that the released gel may act through the influence of newly synthesized basement membrane components. To test the mechanism of cell–ECM interaction more directly, we have analyzed the consequences of culturing PMME cells on a reconstituted basal lamina derived from Engelbreth–Holm–Swarm (EHS) tumor (15) and on some of its individual components.

MATERIALS AND METHODS

PMME from 14- to 17-day pregnant BALB/c mice and collagen from rat tail tendon were prepared as described (5, 10, 11). Tumors (EHS) from normal or lathyritic mice were extracted with high salt and 2 M urea as described by Kleinman *et al.* (15). Dialyzed EHS extract (100–200 μl) was spread either directly on 35-mm dishes or on top of rat-tail collagen gels. Collagen gels were released 3–4 days after seeding. Matrigel, an EHS preparation from Collaborative Research (Waltham, MA), was used for some experiments. Laminin and type IV collagen (Bethesda Research Laboratories) were spread at 2.5–10 $\mu g/cm^2$. Heparan sulfate proteoglycan (HSPG; low density form) was prepared from EHS tumors as described by Hassell *et al.* (16) and was spread at 1–5 $\mu g/cm^2$ or was added to the medium at 5 μg/ml every other day. The latter was less toxic but also less effective. EHS (10 μg/ml) and individual substrata other than HSPG were added at 1 μg/ml on days 4 and 6 after culturing.

Antisera preparation, [^{35}S]methionine labeling, DNA measurement, $NaDodSO_4$/PAGE, and immunoblots were as described (11). After [^{35}S]methionine labeling (150 μCi per 35-mm plate for 3 hr; 1 Ci = 37 GBq), medium was removed and, after addition of 2 mM *p*-methylsulfonyl fluoride, was frozen for further analysis. The cells were rinsed with phosphate-buffered saline and used for RNA preparation.

All of the substrata, especially released gels and EHS preparations, interfere with immunofluorescence microscopy. Therefore, it was necessary to remove cells with colla-

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Abbreviations: PMME, primary mouse mammary epithelial cells; ECM, extracellular matrix; EHS, Engelbreth–Holm–Swarm tumor; HSPG, heparan sulfate proteoglycan; SEM, scanning EM; TEM, transmission EM.

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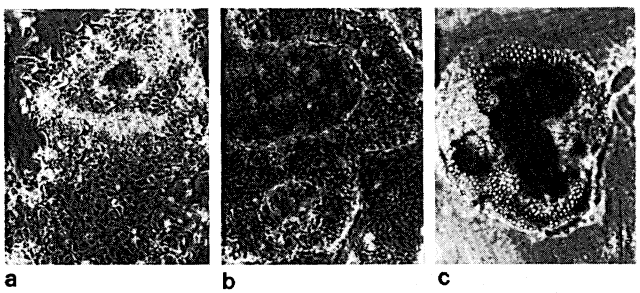

FIG. 1. Phase-contrast micrographs of PMME cultured on plastic and EHS matrix. (*a*) Cells on tissue culture plastic. (*b*) Cells cultured on EHS matrix. (*c*) Cells cultured on a different batch of EHS matrix.

genase treatment (0.3% collagenase for 30 min at 37°C, followed by 0.04% DNase I for 15 min). Cells subsequently were counted by hemocytometer and cytocentrifuged [Shandon; 2×10^6 cells per ml; 10^5 cells per coverslip (no. 1, Thomas) at 10,000 rpm ($1032 \times g$) for 10 min]. Coverslips were fixed with freshly mixed 1:1 (vol/vol) acetone/methanol for 2 min at −20°C. The first antibody was an anti-mouse β-casein monoclonal antibody (17), followed by rhodamine-conjugated goat anti-mouse antibody (Tago, Burlingame, CA). Specimens were photographed on a Zeiss inverted-phase microscope equipped for epifluorescence.

Cytoplasmic RNA was prepared as described (18) with some modification. After addition of Nonidet P-40 and vanadyl–ribonucleoside complex to washed PMME preparations, cells were homogenized and the lysates were centrifuged ($1500 \times g$ for 15 min) to pellet nuclei and debris. The pellet was used to determine the DNA content (19). To determine the RNA content, 4 M guanidine isothiocyanate in 50 mM Tris·HCl/10 mM EDTA/0.1 M 2-mercaptoethanol was added to the supernatant (cytoplasmic RNA) or directly to each 35-mm plate (total cellular RNA), and samples were layered onto 5.7 M CsCl and centrifuged at 15°C for 20 hr (SW 50.1 rotor, 36,000 rpm). Pellets were recovered in Tris/EDTA buffer, and RNA was quantified and stored in ethanol at −70°C. Nick-translation and hybridization were essentially as described by Maniatis *et al.* (20). Dot blots were done in 50% (vol/vol) formamide containing 0.75 M NaCl, 0.075 M sodium citrate (pH 7), 10% dextran sulfate, 0.02% bovine serum albumin, 0.02% polyvinylpyrrolidone, and 0.02% Ficoll. Specificity of β-casein probe preparations was confirmed with blot hybridizations (20).

For scanning electron microscopy (SEM), cells were fixed, dehydrated, dried at the critical point, gold-coated, and observed in a Phillips 500 scanning electron microscope as described (21). For transmission EM (TEM), the cells remaining in the culture wells were dehydrated and embedded in Epon *in situ*. Pieces of hardened Epon were reimbedded so that thin sections cut perpendicular to the dish surface could be prepared.

RESULTS

Different batches of EHS extract prepared by the same method led to distinct morphological characteristics (Fig. 1). Obvious differences between the various EHS preparations were not revealed by $NaDodSO_4$/5% PAGE (not shown). However, there appeared to be an inverse correlation between the degree of cell spreading and the DNA content of the EHS preparation (not shown). Other preparations, including Matrigel, caused morphological characteristics intermediate between those shown. Cells either remained in tight multilayered clusters, with occasional myoepithelial (or fibroblastic-like cells) migrating beneath the surface of the EHS matrix (Fig. 1*c*), or formed domes and duct-like structures (Fig. 1*b*). Most cells were refractile and contained numerous secretory granules. Surface activity of cells on EHS matrix-coated plastic, even in relatively "flat" areas, resembled that on floating gels as reported previously (ref. 5; compare *a* and *b* in Fig. 2). Viewed by TEM, cells on EHS matrix were seen to form right-side-in "lumina" complete with numerous apical microvilli, well-developed rough endoplasmic reticulum and Golgi, and evidence of secretory activity (compare *c* and *d* in Fig. 2). At high magnification (not shown), these lumina were seen to be surrounded by a thin but continuous basal lamina, which separated them from the less-dense matrix in which they were embedded. On some

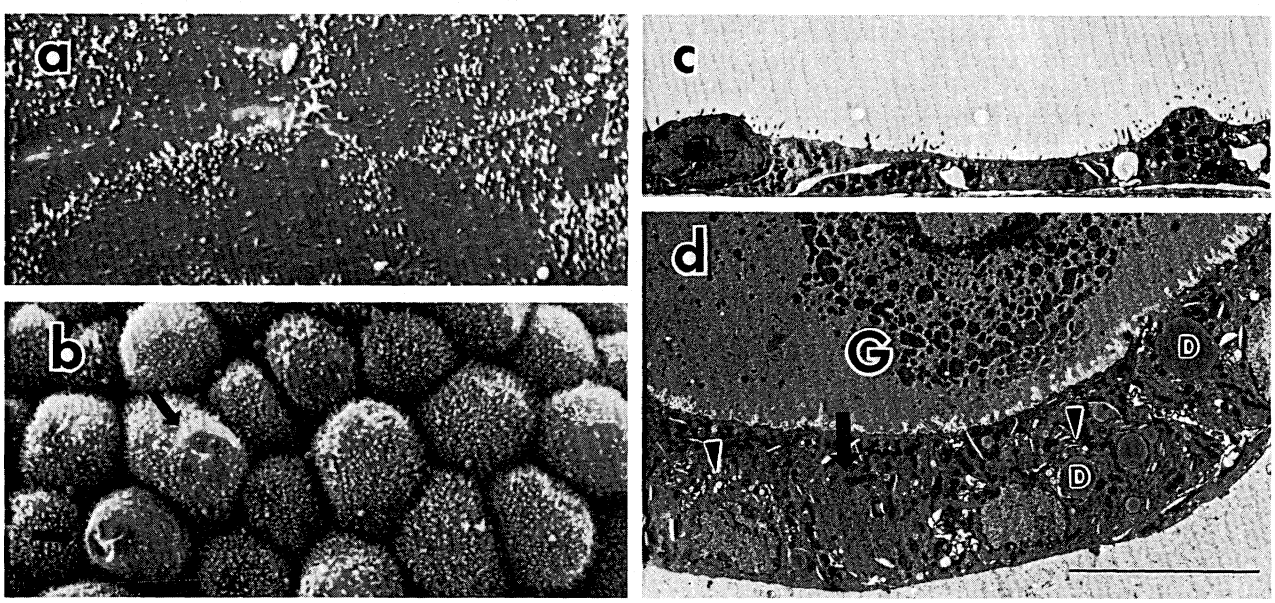

FIG. 2. EM of PMME cultured on EHS matrix for 7 days. Cells on coverslips (*a* and *c*) spread to form a flat epithelial layer, while cells on EHS matrix (*b* and *d*) are rounded and covered with apical microvilli. By TEM, cells cultured on EHS matrix are seen to form hollow alveolar-like structures, with the base of the cells facing out and their apices in. Evidence of secretory activity includes extensive rough endoplasmic reticulum (large arrow), Golgi apparatus (arrowheads), numerous mitochondria, and lipid droplets (D). Some of the granular material (G) that fills the lumen may be secreted milk proteins. (Bar = 10 μM.)

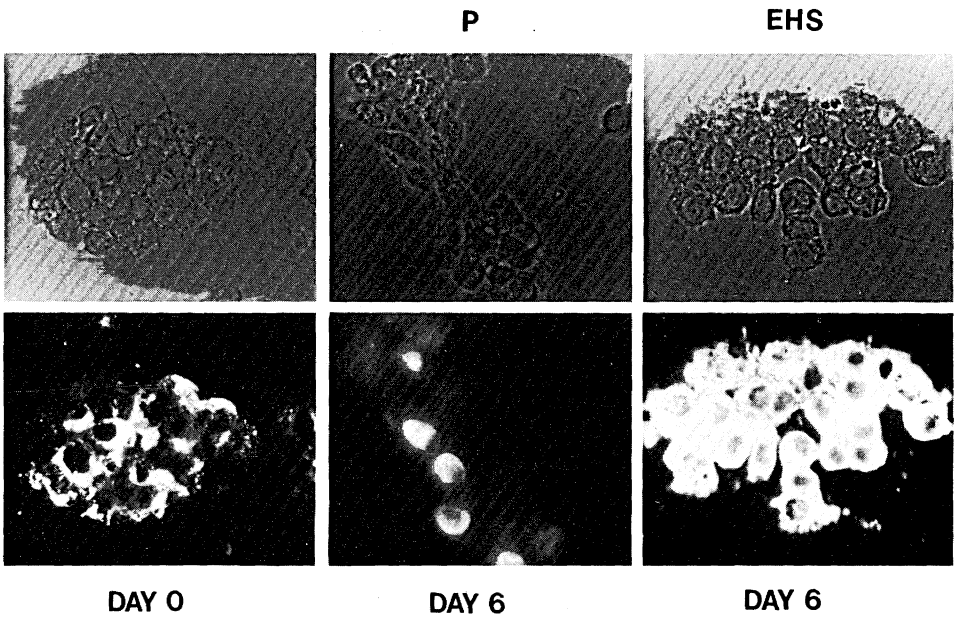

FIG. 3. Immunofluorescent localization of β-casein in PMME. Cytocentrifuged preparations of dissociated cells were stained with anti-β-casein. (*Left*) Day 0, epithelial cells from 16- to 18-day pregnant mouse gland before plating. (*Center*) The same cell preparation after plating on tissue culture plastic (P) for 6 days. (*Right*) The same cells after plating on EHS matrix for 6 days.

EHS preparations, especially Matrigel, cells began to flatten and lose their domes and ductules after 4–5 days in culture. This could be prevented by addition of a small amount of EHS preparation (10 μl/ml) to the medium from day 4 onward.

To determine the percentage of casein-producing cells under the above conditions, we separated PMME from matrix by digestion with collagenase and then treated them with monoclonal antibody to β-casein. Both the level and the number of cells producing β-casein were dramatically increased on EHS matrix compared to plastic (Fig. 3). In this experiment, 40% of cells expressed β-casein at time zero. Six days later the same cells plated on plastic or EHS matrix contained 7% or 95% β-casein-producing cells, respectively. Cells on released collagen gels contained 38% β-casein-positive cells (not shown). Furthermore, as is apparent from Fig. 3, cells cultured on EHS matrix expressed much higher levels of β-casein per cell than did pregnant gland.

Six-day cultures of PMME on plastic and ECM-coated dishes were labeled with [^{35}S]methionine and extracted for analysis of RNA. In general, the basal level of the β-casein message is difficult to control in PMME cultures because it changes as a function of gestation age, length of collagenase treatment, seeding density, quality of isolated cells, and length of culture. Therefore, for each experimental measurement, values are expressed in relation to those from cultures on plastic. HSPG was toxic to cells, especially after prolonged exposure and at levels above 500 ng/cm^2. To ensure sufficient live cells for RNA and protein analysis, experiments reported here were terminated 6 days after plating. The amount of β-casein message decreases in cultures on plastic and increases in cultures on floating gel as a function of time. Thus, earlier harvest means higher levels of message on plastic and lower levels on floating gels (Fig. 4, lanes 1 and 2).

Four basal lamina components were tested. Neither type IV collagen nor fibronectin (not shown) influenced either cell morphology or β-casein message. In contrast, PMME cultured on laminin (2.5 μg/cm^2) had as much β-casein message as did cells cultured on floating gels (compare lanes 2 and 3

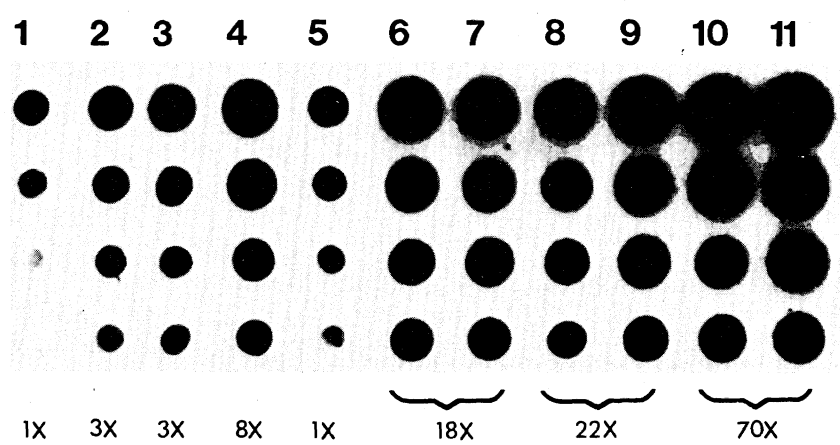

FIG. 4. β-Casein mRNA levels on different substrata. Cytoplasmic RNA was extracted as described 6 days after seeding, dotted onto nitrocellulose, and hybridized with the nick-translated β-casein probe. The top dot is 4 μg of RNA; other dots are sequential 1:2 dilutions. Lanes: 1, plastic; 2, released gel; 3, laminin; 4, HSPG; 5, type IV collagen; 6 and 7, EHS matrix on plastic; 8 and 9, EHS matrix on flat gel; and 10 and 11, EHS matrix on released gel.

in Fig. 4); in other experiments, increases in message compared to that of cells cultured on plastic ranged from 1.5 to 4.0. HSPG induced increases in message levels that, while quite variable, at times reached the largest obtained with any single purified ECM component (Fig. 4, lane 4). EHS matrix (the two preparations shown in Fig. 1 were used) induced a remarkable increase in β-casein message, whether it was spread on plastic (Fig. 4, lanes 6 and 7), on flat type I collagen gels (lanes 8 and 9), or on gels that were released 4 days after seeding (lanes 10 and 11). Thus, PMME maintained on EHS matrix-coated released gels had more than 3 times the message of cells in a lactating gland in this experiment! RNA from yeast was totally negative, whereas that in cells from a 5-day lactating mouse was 22-fold higher than in cells cultured on plastic (not shown). If the EHS preparation did not "gel" properly or if the preparation was stored for more than 1 wk at 4°C, gains in β-casein message levels were much more modest. Cells cultured on Matrigel produced 5- to 12-fold increases in β-casein message over cells cultured on plastic. Expressed in relation to total RNA, this level is comparable to that reached on released gels 3 or more days after flotation.

Except for cells cultured on plastic, the rate of protein synthesis and secretion is roughly proportional to mRNA levels for β-casein (11). However, cells cultured on HSPG showed drastically reduced levels of β-casein in the medium (Fig. 5). In experiment I, β-casein message in cells cultured on HSPG was increased 3-fold over levels in cells cultured on plastic; yet it is clear from both the CCl_3COOH-precipitable bands (Fig. 5 *Lower*, lanes 1) and the immunoblot (Fig. 5 *Upper*, lanes 1) that no β-casein was secreted into the medium. In experiment II,

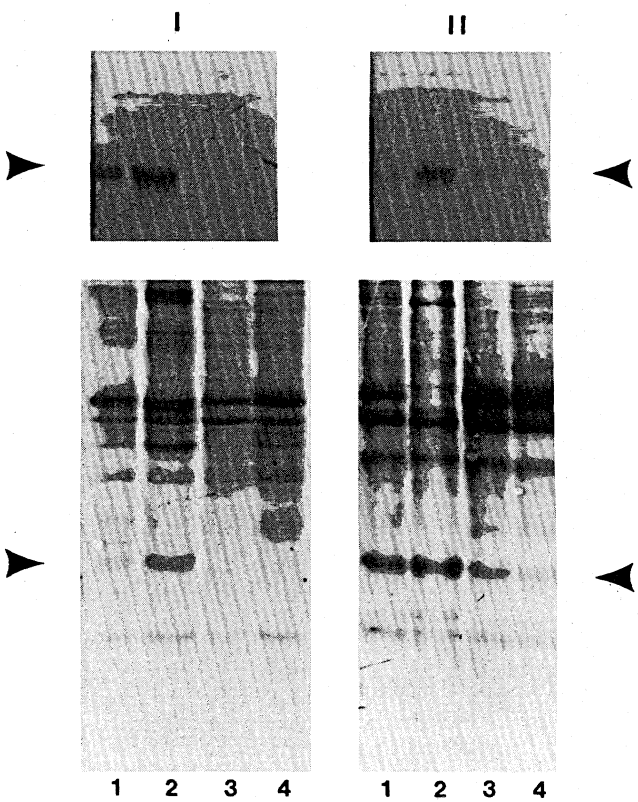

FIG. 5. Comparison of β-casein and other proteins secreted by cells cultured on plastic, EHS matrix, and HSPG. Equivalent amounts of secreted proteins from two separate experiments are shown. (*Lower*) Autoradiograms. (*Upper*) β-casein immunoblot performed on identical samples. Lanes in experiment I: 1, culture on plastic; 2, EHS matrix; 3, HSPG (5 $\mu g/cm^2$); 4, HSPG (2 $\mu g/cm^2$). Lanes in experiment II: 1, culture on plastic; 2, EHS matrix; 3, HSPG (10 μg/ml of medium); 4, HSPG (2 $\mu g/cm^2$). The arrowheads point to the β-casein band.

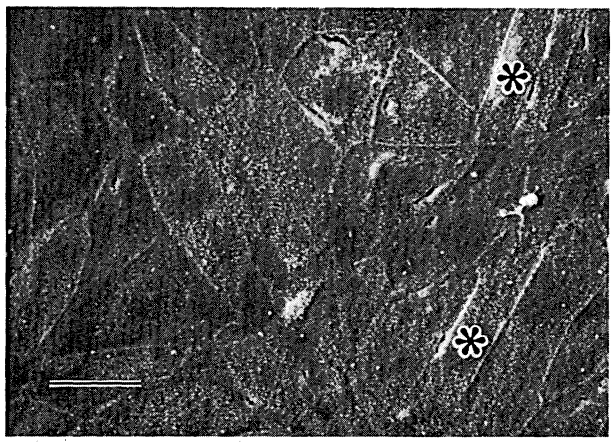

FIG. 6. Morphology of PMME cultured on HSPG for 7 days. These cells form a flat epithelial layer similar to cells cultured on glass alone (Fig. 2*a*), with occasional small clumps of rounded cells (not shown). The unusual rectangular shape of many of these cells (asterisks) is not observed in cells grown on glass or plastic alone. (Bar = 20 μM.)

mRNA levels in cells cultured on HSPG compared to levels in cells cultured on plastic were increased 3- to 5-fold, yet secreted β-casein was much lower in the medium from the former than from the latter (Fig. 5 *Upper*, compare lanes 3 and 4 with lane 1). Inhibition of β-casein secretion appeared to be specific, because other secreted proteins (Fig. 5 *Lower*, lanes 3 and 4 in experiments I and II) were comparable to those from cells cultured on plastic. Interestingly, the morphology of cells cultured on HSPG more closely resembled the flattened cells grown on plastic than the rounded ones observed on EHS matrix or released collagen gels (Fig. 6). β-Casein levels in the medium of cells cultured on EHS matrix were variable, depending on the amount of lumina formed. We suspect that cells secrete vectorially into sealed lumina (Fig. 2*d*), reducing levels measurable in the medium.

DISCUSSION

Most cells in intact higher organisms are in contact with complex ECMs that contain different collagen types, proteoglycans, and glycoproteins. The ECM was postulated to play a role in morphogenesis and differentiation decades ago, but our knowledge of its biochemistry and its supramolecular organization has been arrived at much more recently (for reviews, see refs. 22 and 23). There has also been rapid recognition that ECM must play a fundamental role in development and maintenance of the differentiated state (22–25). Yet a direct demonstration of ECM action on mRNA levels is still limited to the mammary gland and more recently to the liver (for reviews, see refs. 3 and 26). This study is an extension of previous ones in mouse and rabbit mammary glands, where released collagen gels were shown to permit morphological differentiation, higher levels of α- and β-casein mRNA (12, 27), and increased secretion of many milk proteins. β-Casein mRNA levels in cells on released collagen gels continue to increase with time in culture, consistent with the idea that elaboration of a basement membrane is a necessary early step in modulation of β-casein expression. We show here that a reconstituted basement membrane composed of laminin, type IV collagen, HSPG, and entactin (15), which forms an ordered supramolecular structure under our culture conditions, allows (*i*) production of high levels of mRNA for β-casein (and for α-casein and transferrin; M.L., L. Chen, and M.J.B., unpublished data); (*ii*) synthesis of β-casein in a large majority of cells (>90%); and (*iii*) formation in culture of "glandular" structures resembling secretory alveoli *in vivo*. Rat Sertoli cells grown both on and within

EHS matrix also have been shown to undergo striking changes in morphology and secretory activity (28). We further show that HSPG and laminin, but not type IV collagen or fibronectin, also increase mRNA levels for β-casein.

The additional increase in β-casein message observed in PMME on floating EHS matrix-collagen gels (Fig. 4, lanes 10 and 11) [also observed in an established mouse mammary cell strain (unpublished data; ref. 29)] is intriguing and further emphasizes the correlation between changes in cell shape and epithelial function (for reviews, see ref. 3). However, the increase in β-casein message in cells cultured on HSPG without an obvious morphological change argues against a simple correlation between shape and mRNA levels. Cell-associated HSPG has been shown to span the plasma membrane, connecting the ECM and the cytoskeleton (30), and laminin also has been reported to have connections to actin via a cell-surface receptor (31). Perhaps HSPG and laminin in our cultures can increase β-casein mRNA levels by altering cytoskeletal interactions with polysomes, leading to increased message stability (24).

We have shown (12) that β-casein synthesized by cells cultured on plastic was degraded intracellularly, suggesting that routing of some secretory components was dependent on cell shape. Because the morphology of cells cultured on HSPG is similar to cells cultured on plastic (Fig. 6) and because the β-casein pools are comparable (unpublished data), it is possible that most of the newly synthesized β-casein in HSPG cultures is degraded internally as well. On the other hand, the possibility that HSPG-induced toxicity may have increased specific caseinolytic activities cannot be ruled out.

Basement membranes *in vivo* are known to be heterogeneous (32). The complex interactions between, and precise compositional ratios of, ECM components are being analyzed *in vitro* (15, 33). Attachment, spreading, and undoubtedly functional differentiation of cells are influenced by composition, concentration, and spatial interactions of ECM molecules. The aim of our studies is not only to establish that the ECM is an "informational" entity, receiving, imparting, and integrating structural and functional signals, but also to understand the mechanism(s) by which the ECM modulates gene expression. Although we have come a long way in establishing the former, the latter aim will require careful quantitative analysis of the chemistry and biochemistry of the ECM and a detailed kinetic analysis of cellular functions in response to cell–ECM interactions.

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