

Response of Basal Epithelial Cell Surface and Cytoskeleton to Solubilized Extracellular Matrix Molecules

STEPHEN P. SUGRUE and ELIZABETH D. HAY

Department of Anatomy, Harvard Medical School, Boston, Massachusetts 02115

ABSTRACT Corneal epithelium removed from underlying extracellular matrix (ECM) extends numerous cytoplasmic processes (blebs) from the formerly smooth basal surface. If blebbing epithelia are grown on collagen gels or lens capsules *in vitro*, the basal surface flattens and takes on the smooth contour typical of epithelium in contact with basal lamina *in situ*. This study examines the effect of soluble extracellular matrix components on the basal surface. Corneal epithelia from 9- to 11-d-old chick embryos were isolated with trypsin-collagenase or ethylenediamine tetraacetic acid, then placed on Millipore filters (Millipore Corp., Bedford, Mass.), and cultured at the medium-air interface. Media were prepared with no serum, with 10% calf serum, or with serum from which plasma fibronectin was removed. Epithelia grown on filters in this medium continue to bleb for the duration of the experiments (12–24 h). If soluble collagen, laminin, or fibronectin is added to the medium, however, blebs are withdrawn and by 2–6 h the basal surface is flat. Epithelia grown on filters in the presence of albumin, IgG, or glycosaminoglycans continue to bleb. Epithelia cultured on solid substrata, such as glass, also continue to bleb if ECM is absent from the medium. The basal cell cortex *in situ* contains a compact cortical mat of filaments that decorate with S-1 myosin subfragments; some, if not all, of these filaments point away from the plasmalemma. The actin filaments disperse into the cytoplasmic processes during blebbing and now many appear to point toward the plasmalemma. In isolated epithelia that flatten in response to soluble collagens, laminin, and fibronectin, the actin filaments reform the basal cortical mat typical of epithelia *in situ*. Thus, extracellular macromolecules influence and organize not only the basal cell surface but also the actin-rich basal cell cortex of epithelial cells.

The basal cell surface and cortical cytoskeleton of the embryonic corneal epithelium are intimately related to the underlying extracellular matrix (ECM). The cortical cytoskeleton forms a dense mat next to the basal epithelial plasmalemma, and the latter is relatively smooth or flat in configuration. The basal plasmalemma seems to be connected to the lamina densa of the underlying basal lamina (basement membrane) by extracellular filaments that traverse the lamina rara externa (1). The laminae rarae externa and interna of the corneal, and most, if not all, other epithelial basal laminae (2–5) contain proteoglycan granules rich in glycosaminoglycans (GAG), such as heparan and/or chondroitin sulfates (HS, CS) and hyaluronic acid (HA). Laminin and fibronectin occur in the laminae rarae of the glomerular basement membrane (4), and both are found in the embryonic avian corneal basal lamina (Sugrue and Hay, unpublished observations). Type IV collagen is also present in the corneal basement membrane (6). Type IV and other col-

lagens (e.g., type V) may be concentrated in the lamina densa (4) but might also extend in the form of fine filaments (1) to the cell surface in concentrations too dilute to be detected by immunohistochemistry.

The existing evidence that the organization of the basal epithelial cell cytoplasm is dependent on the presence of the underlying ECM is mainly circumstantial. In corneal epithelia or epidermis with well-developed hemidesmosomes, tonofibrils insert in cytoplasmic plaques that seem to be attached to specialized regions of ECM (1). In younger corneal epithelia lacking hemidesmosomes, the mat of cortical filaments in the basal cytoplasm seem to follow the contour of the basal lamina (1). More direct evidence that the configuration of the basal cell surface and cortical cytoplasm depends on ECM derives from studies using enzymes or EDTA to remove the epithelial basal lamina; such epithelia extend cytoplasm-filled blebs from their naked basal surfaces (see references 5 and 7). When

grown on solid collagenous substrata, isolated embryonic avian epithelia withdraw these blebs, become smooth-surfaced again, and step up synthesis of both collagen and GAG (7-9).

In the present paper, we ask whether the reaction of the basal corneal cytoplasm to ECM is due merely to the physical support of the collagenous substratum, or whether solubilized ECM molecules themselves can induce isolated epithelia to withdraw the blebs. Blebbing epithelia impaled on Millipore filters (Millipore Corp., Bedford, Mass.) are exposed to solutions of various collagens, glycosaminoglycans, fibronectin, and laminin to judge the effect of these molecules on the structure of the basal epithelial surface. The appearance of the basal surface on solid but inert substrata is also examined. Finally, we address the question of the organization and nature of the cortical cytoskeleton in blebbing and ECM-treated embryonic corneal epithelia by examining the actin components labeled with S-1 fragments of heavy meromyosin.

MATERIALS AND METHODS

Corneas harvested from chick embryos after 9-11 d of incubation were treated at 25°C with 0.1% trypsin (Sigma Chemical Co., St. Louis, Mo.) and 0.1% collagenase (Sigma Chemical Co.) in Hanks' solution, pH 7.4, for 9-12 min or were soaked in 0.04% EDTA (Sigma Chemical Co.) in calcium-magnesium-free Hanks' solution, pH 7.4, for 30 min. The epithelia were removed with forceps from the stroma as a sheet, transferred to a Millipore filter (HATF 0.45- μ m pore size). Such epithelia are completely clean of contaminating ECM (8, 9). Epithelia on filters were then cultured at the air-medium interface of Falcon culture dishes (Falcon Plastics, Oxnard, Calif.) in Ham's F-12 medium (Gibco Laboratories, Grand Island Biological Co., Grand Island, N. Y.) without serum or with 10% fetal calf serum (Flow Laboratories, McLean, Va.) from which plasma fibronectin (CIG) was removed by affinity chromatography with gelatin. Medium was supplemented with 50 μ g/ml ascorbic acid and 1% antibiotic-antimycotic (penicillin, fungizone, and streptomycin, Gibco Laboratories).

Each isolated corneal epithelium was usually placed basal-side down on a disk (3-mm Diam) made of Millipore filter and transferred within 2 h after isolation to standard medium (above) or to medium containing soluble ECM molecules, albumin, or IgG. Some epithelia were cultured at the air-medium interface on glass cover slips coated with 0.1% poly-L-lysine (Sigma Chemical Co.) or tissue culture plastic (Falcon Plastics). Other epithelia were attached by their apical surfaces to Millipore filter disks coated with poly-L-lysine. The disks were then inverted across holes 2.3-mm Diam in another filter; as a result, a large stretch of

the basal epithelial surface was exposed to the underlying medium without physical support on the basal side. Still other epithelia were grown on Millipore filters that had been soaked for 18 h in collagen (type I, 100 μ g/ml, see below) and washed briefly in three changes of Hanks' solution.

Purified collagens were dissolved in 0.5 M acetic acid at a concentration of 1 mg/ml, then dialyzed against 0.1 M phosphate-buffered saline (PBS), pH 7.4. Type I collagen was denatured by heating at 50°C for 20 min. Type I collagen was purified from rat tail tendons by our laboratory (8, 9), and type II collagen from sternal cartilage by Dr. Thomas Linsenmayer (Department of Anatomy, Harvard Medical School, Boston, Mass.) (10). Type IV collagen and laminin from a murine tumor (11, 12) were provided by Dr. George Martin (National Institute of Dental Research, Bethesda, Md.). Rat tendon α 2(I) chains were also obtained from Dr. Martin. Cellular fibronectin was extracted from NIL8 cells, and plasma fibronectin was purified from human plasma by Dr. Richard Hynes (Massachusetts Institute of Technology, Cambridge, Mass.) (13). HA and CS mixed chains (Sigma Chemical Co.), HS chains (Dr. J. A. Cifonelli, University of Chicago, Chicago, Ill.), and heparin (Upjohn Co., Kalamazoo, Mich.) were dissolved double-strength in H₂O and diluted in double-strength media. Bovine serum albumin (Sigma Chemical Co.) and nonspecific rabbit IgG (Miles Laboratories Inc., Elkhart, Ind.) were dissolved directly into medium in concentrations as stated in Results and in Table I.

After incubation, cultures were routinely fixed for 30 min in 2% paraformaldehyde, 2.5% glutaraldehyde in cacodylate buffer (0.1 M), and postfixed in 1% osmium tetroxide in 0.1 M cacodylate buffer, pH 7.4, at 4°C for 30-60 min. They were stained en block in 1% uranyl acetate, dehydrated, and embedded in Spurr (D. E. R. 736 embedding kit, Tousimis Research Corp., Rockville, Md.).

Myosin S-1 was prepared by the method of Margossian and Lowey (see reference 14) and stored at a concentration of 30 mg/ml in 50% glycerol. Epithelia were extracted with Triton X100 in 0.05-0.1% in 0.1 M PIPES buffer (Sigma Chemical Co.), 2 mM MgCl₂, 2 mM EGTA, 1 mM phenylmethyl sulfonyl fluoride (PMSF), 1 mM *p*-tosyl-L-arginine methyl ester-HCl. S-1 decoration was performed in 0.3 M PIPES buffer containing S-1 fragments, 0.5-1 mg/ml, for 10 min at room temperature. Fixation was carried out subsequently in 1% glutaraldehyde with 0.2% tannic acid (Mallinckrodt, St. Louis, Mo., batch AR 1764) in 0.1 M PIPES buffer (14) for 30 min. After thorough washing, tissue was postfixed in 1% OsO₄ in 0.1 M phosphate buffer, pH 6.0, for 30 min at 4°C, and prepared as described above for electron microscopy.

RESULTS

The avian corneal epithelium at 9-11 d of embryonic development is two to three cells thick. The cells of the basal layer are cuboidal to columnar in shape and possess extensive rough endoplasmic reticulum and prominent Golgi complexes (1). The cells of the middle layer are round in shape and the

FIGURE 1 The tissues shown in Figs. 1-7 were routinely fixed and processed for electron microscopy. Fig. 1 is a micrograph of the basal cytoplasm of a corneal epithelial cell fixed *in situ*. The basal cortex contains a mat (parentheses) of microfilaments (*mf*) running close to the plasmalemma and parallel to the basal lamina (*bl*). The cytoplasm is rich in granular endoplasmic reticulum at this stage (10 d of incubation). Mitochondrion (*m*), nucleus (*n*), collagen fibrils in underlying corneal stroma (*cf*). Bar, 0.5 μ m.

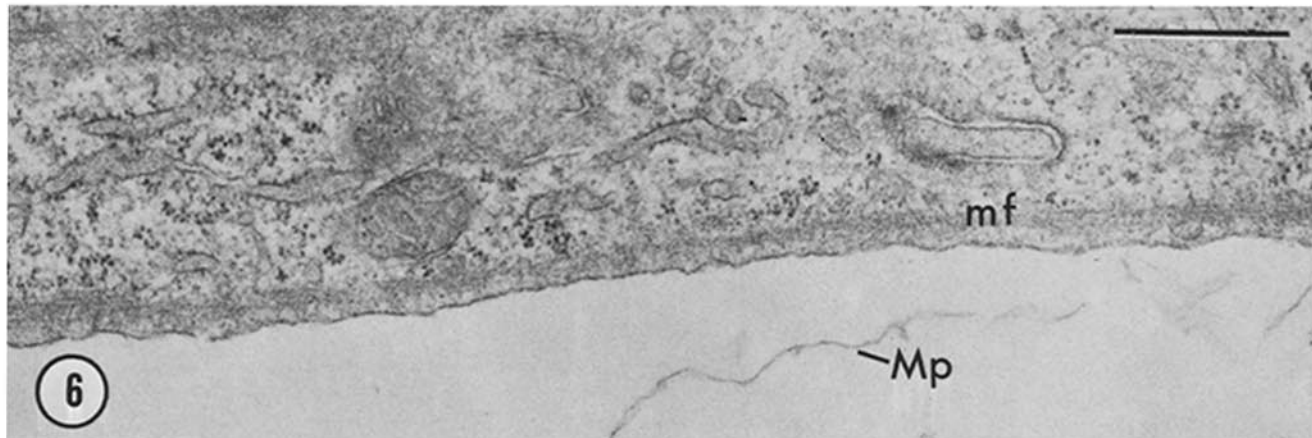
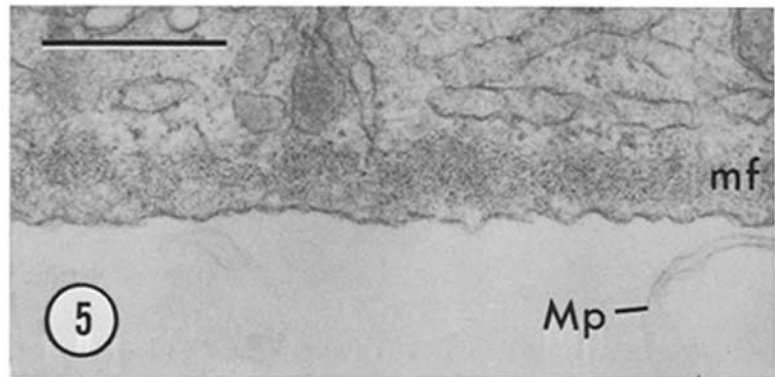
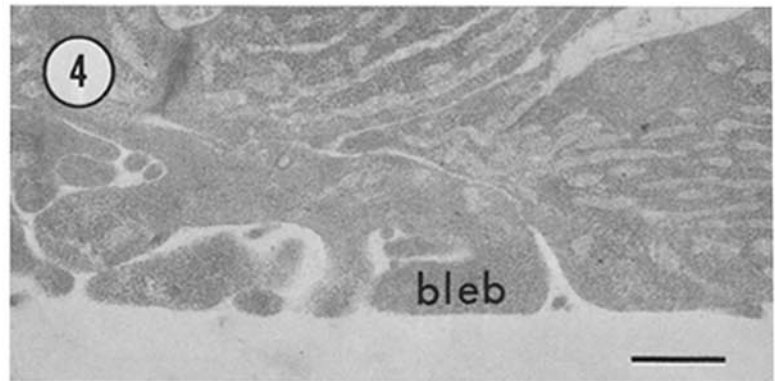
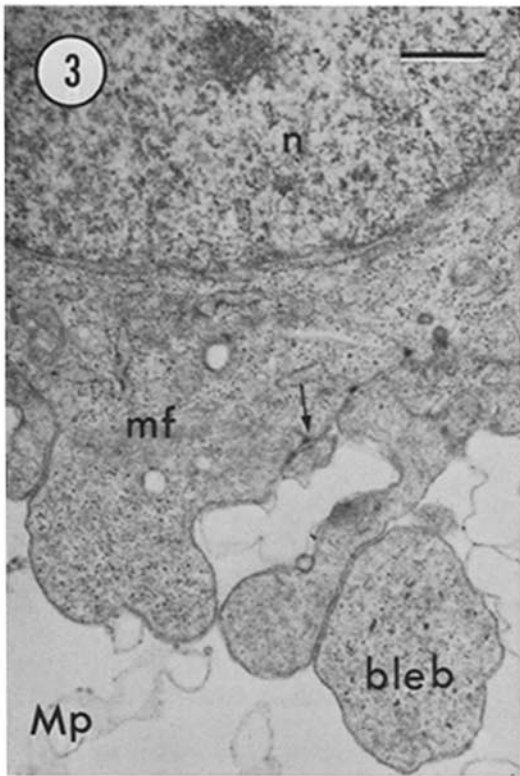
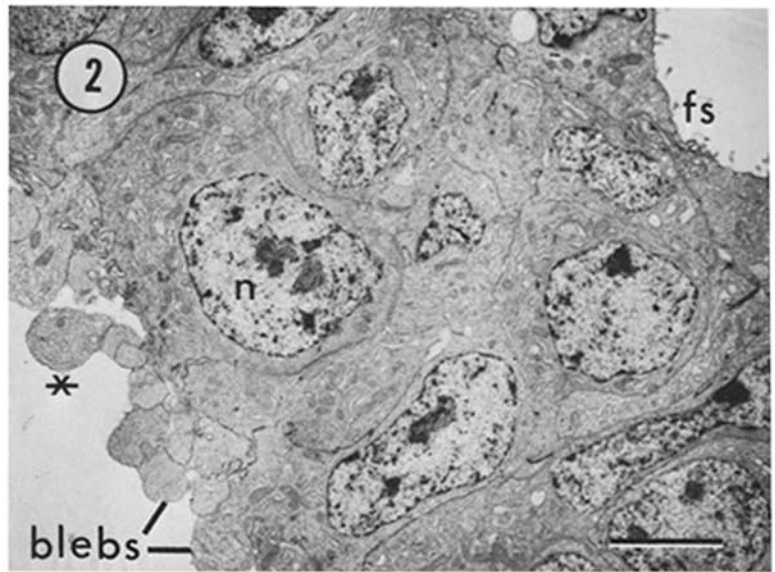
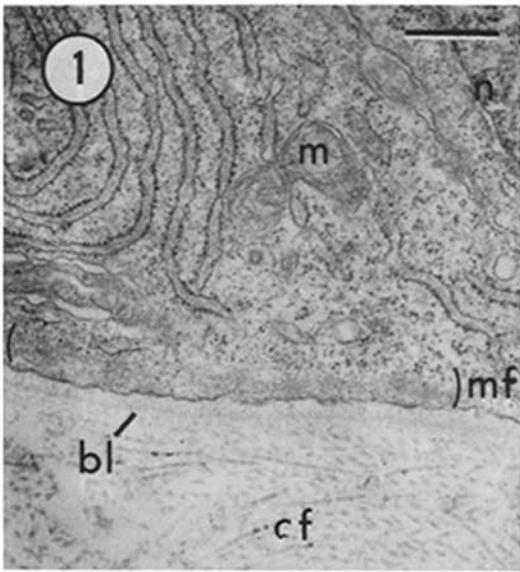
FIGURE 2 The basal surface of the corneal epithelium begins to bleb as soon as the basal lamina is removed. The blebs vary in size and contain filamentous ground substance, ribosomes, and even mitochondria and endoplasmic reticulum (as in the bleb labeled by the asterisk). Neither trypsin-collagenase nor EDTA in the concentrations used here affects the free surface (*fs*), which possesses microvilli and a surface coat. Lateral cell surfaces and junctions also appear unaffected by the treatment. Nucleus (*n*). Bar, 5 μ m.

FIGURE 3 The blebs disrupt the basal cytoskeleton. Microfilaments (*mf*) attached to lateral cell junctions (as at the arrow) may persist, but the cytoplasm extending into the blebs appears unorganized. The enzyme-isolated epithelium shown here was incubated for 6 h on a Millipore filter (*Mp*) before fixation in aldehydes and osmium tetroxide. Nucleus (*n*). Bar, 0.5 μ m.

FIGURE 4 The blebs persist even when isolated epithelia are grown on inert solid substrata such as plastic (shown here) or glass. The blebs are flattened slightly where attached to the plastic. The plastic itself was removed after embedding the tissue. Bar, 0.5 μ m.

FIGURE 5 Isolated epithelia grown on Millipore filters in media containing solubilized collagens lose their blebs as early as 2 h after collagen is added to the medium. The specimen shown here was treated for 4 h with 100 μ g/ml of type IV collagen. The epithelium is flattened and stretches across a pore in the filter. Millipore filter (*MP*), reorganized basal microfilaments (*mf*). Bar, 0.5 μ m.

FIGURE 6 The isolated epithelium shown here was treated with α 2(I) chains (100 μ g/ml) for 6 h. The basal epithelial surface has flattened and reorganized the cortical microfilamentous mat (*mf*). The unsupported basal surface spans distances of 1-2 μ m without filter contact. Millipore filter (*Mp*). Bar, 0.5 μ m.



outermost cells (periderm) are flat. The cells are connected laterally by desmosomes, which are increasing in number at this time, and they are joined at the apical surface by typical junctional complexes (1). The free surface of the periderm is covered with microvilli and a glycoprotein surface coat.

The basal surface of the epithelium is smooth and follows the contour of the basal lamina (Fig. 1). Just above the basal plasma membrane, the cortical cytoskeleton forms a dense mat, 150 nm wide, consisting of numerous microfilaments (*mf*, Fig. 1) running parallel to the plasma membrane. Beneath the basal lamina is a cell-free zone of collagen fibrils (cf, Fig. 1), rich in proteoglycan and fibronectin. Below this are the fibroblasts and orthogonally arranged collagen fibrils of the stroma proper, and, below them, the corneal endothelium.

Epithelia isolated with trypsin-collagenase (Fig. 2) or EDTA exhibit dramatic blebbing of their basal surfaces upon removal of the basal lamina. The blebs persist indefinitely when epithelia are grown on Millipore filters (Fig. 3). The blebs take the form of cytoplasm-filled protrusions from the basal epithelial surface. Their plasmalemma is completely free of visible extracellular material (Figs. 2 and 3). They contain ribosomes (Fig. 3), filaments, and even mitochondria and endoplasmic reticulum (Fig. 2). These protrusions on living cells are not to be confused with empty membrane-bounded blisters (see reference 15), which are most likely an artifact of aldehyde fixation (16).

The cytoskeletal elements associated with the lateral and apical surfaces of isolated epithelia appear unaffected by the treatment, but the microfilamentous cortical mat is partly or completely dispersed. The general configuration of the apical and lateral surfaces of blebbing epithelia (Fig. 2) appears essentially normal. The cells remain connected by desmosomes, and cytoplasmic organelles also seem little affected.

On Millipore filters (Fig. 3), the blebs move into the pores (for a distance of up to 20 μ m). Such epithelia continue to bleb indefinitely in ECM-free media. Isolated epithelia also continue to bleb on solid glass or plastic substrata (Fig. 4), even though attached to the substratum. Coating the dish with polylysine does not cause the blebs to disappear. On solid substrata composed of collagen, however, epithelia quickly withdraw the blebs (8).

To test the hypothesis that collagen molecules themselves interact with the basal epithelial surface, we added solubilized collagen to the medium of isolated epithelia grown on Millipore filters. Within 2–4 h, such epithelia withdraw their blebs from the pores of the filter, reorganize their basal cytoskeleton, and become flattened. Types I, II, and IV (Fig. 5) all have similar activities (Table I). Although some effect is observed at a concentration of 50 μ g/ml, collagen proved to be most effective at concentrations of 100 μ g/ml. Because heat-denatured type I collagen was as effective as intact molecules, we added purified α 2(I) chains to the culture medium. α 2(I) chains were also quite effective, at a concentration of 100 μ g/ml, in inducing blebbing epithelia to reorganize the cortical cytoskeleton (Fig. 6 and Table I). The reorganized basal microfilamentous mat (*mf*, Figs. 5 and 6) is similar to that observed *in situ* (Fig. 1). Because the flattened surface of the basal epithelial cells bridges the pores of the filter, and because no basal lamina reforms (Figs. 5 and 6), it seems likely that it is the cortical cytoskeleton that supports the cells.

The idea that physical support from the substratum is not required for maintenance of the cortical cytoskeleton derives from the following considerations. Although the effective pore

size of the Millipore filter is 0.45 μ m, the actual space between cellulose acetate strands facing the basal epithelial surface ranges from 1–2 μ m. The flattened epithelial surface spanning these spaces thus must be supported by the cytoskeleton in response to collagen molecules on the cell surface. To examine whether or not collagen affected the epithelia by binding to the filter rather than the cells, epithelia were placed on filters that had been previously immersed in collagen solution and then rinsed in Hanks' solution. Such epithelia continued to bleb. To demonstrate further that the reorganization of the basal epithelial surface is independent of attachment to a physical substratum, epithelia were placed on polylysine-coated Millipore filters with apical surface facing the filter and then were grown basal-side down across holes 2.3 mm Diam

TABLE I
Response of Basal Surface to ECM

Substance μ g/ml	Hours after treatment				
	1	2	4	6	12–48
Collagen I					
10–20	0/2*	0/2	0/2	0/5	1/2
50	—	1/2	—	2/4	—
100	0/3	3/4	2/2	6/7	4/4
200	0/3	—	—	—	2/2
Collagen II					
10	0/2	—	0/2	1/5	—
50	0/2	1/3	2/3	2/3	—
100	1/4	2/3	4/5	4/4	—
200	0/4	—	3/3	—	—
Collagen IV					
10–20	0/2	—	0/2	0/4	1/2
50	0/2	—	1/2	3/4	—
100	—	2/4	3/3	2/2	—
200	—	2/2	—	2/2	—
α 2(I) chains					
50	—	0/2	—	1/4	—
100	—	1/3	—	3/4	—
Cellular fibronectin					
10	—	0/3	—	0/6	—
25	—	0/3	—	3/6	2/2
50	0/2	0/7	1/4	7/9	8/8
100	—	0/3	—	2/2	—
Plasma fibronectin (CIG) 50	—	0/4	—	4/5	—
Laminin					
0.5	—	0/2	0/2	1/2	1/2
1	—	2/4	2/3	3/3	—
5	—	1/3	3/3	2/2	2/2
10	0/2	1/4	3/3	3/3	—
20	—	0/4	—	2/2	—
F-12, no serum	—	0/6	—	0/8	0/6
F-12, 10% serum	—	0/3	—	0/4	0/4
F-12 10% serum without CIG	0/4	0/7	0/3	1/18	0/11
Hyaluronic acid 250	—	—	—	0/8	—
Chondroitin sulfate 250	—	—	—	0/4	0/4
Heparan sulfate 250	—	—	0/4	0/5	—
Heparin 250	—	—	—	0/3	—
Serum albumin 250	—	—	—	0/3	—
IgG 250	—	—	—	0/4	—

* The number of epithelia that flattened after the treatment indicated is given as the numerator, and total number of experiments as the denominator. An epithelium is only scored as positive after examination of the entire basal surface.

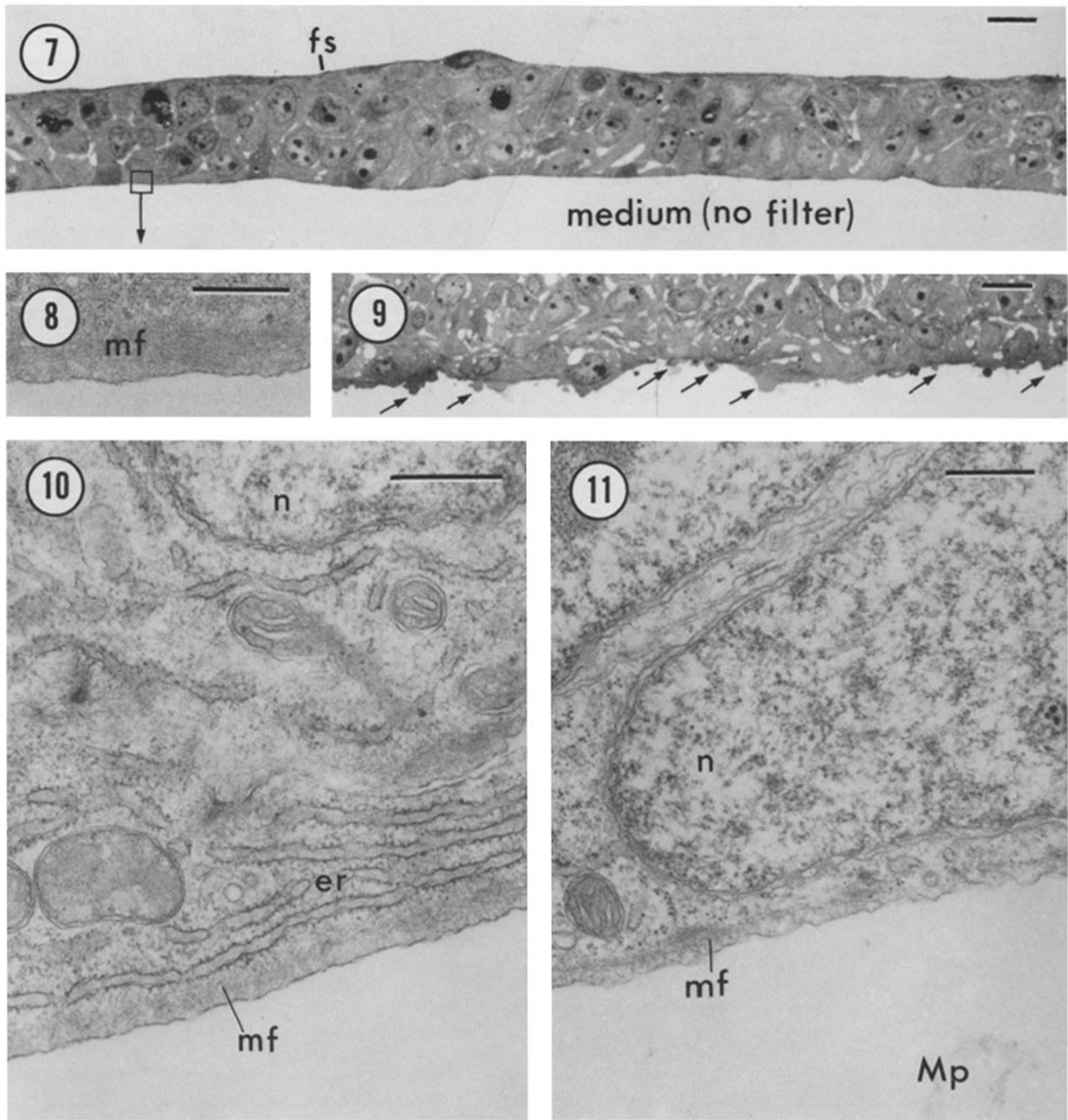


FIGURE 7 The epithelium shown on this light micrograph was cultured for 6 h without any filter support for the stretch of basal surface depicted. The medium contained 100 $\mu\text{g/ml}$ type I collagen. The basal surface has flattened without any physical substrate. Free surface (Fs) square, area shown in Fig. 8. Bar, 10 μm .

FIGURE 8 This electron micrograph shows the area depicted by the square in Fig. 7. The basal surface is flat and no polymerized material can be resolved on the surface. Microfilamentous mat (mf). Bar, 0.5 μm .

FIGURE 9 This light micrograph shows an isolated epithelia suspended as in Fig. 7 for 6 h over media without basal filter support. In this case, no extracellular molecules were added. The basal surface is decorated with numerous blebs (arrows). Bar, 10 μm .

FIGURE 10 Isolated epithelium grown on Millipore filters in the presence of laminin in the medium reorganizes its microfilamentous basal cortical mat (mf) and appears flat in 4-6 h. The specimen shown here was treated for 6 h with 1 $\mu\text{g/ml}$ of laminin. The blebs have retracted. Nucleus (n), endoplasmic reticulum (er). Bar, 0.5 μm .

FIGURE 11 Isolated epithelium cultured in the presence of 50 $\mu\text{g/ml}$ fibronectin flattened after 6 h of incubation and the microfilamentous mat (mf) reorganized. Nucleus (n); Millipore filter (Mp). Bar, 0.5 μm .

(see Materials and Methods). The basal side thus had no physical support over most of its surface and, again, no detectable ECM polymerized on the basal surface (Fig. 8). Yet, these epithelia flattened in response to soluble collagen molecules in medium (Figs. 7 and 9).

We next asked whether or not other molecular components of the basal lamina, if added in soluble form to isolated epithelia on Millipore filters, would also cause the cortical cytoskeleton to organize. When the blebbing epithelia are confronted with laminin or fibronectin, they respond by withdrawing the blebs and flattening the basal surface (Figs. 10 and 11). It is possible to observe the effect even if epithelia are suspended across 2.3-mm holes as described above (Fig. 7). Laminin at concentrations of 1–10 $\mu\text{g}/\text{ml}$ causes epithelia to withdraw blebs from the filters by 2–4 h. At higher concentrations ($>20 \mu\text{g}/\text{ml}$), laminin is not so effective (Table I) and at 100 $\mu\text{g}/\text{ml}$ laminin precipitates from solution.

Fibronectin, in concentrations as low as 50 $\mu\text{g}/\text{ml}$, is effective after 6 h of incubation, but fibronectin does not appear to affect the epithelium at earlier intervals, regardless of the concentration (Table I). Interestingly, although we used CIG-free serum or omitted serum for the experiments, we found that whole fetal calf serum has no effect on blebbing (Table I). The CIG content of commercial serum presumably is too low, because plasma fibronectin is as effective as cellular fibronectin in causing the epithelial surface to flatten (Table I).

Earlier studies demonstrated that the isolated corneal epithelium responds to GAG by increasing its synthesis of GAG (17). To examine the effect of such macromolecules on the blebbing morphology of epithelia, we confronted isolated epithelia on Millipore filters with HA, CS, HS, and heparin. None of the molecules at concentrations of 250 $\mu\text{g}/\text{ml}$ had any effect on the blebbing (Table I).

To examine whether or not the flattening of the basal surface is a nonspecific effect of protein binding randomly to the epithelium, we cultured isolated epithelia on Millipore filters in the presence of bovine serum albumin or IgG (nonspecific rabbit). The epithelia exhibited no response to the addition of these molecules at concentrations of 250 $\mu\text{g}/\text{ml}$ (Table I).

To investigate further the dramatic effects of ECM molecules

on the basal cytoarchitecture of epithelial cells, we decorated the actin filaments with S-1 subfragments of heavy meromyosin. The *in situ* basal epithelial cell (Fig. 12) on basal lamina has a microfilamentous cortical mat composed of a meshwork of actin filaments. Actin filaments can be seen branching out from this meshwork both downward, toward the basal plasma membrane, and upward, intermingling with or attaching to the secretory organelles and ribosomes (Fig. 12). There does not seem to be a unidirectional polarity to the actin filaments in the cortical mat; they resemble stress fibers (14) in this regard. Actin filaments approaching the basal plasma membrane mainly point away from the membrane, as observed in a number of other cells (14).

In striking contrast to their organization *in situ*, the basal microfilaments of isolated epithelia are dispersed into a meshwork in the blebs (Figs. 13–15). The decorated actin filaments frequently approach the plasma membrane and occasionally there appears an electron-dense plaque at the putative insertion site (*dp*, Fig. 14). Interestingly, the polarity of the decorated filaments “inserting” into the plasmalemma is often with arrowheads pointing toward the membrane (Fig. 13 *inset* and Fig. 14). Filaments may also run parallel to the plasmalemma in the blebs (Fig. 15).

If such a blebbing epithelium is confronted with collagens, laminin, or fibronectin, incubated for 6 h, then extracted with detergent and decorated with S-1, a morphology results that is remarkably similar to that seen in the *in situ* epithelium. The actin-rich microfilamentous mat reorganizes (Fig. 16). Filaments descending from the actin complex to approach the basal plasma membrane again more often point away from the membrane. It is difficult to discern the exact relation of actin filaments to the plasmalemma because of extraction and swelling caused by the detergent treatment.

DISCUSSION

The present study demonstrates that embryonic corneal epithelial cells are capable of interacting with, and responding to, three classes of extracellular matrix glycoproteins in soluble form, namely, collagens, laminin, and fibronectin (Fig. 17). In

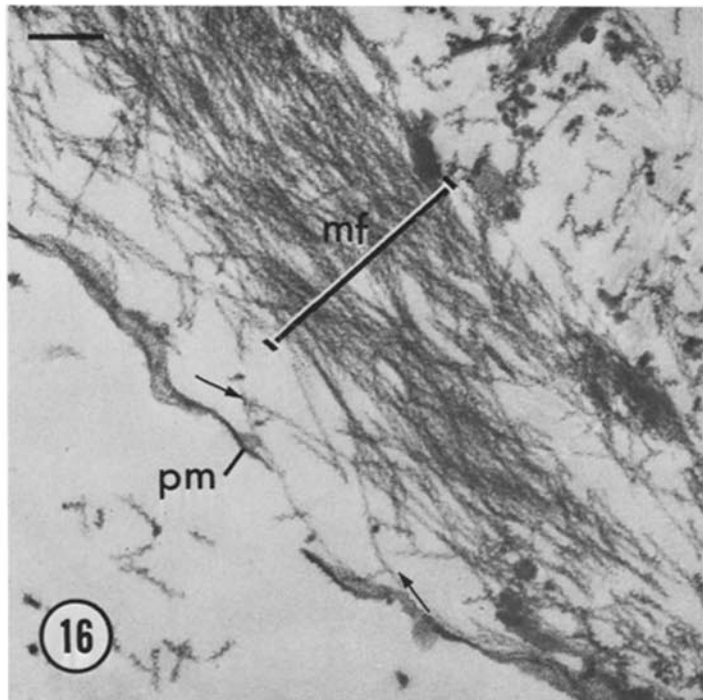
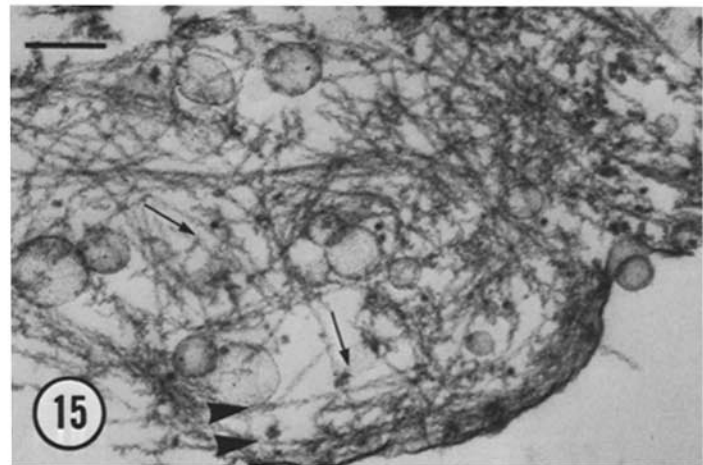
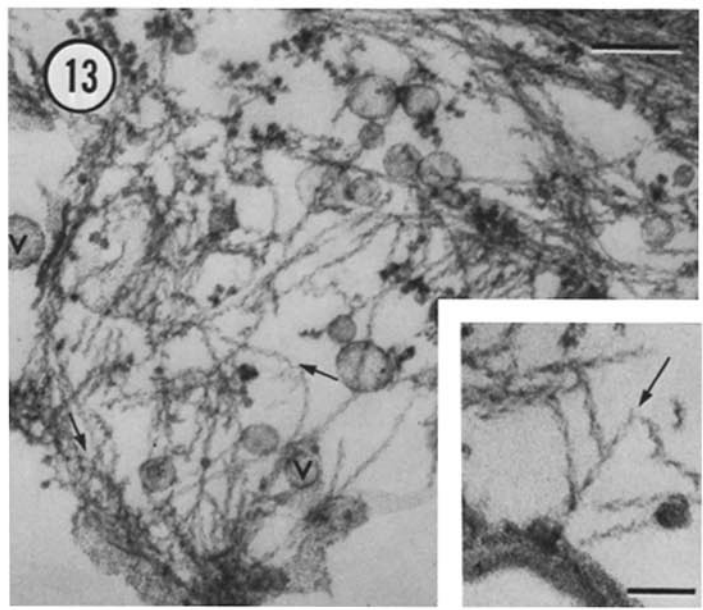
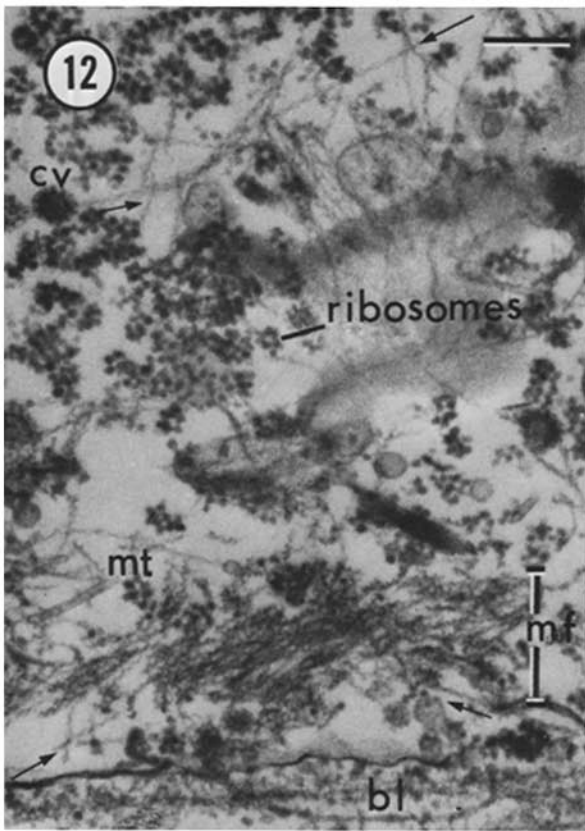
FIGURE 12 The tissues depicted in the micrographs in Figs. 8–12 were detergent extracted and treated with S-1 fragments of heavy meromyosin before fixation in aldehydes, tannic acid, and osmium tetroxide. Fig. 12 shows a section of basal epithelial cytoplasm *in situ*. The microfilamentous basal cortical mat (*mf*) appears swollen due to the detergent treatment. It is well labeled with S-1 fragments, and the arrowheads may point in opposite directions (small arrows). Above the mat, ribosomes seem to be suspended on actin filaments. Coated vesicle (*cv*), microtubule (*mt*), basal lamina (*bl*). Bar, 0.2 μm .

FIGURE 13 In a typical bleb, the direction of the S-1 decoration on actin filaments may appear random (small arrows, *main figure*). The arrowheads, however, often point toward the plasmalemma (small arrow, *inset*). The detergent-treated preparations contain vesicles (*v*) inside or outside of the cells that derive from the solubilized membranes. All the blebs shown in Figs. 9–12 were on the undersurface of enzyme-isolated epithelia grown for 6 h on Millipore filters. Main figure: bar, 0.2; *inset*: bar, 0.1 μm .

FIGURE 14 In the bleb depicted here, several actin filaments are decorated with arrowheads pointing toward the plasmalemma (small arrows). Some appear to insert in a dense plaque (*dp*) in the membrane. The cytoplasm above the bleb shown here is rich in intermediate-sized filaments (*if*), which are associated with nearby desmosomes (not shown). Bar, 0.2 μm .

FIGURE 15 In the bleb depicted here, some actin filaments (large arrowheads) are running parallel to the plasmalemma. Others are running in a perpendicular direction (small arrows). This is a small bleb, which may be partly retracting into the cell. Bar, 0.2 μm .

FIGURE 16 After addition of solubilized collagens, laminin, or fibronectin, the organized actin complex (*mf*) that comprises the basal cytoskeleton re-forms. The epithelium depicted here was treated for 6 h with type IV collagen in solution (100 $\mu\text{g}/\text{ml}$). The section is slightly tangential to the poorly preserved plasmalemma (*pm*). This plane of section and the detergent treatment tend to exaggerate the distance between the cortical mat (*mf*) and the plasmalemma (*pm*). Actin filaments (small arrows) may be oriented in opposite directions. S-1 debris is present in the space (lower left) between the epithelium and filter. Bar, 0.2 μm .



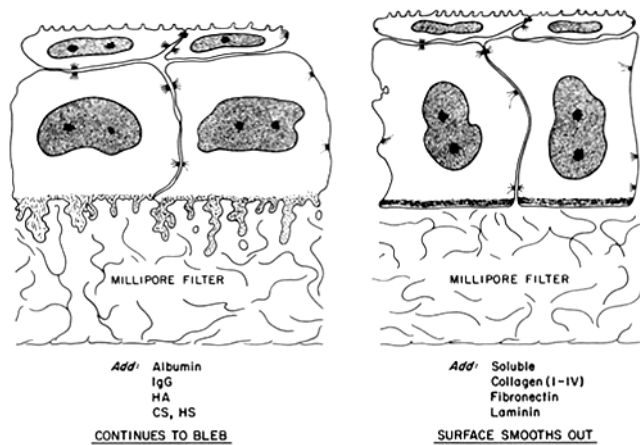


FIGURE 17 Diagram summarizing the effects of several different molecules on the organization of the basal corneal cell surface. The basal surface blebs when the basal lamina is removed by EDTA or enzyme treatment, and this blebbing persists (left) on Millipore filters in the presence of nonmatrix proteins or GAG. Soluble collagens, fibronectin, and laminin added to the medium (right) cause the blebs to retract and the cytoskeleton to reform the basal mat of actin filaments.

response to removal of the glycoprotein-rich basal lamina from the basal surface, the epithelial cells send out numerous round or irregularly shaped cytoplasm-filled protrusions (blebs). Staining of the basal cytoskeleton of the blebbing epithelial cells with S-1 fragments of heavy meromyosin shows that the actin-rich cortical microfilamentous mat, which characterizes the basal cells *in vivo*, is disrupted and that actin filaments flow out into the cell protrusions during blebbing. The epithelia continue to bleb when grown on filters in the presence of IgG, albumin, and GAG (Fig. 17). We also demonstrate that inert solid substrata do not cause retraction of the blebs. We found in this study that solubilized collagens are just as effective as solid collagenous substrata in causing the epithelial surface to flatten and the basal cytoplasm to reorganize. Soluble laminin and fibronectin also cause the basal epithelial surface to resume its *in situ* appearance (Fig. 17). The results suggest that these ECM molecules themselves interact with the basal cell surface, because it is clear that they need not be polymerized into solid substrata to exert their effect on epithelial cytoarchitecture. In the discussion, we will consider the effects of each of the glycoproteins separately, and then speculate on the possible interactions that might occur among these molecules, the cell surface, and the cytoskeleton.

Any type of solid collagenous substratum, including lens capsule (type IV collagen) and collagen gels (type I or type II collagen), induces flattening of the blebbing basal surface of enzyme- or EDTA-isolated corneal epithelium (8, 9, 18). In the present study, a similar nonspecificity was observed. Types I, II, and IV collagens in solution are capable of inducing blebbing epithelia to retract basal cytoplasmic protrusions within 2 h (at concentrations of 100 $\mu\text{g}/\text{ml}$). Heat-denatured type I collagen and purified $\alpha 2(\text{I})$ collagen chains seem to be as effective as undenatured collagen in eliciting the recovery of basal cytoarchitecture. These results indicate that the epithelial-ECM interaction is not dependent on the native helical configuration and is mediated by components of collagen structure that the various molecules share.

Rubin et al. (19) have recently reported that rat hepatocytes attach equally well to all collagens tested (types I-V), as well

as to denatured collagen, $\alpha 1(\text{I})$ chains, cyanogen bromide peptides, and collagenlike synthetic peptides (albeit less efficiently). Epidermal cells show a preference for adhering to type IV collagen, although there is significant attachment to type I collagen (20). A preference for type IV collagen was also demonstrated in mammary gland epithelium attachment *in vitro* (21). Thus, hepatocytes resemble corneal epithelial cells in the nonspecificity of their requirement for collagen, whereas certain epithelia seem to be more demanding.

It is important to point out, however, that our study is not an attachment assay. Indeed, we showed that attachment to a physical substratum is not necessary for the epithelial basal surface to interact with and respond to ECM molecules. Blebbing epithelia suspended over holes 2.3-mm Diam flatten in response to soluble ECM molecules. Additional evidence that the corneal epithelial surface can interact with ECM molecules in soluble form is as follows. Filters presoaked in collagen and washed briefly do not have an effect on the basal epithelial surface. Indeed, if the epithelium had attached to the filter via filter-bound collagen, the epithelium would have followed the contour of the filter pores, rather than spanning the pores. Moreover, neither basal lamina, collagen fibrils, nor other visible ECM polymerizes on the basal epithelial surface during the 2- to 6-h period in which the basal surface flattens.

Laminin may be involved in the attachment to collagen of epidermis, mammary and yolk sac epithelia, and several epithelial cell lines that prefer type IV collagen (20-22), whereas endothelial cells (23), lens epithelium (24), and some hepatocytes (25) seem to use fibronectin to attach to collagen. Therefore, we also studied the effects of these two molecules on the basal epithelial surface. We found that both laminin and fibronectin in soluble form have a dramatic effect on the corneal epithelial basal surface. Laminin in very low concentrations eliminates blebbing in 2-4 h, but both cellular and plasma fibronectin require 4-6 h to begin to flatten the basal cell surface. The effect of laminin, then, is as fast as the effect of collagen, but the effect of fibronectin is somewhat delayed.

It will be of considerable interest to determine the interrelationships, if any, among these ECM molecules during their interaction with the basal epithelial surface. The corneal epithelium synthesizes types I and II collagens (26) and probably also type IV (18). In all likelihood, this epithelium also synthesizes laminin and fibronectin; both glycoproteins are present in the corneal basement membrane. Therefore, the possibility exists that the effect of exogenous collagen and laminin on epithelial blebbing, even though very rapid, uses an endogenous source of one or the other ECM molecules. Terranova et al. (27) demonstrated that certain epithelial cells in the presence of a protein synthesis inhibitor, cycloheximide, fail to adhere to collagen unless exogenous laminin is added. In preliminary studies, however, we find that cycloheximide does not inhibit the effect of either collagen or laminin on corneal epithelial blebbing but does abolish the effect of fibronectin, as do inhibitors of collagen secretion (28). The simplest explanation for our data is that collagen and laminin are capable of independent, direct interaction with the epithelial cell surface to affect the basal cytoskeleton, whereas fibronectin interacts indirectly (e.g., via collagen).

It is tempting to believe that these effects on cell surface and cytoskeletal morphology are mediated via receptors for one or more of these ECM molecules. Kleinman et al. (29) suggested a ganglioside type receptor for fibronectin, which then indirectly binds to collagen; however, fibronectin binding to cells is increased by adding soluble collagen (30). Goldberg (31)

reported that a receptor for collagen itself exists on the cell surface of fibroblasts. When he added labeled type I collagen to the top of epithelial sheets, it failed to bind, suggesting that there is not such a receptor site on the epithelial cell surface (31). Because epithelial cells on culture dishes are polarized with the apical surface up, however, basal surfaces would not have been exposed to collagen in Goldberg's studies. The binding site on the collagen molecule for the fibroblast cell surface is in the helical portion of $\alpha 1$ and $\alpha 2$ chains and seems to be determined by primary structure rather than helical conformation (31). This type of binding would be consistent with the results of the present study, because both collagen chains and heat-denatured collagens interact with corneal epithelial cells.

More recently, Rubin et al. (19) have hypothesized that the attachment of rat hepatocytes to collagen, a process that does not require fibronectin (32, 33), is mediated by receptors that recognize multiple repeating sites (e.g., Gly-Pro-Hyp) along the collagen molecule. They suggest that these receptors have a low affinity for collagen and are mobile in the plane of the cell membrane. The tertiary structure of the collagen molecules may also be important, because native collagen substrates promote hepatocyte attachment better than denatured collagen or synthetic collagenlike substrates (19).

Regardless of the possible mode of interaction of collagen, laminin, and fibronectin with the cell surface, either via independent receptors or by means of molecular complexes, the end result observed in the present study is a dramatic effect on the basal epithelial cytoskeleton. In the presence of solubilized ECM, the basal cytoskeleton is composed of organized actin filaments in parallel array, some of which may attach directly or indirectly (34) to the cell membrane. The arrangement is unlike the relationship of polymerized fibronectin to actin cables in cultured fibroblasts (35), in that whole actin bundles do not appear to insert into the plasmalemma.

The blebs, which form instantly on the basal epithelial surface when basal lamina is removed, contain a disorganized meshwork of actin filaments and are undoubtedly mobile because they move into the pores of filters. Interestingly, individual actin filaments in the blebs may decorate with S-1 fragments in a pattern pointing toward the cell membrane, whereas in microvilli the pattern usually points away from the plasmalemma (14). For an actin-myosin sliding mechanism to work, the actin pattern should point to the membrane if processes are moving away from the cell (15, 36), as seems to be the case for the blebs. Further work is necessary, however, to establish that an actin-myosin interaction occurs in the blebs.

The functional implications of the structural organization of the basal epithelial surface have received little attention in the past, even though blebs have been reported to form on a number of different types of epithelia when isolated by EDTA or trypsin (see references 5, 37, and 38). Our study suggests that one effect of ECM molecules may be to immobilize basal plasmalemmal receptors and cytoskeleton. Along these lines, it is interesting to note that certain malignant epithelial cells, after digesting the basal lamina, become mobile and invade the underlying spaces (39). Another effect, directly or indirectly related to the organization of the cytoskeleton, is the effect of ECM molecules, such as collagen, on epithelial polarity (40), cell shape, and metabolism (9). The present study calls attention to the role of ECM in controlling the organization of the basal epithelial cytoplasm and invites further study of its structurally important and potentially motile cytoskeletal elements.

We thank Dr. George Martin for samples of purified type IV collagen, $\alpha 2(I)$ chains, and laminin; Dr. Richard Hynes for fibronectin; and Dr. Thomas Linsenmayer for type II collagen. We also thank Drs. David Begg and David Albertini for supplying S-1 fragments and for help in using them, Ms. Deborah Almeida and Ms. Nancy Pollack for their technical assistance, and Ms. Susan Hunt for her secretarial talents.

This research was supported by a National Institutes of Health grant, HD-00143, to Dr. Hay. The laboratories supplying purified ECM components were supported by CA-17007 (Dr. Hynes), EY-02261 (Dr. Linsenmayer), and the National Institute of Dental Research (Dr. Martin).

Received for publication 23 April 1981, and in revised form 16 June 1981.

REFERENCES

- Hay, E. D., and J. P. Revel. 1969. Fine Structure of the Developing Avian Cornea. S. Karger, Basel. 144 pp.
- Trelstad, R. L., K. Hayashi, and B. P. Toole. 1974. Epithelial collagens and glycosaminoglycans in the embryonic cornea. Macromolecular order and morphogenesis in the basement membrane. *J. Cell Biol.* 62:815-830.
- Hay, E. D., and S. Meier. 1974. Glycosaminoglycans synthesis by embryonic inductors: neural tube, notochord and lens. *J. Cell Biol.* 62:889-898.
- Farquhar, M. G. 1981. The glomerular basement membrane: a selective macromolecular filter. In *Cell Biology of Extracellular Matrix*. E. D. Hay, editor. Plenum Press, New York. In press.
- Banerjee, S. D., R. H. Cohn, and M. R. Bernfield. 1977. Basal lamina of embryonic salivary epithelia: production by the epithelium and role in maintaining lobular morphology. *J. Cell Biol.* 73:445-463.
- Hay, E. D., T. L. Linsenmayer, R. L. Trelstad, and K. von der Mark. 1979. Origin and distribution of collagens in the developing avian cornea. *Curr. Top. Eye Res.* 1:1-35.
- Hay, E. D. 1973. Origin and role of collagen in the embryo. *Am. Zool.* 13:1085-1107.
- Dodson, J. W., and E. D. Hay. 1974. Secretion of collagen by corneal epithelium. II. Effect of the underlying substratum on secretion and polymerization of epithelial products. *J. Exp. Zool.* 189:51-72.
- Meier, S., and E. D. Hay. 1974. Control of corneal differentiation by extracellular materials. Collagen as a promoter and stabilizer of epithelial stroma production. *Dev. Biol.* 38:249-270.
- Linsenmayer, T. F., and M. J. C. Hendrix. 1980. Monoclonal antibodies to connective tissue macromolecules: type II collagen. *Biochem. Biophys. Res. Commun.* 92:440-446.
- Timpl, R., G. R. Martin, P. Bruckner, G. Wick, and H. Wiedemann. 1978. Nature of the collagenous protein in a tumor basement membrane. *Eur. J. Biochem.* 84:43-52.
- Timpl, R., H. Rohde, P. G. Robey, S. I. Rennard, J. M. Foidart, and G. R. Martin. 1979. Laminin-A glycoprotein from basement membranes. *J. Biol. Chem.* 254:9933-9937.
- Hynes, R. O., A. T. Destree, M. E. Perkins, and D. D. Wagner. 1979. Cell surface fibronectin and oncogenic transformation. *J. Supramol. Struct.* 11:95-104.
- Begg, D. A., R. Rodewald, and L. I. Rebhun. 1978. The visualization of actin filament polarity in thin sections. Evidence for uniform polarity of membrane-associated filaments. *J. Cell Biol.* 79:846-852.
- Trinkaus, J. P. 1980. Formation of protrusions of the cell surface during tissue cell movement. In *Tumor Cell Surfaces and Malignancy*. R. O. Hynes, and C. F. Fox, editors. Alan R. Liss, Inc., New York. 887-906.
- Hasty, D. L., and E. D. Hay. 1978. Freeze-fracture studies of the developing cell surface. II. Formation of particle-free membrane blebs during glutaraldehyde fixation. *J. Cell Biol.* 78:756-768.
- Meier, S., and E. D. Hay. 1974. Stimulation of extracellular matrix synthesis in the developing cornea by glycosaminoglycans. *Proc. Natl. Acad. Sci. U. S. A.* 71:2310-2313.
- Hay, E. D., and J. W. Dodson. 1973. Secretion of collagen by corneal epithelium. I. Morphology of the collagenous products produced by isolated epithelia grow on frozen-killed lens. *J. Cell Biol.* 57:190-213.
- Rubin, K., M. Höök, B. Öbrink, and R. Timpl. 1981. Substrate adhesion of rat hepatocytes: mechanism of attachment to collagen substrates. *Cell.* 24:463-470.
- Murray, J. C., G. Stingl, H. K. Kleinman, G. R. Martin, and S. I. Katz. 1979. Epidermal cells adhere preferentially to type IV (basement membrane) collagen. *J. Cell Biol.* 80:197-202.
- Wicha, M., L. A. Liotta, S. Garbisa, and W. R. Kidwell. 1979. Basement membrane collagen requirements for attachment and growth of mammary epithelium. *Exp. Cell Res.* 124:181-190.
- Kleinman, H. K., R. J. Klebe, and G. R. Martin. 1981. Role of collagenous matrices in the adhesion and growth of cells. *J. Cell Biol.* 88:473-485.
- Gold, L. I., and E. Pearlstein. 1980. Fibronectin-collagen binding and requirement during cellular adhesion. *Biochem. J.* 186:551-559.
- Hughes, R. C., G. Mills, and Y. Courtois. 1979. Role of fibronectin in the adhesiveness of bovine lens epithelial cells. *Biol. Cell.* 36:321-330.
- Berman, M. D., J. G. Eaggoner, J. N. Foidart, and H. K. Kleinman. 1980. Attachment to collagen by isolated hepatocytes from rats with induced hepatic fibrosis. *J. Lab. Clin. Med.* 95:660-665.
- Linsenmayer, T. F., G. N. Smith, and E. D. Hay. 1977. Synthesis of two collagen types of embryonic chick corneal epithelium *in vitro*. *Proc. Natl. Acad. Sci. U. S. A.* 74:39-43.
- Terranova, V. P., D. H. Bohrbach, and G. R. Martin. 1980. Role of laminin in the attachment of PAM 212 (epithelial) cells to basement membrane collagen. *Cell.* 22:719-726.
- Sugrue, S. P., and E. D. Hay. 1981. Effect of protein synthesis inhibitors on interaction of epithelium with extracellular matrix. *Anat. Rec.* 199:249A. (Abstr.).
- Kleinman, H. K., G. R. Martin, and P. H. Fishman. 1979. Ganglioside inhibition of fibronectin-mediated cell adhesion to collagen. *Proc. Natl. Acad. Sci. U. S. A.* 76:3367-3371.
- Rennard, S. I., M. L. Wind, A. T. Hewitt, and H. K. Kleinman. 1981. Effect of collagen and cell shape on binding of fibronectin to cells. *Arch. Biochem. Biophys.* 206:205-212.
- Goldberg, B. 1979. Binding of soluble type I collagen molecules to the fibroblast plasma membrane. *Cell.* 16:265-275.
- Rubin, K., A. Oldberg, M. Höök, and B. Öbrink. 1978. Adhesion of rat hepatocytes to

- collagen. *Exp. Cell Res.* 117:165-177.
33. Rubin, K., S. Johansson, I. Pettersson, C. Ocklind, B. Öbrink, and M. Höök. 1979. Attachment of rat hepatocytes to collagens and fibronectin: a study using antibodies directed against cell surface components. *Biochem. Biophys. Res. Commun.* 91:86-94.
 34. Geiger, B., K. T. Tokuyasu, A. H. Dutton, and S. J. Singer. 1980. Vinculin, and intracellular protein localized at specialized sites where microfilament bundles terminate at cell membranes. *Proc. Natl. Acad. Sci. U. S. A.* 77:4127-4131.
 35. Singer, I. I. 1979. The fibronexus: a transmembrane association of fibronectin-containing fibers and bundles of 5 nm microfilaments in hamster and human fibroblasts. *Cell.* 16: 675-685.
 36. Pollard, T. D. 1977. Cytoplasmic contractile proteins. In *International Cell Biology, 1976-1977*. B. R. Brinkley and K. R. Porter, editors. The Rockefeller University Press, New York. 378-387.
 37. Dodson, J. W. 1967. The differentiation of epidermia. I. The interrelationship of epidermis and dermis in embryonic chicken skin. *J. Embryol. Exp. Morphol.* 17:83-105.
 38. Jensen, H. M., and N. K. Mottet. 1970. Ultrastructural changes in keratinizing epithelium following trypsinization, epidermal detachment and apposition to mesenchymes. *J. Cell Sci.* 6:511-535.
 39. Liotta, L. A., S. Abe, P. Gehron-Robey, and G. R. Martin. 1979. Preferential digestion of basement membrane collagen by an enzyme derived from a metastatic murine tumor. *Proc. Natl. Acad. Sci. U. S. A.* 76:2268-2272.
 40. Mauchamp, J., A. Margotat, M. Chambard, B. Charrier, L. Remy, and M. Michel-Bechet. 1979. Polarity of three-dimensional structures derived from isolated hog thyroid cells in primary culture. *Cell Tissue Res.* 204:417-430.