The Identification of Extracellular Matrix (ECM) Binding Sites on the Basal Surface of Embryonic Corneal Epithelium and the Effect of ECM Binding on Epithelial Collagen Production

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Abstract. Previously, we have shown that embryonic corneal epithelia can interact with, and respond to, soluble extracellular matrices (ECM) (laminin, collagen, and fibronectin). The basal surface of epithelia isolated free of the underlying ECM can be seen to be disrupted by numerous blebs that sprout from this formerly smooth surface. Laminin, collagen, or fibronectin added to the culture medium cause the epithelium to reorganize its cytoskeleton and flatten its basal surface. We show here that ECM molecules at concentrations that reorganize epithelial cytoskeletal morphology also increase the amount of collagen produced by the epithelial cells. However, molecules that do not reorganize basal epithelial morphology (con-

canavalin A, heparin, bovine serum albumin) have no effect on collagen production. We also report that fluorescently labeled laminin, collagen, and fibronectin, when added to the medium surrounding isolated corneal epithelia, bind to and flatten the basal epithelial cell surface. The binding site on the basal surface is protease sensitive and is specific for each ECM molecule. These results are compatible with the idea that the basal epithelial plasmalemma possesses a diverse population of binding sites for ECM that link cell surface matrix to the cytoskeleton, causing a dramatic cytoskeletal reorganization which in turn results in enhanced production of collagen by the cells.

EPITHELIAL cells exhibit highly polarized apical and basal cell surfaces. The apical surface expresses enzymes and other molecules that do not appear on the basolateral surface, whereas the basolateral surface is known to have sodium potassium ATPase, binding sites for hormones, and other molecules that do not occur on the apical surface (26). Epithelia confront the extracellular matrix (ECM)[1] only at their basal surfaces. From the work of Sabatini et al. (26), it seems that the contact of the epithelial cells with the substratum is one of the key events in the determination of the apical–basal polarity of the cells. In a study of hepatocytes attaching to collagen, Rubin et al. (25) postulated that receptors in the cell membrane for collagen are mobile and upon attachment to ECM, repolarize to the basal cell surface. Recently, specific laminin-binding, fibronectin-binding, and collagen-binding proteins have been reported for several cell types, as well as a heparan sulfate proteoglycan that seems to be membrane intercalated (for review see references 10, 28, 33).

We have been investigating the interaction of ECM components with embryonic corneal epithelium and the role of this interaction in the maintenance of epithelial cytoarchitecture and differentiation. During development of the eye, the avian corneal epithelium secretes the primary stroma, which

consists of an orthogonal array of striated fibrils containing several collagens, including types I and II (4, 8). If the corneal epithelium is removed from basal lamina and underlying stroma by EDTA or trypsin collagenase, the basal surface extends cell processes called blebs (4, 20). On collagenous substrata, the basal surface flattens, whereas it continues to bleb on bare Millipore filters. The epithelium cultured on lens capsule or collagen gel produces over twice as much collagen as does comparable epithelium grown on noncollagenous substrate, as measured by accumulation of radiolabeled collagen (20).

Recently, we have shown (29) that enzymatically or EDTA-isolated epithelial sheets are capable of interacting with soluble ECM (laminin, collagen, and fibronectin). Upon isolation of embryonic corneal epithelium from the underlying basal lamina and stroma, there is, as noted above, an immediate and marked disruption of the formerly smooth basal cell surface. Using heavy meromyosin subfragment (S1), we showed that the cortical mat of actin filaments, which normally runs parallel to the flattened basal cell surface in contact with ECM, becomes disorganized and the actin filaments flow into the blebs (29). We demonstrated that when such denuded epithelia are confronted with soluble collagen, laminin, or fibronectin in the culture medium, there is a reorganization of the basal cytoskeleton and a flattening of the basal cell surface (29). We show here that the dramatic effect of soluble

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1. *Abbreviations used in this paper:* con A, concanavalin A; ECM, extracellular matrix; HBSS, Hanks' balanced salt solution; OH-proline, hydroxyproline.

ECM molecules on cytoskeletal morphology is accompanied by a twofold increase in collagen produced by the epithelium.

These results led us to speculate that the solubilized ECM molecules bind to the basal epithelial surface at specific binding sites or receptors, and that this interaction results in reorganization of the basal actin cytoskeleton, which in turn leads to stimulation of epithelial metabolism (7, 27). It was not possible, however, to visualize the interacting exogenous ECM molecules by routine electron microscopy. In the present study, we report that fluorescently labeled laminin, collagen, and fibronectin, when added to the medium surrounding embryonic corneal epithelia in culture, bind to and flatten the basal epithelial cell surface. These results are compatible with the idea that the basal epithelial surface possesses a diverse population of binding sites for ECM. Localization of ECM binding sites on the basal epithelial cell surface provides a new example of polarized expression of cell surface proteins by epithelia. These binding sites may be the link between matrix at the cell surface and the cytoskeleton and thus be involved in the enhancement by ECM of collagen production by the corneal epithelium.

Materials and Methods

Tissue Isolation

Corneal epithelia from day 10 chicken embryos were harvested as previously described (29). Briefly, corneas were treated at 37°C for 8–12 min with 0.1% trypsin (Sigma Chemical Co., St. Louis, MO), 0.1% collagenase (Sigma Chemical Co.) in Hanks' balanced salt solution (HBSS) (pH 7.4), or with 0.04% EDTA in calcium-magnesium free HBSS (pH 7.4). Epithelia were then dissected free of the underlying basal lamina and floated in F-12 media, with no serum or with 10% fetal calf serum (GIBCO, Grand Island, NY) which had previously been depleted of plasma fibronectin by gelatin affinity chromatography. All media was supplemented with 50 μg/ml fresh ascorbic acid and 1% antibiotic-antimycotic (penicillin, fungizone, streptomycin; GIBCO).

For experiments requiring inhibition of protein synthesis, corneas were soaked in HBSS containing 25 μg/ml cycloheximide (Sigma Chemical Co.) for 4 h before isolation and for the duration of the experiment. We have previously reported the effectiveness of this dose of cyclohexamide in inhibiting protein synthesis by the corneal epithelium (30).

Protein Production by Epithelia

Corneal epithelia were cultured in the presence of [^{3}H]proline (30–45 μCi/ml, L-[2,3^{3}H]proline, 23.7 Ci/mmol, New England Nuclear, Boston, MA) or [^{3}H]-leucine (30 μCi/ml, L-[2,3,4,5-^{3}H]leucine, 120 Ci/mmol, ICN Biomedicals Inc., Irvine, CA). After a 16–24-h incubation, the tissue and media were harvested in 0.01 M Tris (pH 7.4) with 2 mM EDTA and EGTA and protease inhibitors, phenylmethylsulfonyl fluoride, n-ethyl maleimide, and amino caproic acid.

Unincorporated ^{3}H-amino acids were removed by (a) dialyzing the samples extensively against buffer containing 100 mM l-proline or l-leucine or (b) precipitating the samples with 10.0% trichloroacetic acid in the presence of 100 mM l-proline or l-leucine, followed by washes in 5% trichloroacetic acid and 0.1 M l-proline or l-leucine. The two methods of eliminating non-incorporated ^{3}H-amino acids gave comparable results for total ^{3}H-amino acids incorporated into protein.

Background levels of unincorporated ^{3}H-amino acids were determined by adding ^{3}H-amino acids to cultures immediately before processing as above. The amount of radiolabeled amino acids adsorbed to nondialyzable or precipitated material was then assayed for both tissue and medium. For tissue samples the background (proline 30–45 cpm/μg DNA; leucine 63–102 cpm/μg DNA) was <1% of counts incorporated into total protein (see above). The background was in medium samples ranged from 3–4.5% of incorporated counts for proline (104–167 cpm/μg DNA) and 10–13% for leucine (230–319 cpm/μg DNA). These relatively low background levels of free ^{3}H-amino acids have little impact on the interpretation of total protein production by the epithelium. No difference was observed between background levels of control and treated groups.

Hydroxyproline Assay, Index of Collagen Production

Taking advantage of the fact that hydroxyprolines (OH-prolines) are found almost exclusively in the collagens of vertebrate tissues, we used the incorporation of ^{3}H into OH-proline as an index of collagen production. Samples were concentrated to a final volume of 0.5 ml and hydrolyzed in 6 N HCl at 110°C for 18 h in vacuum-sealed vials. The levels of newly synthesized OH-proline were determined by the high voltage electrophoretic method described by Tseng et al. (32). High voltage electrophoresis (32) of the hydrolyzed samples from the corneal epithelia resolved not only OH-proline separately from proline but also 3-OH-proline from 4-OH-proline.

Hydrolyzed samples were spotted on Whatman 3MM paper along with proline and OH-proline amino acid standards, and the paper was wetted with running buffer (1.4 M acetic acid, 0.47 M formic acid, pH 1.9). Electrophoresis was carried out in a model Lt 48 high voltage tank (Savant Instruments, Inc., Hicksville, NY, at 5,000 V, 115 mA for 45–60 min. After the electrophoretic separation, the amino acids were visualized by ninhydrin stain, and the corresponding sample strips were cut out for scintillation counting. The paper strips were eluted in 75% ethanol for 40 min at 40°C. Scintillation counting was performed in Beckman LS100 with maximum efficiency 58% under quenched conditions in Aquasol (New England Nuclear). For comparison, the data has been normalized to represent measured incorporation of ^{3}H into OH-proline per μg DNA. DNA was determined by the method of Kissane and Robbins as modified by Hinegardner (9).

Fluorescent Tagging of Molecules

Laminin from the EHS mouse tumor was a generous gift from Drs. Hynda Kleinman and George Martin (National Institute of Dental Research, Bethesda, MD). Collagen type I was prepared from rat tail tendons as previously described (29). Purified collagen, in 0.5 M acetic acid, was dialyzed against phosphate-buffered saline (PBS), pH 7.4, then denatured by heating at 60°C for 30 min. Fibronectin was purified from human plasma or cultures of fibroblasts by gelatin affinity chromatography, and hamster cellular fibronectin was provided by Dr. Richard Hynes (Massachusetts Institute of Technology, Boston, MA). Heparin and chondroitin sulfate (mixed isomers) were obtained from Sigma Chemical Co. Concanavalin A (con A) and wheat germ agglutinin were purchased from Cappel Laboratories (Cochranville, PA).

The preceding molecules were fluorescently labeled by one of the following methods. Fluorescein isothiocyanate or tetramethyl rhodamine isothiocyanate was coupled to purified protein in 0.1 M carbonate–bicarbonate buffer (pH 9.3) at a concentration of 0.5 mg protein/mg of dye in a total volume of 1.0 ml. [5-(4,6-Dichloro triazanyl)] amino fluorescein (Molecular Probes Inc., Junction City, OR) was coupled to the ligands at 1:100 protein to dye ratio in phosphate buffer (pH 7.0). Protein-bound dyes were separated from unbound dyes by gel filtration on a 5-ml column of G25-150 Sephadex. To control for nonspecific adsorption of fluorescent proteins to the epithelial cell surface, fluorescent bovine serum albumin (BSA) and bovine hemoglobin were prepared by the same methods.

We have also used Covaspheres (Covalent Technologies Inc., Ann Arbor, MI). There are intensely fluorescent latex spheres 0.4–0.6 μm in diameter. Preactivated spheres (Covaspheres-mx) can be readily coupled to proteins via amino and sulfhydryl groups. Alternatively, inert spheres (Covaspheres-fx) were coupled to protein by carbonyl linkages with carbodiimide. Covaspheres were added to protein solutions at a concentration of 10 μg microspheres/10 μg ECM molecules in 100 μl of phosphate-buffered saline (pH 7.4). Unbound reactive sites on the spheres were blocked by addition of BSA or fetal calf serum (depleted of plasma fibronectin). As controls for nonspecific binding of microspheres to the isolated epithelia, inert unreactive spheres and Covaspheres coated with only BSA were prepared.

Labeling and Fixation of Epithelia

Isolated epithelia were free floated in 2 ml of media containing one of the above preparations, so that the apical as well as the basal surfaces of the epithelia had equal exposure to the fluorescent probes. Incubation was carried out at 4°C or 37°C. Some of the epithelia were treated with additional trypsin (50 μg/ml) for 10 min at 37°C before or after exposure to the tagged molecules.

After a 1-, 2-, 4-, or 6-h incubation, epithelia were washed in HBSS (three times 5 min) and either frozen unfixed or fixed in 1% paraformaldehyde in 0.1 M cacodylate buffer (pH 7.4), then frozen, for cryomicrotomy. Fixed and unfixed cryostat sections, 5–8 μm thick, were viewed by epifluorescence with a Zeiss Universal II photomicroscope. Epithelia to be examined by electron microscopy were fixed for 30 min in 2% paraformaldehyde, 2.5% glutaraldehyde in cacodylate buffer, and postfixed in 1% osmium tetroxide in 0.1 M

cacodylate buffer (pH 7.4 at 4°C) for 45 min. They were then stained en bloc in 1% uranyl acetate, dehydrated, and embedded in Spurr (Tousimis Research Corp., Rockville, MD).

Results

ECM Stimulation of Epithelial Collagen Production

Corneal epithelia cultured in the presence of soluble ECM (laminin, collagen, and fibronectin) produced and secreted higher levels of collagen than did control epithelia. At concentrations of ECM molecules that were previously shown to cause blebbing epithelia to withdraw the blebs and flatten, there is approximately a twofold increase in the amount of collagen produced by the epithelial cells as judged by accumulation of [³H]proline into OH-proline (Fig. 1). Accumulation is believed to measure collagen production, not turnover, because corneal epithelium does not produce collagenase (13), nor digests the substratum when cultured on collagen
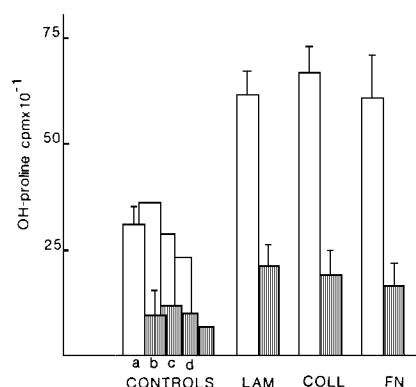
Figure 1. Collagen production by corneal epithelia cultured in the presence of exogenous ECM was monitored by the incorporation of [³H]proline into OH-proline. Data are expressed as cpm OH-proline/μg DNA and open bars represent epithelial tissue-associated counts, whereas the shaded bars represent counts recovered from the medium. The epithelia were cultured in [³H]proline for 24 h. Controls include epithelia cultured in F12 media with (a) no additions; (b) chondroitin sulfate (200 μg/ml); (c) heparin (200 μg/ml); (d) con A (50 μg/ml). There can be seen an approximately twofold increase in OH-proline incorporation by epithelia cultured in the presence of laminin (LAM, 2 μg/ml); collagen (COLL, 100 μg/ml); fibronectin (FN, 50 μg/ml). T = SD.

gels (20). This increase could be demonstrated in the collagen found in the medium as well as that associated with the epithelium (Table I). Total protein synthesis, demonstrated by incorporation of [³H]proline or [³H]leucine, increased by only 20%, suggesting that the soluble ECM was not stimulating protein synthesis in general, but only ECM synthesis (Table I).

Interestingly, each ECM molecule (laminin, collagen, and fibronectin), when administered at the concentration known to flatten the basal surface (29), causes about the same increase in collagen production (Fig. 1). Analysis of collagen production by epithelia cultured in decreasing amounts of ECM demonstrated a clear dose-response curve for each of the soluble ECM molecules (Fig. 2). Glycosaminoglycans, such as heparin or chondroitin sulfate, BSA, or the lectin con A, all known not to inhibit the epithelial blebbing (29), had no effect on the amount of collagen produced by the epithelia (Fig. 1).

The increase in collagen production by epithelia cultured in soluble ECM is reflected in both the 3-OH- and 4-OH-proline levels. Analysis of the 3-OH- and 4-OH-proline levels, however, showed that a higher 3-OH/4-OH ratio was associated with the epithelia than with the media. There are elevated levels of 3-OH-proline in type IV collagen (10-15/alpha chain) as compared to that (1-2/alpha chain) present in the stromal collagens (types I, II, and III). These data are compatible with the idea that more type IV collagen than stromal collagen remains associated with the epithelia than with the media, but the point requires more study.

Binding of Labeled ECM Molecules

To examine the site of ECM-epithelial cell interaction, we have coupled the soluble ECM molecules to fluorescent probes. The ECM components were coupled either to soluble fluors, such as fluorescein isothiocyanate, or to insoluble beads (Covaspheres, size ranging from 0.3-0.5 μm). The spheres have an advantage of quick recognition with no background due to autofluorescence and can be easily coupled to the ECM moieties. We have been successful in tagging ECM molecules with these fluorescent labels (Fig. 3, A–F) without interfering with the ability of these molecules to bind to epithelial cells and flatten the basal epithelial surface (Fig. 4A). Laminin, collagen type I, or fibronectin labeled with fluorescein or

Table I. Counts Incorporated into Protein and Collagen by 10-d-old Corneal Epithelium Cultured in the Presence of Soluble ECM*

	Leucine	Proline	OH-proline	Ratio 3OH/4OH
Control				
epithelium	6,253 ± 458	9,088 ± 126	310 ± 37	0.091
medium	1,877 ± 430	3,229 ± 147	90 ± 35	0.024
Laminin (2 μg/ml)				
epithelium	6,870 ± 620	11,844 ± 178	627 ± 48	0.080
medium	2,448 ± 544	3,840 ± 106	207 ± 45	0.028
Collagen (100 μg/ml)				
epithelium	6,460 ± 374	10,290 ± 132	666 ± 54	0.076
medium	1,924 ± 466	3,495 ± 164	194 ± 50	0.018
Fibronectin (50 μg/ml)				
epithelium	5,466 ± 638	12,604 ± 260	612 ± 70	0.098
medium	1,433 ± 377	3,216 ± 315	178 ± 36	0.022

* Counts per minute per microgram DNA ± SD; n = 3 for leucine, n = 5 for proline and OH-proline. Tissue samples had <1% background of unincorporated ³H-amino acids (leucine and proline), whereas the media samples had background levels of 3-4.5% for ³H-proline and 10-13% for ³H-leucine.
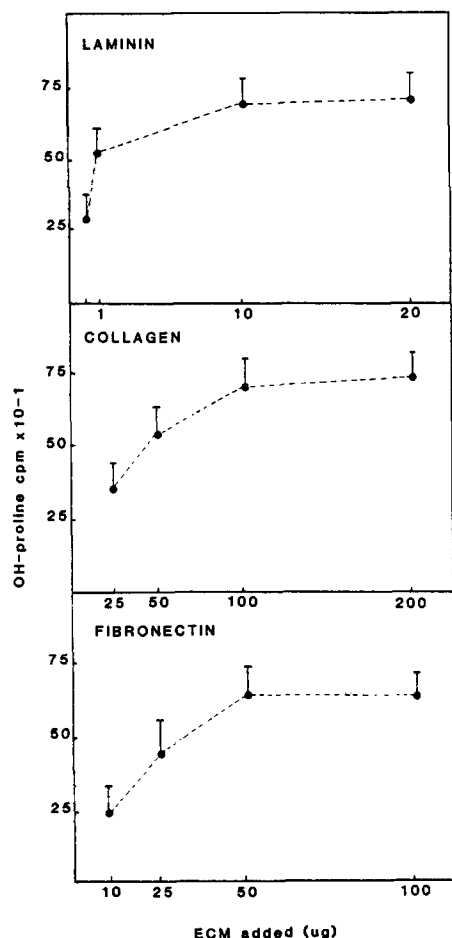
Figure 2. Incorporation of [^{3}H]proline into OH-proline by epithelia cultured in the presence of increasing concentrations of exogenous ECM to demonstrate dose–response relationships. In each case, the epithelium obtains a maximum collagen production at concentrations that correspond to those which cause a blebbing epithelium to flatten completely. Data expressed as cpm OH-proline/μg DNA; n = 5; $\top$ = SD.

attached Covaspheres were added to embryonic corneal epithelia suspended in culture medium. Each of these three molecules binds to the basal epithelial cell surface only, even though the apical surface of the epithelium has equal access to the fluorescent probe (Fig. 3, *A–F*).

Other fluorescein-labeled ligands tested include BSA, heparin, and the lectin con A. Con A binds to both the apical and basal surfaces of the epithelia (Fig. 3 *G*) and has no effect on the epithelial blebbing (Fig. 3 *B*). Fluorescein-labeled hemoglobin does not bind to the epithelium. BSA does not

bind to the epithelium with any regularity and does not stop the blebbing. When seen to bind, BSA forms a patchy pattern with no apical or basal preference. Heparin binds only to the basal cell surface (Fig. 3 *H*), but does not affect epithelial blebbing (Fig. 4 *C*). These results suggest that the basal epithelial cell surface contains or expresses a population of binding sites for specific ECM molecules.

To further examine the nature of the ECM–basal cell surface interaction, we assayed the role of endogenous protein synthesis in the binding of fluorescent ECM to the cells. Subsequent to inhibiting protein synthesis by the epithelia with a known effective concentration of cycloheximide (30), we confronted the cells with fluorescent ECM molecules that normally bind to the basal cell surface. Inhibition of protein synthesis did not affect the ability of the epithelia to bind either collagen or laminin, but did abolish the ability of epithelia to bind fluorescent fibronectin. These results are consistent with our previous study (30), which indicated that cycloheximide-treated or 1-azetidine carboxylic acid–treated epithelia do flatten the basal surface in response to laminin and collagen, but not to fibronectin, which seems to require helper protein to bind to the cells.

In contrast, when fibronectin is linked to Covaspheres, there is binding to the basal epithelial surface in the presence of cycloheximide. We interpret these results to mean that the spheres are acting as substrata for the fibronectin molecules, thereby activating the molecules for binding (see Discussion).

The specificity of the binding was examined by assaying the binding of fluoresceinated ECM in the presence of different concentrations of cold or unlabeled ECM molecules. From these experiments, it was determined that only laminin could compete successfully for the laminin-binding site. Elevated concentrations (50–300 μg/ml) of unlabeled collagen, fibronectin, or heparin had no visible effect on the amount of labeled laminin bound (Table II). Likewise, only collagen could compete for the collagen-binding site and fibronectin for the fibronectin-binding site. The competition of collagen for the binding site seemed independent of collagen type. Type I (denatured), type II, and native type IV were all capable of competing for the binding site of fluoresceinated type I collagen. However, type IV collagen was successful in competing for the binding site at lower concentrations than types I or II (Table II).

Competition experiments using collagen and fibronectin together were complicated by the aggregation of fibronectin to the collagen. This was very evident when denatured type I collagen-coated Covaspheres were used in the presence of >20 μg/ml fibronectin; under these conditions, large aggregates of spheres were formed, presumably the result of cross-linking (collagen–fibronectin–collagen). Type IV collagen

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Figure 3. Day 10 embryonic corneal epithelia were isolated from the underlying ECM and free-floated in media containing various fluorescently labeled molecules. (*A*) Epithelium cultured for 3 h in the presence of fluorescein-labeled laminin. Notice the laminin only binds to the basal epithelial surface (apical surface is toward top of page). (*B*) Epithelium cultured in media containing laminin-coated fluorescein Covaspheres. Again laminin only binds to the basal surface. (*C*) Epithelium cultured in fluorescein-tagged type I collagen. Fluorescence can only be detected on the basal cell surface. (*D*) Same epithelium as shown in *B*, but here the collagen-coated rhodamine Covaspheres that were added to the cells were photographed. The epithelium can simultaneously bind two different ECM moieties. (*E*) Fluorescein-labeled fibronectin and (*F*) fibronectin-coated Covaspheres bind only to the basal surface. (*G*) Fluorescein-con A binds to both apical and basal epithelial surfaces. (*H*) Fluorescein isothiocyanate–labeled heparin can be seen to bind only to the basal epithelial surface. Bars, 20 μm.

A fluorescein laminin added

B con-A

C heparin

2μm
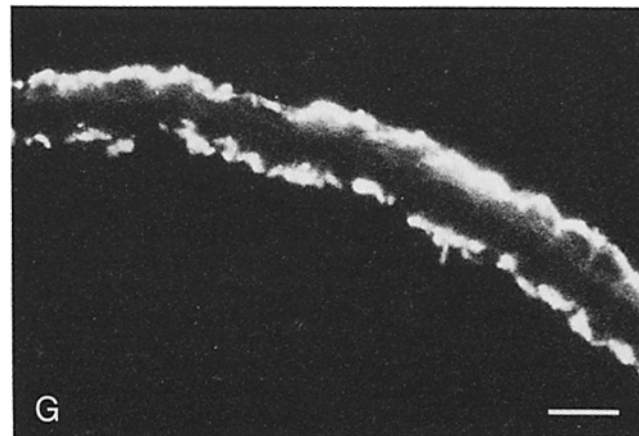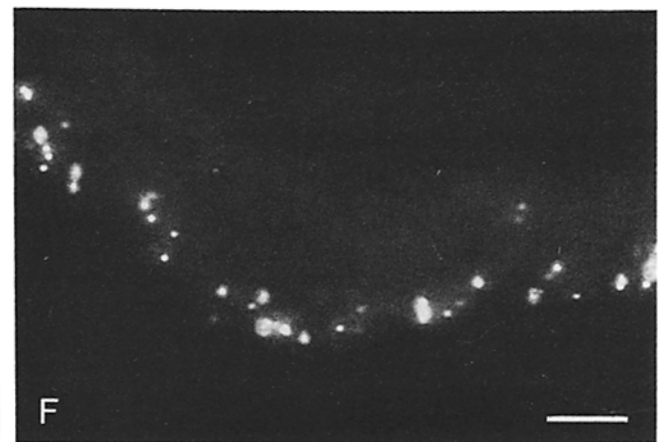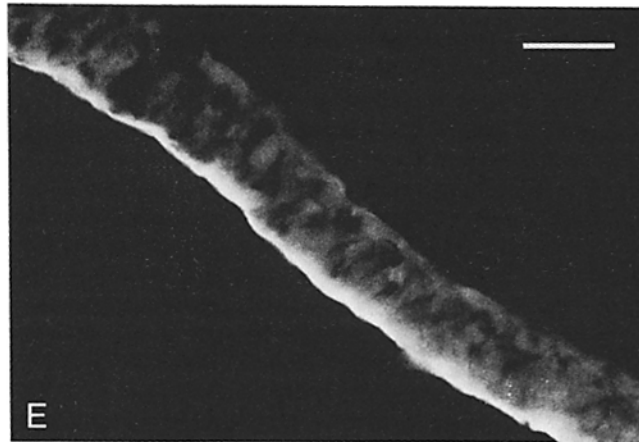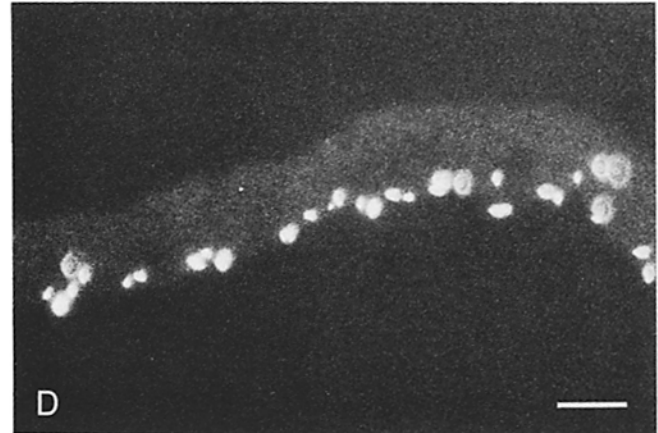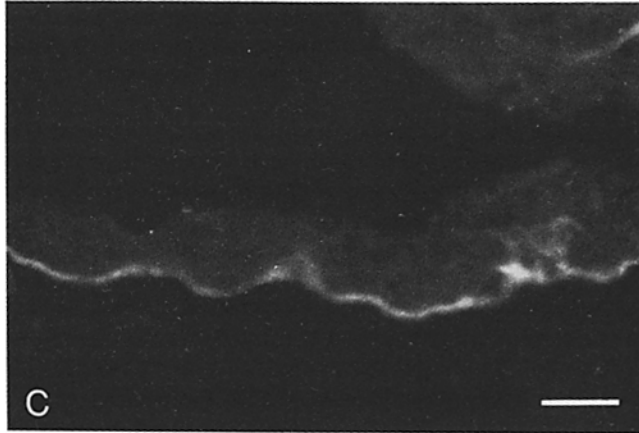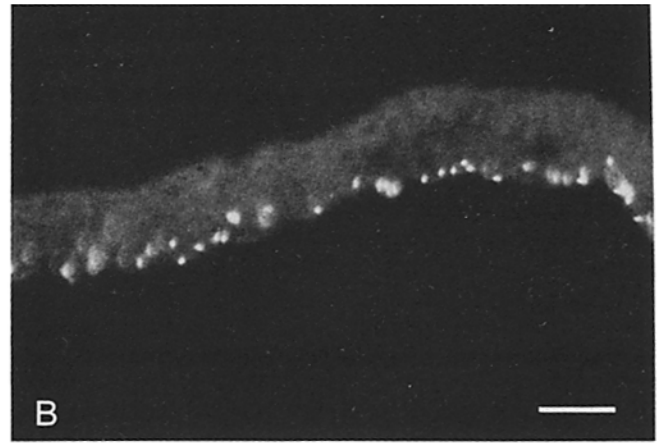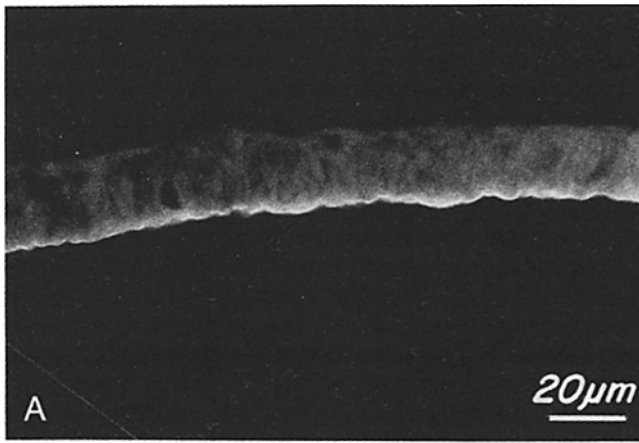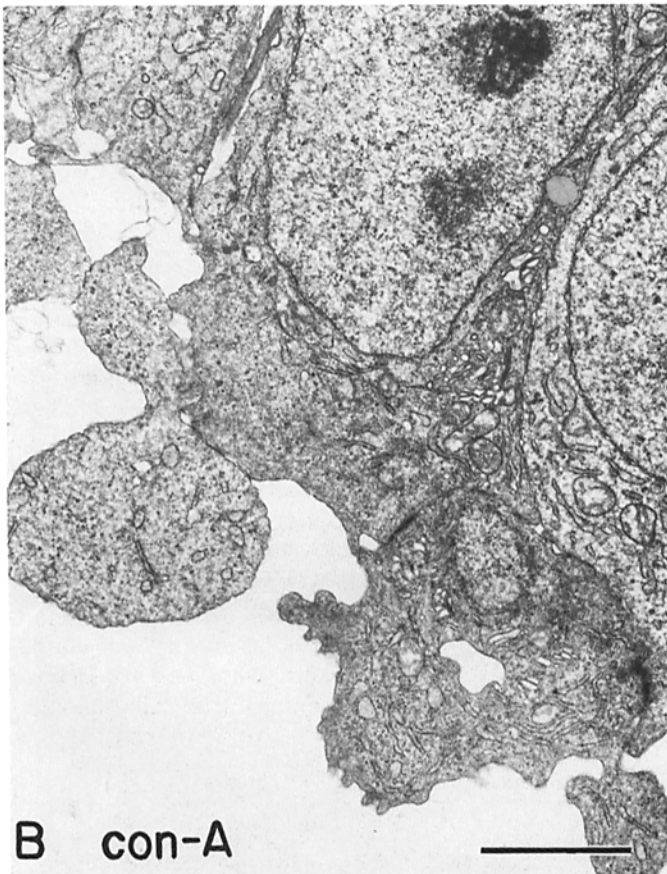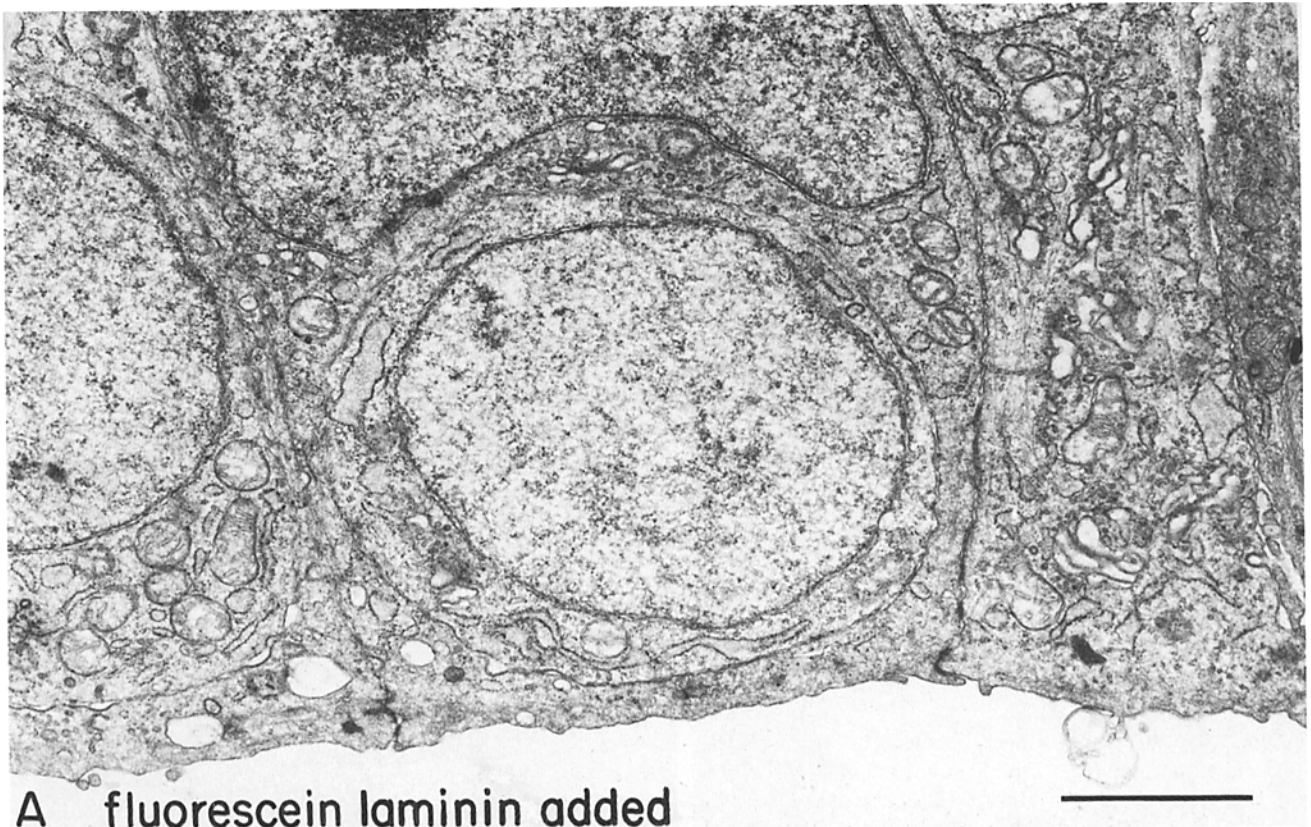
*Table II. Competition for Binding to Corneal Epithelium of Fluorescent Covasphere-labeled ECM with Unlabeled ECM**

Labeled	Laminin	Collagen (type I)	Fibronectin
Unlabeled			
Laminin			
1 µg/ml	+/−	+	+
10 µg/ml	−	+	+
Collagen (type I)			
50 µg/ml	+	+/−	+
200 µg/ml	+	−	+
Collagen (type II)			
50 µg/ml	ND	+/−	ND
200 µg/ml	ND	−	ND
Collagen (type IV)			
50 µg/ml	+	−	+
200 µg/ml	+/−	−	+
Fibronectin			
25 µg/ml	+	ND	+/−
100 µg/ml	+	ND	−
Heparin			
100 µg/ml	+	+	+
300 µg/ml	+	+	+

* Scored as +, epithelium with >12 spheres/100 µm of basal surface; +/−, epithelium with <12 spheres/100 µm of basal surfaces; −, epithelium without label; ND, no data.

does not aggregate the fibronectin-coated spheres and does not compete successfully for the fibronectin-binding site (Table II). Likewise, unlabeled fibronectin does not compete for the collagen site (Table II). These results are consistent with the existence of separate collagen- and fibronectin-binding sites on the basal epithelial surface.

The binding sites for these three ECM molecules are protease sensitive. Treatment of the isolated epithelia with trypsin (50 µg/ml for 10 min) abolishes the subsequent binding to the cell surface of all the tagged ECM (laminin, collagen, and fibronectin directly fluoresceinated or on Covaspheres), suggesting that the binding sites are at least in part proteinaceous (Fig. 5). The epithelia are capable of restoring the binding sites within 3–6 h and this restoration can be blocked by cycloheximide (25 µg/ml), indicative of prerequisite protein synthesis for replenishment of binding sites on the basal epithelial surface. It is interesting to note, however, that routine isolation of the epithelia from whole corneas with a combination of trypsin/collagenase (even at the high concentration of 1.0 mg/ml) leaves the binding sites intact as judged by the binding of tagged molecules to the basal surface immediately upon isolation (see Discussion). Trypsin treatment of isolated epithelia, which had been incubated in fluorescent ECM, was successful in dislodging most of the bound ECM from the basal cell surface (Fig. 5 C).

Discussion

Here, we report that exogenously added soluble ECM molecules, such as laminin, collagen, and fibronectin, stimulate

epithelial collagen production and bind preferentially to the basal surface of isolated epithelial sheets. Using fluorescein-ated molecules and molecules bound to Covaspheres, we have examined the distribution and protease sensitivity of these ECM binding sites. The data suggest that specific binding sites are present for each of these three ECM components (as judged by competition assays) and that the same basal epithelial cell surface contains different binding sites for various ECM moieties. These results are supportive of earlier work showing that the isolated embryonic epithelia are capable of interacting with and responding to either laminin, collagen, or fibronectin administered in solution (29). Based on sensitivity of all three of the binding sites to protease digestion and the requirement for protein synthesis to restore the binding of laminin, collagen, or fibronectin, we conclude that the binding sites are, at least in part, protein. However, these results do not rule out the possible involvement of other membrane components such as glycolipids (14, 34).
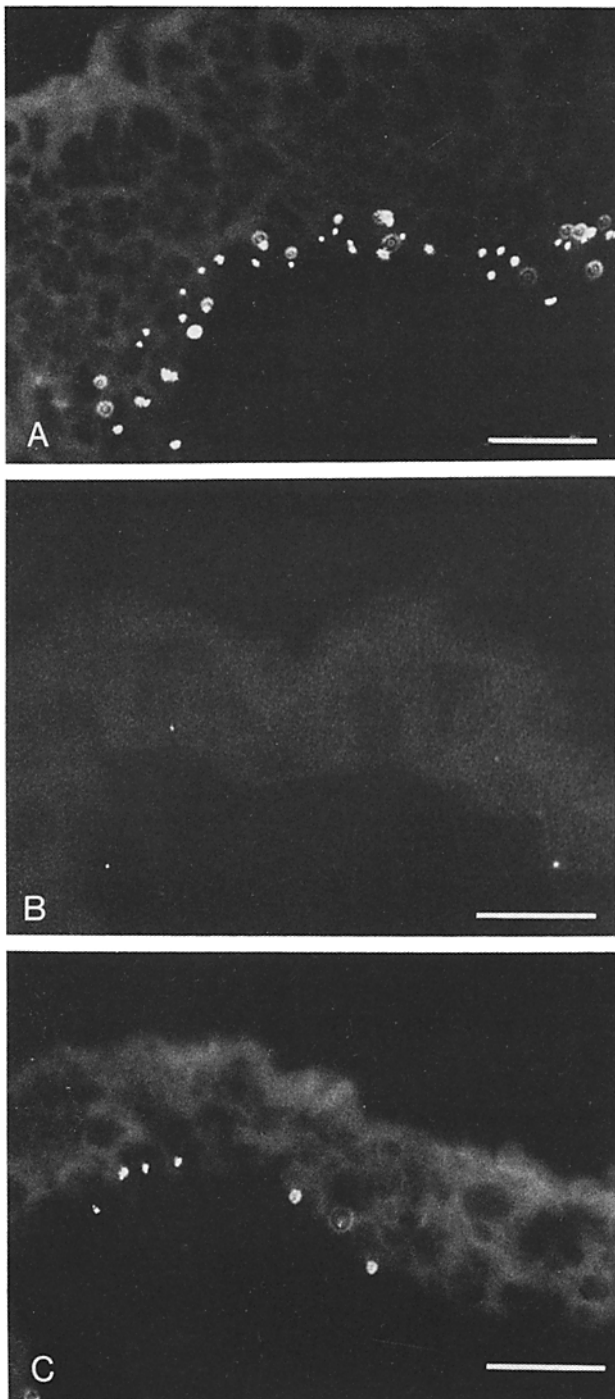
The basal cell surface of corneal epithelia also binds con A and heparin, but neither of these molecules causes a blebbing epithelium to flatten and neither stimulates collagen synthesis. While the con A bound to the apical surface as well as the basal, heparin demonstrated binding to the basal surface only. The binding sites on the basal cell surface for heparin and con A appear to be distinct from the ECM binding sites that stabilize the basal surface and cause withdrawal of the blebs.

The corneal epithelium can be isolated with trypsin and collagenase and the binding sites for soluble ECM (laminin, collagen, fibronectin) are still intact. Yet, the binding sites are sensitive to further proteolytic attack. These results could be explained by the possibility that the trypsin-sensitive site on the binding proteins are masked in situ, but are available to degradative enzymes on the isolated epithelia. ECM molecules can protect other ECM molecules from degradation. For example, David and Bernfield (3) have reported that the turnover of proteoglycans at the basal cell surface of mammary epithelia is slowed by the presence of type I collagen.

We show here that soluble ECM molecules, in concentrations that cause withdrawal of the blebs and reorganization of the basal surface (29), bring about an increase in collagen production by the epithelial cells. The enhancement of collagen accumulation measured here is not quite as high as that reported by Meier and Hay (20) for corneal epithelia grown on polymerized collagenous substrata, but the difference could reflect the different methods used to assay collagen. Alternatively, polymerization produces a thick coat of collagen on the substratum that may interact more effectively with the cells.

It is tempting to think that ECM molecules stimulate epithelial metabolism by their effect on the cytoskeleton. Withdrawal of the blebs and enhancement of collagen synthesis is correlated with a reorganization of the actin cytoskeleton as judged by heavy meromyosin staining (29). There is considerable precedence for the idea that fibronectin interacts

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Figure 4. Tissues shown in this figure were routinely fixed and processed for electron micrscopy. (*A*) Electron micrograph of a section showing the basal surface of an epithelia cultured in the presence of fluorescein-labeled laminin (2 µg/ml). Note that the basal epithelial surface is flattened and the cytoskeleton reorganized. (*B*) Epithelium cultured in the presence of con A (50 µg/ml). The basal surface is still disrupted and blebbing. (*C*) Epithelium cultured in heparin (200 µg/ml). The epithelium continues to bleb from the basal surface. Bars, 2 µm.

Figure 5. The sites of ECM binding on the epithelial cell surface are protease sensitive. (A) Corneal epithelia free floated in media containing laminin-coated Covaspheres binds the fluorescent spheres on the basal surface (section is cut slightly tangentially). (B) There is no binding of laminin Covaspheres to epithelium treated with trypsin (50 μg/ml) after isolation. (C) Section of an epithelia cultured in laminin-coated Covaspheres, then treated with trypsin, which removes most of the spheres from the basal epithelial surface. Bars, 20 μm.

across the plasmalemma directly or indirectly with the cytoskeleton (11). There is also precedence for the view that cytoskeletal organization can regulate protein synthesis (22), but the mechanisms by which the cytoskeleton might be

involved in enhancing synthetic mechanisms are unknown. Penman et al. (22) have speculated that it may be necessary for mRNA and ribosomes to attach to the cytoskeleton to translate efficiently. Alternatively, the cytoskeleton may stabilize attached mRNA and/or newly produced protein by preventing turnover. Secreted collagen is not turned over significantly (20), but we do not yet have data on mRNA or intracellular protein.

The dose–response to ECM molecules reported here may reflect differing degrees of reorganization of the cytoskeleton by the lower doses, resulting in differing levels of mRNA translation and/or turnover. We have not succeeded in experiments combining ECM molecules to determine whether or not their effects would be additive, because collagen and fibronectin, for example, interact with each other in solution and precipitate from the medium underlying the epithelium. The dose–response data reported here show that each molecule (collagen, laminin, fibronectin) produces the same maximum response, as judged by collagen production. Morphological data (29) suggest that each molecule has the same cytoskeletal effect at its most effective concentration. One would, perhaps, not expect an additive effect if the maximal cytoskeletal and metabolic response can be reached independently by each molecule.

Recently, a number of different laboratories have isolated glycoproteins (65–70 kD) from several cell types that may represent the binding proteins or receptors for laminin (17, 18, 24, 31). A similar protein has been isolated from the avian corneal epithelium (28). It is tempting to think that this protein provides a transmembrane link to the actin cytoskeleton, permitting ECM to organize the cytoskeleton and thus be involved in the enhancement of collagen production discussed above. Brown et al. (1) reported that the 68-kD laminin-binding protein isolated from fibrosarcoma binds directly to actin in vitro. However, it is equally possible that intermediate actin-binding proteins link the putative receptor to the actin cytoskeleton of the corneal epithelium.

Collagen, like laminin, appears to interact directly with the epithelial basal cell surface and it has been speculated that enhancement of collagen production by collagen is likewise mediated by a transmembrane link to the cytoskeleton (7, 27). Cell surface binding sites for collagen have been reported in a number of different cells. They seem to be small to medium sized (31 kD, 47 kD, 70 kD) proteins (2, 16, 21). We have isolated two cell surface proteins (70 kD and 47 kD) from corneal epithelial membranes that exhibit similar binding qualities to both denatured type I collagen and native type IV collagen (28). In the present report, we showed that collagen, like laminin, exhibits specific binding to the basal surface of isolated corneal epithelia. It seems likely that either of these two molecules is capable, by transmembrane link to the actin cytoskeleton, of enhancing collagen production by organizing the cytoskeleton.

All collagens tested (types I, II, IV) are able to bind to the basal epithelial cell surface, seemingly to the same sites, for type I competes with type IV and so on. The possibility that a single collagen-binding site exists that recognizes to some degree all collagen alpha-chains would explain our previous work showing that epithelia stop blebbing when cultured in the presence of any type of collagen, even purified type I alpha-chains. Rubin et al. (25) reported that various types of

collagen, alpha-chains, and even synthetic peptides resembling the primary structure of collagen mediate hepatocyte attachment.

In the literature on epithelial attachment to ECM, such direct mediation of cell adherence to collagen (25) has not often been reported. For example, laminin has been described in several cases as a necessary linker protein for epithelial cells to bind to collagen (15). Only recently has it been appreciated that at least some epithelial cells are capable of attaching directly to collagen, judged by the ability of hepatocytes to adhere to collagen without linker ECM (25). While this epithelium–collagen interaction may be of low affinity and may be overlooked in many assays of cell attachment, it is likely to have biological relevance. Indeed there is a growing awareness that cells which adhere to collagen via linker molecules, such as fibronectin and laminin, may also be capable of binding directly to collagen (see references 2, 16, 21, 25, 33).

In addition to laminin and collagen, fibronectin binds to the basal surface of corneal epithelia and stimulates collagen production by the corneal epithelium. Putative receptors in the plasmalemma, including a 140-kD protein (see reference 23 for review), have been identified as fibronectin-binding sites, but it is not clear exactly how fibronectin interacts with cells. We found inhibition of collagen production by l-azetidine carboxylic acid inhibits the interaction of fibronectin with isolated corneal epithelium, as judged by epithelial morphology (30). Recently, Mattey and Garrod (19) demonstrated a similar result with cultured corneal epithelial cells; cells inhibited from secreting collagen are unable to interact with a fibronectin matrix.

Complicating the problem of identifying the manner in which fibronectin interacts with cells is the observation, in the present paper and by previous workers, that interaction of fibronectin with solid substrata (such as beads), as well as with collagen or heparin, promotes the binding of fibronectin to its presumably specific receptor (5, 6, 12). Fibronectin may need a conformational change or, alternatively, there may be a requirement for a multivalent interaction at the binding site.

In conclusion, we have in this paper demonstrated that collagen, laminin, and fibronectin do bind to corneal epithelium, and in polarized fashion, to the basal surface. Soluble ECM molecules enhance epithelial production of collagen, each to the same degree, presumably because each has the same effect, directly or indirectly, on the organization of the cortical epithelial cytoskeleton. The correlation between cytoskeletal organization and collagen production suggests that binding of ECM to the basal cell surface promotes a kind of cytoskeletal architecture that enhances the synthetic capacities of the cell. In a subsequent paper, we examine the possibility that transmembrane receptors connect ECM molecules to the corneal epithelial cytoplasm.

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