BASAL LAMINA OF EMBRYONIC SALIVARY EPITHELIA

Production by the Epithelium and Role in Maintaining Lobular Morphology

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ABSTRACT

The role of the basal lamina in maintaining the normal morphology of mouse embryo submandibular epithelia was assessed by examining its production as well as the cellular and organ culture changes associated with its removal and replacement. The lamina was removed from epithelia isolated free of mesenchyme by brief treatment with testicular hyaluronidase in the absence of calcium. The treatment causes rounding-up of the cells, loss of cellular cohesion, appearance of microvilli, and changes in the organization of cytoskeletal structures. The lamina is not removed and the cellular alterations do not occur in the absence of hyaluronidase in calcium-free medium or when both enzyme and calcium are present, possibly because digestion of chondroitin sulfate, a component of the lamina, is inhibited by calcium. Within 2 h after treatment, in the absence of mesenchyme or biological substrata, the epithelia deposits a new lamina, which is identical by several criteria to the preexisting lamina, and reverses the cellular alterations. Epithelia treated with hyaluronidase lose lobular morphology during culture with mesenchyme. Delaying culture with mesenchyme, to allow restoration of the lamina and of normal cellular architecture, prevents the loss of lobular morphology. The results indicate that the basal lamina imposes morphologic stability on the epithelium, while the mesenchyme apparently affects processes involved in changes in morphology, possibly by selective degradation of the basal lamina.

The molecular mechanisms which underlie the development of the characteristic morphology of tissues and organs are poorly understood. Substantial evidence supports the view, however, that extracellular materials associated with tissue surfaces are involved in epithelial morphogenesis. With regard to the basal lamina, for example, an intact lamina is required for proliferation and orientation of cells in the epidermis (reviewed in reference 49) and developing tooth (reviewed in reference 39), and removal of the lamina causes a loss of lobular morphology of the submandibular gland (5). Embryonic basal laminae may contain glycosaminoglycan (GAG) (4, 11, 22, 23, 27, 30, 44), collagen of a specific type (8, 43, 45, 46) and, by analogy with adult organs, likely contain other components, principally glycoprotein (26).

Studies on the tissue of origin of the epithelial basal lamina have revealed that the epithelial cells themselves are the predominant source (21, 32, 36, 44), although certain embryonic epithelia apparently either do not produce collagen or pro-

duce collagen at exceedingly low rates (3, 23, 34). Extensive studies of the embryonic cornea indicate that production of its lamina requires the close proximity of a collagenous substratum: Culture on noncollagenous substrata does not result in the appearance of laminar materials (15, 16, 31, 32).

We have used the mouse embryonic submandibular gland to examine the role that extracellular materials may play in its characteristic morphology. The 13-day gland consists of a multilobular epithelial bud surrounded by condensed mesenchyme which is required for normal epithelial morphogenesis. The mesenchyme is separated from the epithelium by fibrillar collagenous and amorphous materials and a closely associated epithelial basal lamina. Fibrillar collagen is present in large amounts along the epithelial stalk and deep within the clefts between the lobules, but in small amounts on the tips of the lobules (20). By treatment of the rudiment with collagenase and microdissection, the mesenchyme as well as the fibrillar collagen and amorphous materials can be removed, leaving the epithelium with its basal lamina (5). Such epithelia maintain their lobular shape and continue uninterrupted morphogenesis when cultured in association with mesenchyme. The basal lamina can be removed by briefly treating collagenase-isolated epithelia with enzymes which degrade GAGs. When epithelia devoid of a basal lamina are placed in culture with mesenchyme, however, the lobular morphology is lost and a spherical epithelial rudiment forms in which branching morphogenesis subsequently resumes (5).

Several studies suggest that the multilobular shape of submandibular epithelia is dependent upon the function of intracellular microfilaments. These microfilaments bind heavy meromyosin (40) and are thought to have properties characteristic of actin (reviewed in reference 37). Clefts and lobules are lost within a few minutes after treatment of the glands with cytochalasin B (41), an agent which alters the organization of the microfilaments, and are lost over several hours after treatments by agents presumed to inhibit microfilament contractility: i.e., papaverine, an inhibitor of smooth muscle contraction, and calcium-free medium (1). Epithelia maintain their morphology and recover from cytochalasin treatment in the presence of levels of colchicine which disrupt microtubules, suggesting that microtubules are not involved in maintaining lobular shape (41).

Our ability to isolate submandibular epithelia either free of mesenchyme but retaining the lamina or free of the lamina itself has allowed us to determine the tissue of origin of the lamina and to assess the function of the lamina and the role of the mesenchyme in the maintenance of epithelial morphology. Preliminary reports of this work have previously appeared (2, 6).

MATERIALS AND METHODS

Chemicals and Enzymes

Clostridium histolyticum collagenase type A (CLSPA lots 9EA and 8BA) was obtained from Worthington Biochemical Corp. (Freehold, N. J.). Contaminating enzyme activities of these collagenase preparations have been published (5). Testicular hyaluronidase (Type VI) obtained from Sigma Chemical Co. (St. Louis, Mo.) and from Leo Pharmaceutical Co., Helsingborg, Sweden, as well as chondroitinase ABC from Miles Laboratories (Miles Research Products, Elkhart, Ind.), were free of detectable protease activity (5). Hyaluronic acid (K salt, grade III-P) and crude chondroitin sulfate were obtained from Sigma. [6-^{3}H]glucosamine (6.7 Ci/mmol) and [2,3-^{3}H]proline (24 Ci/mmol) were obtained from New England Nuclear (Boston, Mass.) Eagle's basal medium (BME) was obtained from Grand Island Biological Co. (Grand Island, N. Y.) and ruthenium red from Chroma Gesellschaft.

Dissection and Culture Techniques

Embryos were obtained from the hybrid of C57BL/6 × DBA/2 mice at approximately 2:00 p.m. of the thirteenth gestational day, the day of discovery of the vaginal plug (8:00 a.m.) being counted as day 0. Submandibular rudiments were obtained as previously described (5), except that only glands containing three to four lobules were used. Glands were transferred to horse serum:Tyrode's solution (1:1) (HST) and, without injuring the epithelium, as much mesenchyme as possible was removed surgically. Epithelia were isolated free of residual mesenchyme by microdissection during 10-min incubation of the rudiment in collagenase (CLSPA; lot 9EA; 200 μg per milliliter; or lot 8BA, 100 μg per milliliter) dissolved in Mg-free Tyrode's solution (MFT). Isolated epithelia were washed in Tyrode's solution, transferred to HST, and stored in 5% CO_2 in air. For a second enzyme treatment, isolated epithelia were washed 5 times in Ca-Mg-free Tyrode's solution (CMFT) and incubated for 10 min at room temperature with testicular hyaluronidase in CMFT (Sigma Type VI, 0.07 μg per milliliter, or Leo, 0.05 μg per milliliter, both fortified with 1 μg/ml bovine serum albumin [BSA]). Control epithelia were incubated identically but without hyaluronidase. Following treatment, epithelia were washed in HST, and then in Tyrode's solution before labeling or culturing.

Culture medium contained 10% chicken embryo extract, 10% dialyzed horse serum, 2 mM glutamine, and 1% antibiotic-antimycotic mixture (Grand Island Biological). Glands were cultured either on top of a Millipore filter (Millipore Corp., Bedford, Mass.) (5) or submerged in the media in a glass dish. Living cultures were photographed with Zeiss photomicroscope II.

Labeling and Autoradiography

Labeling with [^{3}H]glucosamine was done at 40 μCi per milliliter in glucose-free BME containing 10% dialyzed horse serum, 2 mM glutamine, 0.1 mM pyruvate, and twice the usual concentration of amino acids and vitamins. Qualitatively identical results were obtained when glucose-containing medium was used. Labeling with [^{3}H]proline was done either in the above medium (which contains no proline) but containing glucose instead of pyruvate, or in full culture medium at levels indicated in Table I. Tissues were incubated with 1 ml labeling medium in submerged culture. After labeling, tissues were washed 5 times in 10 ml Tyrode's solution, or in MFT when mesenchyme was to be removed subsequent to labeling. Tissues were fixed in Carnoy's solution and processed as previously described (4).

Electron Microscopy

Rudiments prepared for ultrastructural analysis were fixed immediately after culture or enzyme treatment and processed as described in the accompanying paper (11). For ultrastructural studies, only Leo hyaluronidase was used.

Enzyme Assays

Testicular hyaluronidase was assayed turbidimetrically and chondroitinase ABC was assayed by the formation of unsaturated disaccharides, as previously described (5), except that hyaluronic acid as well as chondroitin sulfate was used as a substrate. The reactions were carried out at pH 7.4–7.6 in complete or modified Tyrode's solution, as indicated in the figure legends, at substrate concentrations where activity was linearly proportional to enzyme concentration.

RESULTS

Tissue of Origin of the Epithelial Basal Lamina

ULTRASTRUCTURE: Epithelia isolated with collagenase show a normal-appearing basal lamina over most of their surface (Fig. 1 *a* and *d*), and show very few adherent mesenchymal cells by scanning electron microscopy (data not shown). Treatment of collagenase-isolated epithelia with hyaluronidase removed all of the mesenchymal cells and nearly all of the lamina (Fig. 1 *b* and *e*), leaving laminar materials only deep within the clefts. When these latter epithelia were incubated for 2 h in the absence of mesenchyme either directly on a Millipore filter in contact with culture medium or immersed in medium on the bottom of a glass or plastic dish, a well-defined lamina was regenerated (Fig. 1 *c* and *f*). The newly deposited lamina appeared similar to that on epithelia freshly isolated with collagenase and covered 70–80% of the epithelial surface; other portions of the surface showed small amounts of surface material or remained denuded. These data indicate that in the absence of biological substratum or mesenchymal tissue, epithelia from which the lamina has been removed produce an ultrastructurally normal-appearing basal lamina over most of their surface within 2 h.

[^{3}H]GLUCOSAMINE AUTORADIOGRAPHY: Intact submandibular glands, labeled for 2 h with [^{3}H]glucosamine, deposit labeled material at the epithelial-mesenchymal interface (Fig. 2 *a*). Epithelia labeled in intact glands retain label at their surfaces when isolated free of mesenchyme with collagenase (Fig. 2 *b*), while treatment of such isolated epithelia with hyaluronidase removes the surface label (Fig. 2 *c*). The surface glucosamine label represents GAG within the epithelial basal lamina (5, 11).

To determine the source of laminar GAG, both epithelia retaining the lamina and that stripped of the lamina were examined for the presence of surface label after various types of incubation with [^{3}H]glucosamine. After 2 h labeling, regardless of their prior treatment, isolated epithelia accumulated substantial amounts of surface label (Fig. 3 *a* and *b*). When unlabeled epithelia were cultured for 2 h in close association with prelabeled mesenchyme, no radioactivity localized to the epithelial surface despite the presence of label within the mesenchyme (Fig. 3 *c* and *d*). This result suggests that the mesenchyme does not contribute significantly to the epithelial surface label. However, to be certain that the latter result was not due to very rapid turnover in the absence of precursor, epithelia prelabeled as intact glands were isolated and then reincubated for 2 h in medium containing nonradioactive glucosamine (0.1 mM). Collagenase-isolated epithelia (Fig. 3 *g*) showed no apparent loss of surface label (cf. 2 *b*), while hyaluronidase-treated epithelia redeposited label at their surfaces whether cultured in the presence or absence of mesenchyme (Fig. 3 *e* and *f*). Hence, the epithelium alone is the source of laminar GAG,

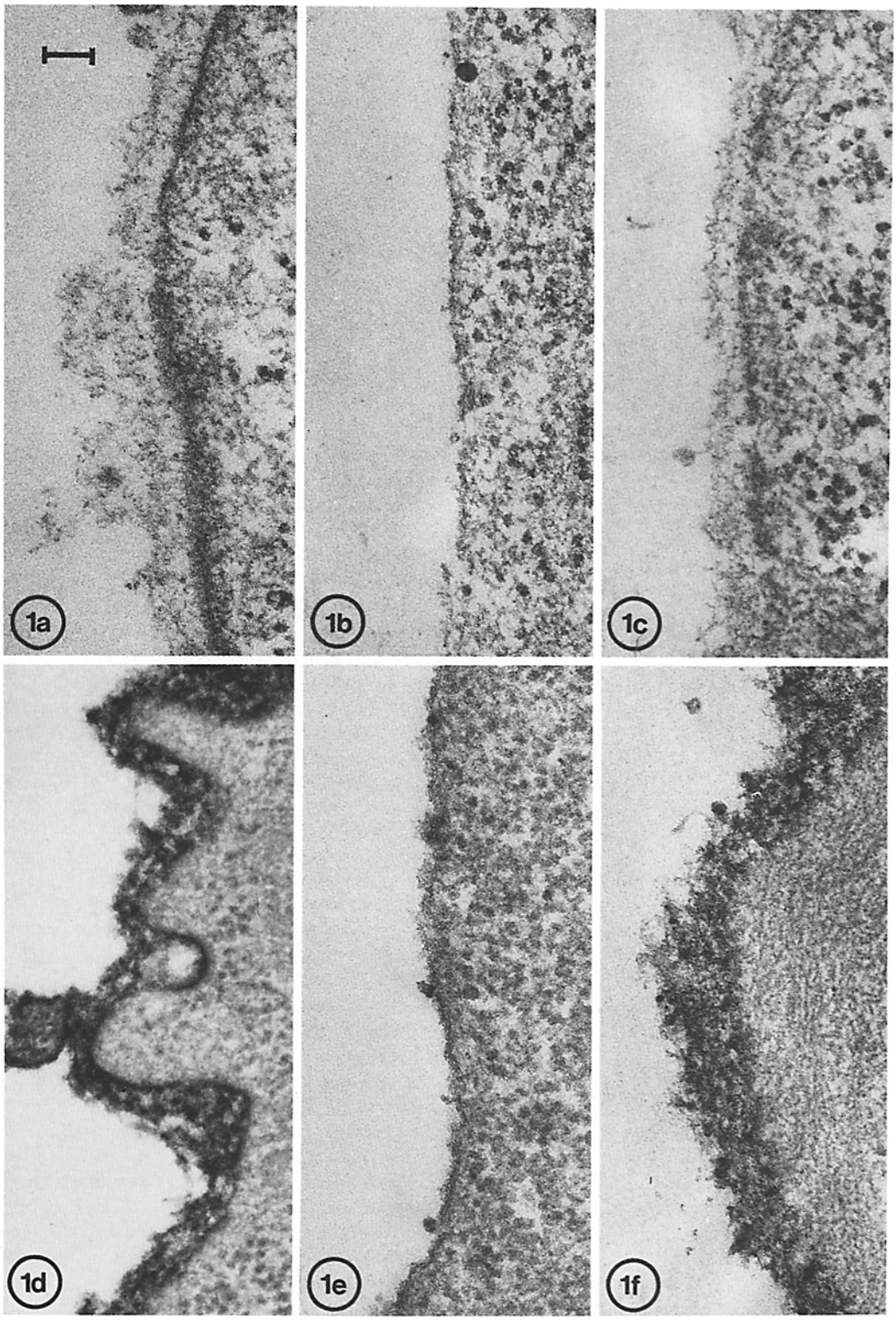


FIGURE 1 Electron micrographs of basal surfaces of submandibular epithelia. Top row (1 *a–c*), routinely fixed and stained. Bottom row (1 *d–f*), fixed and stained with ruthenium red. Bar is 0.1 μm. × 80,000. 1 *a* and *d* Collagenase-isolated epithelia. A basal lamina is present but no collagen-like fibrils are seen at its surface. (1 *b* and *e*) Epithelia as in 1 *a* and *d* treated with hyaluronidase. The lamina is nearly completely removed. Ruthenium red reveals some stain deposits on the plasmalemma. (1 *c* and *f*) Epithelia isolated with collagenase and treated with hyaluronidase (as in 1 *b* and *e*) cultured for 2 h in the absence of mesenchyme. Materials with the ultrastructural appearance and staining properties of the basal lamina are seen.

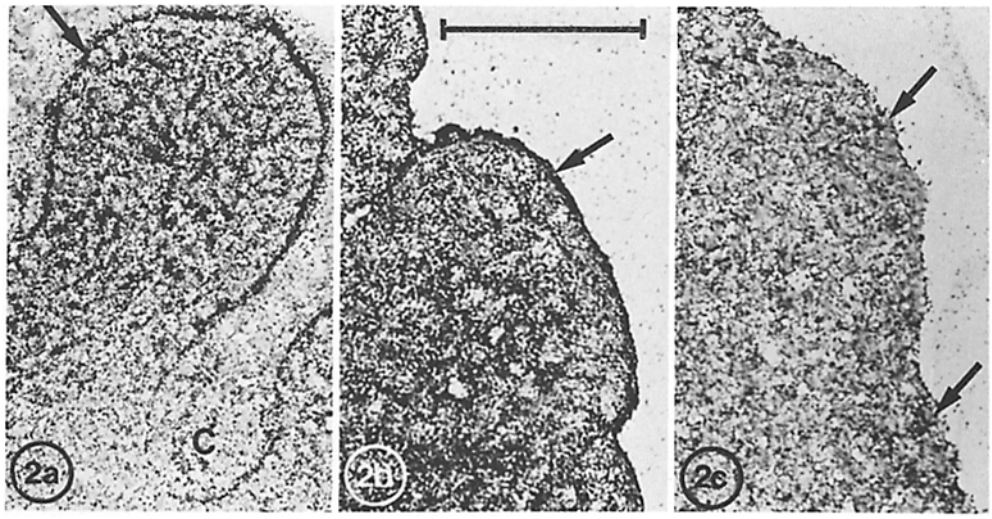


FIGURE 2 [^{3}H]glucosamine autoradiograms of submandibular glands labeled as intact glands for 2 h. Bar is 50 μm. × 540. (2*a*) Intact gland. There is heavy localization of label at the epithelial surface of the distal aspects of the lobule (arrow). Substantially less label is present at the epithelial surface within the cleft (*C*). Label is also present within the epithelium. (2*b*) Epithelium from which mesenchyme was removed with collagenase. Label remains at the epithelial surface (arrow). (2*c*) Epithelium as in 2*b*, briefly treated with hyaluronidase. No label is present at the epithelial surface, but label remains within the epithelium.

and deposition of this GAG on epithelia devoid of a lamina correlates in time with the appearance of an ultrastructurally normal lamina.

[^{3}H]PROLINE AUTORADIOGRAPHY: Evidence for the deposition of collagen within the basal lamina was sought by [^{3}H]proline autoradiography. Under labeling conditions which localize [^{3}H]GAG to the epithelial surface (Experiment 1, Table I), intact glands labeled with [^{3}H]proline did not show surface label, although there was substantial intra-epithelial and mesenchymal label (Fig. 4*a*). After 24 h of continuous labeling, however, small amounts of label were seen at epithelial surfaces deep within clefts (Experiment 2, Table I; Figure 4*b*). This labeling was due to prolonged incubation with the precursor and not due to the longer culture period since no surface label was seen when glands were labeled for 2 h following 22 h of culture (Experiment 3, Table I). As expected, since proline is a nonessential amino acid, identical results were obtained regardless of whether proline-free medium (Experiments 1 and 2, Table I) or full culture medium (Experiments 4 and 5, Table I) was used for labeling. Since the results were the same with 25 or 50 μCi per milliliter [^{3}H]proline, but because extensive intra-epithelial and mesenchymal labeling at 50 μCi per milliliter made surface label more difficult to distinguish, 25 μCi per milliliter was used in subsequent studies.

The possibility that epithelial surface label was not seen because of a delay in the extracellular deposition of label was investigated by labeling glands for 2 h and chasing in medium containing nonradioactive proline (0.1 mM). No epithelial surface label was seen after 2, 18 or 24 h of chase (Experiment 6, Table I). To assess whether surface label might be masked in some way by the mesenchyme, epithelia labeled in intact glands were isolated both retaining and devoid of a lamina and then recultured either alone for 2 h (Experiments 7 and 8, Table I) or in combination with unlabeled mesenchyme for 18 h (Experiments 9 and 10, Table I). In the latter instance, epithelia, isolated devoid of a lamina, lost their lobular morphology and then reformed clefts and lobules. No label localized to the basal surface of any of these epithelia, despite the restoration of lobular morphology in those recultured 18 h. Finally, when isolated epithelia were labeled for 2 h, no surface proline label was seen regardless of whether the epithelia had been isolated retaining or devoid of a lamina (Experiments 11 and 12, Table I; Fig. 4*c* and *d*).

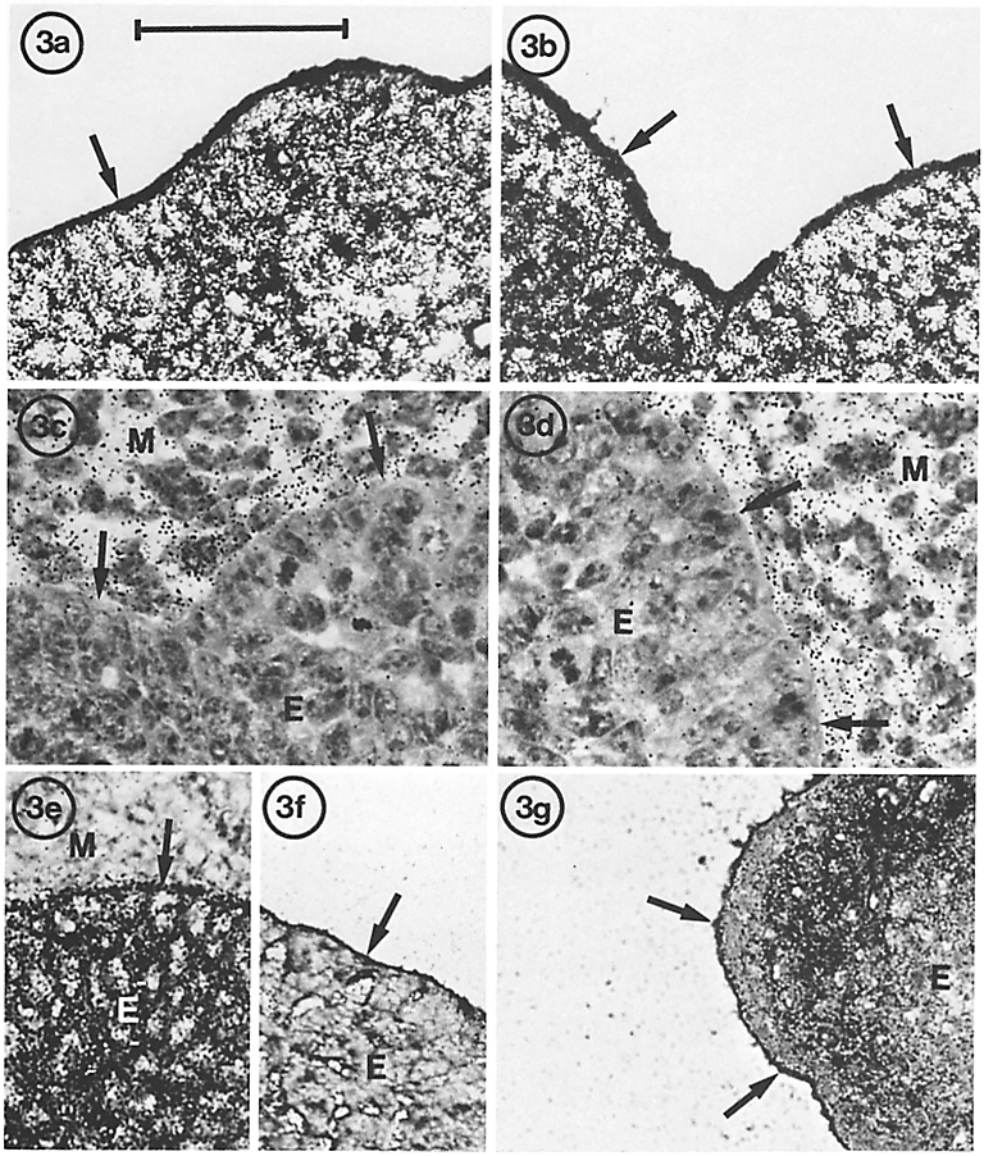


FIGURE 3 [^{3}H]glucosamine autoradiograms of submandibular epithelia isolated free of mesenchyme with collagenase (right column) and of such epithelia treated with hyaluronidase (left column). *M* denotes mesenchyme; *E* denotes epithelium. Bar is 50 μm. × 540. (3*a* and *b*) Isolated epithelia labeled for 2 h. Label accumulates at the surface (arrow) of the rudiments. (3*c* and *d*) Unlabeled isolated epithelia cultured combined with prelabeled mesenchyme for 2 h. No label localizes to the epithelial surface (arrow) of either rudiment, while mesenchyme shows label. (3*e-g*) Epithelia pre-labeled as intact glands before isolation and reculture in medium containing nonradioactive glucosamine for 2 h. Hyaluronidase-treated epithelia redeposit label at their surfaces (arrows) whether recultured in the presence (3*e*) or absence (3*f*) of mesenchyme. Collagenase-isolated epithelium (3*g*) retains surface label (arrow) during reculture period.

TABLE I

[^{3}H]Proline Autoradiographic Experiments

Experiment	Label Period	Post-Labeling Procedures		
		Enzyme*	Chase	Duration
	h			*h*
Intact glands				
1‡	2			
2‡	24			
3‡	2 (after 22 h culture)			
4§	2			
5§	24			
6§	2		proline	2, 18, or 24
7‖	2	C + H	proline	2
8‖	2	C	proline	2
9‖	2	C + H	mesenchyme	18
10‖	2	C	mesenchyme	18
Isolated epithelia§*				
11 C	2			
12 C + H	2			

* C represents collagenase-isolated epithelia; C + H represents collagenase-isolated and hyaluronidase-treated epithelia.

‡ Labeling in proline-free medium at 25 and 50 μCi/ml.

§ Labeling in culture medium at 25 μCi/ml.

‖ Labeling in proline-free medium at 25 μCi/ml.

Morphologic Function of the Basal Lamina

Characteristic lobular morphology is rapidly lost when epithelia isolated devoid of a lamina are immediately cultured in association with mesenchyme (5). The fact that a lamina, complete with GAG, is deposited by such epithelia during a 2-h incubation in the absence of mesenchyme suggested that allowing redeposition of a lamina during a brief preculture period might prevent the loss of lobular morphology. To assess this possibility, collagenase-isolated epithelia stripped of their lamina with hyaluronidase were incubated either on top of a Millipore filter support or immersed in medium for 2 h before culture combined with mesenchyme. Isolated epithelia retaining and devoid of a lamina but not precultured were assessed concurrently as controls.

As previously reported, epithelia retaining the lamina maintain their lobular shape when cultured in association with mesenchyme (Fig. 5), and epithelia stripped of the lamina with hyaluronidase lose their shape and round up within 6 h of culture (Fig. 6), although branching morphogenesis subsequently resumes. Allowing the stripped epithelia to redeposit a lamina before recombination with mesenchyme prevents the loss of lobular morphology in ~ 80% of the cases (Fig. 7, Table II), regardless of whether the 2-hr preincubation was done on a filter or in medium. Lobular morphology is retained after treatment of the preincubated epithelia with collagenase, but is lost after treatment of these epithelia with hyaluronidase (Table II). Thus, in the maintenance of epithelial morphology, the newly deposited, epithelially derived lamina and the lamina produced in intact glands function identically.

Ultrastructural Changes Associated with Removal and Replacement of Surface Materials

To clarify the morphogenetic effects of the enzyme treatments, ultrastructural observations were made on collagenase-isolated epithelia incubated for 10 min in CMFT either with or without hyaluronidase and on hyaluronidase-treated epithelia after 2 h culture in the absence of mesenchyme.

COLLAGENASE-ISOLATED EPITHELIA (FIGURE 8): The lamina retained on epithelia isolated in collagenase has ultrastructural features identical to those seen in intact glands (6) except

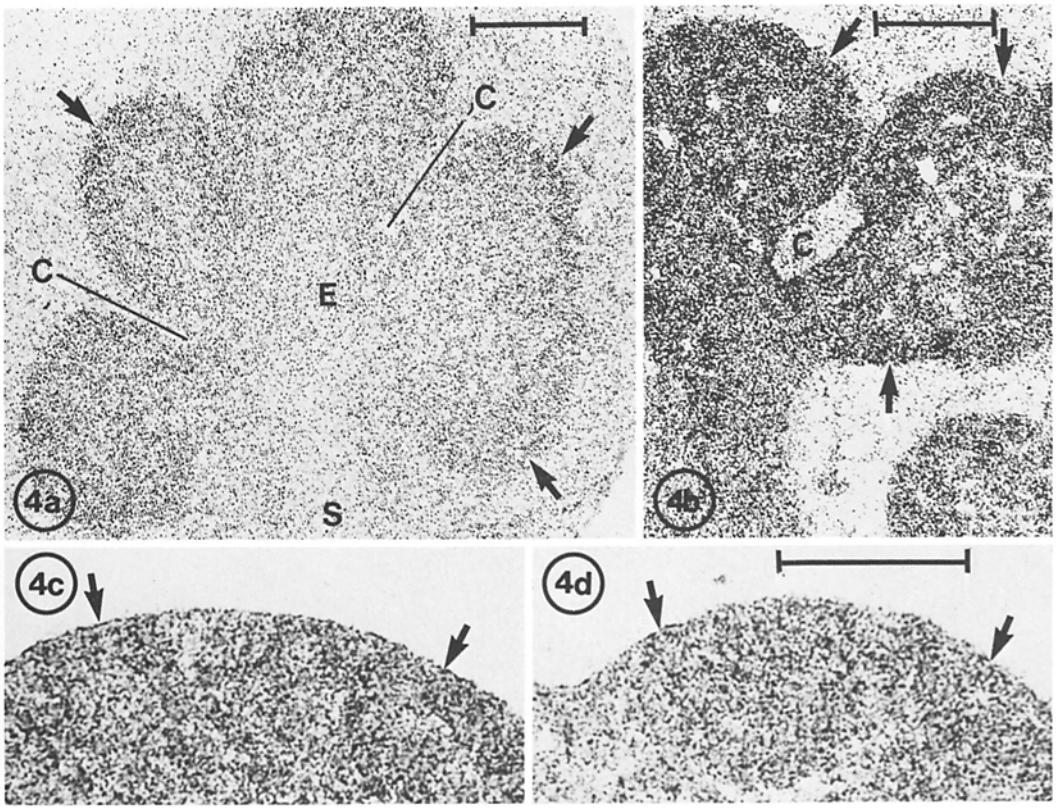
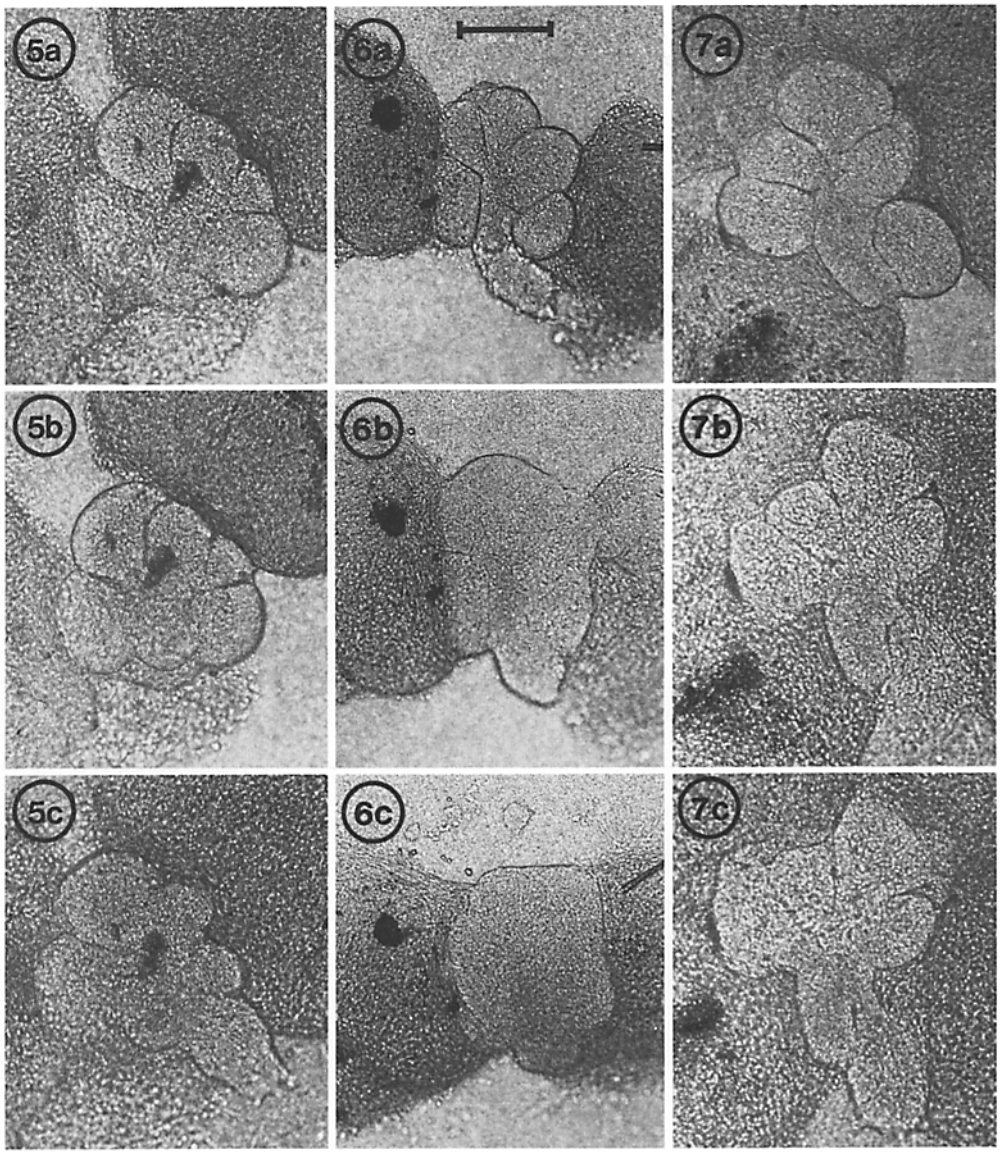


FIGURE 4 [^{3}H]proline autoradiograms of submandibular glands. Tissues were labeled with 25 μCi per ml [^{3}H]-2,3-proline in culture medium. Bars are 50 μm. (4*a*) Intact gland labeled for 2 h. Label accumulates within the epithelium (*E*), but none localizes to the epithelial surface (arrows) either at the distal aspects of the lobules or within the clefts (*C*). *S* is the epithelial stalk. × 190. (4*b*) Intact gland labeled for 24 h. The epithelium is heavily labeled. Label localizes to the epithelial surface (arrows) only deep within clefts (*C*) and at the base of lobules (lower arrow). × 300. (4*c*) Epithelium isolated free of mesenchyme in collagenase and then labeled for 2 h. No label accumulates at the epithelial surface (arrows). Compare with Fig. 3*b*. × 540. (4*d*) Collagenase-isolated epithelium treated with hyaluronidase and then labeled for 2 h. No label accumulates at the epithelial surface (arrows). Compare with Fig. 3*a*. × 540.

that the fibrillar materials are nearly absent, with small amounts being apparent only deep within clefts. The epithelial cells are closely adherent, and within the cells linear arrays of microfilaments are seen as previously described in intact glands (41). The filamentous arrays are up to 0.2 μm in thickness and consist of individual filaments 5–7 nm in diameter. The arrays are present in both apical and basal regions of the cells and are most prominent at the distal ends of the lobules. In the basal region of the cell, they are subjacent to the plasmalemma and can be seen to extend from one side of the cell to the other, appearing to insert into electron-dense junctional complexes. Microtubules were frequently visible and were mostly arranged approximately parallel to the long axis of the cell. A small cellular protrusion frequently seen was invariably associated with a small portion of the adjacent cell which extends over the protrusion. The similarity in the ultrastructure of these epithelia and those of intact glands indicates that removal of mesenchyme in collagenase and brief incubation in CMFT does not appreciably alter cellular architecture.

HYALURONIDASE-TREATED EPITHELIA (FIGURE 9): Epithelia treated with hyaluronidase were almost completely devoid of a basal lamina, and no mesenchymal cells were ever seen in these preparations. The cells were rounded and were loosely adherent to each other. Villus-like projections of the plasmalemma containing 5–7-nm filaments frequently oriented in parallel with



FIGURES 5–7 Living submandibular epithelia isolated free of mesenchyme and then cultured in combination with pieces of fresh mesenchyme on Millipore filters. In each case, (*a*) is immediately after explantation, (*b*) is after 4 h of culture, and (*c*) is after 6 h of culture. Bar is 200 μm. × 62. (5*a–c*) Collagenase-isolated epithelium. Lobular morphology is maintained in culture. (6*a–c*) Collagenase-isolated epithelium treated with hyaluronidase. Lobules are rapidly lost during culture. (7*a–c*) Epithelium isolated with collagenase, treated with hyaluronidase and then pre-incubated for 2 h submerged in medium before culture. Lobular morphology is maintained during culture.

the axis of the projection were prominent at the apical surface and also at the basal surface where junctional complexes had been disrupted. Within the cell cortex, the linear arrays of filaments were no longer seen. Patches of short filamentous material with no apparent orientation were evident near the basal plasmalemma and, with lower frequency, within the surface projections. There was a reduction in the number of longitudinally arranged microtubules but there was no indication of alterations in other cytoplasmic organelles, such as alterations in the organization or structure of

TABLE II

Maintenance of Epithelial Morphology after 6 h Culture Combined with Mesenchyme

Enzyme treatment of collagenase-isolated epithelium	Number of Cultures	Lobular Morphology	
		Maintained	Lost
A. None	35	33	2
B. Hyaluronidase	31	0	31
C. Hyaluronidase plus 2-h preincubation:			
On filter	23	18	5
In medium	46	37	9
D. As (C), then treated with:			
Collagenase	5	4	1
Hyaluronidase	5	1	4

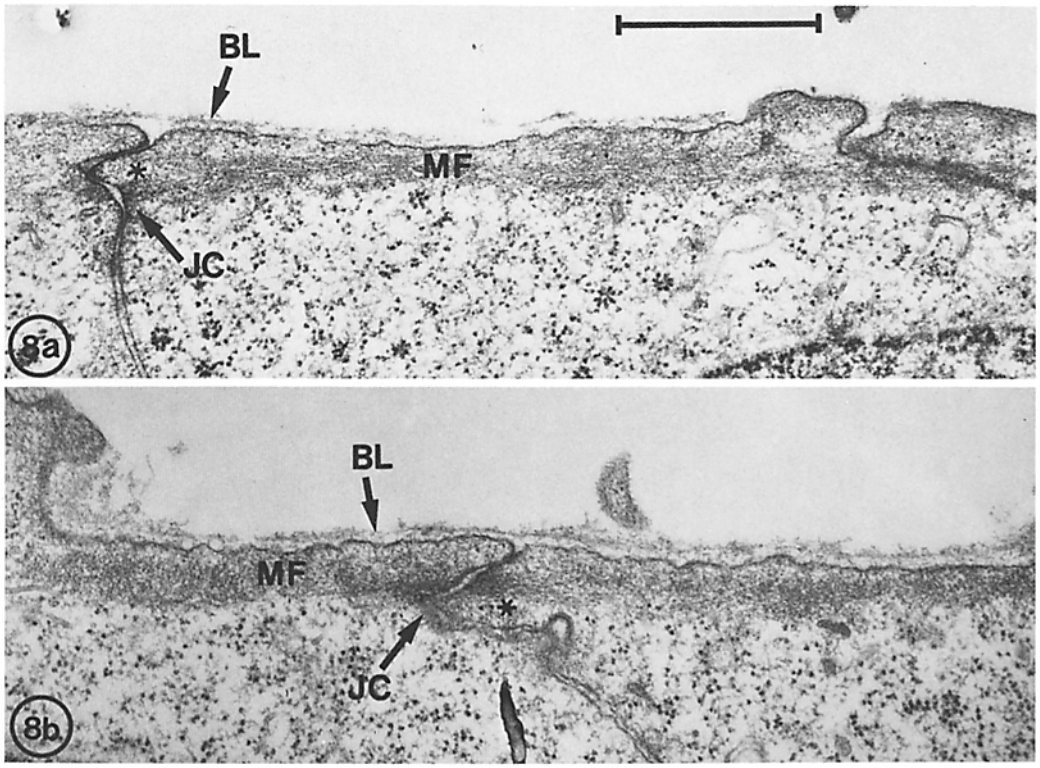


FIGURE 8 Electron micrographs of submandibular epithelia isolated free of mesenchyme with collagenase and then incubated in CMFT for 10 min. *BL* represents the basal lamina, *MF* represents microfilaments, and *JC* is the junctional complex. Bar is 1 μm. × 27,000. (*8a*) Section near the surface of the epithelium showing an area where the basal lamina is sparse. The cells are closely adherent and linear arrays of microfilaments subjacent to the plasmalemma are frequently observed extending from one junctional complex to the other. At regions where the microfilament arrays appear to insert into the junctional complexes, small lateral protrusions (*) of the cell into the adjacent cell are observed. (*8b*) Section showing an area where the lamina completely covers the basal epithelial surface. Microfilament arrays and junctional complex are similar to those in *8a*.

polyribosomes or mitochondria. These observations indicate that, in addition to removal of the lamina, treatment with 50–75 ng per milliliter hyaluronidase in CMFT is associated with rapid changes in cell shape, reduction in cellular apposition, formation of microvilli, and a change in the organization of cytoskeletal structures. Although the epithelia remain multilobular immediately

after the treatment, these alterations might account for the loss of lobular morphology which subsequently occurs when these epithelia are cultured in combination with mesenchyme.

Hyaluronidase treatment was performed in CMFT since treatment in MFT or in Tyrode's solution, even at substantially higher enzyme concentrations, failed to remove the lamina, to cause the cellular alterations, to remove surface GAG and to cause a loss of lobular morphology. Indeed, epithelia treated with hyaluronidase in complete Tyrode's solution appeared and behaved like collagenase-isolated epithelia. This effect of calcium was investigated by examining the influence of calcium on the enzymatic digestion of hyaluronic acid and chondroitin sulfate in Tyrode's solution. In hyaluronidase-catalyzed reactions, hyaluronic acid was digested at identical rates in complete and calcium-free Tyrode's solution. However, the digestion rate of chondroitin sulfate was markedly reduced when calcium was included in the reaction (Figure 10).

To assess the nature of this inhibition, the effect of calcium in Tyrode's solution was studied in reactions catalyzed by chondroitinase ABC, which is assayed differently and has an enzymatic mechanism distinct from that of hyaluronidase. While calcium did not affect the rate of chondroitinase ABC-catalyzed digestion of hyaluronic acid, it inhibited that of chondroitin sulfate. When the reactions were studied at varying calcium concentrations, the rate of digestion of chondroitin sulfate by both enzymes was reduced almost identically (Figure 11), suggesting that the inhibition is due to the interaction of calcium with chondroitin sulfate.

HYALURONIDASE-TREATED EPITHELIA AFTER 2 H CULTURE (FIGURE 12): Culturing of hyaluronidase-treated epithelia for 2 h resulted in epithelia which ultrastructurally closely resembled epithelia freshly isolated in collagenase. The basal surface showed a newly deposited lamina which did not completely encompass the epithelium, and there was no evidence of fibrillar materials. Cells were closely adherent along their lateral and apical surfaces, columnar in appearance, and showed very few villus-like projections. Those projections observed were on the basal surface near junctional complexes. Linear arrays of 5–7-nm filaments were seen appearing to insert into densely stained junctional complexes. Very few patches of nonorganized filamentous material were seen. Intact junctional structures and organized filamentous arrays were seen even at areas where the lamina was incomplete. These findings indicate that the rapid disorganization of cellular architecture induced by hyaluronidase treatment in CMFT is reversible and is temporally correlated with redeposition of a basal lamina.

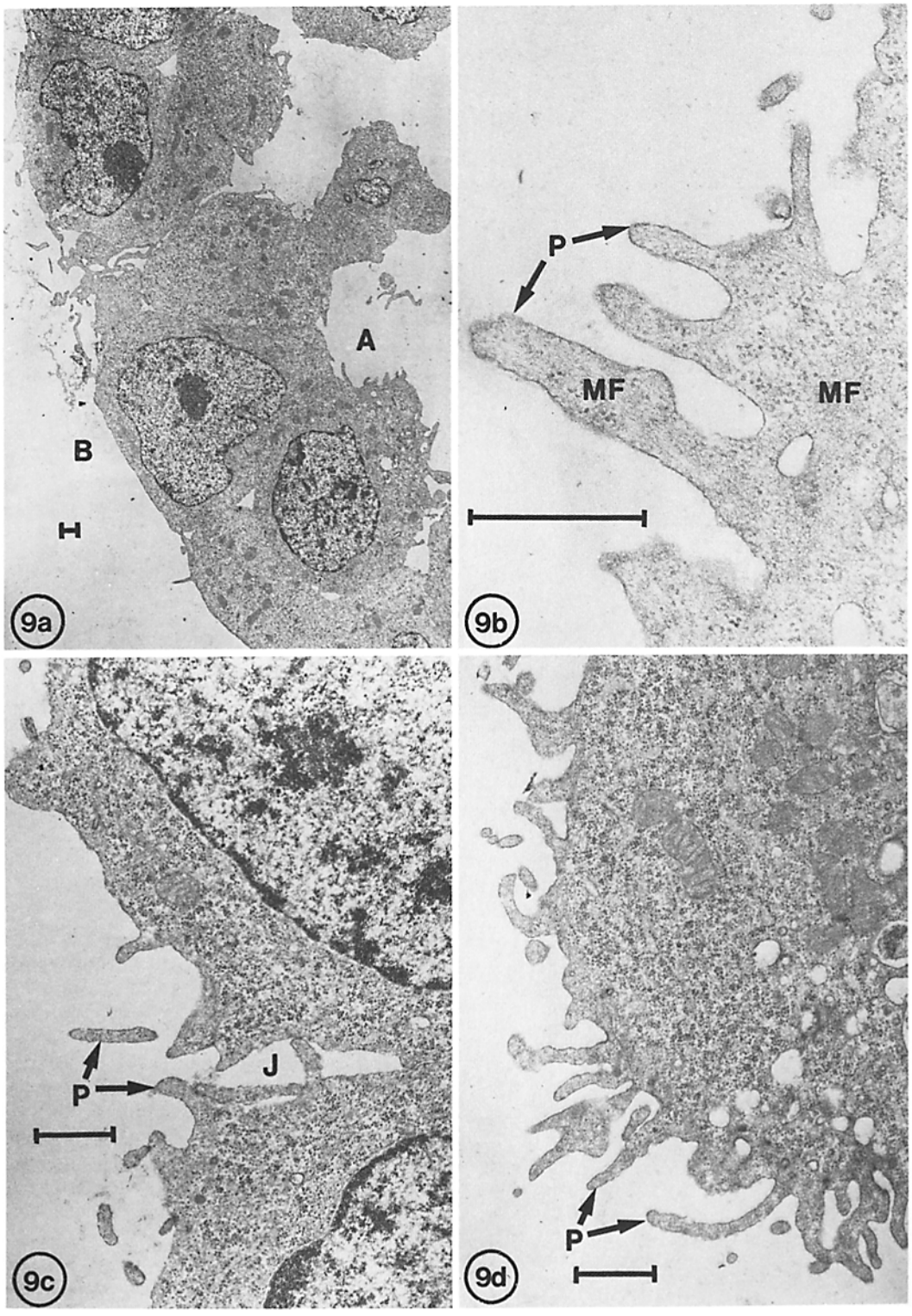
DISCUSSION

This study was performed to assess the origin and role of the basal lamina and the mesenchyme in the maintenance of epithelial morphology. The lamina is removed upon brief treatment of isolated epithelia with small amounts of hyaluronidase and normal cellular architecture is lost. During culture in the absence of mesenchyme or biological substrata, these epithelia restore their cellular architecture and deposit a lamina which is analogous, by ultrastructure as well as by glucosamine and proline autoradiography, to the preexisting lamina. Hyaluronidase-treated epithelia lose their lobular morphology when cultured with mesenchyme, but the loss of morphology is prevented by delaying combination with mesenchyme until the lamina and cellular architecture are restored. The lamina, therefore, is derived from the epithelium and appears to provide it with morphologic stability. The mesenchyme is not directly involved in the synthesis or deposition of the lamina, nor is it required for restoration of the cellular alterations induced by hyaluronidase treatment. These data imply that the mesenchyme is not immediately involved in the stabilization of epithelial morphology. Rather, it is likely that the mesenchyme, as well as inductive influences attributable to it, affects processes involved in the changes in epithelial morphology which occur during development.

Origin of the Basal Lamina

Although an epithelial origin of the basal lamina has been reported previously for several embryonic tissues, including the lens (23), cornea (21), pigmented retina (35), neural tube (9), notochord (27), and early myocardium (24), the rapidity with which a lamina can be produced has not been appreciated. Since labeling of GAG at the epithelial surface seems to be an index of lamina deposition (5, 11), and since several epithelia which undergo branching morphogenesis show surface GAG labeling within 2 h,[1] rapid production of

[1] Banerjee, S. D., and M. R. Bernfield. 1976. Common pattern of distribution of total and newly synthesized glycosaminoglycan at the basal surfaces of embryonic epithelia. Submitted for publication.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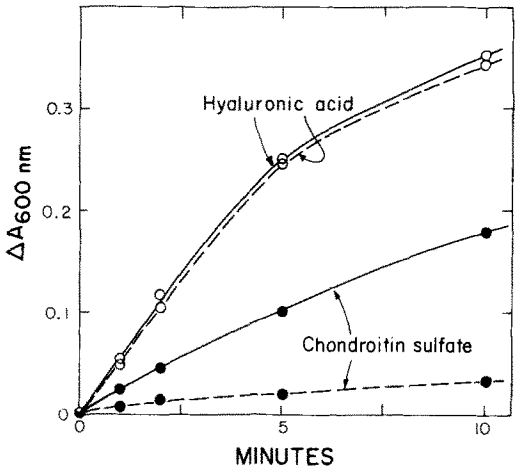


FIGURE 10 Rate of digestion of hyaluronic acid and chondroitin sulfate by testicular hyaluronidase in complete and calcium-free Tyrode's solution. Reactions of the polysaccharides (250 μg/ml) with hyaluronidase (0.425 μg/ml for hyaluronic acid; 6.75 μg/ml for chondroitin sulfate) were followed turbidimetrically. The change in absorbancy at 600 nm (ΔA_{600nm}) was calculated from the turbidity measured at A_{600nm} in a control reaction mixture containing no enzyme. (——) complete Tyrode's solution (1.8 mM calcium); (——) calcium-free Tyrode's solution.

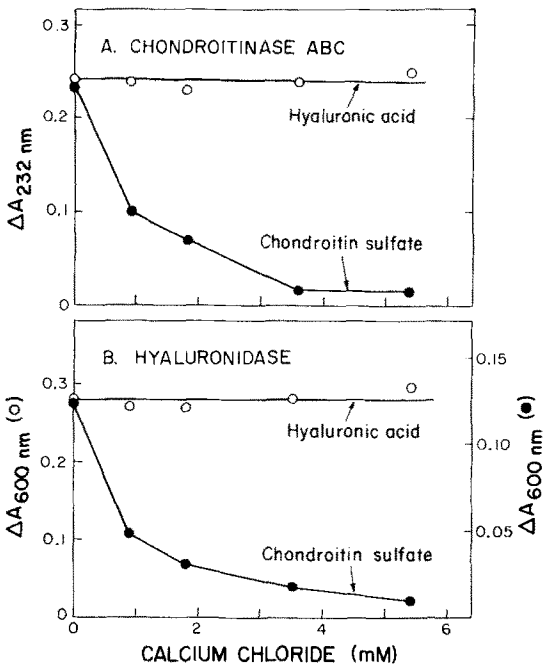
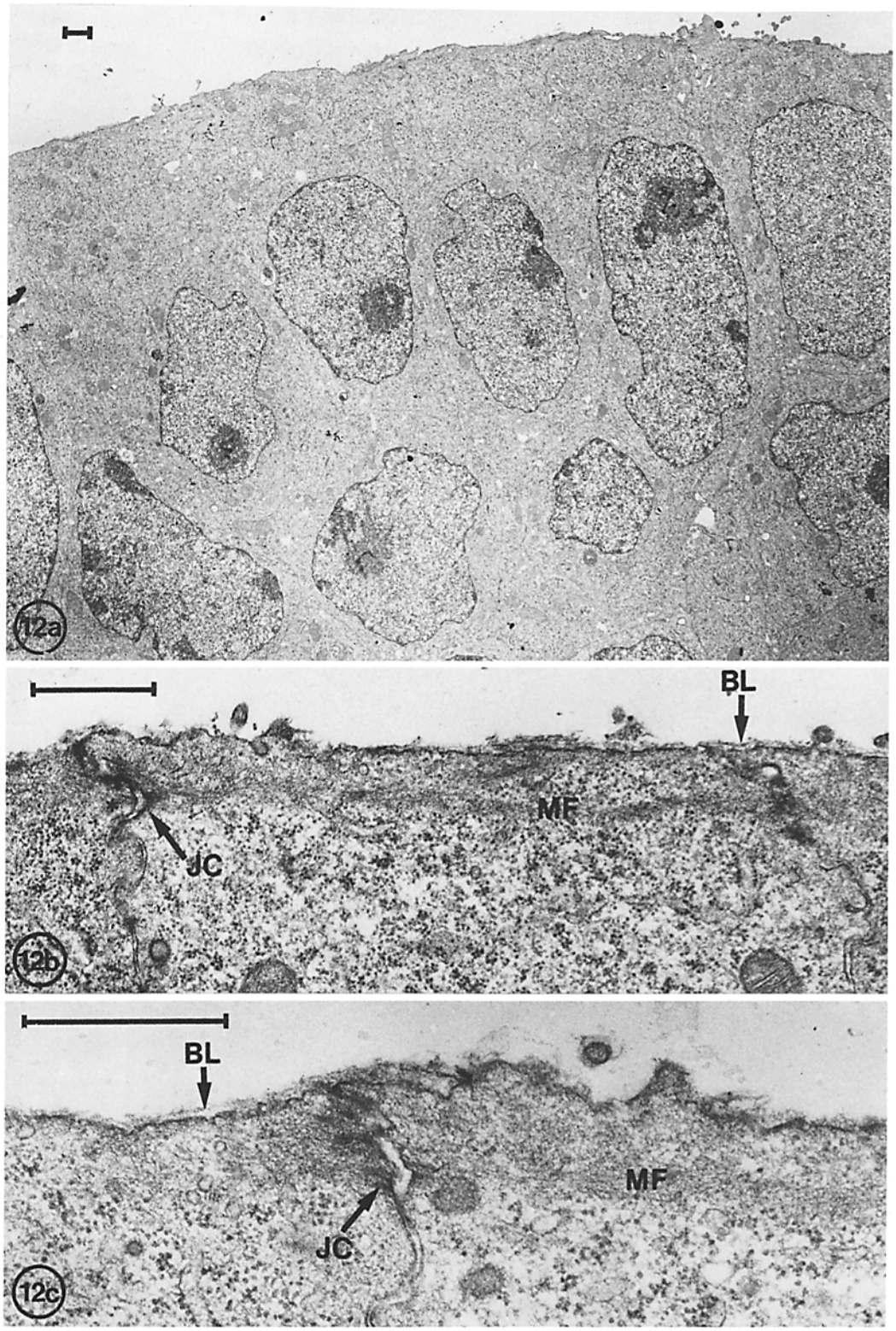


FIGURE 11 Effect of calcium concentration on the digestion of hyaluronic acid and chondroitin sulfate by chondroitinase ABC and testicular hyaluronidase. Incubations were performed in Tyrode's solution containing the indicated $CaCl_2$ concentrations. (A) Reactions of polysaccharides (1 mg/ml) with chondroitinase ABC (0.25 U/ml) were carried out for 3.5 min and were followed by the production of unsaturated disaccharides at 232 nm. (B) Reactions of polysaccharides with hyaluronidase were carried out for 5 min and were assayed as described in Fig. 10.

the basal lamina may be widespread among embryonic organs.

The finding that a basal lamina is produced in the absence of a biological substratum directly conflicts with studies of the chick cornea (16, 21). In these studies, a basal lamina was formed only in the presence of a collagenous substratum. After culture on a Millipore filter, neither basal lamina nor fibrillar collagen were evident near the epithelial surface. These and subsequent studies of the cornea have suggested that corneal epithelial cells may have receptors for collagen since they respond to the presence of collagen with 2-3-fold stimulation of precursor incorporation into collagen and GAG (31, 32). In the absence of stimula-

FIGURE 9 Electron micrographs of submandibular epithelia isolated free of mesenchyme with collagenase and then incubated with hyaluronidase in CMFT for 10 min. Bars are 1 μm. (9*a*) At low magnification, cells at the basal surface (*B*) are seen to be loosely adherent along their lateral and apical (*A*) surfaces and appear round. Small surface projections are seen at both the basal and apical cell surfaces. × 3100. (9*b*) Higher magnification view of the basal epithelial surface. Projections (*P*) of the basal plasmalemma are seen which contain 5-7 nm filaments (*MF*) running parallel to the long axis of the projection. Short patches of filaments (*MF*) are also seen which show less organization than the arrays of filaments seen in Fig. 8. × 28,00. (9*c*) A section at the basal end of the cells. Spaces between cells appear where junctional complexes are usually seen (*J*). Projections (*P*) of the plasmalemma are most numerous at these sites. × 12,100. (9*d*) A section at the apical end of the cells. The apical plasmalemma forms numerous projections (*P*) which appear to contain 5-7 nm filaments. × 12,100.


12a
BL
MF
JC
12b
BL
MF
JC
12c

tion, there is appreciable incorporation, but extracellular matrix materials do not accumulate. In those studies, as in the present work, the epithelia were stripped of their extracellular materials by enzyme treatments, and, as previously indicated (32), the stimulation observed may have been only augmented recovery from the tissue isolation procedure. An alternative explanation, however, is that there is continual turnover of extracellular materials and that when exogenous or preexisting matrix components are absent, newly synthesized materials are rapidly degraded and therefore would not be seen ultrastructurally.

Collagen within the Basal Lamina

Epithelially derived collagen is a component of the basal lamina of nonembryonic epithelia and of epithelia which do not show repetitive folding during development. Our inability to label the basal epithelial surface with [^{3}H]proline under conditions in which epithelia localize [^{3}H]glucosamine to the surface and produce an ultrastructurally and functionally normal lamina is puzzling, but does not necessarily mean that collagen is absent from the submandibular lamina. It is possible that *de novo* proline synthesis produces high intracellular pools that mask our ability to label the lamina. Our findings could also result from slow secretion or processing of procollagen, markedly slower than that required for GAG. Indeed, there is known to be a delay of at least 60 min between the incorporation by embryonic cells of [^{3}H]proline and the secretion of basement membrane collagen (8). We were unable, however, to chase proline label onto the epithelial surface. After an attempt to minimize possible differences in precursor transport rates and pool sizes by labeling for 24 h, proline label at the epithelial surface was found only deep within interlobular clefts. These are the sites of most abundant fibrillar collagen as seen ultrastructurally, suggesting that extracellular deposition of certain types of collagen can be observed by the methods used here.

It is conceivable that organ-specific differences exist in the amount of epithelially derived collagen, in its rate of deposition into the lamina, or in the rate and extent of its degradation, any of which would affect the extent to which this collagen can be studied by [^{3}H]proline autoradiography. There is some evidence, however, that is consistent with the notion that the submandibular basal lamina may not contain an appreciable amount of collagen. As confirmed here, Kallman and Grobstein (25) found that although isolated submandibular epithelia label heavily with [^{3}H]proline and [^{3}H]glycine, no label localized to the epithelial surface. Isolated submandibular epithelia produce no or exceedingly small amounts of collagen measured as polypeptide [^{3}H]hydroxyproline despite incubation with [^{3}H]proline for 40 h (3). Under identical labeling conditions, substantial collagen is synthesized by the mesenchyme. Although collagen within the lamina may have a reduced susceptibility to *Clostridial* collagenase, epithelia isolated with purified collagenase retain a basal lamina, and collagenase treatment of epithelia that have a newly deposited lamina apparently does not remove the lamina as assessed by the subsequent behavior of the epithelia in organ culture. Proline analogues (azetidine-2-carboxylic acid and 3,4-dehydroproline) which inhibit procollagen secretion, or β-aminoproprionitrile, an inhibitor of collagen cross-linking, might be expected to alter the production of a highly collagenous lamina (7). However, neither the site nor the amount of [^{3}H]glucosamine labeling of the epithelial surface is altered by treatment of intact submandibular glands with these drugs at doses and durations of treatment which interfere

FIGURE 12 Electron micrographs of submandibular epithelia isolated free of mesenchyme with collagenase, incubated with hyaluronidase in CMFT for 10 min and then cultured for 2 h in the absence of mesenchyme. Bars are 1 μm. (12*a*) Low magnification view of cells near the basal epithelia surface. The cells appear to be in closer proximity and more columnar than those in Fig. 9*a*. Projections of the plasmalemma are not apparent. × 4300. (12*b* and *c*) Higher magnification views of the basal epithelial surface. A basal lamina (*BL*) is seen covering most of the surface. Linear band-like arrays of microfilaments (*ME*) are seen subjacent to the plasmalemma extending from one lateral junctional complex (*JC*) to the other. Small lateral protrusions (*) of the cell into adjacent cells are frequently observed at the region of the junctional complex. 12*b* × 19,000; 12*c* × 32,00.

with morphogenesis in other systems.[2]

These results are not conclusive, but they do raise the possibility that the laminae of epithelia that undergo repetitive branching, such as submandibular epithelia, may differ from the laminae of other epithelia either in their collagen content or in their rate of collagen accumulation. Because GAG is readily demonstrated in the laminae of branching embryonic epithelia,[1] while collagen predominates in the laminae of adult organs whose branching morphogenesis has been completed (26), this difference could reflect a developmental transition in which the basal lamina changes from being GAG-rich to being collagen-rich.

Effects of Hyaluronidase Treatment

Brief treatment at room temperature of collagenase-isolated epithelia with 50–75 ng per milliliter hyaluronidase in CMFT (pH 7.4–7.6) removes the lamina and causes rounding-up of the cells, loss of cellular cohesion, appearance of microvilli, and changes in the organization of cytoskeletal structures. These effects apparently result from cleavage of highly susceptible bonds because low levels of hyaluronidase were effective at a pH value where the enzyme has substantially reduced activity (33). The absence of calcium is necessary for the effects, since, under identical conditions but in calcium-containing Tyrode's solution, the lamina is not removed and the cellular alterations do not occur. Absence of calcium alone, however, is insufficient. Incubation of collagenase-isolated epithelia in calcium-free Tyrode's solution (under identical conditions but without enzyme) results in neither the removal of the lamina nor any other discernible effect. Therefore, both hyaluronidase and calcium-free medium are required for removal of the lamina and production of the cellular alterations.

The requirement of calcium-free medium may be due to the difference in susceptibility of chondroitin sulfate to enzymatic digestion in the presence and absence of calcium. The rate of chondroitin sulfate digestion by both hyaluronidase and chondroitinase ABC is markedly reduced in Tyrode's solution compared to the rate of digestion by these enzymes in calcium-free Tyrode's solution. Because chondroitin sulfates are *bona fide* components of the lamina (11) and are known to bind calcium (17), the lamina may be stabilized to enzymatic attack in the presence of calcium.

This explanation does not exclude the possibility that the absence of calcium during hyaluronidase treatment may have additional effects on the epithelium. The experiments, however, do not permit us to distinguish between the effects of calcium-free medium and the direct effects of the enzyme. It is possible that the absence of calcium during hyaluronidase treatment allows rapid egress of calcium from the cells with attendant changes in cellular architecture and organization. If this suggestion is correct, the lack of effect of calcium-free medium alone implies that materials removed by hyaluronidase may be involved in maintaining normal intracellular calcium concentrations. Calcium-free medium and papaverine, an agent thought to block calcium entry into cells, cause a loss of lobular morphology after prolonged treatment of intact submandibular glands, but no ultrastructural alterations are seen during papaverine treatment (1). Therefore, regardless of the possible effects of the absence of calcium, maintenance of cellular architecture and the normal appearance of junctions and cytoskeletal elements appear to be dependent on hyaluronidase-susceptible materials.

The Basal Lamina and Morphologic Stability

Assuming that the primary effect of hyaluronidase treatment is the removal of the lamina, the changes induced by hyaluronidase may reveal the function of the lamina in maintaining morphology. The cellular alterations observed following hyaluronidase treatment are comparable to those occurring in single cells during their detachment from a plastic or glass substratum (19). These cells rapidly round-up, form microvilli, show reduced numbers of microtubules, and convert bundles of microfilaments into meshworks of subplasmalemmal filamentous and granular materials (18). These changes reverse within less than an hour upon restoration of cell-substratum contacts. The similarity between the ultrastructural features and the speed of occurrence and reversibility of these changes and those occurring in the epithelia implies that removal of the lamina results in loss of attachment or adhesion sites at the basal surfaces of the epithelial cells. It has been emphasized by Vracko (47) that the basal lamina confers morphologic stability by providing a substratum for

[2] Banerjee and Bernfield, unpublished observations.

cell attachment and adhesion. This role for the lamina is analogous to that proposed for the GAG rich material found at sites of cellular attachment to the substratum (10, 13, 38), and, like components of the lamina, this material is hyaluronidase-susceptible and is thought to bind calcium (42).

Relationship between Mesenchyme, Extracellular Materials, and Epithelial Morphogenesis

Only epithelia that have preexisting laminar materials at their surfaces and normal cellular architecture maintain lobular morphology when cultured in association with mesenchyme. Morphology is lost when hyaluronidase-treated epithelia are combined with mesenchyme before redeposition of a lamina and restoration of normal architecture. Thus, the mesenchyme has some property or activity which seems to inhibit epithelial recovery from the treatment. Although the putative property interferes with the maintenance of morphology after hyaluronidase treatment, it may be involved in the role of the mesenchyme during normal morphogenesis. Direct contact between epithelial and mesenchymal cells is possible when the lamina is absent, but contact per se is probably not the cause of the inhibition because epithelial cell processes normally protrude through the basal lamina into areas where they directly contact mesenchymal cells (12, 14, 29), and because cell contact between epithelium and mesenchyme may be required for morphogenetic interactions (28, 32, 48).

A plausible explanation for the data is that the mesenchyme possesses an activity that degrades the basal lamina, which, in turn, is required for maintaining cellular architecture and lobular morphology. This explanation takes into account the fact that the hyaluronidase-treated epithelia which maintain morphology differ from those which lose morphology by a 2-h preincubation period before culture with mesenchyme. Epithelia cultured without preincubation lack preexisting surface materials, possibly allowing mesenchymal degradation of the new laminar materials produced by the epithelium during culture. Accumulation of a new lamina would be retarded and loss of morphology would result. On the other hand, because of the presence of a preexisting lamina, a much lower proportion of newly deposited surface materials would be degraded on epithelia preincubated for 2 h before culturing with mesenchyme. Degradation would be offset by new deposition, the lamina would be preserved, and loss of lobular morphology would be prevented.

If the degradation involves GAG, this explanation is consistent with our prior suggestion that GAG within the epithelial basal lamina of several embryonic organs undergoes continual turnover.[1] These considerations lead to the hypothesis that the mesenchyme may influence changes in epithelial morphology by degrading extracellular materials. A mechanism for selective degradation should be involved to account for the localized differences in turnover rates previously suggested,[1] and possibly for mesenchymal specificity.

We thank Barbara Gray and Suzanne Tharpe for expert technical assistance.

This work supported by National Institutes of Health grant HD-06763.

Received for publication 3 June 1976, and in revised form 28 December 1976.

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