BASAL LAMINA OF EMBRYONIC SALIVARY EPITHELIA

Nature of Glycosaminoglycan and Organization

of Extracellular Materials

RONALD H. COHN, SHIB D. BANERJEE, and MERTON R. BERNFIELD

From the Department of Pediatrics, Stanford University School of Medicine, Stanford, California 94305. Dr. Cohn's present address is the Department of Medicine, Veterans Administration Hospital, Palo Alto, California 94304.

ABSTRACT

The ultrastructural organization and the composition of newly synthesized glycosaminoglycan (GAG) in the epithelial basal lamina of mouse embryo submandibular glands were assessed. The labeled GAG accumulating in the lamina is distinct from that in its tissue of origin, the epithelium, or from that in the surrounding mesenchyme. In the lamina, hyaluronic acid accounts for ~50% of the labeled GAG, chondroitin-4-sulfate is twice the chondroitin-6-sulfate, and there is a low proportion of chondroitin. This composition is constant regardless of whether the lamina is labeled by whole glands or, in the absence of mesenchyme, by isolated epithelia retaining a lamina and by isolated epithelia generating a lamina de novo. These results indicate that the labeled GAG are bona fide components of the lamina, and suggest that laminar GAG is deposited in units of constant composition. Ultrastructural observations following ruthenium red staining or tannic acid fixation establish that the lamina is a highly ordered specialization of the basal cell surface. Discrete structures in macroperiodic arrays apparently attached to the plasmalemma are visualized. This organization is seen in intact glands and in the laminae produced by epithelia in the absence of mesenchyme or biological substrata. The data are interpreted as indicating that the basal lamina contains supramolecular complexes of hyaluronic acid and proteoglycan which are organized into an extracellular scaffolding which imposes structural form on the epithelium.

Extracellular matrix materials have frequently been implicated in the process of folding, budding, and branching that embryonic epithelial organs undergo during development. The materials include collagen, glycosaminoglycan (GAG) and other components, principally glycoprotein. The well-known interaction between collagen and GAG has led to the suggestion that self-assembly of these materials may play a role in the development of epithelial organ form (15). Additionally, epithelial morphogenesis requires the close association of mesenchymal tissues. Between these interacting tissues lies the epithelial basal lamina, a complex of matrix materials derived from the epithelium and intimately associated with the basal epithelial cell surface. The lamina of several embryonic epithelia contains collagen of a specific type (reviewed in reference 8) as well as GAG

(reviewed in reference 4). We have suggested that the lamina imposes morphologic stability on the epithelium, while the mesenchyme is involved in the changes in morphology which occur during development (2).

In an attempt to understand the morphogenetic role of the basal lamina, we have focused upon GAG within the lamina of mouse submandibular epithelia because, in this rudiment, removal of the lamina by enzymes which degrade GAG is associated with a loss of epithelial morphology, and the amount of GAG and the rate of accumulation of labeled GAG in the lamina vary with changes in morphology (4). This paper reports analyses of the types of GAG deposited in the lamina by isolated epithelia and by epithelia in intact glands, and observations of the ultrastructural organization of the lamina after ruthenium red staining and after tannic acid fixation. The results suggest that the lamina is a highly ordered specialization of the basal cell surface, consisting in part of supramolecular complexes of proteoglycan and hyaluronic acid. The implication for morphogenesis is that this organization and composition, which arise independently of the presence of mesenchyme or biological substrata, make the lamina a pliant and malleable extracellular epithelial scaffolding. Preliminary reports of this work have previously appeared (1, 4).

MATERIALS AND METHODS

Materials

Embryonic submandibular glands from the mouse hybrid C57BL/6 × DBA/2 at 13 days of development were obtained and, where indicated, were isolated free of mesenchyme as described in the accompanying paper (2). Chondroitinase ABC, chondro-4-sulfatase, and chrondro-6-sulfatase from *Proteus vulgaris* and the unsaturated disaccharides, Δdi-4S, Δdi-6S and Δdi-OS,[1] were obtained from Miles Laboratories (Miles Research Products, Elkhart, Ind.). The unsaturated disaccharide derived from hyaluronic acid, Δdi-OHA, was prepared

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[1] The abbreviations used are glcNAc, *N*-acetylglucosamine; galNAc, *N*-acetylgalactosamine; and for the unsaturated disaccharides: Δdi-4S, 2-acetamido-2-deoxy-3-O-(β-D-gluco-4-enepyranosyluronic acid)-4-O-sulfo-D-galactose; Δdi-6S, 2-acetamido-2-deoxy-3-O-(β-D-gluco-4-enepyranosyluronic acid)-6-O-sulfo-D-galactose; Δdi-OS, 2-acetamido-2-deoxy-3-O-(β-D-gluco-4-enepyranosyluronic acid)-D-galactose; Δdi-OHA, 2-acetamido-2-deoxy-3-O(β-D-gluco-4-enepyranosyluronic acid)-D-glucose.

as previously described (5). [3-³H]Glucose (6.7 Ci/mmol) and [6-³H]glucosamine (3.6 Ci/mmol) were obtained from New England Nuclear (Boston, Mass.). Pronase, B grade, was obtained from Calbiochem (San Diego, Calif.). Trypsin (3×, crystalline) was obtained from Worthington Biochemical Corp. (Freehold, N. J.) and tannic acid from Fisher Scientific Co. (Pittsburgh, Pa.). Other enzymes and reagents were as described in the accompanying paper (2).

Electron Microscopy

ROUTINE PREPARATIONS: Tissues were washed in Tyrode's solution and then fixed in filtered (Millipore Corp., Bedford, Mass.) 2% biological grade glutaraldehyde containing 0.075 M phosphate buffer at pH 7.2 (buffer A) for 2 h at room temperature. When necessary after fixation, rudiments were stored at 4°C in 0.18 M sucrose containing buffer A. Post-fixation was carried out for 1.5 h at room temperature in 1% OsO₄ freshly mixed into buffer A containing 0.18 M sucrose. The tissues were washed with water and dehydrated in a graded series of ethanol followed by propylene oxide and embedded in Epon. All tissue sections were obtained on an LKB ultramicrotome II with a diamond knife (LKB Instruments, Rockville, Md.) and examined in a Hitachi HS-8 electron microscope. Routine preparations were placed on 150-mesh Formvar-coated grids and stained with uranyl acetate followed by lead citrate.

RUTHENIUM RED STAINING: Ruthenium red was dissolved at 0.1% in quartz distilled water within 24 h before use and stored at 4°C. Immediately before tissue fixation, a fixing solution was prepared which contained 0.05% ruthenium red and 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.5; buffer B) after which it was passed through a Millipore filter. The tissues were washed in Tyrode's solution, placed in the fixing solution for 4 h at room temperature, rinsed twice for 10 min, and stored in 0.25 M sucrose in buffer B. Post-fixation was carried out in freshly prepared 1% OsO₄ in buffer B containing 0.05% ruthenium red for 1.5 h at room temperature in the dark. During this post-fixation period, the fixative changed from red to brown-black. The tissues were then processed as described for routine preparations; however, sections were stained only with lead citrate. For studies of intact glands, epithelia were teased free of most mesenchymal tissue in a solution of horse serum:Tyrode's solution (1:1), leaving only a few cell layers of mesenchyme.

TANNIC ACID FIXATION: Rudiments were fixed in freshly prepared 1% tannic acid and 1% glutaraldehyde in buffer A for 4 h at room temperature and stored in 0.18 M sucrose at 4°C in the same buffer. The tissues were rinsed 3 times for 10 min in the sucrose solution and post-fixed in 1% OsO₄ in buffer A containing 0.18 M sucrose for 1.5 h. The rudiments were rinsed twice for 10 min in 25% ethanol, dehydrated in a graded series of ethanol and embedded in Epon. Sections were stained with lead citrate.

Labeling and Preparation of
Tissue Fractions

Rudiments were labeled by incubation in glucose-free Eagle's basal medium (BME) (containing twice the usual amino acid and vitamin concentration, 10% dialyzed horse serum, 10 mM sodium pyruvate, and 2 mM glutamine) containing [3-^{3}H]glucose at 300 or 500 μCi per milliliter. Tissues were immersed in the above medium in plastic dishes for 2.5 h at 37.5°C in an atmosphere of 5% CO_2 in air saturated with H_2O. Labeling with [6-^{3}H]glucosamine (200 μCi per milliliter) was performed identically except that glutamine was omitted from the medium. No qualitative differences in labeled GAG were found when intact glands labeled in this glucose-free medium were compared to glands labeled in glucose-containing medium.

ISOLATION OF MESENCHYME, EPITHELIA, AND BASAL LAMINA FROM LABELED WHOLE GLANDS: After labeling, whole submandibular glands were rinsed in magnesium-free Tyrode's solution (MFT), and most of the mesenchyme was trimmed off and quickly frozen. The trimmed rudiments were incubated in MFT containing collagenase (lot 9EA, 200 μg per milliliter or lot 8BA, 100 μg per milliliter) at room temperature for 10 min in 10% CO_2 in air. The remaining mesenchyme was then teased from the epithelia by microdissection and discarded. These epithelia (which are almost totally free of mesenchymal cells but retain an intact basal lamina) were incubated in trypsin (0.001%) in MFT at room temperature for 10 min to remove surface materials, including the basal lamina, and the trypsinate was frozen. The trypsin-treated, denuded epithelia were washed with MFT and also frozen.

LABELING OF ISOLATED EPITHELIA AND PREPARATION OF SURFACE MATERIAL: Epithelia devoid of a lamina were prepared from epithelia isolated from unlabeled whole glands with collagenase by rinsing in calcium-magnesium-free Tyrode's solution (CMFT) and incubating for 10 min in CMFT containing testicular hyaluronidase (0.05 μg per milliliter [Leo Pharmaceutical, Sweden] or 0.07 μg per ml [Sigma Chemical Co., St. Louis, Mo.], both fortified with 1 μg per milliliter bovine serum albumin) at room temperature in 5% CO_2 in air to remove the basal lamina (2). Epithelia retaining a basal lamina were prepared and incubated identically but without hyaluronidase. The isolated epithelia were labeled as described above for 2.5 h, following which they were rinsed with MFT and treated with trypsin as described above to remove newly synthesized surface materials.

Biochemical Methods

PURIFICATION OF POLYSACCHARIDES FOR GAG ANALYSIS: Tissue fractions were thawed, brought to a final volume of 0.5 ml with water, and sonicated for 15 s at 0°C. Carrier hyaluronic acid and crude chondroitin sulfate (125 μg each) were added to each sample and the solutions adjusted to contain 150

mM Tris, pH 8.3, 1.5 mM $CaCl_2$, and either 0.2 mM glucose or glucosamine according to which ^{3}H-precursor was used. The samples were digested with pronase, and polysaccharides were purified as previously described (5).

DETERMINATION OF LABELED GAG: The methodology used was similar to that described by Saito et al. (14), except that more enzyme was used and the pH of the digestion was 7.4 rather than 8.0. The procedure is described in detail by Cohn et al. (5) and briefly involves digestion of isolated polysaccharides with chondroitinase ABC, separation of the unsaturated disaccharide products of GAG by paper chromatography, and elution of these compounds for scintillation counting. Counting efficiency was 48%, measured in a Beckman LS-233 refrigerated counter (Beckman Instruments, Fullerton, Calif.).

The following disaccharides represent their parent GAG: Δdi-6S is derived from chondroitin-6-sulfate; Δdi-4S is derived from chondroitin-4-sulfate and dermatan sulfate; Δdi-OS is derived from chondroitin and nonsulfated residues of the chondroitin sulfates; Δdi-OHA is derived from hyaluronic acid.

DETERMINATION OF THE FRACTION OF DISACCHARIDE LABEL DUE TO N-ACETYLHEXOSAMINE: The methodology employed was identical to that detailed in Cohn et al. (5). Labeled disaccharides were prepared by chondroitinase ABC digestion of polysaccharides (~20,000 dpm) isolated from [3-^{3}H]glucose-labeled whole glands. Sulfated disaccharides were converted to Δdi-OS with chondro-4-sulfatase and chondro-6-sulfatase, and both Δdi-OS and Δdi-OHA were purified by paper chromatography. The amount and radioactivity of the disaccharides were determined prior to their hydrolysis. The resultant glcNAc and galNAc hydrolysis products were isolated by paper chromatography, and, after measuring the radioactivity and recoveries, the percent of disaccharide label in N-acetylhexosamine was calculated.

RESULTS

Utilization of [3-^{3}H]Glucose for GAG Synthesis

Since the relative proportions of various glucose-labeled GAGs were being compared in this study, it was necessary to show that [3-^{3}H]glucose was incorporated equivalently into the various GAGs. It was assumed that the uronic acid moiety of the disaccharides was equivalently labeled regardless of GAG type (10), and the proportion of label derived from hexosamine was determined in the disaccharide derived from hyaluronic acid (Δdi-OHA, which contains glcNAc) and in the disaccharide derived from the chondroitin sulfates (Δdi-OS, which contains galNAc). These propor-

tions were nearly equivalent (Table I), demonstrating that glucose labels glcNAc- and galNAc-containing GAG similarly. Since close to 50% of the label in the disaccharides was found in the amino sugar, this result also indicates that under the conditions of labeling, the specific activities of the precursor nucleotide sugars were nearly equal. When [6-³H]-glucosamine labeling of GAG was analyzed similarly, all of the label in both glcNAc- and galNAc-containing disaccharides was in the hexosamine moiety. These studies also serve to verify the identity of hyaluronic acid as measured here, since the Δdi-OHA isolated from chondroitinase digests contained glcNAc as the only N-acetylated amino sugar.

Characterization of Newly Synthesized GAG

The newly synthesized GAGs characterized in this study do not include heparan sulfate because testicular hyaluronidase, chondroitinase ABC, and chondroitinase AC, enzymes which do not degrade heparan sulfate, remove all the labeled GAG from the basal epithelial surface, as assessed by autoradiography, and hyaluronidase removes essentially all of the basal lamina as assessed ultrastructurally by ruthenium red staining (3). The methodology employed also does not distinguish between chondroitin-4-sulfate and dermatan sulfate, since formation of the disaccharides involves conversion of both glucuronic and iduronic acid to the same keto acid. Dermatan sulfate is not degraded substantially by testicular hyaluronidase or chondroitinase AC, however, and the polysaccha-

TABLE I

[3-³H]Glucose Labeling of Glycosaminoglycans

Disaccharide*	% of Label in N-acetylhexosamine‡
Experiment 1	
Δdi-OHA	49.8
Δdi-OS	46.5
Experiment 2	
Δdi-OHA	48.3
Δdi-OS	44.6

* Labeled disaccharides were prepared by chondroitinase ABC digestion of polysaccharides from [³H]-3-glucose-labeled whole submandibular glands. Sulfated disaccharides were converted to Δdi-OS with specific sulfatases.

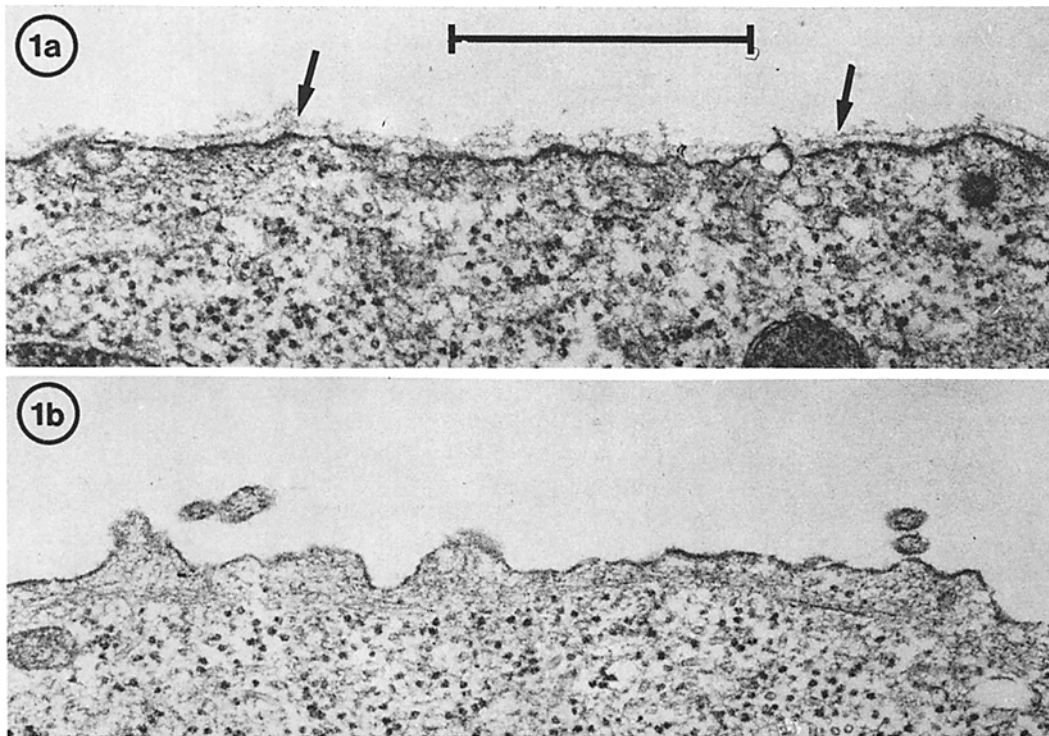‡ Determined by hydrolysis of the disaccharides and correcting for recoveries.

ride represented by Δdi-4S will be referred to in the text as chondroitin-4-sulfate.

LABELED GAG IN THE MESENCHYME, EPITHELIUM, AND BASAL LAMINA PRODUCED IN WHOLE GLANDS: Whole glands were incubated for 2.5 h with [³H]glucose, and three tissue components were prepared: the mesenchyme, representing tissue readily trimmed from rudiments before preparation of isolated epithelia and accounting for approximately 80% of the total mesenchyme; the basal lamina, representing material released by trypsin treatment of epithelia isolated free of mesenchyme with collagenase; and the epithelium, representing the trypsin-treated isolated epithelium.

The basal lamina fraction should not contain GAG associated with fibrillar collagen since collagenase-isolated epithelia are free of fibrillar collagen (Fig. 1a). Trypsin treatment of these epithelia completely removes the laminar materials (Fig. 1b).

The relative incorporation of [³H]glucose into the various GAGs by whole glands is shown in Table II. This incorporation reflects both synthesis and degradation, and does not represent the total amount of GAG present in the fractions. Substantially more labeled GAG was found in mesenchymal tissue than in epithelia, but the proportions of the various types in both tissues were nearly identical; over half of the GAG was from chondroitin sulfates, with chondroitin-6- and chondroitin-4-sulfate being labeled nearly equivalently. The small proportion in chondroitin was likely derived from undersulfated chondroitin sulfates, possibly in the process of biosynthesis. Hyaluronic acid accounted for slightly more than 40% of the GAG label in each tissue.

The labeled GAG in the basal lamina was ~11% of that found in the epithelium, and the proportions of the GAG types were distinctly different from those in mesenchyme or epithelium. Chondroitin sulfates accounted for 40% of the GAG; chondroitin-4-sulfate was nearly twice the chondroitin-6-sulfate and there was a lower proportion of label in chondroitin. Hyaluronic acid accounted for nearly 60% of the GAG. These data demonstrate that the composition of newly synthesized GAG deposited in the basal lamina is distinct from that in its tissue of origin, the epithelium, or from that in the surrounding mesenchyme.

EPITHELIAL AND LAMINAR GAG PRODUCED FOLLOWING REMOVAL OF THE

FIGURE 1 Basal surfaces of submandibular epithelia isolated free of mesenchyme. Bar is 1 μm. × 40,000. (1a) An epithelium isolated in collagenase showing the basal lamina at its surface (arrows). These epithelia are free of fibrillar collagen. (1b) A collagenase-isolated epithelium subsequently exposed to 0.001% trypsin in MFT for 10 min. The basal lamina is removed.

TABLE II

*[³H]Glycosaminoglycan Synthesized by Whole Glands**

Fraction	GAG in polysaccharide§	Total GAG	% of total GAG‡			
			Ch-6-SO₄	Ch-4-SO₄	Chondroitin	Hyaluronic acid
	%	dpm per rudiment‖				
Mesenchyme (3)	53 ± 5	6,450	22.1 ± 1.8	25.2 ± 2.9	8.4 ± 0.9	44.2 ± 4.8
Epithelium (3)	37 ± 4	384	23.7 ± 2.2	27.2 ± 1.7	8.7 ± 1.0	40.4 ± 3.8
Basal Lamina (4)	58 ± 5	42	13.9 ± 1.9	21.6 ± 1.8	4.8 ± 0.4	59.6 ± 3.2

* Glands were labeled for 2.5 h in 300 μCi/ml of [3-³H]glucose before separation of fractions.
‡ Mean ± SE for number of experiments indicated.
§ Chondroitinase-susceptible radioactivity as percent of total polysaccharide; mean ± SE for number of experiments indicated.
‖ Each experiment involved between 16 and 24 rudiments; mean for all experiments.

LAMINA: In the absence of mesenchyme, an ultrastructurally normal lamina is redeposited within 2 h following removal of the lamina (2). To determine whether the composition of labeled GAG in the newly deposited lamina is distinct from that produced in whole glands, isolated epithelia stripped of the lamina with hyaluronidase were labeled with [³H]glucose for 2.5 h in the

absence of mesenchyme or biological substratum.

Table III shows the [³H]GAG produced by the isolated epithelia and deposited in the lamina. These epithelia incorporated substantially more [³H]glucose into GAG than the epithelia of whole glands, but had nearly identical proportions of labeled GAG (cf. Table II). The newly deposited lamina contained ~16% of the GAG label found

in the epithelia and, compared to the lamina of whole glands, showed a small increase in the proportion of the chondroitin sulfates and a corresponding decrease in hyaluronate. The labeled GAG in the lamina, however, remained distinct from that in the epithelium, the lamina showing nearly twice as much chondroitin-4-sulfate as chondroitin-6-sulfate, a low proportion of chondroitin and a predominance of hyaluronic acid. These results indicate that deposition of a new lamina in the absence of mesenchyme does not substantially modify the type and relative proportions of [³H]GAG found in the epithelium or deposited in the lamina.

LABELED GAG IN NEWLY DEPOSITED AND PRE-FORMED LAMINA: To determine whether the GAG accumulating in a preexisting lamina is distinct from that deposited in a newly synthesized lamina, isolated epithelia which retained a lamina and which were devoid of a lamina were labeled with [³H]glucosamine for 2.5 h in the absence of mesenchyme (Table IV).

With glucosamine as a precursor, a substantially greater proportion (~90%) of the labeled polysaccharide in the surface materials removed by trypsin was susceptible to chondroitinase ABC, than when glucose was the precursor (cf. Tables II and III). Regardless of whether glucose (Table III) or glucosamine (Table IV) was the precursor, the proportions of labeled GAG within a newly formed lamina, however, were essentially identical. Since all of the glucosamine label in the GAG disaccharides is in the hexosamine moiety, this result indicates that glcNAc- and galNAc-containing GAG are also labeled equivalently by [³H]glucosamine.

The relative proportions of the various GAG types deposited in the preexisting lamina were identical with those in the newly formed lamina (Table IV). The preexisting lamina contained 21% of the GAG label in the epithelium, and the newly formed lamina had 16% (data not shown), the latter being identical to that previously observed (Table III). Thus, despite differences in the

TABLE III

*[³H]Glycosaminoglycan Synthesized by Isolated Epithelial**

Fraction	GAG in polysaccharide§	Total GAG	% of Total GAG‡			
			Ch-6-SO₄	Ch-4-SO₄	Chondroitin	Hyaluronic acid
	%	dpm per rudiment‖				
Epithelium (3)	42.2 ± 7.0	3,610	24.0 ± 3.1	31.8 ± 2.8	9.2 ± 1.3	35.0 ± 4.2
Basal lamina (3)	63.8 ± 7.8	596	17.0 ± 1.8	32.7 ± 5.5	4.3 ± 1.1	46.0 ± 5.4

* Epithelia isolated in collagenase treatment and stripped of the lamina by hyaluronidase treatment. Labeling was for 2.5 hrs in 500 μCi/ml of [³H]-3-glucose prior to removal of the newly deposited surface materials.
‡ Mean ± SE for number of experiments indicated.
§ Chondroitinase-susceptible radioactivity as percent of total polysaccharide; mean ± SE for number of experiments indicated.
‖ Each experiment involved 13-15 rudiments; mean for all experiments.

TABLE IV

*[³H]Glycosaminoglycan in the Basal Lamina Synthesized by Isolated Epithelia**

Source of lamina‡	GAG in polysaccharide§	Total GAG‖	% of total GAG			
			Ch-6-SO₄	Ch-4-SO₄	Chondroitin	Hyaluronic acid
	%	dpm				
Preexisting lamina	89.4	2,570	16.5	33.6	1.8	48.1
Newly deposited lamina	91.9	1,740	14.8	33.3	1.5	50.4

* Epithelia were labeled for 2.5 h in 200 μCi/ml [6-³H]glucosamine before removal of surface materials with trypsin.
‡ Epithelia with a preexisting lamina were isolated in collagenase; epithelia depositing a new lamina were further treated with hyaluronidase. See Materials and Methods.
§ Chondroitinase-susceptible radioactivity as percent of total polysaccharide.
‖ Represents laminae derived from 8 epithelia.

relative amount of labeled GAG in the lamina, the preformed lamina of isolated epithelia and of whole glands have nearly identical relative proportions of labeled GAG as the lamina formed *de novo*.

Ultrastructure of the Epithelial Basal Lamina

By routine staining procedures, the lamina appears as a three-layered structure containing a possibly filamentous layer of low density adjacent to the plasma membrane (the lamina interna), covered by a less lucent amorphous central layer (the lamina densa) and a superficial layer of varying thickness consisting of fibrils and amorphous materials. Use of ruthenium red, a cationic dye which appears electron dense when combined with OsO_4, has revealed structural elements adjacent to the plasmalemma and associated with the lamina densa (3, 9, 15). An attempt was made to provide contrast and definition to these structures by using tannic acid as a fixative (13).

RUTHENIUM RED STAINING: All layers of the epithelial basal lamina stain intensely with ruthenium red (Fig. 2). At low magnifications, the lamina interna shows deposits of stain with a periodic repeat of ~55 nm, and a similar repeat unit is seen on some of the superficial fibrils (Fig. 2a and 2b). The particles stained within the lamina interna appear to be spherical and are about 40 nm in size. The lamina densa also shows periodic globular deposits of stain. These, however, are less discrete than those in the lamina interna, possibly because of overlapping of stain deposits in adjacent planes. The deposits in the lamina densa are ellipsoid, being from 60 to 90 nm long, and have a period of about 100 nm (Fig. 2b). The distance from the outer surface of these particles to the outer leaflet of the plasmalemma averages 78 nm. At higher magnifications, plaque-like densities are seen associated with the outer leaflet of the plasmalemma (Fig. 2c and 2d). These are invariably associated with the ruthenium red-staining particles of the lamina interna. The plaques measure ~7 nm in thickness, vary in length and usually stain more intensely than the other components of the basal lamina.

The basal lamina deposited in the absence of mesenchyme by epithelia previously stripped of a lamina is free of the superficial fibrils (Fig. 2c). The lamina densa and interna, however, show staining and organizational properties similar to those seen on the surfaces of epithelia in whole glands. This result indicates that the spatial order in the lamina observed here is produced upon deposition of the lamina in the absence of mesenchyme or biological substratum.

TANNIC ACID FIXATION: Compared to routine fixation (e.g. glutaraldehyde; cf. Fig. 1a), fixation in tannic acid-glutaraldehyde results in a distinctly different appearance of the basal epithelial surface (Fig. 3). The lamina appears compact

FIGURE 2 Basal epithelial surfaces of submandibular rudiments fixed and stained with ruthenium red. (2a) A low power view within a cleft of an intact gland. The basal lamina (arrow) stains intensely with ruthenium red. The lamina interna shows densely staining ca. 40 nm-diameter particles which have a repeat of ~55 nm (prominent in bracketed areas). This repeat unit is similar to that on a fiber in the lower left of the micrograph. Bar is 1 μm. × 19,000. (2b) A higher magnification view within a cleft of an intact gland. Globules of stain are also evident within the lamina densa. These ellipsoid-shaped deposits (*) are from 60 to 90 nm in length and demonstrate a repeat of ca. 100 nm. The plasmalemma (arrow) appears densely and uniformly stained at this magnification, and the distance from its outer leaflet to the outer edge of the stain deposits in the lamina densa averages about 78 nm. Bar is 1 μm. × 40,000. (2c) View of the lateral aspect of a lobule of an epithelium previously stripped of the lamina by hyaluronidase treatment and cultured for 2 h in the absence of mesenchyme. The newly deposited lamina has the appearance and staining properties of the laminae of intact glands (cf. 2a and 2b). The globules of ruthenium red in the lamina densa (*) often appear to be lined up with the densely staining particles in the lamina interna (+). Extremely electron-dense plaque-like regions are associated with the outer leaflet of the plasmalemma (M denotes inner leaflet). In some sections, these regions appear to be immediately beneath the particles of the lamina interna. Bar is 0.1 μm. × 167,000. (2d) An enlargement of the boxed region in (2b). The outer surface of the plasmalemma (M denotes inner leaflet) shows the electron-dense plaques (Pl) adjacent to the stain in the lamina interna. The plaques are ~7 nm in thickness and are variable in length, measuring from 50 to 100 nm, and appear in some micrographs to be composed of smaller spherical particles. Bar is 0.1 μm. × 280,000.
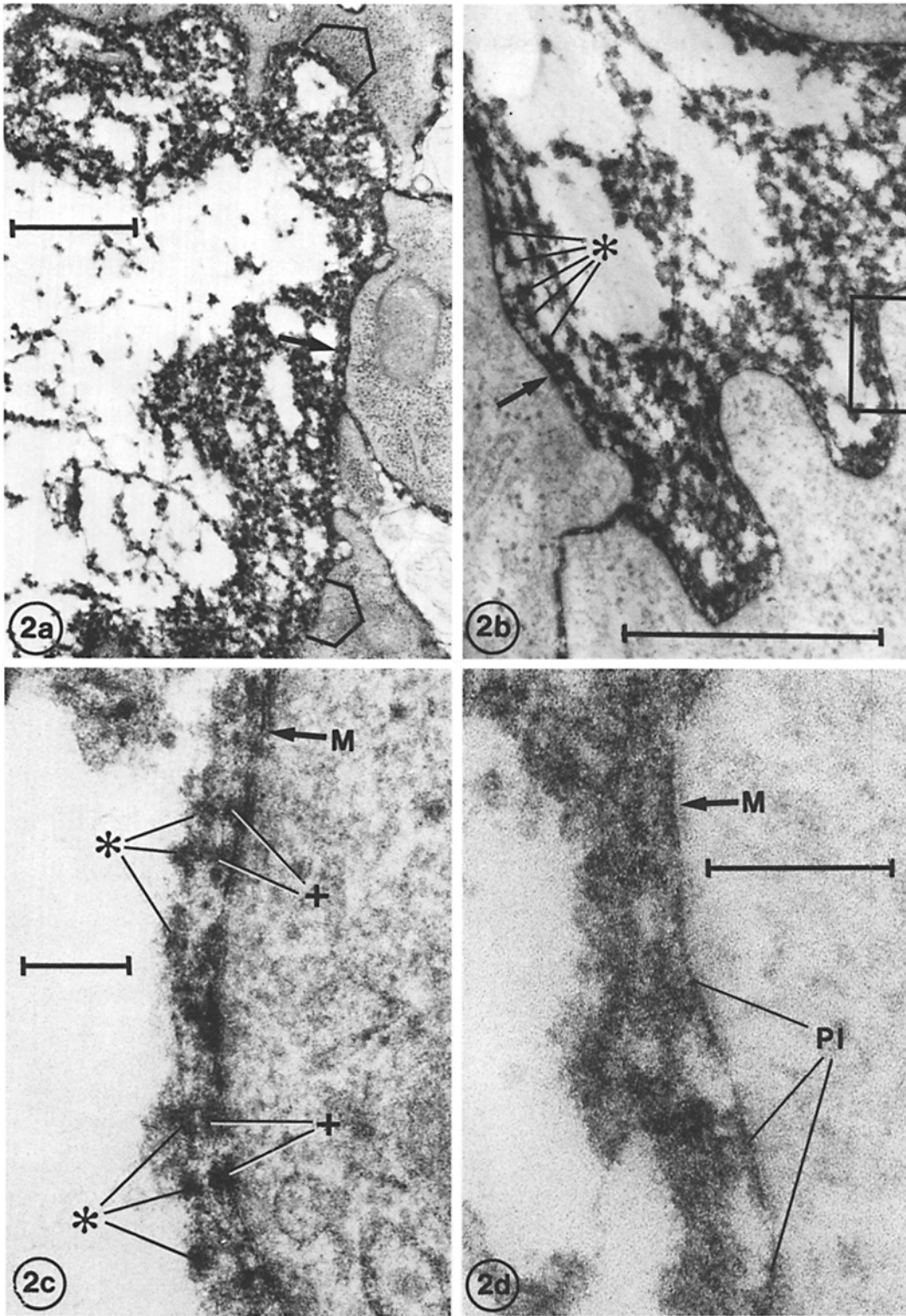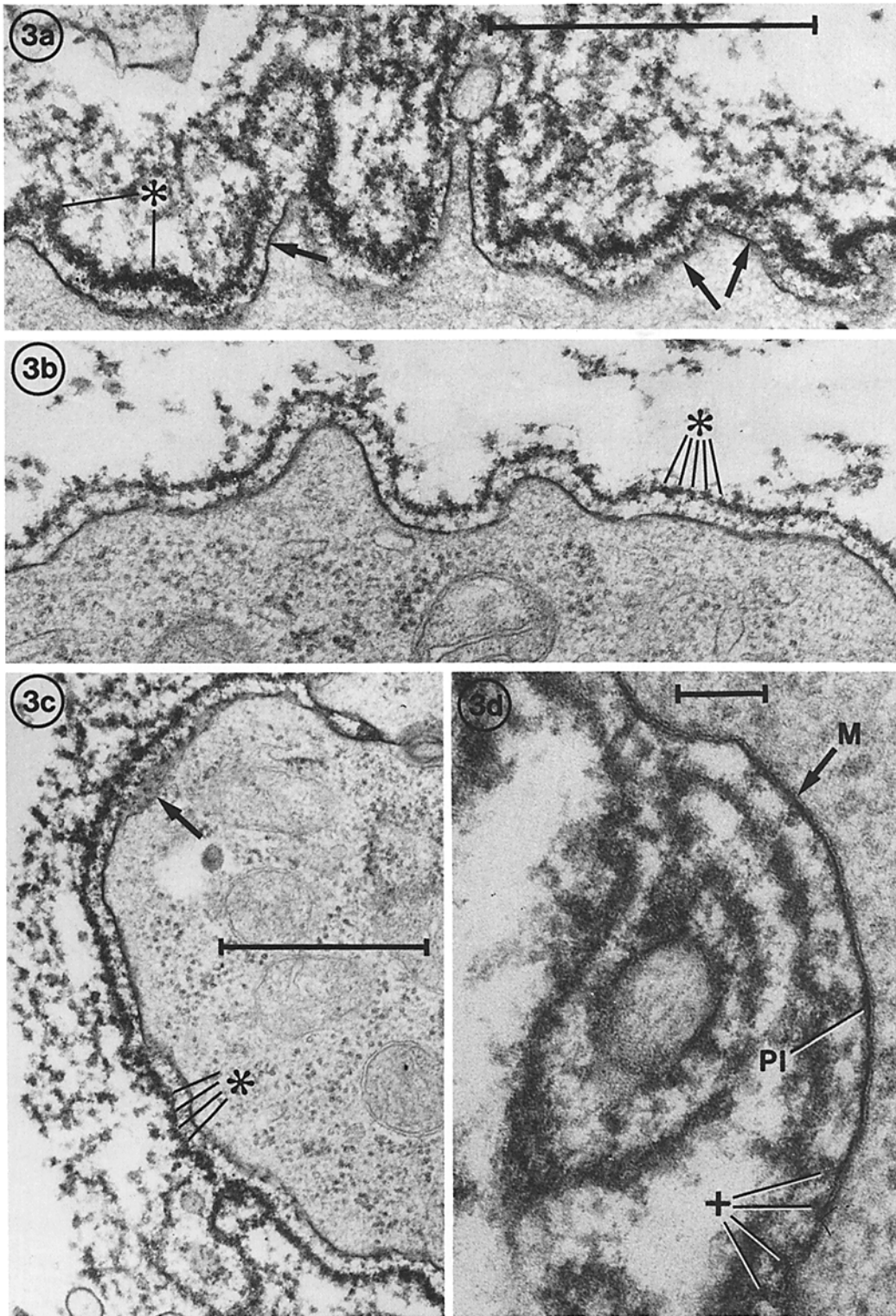
and shows highly ordered arrangements of stain-
ing materials. The lamina densa is composed of
somewhat spherical globules ~30 nm in diameter
arranged in a period of ~32.5 nm (Fig. 3a–c).
Filaments can be seen within the lamina interna
traversing the space between the globules in the
lamina densa and the plasmalemma (Fig. 3a).
These filaments are ~17 nm in thickness and 40
nm in length, while the distance from the plasma-
lemma to the outer surface of the deposits in the
lamina densa averaged 74 nm. In some sections
(Fig. 3a and d), the filaments were arranged in a
47–50 nm period. Tangential sections of the plas-
malemma (Fig. 3c and 4a) showed small elec-
tron-dense plaques associated with the plasma-
lemma. These plaques were about 20 nm in diam-
eter and, although there was no rigorous periodic-
ity, they had a center-to-center spacing of about
50 nm. A higher magnification view (Fig. 3d)
shows that the filaments of the lamina interna
connect to the globular particles of the lamina
densa, perhaps on a one-to-one basis, and that the
filaments appear to insert into the dense plaques
on the plasmalemma.

Tangential sections through the lamina densa
(Fig. 4b) reveal that the periodic arrangement of
30 nm particles seen in cross section can be seen in
other directions. The periodicity was similar to
that seen in sections perpendicular to the plasma-
lemma, being ~32.5 nm. This observation sug-
gests that the lamina densa is composed of a 3-
dimensional array of particles. Thus, when pre-
served with tannic acid, basal lamina appears to
possess a highly ordered geometrically arranged
structure.

DISCUSSION

The present study establishes that the basal lamina
is a specialization of the cell surface having a high
degree of spatial order and a characteristic GAG
composition, notably containing hyaluronic acid as
the major GAG component. Laminae produced
in the absence of mesenchyme or biological sub-
strata also show this characteristic organization
and composition. Taken together with our pre-
vious data (2), these results indicate that the sub-
mandibular epithelium alone synthesizes and de-
posits an epithelial basal lamina which is ultra-
structurally, biochemically with regard to GAG
composition, and functionally analogous, in the
maintenance of morphology, to the lamina pro-
duced in intact glands. The submandibular basal
lamina may represent, in part, an ordered mosaic
of cell surface proteoglycan and hyaluronic acid
which imposes morphologic stability on the epi-
thelial cell population.

Glycosaminoglycans of the Basal Lamina

An objective of this study was to characterize
the GAG deposited in the lamina by the epithe-
lium. The finding of substantial hyaluronic acid in
the lamina is somewhat surprising in view of pre-
vious work on the chick embryo which, based on
enzyme susceptibility, found that hyaluronate is
not a component of the epithelial basal lamina of
the cornea (9, 15) or the notochord (11). Hyalu-
ronate was identified in the present work by sepa-
rating the disaccharide products of chondroitinase
ABC digestion and confirming that the unsatu-
rated disaccharide representing hyaluronate con-

FIGURE 3   Basal epithelial surfaces of intact submandibular glands fixed in tannic acid-glutaraldehyde and
stained with Pb citrate. (3a) View of an area within a cleft. The lamina densa (*) appears electron dense
and globular. Filaments are seen within the lamina interna traversing the space between the plasmalemma
(arrows) and the lamina densa. These filaments are about 17 nm thick and 40 nm long, have a center-to-
center distance of ~50 nm and at areas where the plasmalemma is cut obliquely, appear to insert into the
plasmalemma. Bar is 1 μm. × 51,000. (3b) An area along the lateral aspect of a lobule. The section is
more perpendicular to the plane of the plasmalemma than that of 3a and the periodic nature of the lamina
densa particles (*) is more evident. The particles have a period of ~32.5 nm and measure ~30 nm in
height. The distance from the plasmalemma to the outer edge of the lamina densa particles averages 74
nm. Bar and magnification as in 3a. (3c) A section as in (3b) demonstrating further the periodic nature of
the lamina densa particles (*). A slight tangential cut of the plasmalemma reveals electron densities associ-
ated with it (arrow). These dense sites are about 20 nm in diameter. Bar is 1 μm. × 32,000. (3d) A high
magnification of a section showing the lamina surrounding an epithelial cell process. The filaments of the
lamina interna (+) appear to connect to the globular particles in the lamina densa, perhaps on a 1-to-1
basis, and insert into dense plaques (Pl) on the plasmalemma (M denotes inner leaflet). Bar is 0.1 μm. ×
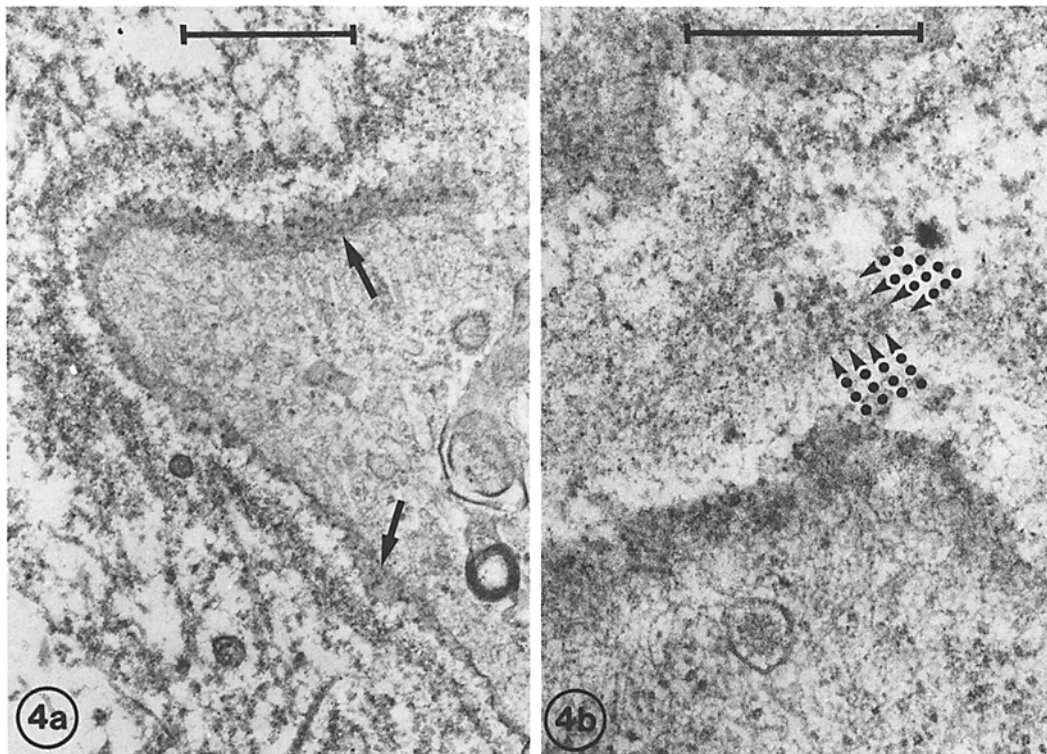135,000.

FIGURE 4 Tangential sections through the basal surfaces of intact submandibular glands fixed in tannic acid-glutaraldehyde and stained with Pb citrate. (4a) A section showing the dense plaque-like structures associated with the plasmalemma. The structures are most prominent at the sites indicated by the arrows and show an average spacing of about 30 nm. Bar is 0.5 nm. × 46,000. (4b) A view of the lamina densa (center) in tangential section and the plasmalemma (top and bottom) in oblique section. A periodicity of about 32.5 nm in the lamina densa can be seen in two directions (arrows), implying that the particles of the lamina densa are organized in 3 dimensions. Bar is 0.5 μm. × 62,000.

tained glcNAc as the only N-acetylated hexosamine. Chondroitinase ABC is well known to have activity against hyaluronic acid although this activity has a lower pH optimum than the activity with the chondroitin sulfates (13). Under the conditions used here, chondroitinase preparations were active against hyaluronic acid and showed no contaminating sulfatases (5). There is little doubt that the epithelial cells produce authentic hyaluronic acid and deposit it in the lamina. The hyaluronate cannot be derived from contaminating mesenchymal cells since the mesenchyme does not contribute GAG to the basal lamina (2) and the isolated, denuded epithelia used here were completely free of associated mesenchymal cells.

The presence of heparan sulfate in the lamina was not investigated because prior studies indicated that the lamina is degraded by enzymes which do not attack heparan sulfate. Synthesis of

heparan sulfate has been detected in other embryonic epithelia (9, 11), however, and it is possible that laminar heparan sulfate might be removed by enzymes which degrade other types of GAG. If present, heparan sulfate is likely not more than 10% of the newly synthesized GAG in the lamina as defined here because ~90% of the [³H]glucosamine-labeled material in the trypsin-solubilized lamina was digested by chondroitinase ABC, which does not attack heparan sulfate.

After labeling whole glands, the proportions of the various GAGs found in the basal lamina were clearly distinct from the proportions in the epithelium or mesenchyme. Whereas there may be differences between the cell types in the rate of incorporation, because the precursor is incorporated equivalently into the various GAGs, the distribution of label among the GAG types should be independent of possible differences in precur-

sor transport or pool sizes. Furthermore, differences between the lamina and epithelium in the proportion of label in the various GAGs are not due to differences in precursor utilization, since the lamina is synthesized by the epithelium. In the basal lamina, hyaluronic acid accounted for more than half of the GAG label, chondroitin-4-sulfate was about twice the chondroitin-6-sulfate, and there was a low proportion of chondroitin. On the other hand, both epithelium and mesenchyme had very similar labeled GAG compositions. Chondroitin-6-sulfate was nearly equivalent to chondroitin-4-sulfate; there was a high proportion of chondroitin, and hyaluronate accounted for ~40% of the total. The similarity of labeled GAG in submandibular mesenchyme and epithelium contrasts with the finding that isolated neural tube, notochord, and lens produce different proportions of the various GAG types than the tissues on which they exert a developmental influence (9, 11).

Similar proportions of labeled GAG derived from proteoglycan (chondroitin, chondroitin-4- and chondroitin-6-sulfate) were found in the lamina, regardless of whether it was derived from labeled whole glands, from epithelia retaining a lamina, or from epithelia devoid of a lamina. While the proportion of label in these GAGs relative to that in hyaluronic acid was nearly identical in the laminae of isolated epithelia, there was a slightly lower proportion in the laminae of whole glands. The most likely explanation for this difference is that the laminae of whole glands were prepared immediately following collagenase treatment of the epithelium, whereas the laminae of isolated epithelia were prepared following a period of labeling in culture. Since the collagenase preparations used have minimal degradative activity against chondroitin sulfates (3) but none detectable against hyaluronic acid,[2] laminae prepared immediately after collagenase treatment might be expected to have reduced amounts of labeled chondroitin sulfates relative to hyaluronic acid.

Relative to the amount of labeled GAG in the epithelium, preexisting laminae of isolated epithelia had slightly more labeled GAG than newly-formed laminae and substantially more than the laminae of whole glands. These results are difficult to interpret because, although the labeling periods were the same, in each situation GAG may accumulate in the lamina at different rates, or at different

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[2] Banerjee, S. D., unpublished observation.

ent sites, or there may be differences in rates of turnover or loss of GAG to the medium. Yet, despite these possible differences, the lamina in each situation showed characteristic proportions of the various GAGs. These results imply that GAG is deposited in the lamina as intact units having a defined composition.

The constancy of laminar GAG labeling was especially evident in the laminae derived from isolated epithelia, where the proportions of labeled GAG in a preexisting lamina were essentially identical with those in a newly-forming lamina. This suggests that each of the GAG components is a bona fide constituent of the lamina, since if a component was found only because it was in transit to the extracellular space, it might be expected to be present in lower proportions in a lamina produced de novo. These data also imply that the proportions of GAG revealed by the incorporation data may represent the actual GAG composition of the lamina.

Spatial Order in the Basal Lamina

A substantial degree of spatial order was found within the lamina, either by ruthenium red staining or by tannic acid fixation. Ruthenium red precipitates anionic polysaccharides (12) and, although not uniquely specific for GAG, it has been frequently used to demonstrate GAG at cell surfaces. In the present study, the staining techniques utilized the facts that ruthenium brown, a readily produced ruthenium red oxidation product which also interacts with OsO_4 to produce electron-dense deposits, precipitates hyaluronic acid equally as well as ruthenium red precipitates chondroitin sulfate (12), and that tannic acid, classically used as a protein fixative, preserves structures not evident with aldehyde and/or OsO_4 fixation alone (13).

Combining the features and measurements of the components seen with ruthenium red staining with those seen by tannic acid fixation strongly suggests that different portions of the same macroperiodic structures are being visualized by both techniques. A simple schematic drawing of these structures is shown in Fig. 5a. Although views of the lamina prepared with both tannic acid and ruthenium red are too dense to provide much detail (Fig. 5b), they are remarkably consistent with this model. The striking feature of the ultrastructure is that the lamina is a highly-ordered, geometrically arranged integral part of the basal cell surface. This organization was observed in
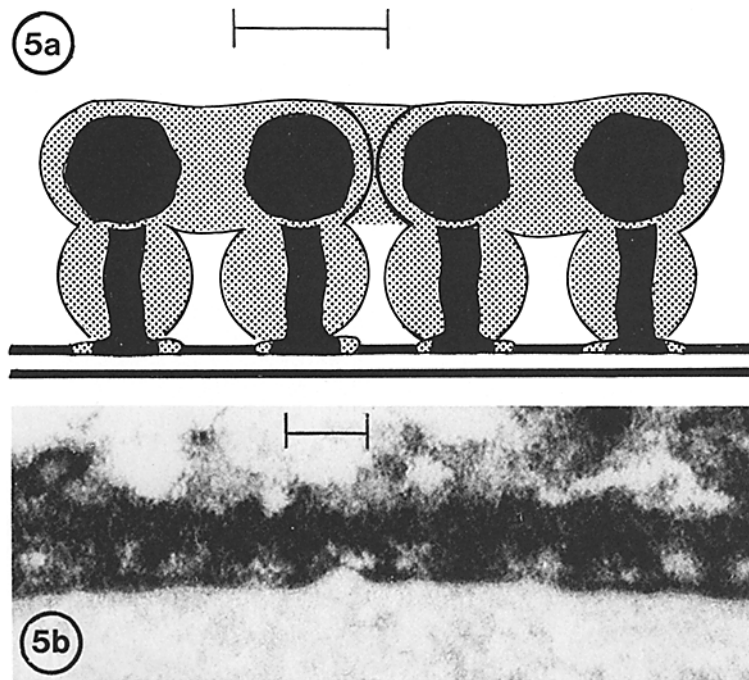
FIGURE 5 (a) A 2-dimensional schematic of materials visualized in the basal lamina based on observations of tannic acid-fixed (filled spaces) and ruthenium red-stained (stippled spaces) specimens. The schematic conforms to the measurements cited in text, and the plasmalemma is included for reference. Bar is 50 nm. (5b) The basal epithelial surface of a whole gland treated for 2 h as described for tannic acid fixation, washed three times in 2% glutaraldehyde in buffer B, and processed as described for ruthenium red staining (see Materials and Methods). Note the similarity between this view and the schematic. Bar is 50 nm. × 200,000.

laminae produced in vivo and in laminae allowed to regenerate in vitro in the absence of mesenchyme.

The structures observed in the lamina are not as well-defined or uniformly regular as some other more precisely arranged biological mosaics. This lack of precise definition may be due to technical limitations. On the other hand, complex extracellular structures, particularly those containing polysaccharides, could have a significant degree of intrinsic microheterogeneity as exemplified by the polydispersity of the cartilage proteoglycan aggregate and its subunit (7).

The spatial order of the submandibular lamina revealed here is somewhat distinct from that seen in the basal lamina of the embryonic chick corneal epithelium (9, 15). Although the plaques associated with the outer leaflet of the plasmalemma observed by Hay and Meier (9) and confirmed by Trelstad et al. (15) are visualized here by both ruthenium red and tannic acid techniques, we do not see ruthenium red deposits of similar size or

spacing, or the periodic arrays localized in the inner and outer faces of the lamina densa seen in the cornea (15). These ultrastructural differences are consistent with the difference in the types of GAG found in these laminae (9, 15) and with the possibility that the submandibular lamina contains substantially less or no collagen (2).

There also are methodological differences. Ruthenium red was used only as a post-fixative stain in one study (9) and therefore was not allowed to precipitate GAG during the aldehydic fixation. The presence of Alcian Blue during fixation (15) cannot account for the difference, because, in studies of the submandibular gland not shown here, inclusion of Alcian Blue in the fixative did not change the appearance of the lamina or reveal fine structure beyond that seen with routine fixation. However, we did find that, since ruthenium red penetrates tissues poorly (12), there is a limited degree of staining and a somewhat different ultrastructure if more than a few layers of mesenchymal cells cover the lamina.

No chemical properties can be definitively ascribed to the structures within the lamina observed here. By analogy with the effects of chondroitinase ABC on the ultrastructure of the corneal epithelial lamina (15), the ruthenium red deposits could represent β1,4-linked GAG chains. The schematic model suggests the possibility that the fibrillar structures revealed by tannic acid mediate the attachment of these deposits to the electron-dense plasmalemmal plaques. In the corneal lamina, the plaques are not removed by testicular hyaluronidase treatment and have been suggested to consist of heparan sulfate (9).

Significance of the Organization and Composition of the Lamina

The highly ordered pattern of organization together with the suggestion that the GAG components accumulate as units of constant composition implies that the lamina contains discrete supramolecular complexes of GAG. This implication supports the suggestion of Trelstad et al. (15) that the lamina may be constructed by the extracellular self-assembly of macromolecules. Evidence for the self-assembly of GAG-containing molecules comes from work on the cartilage proteoglycan aggregate, a supramolecular complex of proteoglycans and noncovalently bound link proteins and small amounts of hyaluronic acid (6, 7). Analogous complexes in tissues other than cartilage are not known to exist and, although GAGs are major components of the carbohydrate at animal cell surfaces, their organization and relationship to the plasmalemma are virtually unknown. The apparent organization and composition of the GAG in the lamina are distinct from those in the cartilage aggregate, but the structure of the cartilage aggregate would seem to serve the function of cartilage in resisting compressive forces but would not be optimal at cell surfaces where rapid shape changes must be accommodated. Therefore, it is reasonable to suspect that the organization and composition of GAG complexes at cell surfaces may be distinct from those in cartilage, and possibly may vary with the type and physiological or developmental state of the cell.

The possibility that the GAG in the lamina exists in highly ordered supramolecular complexes lends credence to the idea that it imposes morphologic stability on the epithelium (2). The ordering and polarity of the laminar materials with respect to the plasmalemma suggest that they provide a pliant and malleable extracellular scaffolding. This supporting framework could impart structural form to the epithelium by several possible mechanisms, from the provision of cell adhesion and attachment sites to supplying membrane stability for the action of force-generating intracellular actin filaments.

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