ACID MUCOPOLYSACCHARIDE (GLYCOSAMINOGLYCAN) AT THE EPITHELIAL-MESENCHYMAL INTERFACE OF MOUSE EMBRYO SALIVARY GLANDS

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ABSTRACT

Acid mucopolysaccharide (glycosaminoglycan) has been demonstrated at the epithelial-mesenchymal interface of mouse embryo submandibular glands by (a) specific staining for polymeric sulfate with Alcian blue 8 GX at various magnesium concentrations, (b) specific staining for polymeric uronic acid by selective oxidation of these residues to Schiff-reactive compounds, (c) electron microscope localization of ruthenium red staining, (d) radioautographic localization of glucosamine-3H and 35SO4, and (e) by susceptibility of the glucosamine radioactivity at the interface to digestion with protease-free hyaluronidase. Moreover, material labeled with glucosamine-3H and 35SO4 and with chemical characteristics identical with those of acid mucopolysaccharide were isolated from the glands. Acid mucopolysaccharide is distributed over the entire epithelial surface. The amount of acid mucopolysaccharide, as revealed by the staining procedures, is nearly equivalent at all sites. In contrast, the rate of accumulation of glucosamine-labeled mucopolysaccharide is greater at the surface of the distal ends of the growing and branching lobules. This distribution of newly synthesized acid mucopolysaccharide at the sites of incipient cleft formation suggests that surface-associated acid mucopolysaccharide is involved in the morphogenetic process. A mechanism of branching morphogenesis is proposed which accounts for the distribution of collagen fibers and total and newly synthesized acid mucopolysaccharide at the epithelial surface.

INTRODUCTION

A variety of observations suggest that the extracellular matrix at the interface between embryonic epithelial primordia and their investing mesenchyme plays an important role in the morphogenetic interaction between these tissues (see reference 1 for review). The matrix materials that are thought to be involved include the epithelial basement membrane, collagen, and acid mucopolysaccharide. Collagen fibers have been identified ultrastructurally at the epithelial-mesenchymal interface of a number of developing tissues (2–4). However, direct evidence is lacking for the presence of acid mucopolysaccharide. The characteristic staining of the epithelial-mesenchymal interface with periodic acid–Schiff (PAS) reagents (1, 5) is not indicative of acid mucopolysaccharide, since these molecules are not PAS-reactive when tissues are stained in the usual manner (6).

The radioautographic studies of Kallman and Grobstein (7) provide suggestive evidence that acid mucopolysaccharides occur at the epithelial surface. These workers showed that salivary epithelia,
freed of mesenchyme by crude trypsin-pancreatin treatment and cultured across a membrane filter from mesenchyme, incorporate glucosamine-3H into a fixable material. The radioactivity was maximally localized at the epithelial surface proximate to the membrane filter. Treatment of the sections with hyaluronidase of unknown purity removed most of the glucosamine radioactivity, but the precise nature of the labeled material was not determined.

Acid mucopolysaccharides occur as large polymers and (with the possible exception of hyaluronic acid) are covalently linked to protein (6, 9). The carbohydrate component of these macromolecules is distinguished from other polysaccharides by (a) a high content of hexosamine, (b) polyanionic properties due to sulfate ester and/or carboxylic acid groups, (c) the presence of uronic acid residues, and (d) susceptibility to digestion by \( \beta \rightarrow 4 \) hexosaminidases.

We have utilized all of these distinguishing features to demonstrate that acid mucopolysaccharide occurs at the epithelial-mesenchymal interface of embryonic salivary glands. The acid mucopolysaccharide is distributed over the entire epithelial surface, but the rate of mucopolysaccharide accumulation is greatest at the surface of the growing and branching lobules. This distribution of newly synthesized acid mucopolysaccharide contrasts with the reported distribution of collagen fibers at the epithelial interface and provides a possible explanation for the role of surface-associated materials in salivary epithelial morphogenesis.

**MATERIALS AND METHODS**

**Reagents and Isotopes**

Alcian blue 8 GX was a gift from I.C.I. America Inc., Stamford, Conn., glucosamine-3H (1150 and 1300 mCi/m mole) and \( \text{Na}_2\text{SO}_4 \) (283–347 mCi/m mole) were obtained from New England Nuclear Corp., Boston, Mass. Bovine testicular hyaluronidase (Type I) was obtained from Sigma Chemical Co., St. Louis, Mo. Assays for ribonuclease (10), collagenase (11), and nonspecific protease (12) detected none of these activities in the hyaluronidase preparation. The minimum concentrations of ribonuclease A (RAF, Worthington Biochemical Corp., Freehold, N. J.), clostridial collagenase (CLSPA, Worthington), and crystalline trypsin (TRL50S, Worthington) detectable in these assays were 0.0002, 0.001, and 0.005, respectively, of the maximum hyaluronidase concentration tested.

**Tissues and Culture Techniques**

Mouse embryo submandibular salivary glands were obtained and cultured as previously described (13), except 13½-day embryos (obtained in late afternoon of day 13) were used, and intact glands were cultured without a clot. Labeling of glands was in 1.0 ml nutrient medium containing either 100 \( \mu \)Ci/ml glucosamine-3H or 100 \( \mu \)Ci/ml \( \text{Na}_2\text{SO}_4 \). The glands were labeled for 2 hr at 37.5°C and then washed five times for 5 min in 10 ml portions of ice-cold Tyrode's solution before fixing.

**Histologic and Radioautographic Techniques**

Glands were fixed in Carnoy's solution (30 min); identical results were obtained after alcohol-formalin fixation (40% aqueous formaldehyde:ethanol, 1:9, at \(-20^\circ\text{C}\) for 72 hr). Tissues were dehydrated in graded alcohols, embedded in paraffin, and sectioned at 4–5 \( \mu \)m by standard methods. Staining with Alcian blue was in 0.025 M sodium acetate (pH 5.0) and at \( \text{MgCl}_2 \) concentrations of 0.1–0.9 M as described by Quintarelli and Dellovo (14). Two-step PAS staining was performed in the dark as described by Scott and Dorling (15). Periodate oxidations were carried out with freshly prepared 2% sodium metaperiodate at 30°C. Reduction was performed with freshly prepared 1% sodium borohydride for 3 min at 24°C. The duration of the oxidations is discussed in the results section.

Slides for radioautography were coated with liquid emulsion (Type K-5, Ilford Nuclear Research, Ilford, England). The slides were exposed for 14 days at 4°C and were developed with Kodak Rapid Fixer. Sections were stained with Mayer's hematoxylin and eosin. Treatment of sections with hyaluronidase was performed by incubating the slides in testicular hyaluronidase (2.0 mg/ml in 0.1 M sodium phosphate containing 0.15 M NaCl, pH 5.3) for 24 hr at 37°C.

**Ruthenium Red Staining for Electron Microscopy**

Most of the mesenchymal tissue was teased away from the epithelium of 13½-day glands, leaving a thin layer of mesenchyme 6–10 cells thick. These rudiments were fixed in 2.5% glutaraldehyde buffered in 0.1 M cacodylate (pH 7.2) containing 0.05% ruthenium red for 3 hr at room temperature. The rudiments were washed in buffer and postfixed in buffered 2% osmium tetroxide containing 0.05% ruthenium red for 3 hr at room temperature in the dark (16). After dehydration in a graded series of ethanol, the rudiments were embedded in Epon. Ultrathin sections were cut on glass knives and the sections were placed on Formvar-coated 150-mesh grids. The sec-
Isolation of Acid Mucopolysaccharide

The procedure used is based upon methods which result in the isolation of acid mucopolysaccharide from other tissues (17). Glands were labeled and washed as described above and disrupted by sonication (0°C, 60 sec) in 1.0 ml 0.1 M potassium phosphate (pH 7.5). In some experiments, the glands were sonicated in methanol and extracted with chloroform: methanol, 2:1. A portion of the homogenate was removed for protein determination (18), and the homogenate was heated to 100°C for 10 min, cooled, and 2 mg chondroitin SO4 “C” (Calbiochem, Los Angeles, Calif.) was added. Pronase (0.3 mg, Calbiochem) was added and the sample was incubated at 35°C. After 8 hr, another 0.3 mg pronase was added. Identical results were obtained with papain digestion (17). After 16 hr incubation, the sample was cooled to 37°C and made 5 mM in MgCl2. RNase A (10 µg, Worthington) and DNase I (10 µg, Worthington) were added and the sample was incubated 30 min. After cooling to 23°C, the mixture received 5 ml of a solution containing 0.1 mM Na2SO4, and 0.1 mM glucoseamine and was clarified by centrifugation (45,000 g, 20 min). To the supernatant, 0.5 ml of a 0.8% solution of cetyl pyridinium chloride (CPC, K and K Laboratories, Inc., Plainview, N.Y.) was added, and the sample was incubated 1 hr at 37°C. The precipitate was collected (45,000 g, 20 min), washed with aqueous 0.08% CPC, and then washed with aqueous 0.08% CPC. The residue readily dissolved in 1.5 ml methanol. A toluene-based fluor was added and the clear solution was counted in a liquid scintillation counter.

RESULTS

Staining of the Epithelial-Mesenchymal Interface

ALCIAN BLUE: Acid mucopolysaccharides are polyanions containing sulfate esters and carboxylic acid residues. These macromolecules may be identified by their interaction with certain organic cations to form an insoluble complex (19). The concentration of magnesium ion that dissociates the complex is determined by the type of anionic groups on the macromolecule (20). Formation of the complex and its dissociation are the bases for a histochemical procedure for acid mucopolysaccharides developed by Scott and coworkers (20–22). The method employs Alcian blue, a polyvalent cationic dye that binds with polyanions in tissues. In the absence of added electrolyte, staining with the dye is nonspecific, but low concentrations of Mg2+ displace the dye from nonspecific binding sites. The dye is displaced from mucopolysaccharides and staining ceases at a characteristic and narrow range of Mg2+ concentration (the “critical electrolyte concentration”). Thus, the anionic groups of acid mucopolysaccharides can be identified histochemically by the concentration of Mg2++ that prevents staining with Alcian blue. Complications arising from masking of anionic sites by protein are minimized by staining at pH 5–6 (22).

Whole salivary rudiments were stained with Alcian blue 8 GX in the presence of various concentrations of Mg2+ (Figs. 1–5). The epithelial-mesenchymal interface stained blue at Mg2+ concentrations of 0.2, 0.3, and 0.4 mM (Figs. 1–3). The stain was nearly uniformly distributed around the surface of the epithelium, and the intensity of the stain was similar at each of these Mg2+ concentrations. At concentrations of 0.6 and 0.7 mM Mg2+ (Figs. 4 and 5), the blue staining of the interface was markedly less intense; the stain remaining did not completely encircle the epithelium. At these Mg2+ concentrations, some sections revealed no appreciable stain at the epithelial surface. At higher levels of Mg2+, no Alcian blue staining material was seen at the interface. Comparison of these results with Alcian blue staining of other tissues (14) strongly suggests that the epithelial-mesenchymal interface contains acid mucopolysaccharide. This suggestion is supported by the marked decrease in Alcianophilic material at the interface observed after treatment of the sections with hyaluronidase. Such sections stained at 0.3 mM Mg2+ appear similar to untreated sections stained at 0.6 or 0.7 mM Mg2++.

Vigorous methylation of tissue sections removes the sulfate esters from acid mucopolysaccharide and esterifies the carboxyl groups (25). Thus, methylation removes the anionic charge from these reactive groups. Methylation of sections of intact glands by either of two procedures (24, 25) prevented the Alcian blue staining of the epithelial surface at 0.4 mM Mg2++, supporting the contention that the Alcianophilic material at the epithelial-mesenchymal interface is acid mucopolysaccharide.

TWO-STEP PAS: Acid mucopolysaccharides are distinct from other polysaccharides (or glycoproteins) in that they contain uronic acid, but do not contain sugars that are readily oxidized by
Salivary glands stained with Alcian blue 8 GX at various magnesium concentrations.

X 166. Alcian blue stains materials at the epithelial-mesenchymal interface (arrows) at 0.2 mM MgCl₂ (Fig. 1), 0.3 mM MgCl₂ (Fig. 2), and at 0.4 mM MgCl₂ (Fig. 3). At higher magnesium ion concentrations (0.6 mM in Fig. 4), the staining of the interface (arrows) has markedly decreased and at 0.7 mM (Fig. 5) the stain at the interface has almost completely disappeared, indicating that the concentration of MgCl₂ at which most of the Alcian blue is displaced from the material at the interface is between 0.4 and 0.6 mM.

Periodic acid Schiff reagents. The 1:4-linked uronic acid residues in mucopolysaccharides are attacked very slowly by periodate to produce Schiff-positive compounds by oxidation of the C₂⁻C₄ glycol group. This difference in rate of oxidation can be used to detect uronic acid-containing mucopolysaccharides. Tissues are oxidized with periodate for a short time and the aldehydes produced from rapidly oxidized compounds are rendered Schiff-negative and stable to further periodate oxidation by reduction with sodium borohydride. A longer periodate oxidation follows, producing Schiff-positive groups from the slowly reactive uronic acid residues.

Whole salivary glands were evaluated by the two-step PAS method (Figs. 6–8). Sections treated with periodate for 15 min followed by Schiff’s reagent revealed a magenta stain which completely encircled the epithelium (Fig. 6). PAS staining of the epithelial-mesenchymal interface is well known and indicates the presence of the basement membrane. To determine whether the 15 min periodate treatment oxidized all of the rapidly reactive glycols at the epithelial surface, the duration of the periodate oxidation was varied (15–60 min). The sections were then reduced with borohydride, oxidized again with periodate for 60 min, and observed for completeness of the initial oxidation by staining with Schiff’s reagent. Since no further Schiff-reactive groups were revealed by this procedure (Fig. 7), it can be concluded that the initial 15 min oxidation period was sufficient.

Sections were examined for the slowly oxidized uronic acid groups by treating with periodate for 15 min, exposing to borohydride, and oxidizing again for 24 hr. In these sections, definite Schiff-reactivity was observed at the epithelial-mesenchymal interface (Fig. 8). The distribution of Schiff-positive material was nearly uniform.
the epithelium and was essentially identical with that of the material staining with Alcian blue at 0.4 M Mg++. These data provide evidence for a uronic acid–containing acid mucopolysaccharide at the epithelial-mesenchymal interface.

**Ruthenium Red**

Ruthenium red (ruthenium oxychloride) is a polyvalent cation which interacts with anions and, when used in combination with osmium tetroxide, produces an electron-opaque material readily observed in the electron microscope. Solutions of polymerized hyaluronic acid, chondroitin sulfate, heparin, and pectin are readily precipitated by ruthenium red (16). Staining by this metallic dye is prevented by prior treatment with either hyaluronidase (27) or quaternary ammonium compounds which bind to mucopolysaccharides (28). Thus, it has been used as an ultrastructural indicator of acid mucopolysaccharide.

Ultrastructural observation of salivary rudiments treated with this stain revealed electron-opaque material in the intercellular spaces of the epithelium and mesenchyme. The epithelial-mesenchymal interface showed an extremely heavy deposit of ruthenium red–staining material, which corresponded in position to the basal lamina of the epithelium (Fig. 9) and appeared to be uniformly distributed on the surfaces of adenomeres, stalks, and clefts, in a fashion similar to the Alcianophilic and two-step PAS-reactive material observed in the light microscope.

**Incorporation of Mucopolysaccharide Precursors**

**Labeling of the Epithelial-Mesenchymal Interface**: The radioautographic localization of glucosamine-3H and 35SO4 was studied to determine whether the distribution of material labeled by these mucopolysaccharide precursors was similar to that of the Alcian blue– and two-step PAS-staining material. Whole salivary glands incubated immediately after explantation for 2 hr in the presence of glucosamine-3H showed a heavy accumulation of label at the epithelial-mesenchymal interface (Fig. 10). Substantial incorporation was noted within the epithelium, primarily within the lobules, but very little label was seen in the mesenchyme. Maximal accumulation of label was at the epithelial surface of the distal ends of the growing and branching lobules. Less radioactivity was observed at the epithelial surface of the stalk and interlobular clefts. Treatment of the sections with protease-free, testicular hyaluronidase (Fig. 11) removed nearly all of the label from the surface of the epithelium and the stalk, whereas the radioactivity within the epithelium was less affected by the enzyme treatment.

Glands incubated immediately after explantation for 2 hr in the presence of 35SO4 showed minimal incorporation of radioactivity. Rudiments labeled for 2 hr after 22 hr of culture revealed in-
creased radiosulfate incorporation (Fig. 13). In contrast, glands labeled with glucosamine-\(^{3}H\) after 22 hr of culture incorporate less precursor (Fig. 12) than the glands labeled immediately after explantation described above. The distribution of \(^{35}SO_4\) was identical with that of glucosamine-\(^{3}H\): the greatest localization of radioactivity was at the junction of the developing epithelium and mesenchyme. Maximal accumulation of glucosamine radioactivity was again noted at the surface of the distal ends of the lobules. This distribution was not as apparent in the glands labeled with \(^{35}SO_4\) probably because of the poor resolution and difficulty in assessing quantitative differences in \(^{35}S\) radioautograms due to the high energy of the \(^{35}S\) \(\beta\)-particle (29). After 46 hr in culture, glands
labeled for 2 hr showed less incorporation of both glucosamine and sulfate radioactivity than rudiments labeled at 22 hr, but again, maximum accumulation of label was at the epithelial-mesenchymal interface.

Mature salivary glands produce mucins containing sialic acid (30) and sulfated glycoproteins (31). The mucins are readily observed within the acini of epithelia from adult animals by Alcian blue and standard PAS stains (32) but were not observed in the embryonic glands. Therefore, since the glucosamine-3H and 35SO4 radioactivity within the epithelial cells was only slightly affected by hyaluronidase treatment, this label probably represents materials other than either acid mucopolysaccharide or salivary mucins.

INCORPORATION OF GLUCOSAMINE-3H AND 35SO4 INTO ACID MUCOPOLYSACCHARIDE: The incorporation of glucosamine-3H and 35SO4 into materials with an identical localization as histochemically identified acid mucopolysaccharide and the susceptibility of the glucosamine-3H label at the epithelial surface to digestion with hyaluronidase provide evidence that the radioactive precursors are in acid mucopolysaccharide. To demonstrate that the precursors could be incorporated into authentic acid mucopolysaccharide, acid mucopolysaccharide was isolated from glands incubated as for radioautography with both glucosamine-3H and 35SO4 (Table I). Glands labeled immediately after explantation or after 22 hr of culture yielded acid mucopolysaccharide containing both 3H and 35S radioactivity. Extracts

| Time of labeling after explantation | Incorporation into acid mucopolysaccharide* (cpm/mg protein) |
|------------------------------------|-------------------------------------------------------------|
|                                   | glucosamine-3H | 35SO4 |
| Experiment 1                      |                |       |
| 0-2                               | 2408           | 704   |
| 22-24                             | 1898           | 690   |
| Experiment 2                      |                |       |
| 0-2                               | 3797           | 591   |
| 0-24 (Lipid extracted)            | 4846           | 415   |

* Four glands were used for each determination. Labeling procedures and method of mucopolysaccharide isolation are described in the text.
tion of lipids before isolation of acid mucopolysaccharides did not substantially alter the amount of acid mucopolysaccharide isolated from glands labeled for 24 hr.

**DISCUSSION**

Several features of the localization and accumulation of acid mucopolysaccharide at the epithelial-mesenchymal interface suggest that this material is involved in the morphogenetic process. As revealed by histochemical procedures, acid mucopolysaccharide is within the basement membrane and is distributed in apparently equivalent amounts over the entire epithelial surface. In contrast, the rate of accumulation of newly synthesized mucopolysaccharide is substantially greater at the surface of the distal ends of the growing and branching lobules than at the surfaces of the stalks and interlobular clefts. Although the distribution of glucosamine-H is identical to that of $^{35}$SO$_4$—the radioactivity being predominantly at the surface of the lobules—glucosamine was maximally incorporated immediately after explantation whereas sulfate incorporation was minimal at this time and increased with further culture.

Newly synthesized mucopolysaccharide accumulates most rapidly at sites showing the least amount of collagen as observed ultrastructurally. In embryonic salivary glands, there are substantially more collagen fibers at the morphogenetically quiescent areas (stalk, interlobular clefts) than at the distal ends of the growing lobules, the morphogenetically most active zones (33; F. Kallman, unpublished observations); collagen fibers are distributed on developing lung epithelia in a similar fashion (34). Acid mucopolysaccharide—protein complexes influence the fibrogenesis of tropocollagen in vitro (35, 36) and have been suggested to serve a similar function in vivo (37). If so, those sites with the greatest amount of collagen fibers might be those at which mucopolysaccharide had been previously deposited, an idea suggested by the slower rate of accumulation of newly synthesized mucopolysaccharide at these sites.

These distributions of extracellular materials may be related to the formation of the epithelial clefts, the pattern of which determines the ultimate organ form. Cleft formation in salivary glands is thought to occur by contraction of microfilaments within the basal ends of the epithelial cells (38). Using the antibiotic cytochalasin B, which reversibly disrupts the microfilaments, Spooner and Wessells have shown that recently formed clefts disappear during cytochalasin treatment and reappear precisely at their prior sites after removal of

**FIGURE 14** Model depicting the relationship between cleft formation and the distribution of extracellular materials. (A) A primary lobule. Newly synthesized mucopolysaccharide (striped areas) accumulates at the surface of the distal end of the lobule. The lateral surfaces of the lobule are sites of low rates of mucopolysaccharide accumulation and show bundles of collagen fibers. Intracellular microfilaments are present at the basal end of each cell. (B) Early cleft formation in a primary lobule. Contraction of microfilament bands within cells at the top of the lobule causes the tissue to bulge inward, initiating the formation of secondary lobules. Tropocollagen derived from the mesenchymal cells begins to undergo fibrogenesis near the surface of the incipient cleft due to the influence of the mucopolysaccharide-protein complexes. This cleft would be sensitive to the action of cytochalasin. (C) Deepening of the cleft and growth of secondary lobules. Mitotic activity of cells within the secondary lobules and continued microfilament contractility cause the cleft to deepen. As the secondary lobules grow, extracellular materials originally at the surface of the primary lobule are incorporated into the cleft, where bundles of collagen fibers are seen. This cleft would be insensitive to the action of cytochalasin. The distal surfaces of the growing secondary lobules show the bulk of newly synthesized mucopolysaccharide, and with further growth undergo cleft formation.
the drug, and that deeper, "older" clefts are not affected by the drug (38, 39). This behavior might be expected if extracellular materials participated in controlling where clefts form and in maintaining clefts after they have formed.

In light of these considerations, a sequence of events can be depicted which accounts for the distribution of total and newly synthesized mucopolysaccharide and that of collagen fibers at the epithelial surface (Fig. 14). Acid mucopolysaccharide–protein complexes are deposited at the surface of the distal ends of the primary lobules. Clefts form at these sites by the action of the putatively contractile microfilaments, and concurrently, tropocollagen, synthesized predominantly by the mesenchyme (13), begins to undergo fibrogenesis at these sites due to the influence of the mucopolysaccharide complexes. Formation of the cleft divides the primary lobule into two secondary lobules, and the cleft deepens as the secondary lobules adjacent to the new cleft grow in size due to mitotic activity. As the secondary lobules grow, an increasing number of collagen fibers, some of which were previously on the surface of the primary lobule, organize into bundles within the deepening cleft and act to stabilize it. Because of the prior deposition of mucopolysaccharide complexes at the surface of what is now the cleft, relatively little newly synthesized mucopolysaccharide accumulates within the cleft. Maximal accumulation is at the surface of the distal ends of the enlarging secondary lobules, the sites of subsequent cleft formation.

In this proposed mechanism of salivary morphogenesis, mucopolysaccharide is involved in initiating collagen fibrogenesis at sites of incipient cleft formation. The proposal implies that the areas of most rapid growth will be associated with the greatest rate of mucopolysaccharide accumulation, and suggests that salivary branching morphogenesis is dependent upon the presence of acid mucopolysaccharide–protein complexes at the epithelial surface. Experiments documenting these suggestions are reported in the accompanying paper (40).

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