/INVOLVEMENT OF EXTRACELLULAR GLYCOCONJUGATES IN BRANCHING MORPHOGENESIS OF EMBRYONIC MOUSE SUBMANDIBULAR SALIVARY GLANDS/

by

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GENERAL INTRODUCTION

Glycoconjugates are abundant throughout the biological domain. These molecules are a diverse group of biopolymers in which simple sugars or one or more carbohydrate chains are covalently linked to polypeptide or lipid (Schachter, 1984). There is increasing evidence that they are involved in many important functions, often associated with cell-cell interactions and cellular communication (Baumann and Doyle, 1984).

In embryonic organ systems, glycosylated proteins of the extracellular matrix at the epithelial-mesenchymal interface, have been implicated in mediating the interactions between the epithelium and mesenchyme. These glycosylated proteins include: (a) collagens, which contain short carbohydrate chains (Schachter, 1984); (b) classic glycoproteins, containing branched and unbranched sugar side chains of variable length (Kornfeld and Kornfeld, 1980); and (c) proteoglycans, with long polysaccharide chains composed of repeating disaccharide units (glycosaminoglycans) (Sharon, 1975), covalently attached to a core protein. Hyaluronic acid is a large glycosaminoglycan with little or no covalently-linked protein.

Interactions between the epithelium and mesenchyme are required for the development of many embryonic tissues into mature organs (Grobstein, 1967). The embryonic mouse submandibular salivary gland is one such system, and attains its mature morphology by branching morphogenesis, a developmental process characterized by recurrent lobule forming events of the epithelium. It has been a useful model for studying the involvement of extracellular matrix macromolecules in development.

One approach used by this laboratory investigates the extracellular matrix by using agents that specifically interfere with the synthesis, secretion, or

deposition of particular classes of extracellular matrix macromolecules, and assessing the effect of such interference on branching morphogenesis. The approach is a useful one, providing information about matrix requirements not obtainable by descriptive correlations, as long as non-specific effects of the inhibitory agents are addressed. Branching morphogenesis is interrupted by cytochalasin B, proline analogs, LACA, and β-xylosides by; disrupting cytoplasmic microfilaments (Spooner and Wessells, 1972), inhibiting the synthesis and secretion of interstitial collagen (Spooner and Faubion, 1980), and inhibiting proteoglycan biosynthesis (Thompson and Spooner, 1983), respectively. Such data have been crucial in demonstrating that, cytoplasmic microfilaments, interstitial collagen, and proteoglycans are all required for morphogenesis of developing salivary rudiments.

The studies reported in this thesis employ β-D-xylosides, to further characterize its inhibitory effect on branching morphogenesis, and tunicamycin, to assess the possible involvement of glycoproteins in the morphogenesis of the embryonic mouse submandibular salivary gland.

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Sulfated Glycosaminoglycan Deposition and Processing at the Basal Epithelial Surface in Branching and β-D-Xyloside Inhibited Embryonic Salivary Glands

ABSTRACT

We investigated whether the inhibition of proteoglycan synthesis and salivary branching morphogenesis by β-D-xyloside was related to the deposition and processing of newly synthesized glycosaminoglycans at the basal epithelial surface that correlates with normal branching activity. Forty eight-hour cultures of control and 0.5 mM β-xyloside-treated submandibular rudiments were labeled for 2 hr with [^{35}S]sulfate and fixed and processed for autoradiography, immediately or after 2, 4, 6, or 8 hr of postlabeling chase in nonradioactive medium. The data demonstrated that deposition of chondroitin sulfate-rich material at the basal epithelial surface was strikingly reduced in β-xyloside-treated rudiments, while patterns of label loss during postlabeling chase were not altered.

INTRODUCTION

The extracellular matrix of developing tissues is thought to be intimately involved in embryonic morphogenesis (see Hay, 1981, for reviews). In organ systems which undergo mesenchyme-dependent branching morphogenesis of the epithelium, there is evidence that both collagen and proteoglycans and glycosaminoglycogans (GAG) of the extracellular matrix are essential macromolecular components (see Spooner, et al., 1985 for review). The embryonic submandibular salivary gland is a well-studied organ rudiment that requires epithelial-mesenchymal tissue interactions (Grobstein, 1953), microfilament-mediated epithelial cell shape changes (Spooner and Wessells, 1972), and interstitial collagen (Spooner and Faubion, 1980) for branching morphogenesis in culture.

The evidence for proteoglycan and glycosaminoglycan involvement in salivary branching morphogenesis comes from two different lines of research. On the one hand, Bernfield and his collaborators have demonstrated the presence of glycosaminoglycans at the basal epithelial surface (Bernfield and Banerjee, 1972), which are components of the epithelially derived basal lamina (Banerjee et al., 1977), and have shown that removal of the glycosaminoglycan-containing basal lamina interrupts morphogenesis (Bernfield et al., 1972). Furthermore, they have shown that the basal lamina synthesized by the epithelium contains both hyaluronic acid and sulfated glycosaminoglycans, i.e., proteoglycans (Cohn et al., 1977), and that mesenchyme-mediated differential turnover of these materials correlates with branching morphogenesis (Bernfield and Banerjee, 1982, Smith and Bernfield, 1982). In a separate series of experiments, we have demonstrated that β-xylosides dramatically inhibit branching morphogenesis (Thompson and Spooner, 1982), and that, while β-xylosides both stimulate synthesis of core protein-free sulfated glycosaminoglycans and

inhibit proteoglycan synthesis, the inhibition of branching directly correlates with the inhibition of proteoglycan synthesis (Thompson and Spooner, 1983; Spooner et al., 1985).

The present study investigates whether the inhibition of proteoglycan synthesis in β-xyloside-treated salivary rudiments results in alterations in sulfated glycosaminoglycan deposition or subsequent loss at the basal epithelial surface. The results show reduced deposition of newly synthesized material, providing a crucial link between the evidence that proteoglycan synthesis is required for branching activity (Thompson and Spooner, 1983) and the evidence that a proteoglycan-rich basal lamina is necessary (Banerjee et al., 1977). However, the pattern of disappearance of sulfated glycosaminoglycan is not altered in β-xyloside-treated cultures, even though the amount deposited is substantially reduced.

MATERIALS AND METHODS

Organ culture. Submandibular salivary gland rudiments were microdissected from embryos taken from pregnant CD-1 mice at Day 13.25 of gestation (day of vaginal plug discovery designated as Day 0). At this time, the epithelium possessed three to five lobes. The rudiments were organ cultured for 48 hr at 37°C, in a 5% CO_2:95% air high-humidity incubator, at the air-medium interface on TH-Millipore filter rafts, as previously described (Spooner and Faubion, 1980; Thompson and Spooner, 1982). The culture medium was modified Ham's F12 containing 10% fetal calf serum. Cultures were fed by a complete change of medium at 24 hr of incubation. p-Nitrophenyl-β-D-xlopyranoside (β-xyloside) was incubated in culture medium at a concentration of 0.5 mM (which inhibits branching, stimulates free sulfated GAG synthesis, and inhibits proteoglycan synthesis), and both control and β-xyloside cultures were observed and photographed through a 4X Planapo objective on a Zeiss photomicroscope.

Pulse-labeling with [^{35}S]sulfate. At 48 hr of culture, the medium was removed and replaced with serum-free medium containg 50 μCi/ml [^{35}S]sulfate, with or without 0.5 mM β-D-xyloside (consistent with the first 48 hr of culture). Following a 2-hr labeling period, the radioactive medium was removed, and the cultures were rapidly rinsed 3-4X with nonradioactive medium and then either fixed immediately or reincubated and fixed after 2, 4, 6, or 8 hr of postlabeling chase in nonradioactive control or β-D-xyloside-containing medium.

Labeling with [^{3}H]glucosamine. Freshly dissected Day 13 rudiments were immediately placed in a sterile plastic petri dish containing serum-free medium and [^{3}H]glucosamine (50 μCi/ml) with or without the addition of 0.5 mM β-D-xyloside. Following a 2 hr labeling period the rudiments were rinsed 3-4X with nonradioactive medium and fixed.

In a separate series of experiments rudiments were cultured for 48 hrs in either control or 0.5 mM β-D-xyloside medium then transferred to a Falcon plastic microtiter test plate by placing each Millipore filter over a well filled with 15 µl of serum-free medium containing 50 µCi/ml [^{3}H]glucosamine. The rudiments were incubated in this medium for 2 hrs with or without the addition of 0.5 mM β-D-xyloside according to their original culture conditions. Immediately after the labeling period each rudiment was rinsed by inverting the Millipore filter (rudiment face down) and floating them in a petri dish of non-radioactive medium for 10 minutes and then fixed.

Fixation and autoradiography. Rudiments were fixed in absolute ethanol: acetic acid (99:1) for 24 hr, dehydrated through 100% ethanol, and embedded in paraffin. The paraffin blocks were sectioned at 7 µm, and the sections were placed on albumin-coated slides. After 24 hr, the sections were deparaffinized and rehydrated, immersed in nuclear track emulsion type NTB_2 (Eastman Kodak) for 5 sec, and air-dried vertically (to drain excess emulsion and obtain uniform emulsion coating). Emulsion-coated slides were exposed for 5 days in light-tight boxes and developed, coverslips were mounted over the sections, and the slides were observed and photographed through bright-field optics on a Zeiss photomicroscope.

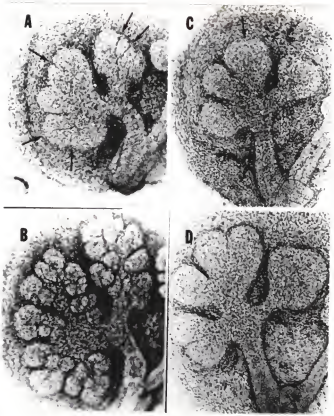
Enzyme treatments. To assess enzyme sensitivity of [^{35}S]sulfate-labeled material, sections were deparaffinized and rehydrated, and then incubated for 24 hr in either testicular hyaluronidase (type III, Sigma), at 2 mg/ml in 0.1 M sodium acetate buffer (pH 5.0), or chondroitin ABC lyase (Miles), at 0.5 U/ml in 0.01 M Tris·HCl (pH 8.0). Following enzyme treatment, or control treatment with buffer alone, the slides were processed for autoradiography as above.

RESULTS

Morphogenesis of Control Versus β-Xyloside-Treated Rudiments

Submandibular salivary rudiments, isolated from 13.25-day mouse embryos, initially possess three to five epithelial lobes (i.e., two to four clefts are present in the epithelium). During a 48-hr organ culture tenure, control rudiments undergo growth and extensive branching morphogenesis. The number of epithelial lobes approximately doubles to triples during the first 24 hr of culture, and again triples during the 24-48 hr culture period (Figs. 1A and B). In contrast, rudiments cultured for 48 hr in the presence of 0.5 mM β-xyloside display inhibited branching activity (Figs. 1C and D). During the 0-24 hr culture period, growth and morphogenesis continue, although branching activity is somewhat retarded relative to controls. That is, the number of lobes variably increases by 25-100% during the first 24 hr of culture, in comparison to a tripling of lobes in controls. However, during the 24-48 hr culture period, branching activity is dramatically inhibited in 0.5 mM β-xyloside-treated rudiments (Fig. 1D). While controls again triple the number of epithelial lobes, few new lobes appear in the β-xyloside-treated rudiments (i.e., 0-15% increase). Thus, branching activity is inhibited, relative to controls, by some 75-80% over the entire 48-hr culture tenure, and by 85-100% during the 24-48 hr culture period. However, clefts and lobes present at 24 hr remain at 48 hr and furthermore, epithelial expansion continues (Figs. 1C and D), resulting in cleft deepening (see also Fig. 1, Thompson and Spooner, 1983). The loss of clefts by lobe fusion that is sometimes observed, particularly at higher β-xyloside concentrations (Thompson and Spooner, 1982), is clearly not an obligatory correlate of the inhibition of branching morphogenesis.

Fig. 1. β-Xyloside inhibition of branching morphogenesis. (A) A rudiment cultured for 24 hr in control medium. (B) The same rudiment after 48 hr of culture. (C) A rudiment cultured for 24 hr in 0.5 mM β-xyloside-containing medium. (D) The same rudiment after 48 hr of culture in the presence of 0.5 mM β-xyloside. Both rudiments possessed 3 lobes at the time of explantation. At 24 hr, the control (A) has 4 major lobes and, additionally, has several narrow clefts (arrows) that define a total lobe number of about 8, while the β-xyloside-treated rudiments (C) has 4 major lobes plus one very shallow early cleft (arrow). At 48 hr, the control (B) has about 30 lobes, while the β-xyloside-treated rudiment has only 6 well-defined lobes. Growth and expansion of the epithelium has continued (compare C and D). Branching has been inhibited in the presence of β-xyloside by some 80% (compare D with B), even though the epithelium retains the lobular structure it had at 24 hr, has formed a few new lobes, and has expanded. A-D, 34X.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The presence of lobules and clefts in β-xyloside-treated rudiments allow an assessment of the deposition, and subsequent processing, of radioactively labeled GAG at the basal epithelial surface, by autoradiographic analysis of [^{35}S]sulfate pulse-chased rudiments, comparing both lobe tips and clefts of actively branching and β-xyloside-inhibited rudiments.

[^{35}S]Sulfate Labeling and Turnover in Control Cultures

Submandibular salivary rudiments, cultured for 48 hr in control medium and then pulse-labeled for 2 hr with [^{35}S]sulfate (50 μCi/ml), incorporate substantial radioactivity that survives fixation, embedding, sectioning, and rehydration and is resolvable by light microscopic autoradioagraphy (Fig. 2A). Intense label localization at the epithelial surface defines the epithelial contour. Label distribution over the basal surface of the epithelium is relatively uniform. With increasing periods of postlabeling chase in non-radioactive medium, label is progressively lost from the epithelial surface (Figs. 2B-E). Initially, label is lost from the tips of lobes and is more stable in the clefts between lobes. With continued label loss, the epithelial contour becomes poorly defined, although radioactivity continues to be present in clefts.

The bulk of the ^{35}S radioactivity localized in these preparations is sensitive to treatment of the sections with testicular hyaluronidase or chondroitin ABC lyase (Fig. 3). Thus, the labeled material deposited at the basal epithelial surface during a 2-hr period is predominantly chondroitin sulfates, on the basis of hyaluronidase sensitivity. Enzyme-resistant material may include dermatan sulfate, heparan sulfate, and/or sulfated glycoproteins. The similar result with chondroitin ABC lyase is consistent with the conclusion that the labeled material is predominantly chondroitin sulfates and dermatan

Fig. 2. Deposition and turnover of surface-associated sulfated GAG in cultured embryonic submandibular salivary rudiments. Rudiments were cultured for 48 hr and then labeled for 2 hr with [^{35}S]sulfate (50 μCi/ml). Autoradiograms of sections of rudiments fixed and processed immediately after labeling (A), and after 2 hr (B), 4 hr (C), 6 hr (D), and 8 hr (E) of postlabeling chase in nonradioactive medium show intense labeling of the basal epithelial surface that is progressively lost over an 8-hr chase period. Arrows indicate the tips of lobes. Only a portion of a section is shown, in each case, in order to illustrate lobe tips and clefts. A-E, 128X.

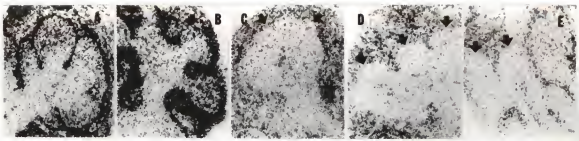
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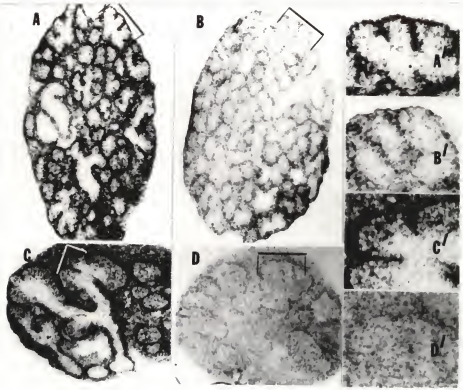
Fig. 3. Surface-associated radioactivity is sensitive to GAG degradative enzymes. Rudiments were cultured 48 hr, labeled with [^{35}S]sulfate for 2 hr (as in Fig. 2), and then fixed (no chase period), embedded and sectioned. Sections incubated, prior to processing for autoradiography, with hyaluronidase buffer alone (A) versus buffer containing hyaluronidase (B) demonstrate that the bulk of the radioactivity is sensitive to this enzyme. These sections also show the extensive degree of morphogenesis that has taken place during 48 hr in culture. The areas marked in (A) and (B) are shown at higher magnification in (A') and (B'), respectively to illustrate a region of lobes and clefts. Equivalent areas of sections are shown in Figs. 2, 4, and 6, to allow evaluation of labeling at lobe tips and in clefts. Sections incubated with chondroitin ABC lyase buffer alone (C) versus buffer containing chondroitin ABC lyase (D) show the sensitivity of the bulk of the radioactivity to this enzyme. These sections are at different angles through the rudiment than those shown in (A) and (B), but the rudiment is at an identical stage of development (i.e., 13.25-day rudiment cultured for 48 hr) and degree of morphogenesis. Marked areas are shown in (C') and (D'), respectively, at higher magnification, equivalent to those shown in (A') and (B'). The bulk of the radioactivity is sensitive to both enzymes and is, therefore, predominantly chondroitin sulfates. Some dermatan sulfate may also be included. A and B, 64X; C and D, 92X; A', B', C', and D', 150X.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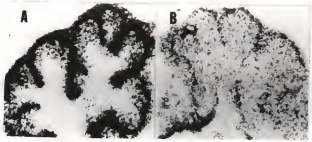
sulfate. Enzyme-resistant material may include heparan sulfate and/or sulfated glycoproteins.

Surface-Associated [^{35}S]GAG Deposition Is Reduced in β-Xyloside-treated Rudiments

Rudiments were cultured for 48 hr in control or β-xyloside (0.5 mM) medium, labeled for 2 hr with [^{35}S]sulfate (50 μCi/ml), and immediately processed for autoradiography, as above. We have evaluated some 100 rudiments in five different experiments and the results are always the same. Sections of controls exhibit intense label localization that defines the basal epithelial surface (Fig. 4A). Sections of β-xyloside-treated rudiments, on the other hand, exhibit a sharply reduced degree of label localization (Fig. 4B). Radioactivity is again present at the basal epithelial surface, defining the epithelial contour, but the intensity is substantially less than that observed in controls. We have used matched photographic exposure times, with the time determined on the basis of both control and β-xyloside maximal exposure times, and the result is always the same. That is, label intensity is greatly reduced in β-xyloside preparations, relative to controls. The decreased radioactivity detected by autoradiography in β-xyloside-treated rudiments is not the result of reduced total sulfated GAG synthesis in these cultures. Previous studies (Thompson and Spooner, 1982, 1983) demonstrated that total sulfated GAG synthesis was dramatically stimulated in the presence of β-xyloside, but the bulk of the excess GAG (relative to controls) was found associated with the medium rather than the tissue. Analysis of the medium following the 2-hr labeling period in the present studies, showed that the medium from β-xyloside-treated cultures contained four times the control medium amount of Sephadex G50-excludable radioactivity, consistent with the results of those earlier studies.

Fig. 4. Deposition of epithelial surface-associated radioactivity is dramatically reduced in β-xyloside-treated rudiments. Submandibular rudiments were cultured in control medium or medium containing 0.5 mM β-xyloside for 50 hr, labeled with [^{35}]sulfate during the final 2 hr of culture, and then immediately fixed and processed for autoradiography. Autoradiograms of control sections (A) show intense radioactivity at the basal epithelial surface (as in Fig. 2A). Sections of β-xyloside-treated rudiments (B) exhibit sharply reduced amounts of surface-associated radioactivity. These sections were identically exposed and developed, photographed under identical exposure conditions, and printed identically. A portion of a section, illustrating lobe tips and clefts, is shown in each case. A and B, 126X.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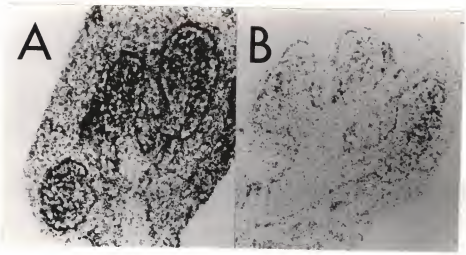
The radioactivity that is localized in the β-xyloside-treated cultures, although less intense than in controls, is similarly sensitive to treatment with testicular hyaluronidase and chondroitin ABC lyase. That is, although there is far less radioactivity localized to begin with, it remains when incubated with buffer alone, and is further diminished by enzyme treatment.

Thus, β-xyloside-treated salivary rudiments exhibit a sharply reduced deposition of newly synthesized sulfated GAG (principally chondroitin/dermatan sulfate) at the basal surface of the epithelium, a result consistent with a documented inhibition of proteoglycan synthesis in this system (Thompson and Spooner, 1983) that correlates with an inhibition of branching morphogenesis (Spooner and Thompson, 1982). The data therefore relate the β-xyloside effect on branching to the proteoglycan-rich basal lamina produced by the epithelium (Cohn et al., 1977; Banerjee et al., 1977).

Bernfield's laboratory has investigated laminar glycosaminoglycan synthesis and degradation by labeling freshly explanted 13 day salivary rudiments with [^{3}H]glucosamine (Bernfield and Banerjee, 1972; Bernfield, 1982). Figure 5A displays an autoradiogram of a freshly dissected 13 day control rudiment labeled with [^{3}H]glucosamine for 2 hrs. There is an intense localization of label at the tips of lobes, and little label within and at the base of the clefts, thus replicating Bernfield's results. When β-xyloside is included in the [^{3}H]glucosamine labeling medium, there is a substantial reduction in the amount of radioactivity at the basal epithelial surface (Fig. 5B), compared to controls and consistent with the results obtained with [^{35}S]sulfate-labeled β-xyloside-cultured rudiments. It appears, therefore, that the inhibition of proteoglycan synthesis occurs swiftly, evident after only 2 hrs of incubation with β-xyloside. In fact, 2 hrs is a conservative estimate since that is the

Fig. 5. Submandibular salivary glands were incubated immediately after dissection in the presence of 50 μCi/ml [^{3}H]glucosamine with or without the addition of 0.5 mM β-xyloside for 2 hr, fixed and processed for autoradiography. Autoradiograms of control sections (A) show substantial amounts of radioactivity within the epithelium and at the distal, epithelial-mesenchymal interface of lobes. Significantly less label is deposited at this boundary deep within the clefts (arrows). Sections of β-xyloside-treated rudiments (B) demonstrate considerably reduced amounts of radioactivity throughout the epithelium and at the basal epithelial surface. Each autoradiogram illustrates a section of an entire submandibular salivary gland. A and B, 200X.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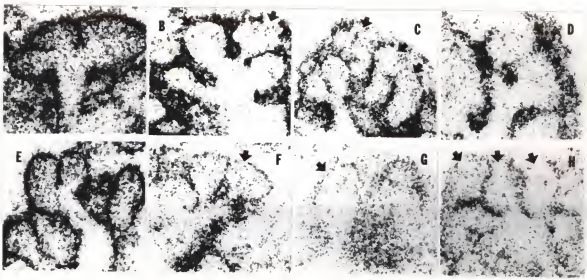
time required for labeled precursor incorporation into laminar glycosaminoglycan and proteoglycan, demonstrable by autoradiography, at the basal epithelial surface of controls.

Turnover of Surface-Associated [^{35}S]GAG in Control Versus β-Xyloside-Treated Rudiments

To determine whether the reduced deposition of newly synthesized [^{35}S]GAG, in the presence of β-xyloside, was followed by perturbations in the pattern of turnover of surface-associated sulfated GAG, pulse-chase studies were conducted. β-Xyloside-treated rudiments and controls were cultured for 48 hr, labeled for 2 hr with [^{35}S]sulfate (50 μCi/ml), and then fixed and processed for autoradiography, immediately or after 2, 4, 6 or 8 hr of postlabeling chase in nonradioactive medium. Autoradiograms of the sections failed to reveal differences in the pattern or time course of disappearance of the surface-associated label (Fig. 6). Definition of the basal epithelial surface was progressively lost with increased chase time. Label was initially lost from the tips of lobes and was lost more slowly from the clefts between lobes.

Fig. 6. Turnover of surface-associated radioactivity is not perturbed in β-xyloside-treated cultures. Rudiments were cultured, labeled, and chased in control or β-xyloside-containing medium and then fixed, embedded, sectioned, and processed for autoradiography immediately or after increasing periods of incubation in nonradioactive (chase) medium. Autoradiograms are shown at 0 (A), 4 (B), 6 (C), and 8 (D) hr of postlabeling chase for controls, and at 0 (E), 4 (F), 6 (G), and 8 (H) hr of postlabeling chase for β-xyloside-treated rudiments. Radioactivity is progressively lost with increasing chase time, initially from the tips of lobes (arrows) and more slowly from clefts between lobes, in both control and β-xyloside-treated rudiments. For an additional pulse-chase control series, compare Fig. 2 with the β-xyloside E-H series. A-H, 144X.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DISCUSSION

Morphogenesis of the embryonic submandibular salivary gland requires an epithelial-mesenchymal tissue interaction (Grobstein, 1953). Interstitial collagen, produced by the mesenchyme (Kallman and Grobstein, 1965; Bernfield, 1970), must be present at the epithelial-mesenchymal tissue interface if epithelial branching is to take place (Spooner and Faubion, 1980). The epithelium is covered by a basal lamina, rich in glycosaminoglycans (Bernfield and Banerjee, 1972), whose experimental removal interrupts morphogenesis (Bernfield et al., 1972). The basal lamina is produced by the epithelium (Banerjee et al., 1977), and the GAG composition of the newly synthesized basal lamina is about 50% hyaluronic acid and 40% chondroitin sulfates, with the remaining 10% probably heparan sulfate (Cohn et al., 1977). Chondroitin, dermatan, heparan sulfates are normally found as proteoglycan, rather than free GAG chains. Thus, the major proteoglycan of the basal lamina appears to be chondroitin/dermatan sulfate proteoglycan, but the non-protein-associated GAG, hyaluronic acid, accounts for some 50% of the total GAG present.

The xylose derivative, p-nitrophenyl-β-D-xylopyranoside, competes with endogenous xylosylated core protein and results in synthesis of core protein-free GAG chains, inhibition of synthesis of proteoglycans, and inhibition of branching morphogenesis (Thompson and Spooner, 1982). Analysis of β-xyloside effects on morphogenesis versus effects on free GAG and proteoglycan synthesis demonstrated a direct relationship between the degree of inhibition of proteoglycan synthesis and the degree of inhibition of branching morphogenesis (Thompson and Spooner, 1983). The present study demonstrates that the depression in proteoglycan synthesis, in the presence of β-xylosides, is resolvable as reduced deposition of sulfated GAG at the basal surface of the epithelium,

i.e., the level of the basal lamina. The experiments also confirm, on the basis of testicular hyaluronidase and chondroitin ABC lyase sensitivity, the observation of Cohn et al. (1977) that chondroitin/dermatan sulfate proteoglycan is the principal proteoglycan class synthesized and deposited at the basal surface of the epithelium. Thus, β-xyloside inhibition of proteoglycan biosynthesis results in reduced deposition of proteoglycan at the basal epithelial surface and branching morphogenesis ceases. The data therefore show a relationship between the requirement for a GAG-rich basal lamina (Bernfield et al., 1972; Banerjee et al., 1977) and the requirement for proteoglycan synthesis (Thompson and Spooner, 1983; Spooner et al., 1985) in submandibular epithelial branching morphogenesis. They do not comment on possible requirements for other basal lamina components (hyaluronic acid, laminin, or basement membrane collagens), because β-xylosides have not been shown to affect those other components and, in fact, a structurally intact basal lamina remains in β-xyloside-treated rudiments (Thompson and Spooner, 1983).

The discussion thus far has suggested that inhibition of branching by β-xyloside results from insufficient amounts of basal epithelial surface-associated proteoglycan. However, it is possible that the actual basis for inhibition is at the level of postdeposition processing of the proteoglycan. It has been shown, by autoradiography of [^{3}H]glucosamine pulse-chased rudiments, that the basal epithelial surface-associated GAG undergoes a distinct pattern of turnover (Bernfield and Banerjee, 1982) and that the mesenchyme is responsible for the GAG degradation (Smith and Bernfield, 1982). Pulse-chase experiments in the present study show a similar pattern of [^{35}S]sulfate-labeled GAG loss, and moreover, demonstrate that sulfated GAG loss in β-xyloside-treated rudiments occurs with a time course and pattern that is not distinguishable from controls. Thus, although less proteoglycan is deposited at the

level of the basal lamina in β-xyloside-treated cultures, it appears to be degraded normally. Therefore, the β-xyloside inhibition of branching appears to stem from decreased deposition of proteoglycan and not from postdeposition processing alterations. The studies of Bernfield and Banerjee (1982) that demonstrated rapid loss of labeled GAG from the tips of lobules also showed that when lobule expansion is blocked with inhibitors of cell proliferation, label loss is markedly slowed. Since lobule expansion continues in the presence of β-xylosides, it is, perhaps, not surprising that label loss occurs apparently normally. It will be of interest to compare the basal laminae of control and β-xyloside-treated rudiments for anionic site presence and distribution for additional insight into proteoglycan involvement in branching morphogenesis.

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Glycoproteins and Epithelial Morphogenesis: Tunicamycin Effects on Embryonic Salivary Gland Growth and Branching Activity

ABSTRACT

The possible involvement of glycoproteins in branching morphogenesis of cultured embryonic mouse salivary glands was examined by the use of tunicamycin, a potent inhibitor of N-asparagine linked protein glycosylation. Glycoproteins such as laminin, fibronectin, and entactin appear to be present in most basement membranes and may play a role in epithelial morphogenesis. Dose-response experiments demonstrated increasing salivary growth inhibition with increasing doses of tunicamycin (to 50 ng/ml), at which point cultures no longer remained viable. However, branching morphogenesis was not affected, even when growth was substantially retarded. Light microscopic autoradiograms of rudiments cultured for 48 hrs in the presence of 25 ng/ml tunicamycin and metabolically labeled with [^{3}H]mannose for 4 hrs, revealed a marked decrease in the amount of label, presumably glycoprotein, incorporated and deposited at the basal surface of the epithelium when compared to controls. While the role served by intact glycoproteins at the basal epithelial surface remains unclear, the data suggest that control levels of N-asparagine linked oligosaccharide synthesis and deposition are not required for ongoing branching of the epithelium.

INTRODUCTION

Extracellular matrix-mediated epithelial-mesenchymal tissue interactions are essential for the development of many embryonic organ systems (for review see Grobstein, 1967). The submandibular salivary gland is representative of such systems and attains its mature morphology by branching morphogenesis, a developmental process characterized by repetitive lobule forming events of the epithelium. Although branching results from forces within individual epithelial cells that generate changes in cell shape to produce branch points between lobes (Spooner and Wessells, 1972), the presence of the mesenchyme is required and regulates the pattern of epithelial morphogenesis (Taderera, 1967; Nogawa, 1981).

The epithelium and mesenchyme are partitioned by an extracellular matrix whose constituent macromolecules have been implicated as important mediators in developmental events. The major classes of matrix macromolecules include collagens (Ekblom, 1981), glycosaminoglycan and proteoglycan (Thompson and Spooner, 1982; Thompson and Spooner, 1983), and glycoproteins (Thesleff et al., 1981). They are present in the extracellular spaces between mesenchymal cells, the interstitial matrix, and in the basal lamina, an epithelially derived extracellular matrix compartment. When interstitial collagen is reduced by inhibiting its synthesis in embryonic mouse lung and salivary glands, branching morphogenesis is blocked, demonstrating a developmental requirement for interstitial collagen in these systems (Spooner and Faubion, 1980). Biochemical studies show that the basal lamina of embryonic mouse salivary glands contains abundant glycosaminoglycan and sulfated proteoglycan, and the existing evidence suggests a major role for these molecules in branching morphogenesis (Cohn et al., 1977; Thompson and Spooner, 1983). For

example, recombination of a basal lamina-free salivary epithelium with its original mesenchyme interrupts branching, as does direct inhibition of proteoglycan synthesis and deposition at the basal surface of the epithelium (Thompson and Spooner, 1983; Spooner et al., 1985). These effects are reversible; in the first case, by allowing the epithelium to deposit a new basal lamina prior to recombination with mesenchyme (Banerjee et al., 1977); in the second case, by removal of the proteoglycan synthesis inhibitor. Additionally, the directed turnover of basal lamina materials, via mesenchymal interactions, correlates with branching morphogenesis (Bernfield and Banerjee, 1982).

Although glycoproteins such as laminin and fibronectin are present in basement membranes and extracellular spaces in various organ systems (Thesleff et al., 1981; Jaskoll and Slavkin, 1984; Parmigiani and McAvoy, 1984), their role in branching morphogenesis has not yet been determined. Tunicamycin is an antibiotic isolated from Streptomyces lysosuperficus which inhibits the biosynthesis of glycoproteins containing N-asparagine linked oligosaccharides (Takatsuki et al., 1971; Lehle and Tanner, 1976). It has been used to investigate the function of N-glycosidically linked oligosaccharide moieties of glycoproteins in a diverse range of biological processes (Olden et al., 1978; Hickman and Kornfeld, 1980; Gipson et al., 1984) including development (Surani, 1979; Thesleff and Pratt, 1980). The application of tunicamycin to systems undergoing branching morphogenesis has the potential to provide insight into the involvement of glycoprotein synthesis in this process.

Embryonic salivary glands cultured in the presence of tunicamycin exhibit inhibited growth, with no apparent effect on branching morphogenesis. Metabolic labeling, with [^{3}H]mannose, and light microscopic histological autoradiography reveals a sharp decrease in the amount of radioactive material, presumably

glycoprotein, localized to the basal surface of the epithelium in rudiments cultured with tunicamycin, relative to controls. These data suggest that N-asparagine linked oligosaccharide-containing glycoproteins, or their constituent carbohydrate moieties, are not required for branching of the salivary epithelium, but may be necessary for growth.

MATERIALS AND METHODS

Organ Culture

Embryos were obtained from cervically dislocated pregnant CD-1 outbred mice (Charles River Mouse Farms) at 13 days of gestation, where Day 0 was marked by discovery of the vaginal plug. Under sterile conditions, submandibular salivary gland rudiments (and sublinguals) were microdissected from these embryos and collected into a petri dish of 37°C Hank's Balanced Salt Solution (HBSS).

Each rudiment was transferred onto the upper surface of a TH-Millipore filter (Millipore Filter Corp.) culture assembly placed in a Grobstein tissue dish (Grobstein, 1956). An air-medium interface was provided by pipetting the culture medium up to the level of the plexiglass raft. The culture medium used was a modified Ham's F12 (Spooner, 1970) containing 10% fetal calf serum (Grand Island Biological Co.). Rudiments were cultured for 48 hrs at 37°C in a humidified atmosphere of 5% CO_2 in air. After 24 hrs of incubation, cultures were fed by a complete change of medium. Tunicamycin was prepared as a stock solution (10 µg/ml in DMSO:medium [1:10]) and stored at -70°C in 0.1 ml aliquots. Stock aliquots were diluted to the desired final concentrations with warm culture medium. Submandibular rudiments were cultured with 0.5 mM β-D-xylosides as previously described (Spooner et al., 1985).

Branching morphogenesis of living control and tunicamycin treated cultures was observed and photographed every 24 hrs under a 4X Planapo objective using bright field optics on a Zeiss photomicroscope II.

Labeling with [^{3}H]mannose

After 48 hrs of culture, the rudiments were transferred to a Falcon plastic microtiter test plate. Each Millipore filter was placed over a well

filled with 15 µl of serum-free medium containing 100 µCi/ml mannose, D-[3,4-3H(N)]-(40.6 Ci/mmole) (New England Nuclear). The rudiments were incubated in this medium, with or without the addition of tunicamycin (25 ng/ml) (Sigma) according to their original culture conditions, for 4 hrs. Immediately after the labeling period, the rudiments were rinsed by inverting the Millipore filter (rudiment face down) and floating them in a petri dish of non-radioactive medium for 10 minutes. Following the rinse period, rudiments were fixed in 95% ethanol:glacial acetic acid (99:1) for 24 hrs.

To monitor the turnover of mannose-containing materials, rudiments were cultured for 48 hrs in control medium and pulse-labeled as above. The Millipore filters, with rudiments intact, were returned to their original Grobstein culture assemblies, rinsed 3-4X and fixed immediately or after additional 2, 4, 8, or 24 hr culture periods in nonradioactive control medium.

Labeling with [^{35}S]sulfate

Following 48 hrs of incubation in either control or tunicamycin (25 ng/ml) medium, rudiments were cultured in serum-free medium containing 50 µCi/ml [^{35}S]sulfate with or without the addition of tunicamycin, consistent with the previous culture conditions. After a 2 hr labeling period, the radioactive medium was removed, the cultures rinsed 3-4X with non-radioactive medium and fixed. Identical labeling techniques were used for 0.5 mM β-xyloside cultures.

Processing and Autoradiography

Processing of the fixed rudiments included dehydration through 100% ethanol with transfers to toluene, toluene:paraffin (1:2), and 100% paraffin. Following an overnight infiltration with 100% paraffin, rudiments were embedded in paraffin blocks, sectioned at 7 µm, and the sections flattened on albumin coated slides. The slides were deparaffinized and rehydrated through distilled

water. In total darkness, two slides, back to back, were submerged for 5 seconds in nuclear track emulsion type NTB2 (Eastman Kodak) and the excess emulsion was drained by orienting the slides vertically. The emulsion coated slides were air dried and exposed for 20 days in a light tight box, containing dessicate, at 4°C.

Exposed slides were developed for 2 min (Dektol:water [1:1]), rinsed for 10 sec in a distilled water stop bath, immersed in fixer for 5 min and washed for 5 min in distilled water (all solutions at 15°C). The resulting autoradiograms were air dried, mounted with coverslips and photographed using the bright field optics on a Zeiss photomicroscope II.

Enzyme Treatment

The sensitivity of the [^{3}H]mannose labeled material to chondroitin ABC lyase (Miles) was tested. Sections were deparafinized, rehydrated, and then incubated with chondroitin ABC lyase, at 0.15 U/ml in 0.01 M Tris.HCl (pH 8.0) for 24 hrs. Following enzyme treatment, or control treatment with buffer alone, the slides were processed for autoradiography as above. In an identical manner, the sensitivity of [^{35}S]sulfate labeled material to chondroitin ABC lyase was tested.

RESULTS

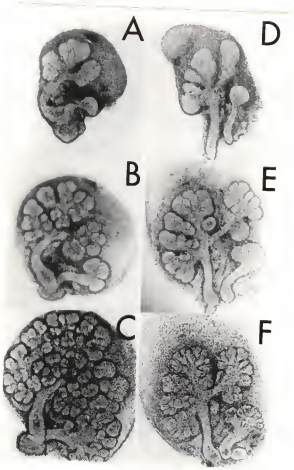
Morphogenesis of Salivary Cultures Under Control Conditions

In vivo, embryonic mouse submandibular salivary gland morphogenesis is characterized by growth and extensive branching of the epithelium within the bed of mesenchyme. Salivary rudiments dissected from day 13 mouse embryos and cultured over a period of 48 hrs exhibit a morphogenetic pattern which simulates development *in vivo* (Fig. 1A, B, C). Rudiments at day 13 routinely consist of 3-5 lobes (Fig. 1A, D), and the number of lobes approximately triples every 24 hrs in culture for at least the first 48 hrs. Growth of the rudiment accompanies branching, evident as expansion of the newly formed epithelial lobes.

Effect of Tunicamycin on Salivary Morphogenesis

Preliminary tunicamycin dose-response experiments established 25 ng/ml as a concentration that was non-toxic, but still elicited an effect on salivary development in organ culture. At 25 ng tunicamycin/ml of culture medium (Fig. 1D, E, F), the number of new branch points that form and deepen is maintained, relative to controls. However, there is little apparent growth of these lobes during the 24 to 48 hr culture period, resulting in an overall decrease in the size of the epithelium, compared to controls. The lack of growth of the epithelium during the 24 to 48 hr culture period, while branching activity continues unabated, results in a rudiment comprised of many miniature lobes. The sublingual salivary gland, which is situated alongside the submandibular gland in these cultures, seems to be affected in the same manner, i.e., retarded growth but normal branching. Tunicamycin at 5 ng/ml has no observable effect on submandibular morphogenesis, and at 50 ng/ml, death of the cultures results during the 24 to 48 culture interval.

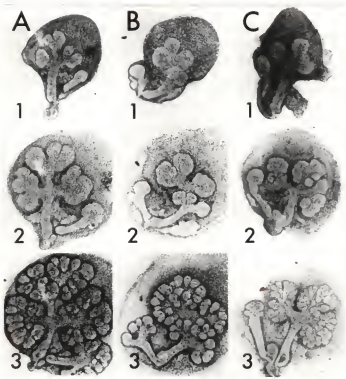
Fig. 1. Morphogenesis of control and tunicamycin-treated salivary glands. Photographs of two living submandibular salivary rudiments (and sublinguals, smaller in size and possessing their own stalk) are shown at 0 hr (A, D), 24 hr (B, E), and 48 hr (C, F) of culture. The epithelium of the rudiment cultured in control medium undergoes extensive branching and growth during the culture period (A-C). Rudiments cultured in 25 ng of tunicamycin/ml of culture medium (D-F) exhibit inhibited epithelial expansion (growth) by 48 hr culture time, however, branching activity is maintained relative to controls. A-F, 28X.


A
D
B
E
C
F

Growth of the epithelium appears only minimally inhibited by tunicamycin (25 ng/ml) during the first 24 hrs of culture, compared to controls (Fig. 2, A2, B2, C2). Day 13 rudiments cultured in the presence of tunicamycin for 24 hrs, briefly rinsed, and cultured in control media for an additional 24 hrs exhibit a morphology which differs from rudiments cultured continuously in either control or tunicamycin-containing media (Fig. 2, A3, B3, C3). Compared to controls, the epithelial component of these "recovery" rudiments after the 48 hr culture period (Fig. 2, B3) appears somewhat smaller in size suggesting that there is an effect which is not significantly expressed morphologically after the first 24 hrs in culture. Additionally, the epithelium of rudiments cultured in tunicamycin for a full 48 hrs appears flatter, that is, expansion occurs to a lesser degree in the direction perpendicular to the horizontal plane of the rudiment (Fig. 2, C3). The lobes of "recovery" rudiments, cultured first in tunicamycin, then in control media, appear to grow in a fashion similar to controls during the second 24 hr culture interval. They exhibit a three-dimensional texture not apparent in rudiments cultured continuously in tunicamycin.

To quantitatively assess the effect of tunicamycin on epithelial growth, the planar expansion of the epithelium was monitored. The approach taken involved cutting out and weighing photocopies of submandibular epithelia, made from photographic prints of living rudiments, at successive culture periods, under control, continuous tunicamycin culture, and recovery conditions. The results are shown in Fig. 3. By 24 hrs of culture, tunicamycin inhibits epithelial expansion by approximately 30%, a degree of inhibition not easily detected by simple visual observation of the rudiments. During the 24-48 hr culture interval, rudiments cultured continuously in tunicamycin-containing medium exhibited a rate of epithelial expansion that was severely retarded

Fig. 2. Morphological aspects of recovery following culture with tunicamycin. Photographs of three living salivary rudiments at 0 hr (A1, B1, C1), 24 hr (A2, B2, C2), and 48 hr (A3, B3, C3) of culture. Control (A series) and tunicamycin-treated cultures, 25 ng/ml (C series), develop as described in Fig. 1. The rudiment depicted in series B was cultured in 25 ng tunicamycin/ml of culture medium for the first 24 hrs, rinsed with control medium, and incubated in control medium for an additional 24 hr. Within 24 hr of culture in control medium, the rudiment shows partial recovery (B3), expressed by increased expansion of the epithelium and acquiring an appearance characteristic of controls. A1-C3, 26X.


A
B
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1
1
1
2
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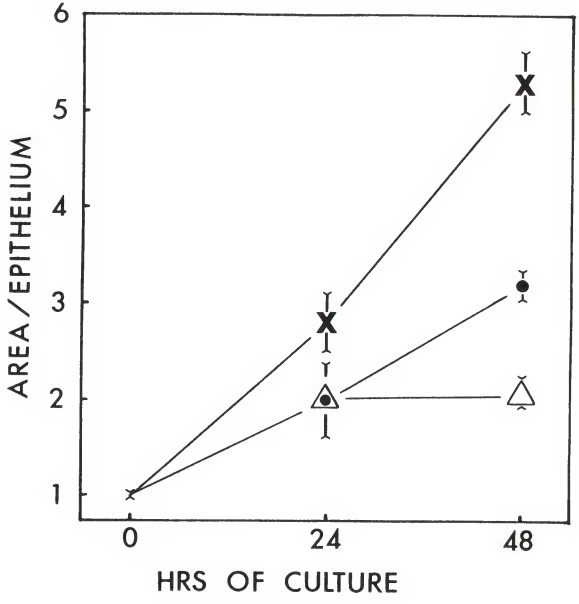
3
3
3

Fig. 3. Quantitative assessment of the tunicamycin inhibition on epithelial growth. As described in the text, photographs of living rudiments at successive culture intervals were photocopied and epithelial portions cut out and weighed. The weights of epithelial "cut-outs" were expressed as epithelial area. All areas were normalized to the area of the epithelium at zero hr of culture. Rudiments cultured in the presence of tunicamycin for the first 24 hrs followed by culture in control medium for the final 24 hrs (●), begin to recover during this period. Their epithelium expanded at a rate that was 84% of the rate of expansion of controls (X). Rudiments cultured continuously in tunicamycin-treated medium (△), exhibited only a 3% increase in epithelial area during the 24 to 48 hr culture interval. Error bars indicate standard error (N = 7-22).


6
5
4
3
2
1
AREA / EPITHELIUM
0
24
48
HRS OF CULTURE

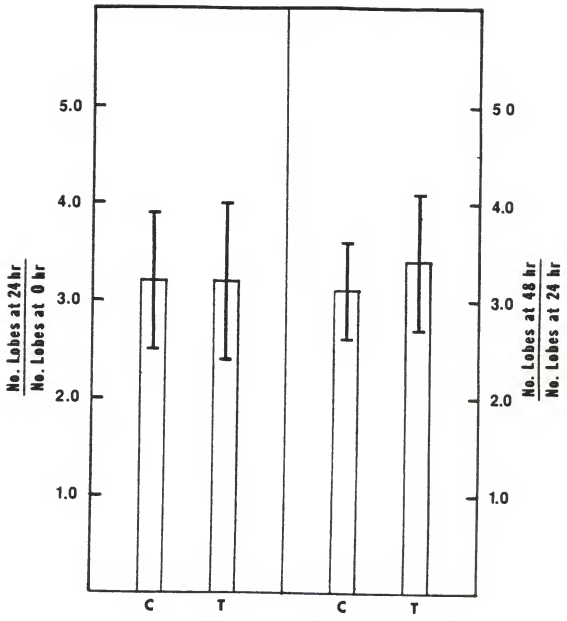
compared to controls. This resulted in an epithelial area that was only 39% of the control value after 48 hrs culture time, supporting the observation of growth inhibition by tunicamycin. It is also evident that the inhibitory effect on growth is reversible. Recovery was tested by first culturing rudiments for 24 hrs in medium containing tunicamycin and replacing it with control medium for the final 24 hr culture interval. After 24 hrs in tunicamycin-free medium (48 hrs after the initiation of the cultures), the rate of epithelial expansion rose to 84% of the control rate, resulting in epithelial areas that were 60% that of controls.

To quantitatively determine if tunicamycin (25 ng/ml) has an effect on the rate of branching, the number of lobes on each submandibular gland were counted at 0, 24, and 48 hrs. A ratio of the number of lobes at successive 24 hr culture intervals was determined for each rudiment and expressed as an integer. The averaged values shown in Fig. 4 reveal an approximate three fold increase in the number of lobes generated in control rudiments within each 24 hr culture period. Salivary rudiments cultured in the presence of tunicamycin continue to branch at a rate equivalent to controls. Thus, while epithelial growth is inhibited, branching of that tissue progresses normally.

Submandibular Salivary Rudiments Incorporate [^{3}H]mannose and [^{35}S]sulfate

The synthesis and distribution of glycoproteins (as well as other mannose bearing molecules) in submandibular salivary glands was monitored by metabolically labeling cultured rudiments with [^{3}H]mannose (100 μCi/ml) for the final 4 hrs of a 48 hr culture routine in control media, then processing the rudiments for light microscopic autoradiography. The resulting autoradiograms show that a substantial amount of radioactivity becomes incorporated during the labeling period, and as seen in section, the bulk of the radioactivity is present throughout the whole of the epithelium (Fig. 5A). Relative to the

Fig. 4. Effect of tunicamycin on epithelial branching activity. The rate of branching during the 2 day culture period is expressed as an average of the ratio of the number of lobes at successive 24 hr culture intervals. Tunicamycin-treated rudiments branch at a rate that is equivalent to control branching activity. C (control cultures), T (tunicamycin-treated cultures). Error bars indicate standard error (N = 20-27).


No. Lobes at 24 hr / No. Lobes at 0 hr
5.0
4.0
3.0
2.0
1.0
C
T
C
T
No. Lobes at 48 hr / No. Lobes at 24 hr
5.0
4.0
3.0
2.0
1.0

epithelium, considerably less label accumulates in the surrounding mesenchyme. Furthermore, there is a heightened and occasionally intermittent localization of label which extends over much of the basal surface of the epithelium. Autoradiographic studies employing [^{35}S]sulfate as a precursor molecule to examine sulfated glycosaminoglycan [i.e. sulfated proteoglycan (Cohn et al., 1977)] synthesis and deposition in the salivary system (Spooner et al., 1985) result in autoradiograms exhibiting a labeling pattern which is somewhat different from [^{3}H]mannose labeled rudiments (Fig. 5B). The conspicuous similarity between the two stems from the localization of label which clearly outlines the basal surface of the epithelium; the site of the basal lamina, rich in sulfated proteoglycan and presumably glycoproteins as well.

Sensitivity of [^{35}S]sulfate and [^{3}H]mannose Label to Chondroitin ABC Lyase

Rudiments labeled with [^{35}S]sulfate and treated with testicular hyaluronidase or chondroitin ABC lyase prior to immersion in nuclear track emulsion for autoradiography exhibit a substantially reduced level of label relative to untreated sections (Fig. 6A, B). Based on the substrate specificities of these enzymes, the ^{35}S-labeled material consists principally of chondroitin sulfates and dermatan sulfate (Spooner et al., 1985), a result consistent with other labeling studies (Cohn, et al., 1977). Heparan sulfate, and sulfated glycoproteins are resistant to these enzymes and probably account for the remaining radioactivity. Dermatan sulfate may partially account for hyaluronidase resistant label. Mannose-incorporated-material, presumably glycoproteins, is insensitive to treatment with chondroitin ABC lyase (Fig. 6C, D). Label remains clearly visible at the basal epithelial surface, suggesting that the label has not incorporated into chondroitin or dermatan sulfates, or hyaluronic acid; major components of the submandibular basal lamina.

Fig. 5. Submandibular salivary rudiments incorporate [^{3}H]mannose and [^{35}S]sulfate label at the basal surface of the epithelium. Submandibular rudiments were cultured in control medium for 48 hrs, labeled with [^{3}H]-mannose for 4 hrs or [^{35}S]sulfate for 2 hrs, and then immediately fixed and processed for autoradiography. Autoradiograms of [^{3}H]mannose labeled rudiments (A) reveal intense radioactivity within the epithelium compared to autoradiograms of rudiments labeled with [^{35}S]sulfate (B). In both cases, a substantial amount of radioactivity is deposited at the basal surface of the epithelium. A and B, 162X.

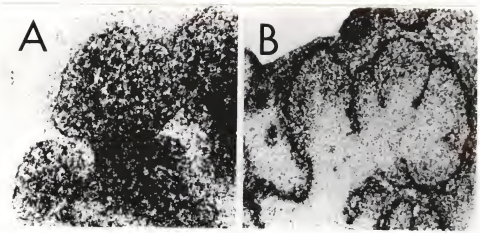
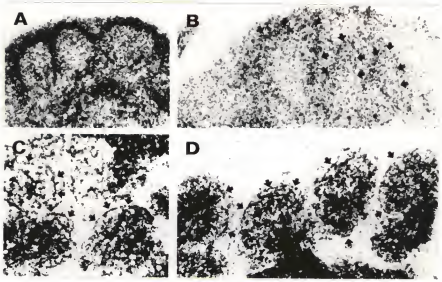

A
B

Fig. 6. [^{35}S]Sulfate, but not [^{3}H]mannose surface-associated radioactivity is sensitive to chondroitin ABC lyase. Autoradiograms of sections of [^{35}S]sulfate-labeled rudiments, incubated in chondroitin ABC lyase buffer alone (A) versus buffer containing chondroitin ABC lyase (B), prior to processing for autoradiography, show that the bulk of the radioactivity is removed, demonstrating a sensitivity of the sulfated-material to this enzyme. Autoradiograms of rudiments labeled with [^{3}H]mannose, sectioned, and sections incubated in chondroitin ABC lyase buffer alone (C) or buffer containing chondroitin ABC lyase (D), prior to processing for autoradiography, reveal that mannose-labeled material is insensitive to this enzyme. The arrows highlight the surface-associated radioactivity. A-D, 173X.


A
B
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D

Pulse-Chase Studies Demonstrate Lability of [^{3}H]mannose Labeled Material

The dynamic nature of the basal lamina in developing submandibular salivary glands is well illustrated by autoradiographic pulse-chase experiments using [^{35}S]sulfate (Spooner, et al., 1985) and [^{3}H]glucosamine (Bernfield and Banerjee, 1982) to label laminar glycosaminoglycan. After the pulse labeling period, radioactivity, localized at the basal surface of the epithelium rapidly (6-8 hrs) turns over. The turnover of this laminar glycosaminoglycan correlates with branching of the epithelium (Bernfield and Banerjee, 1982). Rudiments cultured for 44 hrs under control conditions, pulse labeled 4 hrs with [^{3}H]-mannose, then fixed after 0, 2, 4, 8, or 24 hrs of chase in non-radioactive medium and processed for light microscopic autoradiography, reveal a loss of label at the basal epithelial surface which is relatively slow compared to laminar glycosaminoglycan turnover. The intensity of the label at the basal surface of the epithelium does not appreciably diminish during the first 4 hrs of chase (Fig. 7B, C). Even after 8 hrs of chase, label at this site is quite recognizable (Fig. 7D). Within 24 hrs however, little if any label remains at the basal surface, whereas a moderate amount of label remains throughout the rest of the epithelium (Fig. 7E). These results clearly show that the labeled material is not static, suggesting a process in which it may be degraded and replaced by newly synthesized material (turnover).

Effect of Tunciamycin on [^{3}H]mannose Incorporation

Submandibular salivary rudiments cultured in the presence of tunicamycin for 44 hrs, labeled with [^{3}H]mannose for 4 hrs and processed for autoradiography yield autoradiograms that exhibit a considerable reduction in the amount of label present compared to controls (Fig. 8A, B). The decreased level of incorporated label within the epithelium and particularly at its

Fig. 7. Turnover of surface-associated mannose-labeled-material in cultured embryonic salivary rudiments. Rudiments were cultured for 48 hrs in control medium, labeled with [^{3}H]mannose and fixed after 0 (A), 2 (B), 4 (C), 8 (D) or 24 (E) hrs of "chase" in non-radioactive medium. The fixed rudiments were embedded, sectioned, and processed for autoradiography. Autoradiograms show that the radioactivity at the basal surface of the epithelium (arrows) is most intense when rudiments are fixed immediately after the labeling period. Label is still evident at this site 8 hrs after the onset of the chase period. By 24 hrs postlabeling chase time, most of the radioactivity at the basal surface of the epithelium (arrows) has been lost. Furthermore, loss of surface-associated radioactivity seems to occur uniformly, that is, label at the distal ends of lobes and within clefts turns over with an apparently equal time course. A-E, 189X.

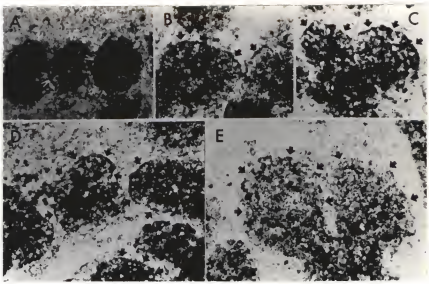
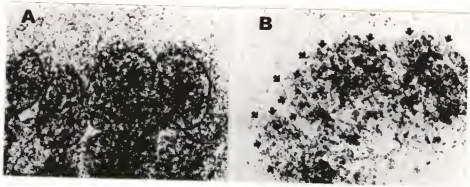

A
B
C
D
E

Fig. 8. Tunicamycin inhibits deposition of [^{3}H]mannose-containing material at the basal epithelial surface. Submandibular rudiments were cultured in control medium or medium containing 25 ng/ml tunicamycin for 52 hrs, the final four hrs included 100 μCi/ml [^{3}H]mannose in both control and tunicamycin medium. Autoradiograms of control sections (A) show a localization of label within the epithelial compartment and at the basal surface of the epithelium (as in Fig. 5A). Sections of tunicamycin-treated rudiments (B) exhibit a substantially reduced level of radioactivity, particularly at the basal epithelial surface (arrows). A and B, 173X.


A
B

basal border is such that it is difficult to discern the epithelium from the mesenchyme. The data demonstrate the ability of tunicamycin to effectively inhibit the incorporation of mannose into those molecules which normally contain this monosaccharide. Thus, the inhibition of mannose incorporation caused by tunicamycin correlates with an inhibition of growth, but not branching.

Effect of Tunicamycin on [^{35}S]sulfate Incorporation

The utilization of [^{35}S]sulfate has proven to be an effective and accurate means by which to visualize sulfated glycosaminoglycan deposition at the site of the basal lamina. While tunicamycin should not directly interfere with their synthesis it might well inhibit the production of glycosyltransferases, glycoproteins responsible for the synthesis of glycosaminoglycans. To examine this possibility, autoradiograms were prepared from rudiments cultured in tunicamycin-containing-media for 48 hrs then labeled with [^{35}S]sulfate for 2 hrs. The resulting autoradiograms do reveal a small decrease in the amount of label associated with the basal epithelial surface when compared with controls (Fig. 9A, B). However, when compared with rudiments cultured in ß-xyloside, a recognized inhibitor of proteoglycan synthesis and branching morphogenesis (Thompson and Spooner, 1983), then labeled with [^{35}S]sulfate for 2 hrs and processed for autoradiography, a drastic reduction in the amount of label deposited in this region is exhibited by the autoradiograms (Fig. 10A, B). Thus, while tunicamycin might be interfering with glycosaminoglycan synthesis to some degree, the effect is not sufficiently severe to inhibit branching of the epithelium. Furthermore, the decrease in label may well be due to a reduced synthesis of sulfated glycoproteins, a plausible source of testicular hyaluronidase and chondroitin ABC lyase resistant material referred to in Fig. 6B.

Fig. 9. Deposition of epithelial surface-associated [^{35}S]sulfate is slightly reduced in tunicamycin-treated rudiments. Submandibular rudiments were cultured in control medium or medium containing 25 ng tunicamycin/ml for 48 hrs, labeled with [^{35}S]sulfate for an additional 2 hrs, and then fixed and processed for autoradiography. Autoradiograms of control sections (A) show a localization of label at the epithelial-mesenchymal boundary. Tunicamycin-treated sections (B) reveal an intensity of radioactivity at this site, which is only slightly less than controls. A and B, 221X.

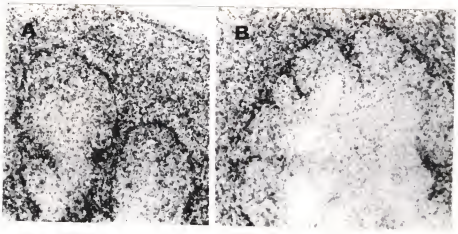
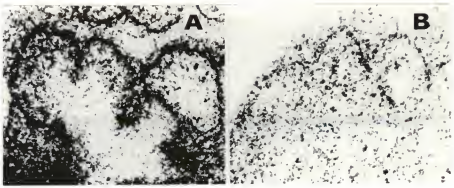

A
B

Fig. 10. Deposition of epithelial surface-associated radioactivity is dramatically reduced in β-xyloside-treated rudiments. Submandibular rudiments were cultured in control medium or medium containing 0.5 mM β-xyloside for 48 hrs, labeled with [^{35}S]sulfate for an additional 2 hrs of culture, and fixed and processed for autoradiography. Autoradiograms of control sections (A) reveal a considerable amount of radioactivity deposited at the basal surface of the epithelium. Considerably less label is deposited at the basal epithelial surface of β-xyloside-treated rudiments (B). A and B, 176X.


A
B

DISCUSSION

The embryonic submandibular salivary gland is one of many organ systems whose development requires an interaction between epithelium and mesenchyme (Grobstein, 1967). An extracellular matrix composed of collagens, proteoglycans and glycosaminoglycans, and glycoproteins is situated between these tissues and has been implicated in mediating developmental events. The dynamic nature of the developing salivary rudiment lends itself as a useful tool in studying these interactions and the involvement of matrix macromolecules.

Mesenchymally-synthesized interstitial collagens (Bernfield, 1970) have been biochemically demonstrated, and type IV collagen, an epithelially produced basal laminar component, has been observed by indirect immunofluorescence, lining the stalk, within clefts, and at the epithelial-mesenchymal boundary. They are present in differing amounts and distributions, presumably reflecting the particular morphogenetic state of that compartment (Bernfield, et al., 1984). Numerous collagen fibrils are present in the extracellular spaces between mesenchymal cells (Spooner and Faubion, 1980) and, as shown by light microscopic autoradiography, the mesenchyme labels densely with [^{3}H]proline (Bernfield, 1970). Inhibition of interstitial collagen secretion by L-azetidine-2 carboxylic acid correlates with a retardation of branching morphogenesis, thus demonstrating a developmental requirement for interstitial collagen (Spooner and Faubion, 1980).

In addition to collagen, glycosaminoglycans and proteoglycans are obligatory macromolecular species associated with branching of the epithelium throughout embryonic submandibular salivary gland morphogenesis. Histochemical (Bernfield and Banerjee, 1972) and autoradiographic (Cohn, et al., 1977; Spooner, et al., 1985) studies have demonstrated the presence of glycosaminoglycan at the epithelial-mesenchymal interface, the level of the basal lamina.

Removal of the glycosaminoglycan-rich basal lamina impedes morphogenesis (Bernfield, et al., 1972). The epithelium has the ability to resynthesize a basal lamina (Banerjee, et al., 1977) in which approximately 50% of the total glycosaminoglycan exists as hyaluronic acid (Cohn, et al., 1977), a non-protein-associated-glycosaminoglycan. The remaining glycosaminoglycans include chondroitin sulfates (approximately 40%), dermatan sulfate, and heparan sulfate, glycosaminoglycan species that are normally present as components of proteoglycan rather than as free glycosaminoglycan chains (Cohn, et al., 1977). β-D-Xyloside inhibits the synthesis of proteoglycans and branching morphogenesis (Thompson and Spooner, 1983). The concommitant increase in free glycosaminoglycan chains does not replace the requirement for proteoglycan-associated glycosaminoglycans (Thompson and Spooner, 1983). In addition to the synthesis and deposition of laminar glycosaminoglycan and sulfated proteoglycan (Spooner, et al., 1985), a distinct mesenchymally mediated (Smith and Bernfield, 1982) turnover pattern of these macromolecules exists, which correlates with branching morphogenesis.

Thus far, the major classes of extracellular macromolecules deemed necessary for ongoing branching morphogenesis include the collagens, glycosaminoglycan, and proteoglycan. The present study has focused on the possible involvement of extracellular glycoproteins in branching morphogenesis of the embryonic mouse salivary gland. In the broadest sense of the term, glycoproteins are defined as proteins to which carbohydrate is covalently attached. This investigation however, is limited to N-linked glycoprotein species.

Recently, considerable advances have been made in understanding the mechanisms involved in glycoprotein biosynthesis (for review see Struck and Lennarz, 1980; Schachter, 1984). Initially, a core oligosaccharide (common to

most N-linked glycoproteins) is synthesized by the addition of N-acetylglucosamine-1-phosphate to dolichol phosphate, a lipid carrier. A series of specific glycosyltransferases sequentially add an additional N-acetylglucosamine, nine mannose, and three glucose residues. The completed activated core oligosaccharide is transferred en bloc from dolichol pyrophosphate to a specific asparagine residue in an Asn-X-Ser(Thr) sequence within a polypeptide backbone (Schachter, 1984). These core oligosaccharides undergo additional processing (deletion and addition of specific monosaccharides) in the Golgi apparatus, thus accounting for the heterogeneity of the carbohydrate moeities of glycoproteins, a characteristic feature of this class of molecules (Spiro, 1973). Tunicamycin is a potent antibiotic isolated from Streptomyces lysosuperficus which inhibits N-asparagine-linked glycoprotein biosynthesis. Tunicamycin blocks the addition of N-acetylglucosamine-1-phosphate to dolichol phosphate, the first step in the synthesis of the core oligosaccharide (Lehle and Tanner, 1976), thus inhibiting N-linked glycoprotein biosynthesis. Tunicamycin has successfully been used to inhibit the synthesis of glycoproteins in a diverse number of biological systems such as yeast (Lehle and Tanner, 1976), rat hepatocytes (Caro, et al., 1983), fetal rat cerebral neurons (Yavin, et al., 1984),and pig ear skin slices (King and Tabiowo, 1981).

Tunicamycin, like many agents which perterb anabolic or catabolic pathways may cause undesirable side effects; either indirect or non-glycoprotein-specific. The extent of these effects by tunicamycin seems to depend on the dose employed and the particular biological system under investigation. Thus, it is possible that sulfated glycosaminoglycan synthesis is inhibited in pig epidermis (King and Tabiowo, 1981) but not sea urchin embryos (Heifetz and Lennarz, 1979), with glycoprotein synthesis being inhibited in both cases. Macromolecules which are produced via the dolichol pathway or depend on

glycosyltransferases for their synthesis, are likely to be inhibited by tunicamycin. In fact, the inhibition of glycolipid biosynthesis by tunicamycin has been reported in developing cerebral neuron cultures (Yavin et al., 1984). General protein synthesis is also inhibited by tunicamycin (Duksin and Mahoney, 1982). Nevertheless, tunicamycin has been instrumental in assessing general glycoprotein involvement in many biological processes.

The present study demonstrates a direct correlation between salivary gland growth inhibition and increasing doses of tunicamycin. Concentrations of tunicamycin nearing 50 ng/ml were toxic as revealed by a darkening of the tissue. During a 48 hr culture period, the expansion of submanidublar epithelia of rudiments cultured in 25 ng/ml tunicamycin progressed to only 54% of controls, and interestingly, their rate of growth over the final 24 hrs of culture was nearly zero. In spite of the marked inhibition of growth, branching of the epithelium continues at the same rate as control rudiments. Therefore, 25 ng/ml tunicamycin appeared to be a useful dose with which to further study its effects. The inhibitory effect on growth appears reversible as the morphology and rate of growth approach that of controls after 24 hrs of recovery culture in tunicamycin-free medium. The possibility exists that tunicamycin is inhibiting general protein synthesis, and therefore growth and epithelial expansion. This possibility will be addressed in future experiments by examining the incorporation of [^{3}H]leucine into TCA-insoluble-material in control and tunicamycin-related rudiments.

As revealed by light microscopic autoradiography, salivary rudiments labeled with [^{3}H]mannose accumulate a moderate amount of radioactivity at the basal surface of the epithelium, the site of the basement membrane. The radioactivity is resistant to chondroitin ABC lyase treatment, suggesting that the label has not substantially been interconverted and/or incorporated into

chondroitin sulfate proteoglycan, a major, or dermatan sulfate proteoglycan, a minor, component of the basal lamina. Studies with Hela S_3 cells and human diploid fibroblasts have demonstrated that [^{3}H]mannose can be utilized for up to 24 hrs with only minimal relocation into other compounds (Yurchenco, P.D., et al., 1978). Compared to controls, there is a substantial reduction in the amount of label localized at the epithelial-mesenchymal interface by rudiments cultured in tunicamycin. Other studies, although not autoradiographic, have previously demonstrated decreased [^{3}H]mannose incorporation into glycoproteins by tunicamycin (Heifetz and Lennarz, 1979; Seehafer, J. et al., 1984).

Turnover of sulfate-labeled-glycosaminoglycan follows a pattern in which label is lost first at the distal tips of the lobes and subsequently from clefts between lobes (Spooner, et al., 1985). Furthermore, the bulk of the radioactivity is lost within 8 hrs of postlabeling chase time. The pattern and time course of label loss that occurs following a 4 hr pulse period with [^{3}H]mannose contrasts that of sulfate-labeled-glycosaminoglycan turnover. Label, localized at the basal surface of the epithelium, appears to be turned over uniformly and requires longer periods of postlabeling chase time, approximately 24 hrs, for its removal.

Autoradiograms of tunicamycin-cultured, [^{35}S]sulfate-labeled-rudiments exhibit a decreased localization of radioactivity into their basal epithelial surfaces compared to controls. The decrease is slight however, when compared with the drastic reduction in deposition of sulfated material that results from treatment with β-xyloside, an inhibitor of proteoglycan synthesis, suggesting that sulfated proteoglycan synthesis is not significantly depressed by tunicamycin. In fact, the oligosaccharide moieties of many proteoglycans, including chondroitin sulfate and dermatan sulfate proteoglycans, are attached to core proteins by O-glycosidic, xylosyl-serine linkages (Schachter, 1984),

thus their synthesis should not be directly affected by tunicamycin. Furthermore, the decreased incorporation of [^{35}S]sulfate label may be due to an inhibition of sulfated glycoprotein synthesis. Entactin is a sulfated glycoprotein which has been identified as a basement membrane component in a variety of tissues (Carlin, et al., 1981) including the lactating rat mammary gland (Warburton, et al., 1984). Nidogen, a recently identified, and possibly sulfated glycoprotein, is present in embryonic as well as adult basement membranes.

The data reported in this study suggests that mannose-containing/tunicamycin-susceptible material, presumably glycoproteins, or their constituent carbohydrate moieties, play a crucial role in epithelial growth and expansion, yet do not seem obligatory for ongoing branching of that same tissue. The experimental independence of epithelial growth and epithelial branching does not support models of morphogenesis that propose obligatory coupling of these activities (Bernfield et al., 1984), an observation that will necessitate new views of the regulation of the morphogenetic process.

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INVOLVEMENT OF EXTRACELLULAR GLYCOCONJUGATES IN BRANCHING MORPHOGENESIS OF EMBRYONIC MOUSE SUBMANDIBULAR SALIVARY GLANDS

by

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ABSTRACT

Interactions between the epithelium and mesenchyme are required for branching morphogenesis of embryonic mouse submandibular salivary glands. Collagens, proteoglycans, and glycoproteins are extracellular matrix macromolecules which have been implicated as mediators of these interactions, and although a requirement for interstitial collagen and basal laminar glycosaminoglycan and proteoglycan has been demonstrated for ongoing branching of the epithelium, the precise nature of their involvement is not well understood.

β-D-Xyloside inhibits proteoglycan synthesis and salivary branching morphogenesis. Whether this effect is related to the deposition and processing of newly synthesized glycosaminoglycans at the basal epithelial surface that correlates with normal branching activity was investigated. Forty eight-hour cultures of control and 0.5 mM β-xyloside-treated submandibular rudiments were labeled for 2 hrs with [^{35}S]sulfate and fixed and processed for autoradiography immediately or after 2, 4, 6, or 8 hrs of postlabeling chase in nonradioactive medium. The data demonstrated that deposition of chondroitin sulfate-rich material at the basal epithelial surface was strikingly reduced in β-xyloside-treated rudiments, while patterns of label loss during postlabeling chase were not altered.

The presence of matrix glycoproteins in embryonic mouse salivary glands has previously been observed in other studies, but their requirement in branching morphogenesis is presently unknown. As an initial assessment of the general role of glycoproteins, the effects of tunicamycin, an inhibitor of N-linked glycoprotein biosynthesis, on *in vitro* salivary gland morphogenesis, was examined. At a concentration of 25 ng/ml of culture medium, tunicamycin dramatically inhibits the incorporation of [^{3}H]mannose into intraepithelial

and basal epithelial surface-associated compartments, as resolved by light microscopic autoradiography. Such treatment also strikingly inhibits epithelial growth and expansion, events thought to be essential for branching activity. However, branching continues unabated in these rudiments, resulting in control numbers of branch points and lobes. Lobe size is reduced and branch points are closer due to the reduced epithelial area, but growth and expansion resume following removal of tunicamycin. The data demonstrate that tunicamycin-sensitive material (presumably glycoproteins) are required for epithelial growth and expansion. However, they seem not to be necessary for branching morphogenesis. Thus, epithelial branching activity, a process requiring interstitial collagen and basal laminar proteoglycan, is independent of control levels of glycoprotein synthesis and control degrees of epithelial growth and expansion.