Scanning electron microscopic observation of mouse embryonic submandibular glands during initial branching: preferential localization of fibrillar structures at the mesenchymal ridges participating in cleft formation

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SUMMARY
Branching submandibular glands of 12-day mouse embryos and those cultured in the presence and absence of a collagenase inhibitor from the culture medium of bovine dental pulp or a Clostridial collagenase were examined with the scanning electron microscope. Fracturing of fixed and dried glands with the tip of a fine needle succeeded in exposing the surfaces of the lobules and of their mesenchymal replicas at different stages of branching. At the beginning of branching, corresponding parts of the mesenchyme formed ridges on or in which the fibrillar structures were often found. At the stage forming deeper clefts thicker fibres, 0.5–2.5 μm in diameter, were observed between two adjacent lobules. On the contrary, no apparent differences in the fibrillar structures on the epithelial surfaces were detected between the shallow cleft and noncleft regions at the initial phase of branching. These fibrillar structures were very abundant in glands cultured with collagenase inhibitor and were completely lost in glands cultured with bacterial collagenase, strongly indicating that these materials consisted of collagen. The possible involvement of mesenchyme in epithelial branching is discussed with special reference to mesenchymal traction forces that would be elicited by fibrillar collagens.

INTRODUCTION
Submandibular gland branching at early morphogenetic stages of the embryo is initiated by the formation of sharp furrows in the epithelium followed by their progressive broadening. This type of epithelial branching is characteristic of submandibular organogenesis and is different from other organs such as lung. Rodent embryonic submandibular glands provide an excellent system to study

Key words: mouse submandibular gland, epithelial branching, collagen fibril, scanning electron microscopy, collagenase inhibitor, bacterial collagenase, mesenchymal ridges, cleft formation, fibrillar structures.
branching morphogenesis in vitro since cleft formation in the epithelium can be easily observed (Grobstein, 1954).

Collagen, one of the major constituents of extracellular matrix, is thought to be important in cleft formation due to the following facts: (1) the presence of commercial bacterial collagenase preparations in the culture medium inhibited epithelial branching (Grobstein & Cohen, 1965; Wessells & Cohen, 1968), and (2) a proline analogue, L-azetidine 2-carboxylic acid, in the culture medium suppressed epithelial morphogenesis (Spooner & Faubion, 1980). The former experiments were, however, hampered by a possible contamination of glycosaminoglycan degrading enzymes and proteases (Bernfield, Banerjee & Cohn, 1972). Although the latter authors demonstrated that L-azetidine 2-carboxylic acid inhibited total protein synthesis to a much lesser degree than the collagen synthesis of 13-day salivary glands, it was possible that the impaired noncollagenous proteins synthesized in the presence of the drug might affect the proper morphogenesis. To overcome the above possibilities, we have recently used a highly purified Clostridial collagenase and an interstitial collagenase-specific inhibitor (CI) obtained from the culture medium of bovine dental pulp, the use of which resulted in modulation of epithelial branching in mouse submandibular glands. The collagenase in the medium completely inhibited the branching, whereas the collagenase inhibitor dramatically stimulated it within 14 h culture of 12-day glands without affecting rates of cell proliferation (Nakanishi, Sugiura, Kishi & Hayakawa, 1986a). This result confirmed earlier findings that collagen plays a crucial role in the branching morphogenesis of mouse embryonic submandibular gland.

There have been a few reports documenting the presence and distribution of collagen in salivary gland by using electron microscopy and immunostaining (Bernfield & Wessells, 1970; Spooner & Wessells, 1972; Spooner & Faubion, 1980; Bernfield, Banerjee, Koda & Rapraeger, 1984). These studies were, however, not sufficient in describing the localization and the three-dimensional orientation of collagen fibrils during early morphogenetic stages.

How does collagen act as a morphogenetic component? Recently, embryonic chick heart mesenchyme and several other fibroblastic cells have been shown to contract silicone rubber as well as collagen gel (Bell, Ivarsson & Merrill, 1979; Steinberg, Smith, Colozzo & Pollack, 1980; Harris, Stopak & Wild, 1981; Stopak & Harris, 1982). It is, therefore, reasonable to assume that some insoluble or fibrillar components of the substratum could be prerequisites for such cells to exert traction. To examine a possible involvement of such traction force in branching process, it is of importance to know the three-dimensional alignment of fibrillar architectures at the epithelial–mesenchymal interfaces of the salivary gland. In the present study, we examined control specimens of 12-day glands and those cultured in the presence and absence of either CI or a Clostridial collagenase with the scanning electron microscope. We present evidence showing the preferential localization of fibrillar architectures at the mesenchymal ridges participating in cleft formation.
MATERIALS AND METHODS

Materials

A collagenase inhibitor (CI), which is known to be specific for the interstitial collagenases, was purified from the culture medium of bovine dental pulp by the method of Kishi & Hayakawa (1984).

A highly purified Clostridial collagenase devoid of caseinolytic activity was kindly donated by Drs T. Ohya and N. Yokoi of Amano Pharmaceutical Co., Japan. No hyaluronidase or chondroitinase activities were detected.

Embryos

12-day embryos were obtained from DDY mice. The day of discovery of the vaginal plug was designated as day 0.

Organ culture

Embryonic salivary glands were dissected from 12-day embryos in Tyrode's solution. Organ culture was routinely carried out on ultrathin Millipore filter (THWP, 0.45 μm pore size) assemblies (Banerjee, Cohn & Bernfield, 1977) in BME medium supplemented with ascorbic acid (50 μg ml⁻¹) and 10% foetal calf serum. When CI- and collagenase-treated glands were prepared, the final concentration of either CI or the bacterial collagenase in the medium was 5 μg ml⁻¹ (Nakanishi et al. 1986a). Living cultures of salivary glands were monitored by taking photographs at appropriate intervals on an Olympus light microscope BH-2 equipped with an automated exposure unit.

Processing for scanning electron microscopy

Intact 12-day glands or those cultured under various conditions were fixed in 1.6% glutaraldehyde and 1.6% paraformaldehyde in 0.1 M-phosphate buffer, pH 7.2, and postfixed in 2% OsO₄ in 0.1 M-phosphate buffer, pH 7.2. The specimens were then dehydrated with ethanol, transferred to isoamyl acetate and dried in a critical-point drier using CO₂ as transitional fluid. The dried specimens thus prepared were mounted onto aluminium stubs, cracked with the tip of a fine needle under the microscope, coated with gold and examined with an Akashi ALPHA-30 scanning electron microscope at 30 kV.

RESULTS

Scanning electron microscopy was employed to determine if regional differences in fibrillar structures exist on the surfaces of the lobular epithelia and of their complementary mesenchyme during the first cleft formation. We therefore examined 12-day glands, which were at the stage just before forming clefts, and those cultured under normal conditions for 17 h, reaching the mid 13-day stage. In addition, glands cultured in the presence of either CI or Clostridial collagenase for 11 h were also examined.

Intact 12-day gland

An intact 12-day gland was cracked and the exposed surfaces of the epithelium including lobular and stalk regions and its complementary mesenchyme were examined with the scanning electron microscope (Figs 1–6). An overall view of the specimen is shown in Fig. 1, in which epithelia of lobular and stalk regions and their mesenchyme are clearly seen. The lobule had several indentations (Figs 2, 3)
as reported by Nogawa (1983). Some of these preclefts grew to definitive clefts while others disappeared during the subsequent cultivation (Nakanishi et al. 1986b). Fine fibrils, 80–150 nm in diameter, were scattered over the distal end of the lobule and similar fibrils were seen within the preclefts (Figs 3, 4).

Much denser distribution of fibrillar materials was found on the stalk region adjacent to the lobule (Figs 2, 5). Their diameter, 100–200 nm, was greater than that of the lobular surface (Fig. 4). This observation is entirely consistent with the statement by Grobstein & Cohen (1965) that the morphogenetically inactive stalk region is covered with dense collagen fibrils.

Likewise, on the surface of stalk mesenchyme we saw random arrays of fibrils (Fig. 1). The arising mesenchymal ridges complementary to the preclefts in the epithelium had fibrillar material attached to the mesenchyme (Fig. 6), suggesting that these structures were synthesized by the mesenchyme. However, mesenchyme that was not confronted with epithelium did not contain so many fibrillar structures (Figs 1, 2), implying that there would be some specific interactions between epithelium and mesenchyme leading to the deposition of fibrillar material at the interface (Kallman & Grobstein, 1965; Grobstein & Cohen, 1965; Bernfield, 1970; von der Mark, 1981).

**Cultured 12-day glands**

12-day glands were cultured in the normal medium for 17 h, during which period two clefts were formed on average, and the glands were processed as described in Materials and Methods. Scanning electron micrographs of the two specimens are presented (Figs 7–12). An overall view of the lobule and its mesenchymal counterpart is shown in Fig. 7. Judging from the top view of the specimen (Fig. 8), together with the picture in Fig. 7, it is reasonable to conclude that the mesenchyme at the top had covered the lobule seen at the bottom in Fig. 7. It is of particular interest that a narrow but long indentation like a tract runs over the lobule (Fig. 8), which would probably be growing to a new cleft. There were no obvious differences in the fibrillar structures between the lobular surfaces of the shallow cleft and noncleft regions (Fig. 9). The indentation in the epithelium in

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**Fig. 1.** Overall view of the specimen. Lobular and stalk epithelia and their mesenchymal replica are clearly seen, in addition to a part of the broken stalk at the bottom. ×250.

**Fig. 2.** Exposed surfaces of the epithelium and mesenchyme of the 12-day gland seen at the right-hand side in Fig. 1. Several indentations in the lobule and dense fibrils on the stalk can be seen. ×340.

**Figs 3, 4.** Top view of the lobule in Fig. 2. There are several indentations and fibrils with different diameter in the higher magnification view (Fig. 4) of the area indicated by an arrow in Fig. 3. Fig. 3, ×660; Fig. 4, ×3370.

**Fig. 5.** High magnification view of the area indicated by an arrow in Fig. 2, in which a dense fibrillar network is visible. ×4680.

**Fig. 6.** High magnification view of the rectangular area in Fig. 1. Numerous fibrils are attached to the ridges corresponding to the indentations in Figs 2, 3. ×1240.
Fig. 9 corresponds to the ridge in the mesenchyme in Fig. 10. Collagen-like fibrillar structures are seen preferentially very close to or on the mesenchymal ridge (Figs 10, 11). Essentially the same results were obtained when intact mid 13-day glands were examined with the scanning electron microscope.

In a micrograph of another specimen of the 12-day gland cultured in normal medium (Fig. 12), bundles of fibrils, indicated by arrows, were detected at the neck of the lobules and between lobules. The maximal diameter of such bundles was approximately 2-0 μm. These results suggest that the presence of large bundles of fibrils is related to epithelial clefting induced by mesenchyme.

12-day glands cultured in the presence of CI

As reported previously, CI from the culture medium of bovine dental pulp when included into the culture medium strongly enhanced cleft formation in submandibular glands, i.e. five clefts were formed on the average compared with two in control cultures (Nakanishi et al. 1986a). Therefore, the relationship between the localization of the fibrillar structures and the cleft formation was examined.

As is evident in Fig. 13, two shallow clefts are visible on one of the four lobular surfaces of the gland cultured for 11 h with CI (5 μg ml⁻¹) and the corresponding ridges are observed in the mesenchymal replica (Fig. 14). Micrographs of these areas taken at higher magnifications (Figs 15, 16) indicate that the mesenchymal ridges contain thick fibrils along them and are consistent with Figs 6 and 11.

Figs 17–21 show a gland cultured for 11 h in the presence of CI (5 μg ml⁻¹), which was fractured at the base of the clefts. These figures provide relevant information about the three-dimensional alignment of the fibrillar structures. In Fig. 17, lateral surfaces of three lobular epithelia, consisting of columnar and rather round cells, are seen and many fibrils are associated with the basal surfaces of the epithelia facing the mesenchyme (Fig. 18). The most important finding is that upper and lower parts of the mesenchyme are connected by large bundles of fibrils.
Figs 13–16. Scanning electron micrographs of a specimen of 12-day glands cultured in the presence of CI (5 μg ml⁻¹) for 11 h.

Fig. 13. Surface view of the lobule having two shallow clefts indicated by arrows a and b. ×1050.

Fig. 14. Surface view of the mesenchymal replica of the lobule in Fig. 13. Two ridges (b and a), corresponding to the shallow clefts (a and b), respectively, in Fig. 13, are arising from the mesenchymal surface. ×1000.

Figs 15, 16. Fibrillar structures at the mesenchymal ridges observed in Fig. 14. Figs 15 and 16 are higher magnifications of the ridges b and a, respectively. Fig. 15, ×4950; Fig. 16, ×5020.

Fibrils (0.5–2.5 μm in diameter) running vertically between two adjacent lobules (Figs 17, 20, and see also Fig. 12). Evidence against the possibility that such large fibres might be formed by artefactual collapsing of the fibre meshwork is provided by the finding of similar fibres between two lobules (Fig. 19, and see also Fig. 12). The figure clearly shows that the fibre consists of much thinner fibrils gathering together from the lower part of the mesenchyme (Figs 17, 19–21). These observations indicate that there must be some unknown mechanism for the formation of such bundle structures working between two adjacent lobules.

Some glands that were cultured for 17 h in the presence of CI were found to accumulate heavy fibrillar structures at the epithelial–mesenchymal interfaces
Figs 17–21. Scanning electron micrographs of a specimen of 12-day glands cultured in the presence of CI (5 μg ml⁻¹) for 11 h. e, epithelium; m, mesenchyme.

Fig. 17. Lateral surfaces of the mesenchyme and epithelial exposed after vertical cracking of the specimen at the base of the clefts. ×570.

Fig. 18. High magnification view of the epithelial–mesenchymal interface and the lateral surface of the lobule of the right rectangular area in Fig. 17. It is clear that lobular epithelia consist of both columnar cells outside and rather round cells inside. ×1710.

Fig. 19. High magnification view of the middle rectangular area in Fig. 17, showing the presence of a bundle of fibrils between two adjacent lobules. ×2430.

Fig. 20. High magnification view of the left rectangular area in Fig. 17, showing a long fibre connecting the mesenchyme at the upper and lower parts of the figure. ×1610.

Fig. 21. High magnification view of the rectangular area in Fig. 20, indicating that the fibre consists of many thinner fibrils. ×4030.
Figs 22–25. Scanning electron micrographs of specimens of 12-day glands cultured in the presence of Clostridial collagenase (5 μg ml⁻¹) for 11 h. e, epithelium; m, mesenchyme.

Fig. 22. Surface view of the lobule with the smooth contour. ×750.

Fig. 23. High magnification view of the rectangular area in Fig. 22. No fibrillar structures are found on the lobular epithelium. ×5340.

Fig. 24. Surface view of the mesenchyme originally apposed to the lobule in Fig. 22. ×2120.

Fig. 25. Surface view of the mesenchyme obtained from another specimen. No fibrillar structures are observed as in Fig. 24. ×3760.

(Nakanishi et al. 1986a), consistent with the presence of collagenase activity in 12-day glands and also with its inhibition by CI in vitro (Nakanishi et al. 1986a).

12-day glands cultured in the presence of bacterial collagenase

We then analysed the Clostridial collagenase-treated glands (5 μg ml⁻¹, 11 h), which have no clefts or smooth contours (Nakanishi et al. 1986a,b). The cracking method used in this study was only occasionally successful in obtaining specimens that were fractured at the epithelial–mesenchymal interface. In contrast to those of normal and CI-treated glands, fibrillar structures were not found on the epithelial surfaces and short, microvilli-like cell processes were seen (Figs 22–25). The mesenchymal replica of the lobule (Fig. 24) and that from another specimen
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(Fig. 25) did not have a significant number of fibrillar structures either. The fibrillar material in the collagenase-treated glands was extremely scarce, indicating that such specific structures found at the epithelial and mesenchymal surfaces of control and CI-treated glands consisted of collagenase-susceptible materials. Since the collagenase-treated glands did not as frequently expose epithelial and mesenchymal surfaces originally apposed, it is likely that the presence of these fibrillar materials at the epithelial–mesenchymal interfaces (Figs 17, 18) facilitated the separation of the two different tissues by a needle tip.

DISCUSSION

The process of the cleft formation in epithelium consists of two major parts: initiation and stabilization. Two findings in the present study seem to be important...
in the initiation process. First, at the beginning of branching no defined arrays of fibrillar structures were observed on the surfaces of the lobules (Figs 9, 13, and see also Spooner & Faubion, 1980). Rather, the fibrillar structures located either on or in the mesenchyme ran mostly along the preclefts or clefts (Figs 6, 11, 15, 16). Secondly, we found fibres, 0.5–2.5 μm in diameter, consisting of many thinner fibrils between two adjacent lobules with relatively deep clefts (Figs 12, 17, 19–21), strongly supporting an earlier result obtained by transmission electron microscopy in which a large bundle of collagen fibrils (diameter 0.8 μm) was seen at the very base of the deep cleft (Bernfield & Wessells, 1970).

These observations are reminiscent of the recent findings that mesenchyme and several fibroblastic cells are able to contract silicone rubber as well as collagen gel (Bell et al. 1979; Steinberg et al. 1980; Harris et al. 1981; Stopak & Harris, 1982). Judging from the localized and oriented distribution of fibrillar collagens described so far, it is highly probable that the mesenchyme initiates clefting by establishing ridges containing fibrillar architectures on the surfaces of lobular epithelium. A possible model for cleft formation in the submandibular epithelium is presented in Fig. 26. At the beginning of the cleft formation mesenchymal cells align collagen fibrils by their traction to form ridges which cut into the lobular epithelium in the region of the forming clefts. This process may accelerate the bundle formation which facilitates the production of a narrow, deep cleft (Fig. 26). In this case, the collagen fibres or fibrils may provide the substratum for the mesenchymal cells, consequently generating the deforming force on the epithelial surface. The alignment of fibrils by fibroblastic cells is supported by the finding of Harris et al. (1981) that collagen fibrils in the gel, which show random arrays at the onset of culture, become axially oriented between two cultured cell masses, possibly resulting in the formation of fibres. However, important questions as to whether the submandibular gland mesenchyme can contract collagen gel and as to how the lobular epithelium containing basal lamina behaves in the initiation process remain to be resolved.

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REFERENCES


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