Determination of the curvature of epithelial cell mass by mesenchyme in branching morphogenesis of mouse salivary gland

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SUMMARY

Mouse submaxillary epithelium undergoes branching morphogenesis with increase in the curvature of its surface in vivo. Recombination experiments in vitro of the epithelium and mesenchyme between 13- and 14-day rudiments showed (1) that the 14-day mesenchyme more actively induced the epithelium to branch than the 13-day mesenchyme, (2) that the 14-day mesenchyme could produce clefts on smaller epithelial lobes than the 13-day mesenchyme, (3) that the 14-day mesenchyme produced lobes with similar diameter to lobes of the 14-day intact rudiment, and (4) that the lobular morphology of assembled 14-day lobes became obscure in recombinates with the 13-day mesenchyme while it was well maintained in recombinates with the 14-day mesenchyme. From these results it is concluded that the mesenchyme determines the curvature of epithelial surface, and that clefts are formed on the epithelial surface as a result of increase in the epithelial curvature.

INTRODUCTION

Mechanisms of branching morphogenesis of mouse salivary gland have been investigated by many workers since introduction of in vitro culture of this organ by Borghese (1950). Spooner & Wessells (1972) and Spooner (1973) presented a model of salivary branching morphogenesis in which clefts were formed by changes of the shape of epithelial cells which were caused by shortening or lengthening of epithelial microfilament bundles, and deeper clefts were stabilized by collagen fibrils at the epitheliomesenchymal interface. Thereafter, this model was modified by Banerjee, Cohn & Bernfield (1977), taking account of basal lamina which consisted of proteoglycans and had a role of maintaining epithelial morphology. Spooner & Faubion (1980) and Thompson & Spooner (1982) reconfirmed the role of collagen and proteoglycans as stabilizers of the epithelial morphology using inhibitors of synthesis of these substances respectively.

Studies on epitheliomesenchymal interactions in mouse salivary morphogenesis have shown that only a few kinds of other mesenchymes than the...
salivary mesenchyme can support branching of the salivary epithelium (Grobstein, 1953; Sherman, 1960; Cunha, 1972; Lawson, 1974), and that the salivary mesenchyme can instructively influence mammary epithelium to branch in salivary-like fashion (Kratochwil, 1969; Sakakura, Nishizuka & Dawe, 1976). We have introduced the elongating and branching morphogenesis of quail salivary glands to study tissue interactions in the epithelial morphogenesis (Nogawa, 1978 & 1981; Nogawa & Mizuno, 1981). Recombination experiments of the epithelium and mesenchyme between elongating-type (quail anterior submaxillary) and branching-type (quail anterior lingual and mouse submaxillary) salivary glands showed that the elongating or branching morphogenesis of quail salivary epithelium was controlled by the associated mesenchyme. Yet, it remains unclear how the salivary mesenchyme participates in controlling the epithelial morphogenesis.

Comparative observations of the branching and elongating morphogenesis in vivo let us notice that the curvature of branching-type epithelium became larger with progress of the development (Fig. 1), while that of elongating-type epithelium was nearly constant during elongation (see Figs 2 to 4 in Nogawa, 1981). This may suggest that ‘branching’ can be regarded as increase in the epithelial curvature. In the present study, branching morphogenesis of mouse submaxillary gland is analysed with recombination experiments in vitro of the epithelium and mesenchyme between two submaxillary rudiments whose epithelial curvature is different, and a new model of branching morphogenesis is presented from a viewpoint of the epithelial curvature determined by the associated mesenchyme.

**MATERIALS AND METHODS**

*Isolation of submaxillary rudiments*

ICR mice were mated during the night, and the morning of discovery of the vaginal plug was counted as day 0. Submaxillary rudiments with sublingual rudiments were isolated from 13-, 14- and 15-day foetuses according to the procedure of Borghese (1950), and accompanied sublingual rudiments were removed.

*Separation of epithelium and mesenchyme*

Isolated 13- and 14-day submaxillary rudiments were exposed to 0.25% trypsin (1:250) in Ca²⁺-, Mg²⁺-free Hanks' balanced salt solution (GIBCO Laboratories) at 4°C for 15 min. Since the distal part of the rudiments consisted of only mesenchymal component, the submaxillary mesenchyme was taken up from the distal part. The submaxillary epithelium was cleared of mesenchymal cells with very fine forceps, and a whole part of the 13-day epithelium or lobes of the 13- and 14-day epithelium were used in recombination experiments.
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Separated epithelia and mesenchymes were rinsed twice in a mixture of Hanks' balanced salt solution (HBSS) and horse serum (1:1), and stored in HBSS with 20% horse serum at room temperature. Before being recombined with the mesenchyme, 13- and 14-day epithelial lobes were measured, and 13-day lobes with diameter 135–150 μm and 14-day lobes with diameter 95–110 μm were used.

Organ culture

Explants were cultivated at 37.5 °C according to the method of Wolff & Haffen (1952). The medium was composed of medium 199 (GIBCO Laboratories) with 20% horse serum (Flow Laboratories), 0.5% agar (DIFCO Laboratories) and penicillin G potassium (100 units/ml). Three pieces of 13- or 14-day submaxillary mesenchyme were assembled on the semisolid medium, and submaxillary epithelia were placed in the centre of the mesenchymal mass.

Measurement

Recombinates were photographed 20 or 44 h after cultivation. All the clefts formed on the epithelial surface in each recombine were classified as deep clefts (deeper than 50 μm) and shallow clefts, and the value of cleft formation (Vc) was given to each recombine by counting one deep cleft as 1 and one shallow cleft as 0.5.

In order to compare the size of epithelial lobes with shallow clefts and those without clefts, the area of epithelium which was traced on the thick paper was weighed, and diameter of the lobe was calculated as a circle from the weight.

RESULTS

Increase of epithelial curvature in normal development

A submaxillary epithelium of the 13-day rudiment consists of three to five spherical lobes and a cylindrical stalk (Fig. 1A). Diameter of the lobes is 120–180 μm, and that of the stalk is 80–100 μm. On the 14th day, the epithelium undergoes branching and the number of lobes becomes 30–40 (Fig. 1B). Diameter of the lobes decreases (70–130 μm), and that of the stalk also decreases (40–50 μm). On the 15th day, the number of lobes reaches 150–200 (Fig. 1C). Diameter of the lobes continues to decrease (40–100 μm), but that of the stalk is constant. These observations show that the curvature of lobular epithelium increases with developmental stages, and that lobes with diameter over 130 μm, which are present in the 13-day rudiment, are absent in the 14-day rudiment.

Morphogenesis of 13-day epithelium in recombinates

In intact rudiments, the 14-day mesenchyme surrounded smaller lobes than the 13-day mesenchyme. When a whole 13-day epithelium was cultured in recombination with the 14-day mesenchyme for 20 h, the epithelium formed
smaller and more lobes than the epithelium did when cultured in recombination with the 13-day mesenchyme (Figs 2A and B).

In order to simplify the experimental system, one of 13-day epithelial lobes with diameter 135–150 μm was recombined with the 13- or 14-day mesenchyme and cultured (Fig. 3A, Table 1). The value of cleft formation (Vc) of the lobe recombined with the 13-day mesenchyme was 1.1 ± 1.1 at 20 h of cultivation, and the epithelium formed shallow clefts in two thirds of the recombinates (Fig. 3B). In contrast, the Vc of the lobe recombined with the 14-day mesenchyme was 3.4 ± 1.1 at 20 h of cultivation, and the epithelium cleaved deeply to form new lobes in most of the recombinates (Fig. 3C). Diameter of the newly formed lobes was 110 ± 20 μm (n = 54, min. 50 μm and max. 160 μm) and was similar to that of one lobe of the 14-day intact rudiment. A question then arose as to how the morphogenesis of a smaller epithelial fragment of the 13-day lobe proceeded.
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Fig. 2. Recombinates of a whole 13-day epithelium with the 13-day mesenchyme (A) and with the 14-day mesenchyme (B) at 20 h of cultivation.

Fig. 3. Recombinates of one 13-day lobe with the mesenchymes. The lobe (arrow) at the beginning of cultivation (A). The lobe recombined with the 13-day mesenchyme (B) forms only a shallow cleft at 20 h of cultivation, while the lobe recombined with the 14-day mesenchyme (C) forms deeper clefts and consists of several lobes.

Fig. 4. Recombinates of the one-third 13-day lobe with the 13-day mesenchyme (A) and with the 14-day mesenchyme (B) at 20 h of cultivation. Two shallow clefts are formed in B.

Bar = 200 μm.

when cultured in recombination with the mesenchymes. One third of the one 13-day lobe was cut off and cultured in recombination with the 13- or 14-day mesenchyme (Table 1). In most of the recombinates with the 13-day mesenchyme
Table 1. Cleft formation of the 13-day epithelium in recombinates at 20 h of cultivation

<table>
<thead>
<tr>
<th>Epithelial lobe</th>
<th>Age of mesenchyme (day)</th>
<th>No. of recombinates (diameter of epithelium, μm)*</th>
<th>Value of cleft formation (Vc)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>with deep and shallow clefts</td>
<td>with only shallow clefts</td>
</tr>
<tr>
<td>one</td>
<td>13</td>
<td>4 (240 ± 30)</td>
<td>17 (240 ± 20)</td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>20</td>
<td>4 (230 ± 20)</td>
</tr>
<tr>
<td>1/3</td>
<td>13</td>
<td>0 (180 ± 10)</td>
<td>3 (180 ± 20)</td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>1 (170 ± 20)</td>
<td>11 (170 ± 20)</td>
</tr>
</tbody>
</table>

* Mean ± s.d.

the epithelial fragment remained round at 20 h of cultivation (Fig. 4A), but underwent branching at 44 h. In contrast, half of the recombinates with the 14-day mesenchyme formed shallow clefts at 20 h (Fig. 4B), while the rest half remained round.

Recombinates with the 13-day mesenchyme and those with the 14-day mesenchyme were compared on diameter of lobes which were forming shallow clefts (Table 1). When the 13-day mesenchyme was associated, the lobe with diameter ca. 240 μm 20 h after cultivation could form shallow clefts, but the lobe with diameter ca. 170 μm could not. However, when the 14-day mesenchyme was associated, even the lobe with diameter ca. 170 μm 20 h after cultivation could form shallow clefts.

Morphogenesis of 14-day epithelium in recombinates

One of the 14-day epithelial lobes with diameter 95–110 μm was recombined with the 13- or 14-day mesenchyme and cultured (Fig. 5A, Table 2). In most of the recombinates with the 13-day mesenchyme the lobe remained round at 20 h of cultivation (Fig. 5B), but underwent branching at 44 h. In contrast, the lobe formed shallow clefts at 20 h of cultivation in most of the recombinates with the 14-day mesenchyme (Fig. 5C), and the Vc was 1.0 ± 0.7, which was similar to the Vc of one 13-day lobe with the 13-day mesenchyme. Diameter of the 14-day lobe forming shallow clefts in recombinates with the 14-day mesenchyme was ca. 180 μm, which was similar to that in recombinates of the one-third 13-day lobe with the 14-day mesenchyme. These data indicated that the 14-day mesenchyme could produce shallow clefts on the lobe with diameter ca. 180 μm irrespective of age of the epithelium.
Fig. 5. Recombinates of one 14-day lobe with the mesenchymes. The lobe (arrow) at the beginning of cultivation (A). The lobe recombined with the 13-day mesenchyme (B) remains round at 20 h of cultivation, while the lobe recombined with the 14-day mesenchyme (C) forms three shallow clefts.

Fig. 6. Recombinates of an assembly of two 14-day lobes with the mesenchymes. The assembled two lobes (arrows) at the beginning of cultivation (A). The two original lobes fuse into one larger lobe without clefts in recombinate with the 13-day mesenchyme at 20 h of cultivation (B), while the fusing lobes have a few clefts besides two deep clefts in recombinate with the 14-day mesenchyme (C).

Fig. 7. Recombinates of an assembly of three 14-day lobes with the mesenchymes. The assembled three lobes (arrows) at the beginning of cultivation (A). The three original lobes fuse into one larger lobe with three clefts in recombinate with the 13-day mesenchyme at 20 h of cultivation (B), while the fusing lobes have a few clefts besides three deep clefts in recombinate with the 14-day mesenchyme (C).

Bar = 200 μm.
Table 2. Cleft formation of the 14-day epithelium in recombinates at 20 h of cultivation

<table>
<thead>
<tr>
<th>Epithelial lobe</th>
<th>Age of mesenchyme (day)</th>
<th>No. of recombinates (diameter of epithelium, μm)*</th>
<th>Value of cleft formation (Vc)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>with deep and shallow clefts</td>
<td>with only shallow clefts</td>
</tr>
<tr>
<td>one</td>
<td>13</td>
<td>0</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>1</td>
<td>19</td>
</tr>
<tr>
<td>two</td>
<td>13</td>
<td>5</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>13</td>
<td>1</td>
</tr>
<tr>
<td>three</td>
<td>13</td>
<td>8</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>16</td>
<td>(270 ± 20)</td>
</tr>
</tbody>
</table>

* Mean ± s.d.

Since the 13-day mesenchyme could not have a morphogenetic effect on one 14-day lobe 20 h after cultivation which was smaller than one 13-day intact lobe at the beginning of cultivation, it was investigated whether the 13-day mesenchyme could influence the morphogenesis of assembled 14-day lobes (Figs 6A and 7A, Table 2). When two lobes were assembled and cultured in recombination with the 13-day mesenchyme, the lobes fused together in all the cases. At 20 h of cultivation, the fusing lobes rarely had more than two shallow clefts and a few of them became one larger lobe without clefts (Fig. 6B). When an assembly of three lobes was cultured in recombination with the 13-day mesenchyme, the fusing lobes mostly had only three clefts at 20 h of cultivation which consisted of a mixture of shallow and deep clefts (Fig. 7B). However, when an assembly of two or three lobes was cultured in recombination with the 14-day mesenchyme, it formed new clefts in addition to two or three deep clefts at 20 h of cultivation (Figs. 6C and 7C). These results suggested that since the 13-day mesenchyme could not maintain the morphology of individual 14-day lobes differently from the 14-day mesenchyme, the boundaries between the lobes disappeared after fusion or became shallow clefts in recombinates with the 13-day mesenchyme while they developed into deep clefts in recombinates with the 14-day mesenchyme.

DISCUSSION

In the previous study it was demonstrated that mouse submaxillary mesenchyme induced the elongating-type quail salivary epithelium to branch (Nogawa
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& Mizuno, 1981), but the nature of the instructive influence of the mesenchyme remained unclear. The present study demonstrated that each lobe of mouse submaxillary epithelium became smaller with progress of the development in vivo. From this fact it was expected that making the curvature of lobes larger resulted in cleaving lobes. Then, recombination experiments in vitro of the epithelium and mesenchyme between 13- and 14-day rudiments showed that the 14-day mesenchyme more actively induced the epithelium to branch than the 13-day mesenchyme. The 14-day mesenchyme, however, did not more actively stimulate the epithelial growth since there was no difference in diameter between the lobes forming shallow clefts in recombinates with the 14-day mesenchyme and the round lobes without clefts in recombinates with the 13-day mesenchyme. Although Alescio & Di Michele (1968) reported that in the development of mouse embryonic lung the branching activity of epithelium correlated to the growth rate of epithelium, it is not appropriate to this case. The higher branch-inducing activity of the 14-day mesenchyme seems to originate from the fact that the mesenchyme has the ability to make the curvature of epithelial surface as large as that of the intact lobe of the mesenchyme-dependent age: in the lobe with diameter ca. 170 μm, the 14-day mesenchyme could produce shallow clefts while the 13-day mesenchyme could not, and in the lobe with diameter ca. 240 μm, the 14-day mesenchyme produced several lobes as small as the 14-day intact lobes while the 13-day mesenchyme produced only shallow clefts. Probably, clefts may be formed on the epithelial surface as a result of that the mesenchyme increases the epithelial curvature. This supposition is supported by the results in the further recombination experiments that the lobular morphology of an assembly of two or three 14-day lobes was maintained by two or three deep clefts in recombinates with the 14-day mesenchyme while it became obscure in recombinates with the 13-day mesenchyme. The present study strongly suggests that submaxillary mesenchyme causes the epithelium to branch by determining the curvature of epithelial surface, but actually the mesenchyme will determine the curvature of the basal lamina. Banerjee et al. (1977) reported that mouse submaxillary epithelium came to project processes when the basal lamina was torn off from the epithelial surface, and that such epithelium became round with disappearance of clefts when cultured in recombination with the mesenchyme. The mesenchyme may not be able to affect the epithelial surface whose curvature is irregular with many processes, and to smooth the epithelial surface may be the most important role of the basal lamina in branching morphogenesis.

By what mechanisms will submaxillary mesenchyme determine the curvature of epithelial surface? Reports on contact guidance by Dunn & Heath (1976) and Fisher & Tickle (1981) may suggest a possible mechanism: fibroblastic cells cannot hold firmly enough to a substratum whose curvature is larger than a critical curvature, and their microfilament bundles play an important role in this cell behaviour. Submaxillary mesenchymal cells may have stage-dependent
increasing critical curvature, and they may be able to affect the epithelial morphogenesis only when they can hold the epithelial surface. Spooner & Wessells (1972) and Spooner (1973) postulated that branching morphogenesis was caused by changes of epithelial microfilament bundles, but a possibility remains still that mesenchymal microfilament bundles play an important role in determining the curvature of epithelial surface.

It was also observed in the present study that several shallow clefts appeared on the epithelial surface at a short interval in early 13-day mouse submaxillary rudiments before branching (Fig. 8). From this finding and the results in the recombination experiments a new model of branching morphogenesis is proposed (Fig. 9). The curvature-increasing model, in which the branching morphogenesis is regarded not as formation of clefts at particular points of the epithelial surface but as increase in the curvature of all the epithelial surface, seems to explain well the dynamic aspect of branching morphogenesis. In normal development of mouse submaxillary rudiments, the initiation of branching will be ensured by the increase in the mesenchyme-determining curvature with developmental stages. Then, how will the epithelial morphogenesis take place when the mesenchyme-determining curvature is constant during development? The epithelium will form a cylindrical stalk without branching, since quail anterior submaxillary rudiments elongate keeping the epithelial curvature constant.

Fig. 8. A submaxillary rudiment of the early 13-day foetus. Several shallow clefts (arrows) are formed at a short interval. Bar = 50 μm.
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![Diagram of curvature model](image)

Fig. 9. Curvature-increasing model of branching morphogenesis. A: when the radius of epithelial curvature ($r_e$) is smaller than that of mesenchyme-determining curvature ($r_m$), the lobe remains round. Thereafter, $r_e$ increases by epithelial cell proliferation, and $r_m$ decreases with developmental stages. B: when $r_e$ becomes larger than $r_m$, many arcs with $r_m$ appear on the epithelial surface under the influence of surrounding mesenchyme, and consequently many shallow clefts are formed. B': when the mesenchyme influences also shallow clefts to take the curvature of $r_m$, some clefts disappear and others deepen. C: finally, spherical lobes with $r_m$ are formed, and the number of lobes formed is determined by a ratio of total volume of epithelium to the volume of one lobe with $r_m$. The curvature of epithelial surface is constant during stages B and C.

In vivo (Nogawa, 1981). If this consideration is added to the curvature-increasing model, the model will design various patterns of branching morphogenesis of glandular organs.

We have grasped in the present study the branching morphogenesis of mouse submaxillary gland as increase in the curvature of epithelial surface under the control of mesenchyme. Recently, Yang, Larson & Nandi (1982) succeeded in making mouse submaxillary epithelial cells grow three dimensionally in mesenchyme-free condition by using collagen gel, but the morphology of the cell mass was quite different from the lobular morphology observed both in the intact rudiment and in the recombinant. We will elucidate the mechanisms of mesenchymal control over the curvature of salivary epithelium in future studies.

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REFERENCES


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