Substitution for mesenchyme by basement-membrane-like substratum and epidermal growth factor in inducing branching morphogenesis of mouse salivary epithelium

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Summary

Mouse salivary epithelium cannot undergo branching morphogenesis in the absence of the surrounding mesenchyme. To clarify the nature of the mesenchymal influence on the epithelium, we have investigated the culture conditions in which the epithelium could normally branch in the absence of mesenchymal cells. Combination of basement-membrane-like substratum (Matrigel) and epidermal growth factor (EGF) could substitute for the mesenchyme, the epithelium showing typical branching morphogenesis. Transforming growth factor \(\alpha\) had the same effect as EGF. Matrigel plus basic fibroblast growth factor or transforming growth factor \(\beta1\) and collagen gel plus EGF were not sufficient to support the branching of the epithelium. These results clearly reveal that the role of mesenchyme in salivary morphogenesis is both to provide the epithelium with an appropriate substratum and to accelerate growth of the epithelium.

Key words: mouse salivary gland, branching morphogenesis, Matrigel, epidermal growth factor.

Introduction

Tissue interactions between epithelium and mesenchyme are necessary for organ development. Branching organs such as salivary gland and lung are excellent material for studying tissue interactions, since they reproducibly undergo branching morphogenesis in vitro, and the extent of branching is easily estimated by the observation of living explants. Mouse salivary epithelium branches only in the presence of its own or a few other kinds of mesenchyme (Grobstein, 1953; Cunha, 1972; Lawson, 1974). Attempts to make the salivary epithelium branch in the absence of mesenchyme have failed. For instance, when the salivary epithelium was cultured alone within a collagen gel, it formed many processes but its morphology was quite different from the normal branching (Yang et al. 1982).

The nature of the mesenchymal influence on the epithelium has been unclear. We have already reported that the salivary epithelium could not branch when separated from its mesenchyme by a membrane filter, but branched only when the epithelium was covered with basement-membrane-like substratum (Matrigel) in the same culture system (Takahashi and Nogawa, 1991). It was further shown that membrane filters with pore size 0.05 \(\mu\)m hardly diminished the effect of mesenchyme. Thus, it was suggested that some diffusible substance transfers from the mesenchyme to the epithelium through the filter. In the present study, we examined what substance induced the epithelium covered with Matrigel to branch in the absence of mesenchyme, and elucidated the role of mesenchyme in the branching morphogenesis of salivary epithelium.

Materials and methods

Preparation of salivary rudiments and mesenchyme

ICR mice were mated during the night and the day of the discovery of the vaginal plug was counted as day 0. Submandibular rudiments were isolated from 13-day foetuses in Hanks' balanced salt solution (HBSS). The mesenchyme, free from the epithelium, was cut out of the distal part of the rudiment (Fig. 1), and stored in HBSS with 20 % horse serum (Gibco Lab.). The rest of the rudiment was treated with dispase (1000 protease units ml\(^{-1}\) in HBSS; Godo Shusei Co., Japan) at 37.5°C for 30 min, and the epithelium was separated from the mesenchyme with fine forceps. The separated epithelium was washed twice with HBSS containing 20 % horse serum. The separated epithelium for the experiments of serum-free culture was washed twice with only HBSS.
Culture of the epithelium with the mesenchyme

Three pieces of the isolated mesenchyme were placed on the semisolid medium prepared in a Falcon 3037 culture dish. Direct recombinates were made by placing epithelium in contact with the mesenchymes. Transfilter recombinates were made according to the method in the previous paper (Takahashi and Nogawa, 1991). Nuclepore filter with pore size 0.1 µm (Nuclepore Corp.) and Matrigel were used in the present experiments. The semisolid medium was composed of medium 199 Earle's balanced salt solution (EBSS) with 20% horse serum, 0.5% agar and penicillin G potassium (100 units ml⁻¹), and the recombinates were incubated at 37.5°C in 5% CO₂/95% air.

Culture of the epithelium without the mesenchyme

The isolated epithelium was placed on Nuclepore filter (diameter 13 mm and pore size 0.1 µm) in the centre well of a Falcon 3037 culture dish, and covered with Matrigel or collagen gel. After gelling, 0.3 ml of the liquid medium was poured into the well, detaching the explant with the filter from the bottom of the well. For the experiments without gel, the epithelium was placed on the filter on a stainless steel grid, and the medium was poured to the level of the filter so as to cover the epithelium. The control medium was composed of medium 199 EBSS with 20% horse serum and penicillin G potassium (100 units ml⁻¹), and the experimental medium contained excess of a growth factor or antibody to a growth factor at various concentrations (see below for details). All the explants were incubated at 37.5°C in 5% CO₂/95% air and photographed. The number of samples in each experiment was more than 9 and each experiments was repeated at least twice.

Gels
Matrigel was purchased from Collaborative Res. Inc. Collagen gel was prepared with a mixture of 8 volumes of Cellmatrix type I-A (acid soluble fraction of type I collagen from porcine tendon, 3.0 mg ml⁻¹; Nitta Gelatine Co., Japan), 1 volume of 10× medium 199 EBSS and 1 volume of 200 mM Hepes buffer solution.

Growth factors
Epidermal growth factor (EGF) was purchased from Collaborative Res. Inc. (from adult mouse submandibular glands, receptor grade). Fibroblast growth factor (FGF) was from Biomedical Tech. Inc. (from bovine pituitary, basic). Transforming growth factor α (TGFα) was from Biotope Inc. (rat, synthetic, res 1–50). Transforming growth factor β1 (TGFβ1) was from R&D systems (from porcine platelets).

Antibodies to a growth factor
Anti-EGF (Collaborative Res. Inc.) was the IgG fraction of rabbit polyclonal antibody, and immunizing antigen was from the same source as the EGF used in the present experiments. Anti-TGFα (Oncogene Sci. Inc.) was the IgG fraction of mouse monoclonal antibody against recombinant human TGFα, and was characterized by the makers as neutralizing TGFα at a ratio of 1 µg antibody to 0.5 ng TGFα.

Results
Branching morphogenesis of epithelium in the presence of mesenchyme
Submandibular epithelium always grew and underwent branching morphogenesis when directly recombined with the mesenchyme and cultured (Fig. 2). Epithelium never branched when separated from the mesenchyme with Nuclepore filter alone. When covered with Matrigel, the epithelium also showed extensive growth and branching morphogenesis in transfilter recombinates, but the extent of branching was inferior to that in the direct recombinates (Fig. 3). Besides the quantitative difference, branching morphogenesis occurred two-dimensionally in the transfilter recombinates, but three-dimensionally in the direct recombinates. Detailed results in transfilter experiments including the effect of pore size were described in the previous paper (Takahashi and Nogawa, 1991).
Substitutes for salivary mesenchyme

Figs 4–7. An epithelium covered with Matrigel and cultured for 2 days in medium containing EGF. Fig. 4: 10 ng ml⁻¹, the epithelium underwent extensive branching, and each lobule was a spherical swelling. Fig. 5: 1.0 ng ml⁻¹. Fig. 6: 0.1 ng ml⁻¹, the epithelium formed short, straight protrusions. Fig. 7: 0 ng ml⁻¹ (control medium), the outline of the epithelium was uneven.

Fig. 8. An epithelium not covered with Matrigel and cultured for 2 days in medium containing EGF (10 ng ml⁻¹). No branching morphogenesis occurred. Bar: 200 μm.

Branching morphogenesis of epithelium in the absence of mesenchyme

The submandibular epithelium was covered with Matrigel, and cultured in the medium containing EGF in the absence of any mesenchymal cells for 2 days. Depending on the concentration of EGF, the epithelium grew and underwent branching morphogenesis (Figs 4–7). At 10 ng ml⁻¹, the epithelium underwent branching morphogenesis in 27 out of 30 cases, and its morphology could not be distinguished from the one directly surrounded by the mesenchyme (compare Fig. 4 with Fig. 2). The concentration of EGF affected not only the number of lobules formed but also the shape of the lobules. Each lobule swelled spherically at 10 ng ml⁻¹ as in the normal branching morphogenesis (Fig. 4), whereas only short, straight protrusions were formed at 0.1 ng ml⁻¹ (Fig. 6). In the control medium with no EGF added, the epithelium often had an uneven outline within the Matrigel (Fig. 7). The incidence of extensive branching morphogenesis decreased at concentrations higher than 10 ng ml⁻¹: occurring in 9 out of 16 cases at 100 ng ml⁻¹ and 6 out of 19 cases at 1000 ng ml⁻¹, which suggested that EGF had an inhibitory effect on the epithelial morphogenesis at the higher concentrations. The epithelial branching morphogenesis never occurred without Matrigel, even in the presence of EGF (Fig. 8).

Effects of other growth factors and substratum

To examine the effects of growth factors other than EGF, the submandibular epithelium was covered with Matrigel and cultured in the medium containing EGF (0.01, 0.1, 1.0, 10 and 100 ng ml⁻¹), TGFα (the same graded concentrations) or TGFβ1 (the same graded concentrations) for 2 days. TGFα was effective at the same concentration as EGF, and the epithelial morphogenesis was almost identical to that with EGF (Fig. 9). FGF had little effect on epithelial morphogenesis, and the outline of epithelium was a little more uneven at 10 and 100 ng ml⁻¹ than that in the control medium (Fig. 10). TGFβ1 had an inhibitory effect on epithelial morphogenesis at concentrations higher than 0.1 ng ml⁻¹, and the epithelium remained round (Fig. 11), while at lower concentrations the epithelium had an uneven outline as in the control medium (Fig. 12).

As to substrata, when the epithelium was covered with collagen gel instead of Matrigel and cultured in medium containing EGF for 2 days, it spread and formed several pointed protrusions, but its morphology was quite different from the normal branching morphology (Figs 13, 14).

Blocking with antibody and serum-free culture

It was examined whether antibodies to EGF or TGFα inhibited the branch-inducing activity of EGF or TGFα. When the epithelium with Matrigel was cultured in the medium containing both EGF (10 ng ml⁻¹) and anti-EGF IgG (10 μg ml⁻¹), epithelial branching morphogenesis was completely inhibited in 9 out of 10 cases (Figs 15, 16; Table 1). In order to test the nonspecific toxicity of anti-EGF or EGF–anti-EGF complex, we
Fig. 9. An epithelium covered with Matrigel and cultured for 2 days in medium containing TGFα (10 ng ml⁻¹). The epithelium underwent extensive branching.

Fig. 10. An epithelium covered with Matrigel and cultured for 2 days in medium containing FGF (10 ng ml⁻¹). The outline of the epithelium was a little more uneven than that in controls (compare with Fig. 7).

Figs 11–12. An epithelium covered with Matrigel and cultured for 2 days in medium containing TGFβ1. Fig. 11: 1.0 ng ml⁻¹, the outline of the epithelium was even and round. Fig. 12: 0.01 ng ml⁻¹, the outline of the epithelium was uneven, as in controls.

Figs 13–14. An epithelium covered with collagen gel and cultured for 2 days in medium containing EGF. Fig. 13: 10 ng ml⁻¹, the epithelium spread and formed some pointed protrusions responding to EGF. Fig. 14: 0 ng ml⁻¹ (control). Bar: 200 μm.

Figs 15–18. An epithelium covered with Matrigel and cultured for 2 days in medium containing growth factors and anti-EGF IgG. Fig. 15: EGF 10 ng ml⁻¹ and 10% phosphate-buffered saline (control). In treated groups, an equal volume of phosphate buffered saline containing anti-EGF IgG was added to the medium. Fig. 16: EGF 10 ng ml⁻¹ and anti-EGF IgG 10 μg ml⁻¹. Fig. 17: TGFα 10 ng ml⁻¹ and anti-EGF IgG 10 μg ml⁻¹. Fig. 18: EGF 10 ng ml⁻¹, TGFα 10 ng ml⁻¹ and anti-EGF IgG 10 μg ml⁻¹. Bar: 200 μm.
Table 1. Effects of anti-EGF or anti-TGFα on branching morphogenesis

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*EGF (10 ng/ml−1), TGFα (10 ng/ml−1), anti-EGF (100 ng/ml−1) and anti-TGFα (20 μg/ml−1) were added to the medium in +.
†No. of explants. Extent of branching morphogenesis was estimated as the number of lobules formed after 2 days of cultivation. ++, more than 10 and 10; +, less than 10; −, 0.

examined the recovery of branching morphogenesis with TGFα. When TGFα (10 ng/ml−1) was added, instead of EGF or in addition to EGF, to the medium containing anti-EGF IgG (10 μg/ml−1) at the beginning of cultivation, the epithelium with Matrigel showed branching morphogenesis in both cases (Figs 17, 18; Table 1).

The inhibitory effects of anti-TGFα IgG (20 μg/ml−1) on TGFα (10 ng/ml−1) and the recovery of branching morphogenesis with EGF were investigated in the same program as that with anti-EGF, and similar results were obtained (Table 1).

Since many undefined factors are involved in serum at undefined concentrations, it is unclear whether EGF alone or a combination of EGF and other factors was effective in inducing branching morphogenesis of the epithelium. We cultured the epithelium with Matrigel for 2 days in the serum-free medium composed of medium 199 and EGF (10 ng/ml−1) or TGFα (10 ng/ml−1) with no other supplement. The epithelium remained round in medium 199 alone, different from the epithelium in serum-containing medium 199 which had an uneven outline (compare Fig. 19 with Fig. 7). When only EGF was added to medium 199, the epithelium clearly showed branching morphogenesis in all 10 cases, though to a slightly lesser degree than that in the presence of serum (Fig. 20). The epithelial branching morphogenesis was similarly observed in all 12 cases in medium 199 with TGFα (Fig. 21).

Discussion

In the present study, it was demonstrated that the combination of Matrigel and EGF or TGFα could substitute for mesenchyme in inducing the branching morphogenesis of salivary epithelium. The extent of branching with 10 ng/ml−1 EGF was identical to that in the direct recombinates. These results suggested that epithelial growth was indispensable for branching morphogenesis, although it was not required for the initiation of early cleft formation (Nakanishi et al. 1987). TGFα was expected to be as effective as EGF, since TGFα has a structural similarity to EGF and can bind to EGF receptors (Marquardt et al. 1984; Massagué, 1983). EGF has been suggested as one of the morphogenetic factors in the development of several organs. In lung and mammary gland, locally applied EGF induced epithelial morphogenesis (Goldin and Opperman, 1980; Vonderhaar, 1987; Coleman et al. 1988), but mesenchyme existed around the epithelium in these experimental conditions. The present mesenchyme-free culture system showed that EGF directly affected salivary epithelium and induced its morphogenesis. A similar culture system was reported to support tubulogenesis of baby mouse kidney epithelial cells in primary culture (Taub et al. 1990).

In the present study salivary epithelium underwent branching morphogenesis not three-dimensionally but two-dimensionally along the surface of the filter in the transfilter culture with the mesenchyme (Fig. 3). These results suggested that a particular growth factor was secreted by the mesenchyme and received by the epithelium with little diffusion after passing through the

Figs 19-21. An epithelium covered with Matrigel and cultured for 2 days in serum-free medium. Fig. 19: no growth factors. Fig. 20: EGF 10 ng/ml−1. Fig. 21: TGFα 10 ng/ml−1. Bar: 200 μm.
filter. EGF or TGFα effectively induced branching morphogenesis of the epithelium covered with Matrigel both in the presence and the absence of serum, suggesting that each growth factor could function alone in vivo where undefined serum factors are present. EGF is synthesized in large quantities by epithelial cells of granular convoluted tubules of adult mouse submandibular gland (Gresik et al. 1985; Rall et al. 1985). However, it is not evident whether EGF or TGFα is synthesized by embryonic salivary mesenchymal cells. Antibodies to EGF or TGFα were shown to abrogate the branch-inducing effect of the corresponding growth factor on the epithelium covered with Matrigel in the present study, but the same antibodies could not inhibit branching morphogenesis of epithelium covered with mesenchyme (unpublished observation). It is unclear whether the inability of the antibodies to inhibit the effect of EGF and TGFα is due to nonspecific binding of the antibodies to mesenchymal components or due to other undefined growth factors secreted by the mesenchyme. In the present study, the epithelium grew little but often showed signs of initiation of branching with its uneven outline when cultured within Matrigel in the control medium (Fig. 7). In this case, the epithelium probably responded to a trace of EGF-like growth factors originally present in serum (Fig. 19) and Matrigel (Taub et al. 1990).

Matrigel is mainly composed of laminin, type IV collagen, heparan sulfate proteoglycan, nidogen and entactin (Kleinman et al. 1986), and these components are present at the epithelial–mesenchymal interface of salivary rudiments (Bernfield and Banerjee, 1982; Nakanishi et al. 1988; Kadoya and Yamashina, 1989). It is, however, unclear whether embryonic salivary mesenchymal cells synthesize these substances and provide the epithelium with them, although type IV collagen was reported to be produced by mesenchymal cells of mouse lung and intestine (Chen and Little, 1985; Simon-Assmann et al. 1990). It may be possible that the epithelium begins to produce these substances endogenously only when it can interact with the mesenchyme in vivo, and that Matrigel compensates for them in the present experimental conditions.

Establishment of a culture system in which the salivary epithelium undergoes the typical branching morphogenesis without any mesenchymal cells answers questions remaining from previous studies: the salivary epithelium can branch without direct contacts with the mesenchymal cells (Cutler, 1977), without the basement-membrane-degrading enzyme secreted by the mesenchymal cells (Smith and Bernfield, 1982) and without the traction force of the mesenchymal cells via collagen fibrils (Nakanishi et al. 1986). In the present study, the epithelium showed various patterns of morphogenesis according to the type of substratum and the level of EGF. The typical branching morphogenesis was observed when the epithelium was cultured within Matrigel with 10 ng ml⁻¹ EGF. Within collagen gel, the epithelium formed pointed protrusions at 10 ng ml⁻¹ EGF. At 0.1 ng ml⁻¹ EGF, the epithelium formed short, straight protrusions within Matrigel, which was rather similar to the early morphogenesis of mammary gland. The living mesenchyme must dynamically control both the components of extraepithelial matrices and the levels of growth factors in vivo, and thereby the mesenchyme may be able to exert an instructive influence upon the epithelial morphogenesis (Alescio and Cassini, 1962; Kratochwil, 1969; Nogawa and Mizuno, 1981; Nogawa, 1983). The present culture system for inducing the salivary epithelium to branch seems to be an excellent system for studying the interaction of epithelial cells with basement membrane components, and the subsequent changes in epithelial shape.

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References


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