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Branching morphogenesis of embryonic mouse lung epithelium in mesenchyme-free culture

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SUMMARY

Embryonic mouse lung epithelium was separated from its mesenchyme and cultured under mesenchyme-free conditions. When covered with Matrigel, the cultured epithelium underwent branching morphogenesis in medium containing acidic fibroblast growth factor (aFGF), in which the epithelial cells constructed a simple columnar cell layer forming a lumen, as seen in normal development. The epithelial growth and branching morphogenesis induced by aFGF was completely inhibited by an antibody against aFGF. Heparin caused extra epithelial growth in cooperation with aFGF, but its use resulted in luminal expansion instead of enhanced branching. Basic FGF induced abnormal morphogenesis of the epithelium, though the lumen formed was lined by a simple columnar cell layer. Epidermal growth factor could not maintain epithelial cell growth, and the epithelium became a smaller and smoother ball than that at the start of cultivation. When covered with a collagen gel instead of Matrigel, the epithelium remained in its initial form, neither newly branching nor becoming a smooth ball, in the presence of aFGF. These results show that the epithelium of lung rudiments was able to branch under mesenchyme-free culture conditions in which a basement membrane matrix and aFGF were substitutes for the mesenchyme.

Key words: branching morphogenesis, lung development, acidic FGF, Matrigel, mouse

INTRODUCTION

Epithelial morphogenesis proceeds through interactions with mesenchymal tissue. Tissue interactions have been studied mainly by the method of recombination experiments in which the epithelium of some organ is separated from its own mesenchyme and cultured in recombination with the mesenchyme of other organs. These studies revealed that there were organ-specificities and instructive- and permissive-type induction in tissue interactions (Grobstein, 1953; Lawson, 1974; Wessells, 1977). The question subsequently arose as to what chemicals or mechanics would work in tissue interactions. One answer to the question seems to be establishment of the culture system in which the epithelium would undergo the three-dimensional morphogenesis without any support of the mesenchymal tissue. Trials on certain epithelia were reported to be successful: a combination of collagen gels, mammogenic hormones, and epidermal growth factor (EGF) was a good condition for morphogenesis of mouse mammary epithelial cells (Yang et al., 1979; Imagawa et al., 1985); that of Matrigel (basement membrane matrices) and EGF, for baby mouse kidney epithelial cells (Taub et al., 1990); that of collagen gels and hepatocyte growth factor, for Madin-Darby canine kidney epithelial cells (Montesano et al., 1991) and that of Matrigel and EGF, for embryonic mouse salivary epithelium (Nogawa and Takahashi, 1991). These results demonstrated that both an extracellular matrix and a particular growth factor were essential for the naked epithelium to undergo morphogenesis.

Rodent lung rudiments have been used by many workers as a material as suitable as salivary rudiments for studying branching morphogenesis of epithelium. Recombination experiments showed that the difference in morphogenesis between branching bronchial epithelium and non-branching tracheal epithelium was derived from the difference not in the epithelial but in the mesenchymal component (Alescio and Cassini, 1962; Wessells, 1970) and that branching morphogenesis of lung epithelium was supported also by salivary mesenchyme (Lawson, 1983). Some recent reports have identified some of the substances taking part in tissue interactions during morphogenesis of lung rudiments: EGF, laminin, integrins, and epimorphin. EGF was reported to have a stimulatory effect on epithelial branching morphogenesis when added to the culture medium, whereas antisense oligodeoxynucleotide against EGF was reported to have an inhibitory effect (Warburton et al., 1992; Seth et al., 1993). Antibody against laminin was reported to perturb epithelial branching when added to the culture medium (Schuger et al., 1990; 1991). Inhibitors of ligand binding to integrin receptors were reported to diminish branching and result in an abnormal morphology (Roman et al., 1991). As to epimorphin, it was shown that NIH 3T3 cells transfected with epimorphin cDNA

supported tubular morphogenesis of the epithelium much better than untransfected NIH 3T3 cells (Hirai et al., 1992). All these experiments, however, were done in culture systems where lung epithelium was surrounded by mesenchyme or other supporting cells having unknown biological activities. It is therefore unclear whether the factor in question affected the epithelium directly or indirectly after transduction to a different form by the mesenchyme. In the present study, we developed a simple culture system in which the lung epithelium underwent branching morphogenesis alone in the absence of external supporting cells.

MATERIALS AND METHODS

Preparation of lung epithelium

ICR mice were mated during the night, and the day of the discovery of the vaginal plug was counted as day 0. Lung rudiments were isolated from 11-day fetuses in Hanks' balanced salt solution (HBSS). The rudiments were treated with dispase (1000 protease Units/ml in HBSS; Godo Shusei Co., Japan) at 37.5°C for 60 minutes, and epithelial lobes were separated from the mesenchyme with fine forceps, in HBSS. Only left epithelial lobes (Fig. 1A) were used in experiments, since it was difficult to isolate the intact whole epithelium of right lobes due to its more advanced branching. The separated epithelial fragments were stored in HBSS without serum.

Culture of epithelium

The isolated epithelium was placed on a Nuclepore filter (diameter of 13 mm and pore size of 0.1 μm) in the center well of a Falcon 3037 culture dish, and covered with Matrigel or collagen gel or left uncovered. After gelling, 0.4 ml of medium was poured into the well. The control medium was composed of medium 199 Earle's balanced salt solution (EBSS) with penicillin G potassium (100 units/ml) and no serum, and the experimental medium contained the desired growth factor at various concentrations. All the explants were incubated at 37.5°C in 5% CO_2/95% air.

Gels

Matrigel was purchased from Collaborative Biomedical Products, USA. Collagen solution was purchased from Nitta Gelatine Co., Japan (Cellmatrix type I-A: acid-soluble fraction of Type I collagen from porcine tendon, 3.0 mg/ml), and collagen gels were prepared from a mixture of Cellmatrix, 10× medium 199 EBSS, and 200 mM Hepes buffer solution (8:1:1).

Growth factors

Acidic fibroblast growth factor (aFGF) and basic fibroblast growth factor (bFGF), human recombinant products having a single band at 18×10^3 and 17×10^3 M_r, respectively, in SDS-PAGE, were purchased from Gibco BRL, USA and used after having been diluted to appropriate concentrations with the control medium containing 0.1% bovine serum albumin. EGF was purchased from Collaborative Biomedical Products (from adult mouse submandibular glands, receptor grade). The number of samples was more than 9 for each concentration of growth factor tested.

Antibody

Anti-aFGF, IgG fraction of rabbit polyclonal antibody against bovine aFGF was purchased from Sigma Chem. Co. USA. We confirmed in immunoblot assay that this antibody detected human aFGF but showed no cross-reactivity with human bFGF. Anti-aFGF in phosphate-buffered saline (PBS) was added to medium at 100 μg/ml with PBS at 1% in experimental groups, and non-specific IgG from rabbit serum (Cappel, USA) was added to medium at the equal concentration in the control groups.

Histological technique

Explants were fixed in Bouin's fluid. After dehydration, they were embedded in paraffin, sectioned at 5 μm, and stained with hematoxylin and eosin. Histological sections were examined with special attention to the types of epithelium, formation of a lumen, necrosis of epithelial cells and contamination of mesenchymal cells.

RESULTS

Morphogenesis in Matrigel in the presence of aFGF

The lung epithelium was covered with Matrigel and cultured in medium containing aFGF (10, 100, 200, 500 or 1000 ng/ml) for 2 days. At 10 ng/ml, the epithelium showed no sign of branching morphogenesis, having only a little trace of its initial form similar to the epithelium within Matrigel in the control medium (Fig. 1B,C). Living epithelial cells constructed a simple columnar cell layer, but the lumen was filled with necrotic cells (Fig. 1D). Necrotic cells were also observed to be left in the periphery of the main cell mass. At 100 ng/ml, the initiation of branching was observed in 9 out of 12 cases, but a small number of necrotic cells were yet present in the lumen, which made it difficult to recognize the lumen in living explants (Fig. 1E). The extent of branching increased at 200 ng/ml (Fig. 1F). At 500 ng/ml, remarkable branching morphogenesis was observed in 24 out of 27 cases, and the lumen was easily recognized inside the epithelial layer in living explants (Fig. 1G). Histological sections showed a simple columnar cell layer similar to that in normal development and no necrotic figures (Fig. 1H). At 1000 ng/ml, the lumen was much expanded, in the central part of the explants, and the extent of branching decreased, probably due to the fact that the expansion of the lumen smoothed a branching, uneven cell layer (Fig. 1I). Because of inferior branching at 200 ng/ml and the over-developing lumen at 1000 ng/ml, the optimum concentration of aFGF for the epithelial branching morphogenesis was determined to be 500 ng/ml.

When the epithelium was cultured without Matrigel in the medium containing aFGF at 500 ng/ml, disconnected epithelial cells, probably dead, drifted away from the main cell mass, and consequently a smaller cell mass with an uneven outline was left 2 days after the start of cultivation (Fig. 1J). All the epithelial cells dispersed, and no cell mass remained when the epithelium was cultured with neither Matrigel nor aFGF.

Morphogenesis in Matrigel in the presence of aFGF plus heparin

Since heparin was reported to cooperate with aFGF or bFGF to stimulate cell proliferation of vascular and non-vascular cells (Thornton et al., 1983; Uhlrich et al., 1986), we investigated how heparin affected the morphogenesis of lung epithelium within Matrigel in cooperation with aFGF. Heparin was added to medium at a final concentration 10, 30 or 100 μg/ml, and its effect was examined with three concentrations of aFGF (10, 100, and 500 ng/ml). Heparin itself had no effect on growth and morphogenesis of the epithelium since there was no difference between the epithelial morphology in the

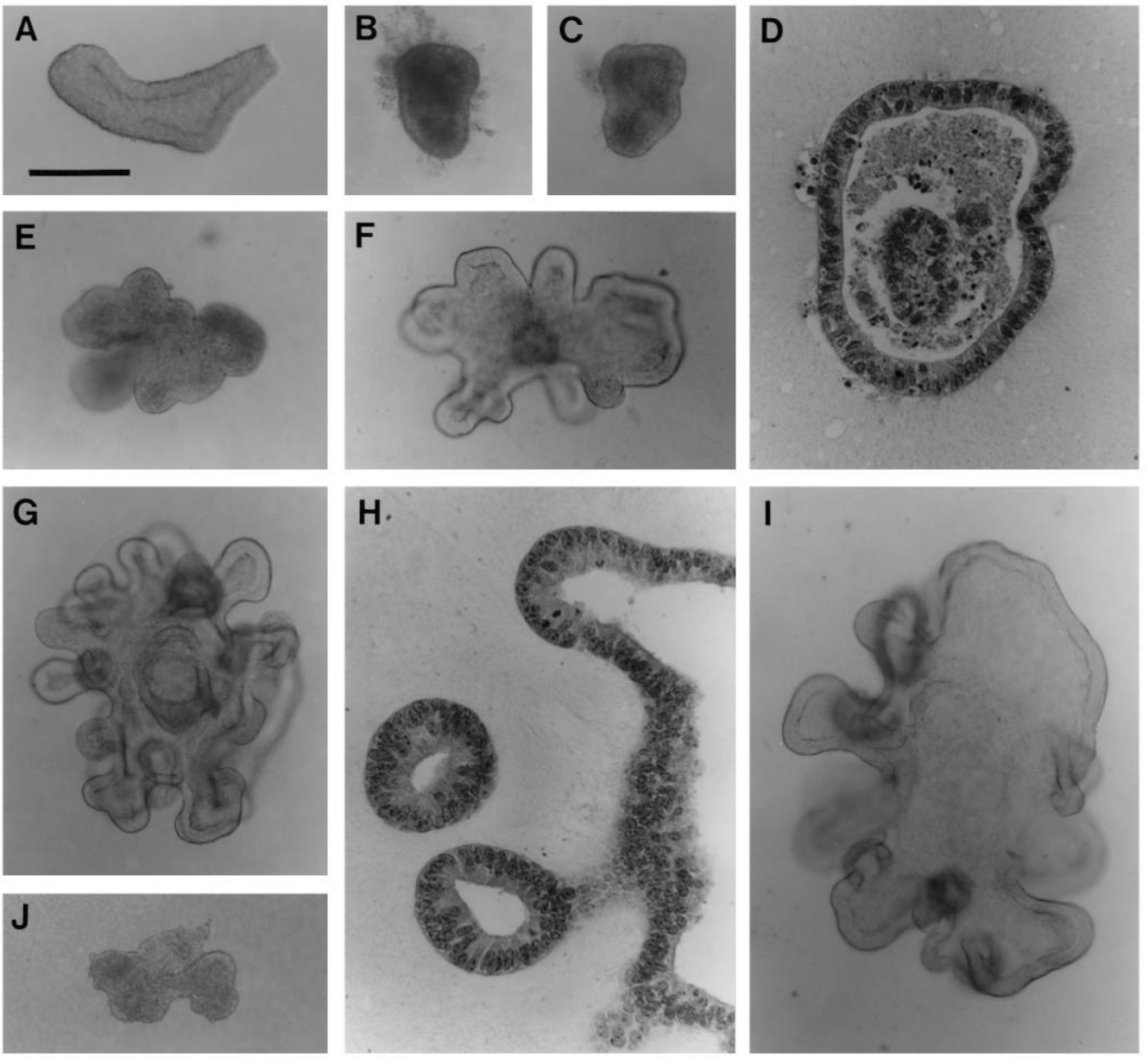

Fig. 1. Morphogenesis of lung epithelium cultured for 2 days within Matrigel in medium containing various concentrations of aFGF. (A) An isolated epithelium of 11-day left lobe at the start of culture. (B) Without aFGF as a control; cells outside the main part were necrotic. (C) 10 ng/ml. (D) Paradermal section of C; a simple columnar epithelium surrounds a lumen filled with necrotic cells. (E) 100 ng/ml. (F) 200 ng/ml. (G) 500 ng/ml; remarkable branching is observed. (H) Paradermal section of G; a simple columnar epithelium lines a lumen. (I) 1000 ng/ml; a larger lumen and less branching than in G is observed. (J) 500 ng/ml without Matrigel; the epithelium having an uneven outline was photographed after necrotic cells around it were removed. Bar, 200 μm for A-C, E-G, I,J; and 50 μm for D,H.

control medium and that in the control medium plus heparin alone (Fig. 2A; compare with Fig. 1B). With aFGF, heparin at 10 μg/ml did not have any cooperative effect on the epithelial morphogenesis, but had some odd influence on it at higher concentrations. At 10 or 100 ng/ml aFGF, heparin (30 or 100 μg/ml) caused extra epithelial growth, but a strangely large lumen was formed instead of enhanced branching morphologenesis (Fig. 2B-E; compare with Fig. 1C,E). Despite the large lumen, the epithelial cells constructed a simple columnar cell layer, not forming a cyst of a simple squamous cell layer (Fig. 2F). At 500 ng/ml aFGF, the epithelium took on a complex folded structure (Fig. 2G,H) or a double-walled cup-like structure (Fig. 2I), quite different from the branching morphology (compare with Fig. 1G). These results suggest that heparin accelerated not the branching morphogenesis but the lumen expansion of the lung epithelium within Matrigel in cooperation with aFGF.

Morphogenesis in Matrigel in the presence of bFGF or EGF

To examine the effect of another member of the FGF family, we covered lung epithelium with Matrigel and cultured it in

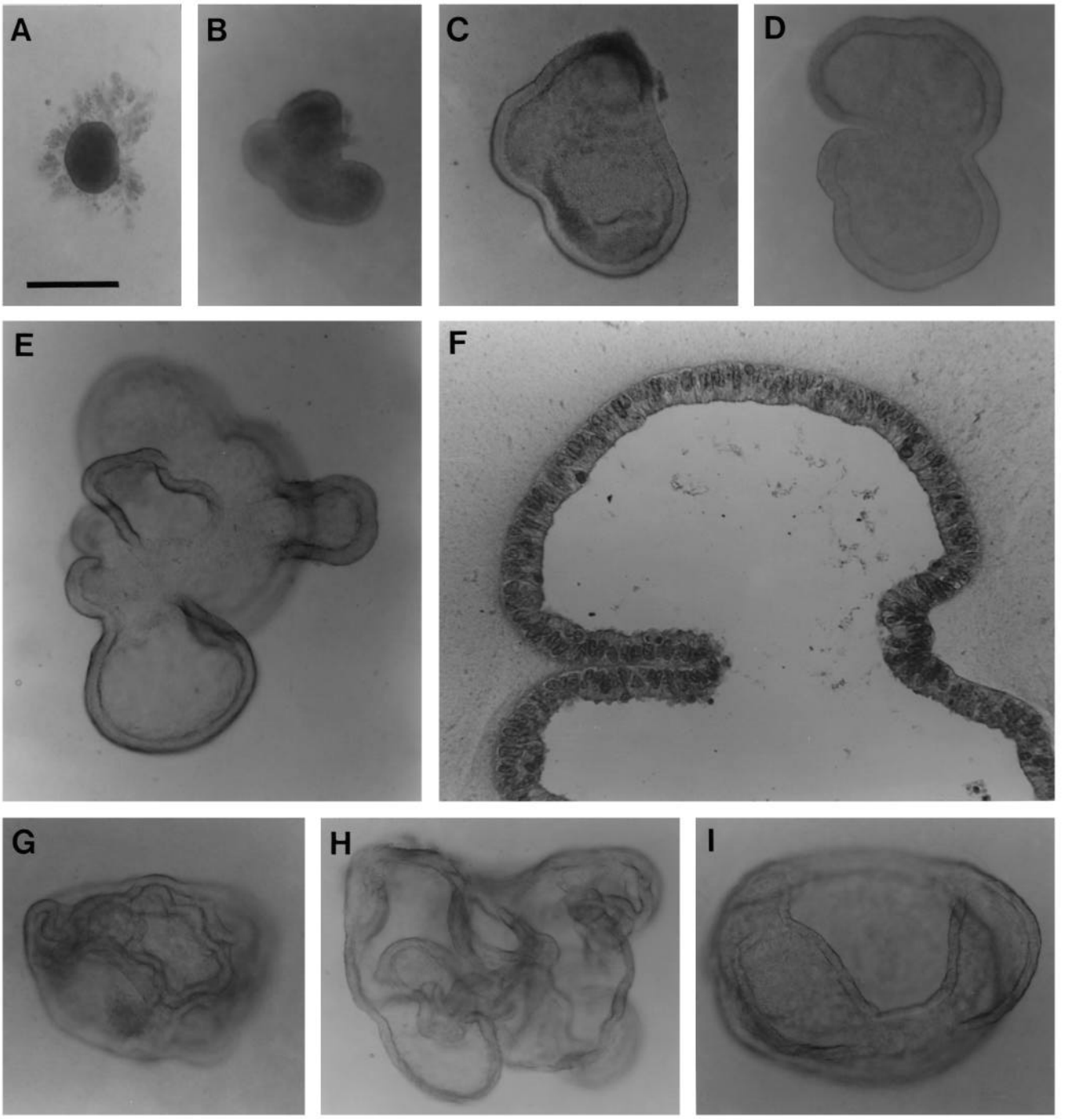

Fig. 2. Effects of heparin on morphogenesis of lung epithelium cultured for 2 days within Matrigel in medium containing various concentrations of aFGF. (A) No aFGF and heparin 100 μg/ml. (B) aFGF 10 ng/ml and heparin 30 μg/ml. (C) aFGF 10 ng/ml and heparin 100 μg/ml. (D) aFGF 100 ng/ml and heparin 30 μg/ml. (E) aFGF 100 ng/ml and heparin 100 μg/ml. Extra epithelial growth is observed in B-E, but there is formed a larger lumen without acceleration of branching (compare with Fig. 1C,E at corresponding concentrations of aFGF). (F) Paradermal section of E; even when a large lumen is formed, the epithelium is simple columnar. (G) aFGF 500 ng/ml and heparin 30 μg/ml. (H,I) aFGF 500 ng/ml and heparin 100 μg/ml. The epithelium takes a complexly folded structure in G and H, and a double-walled cuplike structure in I. Bar, 200 μm for A-E, G-I; and 50 μm for F.

medium containing bFGF (10, 100, or 1000 ng/ml) for 2 days. There was no difference between the epithelial morphology at 10 ng/ml bFGF and that in the control medium, and the size of the epithelium decreased to become smaller than the initial size due to necrosis (Fig. 3A). At 100 ng/ml, the initial size of the epithelium was almost maintained but no branching was initiated (Fig. 3B). At 1000 ng/ml, the epithelium became a

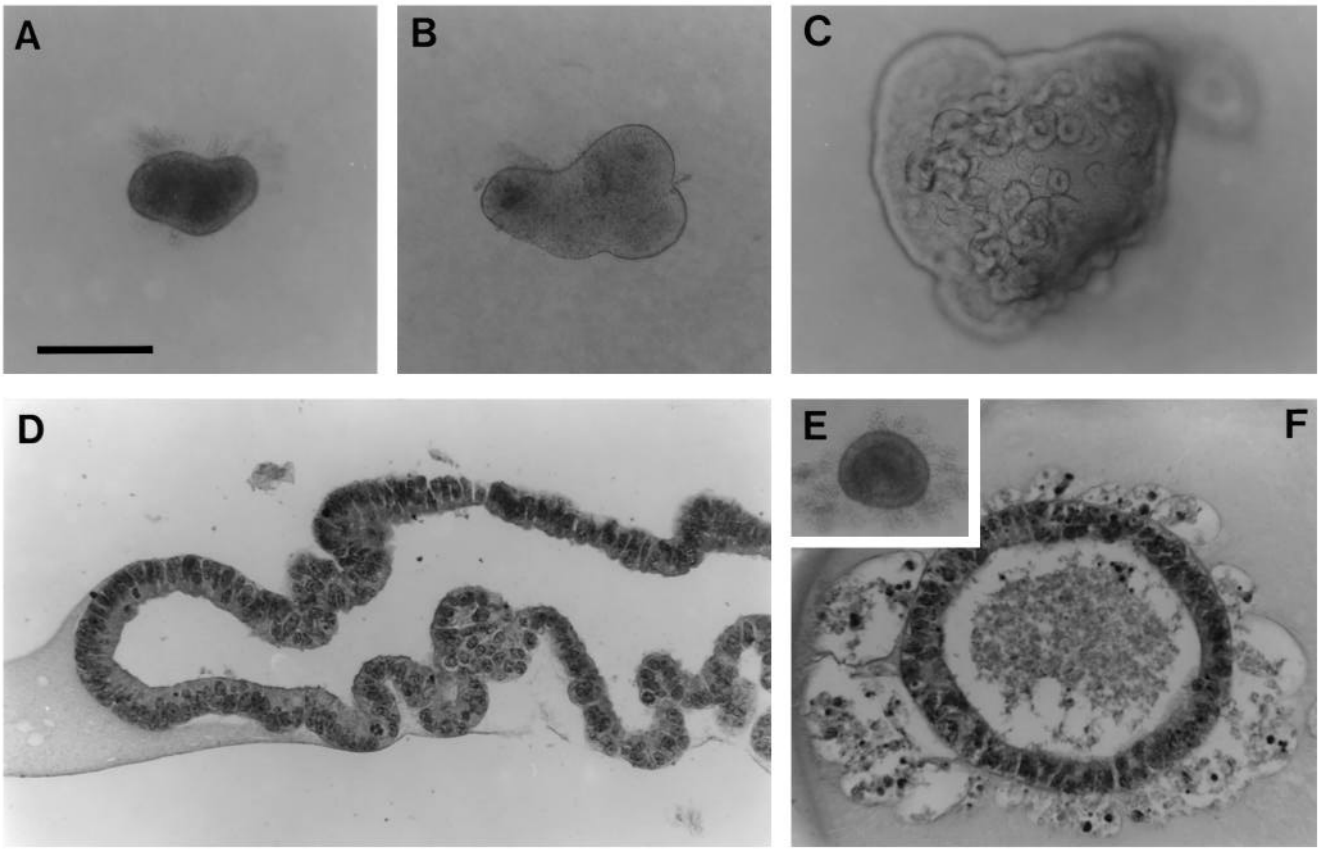

Fig. 3. Morphogenesis of lung epithelium cultured for 2 days within Matrigel in medium containing bFGF or EGF. (A) bFGF 10 ng/ml. (B) bFGF 100 ng/ml. (C) bFGF 1000 ng/ml, and (D) transverse section of C; many short buds are observed. (E) EGF 100 ng/ml, and (F) paradermal section of E; necrotic cells are present both inside and outside the small and smooth epithelial ball. Bar, 200 μm for A-C,E; and 50 μm for D,F.

large spherical ball with many short buds, which projected outwardly and inwardly (Fig. 3C,D).

The epithelium covered with Matrigel was also cultured in medium containing EGF (1, 10 or 100 ng/ml) for 2 days. EGF neither stimulated epithelial cell growth nor kept epithelial cells alive in any of the cases, and thus the epithelium became a smaller and smoother ball than that at the start of culture (Fig. 3E,F).

Morphogenesis in a collagen gel in the presence of aFGF

To examine whether Matrigel was essential to the branching morphogenesis of lung epithelium, we covered the lung epithelium with a collagen gel instead of Matrigel and cultured it for 2 days. In the absence of aFGF, most cells, dying, occupied the lumen and the outlying layer, while minor living cells remained between them but did not construct a simple columnar cell layer (Fig. 4A,B). In the presence of aFGF at 500 ng/ml, the epithelium retained its initial form well, neither newly branching nor becoming a smooth ball (Fig. 4C; compare with Fig. 1A). Histological sections showed a simple columnar cell layer surrounding a lumen (Fig. 4D). These results suggest that the lung epithelium was morphogenetically inactive within a collagen gel even in the presence of aFGF.

Blocking of morphogenesis by anti-aFGF

It was examined whether neutralizing antibody against aFGF abolished the branch-inducing activity of aFGF. When lung epithelium was covered with Matrigel and cultured in medium containing both aFGF (500 ng/ml) and anti-aFGF (100 μg/ml), epithelial growth and branching morphogenesis was completely inhibited in all 13 cases tested in contrast to the control epithelium (Fig. 5A,B). In order to check the non-specific toxicity of anti-aFGF used, we cultured the epithelium covered with Matrigel in medium containing anti-aFGF (100 μg/ml) and bFGF (500 ng/ml). The epithelium showed growth, though no branching, in response to bFGF in the presence of anti-aFGF, which was similar to the control epithelium cultured in the absence of anti-aFGF (Fig. 5C,D). These results suggest that aFGF had a crucial role in branching morphogenesis of lung epithelium in Matrigel.

DISCUSSION

The present study showed that embryonic mouse lung epithelium underwent branching morphogenesis alone when cultured, covered with Matrigel, in medium containing aFGF. We previously reported that embryonic mouse salivary epithelium displayed branching morphogenesis by itself when cultured, covered with Matrigel, in medium containing EGF (Nogawa and Takahashi, 1991). Both of these results demonstrate that branching morphogenesis of an embryonic epi-

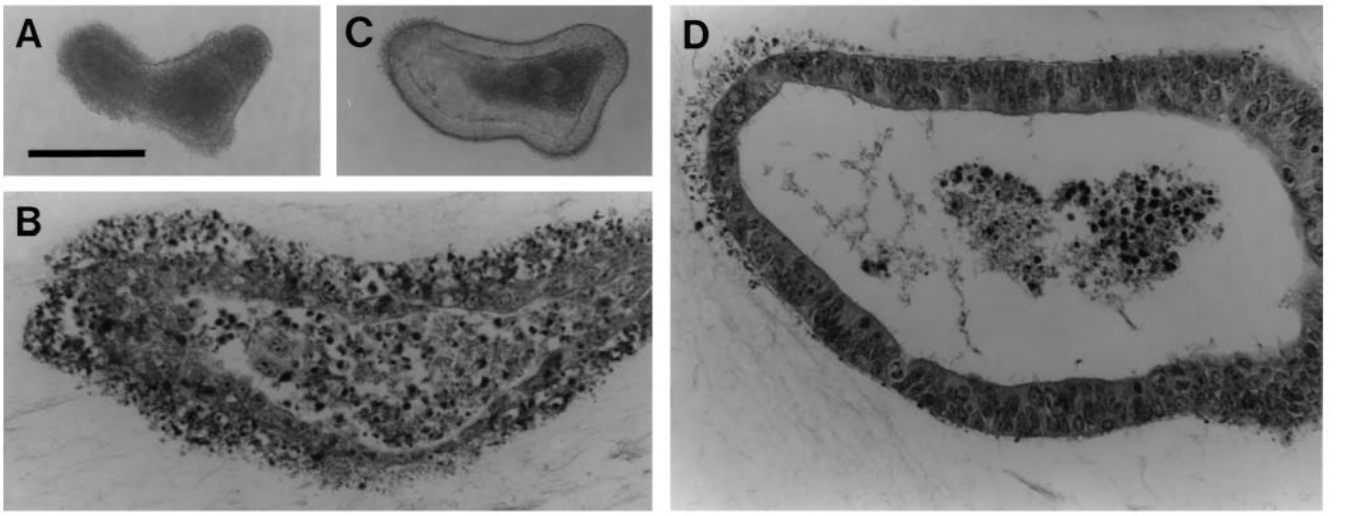

Fig. 4. Morphogenesis of lung epithelium cultured for 2 days within a collagen gel in medium containing aFGF. (A) 0 ng/ml as control. (B) Paradermal section of A; the simple columnar epithelium is absent, and most cells are necrotic. (C) 500 ng/ml; the initial shape is well maintained (compare with Fig. 1A). (D) Paradermal section of C; a simple columnar epithelium lines a lumen, but a small number of necrotic cells are present in the lumen and the periphery. Bar, 200 μm for A, C; and 50 μm for B,D.

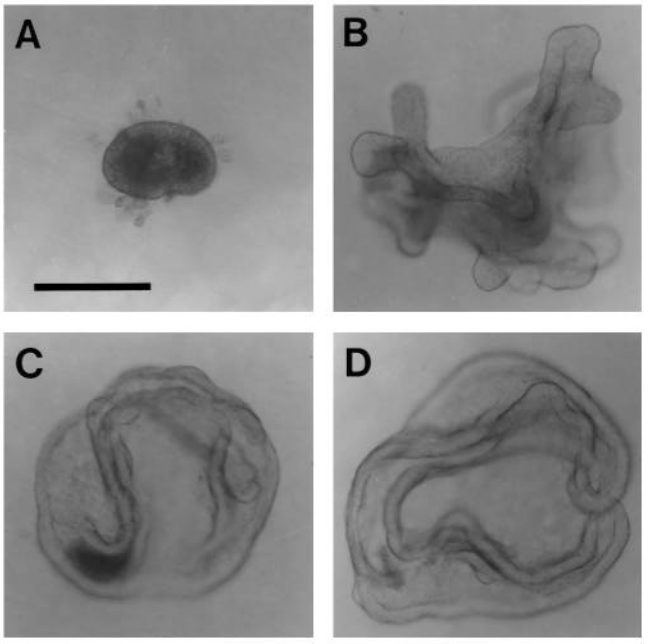

Fig. 5. Effects of anti-aFGF (100 μg/ml) on morphogenesis of lung epithelium cultured for 2 days within Matrigel in medium containing aFGF (500 ng/ml) or bFGF (500 ng/ml). (A) aFGF plus anti-aFGF; epithelial growth and branching morphogenesis are completely inhibited. (B) aFGF plus non-specific antibody (100 μg/ml) as control of A. (C) bFGF plus anti-aFGF; epithelial growth induced by bFGF is not inhibited by anti-aFGF. (D) bFGF plus non-specific antibody (100 μg/ml) as control of C; epithelial growth without acceleration of branching is induced by bFGF. Bar, 200 μm.

thelium can be induced by a combination of a basement membrane matrix and a diffusible growth factor.

Embryonic lung epithelium undergoes branching morphogenesis, forming a lumen with a simple columnar cell layer in normal development. A lumen surrounded by a simple columnar cell layer was well reproduced when the epithelium was cultured under the conditions of Matrigel plus aFGF or bFGF and of a collagen gel plus aFGF. However, the epithelium within Matrigel developed a larger lumen and was less branched at 1000 ng/ml aFGF than at 500 ng/ml, and heparin, in cooperation with aFGF, accelerated the lumimal expansion of the epithelium without enhanced branching. These results suggest that the lumen formation and the branching are different events in the morphogenesis of lung epithelium. When a ball with a simple columnar cell layer grows, the increase in its surface area may lead to two forms. One is a uneven form with branching, and the other is a smooth ball with a large lumen. A third, intermediate form may also be possible. Since the excessive expansion of a lumen smooths a branching, uneven cell layer, the expansion of a lumen works rather as a negative factor to branching morphogenesis. Hirai et al. (1992) reported that the molecule ‘epimorphin’ played a central role in epithelial-mesenchymal interactions of lung epithelial morphogenesis: they used the term ‘tubular morphogenesis’ for the lung epithelial morphogenesis influenced by epimorphin. Since ‘tubular morphogenesis’ corresponds to the construction of a simple columnar cell layer with a lumen, epimorphin seems to be the molecule that plays a crucial role in lumen formation of lung epithelium and probably not in branching morphogenesis.

It is known that aFGF and bFGF have similar effects on cell morphology, proliferation and differentiation in some cases and different effects in other cases, according to the type of cell (Gospodarowicz et al., 1986; Burgess and Maciag, 1989). The present study showed that bFGF reduced necrosis of the epithelium within Matrigel and enabled it to form a lumen as did aFGF, but induced many short buds instead of branching of the epithelium, in contrast to aFGF. Comparative study of them might give a good clue to the mechanisms controlling the process from budding to branching.

EGF was able neither to rescue necrosis nor to induce branching of lung epithelium within Matrigel at 1, 10, and 100

ng/ml. Our previous report revealed that EGF induced branching of salivary epithelium within Matrigel at 10 ng/ml (Nogawa and Takahashi, 1991). These results suggest that lung and salivary epithelia have a common nature of branching, but are specific in their responses to growth factors. The recombination experiments of epithelium and mesenchyme between lung and salivary rudiments showed that each mesenchyme supported branching of the other's epithelium (Lawson, 1974, 1983). It is probable that the mesenchyme may give various types of growth factors to the epithelium and that the epithelium utilizes only a few of them; i.e., both EGF and aFGF might be secreted by salivary and lung mesenchyme, and the salivary epithelium might utilize EGF whereas the lung epithelium might utilize aFGF. Warburton et al. (1992) and Seth et al. (1993) reported that branching morphogenesis of lung epithelium was stimulated by EGF and inhibited by antisense oligodeoxynucleotide against EGF in an organ culture system where the epithelium was surrounded by the mesenchyme, which contradicts the present result that EGF had no effect on morphogenesis of lung epithelium cultured within Matrigel. There are two possible explanations for this. One is that the epithelium within Matrigel was somewhat different from that within the mesenchyme, and receptors for EGF on the cell surface abnormally worked in the former situation. Another is that EGF directly stimulated some activity of the mesenchyme and thereby indirectly exerted its effect on the epithelium. Which answer is the case remains to be resolved.

The epithelial-mesenchymal recombination experiments between branching bronchial region and non-branching tracheal region of lung rudiments showed that the tracheal mesenchyme could not support branching of the bronchial epithelium but that the bronchial mesenchyme could induce the tracheal epithelium to branch (Alescio and Cassini, 1962). Further study showed that collagen fibrils were abundant at the epithelial-mesenchymal interface of the tracheal region, suggesting that collagen fibrils may have the ability to regulate the morphogenetic activity of lung epithelium (Wessells, 1970). This possibility is supported by our findings that the lung epithelium was morphogenetically inactive in a collagen gel, while active in Matrigel, in the presence of aFGF (Figs 1G, 4C).

The distribution of aFGF in developing whole embryos has already been investigated, and there were descriptions about lung rudiments: aFGF was immunohistochemically positive in the cytoplasm and the extracellular matrix of the mesenchymal tissue but negative in the epithelium of embryonic rat lung (Fu et al., 1991). As to receptors for aFGF, Orr-Urtreger et al. (1991), Stark et al. (1991) and Peters et al. (1992, 1993) investigated the expression of FGF receptor genes (FGFRs 1-4) in developing mouse embryos, among which FGFR-2 was specifically expressed in lung epithelium (Peters et al., 1992). Recently, Peters et al. (1994) reported that branching morphogenesis of lung epithelium was completely blocked in transgenic mice with targeted expression of a dominant negative FGFR2 exclusively to lung bud epithelium. This result and our result complement each other since these workers used a deleted cDNA of FGFR2-IIIb, a splice variant of FGFR-2, having high affinity to aFGF and keratinocyte growth factor.

Matrigel is composed of laminin, type IV collagen, heparan sulphate proteoglycan, and other minor components of basement membrane matrices, but it was recently reported that various growth factors are also present in trace amounts: TGFβ, EGF, IGF1, bFGF, and PDGF (Vukicevic et al., 1992). It must therefore be clarified in a future study which substances in Matrigel have a crucial role in the present mesenchyme-free culture system, and how such substances and aFGF work in epithelial branching morphogenesis of normal lung development. The present mesenchyme-free culture system in which Matrigel and aFGF can substitute for the mesenchyme may be useful for studying the mechanisms of branching morphogenesis of lung epithelium since it will avoid the need to consider possible additional activities of mesenchymal cells.

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