

## Histological and Immunohistochemical Analysis of Fetal Hypoplastic Lungs: Preliminary Study

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To investigate cellular growth and differentiation of the airway epithelium of the human fetal lung using immunohistochemistry, with a particular focus on cases of pulmonary hypoplasia. A total of 25 autopsy cases of stillbirth and early neonatal death were examined at Nagasaki University Hospital from 1986 to 1997. Using immunohistochemistry, we determined the expression of a variety of growth factors (EGFR, HGFR, GRP and SAPA) in lung tissues with or without pulmonary hypoplasia. A significant decrease in radial alveolar count was detected in hypoplastic lungs compared with normal lungs. The expressions of EGFR, HGFR and GRP in tissues from hypoplastic lungs were significantly lower than in tissues from normal lungs, but the expression of SAPA was not different between the two groups. Our results indicated that significant abnormalities of cellular growth and differentiation are present in pulmonary hypoplasia. However, lung maturation in pulmonary hypoplasia was not significantly different to that in normal lungs.

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**Key Words:** immunohistochemistry, pulmonary hypoplasia, growth factor, GRP, surfactant apoproteins

### Introduction

Fetal pulmonary hypoplasia is a condition characterized by a decrease in the number of airway epithelial cells, airways, and alveoli with a resulting decrease in organ size and weight. The condition usually develops as a complication of prolonged severe oligohydramnios caused by bilateral renal agenesis, infantile polycystic

kidneys, posterior urethral valve or prolonged leakage of amniotic fluid. Pulmonary hypoplasia can be potentially fatal because it is associated with an inadequate surface area for gas exchange. Cases of pulmonary hypoplasia defined as low lung weight to body weight ratio<sup>1)</sup> account for about 10% of all autopsy cases of stillbirth and neonatal diseases<sup>2)</sup>. Therefore, it is important to clarify the pathogenic mechanisms of pulmonary hypoplasia.

Fetal lung growth following tracheal obstruction, once believed to result in overdistension and hypertrophy of the lungs, seems to represent true lung hyperplasia. However, recently, Papadakis *et al.*<sup>3)</sup> reported that replacement of tracheal fluid with saline inhibits lung hypertrophy seen after tracheal ligation. Indeed, lung weight after ligation and saline replacement was not significantly different from that of unobstructed control. This indicates that tracheal fluid composition, rather than intratracheal pressure, is critical in the development of pulmonary hypoplasia. DiFiore and Wilson<sup>4)</sup> have previously shown that lung fluid after tracheal ligation is mitogenic to pneumocytes *in vitro*. Although cell proliferation and lung fluid production is likely to be mediated by one or more growth factors<sup>5,6)</sup>, their study did not establish whether humoral factors are primary or secondary effectors. Elevated intratracheal pressure and pulmonary stretch could induce such increase in growth factor concentration.

The present study focused mainly on cellular differentiation of the airway epithelium of human lung using immunohistochemistry, with a particular emphasis on cases of pulmonary hypoplasia. Pulmonary hypoplasia was studied to determine whether structural development of the lung is essential for epithelial cell differentiation, since pulmonary hypoplasia is associated with in the number of bronchial branches and/or radial alveolar count (RAC)<sup>1)</sup>.

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## Materials and Methods

In the cases of stillbirth and early neonatal death were autopsied at Nagasaki University Hospital from 1986 to 1997, excluding those cases that were not suitable for the study (cases with severe pneumonia, hemorrhage, fibrosis, or hyaline membrane disease), lung tissues from 25 cases (pulmonary hypoplasia; 15 cases, normal lungs; 10 cases) were prepared for morphological examination as well as immunohistochemical staining.

For assessment of lung hypoplasia, the condition was defined in this study as a lung weight to body weight ratio of less than 0.012 at > 28 weeks of gestation or less than 0.015 at < 28 weeks of gestation, using the criteria defined by Askenazi and Perlman<sup>1)</sup>. At autopsy, lungs were prepared by transbronchial fixation with neutral formalin. The paraffin-embedded left or right lung was cut into 3  $\mu$ m-thick sections. For histological examination, tissues were stained with hematoxylin-eosin (HE). RAC was determined by the method of Emery and Mithal<sup>2)</sup>. Ten to 20 counts were performed in each case.

### Immunohistochemistry

Immunohistochemical studies were performed by enzyme histochemistry using indirect avidin-biotin complex (ABC) method on formalin-fixed paraffin-embedded sections. Briefly, deparaffinized sections were incubated in 3% H<sub>2</sub>O<sub>2</sub> in phosphate-buffered saline (PBS). After incubation with normal goat serum for mouse monoclonal antibody (MONO) or horse serum for rabbit polyclonal antibody (POLY), sections were incubated overnight at 4°C with various antibodies. Antibodies used included rabbit anti-human epidermal growth factor receptor (EGFR, NCL-EGFR, Novocastra, Newcastle Upon Tyne, UK), mouse anti-human hepatocyte growth factor (HGF, NCL-c-MET, Novocastra), monoclonal mouse anti-human surfactant apoprotein A (SAPA, DAKO, Japan) and rabbit anti-human Gastric-Releasing Peptide (GRP, DAKO). As negative control, non-specific mouse IgG for MONO or rabbit IgG for POLY was used instead of each antibody. All sections were incubated with biotinylated goat anti-mouse IgG for MONO or biotinylated horse anti-rabbit IgG for POLY (both from Vector Laboratories Inc., Burlingame, CA) and then with a solution of avidin D-biotinylated horseradish peroxidase (Vector Laboratories, Inc.). Peroxidase activity was examined using 3,3'-diaminobenzidine tetrahydrochloride (Katayama Chemicals Co., Osaka, Japan) in PBS, containing 0.01% H<sub>2</sub>O<sub>2</sub>. Sections were counterstained with hematoxylin.

For evaluation of the results, immunostained sections were scored into one of three groups according to the frequency and intensity of positively stained epithelial cells. Frequency was defined as - and +, when the number of positive cells in the normal bronchus or alveoli in the section was <10% and >10%, respectively. Intensity was defined as + when staining was intense. Scoring of tissue sections was performed by three investigators who reached an agreement on the areas to be analyzed.

Statistical analysis was performed using StatView software (version 4.5, Abacus Concept, Inc, Berkeley, CA). All data were expressed as mean  $\pm$  SD. Differences between continuous data were examined for statistical significance using unpaired *t*-test. Categorical data were analyzed with the Fisher exact test or chi-square test as appropriate. A *P* value less than 0.05 denoted the presence of a statistically significant difference.

## Results

### Clinical Characteristics

Ten cases were assessed to be associated with normal lung growth, while 15 cases were labeled as pulmonary hypoplasia. Fetuses of the latter group were more mature than those of the former group, although the mean gestational age of the two groups was not significantly different (Table 1). The causes of stillbirth and neonatal death were respiratory insufficiency in cases with pulmonary hypoplasia and multiple anomalies due to trisomy-18 in cases without pulmonary hypoplasia.

### Expression of EGFR

Staining for EGFR was detected in the basement membrane of epithelial cells of bronchioles (Figure 1). Histopathologic and immunohistochemical examination of lungs with pulmonary hypoplasia showed a significant decrease in RAC and expression of EGFR relative to those in normal lungs (Tables 1 and 2).

### Expression of HGFR(c-met)

Staining for HGFR was detected in the cytoplasm of epithelial cells of bronchioles (Figure 2). Immunohistochemical examination of lungs with pulmonary hypoplasia showed a significantly low expression of HGFR relative to that in normal lungs (Table 2).

**Table 1.** Clinical characteristics of the two groups

	Pulmonary hypoplasia	Normal lungs	p
n	15	10	
Gestational age at delivery(wk)	35.0±4.3	30.0±2.4	NS
Body weight at birth(g)	1810±565	1224±113	<0.05
Lung weight/body weight ratio	0.008±0.002	0.022±0.004	<0.01
Radial alveolar count	1.77±0.17	2.96±0.37	<0.01

Data are mean ± SD. NS: not significant.



**Figure 1.** *Top:* A representative case of pulmonary hypoplasia. Note the expression of EGFR in the bronchiole. The intensity of staining was judged as +. *Bottom:* A representative case without pulmonary hypoplasia. Note the lack of EGFR expression. (Immunoperoxidase and hematoxylin, magnification × 200).



**Figure 2.** *Top:* A representative case of pulmonary hypoplasia. Note the expression of HGF-R in the bronchiole. The intensity of staining was judged as +. *Bottom:* A representative case without pulmonary hypoplasia. Note the lack of HGF-R expression. (Immunoperoxidase and hematoxylin, magnification × 200).

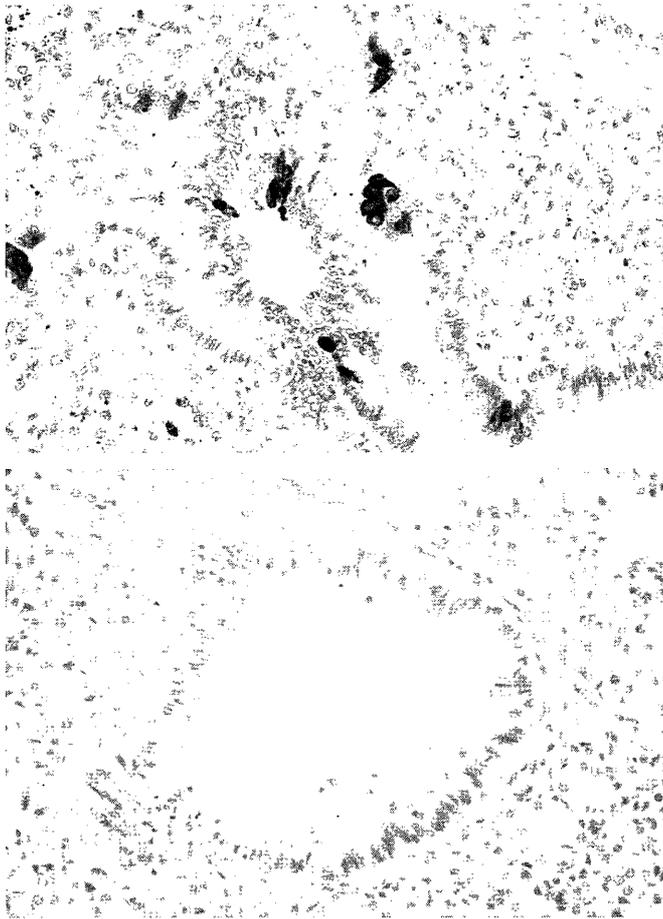
#### Expression of GRP

GRP-containing cells formed corpuscular apparatuses either in bronchi lacking cartilage or in bronchioles, although some GRP-containing cells were present as single cells (Figure 3). Morphologically, the corpuscles were round, elongated, or irregular in shape and consisted of four or more GRP-containing cells. Characteristically, corpuscle cells protruded into the subepithelial connective tissue. The unique histologic features of

these corpuscles resembled those of “neuroepithelial bodies” described by Lauweryns *et al.*<sup>8)</sup>. Tissues from hypoplastic lungs showed a significant decrease in the expression of GRP relative to that in normal lungs (Table 2).

#### Expression of SAPA

SAPA positive cells in alveoli stained as strong as those in the bronchial epithelium (Figure 4). The



**Figure 3.** *Top:* A representative case of pulmonary hypoplasia. Note the expression of GRP in the bronchiole. The intensity of staining was judged as +. *Bottom:* A representative case without pulmonary hypoplasia. Note the lack of GRP expression. (Immunoperoxidase and hematoxylin, magnification \_\_ 200).



**Figure 4.** *Top:* A representative case of pulmonary hypoplasia. Note the expression of SAPA in the alveoli. The intensity of staining was judged as +. *Bottom:* A representative case without pulmonary hypoplasia. Note the lack of SAPA expression. (Immunoperoxidase and hematoxylin, magnification \_\_ 200).

**Table 2.** Expression of each factors in hypoplastic and normal lungs.

	Pulmonary hypoplasia (%)	Normal lungs (%)	p*
n	15	10	
EGF-R			
Intensity	4(27)	8( 80)	<0.05
Frequency	1( 7)	7( 70)	<0.01
HGF-R			
Intensity	7(47)	10(100)	<0.05
Frequency	3(20)	10(100)	<0.01
GRP			
Intensity	12(80)	10(100)	NS
Frequency	8(53)	10(100)	<0.05
SAPA			
Intensity	7(47)	8( 80)	NS
Frequency	5(33)	6( 60)	NS

Data are number of cases while numbers in parentheses represent the percentage of cases.

\*Fisher exact and  $\chi^2$  tests

NS: not significant.

expression of SAPA in tissues from hypoplastic lungs tended to be lower than that in normal lungs, although the difference was not statistically significant (Table 2).

## Discussion

Epidermal growth factor (EGF) is a biologically active polypeptide first described in 1962 by Cohen<sup>9)</sup>. The biologic effects of EGF are primarily those of generalized epithelial growth and keratinization. In 1975, EGF was considered as a growth hormone capable of stimulating the growth of epithelial cells in fetal<sup>10)</sup>. It was shown that constant infusion of EGF into fetal lamb for 3-5 days stimulated epithelial growth in many sites, including upper and lower airways. In addition, EGF appeared to protect the hyaline membrane against the development of hyaline membrane disease when administered in utero at 123-130 days of gestation<sup>11)</sup>, suggesting that EGF stimulated cell differentiation in addition to promoting cell growth.

GRP, first extracted from porcine gastric mucosa by McDonald *et al*<sup>12)</sup> in 1978, consists of 27 amino acid residues, and its biologically active C-terminal tetradecapeptide amide possesses a very similar amino acid sequence to amphibian bombesin<sup>13)</sup>. GRP has now been added to a growing list of brain-gut peptides. The biologic actions of GRP, which are similar to those of bombesin and have only been partially characterized<sup>14)</sup>, include stimulation of secretion of gastrin, insulin, pancreatic polypeptide, and enteroglucagon; as well as contraction of smooth muscles of blood vessels and gallbladder; and stimulation of pancreatic enzyme secretion. Bombesin also stimulates bronchial contraction<sup>15)</sup>.

To our knowledge, the influence of these factors on fetal pulmonary growth has not been previously reported. Therefore, in the present study, we focused on cellular growth and differentiation of the airway epithelium of human fetal lung using immunohistochemistry, focusing particularly on cases of pulmonary hypoplasia. Our results showed that in spite of advanced gestational age, tissues from hypoplastic lungs showed significantly low expressions of EGFR, HGFR and GRP relative to those in lungs without hypoplasia. These results suggest that intrapulmonary epithelial cell differentiation is closely related to the formation of lung structure.

Endo and Oka<sup>16)</sup> reported the results of histological and immunohistochemical analysis of lung development in lung hypoplasia using antibody for epithelial membrane antigen, keratin, Leu-7, Ca 19-9, secretory component and SAPA. In their study, two of six cases

with lung hypoplasia showed deviation from normal in the pattern of immunohistochemical staining, which was probably related to abnormal cellular differentiation, similar to the results reported in our study. However, in the same study, four of their cases with lung hypoplasia showed a normal or near-normal immunohistochemical staining.

In conclusion, we demonstrated in the present study that pulmonary hypoplasia was associated with abnormal cellular growth and differentiation as demonstrated by low expression of a variety of growth factors. However, lung maturation in pulmonary hypoplasia was not significantly different to that in normal lungs. Because the conclusions of this study this study are weakened by the small sample size and few antibody for immunohistochemical staining, more studies are needed to explain the abnormal cellular growth and differentiation in pulmonary hypoplasia.

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