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Effect of Insulin Like Growth Factors on the Development of Hypoplastic Lungs in Connexin 43 Knockout Mice

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Abstract

Aim: The prognosis of patients with hypoplastic lungs is very severe. Since the postnatal treatment is not effective for improving the hypoplastic lungs, prenatal treatment is thought to be required. Insulin-like-growth factors（IGFs）are known to accelerate the maturation of the fetal lungs. Therefore, we investigate whether the administration of IGFs can improve fetal hypoplastic lungs in Cx43 knockout mice.

Method: Male and female heterozygous Cx43 mice（$Cx43^{+/-}$）were mated overnight and the day that the vaginal plug was confirmed was designated as embryonic day 0. Fetuses were obtained by cesarean section on the embryonic day 17, and the fetal lungs were dissected out and divided into the following three groups: IGF-I group, IGF-II group, and Control group. These fetal lungs were incubated for 48 or 72 hours in three types of mediums supplemented with IGF-I, IGF-II, or in the absence of IGFs. The lungs from homozygous Cx43 fetuses（$Cx43^{-/-}$）were investigated by immunohistochemistry and for the mRNA expression of T1 alpha protein, surfactant protein-C, and alpha smooth muscle actin.

Result: Both the number of positive cells and the mRNA expression of all markers were observed to increase in the IGF-I group compared to control group, although there was no significance between the IGF-II and control group. In addition, there were no significant differences between the IGF-I and IGF-II groups.

Conclusions: Based on our result, the administration of IGF-I to the fetus during the late gestational period might be effective for improving the severe hypoplastic lungs.

Key words: Connnexin43, IGFs, lung development, CDH

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Introduction

The induction for prenatal maturation of hypoplastic lung in fetal period has been considered to be one of definite therapies for improving the hypoplastic lungs. Some growth factors are known to play important roles associated with fetal lung development in the process of proliferation, migration, and differentiation of embryo cells. In some growth factors, insulin like growth factors (IGFs) has also been known as one of accelerating factors for the maturation of hypoplastic lung in the fetal period .[1-4] The mRNA of IGF-I is expressed in the mesenchymal tissue of the lung and the mRNA of IGF-II is expressed in the epithelial cell of the lung. These IGFs are thought to affect both proliferation and differentiation of the lung. In addition, prenatal glucocorticoid therapy has been suggested to induce the maturation of the lung through the IGF system.[5] We have previously observed the differentiation of the mouse fetal lung and the expression of IGFs and their receptors were increased in the late stage of fetal period.[6, 7]

Connexin43 (Cx43), which is encoded by the gene Gja1, is one of the major gap junction proteins which compose the gap junction channel.[8-11] Cx43 is a main gap junction protein in the heart and expressed in other organs such as brain, liver, skin and so on. Cx43 is also expressed in the type I and type II epithelial cells of lung and is thought to have the physiological function in the development of fetal lung. In addition, Cx43 knockout mice die at birth, as a result of a failure in pulmonary gas exchange.[11] Therefore, we previously investigated fetal lungs of Cx43 knockout mice and compared to those of wild type mice and confirmed that the fetal lung of Cx43 knockout mice had severe hypoplastic lung.[12]

Based on our previous results, we investigated Cx43 knockout mice as an animal model of hypoplastic lung to determine whether the maturation of fetal lungs could be induced by administration of IGFs as growth factors.

Materials and Methods

Experimental animals

Cx43 heterozygous mice were maintained in appropriate cages under controlled conditions and fed with commercial solid food. Male and female heterozygous were mated overnight and checked daily for vaginal plug. The day the vaginal plug was confirmed was designated as embryonic day 0. Fetuses were harvested by cesarean section on the embryonic day 17, and the fetal lungs were excised and randomly assigned to three experimental groups: IGF-I, IGF-II, and Control group. These lungs were incubated for 48 or 72 hours in three types of mediums supplemented with IGF-I (IGF-I group), IGF-II (IGF-II group), or without IGFs (Control group). After the beginning of the lung cultures, the genotypes of fetuses were determined by polymerase chain reaction (PCR) analysis of tail DNA. Although interbreeding of heterozygous Cx43 mice can provide wild-type, heterozygous, and homozygous Cx43 knockout mice, the only lung samples of fetuses detected as homozygous Cx43 were collected for analysis. The random lung samples from each group were fixed overnight in 4% paraformaldehyde at 4℃ and embedded in paraffin for H&E staining and immunohistochemistry, whereas other samples were immersed for RNA sampling (Qiagen, Hieden, Germany) and stored for total RNA extraction and PCR analysis.

This animal experiment was conducted in compliance with the "Guidelines for the care and use of laboratory animals" established by both Fukuoka University and Kyushu University.

Fetal lung cultures

The fetal lungs harvested from embryonic day 17 mice were placed on polyethylene terephthalate filters (Falcon 3093 cell-culture insert: Becton Dickinson and Company, Franklin Lakes, USA: 8 μm pore size) and incubated in a 6-well culture plates (Falcon 3502 multiwell 6 well: Becton Dickinson and Company, Franklin Lakes, USA) with following three types of mediums in each well: Control group; the chemically defined BGjb medium (Gibco, Grand Island, NY, USA) supplemented with penicillin-streptomycin (Gibco: 100 U and 100 mg/ml), L-ascorbic acid (NACALAI TESQUE Inc., Kyoto, Japan: 150 μg/ml) and transferrin (SIGMA, ST.Louis, USA: 50 μg/ml), IGF-I group; 500 ng/ml of recombinant mouse IGF-I (TECHNE Cor., Minneapolis, USA) was added to the medium in Control group, IGF-II group; 500ng/ml of recombinant mouse IGF-II (TECHNE Cor., Minneapolis, USA) was added to the medium in Control group. The fetal lung explants were incubated in a 5% CO_2 incubator at 37℃ for 48hr and 72hr, and the medium was replaced every 24hr.

Total RNA extraction and reverse transcriptase PCR analysis

Total RNA was extracted from lung samples using

Table 1 The primers used for typing

Gene	Forward Primer	Reverse Primer
Cx43	5'-CCCCACCTATGTCTCC-3'	5'-ACTTTTGCCGCCTAGCTATCCC-3'
Neo	5'-GGCCACAGTCGATGAATCCAG-3'	5'-TATCCATCATGGCTGATGCAA-3'

Table 2 Real time PCR primers

Gene	Forward Primer	Reverse Primer
T1α	5'-CACCTCAGCAACCTCAGAC-3'	5'-ACAGGGCAAGTTGGAAGC-3'
Sp-C	5'-TCGTGGTTGTGGTGGTGGTC-3'	5'-TCCTCCTGGCCCAGCTTAGA-3'
αSMA	5'-GTGCTATGTCGCTCTGGACTTTGA-3'	5'-ATGAAAGATGGCTGGAAGAGGGTC-3'
β-actin	5'-CTGAGAGGGAAATCGTGCG-3'	5'-GGTGGTACCACCAGACAAC-3'

RNeasy Mini kit (Qiagen, Hieden, Germany) according to the manufacturer's instruction. Total RNA quantification was done by spectrophotometry (Gene Quant pro, Amersham Biosciences, UK). First-strand cDNA was synthesized at 42 ℃ for 90 minutes using an oligo $(dT)_{20}$ primer with Superscript III (Invitrogen, Carlsbad, California, USA). This was designed to convert 1μg of total RNA into 20μL of first-strand cDNA.

All cDNA samples were used for relative quantification by Real time PCR (LightCycler, Roche) Each run was performed with 10 min hot-start (95℃) and 35 cycles (95℃, 10sec; 60℃, 10sec; 72℃, 10sec). All PCR reactions were performed using the LightCycler Fast Start DNA Master SYBR Green I kit (Roche Applied Science, Manheim, Germany). The following protocol was used: 16μl of the LightCylcer Fast Start DNA Master SYBR Green I kit, 2μl of cDNA, and 10 pmol of each primer were added in a total volume of 20μl. All primers used are listed in Table 2. T1α is a differentiation gene abundantly expressed in the type I alveolar epithelial cells of lung. Therefore, T1α used as one of the first marker genes for the type I alveolar epithelial cells. SP-C is the only gene expressed in type II alveolar epithelial cells and used as a marker of type II alveolar epithelial cells. α SMA is well know to be a maker of smooth muscle and located in the smooth muscle of both artery and airway tract. In all samples, mRNA expression of podoplanin (T1α), surfactant protein-C, and α-smooth muscle protein (αSMA) was normalized for β-actin.

Immunohistochemistry

Lung tissue specimens from fetal mice were fixed in 4% paraformaldehyde at 4℃ for 24 h and embedded in paraffin. All paraffin sections were cut to 5μm by a microscope. Paraffin sections were dewaxed in xylen, rehydrated in ethanol and incubated in methanol with 3% H_2O_2 to block endogenous peroxidase. All specimens were boiled in 10mM citrate buffer for 15 min using a 500W microwave followed by cool down at room temperature for antigen retrieval. Sections were then incubated overnight with primary antibodies diluted with Dako REAL™ Antibody Diluent (Dako, Glostrup, Denmark) at 4℃. The following antibodies were used as primary antibody: T1α; hamster monoclonal anti-podoplanin antibody (Relia Tech, Wolfenbuttel, Germany: dilution 1:500), SP-C: rabbit polyclonal anti-SP-C antibody (Santa Cruze Biotechnology, Inc, CA, USA: dilution 1:50), and αSMA: rabbit polyclonal anti-αSMA antibody (Abcam Ltd, Cambridge, UK: dilution 1:2000). These sections were then washed in phosphate-buffered saline (PBS). The sections incubated with primary antibodies of SP-C and αSMA were incubated with labelled polymer of EnVision™ kit (Dako, Glostrup, Denmark) according to manufacturer's instructions. The sections incubated with T1α primary antibody were incubated with biotinylated goat anti-mouse immunoglobulin G antisera and then incubated with peroxidase-conjugated streptavidin. Immunohistochemical signals were visualized using 3,3'-di-aminobenzidine substrate.

Statistical analysis

For real-time PCR studies, all quantitative data are presented as mean±SD. The data were analyzed using Student's t test. A value of $P < 0.05$ was considered to be statistically significant.

Result

The number of fetal lungs which were cultured for 72 hours were 6 in the IGF-I group, 5 in the IGF-II group, and 5 in the Control, respectively.

Relative mRNA expression level of T1α, SP-C, and αSMA

Figure 1 showed the result of relative mRNA expression level of all markers, T1α, SP-C, and αSMA. The levels of T1α mRNA were 3.8 ± 0.5 in the IGF-I group, 3.1 ± 1.0 in the IGF-II group, and 2.0 ± 0.3 in the Control, respectively. The levels of SP-C mRNA were 0.019 ± 0.003 in the IGF-I group, 0.014 ± 0.003 in the IGF-II group, and 0.010 ± 0.002 in the Control, respectively. In addition, the levels of αSMA mRNA were 2.0 ± 0.5 in the IGF-I group, 1.9 ± 0.8 in the IGF-II group, and 0.9 ± 0.2 in the Control, respectively. An increase in the mRNA expression of all T1α, SP-C, and αSMA in both IGF-I and IGF-II groups was observed, compared to that in the Control. Especially, there was a significant difference in mRNA expression between IGF-I group and the Control regarding the mRNA expression of all T1α, SP-C, and αSMA. No significant difference was observed between IGF-I and IGF-II group in all investigated markers.

Immunohistochemical analysis of the lungs after 72 h of culture

The T1α protein was distributed on the surface of the type I alveolar epithelial cells in distal lung buds (Fig. 2). There was an increase in number of T1α positive cells in both IGF-I and IGF-II groups, compared to the Control.

The SP-C protein was localized in the type II alveolar epithelial cells in distal lung buds (Fig. 3). The SP-C positive cells showed a slight increase in number in both IGF-I and IGF-II groups, compared to the Control.

The αSMA was mainly distributed in the nuclei layers of both arteries and bronchi. The protein was also located in the mesenchymal cells of the alveolar septa. There was no significant difference in αSMA positive cell's distribution among three groups.

Discussion

We have focused on the effect of prenatal administration of insulin like growth factors (IGFs) on the development of hypoplastic lungs in the fetal period.

IGFs we used are polypeptides, having the structure similar to insulin, and have a close connection with the precursor of the insulin. IGFs consist of IGF-I and IGF-II and promote proliferation and differentiation of cells during embryogenesis through either paracrine or autocrine pathways.[13] IGFs, their receptors, and binding proteins, are expressed in the fetal lung of humans, rodents, and other species.[14] Some studies have revealed a significant role of both IGF-I and IGF-II in development of fetal lungs.[15), 16] In addition, both IGF-I and IGF-II mRNAs were expressed throughout the developmental period.[17] Regarding their localizations, IGF-I mRNA is expressed in mesenchymal cells, predominantly those areas surrounding the airway epithelium. In addition, IGF-I mRNA is not expressed in the vessels and smooth muscles.[18] In contrast, IGF-II mRNA is expressed in the undifferentiated mesenchyme of the distal lung buds in addition to connective tissue of the main airways, interlobular septa, pleura, and developing cartilage. Mice carrying a null mutation of the IGFs gene demonstrate the hypoplastic lung or growth retardation of lung. IGF-I

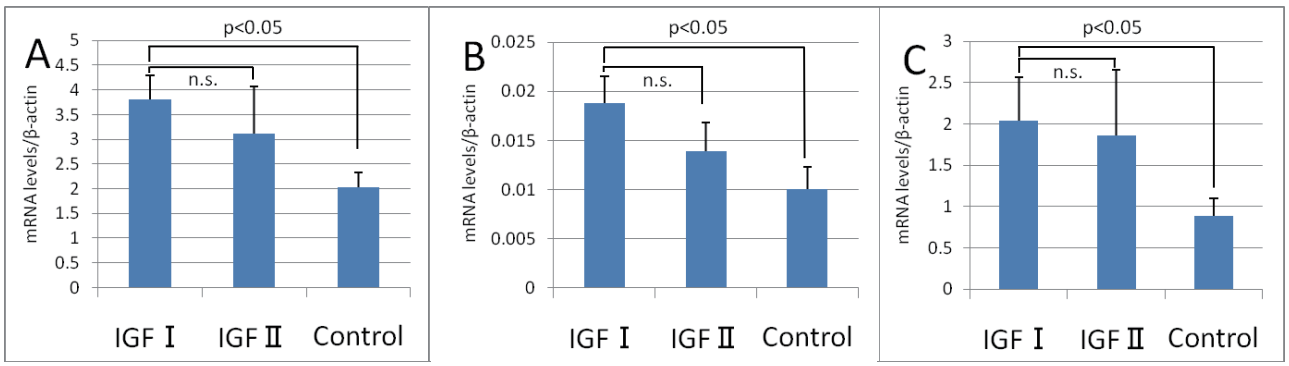

Fig. 1 Relative mRNA expression levels of T1α (A), SP-C (B), and, αSMA (C) in the fetal lung of Cx43 knockout mice at 72 h of culture. The expression levels of all T1α, SP-C, and αSMA were significantly increased in the IGF-I group, compared to the controls. No significant difference was observed between IGF-I and IGF-II group.

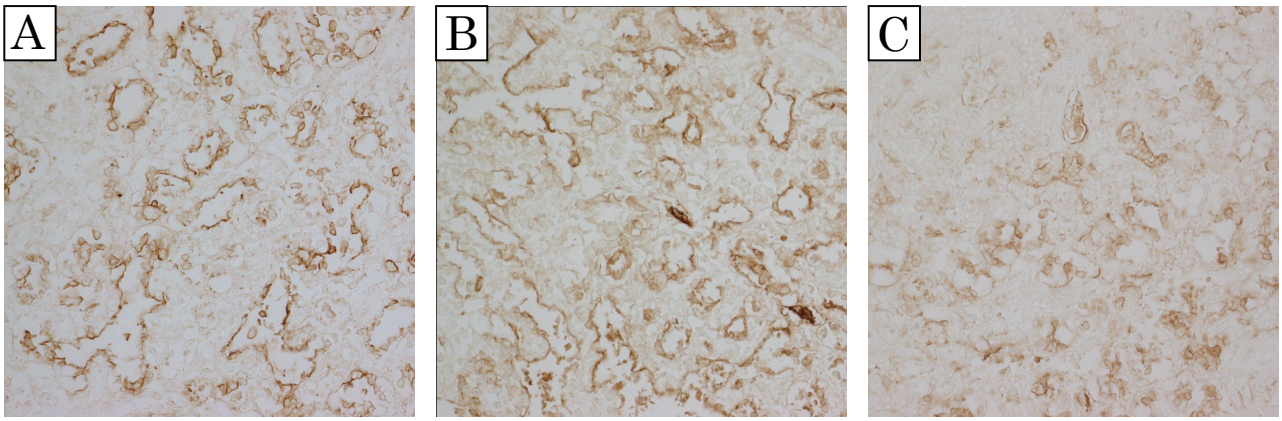

Fig. 2 The distribution of T1α positive cells.
Regarding the distribution of T1α positive cells, in both IGF-I（A）, IGF-II（B）groups, the mild increase of number of positive cells was observed, compared to that of the control（C）.

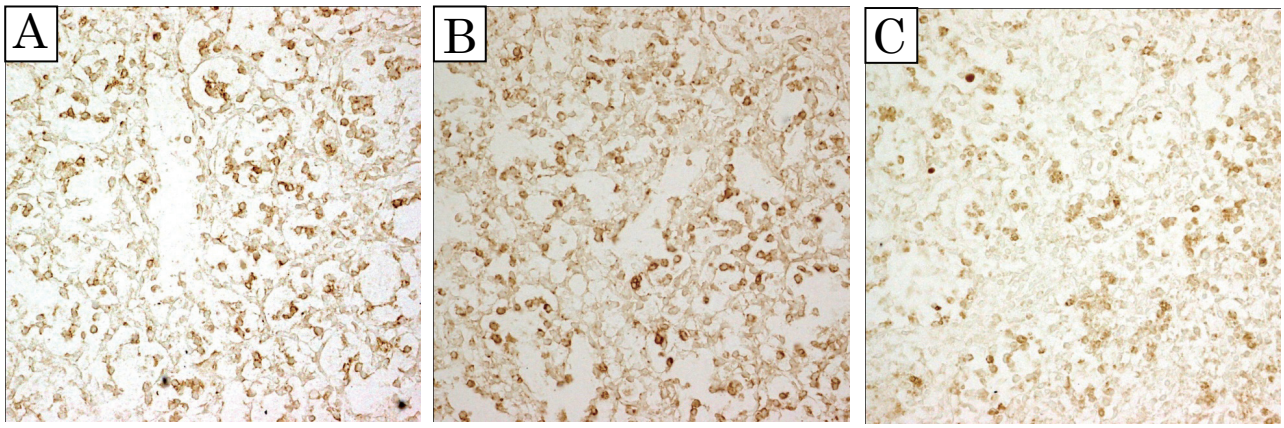

Fig. 3 The distribution of SP-C positive cells.
In both IGF-I（A）, IGF-II（B）groups, the mild increase of number of SP-C positive cells was observed, compared to that of the control（C）.

knockout mice（IGF-I $^{-/-}$）show a decrease of birth weight and their lungs show a retardant epithelial growth with a higher proportion of type II alveolar epithelial cells, less differentiated type I alveolar epithelial cells, and a failure of capillary remodeling, compared to the wild type mice.[15), 17)] On the other hand, IGF-II knockout mice fetuses had altered lung morphology during late gestation with a thicker alveolar septa and poorly organized alveoli, compared to the lungs of the wild type mice.[19)]

Cx43 is one of the major gap junction proteins encoded by the gene Gja1. The gap junctions consist of cell membrane channels.[12)] The gap junctional hemichannel, or connexon is composed of a hexameric array of connexin monomers.[20)] Two connexons from neighboring cells adhere in a mirrors symmetric fashion to form the intercellular membrane channels of gap junctions, which are aqueous conduits for direct passage of ions and small molecules up to 1.2 kDa between neighboring cells.[20-22)] The gap junctions have a significant role for many phases of embryonic development and patterning.[21), 22)] In developing lungs, various cells are assumed to need positional information before their developmental fate is determined. Gap junctions may be responsible for establishment and maintenance of positional information from the substances which pass through gap junction channels.[23)] Cx43 is expressed in many tissues, such as brain, intestine, skin, lungs, kidney, and limbs, throughout development. In lungs, Cx43 is reported to be expressed abundantly in distal lung buds.[24)] The investigation to development of mice lungs has revealed gradual increased expression of Cx43 mRNA in the late stage of gestation.[24)] Our previous study in which fetal lungs of both Cx43 knockout mice and wild type mice were investigated, Cx43 knockout mice showed significant hypoplastic lungs compared to wild type mice.[25)]

In our current study, hypoplastic lungs in Cx43

knockout mice showed a mild increase of number in positive cells of all markers in immunochemistry and an increase of mRNA expression of all markers in reverse transcriptase PCR analysis in IGFs groups, compared to those in the Control. Especially, a significant increase in all T1 α, SP-C, and α SMA mRNA expressions was found between IGF-I group and control group. This suggests that the IGF system, especially IGF-I, might induce the development of both types of alveolar cells and the differentiation of type II alveolar epithelial cell to type I alveolar epithelial cell in the hypoplastic lungs. However, in current study, the morphological change in the hypoplastic lungs was observed to be mild, although all types of mRNA expression in T1 α, SP-C, and α SMA were increased, thus suggesting that the protein production in all markers of investigated lungs by interposition of IGF-I might be not enough. Lallemand [18] investigated human fetal lungs and reported that expression of both IGF-I and IGF-II mRNA throughout gestation progressively decreased with gestation age. The similar tendency has been observed in studies with rodents used as an animal model. In our previous study, similarly to those studies, the expression of IGF-I and IGF-II mRNA peaked in the middle stage of gestation and decreased in the late stage while IGF-IR and IGF-IIR increased with gestation age. [6] Totally thinking with previous studies, the production of IGF-I and IGF-II proteins occur at an earlier stage than the stage occurring formation of both IGF-IR and IGF-IIR in the cell membrane during the fetal period, and combine with both the IGF-IR and IGF-IIR in the late stage, thus resulting in inducing the alveolar formation. Therefore, the mild increase of distribution in both type I and type II alveolar cells in our results might be related to the delay of the formation of both the IGF-IR and IGF-IIR of cell membrane in hypoplastic lungs we used. In addition, the reason for mild morphological change may also occur due to the shortage of tissue culturing time in our study. Further investigations, by extending the culturing time and the chronological change of protein and mRNA expression in both the IGF-IR and IGF-IIR in hypoplastic lungs of Cx43 knockout mice during the tissue culturing period, are considered to be necessary.

In conclusion, the current results suggest that IGFs, especially IGF-I, might induce the maturation of severe fetal hypoplastic lungs in Cx43 knockout mice in the late stage of gestation, although the increase of proteins in T1α, SP-C, αSMA was mild during the observation period. Therefore, the administration of IGF-I to the fetus during the late gestational period might be a possible effective treatment for improving the development of severe hypoplastic lungs.

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