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Connexin43 plays an important role in lung development

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Abstract

Objectives: Connexin43 (Cx43) is one of the proteins associated with gap junction. Connexin43 knockout mice die after birth owing to hypoplastic lungs. The purpose of this study was to analyze the hypoplastic lung of Cx43 knockout mice to clarify the role of the Cx43 during lung development.

Methods: Adult hetero Cx43 mice were mated. Newborn mice were divided into the following groups: wild, hetero, and knockout. Total RNA was extracted from the right lung, and the left lung was fixed for immunohistochemical staining. The mRNA expression of surfactant protein C, aquaporin-5, and α-smooth muscle actin were analyzed by reverse transcriptase polymerase chain reaction. H&E and immunohistochemical staining for those markers were performed.

Results: The mRNA expression of aquaporin-5, surfactant protein C, and α-smooth muscle actin was significantly lower in knockout mice than that in the wild and hetero mice. H&E staining in the knockout mice showed narrow airspaces and thicker interalveolar septae. Immunohistochemical staining in all markers showed the formation of alveoli to be delayed in the knockout mice.

Conclusion: Based on these findings, Cx43 is closely related to alveolar and vascular formation during lung development.

Connexin 43 (Cx43) is a major gap junction protein encoded by the gene *Gja1* [1]. Gap junctions contribute not only to cellular homeostasis, including ion exchange and volume control, but also to morphogenesis, proliferation, and differentiation of several organs [1-6] A recent study showed that Cx43 was associated with the normal development of several important organs such as the brain, heart, kidney, tooth, bone, gut, epidermis, and lung [3-6]. Representative feature of Cx43 knockout mice was pulmonary outflow obstruction and heart malformations [5]. However, there are few studies focusing on Cx43 in pulmonary development.

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A functional analysis of gap junctions shows that Cx43 is expressed between the alveolar epithelial cells (AECs) and proteins of the extracellular matrix or basement membrane that mediates the biologic lung functions [7,8]. In addition, gap junctions also coordinate vascular cell function by providing a pathway for movement of electric charge and small signaling molecules directly from one cell to another [9,10]. Taking the basic evidence into consideration, the Cx43 also may play an important role in lung development. Therefore, the lungs of Cx43 knockout mice were investigated to determine whether Cx43 plays a critical role in lung development.

1. Materials and methods

1.1. Experimental animals

Cx43 hetero mice were kindly provided by our collaborator, Dr Satoshi Fukumoto. Interbreeding of the heterozygous Cx43 knockout mice provided wild-type, heterozygous, and homozygous Cx43 knockout mice. The genotypes of newborn mice were determined by polymerase chain reaction (PCR) analysis of tail DNA. Polymerase chain reaction analysis using primers to the wild-type Cx43 gene and neo-insert in the Cx43 knockout allele allowed the identification of wild-type, heterozygous, and homozygous Cx43 knockout mice [5]. Tissue samples were collected immediately after birth. Lungs were dissected from the thoracic cavity and inflated with 4% paraformaldehyde under pressure (15 cm H_2O pressure) via the tracheal cannula of 25-gauge catheter. After inflation, the right lung was removed, stored at −70°C until RNA extraction was performed, and the left lung was fixed into the 4% paraformaldehyde for immunohistochemistry. This animal experiment was conducted in compliance with the "Guidelines for the care and use of laboratory animals" established by this university (H18-42).

1.2. Total RNA extraction and reverse transcriptase PCR analysis

Total RNA was extracted from lungs using TRIzol reagent (Invitrogen, Carlsbad, California, USA). First-strand cDNA was synthesized at 42°C for 90 minutes using an oligo$(dT)_{20}$ primer with Superscript III (Invitrogen). This was designed to convert 1 μg of total RNA into 20 μL of first-strand cDNA. Polymerase chain reaction amplification was performed using the primers listed in supplemental Table 1. The PCR products were separated on a 1.5% agarose gel. The relative gene expression level was deduced to standard curve constructed using the positive control sample and normalized against the expression level of hypoxanthine guanine phosphoribosyl transferase in each sample.

1.3. H&E staining and Immunohistochemistry

Lung tissue specimens from neonatal mice were fixed overnight in 4% paraformaldehyde at 4°C and embedded in paraffin. All paraffin sections were cut to 5 μm by a microtome. H&E staining was performed according to the standard procedure. For immunohistochemistry, paraffin sections (5 μm) were dehydrated in xylene for 10 minutes, rehydrated through ethanol for 10 minutes, and incubated for 20 minutes with 3% H_2O_2 in methanol to block endogenous peroxidase. All specimens were then pretreated in 10 mmol/L citrate buffer (pH 6.0) for 15 minutes using a microwave. The sections were then incubated with 10% normal goat serum for 20 minutes in a moist chamber and then were incubated overnight at 4°C with the primary antibody. Aquaorin-5 (AQP-5; anti–AQP-5 rabbit polyclonal antibody; Abcam Ltd, Cambridge, Mass, diluted in 1:200) was stained to clarify the expression patterns of the differentiated type I AECs. The lung specimens were stained with surfactant protein C (Sp-C; anti Sp-C rabbit polyclonal antibody, diluted in 1:100; Santa Cruz Biotechnology, Inc, Santa Cruz, Calif) to detect the differentiated type II AECs. In addition, α-smooth muscle actin (α-SMA; anti–α-SMA rabbit polyclonal antibody, diluted in 1:200; Abcam Ltd) was also stained to determine the smooth muscle cells in the fetal mice lung.

Next, the sections were incubated in biotinylated secondary antibody and then processed using peroxidase-conjugated streptavidin. Immunohistochemical signals were visualized using the 3,3′-diaminobenzidine substrate. Slides were counterstained with hematoxylin and examined and photographed by standard microscopy.

1.4. Statistical analysis

The data were analyzed using Student's t test. The results are expressed as the mean ± SD. A P value of less than .05 was considered to be statistically significant.

Table 1 GenBank accession numbers and nucleotide sequences of the PCR primers used to reverse transcriptase PCR

Gene	Accession no.	Forward primer	Reverse Primer	Annealing temperature (°C)	Product size (bp)
AQP-5	NM_009701	5′-ggccctcttaataggcaacc-3′	5′-ccagaagacccagtgagagg-3′	67	400
Sp-C	NM_011359	5′-tggagagtccaccggattac-3	5′-cacagcaaggcctaggaaag-3′	65	515
α-SMA	NM_007392	5′-gcctagcaacactgattc-3′	5′-caagtcctatacgctctc-3′	65	196
HPRT	NM_013556	5′-gcgtcgtgattagcgatgatga-3′	5′-gtcaagggcatatccaacaaca-3′	55	563

HPRT indicates hypoxanthine guanine phosphoribosyl transferase.