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Down-regulation of Wnt signal pathway in nitrofen-induced hypoplastic lung

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Abstract

Purpose: The pathogenesis of pulmonary hypoplasia associated with congenital diaphragmatic hernia is poorly understood. Recently, it has been reported that Wnt signaling pathway plays a critical role in branching lung morphogenesis. Mice lacking Wnt7b gene die soon after birth because of respiratory failure and display severe lung hypoplasia. Wnt2 gene is expressed in the distal airway during development. To test the hypothesis that Wnt-mediated signaling is altered in nitrofen-induced hypoplastic lungs, we examined the expression of Wnt genes and Wnt target gene, BMP4 in normal and nitrofen-treated lungs.

Materials and Methods: Fetal rat lungs of normal (n = 24) and nitrofen-treated (n = 24) dams were harvested on embryonic day (E)15, E17, E19, and E21. The expression of GATA6, the Wnt genes (Wnt7b, Wnt2), and BMP4 was analyzed in each lung by real-time reverse transcription polymerase chain reaction.

Results: The gene expression of Wnt7b, Wnt2, and BMP4 on E15 was significantly reduced ($P < .05$) in lungs from nitrofen-treated animals compared with normal lungs. The expression level of GATA6, which has been reported to transactivate Wnt7b expression, was also significantly reduced ($P < .05$) in lungs from the nitrofen group.

Conclusion: Our results provide evidence for the first time that the Wnt signaling pathway is down-regulated in nitrofen-induced hypoplastic lungs in the early stages of lung development. Decreased expression of GATA6 may account for the down-regulation of Wnt signal pathway. These data suggest that the down-regulation of Wnt signaling pathway may disrupt branching lung morphogenesis, resulting in pulmonary hypoplasia in the nitrofen rat model of congenital diaphragmatic hernia.

Despite prenatal diagnosis and new postnatal treatment strategies, the mortality rate of congenital diaphragmatic hernia (CDH) is still high. The high mortality in patients with CDH has been attributed to pulmonary hypoplasia and persistent pulmonary hypertension [1-3]. Hypoplastic lungs in CDH have fewer alveoli, thickened alveolar walls, increased interstitial tissue, and markedly diminished alveolar airspace [4-6]. The pathogenesis of pulmonary hypoplasia associated with congenital CDH is not fully understood. In the nitrofen-induced CDH animal model, some investigators have reported that nonmechanical factors directly mediated by nitrofen may play a role in the pathogenesis of lung hypoplasia [7,8]. Keijzer et al [8]

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proposed the *dual-hit* hypothesis to explain the observations on pulmonary hypoplasia in this model. This hypothesis proposes that the early retardation in lung development that occurs before the development of the diaphragmatic defect is caused by nitrofen, whereas the late-gestational increase in lung hypoplasia is caused by mechanical compression from herniated viscera [8].

Recently, a role for Wnt signaling in lung development has been suggested by observation that several Wnt genes are expressed in the developing lung mesenchyme and/or epithelium [9-11]. The Wnt growth factor family is composed of at least 19 different secreted ligands that interact with 10 known frizzled receptors [11]. Studies in many model systems have shown that Wnt signaling through the pathway downstream of ligands of the Wnt family can regulate multiple steps in organogenesis, including cell proliferation, differentiation, and lineage specification [11]. Wnt genes play as mediators of epithelial-mesenchymal interactions in the developing lung [11]. Especially, Wnt7b is required for normal lung mesenchymal proliferation in a narrow window of development before embryonic day (E)15.5 in mice [12,13]. Wnt7b inactivation decreased airway branching, caused pulmonary hypoplasia, and decreased lung muscular smooth muscle [12,13]. *Wnt7b* null mutant mice die of respiratory failure at birth [12]. Other Wnt genes, such as Wnt2, Wnt5a, and Wnt11, are expressed in the mesenchyme of the developing lung [11,14,15]. Furthermore, Wnt signaling has been reported to be a critical upstream regulator of proximal-distal patterning in the lung, in part, through regulation of N-*myc*, BMP4, and FGF signaling [9]. Ligands and receptors for BMP signaling are expressed at high levels in the distal airways, and several studies have demonstrated a critical role of this pathway in lung airway branching [15,16]. Transgenic mice expressing inhibitors of BMP signaling, such as gremlin, noggin display a disruption of proximal-distal patterning in the lung where distal epithelial differentiation is inhibited while proximal differentiation is promoted [15,16].

We hypothesized that Wnt signaling pathway is altered in early-gestational stage in nitrofen-induced lung hypoplasia. To test this hypothesis, current study was designed to investigate mRNA expression of Wnts (Wnt7b, 2) and the BMP4 as target genes in CDH lung on E15, E17, E19, and E21. We also investigated the expression of GATA6, which is reported to be a regulator of Wnt7b in the lung epithelium [17].

1. Materials and methods

1.1. Animal model

Fetal pulmonary hypoplasia with coexistent diaphragmatic hernia was created by gavaging time dated pregnant Sprague-Dawley rats. Rats were mated, and the females were checked daily for plugging. Observation of positive smears was considered a proof of pregnancy; the day of observation determined day 0. At 9.5 days gestation (term, 22 days), 100 mg of nitrofen (WAKO Chemical, Osaka, Japan) dissolved in olive oil was given as a single dose via stomach tube under short anesthesia. In control animals, the same dose of olive oil were given without nitrofen. Cesarean delivery was performed on day 15 (E15), 17 (E17), 19 (E19), and 21 (E21) of gestation under general anesthesia. The Department of Health and Children approved all the animal experiments (ref. B100/3697) under the Cruelty to Animal Act, 1876, as amended by European Communities Regulations 2002.

1.2. mRNA isolation and real-time reverse transcription polymerase chain reaction

Lungs dissected from the thoracic cavity under the microscope were immediately suspended in RNA*later* solution (Ambion, UK) and stored at −20°C. The total RNA of each lung was extracted using TRIZOL reagent (Life Technologies, Railey, UK) according to recommended protocol. Reverse transcription polymerase chain reaction (RT-PCR) was performed using Quanti Tect SYBR green RT-PCR kit (Qiagen, UK) according to the manufacturer's protocol. The primer sets used in this study and estimated size of the PCR products are listed in Table 1. The PCR mixture (total, 25 μL) contained 0.5 μmol of each primer, 12.5 μL of Quanti Tect SYBR Green RT-PCR Master Mix, and 0.25 μL of Quanti Tect RT Mix. Relative levels of gene expression were measured by RT-PCR (iCycler iQ Multicolor real-time PCR detection System, Bio-Rad Laboratories, Calif). After RT at 50°C for 30 minutes, 45 cycles of amplification were carried out (denaturation at 95°C for 15 seconds, annealing at 58°C for 30 seconds, and extension at 72°C for 30 seconds). Serial dilution of one sample RNA was prepared to create a standard curve for the relative quantification of mRNA in the samples. Experiments were carried out in triplicate for each data point. The relative changes in levels of specific genes were expressed in percent of the control values that were set equal to 100% after the normalization by the level of β-actin expression in each sample.

Table 1 Quantitative real-time RT-PCR primers

Gene		Sequence (5′-3′)	Product size (bp)
β-actin	Forward	ttg ctg aca gga tgc aga ag	108
	Reverse	tag agc cac caa tcc aca ca	
Wnt7b	Forward	agc caa cat cat ctg caa ca	122
	Reverse	ggc att cat cga tac cca tc	
Wnt2	Forward	ctc cct ctg ctc ttg acc tg	106
	Reverse	gac ctg gca cat tgt cac ac	
GATA6	Forward	gcc aac tgt cac acc aca ac	86
	Reverse	ttc ata taa agc ccg caa gc	
BMP	Forward	cag agc caa cac tgt gag ga	121
	Reverse	cac ctc att ctc tgg gat gc	