Journal of Pediatric Surgery (2007) **42**, 415–419

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Journal of Pediatric Surgery

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Reduced expression of aquaporin 5 water channel in nitrofen-induced hypoplastic lung with congenital diaphragmatic hernia rat model

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Index Words:
Congenital diaphragmatic hernia;
Pulmonary hypoplasia;
Aquaporin 5

Abstract

Purpose: Pulmonary hypoplasia remains the principal cause of high morbidity and mortality in patients with congenital diaphragmatic hernia (CDH). The precise mechanisms causing lung hypoplasia remains unclear. Aquaporins (AQPs) are reported to constitute a family of water channels that facilitate membrane water permeability in various tissues of animals. Aquaporin 5 has been reported to be an important marker expressed in type I alveolar epithelial cells in late gestation and mediates water transport across the human airway epithelium. We hypothesized that AQP5 is reduced in hypoplastic lungs and therefore designed this study to determine AQP5 expression in normal and hypoplastic lungs.

Methods: Fetal rat lungs of control (n=23) and nitrofen-treated (n=37) dams were harvested on embryonic day (E) 15, E17, E19, and E21. The expression of the AQP5 was analyzed in each lung by real-time reverse transcriptase–polymerase chain reaction. Immunohistochemical studies were performed to evaluate the protein expression level of AQP5.

Results: Aquaporin 5 messenger RNA levels on E21 were significantly reduced in lungs from the nitrofen with CDH group (11.8 ± 2.3) compared with normal controls (23.5 ± 11.8) and nitrofen without CDH group (26.9 ± 13.0) ($P < .05$). Aquaporin 5 immunohistochemistry demonstrated AQP5 strongly expressed at the apical membrane of type I alveolar epithelial cells in the normal and nitrofen without CDH groups. By contrast, the AQP5-positive cells were markedly reduced in hypoplastic lungs in the nitrofen with CDH group.

Conclusion: Our results show that the expression of AQP5 is down-regulated in hypoplastic lungs with CDH. Down-regulation of AQP5 may result in abnormal pulmonary fluid metabolism in perinatal period and may be one of the mechanisms disturbing the pulmonary development in late stage in the CDH model.

Congenital diaphragmatic hernia (CDH) occurs once in 3000 births and causes pulmonary hypoplasia and hypertension leading to high mortality rates [1,2]. The mortality rate for newborn infants with CDH is still high despite significant advances in neonatal resuscitation and intensive care such as

Presented at the British Association of Paediatric Surgeons 53rd Annual International Congress, Stockholm, Sweden, July 18-22, 2006.

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doi:10.1016/j.jpedsurg.2006.10.029

high-frequency ventilation and extracorporeal membrane oxygenation [1,2]. Hypoplastic lung and persistent pulmonary hypertension are the principle causes of the high morbidity and mortality in infants with CDH [3-5].

In CDH, it was recently shown that early and late gestational lung underdevelopment is caused by nonmechanical and mechanical factors, respectively [6,7]. This hypothesis is proposed by using animal models induced by nitrofen administration [7]. To elucidate the mechanisms involved especially during late gestation will provide us guides to cure the lung hypoplasia. However, the precise mechanism is unclear.

Recently, aquaporin (AQP) 5, a member of the large family of aquaporin proteins, most of which are water channels, has been reported to be a specific marker for type I alveolar epithelial cells (AECs-I) [8-10]. The lung expresses several AQPs: AQP1 in microvascular endothelia, AQP3 in large airways, AQP4 in large- and small-airway epithelia, and AQP5 in AECs-I [8,9]. Exactly when AQP5 expression commences in development is not clear, but late in gestation, AQP5 expression increases substantially, consistent with the behavior of other AEC-I genes [8,10,11]. This increase is mostly likely related to the large expansion of AEC-I surface area in late lung development. Fluid movement between the air space and vascular compartments in lung plays an important physiologic role in many processes such as regulation of airway hydration, reabsorption of alveolar fluid in the neonatal period in preparation for alveolar respiration, and the resolution of pulmonary edema [9,12]. Aquaporin water channels may be important in the clearance of fluid from the newborn lung [9,11,12]. Osmotic water permeability in lungs in AQP5 knockout mouse models is markedly reduced [13].

In this study, we investigated the expression pattern of AQP5 in nitrofen-induced CDH rat model to elucidate the precise mechanisms involved in lung hypoplasia during late gestation.

1. Materials and methods

1.1. Animal model

Sprague-Dawley rats were mated, and the females were checked daily for plugging. Observation of positive smears was considered as a proof of pregnancy; the day of observation was determined day 0. At 9.5 days of pregnancy (term, 22 days), 100 mg nitrofen (WAKO Chemical, Osaka, Japan) dissolved in olive oil was given as a single dose via a stomach tube under short anesthesia. In control animals, the same dose of olive oil was given without nitrofen. Cesarean delivery was performed on days 15 (embryonic day [E] 15), 17 (E17), 19 (E19), and 21 (E21) of gestation under general anesthesia. Fetuses were harvested by laparotomy and freed of their extraembryonic membranes. Under surgical microscopy, a laparotomy was performed, and all the fetuses were examined for pulmonary hypoplasia and presence or absence of coexisting diaphragmatic hernias. For E19 and E21 fetuses, fetuses with left diaphragmatic hernia defects were defined as CDH group, whereas the fetuses exposed to nitrofen with an intact diaphragm were defined as non-CDH group. For E15 and E17 fetuses, the nitrofen-treated fetuses were not divided into 2 groups as above because the diaphragm formation occurs in rat from E14 through E17. The control group consisted of animals that did not receive nitrofen. The number of each group in E15, E17, E19, and E21 was 5, 6, 6, and 7, respectively. The Department of Health and Children approved all the animal experiments (reference no. B100/3697) under the Cruelty to Animal Act, 1876, as amended by the European Communities Regulations 2002.

1.2. Messenger RNA isolation and real-time reverse transcriptase–polymerase chain reaction

Lungs dissected microsurgically from the thoracic cavity were immediately suspended in RNA*later* solution (Ambion, Huntingdon, Cambridgeshire, UK) and stored at −20°C. The total RNA of each lung was extracted by the guanidine isothiocyanate procedure using TRIZOL reagent (Life Technologies, Paisley, UK), according to the recommended protocol. The pelleted RNA was dissolved in sterile water and quantified by absorbance at 260 nm using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). Oligonucleotide primers were synthesized using Web-based software Primer3 (http://frodo.wi.mit.edu/primer3/primer3_code.html) based on the AQP5 (NM012779) and β-actin (NM031144) sequences. The sequences for the sense and antisense primers were 5′-TCT GGG TAG GGC CTA TTG G-3′ and 5′-CAT GGA GGC TCA GAG AGG AG-3′, respectively, for AQP5. As controls, the housekeeping gene β-actin was also reverse transcribed and amplified by PCR. The sequences of the primers used for rat β-actin was 5′-TTG CTG ACA GGA TGC AGA AG-3′ and 5′-TAG AGC CAC CAA TCC ACA CA-3′. Reverse transcriptase–polymerase chain reaction (RT-PCR) was performed using Quanti Tect SYBR green RT-PCR kit (Qiagen, West Sussex, UK) according to the manufacturer's protocol. The PCR mixture (total, 25 μL) contained 0.5 μmol/L of each primer, 12.5μL of Quanti Tect SYBR Green RT-PCR Master Mix, and 0.25μL 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, Hercules, CA). 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 messenger RNA (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 percentage of the control values that were set equal to 100%, after the normalization by the level of β-actin expression in each sample.

1.3. Immunohistochemistry

Lungs from fetal rat of E21 were immersion fixed in 4% paraformaldehyde for 24 hours and processed for sectioning. The paraffin-embedded tissues were sectioned at a thickness of 6 μm, and the sections were deparaffinized with xylene and then rehydrated through ethanol and distilled water. Tissue sections were pretreated with target retrieval solution (DAKO Ltd, Cambridgeshire, UK) for 10 minutes at 121°C followed by incubation in 0.3% H_2O_2 for 30 minutes to block endogenous peroxidase activity. Immunohistochemistry was performed using primary antibody to rat AQP5 (Lot AN-1; Alomone Labs, Jerusalem, Israel) to detect AQP5 in the lung. To control for nonspecific staining, sections were also processed with omission of the primary antibody. Sections were incubated with primary antibody overnight at 4°C. Sections were incubated in biotinylated secondary antibody and then processed using Vectastain Elite ABC kit followed by detection with DAB substrate kit for peroxidase (both from Vector, Peterborough, UK) and counterstained with hematoxylin.

1.4. Statistical analysis

Data were analyzed by unpaired, nonparametric Mann-Whitney test. Statistical significance was set at $P < .05$.

2. Results

As shown in Fig. 1, mRNA expression of AQP5 was increased between E19 and E21 with advancing gestational stage. Aquaporin 5 mRNA levels on E21 were significantly suppressed in lungs from the CDH group comparing with the other groups ($P < .05$). To determine whether the decreased amounts of AQP5 transcripts were reflected in the change in the amount of the protein themselves, a

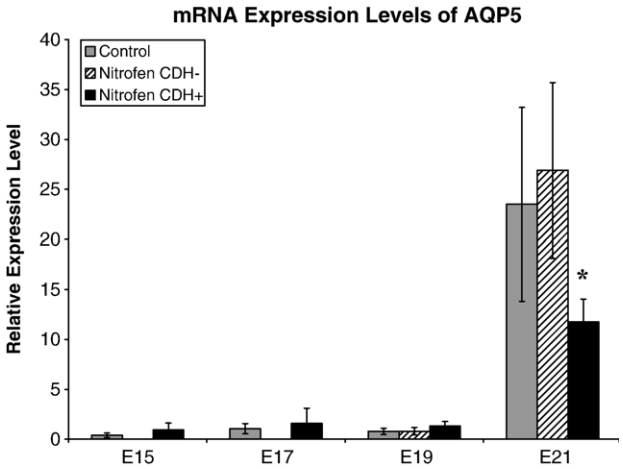

Fig. 1 The graph shows the developmental difference in the AQP5 mRNA levels in control and nitrofen-exposed hypoplastic lungs with or without CDH. Aquaporin 5 expression level was increased dramatically between E19 and E21 in every group. At E21, AQP5 expression level was significantly higher in lungs from control and non-CDH group compared with lungs from the CDH group ($P < .05$, marked by asterisks).

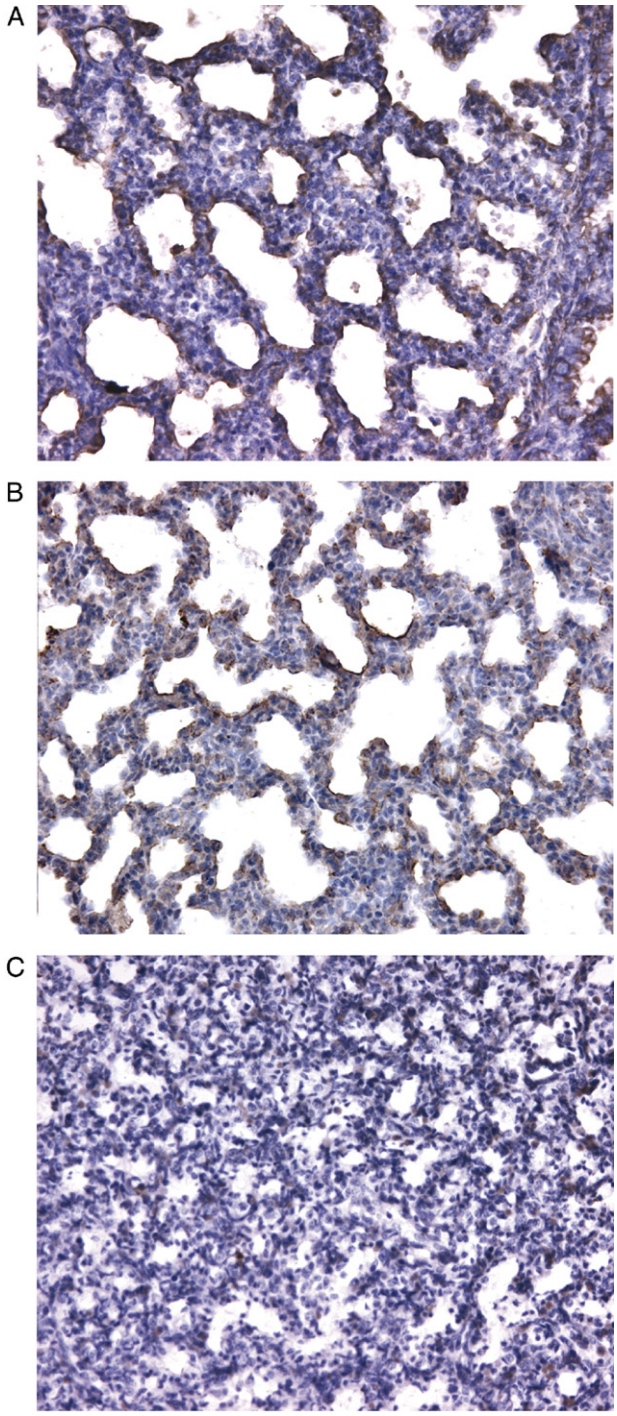

Fig. 2 Immunohistochemistry of AQP5. Immunoperoxidase demonstration of AQP5 in (B) the control group and (A) hypoplastic lung from the CDH group. A and B, Immunoreactivity of AQP5 was detected along the luminal surface of the alveolar epithelium in the lung from control and non-CDH group. C, Decreased immunoreactivity with immature histology was observed in lungs from the CDH group (original magnification of A-C ×40).

immunohistochemical study was performed using lung specimens harvested on E21. Marked AQP5 expression was observed in the apical membrane of AECs-I of lungs from control and nitrofen-treated non-CDH group (Fig. 2A and B). In contrast, the immunoreactivity for AQP5 was very weak in alveolar cells of CDH lung with morphologic immaturity, thickened alveolar walls, increased interstitial tissue, and diminished alveolar air space (Fig. 2C). Taken together, the expression of AQP5 was significantly decreased in the lungs from the CDH group.

3. Discussion

In CDH, recent animal experiments have shown that early and late gestational lung underdevelopment is caused by nonmechanical and mechanical factors, respectively [6,7]. This hypothesis proposes that the early defect 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 [7]. Although the mechanisms causing CDH and associated lung hypoplasia have been well studied [1,14], the precise mechanisms involved in late gestation are not ascertained. Especially, in a review of the recent literature, the precise mechanisms involved in AEC differentiation in hypoplastic lung are still unclear and controversial [1,14,15]. The status of AEC differentiation is not well studied in CDH.

Recently, AQP5, a member of the large family of AQP water-channel proteins, has been reported as a marker for AEC-I [9-11]. Aquaporins facilitate water transport across the epithelia and play an important role in normal physiology and disease in the human airways [9-13]. Aquaporin 5 has been found primarily in the apical membrane of AEC-I and submucosal gland acinar cells in the upper airways [9]. Aquaporin 5 expression increases substantially in late gestation, and the increase relates to the large expansion of AEC-I surface area [10,11]. Thus, AQP5 has been thought to be one of the important AEC-I–specific molecular markers. We tested the hypothesis that expression of AQP5 is suppressed in CDH hypoplastic lungs to elucidate the pathogenetic events resulting in lung hypoplasia involved in late gestation. Similar to previous observations, this study showed that expression of AQP5 increased dramatically between E19 and E21, the very late stage of gestation (Fig. 1). Aquaporin 5 expression on E21 was significantly reduced in hypoplastic lungs associated with CDH (Figs. 1 and 2). The reduced expression of AQP5, which is a marker of AEC-I, may be affected by mechanical compression of lungs from herniated viscera in the late-gestational stage. In the fetus, it has been reported that increased expansion promotes differentiation into the AEC-I phenotype, whereas reduced lung expansion promotes the type II alveolar epithelial cells (AECs-II) phenotype [16]. Type II AECs are thought to be progenitor for AECs-I, and the differentiation from AECs-II to AECs-I is one of the most important event in lung development during late gestation [8,16,17]. Some reports have pointed out that the hypoplastic lungs associated with CDH have thick-walled terminal air spaces, no true alveoli, increased frequency of AECs-II, and decreased expression of AECs-I associated protein, RTI_{40} [15]. Our findings of decreased expression of AQP5, one of the important AEC-I–specific marker, found in the hypoplastic lungs associated with CDH, suggest that mechanical compression of lungs from herniated viscera affects the differentiation of AECs in CDH hypoplastic lung in the late gestational stage. Studies examining the alveolar epithelial cell differentiation in this model may provide further information in understanding the pathogenesis of pulmonary hypoplasia in CDH.

Although the pathway for the flow of water across the endothelial and epithelial lung barrier is not completely elucidated, AQP5 is reported to be important in the clearance of fluid from the newborn lung. For example, decreased expression of AQP5 has been associated with pulmonary edema and inflammation in acute lung injury caused by adenoviral infection [9,18]. Aquaporin 5 knockout mice have a 90% decrease in airspace-capillary water permeability in the lungs [13] and airway hyperresponsiveness to cholinergic challenge [19]. Recently, it has been also pointed out that the expression of epithelial Na^+ channel was significantly reduced in hypoplastic lungs in nitrofen-induced CDH rodent models, which may contribute to impaired distal airspace fluid absorption in hypoplastic lungs [20]. The attenuated expression of the epithelial Na^+ channel and AQP5 may result in fluid-filled lungs at birth with reduced capacity to establish postnatal breathing.

In summary, the results of this study showed, for the first time, that CDH hypoplastic lung is associated with decreased protein and mRNA expression of AQP5. Our findings suggest that down-regulation of AQP5 may result in abnormal pulmonary fluid metabolism in the neonatal period, which may contribute to the respiratory insufficiency seen clinically in infants with CDH.

4. Q&A

Parikh, Birmingham

Did you examine the lungs, were they wet or condensed or fibrosed when in a CDH of nitrofen model?

A.

Unfortunately, I have not done that. I think so but I did not evaluate critically.

Parikh, Birmingham, UK

What happens when you do a tracheal occlusion? Does the AQP go up, down? Have you done that study?

A.

It is a very good and to-the-point question. I have not done that, but I am keen to do it. I presume that tracheal occlusion might increase the expression of AQP5 and,

actually, we are now investigating fetal therapy including tracheal occlusion or using retinoic acid, and I think that this is the good marker for that study.

Tran, Stockholm, Sweden

I enjoyed your talk. Are there any other isotypes of aquaporins that are expressed by these alveolar epithelial cells and have you looked at it?

A.

I have not done this. I have read some reports of this, but I have not done any work on this.

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