Congenital diaphragmatic hernia, tracheal occlusion, thyroid transcription factor-1, and fetal pulmonary epithelial maturation

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Congenital diaphragmatic hernia (CDH) occurs in ~1:2,500 human births and has high morbidity and mortality rates, primarily due to pulmonary hypoplasia and pulmonary hypertension (21, 29). The timing and duration of TO influence its effects on the fetal lung. In fetal sheep with diaphragmatic hernia (DH), long-term TO accelerates lung growth and corrects some of the pulmonary vascular anomalies associated with DH (8, 26, 41) but adversely affects surfactant production (4, 8) and decreases the number of type II alveolar epithelial cells (5). Conversely, with short-duration TO, lung growth is not accelerated, yet postnatal lung function is improved, pulmonary vascular anomalies are corrected, and type II cell numbers are preserved (41, 46, 53). In addition, Wu and associates (54) showed an inverse relationship between the duration of TO and type II cell density in late gestation fetal rabbits with DH.

The rodent model of nitrofen-induced CDH is being increasingly used to study the effects of CDH on lung development (35). Because the diaphragmatic defect is present in early gestation, this model system more closely mimics human CDH than the surgically produced models in fetal sheep and rabbits. Kitano and associates (31) showed that TO causes increases in lung weight, DNA content, and protein content and that TO of CDH fetuses reversed the increases in pulmonary arterial medial and adventitial thickness, which is associated with CDH. Although these studies show that TO tends to correct the pulmonary parenchymal and vascular abnormalities associated with CDH, relatively little is known about how TO affects pulmonary epithelial maturation in rat fetuses with CDH.

Numerous factors participate in the control of lung development and maturation (reviewed in Ref. 36). Of particular interest is the homeobox-containing transcription factor, thyroid transcription factor-1 (TTF-1). Early in fetal development, TTF-1 regulates branching morphogenesis and proximal distal patterning in the lung (43). Later in gestation and postnatally, TTF-1 directs expression of the surfactant proteins (SP), the Clara cell-specific protein CC10 (52), and differentiation of type I cells (49). Thus alterations in TTF-1 during gestation affect lung maturation, both architecturally and biochemically. In the extreme case of the TTF-1 knockout mouse, lung branching morphogenesis is halted at the formation of the mainstem bronchi and a proximal phenotype, epithelium with tracheobronchiolar characteristics, is maintained (56). As noted above, increased distension of the fetal lung due to TO and
decreased distension (e.g., due to CDH) have opposite effects on pulmonary epithelial development. Because TTF-1 participates in development of both type I and type II alveolar epithelial cells, we hypothesize that changes in lung distension may influence pulmonary epithelial maturation through effects on TTF-1.

In the current study, we have examined the effects of CDH and of short-duration TO in CDH in late gestation on indicators of lung growth, type I and type II cell maturation, and on TTF-1. Because nitrogen has primary effects on the lung, we have used nitrogen-exposed litters as controls (12, 20, 27).

**MATERIALS AND METHODS**

**Animals.** All studies were approved by the Committee on Animal Research of the University of California, San Francisco, and all procedures conformed with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Timed-pregnant Sprague-Dawley (SD) rats were obtained from Charles River Laboratories (Hollister, CA) and were provided feed and water ad libitum. Dams were housed in a laminar flow facility that maintained ambient temperature at 21°C.

**CDH and TO.** On day 9.5 of gestation (presence of a vaginal plug indicated day 1), timed-pregnant SD rats were gavage-fed 100 mg of Nitrofen (2,4-dichloro-4’-nitrodiphenyl ether; Wako Chemicals, Osaka, Japan) dissolved in 1 ml of olive oil to produce CDH in the fetuses. On day 20 of gestation, dams and fetuses were anesthetized with an intramuscular injection of ketamine (180 mg/kg) and xylazine (2.5 mg/kg). Through a maternal midline laparotomy, TO was performed on fetuses as previously described (55). In brief, a small section of one uterine horn was exposed. Near the head of a fetus, a purse-string suture was placed in the uterus, and the fetal head and neck were exteriorized through a small hysterotomy. The neck was maintained in an extended position with wet gauze. With the use of an operating stereomicroscope (Leica Wild M691, Leica Microsystems), the trachea was exposed through a midline cervical incision. The trachea was cauterized using a handheld cautery unit equipped with a microtip (Roboz Surgical, Rockville, MD). The fetuses were then returned to the uterus, and the maternal abdomen was closed in two layers. Four to six fetuses per dam received TO by microcautery. On day 22 (term), the dam and fetuses were again anesthetized with an intramuscular injection of ketamine (180 mg/kg) and xylazine (2.5 mg/kg). The fetuses were delivered and weighed, the abdominal cavity was opened, and the presence or absence of CDH was determined by visual inspection of the diaphragm. The lungs were then removed, split into left and right lung, weighed, either snap-frozen or fixed, and stored at −80°C for future study. After all fetuses had been removed, dams were killed by intracardiac injection of pentobarbital (1 ml, 390 mg/ml solution, Euthanasia Solution; Schering-Plough, Union, NJ), visualized by autoradiography, and quantified using the Storm 840 phosphorimager equipped with a blue fluorescence/chemiluminescence detector and Imagequant software (Molecular Dynamics, Sunnyvale, CA).

**Immunohistochemistry.** Fetal rat lungs were immersion fixed in 4% paraformaldehyde for 24 h and processed for cryosectioning. Immunohistochemistry was performed on 3-μm sections using primary antibodies to RTI40 and RTI70, as indicated above, and were detected using indirect immunofluorescence, as previously described (34). Briefly, sections were incubated with both primary antibodies simultaneously and extensively washed before incubation with isotype-specific secondary antibodies, fluorescein-conjugated goat anti-mouse IgM (Cappel, Aurora, OR) and biotinylated goat anti-mouse IgG3 (Santa Cruz Biotechnology, Santa Cruz, CA) followed by Texas red-conjugated streptavidin (Zymed, South San Francisco, CA) for RTI40 and biotinylated goat anti-mouse IgG3 (Santa Cruz Biotechnology, Santa Cruz, CA) followed by Texas red-conjugated streptavidin (Zymed, South San Francisco, CA) for RTI70. Sections were mounted with Prolong antifade kit (Molecular Probes, Eugene, OR), and images were acquired using a Leica TCS SP confocal microscope (Leica Microsystems). To detect TTF-1 in the lung, immunohistochemistry was performed using primary antibody to rat TTF-1 (Clone 8G7G3/1; Dako, Carpenteria, CA). TTF-1 was detected in sections pretreated with target retrieval solution (Dako) for 10 min at 95°C followed by incubation in 1% H2O2 for 10 min to block endogenous peroxidase activity. To control for nonspecific staining, sections were also processed with omission of the primary antibody. Sections were incubated in biotinylated secondary antibody and then processed using Vectastain Elite ABC kit followed by detection with 3,3’-diaminobenzidine substrate kit for peroxidase (both from Vector, Burlingame, CA) and counterstained with Gill’s No. 2 hematoxylin (Sigma). Sections were mounted with Glycergel (Dako), and images were captured using a Leitz Orthoplan 2 microscope (Leica Microsystems).

**RT-PCR Analysis.** Because of the limited number of fetuses available, we used fetuses with either left- or right-sided CDH for the TTF-1 studies. Whole frozen lungs were dounce homogenized on ice in 1 ml of cold nuclear extract buffer B (7), and the extract was transferred to a sterile microfuge tube and ultrasonicated at 30% power for 30 s on ice. Insoluble material was pelleted by microcentrifugation at 16,000...
Both ipsilateral (i.e., left) and contralateral (i.e., right) lung weights were significantly decreased by CDH and increased by CDH+TO, both in absolute terms and expressed as a percent of body weight (Table 1 and Fig. 1). CDH retarded growth of the ipsilateral lung (67% of NC) more than the contralateral lung (85% of NC). In contrast, CDH+TO increased lung weight more in the contralateral lung (156% of NC) than the ipsilateral lung (130% of NC). CDH significantly reduced DNA in the ipsilateral lung to 72% of the NC value, and CDH+TO tended to increase DNA in the ipsilateral lung (to 85% of NC; Table 2). Neither CDH nor CDH+TO affected DNA content of the contralateral lung or protein content of either lung. Protein to DNA ratios did not differ between experimental groups in both lungs (data not shown).

### mRNA expression of type I and II cell markers.

To examine more closely the effects of nitrogen-induced CDH and subsequent TO on distal epithelial cell differentiation and maturation, we measured mRNA expression of RTI40, an indicator of the type I cell phenotype, and of SP-A, SP-B, and SP-C, indicators of the type II cell phenotype, normalized them to 18S ribosomal RNA using multiplex RT-PCR, and expressed the values as percent of the same side lung of littermate fetuses. To examine the effects of CDH and CDH+TO on type I and type II cell markers in an individual lung, all genes were measured from the same RNA isolation for each lung in each animal.

**Ipsilateral lung.** As shown in Fig. 2, A and B, CDH did not affect expression of RTI40, SP-A, or SP-B, but significantly increased SP-C to 126% of NC (P < 0.05). CDH+TO significantly increased expression of RTI40 to 149% of NC (P < 0.05) and 168% of CDH (P < 0.01). In contrast to CDH, CDH+TO significantly reduced expression of SP-A, SP-B, and SP-C. SP-A was reduced to 58% of NC and 60% of CDH (P < 0.05 for both), SP-B was reduced to 68% of NC (P < 0.01), and SP-C was reduced to 62% of NC and 50% of CDH (P < 0.0001 and 0.01, respectively).

**Contralateral lung.** As shown in Fig. 2C, CDH did not significantly affect the level of mRNA in any of the genes examined. Effects of CDH+TO were similar to those in the ipsilateral lung, but the magnitudes of the effects were less marked.

**Protein expression of type I and II cell markers.** To evaluate how the changes in mRNA expression translate into changes at the protein level in fetuses with nitrogen-induced CDH and CDH+TO, we performed quantitative Western dots blots for RTI40, SP-A, SP-B, pro-SP-C, and the type II cell-specific apical epithelial marker RTI170. To examine the effects of CDH and CDH+TO on type I and type II cell markers in an

### Results

#### Incidence of CDH.

CDH occurred in 31.3% of the nitrogen-exposed fetuses. Of those, left-sided CDH occurred in 57.5%, right-sided defects occurred in 30.6%, and bilateral hernias occurred in 11.9%. In nitrogen-exposed fetuses with TO, there was a 49.3% survival rate, with 25% of these animals having a diaphragmatic defect. We studied 189 NC fetuses, 62 fetuses with left-sided CDH, and 15 left-sided CDH+TO fetuses from 54 pregnant dams.

**Body and lung weights, DNA, and protein content.** Both CDH and CDH+TO had effects on growth of the fetal body and lungs (Table 1). Fetal body weights were slightly, but significantly, reduced in fetuses with CDH or CDH+TO compared with NC, whereas body weights from CDH and CDH+TO fetuses did not differ significantly from one another.

### Table 1. Effects of CDH and subsequent TO on body weight and lung weight in fetal rats

<table>
<thead>
<tr>
<th>Experimental Group</th>
<th>Left/Ipsilateral Lung Wet Weight</th>
<th>Right/Contralateral Lung Wet Weight</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Body Weight, g</td>
<td>% body wt</td>
</tr>
<tr>
<td>NC</td>
<td>3.90 ± 0.52 (n = 189)</td>
<td>0.038 ± 0.01 (n = 187)</td>
</tr>
<tr>
<td>CDH</td>
<td>3.74 ± 0.58* (n = 62)</td>
<td>0.025 ± 0.011† (n = 59)</td>
</tr>
<tr>
<td>CDH+TO</td>
<td>3.56 ± 0.58 (n = 15)</td>
<td>0.045 ± 0.019† (n = 15)</td>
</tr>
</tbody>
</table>

Data are means ± SD. Experimental group: NC, nitrofen-exposed fetus without congenital diaphragmatic hernia; CDH, fetus with a left-sided CDH; CDH+TO, fetus with a left-sided CDH and tracheal occlusion. %BW, lung weight as a percentage of fetal body weight; n, number of fetuses. *P < 0.05 to NC; †P < 0.0001 to NC; ‡P < 0.0001 to CDH.
Fig. 1. Effects of congenital diaphragmatic hernia (CDH) and CDH + tracheal occlusion (TO) on fetal lung size. Fetuses were delivered, the diaphragmatic defect was photographed, and the diaphragm and rib cage were removed to facilitate visualization of the lungs. A: left-sided CDH. B: lungs of fetus with left-sided CDH. C: lungs of fetus with left-sided CDH and TO at embryonic day 20. Arrow in A points to large diaphragmatic defect on the left side. The lungs are outlined for clarity. The front of the animal is shown. h in B and C indicates the position of the heart. Note that the lungs of the CDH + TO fetus are much larger than those of the fetus with CDH alone.

Table 2. Effects of CDH and subsequent TO on DNA and protein content in lungs of fetal rats

<table>
<thead>
<tr>
<th>Experimental group</th>
<th>Total Content in Lungs Normalized to Body wt (mg/g body wt) Protein</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Left/ipsilateral</td>
</tr>
<tr>
<td>NC</td>
<td>0.067±0.022 (n=12)</td>
</tr>
<tr>
<td>CDH</td>
<td>0.048±0.014* (n=8)</td>
</tr>
<tr>
<td>CDH + TO</td>
<td>0.057±0.006 (n=5)</td>
</tr>
</tbody>
</table>

Data are means ± SD. Experimental group: NC, nitrofen-exposed fetus without CDH; CDH, fetus with a left-sided CDH; CDH + TO, fetus with a left-sided CDH and TO. Body wt, DNA, or protein content normalized to fetal body weight; n, number of fetuses. *P < 0.05 to NC.
Animals were compared only to littermates, and only fetuses with left-sided CDH were included (except for analysis of TTF-1).

Administration of nitrofen to pregnant dams at 9.5 days of gestation produced primarily left-sided DH, consistent with previous observations (11, 35). Body weights for both CDH and CDH/H11001 TO fetuses were decreased. Previous reports by...
others and by us had shown no differences in body weights with CDH and CDH + TO (9, 31). Those studies involved relatively small numbers of animals, whereas the current study includes much larger numbers, a factor that probably accounts for the statistical differences between the studies. It is likely that the decreased fetal weight with CDH + TO was due to the stress of surgery. However, the reasons for the decreased fetal weight with CDH are not apparent and cannot be accounted for by the decreased lung weights.

CDH lungs appeared relatively small, whereas CDH + TO produced visibly larger lungs (Fig. 1), findings reflected in the lung weights (Table 1). On the basis of lung weights and DNA content, CDH retarded lung growth more in the ipsilateral than in the contralateral lung, a finding previously noted in other species (2, 15).

Although it markedly retarded lung growth, CDH had relatively minor effects on most indicators of maturation of type I and type II cells, with the exception of SP-C mRNA and RTI40 protein. In the ipsilateral lungs, CDH increased SP-C mRNA by 26% but did not affect mRNA for other SP or RTI40, a protein specific in lung to the apical membrane of type I cells (Fig. 2). In the contralateral lung, none of these genes showed significant changes in mRNA expression. These results are similar to previous reports that showed little or no change in mRNA for SP-A, SP-B, or SP-C with nitrofen-induced CDH (12, 44, 50, 51). Although CDH tended to increase the 21-kDa pro-SP-C protein in both lungs, it did not affect the concentration of SP-A and SP-B proteins in either lung (Fig. 3), findings consistent with the mRNA data and with the report by Van Tuyl and associates (51). Furthermore, CDH had no effect in either lung on RTI70, a protein specific to the apical membrane of type II cells. In contrast, CDH decreased the type I cell protein, RTI40, a finding that we previously noted in hypoplastic lungs due to oligohydramnios (34). Therefore, based on these data, CDH has little effect on indicators of type II cell maturation, but does decrease expression of the type I cell-associated protein, RTI40.

As previously reported by Kitano and associates (31), CDH + TO increased lung weight (Table 1). In the current study, this effect was greater on the contralateral lung than on the ipsilateral (see RESULTS), a finding not previously described. The reason for this is not known but may relate to the portion of the liver that has herniated into the thorax, impeding distension of the lung ipsilateral to the CDH (37). In the ipsilateral lung, TO of CDH fetuses tended to increase DNA content, but
not to the level of NC lungs (Table 2). Previously, Kitano and associates (31) reported that CDH + TO increased lung DNA to control levels. The most likely explanation for the differences between our results and theirs is that they produced TO earlier in gestation and for a longer duration, factors known to influence the effects of TO on lung growth (41, 53). Other possible factors include the method of TO (tracheal cautery in the current study vs. tracheal ligation) and the method of comparison of experimental animals to controls (paired comparisons between littermates in the current study vs. unpaired comparisons of nonlittermates). Several investigators have shown that prolonged TO in normal fetuses and in those with DH, in a variety of species, decreases production of surfactant components (4, 25, 39, 47). Furthermore, TO decreases the density of type II cells (47) and the ratio of type II to type I cells (17). This deleterious effect on type II cells can be lessened by performing TO later in gestation and for a shorter period (13, 14, 42, 46, 54, 55). In the current study, we performed TO relatively late in gestation (20 days) in fetuses with left-sided CDH. Our results were concordant with previous studies in that the mRNAs for SP-A, SP-B, and SP-C were decreased in the ipsilateral CDH + TO lung compared with CDH and/or NC lungs. There was a similar, but less marked, effect in the contralateral lung. In an apparent contrast to the mRNAs, only pro-SP-C protein tended to decrease \((P = 0.1)\) in the ipsilateral lung and was significantly decreased in the contralateral lung \((P = 0.03)\) of CDH + TO animals. Of note is that, despite the decreases in mRNA, CDH + TO did not affect lung concentration of SP-A and SP-B proteins. In addition, the type II cell-specific membrane protein RTII\(_{70}\) was also unchanged \((\text{mRNA for RTII}_{70}\text{ was not measured as this protein has not been purified or cloned to date})\). In a previous report (55), we saw similar discrepancies between type II cell-specific mRNA and protein levels and speculated that they were due to entrapment of secreted SP in the fetal lung fluid in animals with TO. These results are similar to those of Kitano and associates (30), who reported that TO of CDH fetuses in the rat impaired SP mRNA expression but had little effect on SP-A and SP-B proteins. Similar findings were reported in normal sheep fetuses with short-term TO by Lines et al. (39).

CDH + TO increased mRNA of RTI\(_{40}\), a protein specific in the lung to type I cells (18) and essential for normal lung development and epithelial differentiation (48). In addition, the concentration of RTI\(_{40}\) protein was increased to \(\geq 250\%\) of the value in CDH lungs, although this change did not quite reach statistical significance (Fig. 3). We have previously shown that expression of RTI\(_{40}\) correlates closely with the surface area covered by type I cells (34). These biochemical results are supported by the immunohistochemical findings that CDH caused an apparent decrease in RTI\(_{40}\) compared with NC, and CDH + TO caused an apparent increase so that these lungs qualitatively were similar to NC lungs; the RTII\(_{70}\) staining pattern appeared similar in all lungs (Fig. 4).

The expression pattern of TTF-1, an important regulator of proximal-distal patterning in early embryonic development and, in late gestation, a transcriptional regulator of the SP, the Clara cell secretory protein, and type I cell differentiation have been examined in CDH lungs by several investigators with mixed results. Losada et al. (40) showed that TTF-1 is down-regulated in the lungs of fetuses exposed to nitrofen independently of the presence of CDH and confirm this in vivo finding.
with in vitro data showing that nitrofen reduces TTF-1 expression in a time- and dose-dependent manner in cultured H441 cells. In addition, Leinwand et al. (38) showed in whole organ culture that nitrogen-exposed mouse lungs have reduced expression of TTF-1. These studies show that nitrofen has a primary effect on TTF-1 expression in lung and must be considered when making comparisons in expression levels between experimental groups.

Several investigators have examined the effect of pulmonary hypoplasia on TTF-1 in lung. CDH caused no change in total TTF-1 mRNA expression in late gestation mice with nitrogen-induced CDH (10, 12), in TTF-1 protein content in sheep with surgically produced CDH (3), or in distribution of TTF-1 protein assessed by immunohistochemistry in human infants with CDH (23). However, in hypoplastic lungs there appears to be disruption of the normal developmental proximal-distal gradient of TTF-1 expression in which there is decreasing expression in proximal airway epithelium. TTF-1 expression persists in proximal airway epithelium, whereas expression patterns in the distal epithelium were normal in CDH lungs (12, 57) and in MyoD knockout mice in which the diaphragm muscle is markedly thinned and nonfunctional, resulting in pulmonary hypoplasia due to lack of fetal breathing movements (24). The latter studies suggest that, for normal fetal lung development, the expression pattern of TTF-1 may be as important as expression levels. We hypothesized that, because TO appears to reverse abnormalities of growth and maturation of the fetal lung that occur with CDH, it may also affect TTF-1 expression. Therefore, we examined TTF-1 protein levels in the lungs of animals with CDH, CDH+TO, and NC.

In the current study, TTF-1 protein levels were increased in CDH lungs compared with NC lungs (Fig. 5). This finding is consistent with the increase in SP-C expression seen with CDH, as TTF-1 is a transcriptional regulator of the SP-C gene (28). Although TTF-1 also regulates transcription of SP-A and SP-B (6), CDH did not affect expression of these genes. Therefore, factors other than decreased lung distension and increased TTF-1 must be necessary to upregulate SP-A and SP-B. In contrast to CDH, CDH+TO decreased TTF-1 expression to the level of NC lungs (Fig. 5). The decrease in expression of the mRNAs for the three SP suggests that a reduction in TTF-1 may have a negative influence on surfactant gene transcription. By immunohistochemistry, TTF-1 was increased in CDH lungs, both in distal airway and in distal parenchymal epithelium; CDH+TO had the opposite effect. Because we did not examine proximal airways, we do not have information on the effects of CDH and CDH+TO on the proximal-distal gradient expression pattern of TTF-1 seen in normally developing fetal lungs.

Our results indicate that changes in distension affect expression of TTF-1. These changes in TTF-1 may be responsible for the observed changes in maturation of type I and type II cells. Alternatively, changes in fetal lung distension may affect indicators of type I and type II cell maturation through other mechanisms independent of TTF-1. Additional studies will be needed to resolve this point.

In the summary, our results show that decreased distension of the fetal lung due to CDH caused pulmonary hypoplasia, retarded type I cell maturation, and increased expression of SP-C and TTF-1. Increased distension of CDH lungs (i.e., CDH+TO) reversed the pulmonary hypoplasia, promoted maturation of type I cells, and reduced expression of SP and TTF-1. These studies do not allow us to determine whether the changes in the type I cell-specific protein RTI40 were due to changes in type I cell number, size, or both. In conclusion, TO of the CDH lung reverses several of the effects of CDH alone, some of which may be mediated in modulations in TTF-1 expression.

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REFERENCES

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