Lung hypoplasia in the nitrofen model of congenital diaphragmatic hernia occurs early in development

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Guilbert, Theresa W., Sarah A. Gebb, and John M. Shannon. Lung hypoplasia in the nitrofen model of congenital diaphragmatic hernia (CDH) and pulmonary hypoplasia in rodent fetuses that closely parallel observations made in humans. We hypothesized that these changes may be due to primary pulmonary hypoplasia and not herniation of the abdominal contents. Timed-pregnant rats were given nitrofen on day 9, and fetuses were harvested on days 15 through 21. Initial evagination of lung buds on gestational day 11 was not delayed in nitrofen-treated fetuses. On gestational day 13, however, there was a significant decrease in the number of terminal end buds in the lungs of nitrofen-exposed fetuses vs. controls. Thymidine-labeled lung epithelial and mesenchymal cells were significantly decreased in nitrofen-treated lungs. Lungs from nitrofen-treated fetuses exhibited wide septae with disorganized, compacted tissue, particularly around the air spaces. Expression of surfactant protein B and C mRNAs was significantly decreased in the nitrofen litters. In situ hybridization of fetal lung tissue at all gestational ages showed no difference in the expression of vascular endothelial growth factor, Flk-1, or Flt-1 mRNAs. Because closure of the diaphragm is completed on gestational day 16 in the rat, our results suggest that lung hypoplasia in this model of CDH is due at least in part to a primary effect of nitrofen on the developing lung.

Lung branching; pulmonary hypoplasia; surfactant proteins; vascular endothelial growth factor; Flk-1; Flt-1; development

CONGENITAL DIAPHRAGMATIC HERNIA (CDH) is a significant clinical problem, occurring once in every 2,500–3,000 human births (51). Despite surgical advances and advances in neonatal intensive care, mortality has remained high at 40–50% (3, 22). CDH infants die primarily from pulmonary hypoplasia and severe persistent pulmonary hypertension, although concomitant cardiac defects contribute to mortality in a subpopulation of infants (50). Eighty-five percent of infants born with CDH have left-sided posterolateral diaphragmatic defects that cause herniation of the abdominal contents into the chest cavity. Microscopically, the lungs have a reduced number of bronchial branches, alveoli, and blood vessels (26). In addition, there is increased smooth muscle mass in vessels, with the greatest vessel abnormalities occurring in those with the worst pulmonary hypoplasia (19). Pulmonary hypoplasia is bilateral but is worse on the side with the diaphragmatic defect. Ten percent of CDH infants have other congenital abnormalities that include chromosomal and neurological defects, as well as cardiac defects such as a small left ventricle or atrial septal defect (16, 38, 52). To date, therapies directed at decreasing pulmonary hypertension in CDH infants, such as vasodilators (nitric oxide and talazoline), prostaglandins, hyperventilation, and exogenous surfactant have met with limited success (see Ref. 50 for review).

Normal diaphragm development is initiated in humans between the third and fourth weeks of gestation. The diaphragm begins as a mass of mesoderm bordered on all sides by epithelium. At 4–8 wk of gestation, the primitive diaphragm consists of three parts: 1) the transverse septum and the 2) “posthepatic mesenchymal plate” (PHMP) described by Iritani (23) are found ventrally and 3) the pleuroperitoneal canals are found caudally. The PHMP expands down and across the liver, and the diaphragm is completed when this structure reaches the pleuroperitoneal canals (23, 28). A long-held view of the pathogenesis of CDH is that it occurs between weeks 8 and 12, when the pleuroperitoneal canals fail to close. The resultant herniation of the abdominal viscera into the chest cavity and subsequent compression of the developing lung are thought to cause pulmonary hypoplasia, which is bilateral, although usually less severe on the side contralateral to the hernia. The potential role of physical compression in inducing pulmonary hypoplasia is supported by studies in fetal lambs (12). In these studies, a diaphragmatic hernia was created surgically, and the bowel was pulled into the chest to create compression, which then resulted in pulmonary hypoplasia; the lungs from these lambs had the morphological, hemo-
A model of CDH induced in rats and mice by the herbicide nitrofen (2,4-dichloro-p-nitrophenyl ether) produced CDH in the offspring that closely resembled the human disease; nitrofen treatment resulted in bilateral pulmonary hypoplasia and an immature lung architecture, along with other developmental abnormalities in the heart (35), eye (21), and kidney (53). Nitrofen must be given at a specific dose and time point in gestation so that lung hypoplasia and CDH occur, suggesting that a teratogenic insult at a critical point in development causes the congenital disease.

In the laboratory mouse, the lungs buds appear on gestational day 9.5 and the diaphragm closes on gestational day 13, which corresponds to the human embryonic period at weeks 4–10. Nitrofen (25 mg) given to pregnant dams on day 8 results in significant pulmonary hypoplasia and CDH in 50–80% of the offspring but does not result in these defects if given at other gestational ages or at lower doses (8). Using this model, Iritani (23) found that on day 11 the diaphragm was not yet closed, but the lung buds already appeared hypoplastic. He suggested that the diaphragmatic defect and lung hypoplasia occurred simultaneously and that complete closure of the diaphragm required normal lung and liver development.

The nitrofen model also has been studied extensively in rats by Tibboel and colleagues (6, 27, 49), who observed smaller lungs, smaller air spaces, thicker septa, and immature alveolar type II cells in treated fetuses (6); as in human CDH, all of these defects were present bilaterally. Ultrastructural analysis revealed abnormal clusters of liver cells and PHMP cells as the diaphragm began to form (27). However, no observations on the effects of nitrofen on lung growth and differentiation in these studies were made until day 16, when the diaphragm has normally closed in the rat (28).

In contrast to the data derived from the surgical model in fetal lambs, the observations described above suggest that the pulmonary hypoplasia in CDH may occur at the same time or even before the diaphragmatic defect. Iritani (23), Kluth et al. (27, 28) and Losty et al. (31) have all observed lung hypoplasia in nitrofen-treated fetuses that lacked a diaphragmatic hernia. The early, bilateral pulmonary hypoplasia and presence of other congenital defects suggest that a fundamental developmental defect may exist in CDH that simultaneously affects the growth of the lung and diaphragm as well as of other organs. To address this question, we studied the gross morphology, cell proliferation, and epithelial differentiation in nitrofen-treated and control fetal rat lungs before and after diaphragm development. Because of the pulmonary vascular abnormalities present in CDH, we have also examined the expression and spatial localization of mRNAs for vascular endothelial growth factor (VEGF) and its receptor Flk-1, which are involved in vascular development. Our results demonstrate that nitrofen produces pulmonary hypoplasia very early in development, before normal closure of the diaphragm. These data suggest that pulmonary hypoplasia is due at least in part to a direct teratogenic insult in the nitrofen model of CDH.

MATERIALS AND METHODS

Animals. Timed-pregnant Sprague-Dawley rats were obtained from Charles River Laboratories (Raleigh, NC). A sperm-positive vaginal smear confirmed mating and represented day 0 of gestation (day of birth, day 22). Pregnant females were given 100 mg of nitrofen (Chem Service, West Chester, PA) in 1 ml of olive oil by gavage on day 9 of gestation; controls were given 1 ml of olive oil alone. Pregnant dams were killed with a lethal intraperitoneal dose of pentobarbital sodium and transection of the aorta, and the fetuses were quickly removed by hysterotomy. The fetuses were weighed and killed by decapitation. All of the animal procedures utilized in this study were approved by the Institutional Animal Care and Use Committee of National Jewish Medical and Research Center (Animal Welfare Assurance Number A3025–1). After the fetuses were examined for a diaphragmatic hernia, the heart, trachea, and lungs were removed en bloc from the chest cavity. The lobes of the lung were dissected free from the major airways with Moria surgical knives (Fine Science Tools, Foster City, CA) and maintained on ice in Hanks’ balanced salt solution. Lungs from littermates were pooled, but individual litters were analyzed independently in all experiments except those for RNA analysis, which required pooling lungs from 2–3 litters to obtain sufficient quantities of RNA.

The average fetal weight per litter for the treatments was compared by a two-way ANOVA, with treatment, gestational age, and their interaction as predictors, with contrasts to test for treatment differences within each age group. All statistical tests were two-sided and conducted using $\alpha = 0.05$. The fetal weight was natural log transformed for this analysis.

Gross morphology and terminal bud counts. Lungs were photographed to document overall growth and morphology for each condition at each gestational age. To determine whether there were differences in lung branching between control and nitrofen-treated embryos, we counted the number of terminal buds of fetal lungs on gestational days 12 and 13. The lungs were removed and placed on ice, and then the number of terminal buds was determined for each embryo. Buds were scored as positive if the evagination from the lung wall comprised at least one-half of a sphere. Terminal bud counts in several litters were made by a second observer to verify results.

The statistical analysis of the average number of terminal lung buds in control vs. nitrofen-treated embryos was performed using a mixed model, with litter and treatment as predictors of effect. A second observer counted terminal buds in eight litters (4 control and 4 nitrofen treated), and these results were compared with those found by the first observer using an intraclass correlation coefficient. All statistical tests were two-sided and conducted using $\alpha = 0.05$.

Immunohistochemistry and morphometry. To distinguish mesenchyme from epithelium, a specific antibody against the intermediate filament protein vimentin, which is not expressed in lung epithelium, was used. Lungs were fixed in acid alcohol overnight at 4°C, dehydrated, and embedded in paraffin. Sections 4 μm thick were deparaffinized, rehydrated, and washed with PBS containing 0.5% hydrogen peroxide to inhibit endogenous peroxidase. Sections were blocked in PBS containing 3% horse serum (GIBCO BRL, Grand Island, NY) for 20 min and then incubated overnight with a 1:400 dilution of anti-vimentin (clone V-9; Boehringer
mannheim Biochemica, Indianapolis, IN) in PBS plus 3% horse serum at 4°C. After two washes with PBS, a 1:250 dilution of biotinylated horse anti-mouse IgG (Vector Laboratories, Burlingame, CA) in PBS plus 3% horse serum was added for 30 min. Sections were washed three times in PBS and then incubated in a 1:250 dilution of streptavidin-biotin-horseradish peroxidase (Amersham Pharmacia Biotech, Piscataway, NJ) in PBS plus 3% horse serum for 1 h at 4°C. After incubation in diaminobenzidine in 50 mM Tris buffer (pH 7.4) plus 0.5% hydrogen peroxide for 10 min, the sections were washed in tap water and counterstained lightly with hematoxylin.

The ratio of mesenchyme to epithelium across different gestational ages was determined using the National Institutes of Health Image software analysis program (Research Services Branch of the National Institute of Mental Health, Bethesda, MD). Slides were examined at \( \times 40 \) on a Zeiss microscope equipped with a JVC television camera. The image of the section was loaded into the Image program and analyzed by density profiling, a standard image processing function. In the density slice mode, all pixels between a lower and upper threshold were highlighted in red. Density calibration was performed using an optical density standard to match the intensity of the stained tissue so that only identifiable cells on the slide were highlighted. The images were calibrated to spatial standards by using the set scale command, which was set to a known value in square millimeters using a stage micrometer. The total area of stained cells within a randomized area of the lung was then measured starting at the top center of the tissue and proceeding in a clockwise fashion, moving two microscope fields at a time. The unstained areas of epithelium were removed using the eraser editor program, and the total area of stained cells minus the area of epithelium was remeasured. This measurement divided by the total area of stained cells gave a percentage of mesenchyme to whole lung.

The statistical analysis of the average vimentin area percentage was compared between nitrofen-treated and control litters using a two-way ANOVA. Treatment and gestational age and their interaction were used as predictors, with litters using a two-way ANOVA. Treatment and gestational age and their interaction were used as predictors, with control and nitrofen-treated lungs using a mixed model, with litter and treatment as predictors. All tests were two-sided and conducted using \( \alpha = 0.05 \).

**In situ hybridization.** Lungs were fixed in freshly prepared 4% paraformaldehyde in ribonuclease-free PBS overnight at 4°C, dehydrated, and embedded in paraffin. Sections 4 \( \mu \)m thick were hybridized with antisense and sense riboprobes for rat surfactant protein (SP) C, rat Fli-1, rat Flk-1, and rat VEGF. The SP-C riboprobes were transcribed from a full-length rat cDNA. Fli-1, Flk-1, and VEGF riboprobes were generated from a 1.5-kb Fli-1 fragment, a 1.7-kb Flk-1 fragment, and a 425-bp VEGF fragment, respectively, generated by RT-PCR of total RNA from day 19 fetal rat lung. The primers used to isolate the Fli-1, Flk-1, and VEGF fragments contained BamHI and EcoRI sequences at their ends to facilitate directional cloning. The rat Fli-1 primers corresponded to nucleotides 450–473 (5'-CGGATCCAAAAAGCT-GAGGCTCTACTAGTGC-3') and 1936–1957 (5'-GGAATTC-TTCAGGCCTCTCTTCGGGTG-3'). The rat Flk-1 primers corresponded to nucleotides 736–756 (5'-CGGATCCAAA-GATTTCTGGGACAGC-3') and 2457–2477 (5'-GGAATTC-TCAATAATAGGAGTGGCAGG-3') of the published rat Fli-1 cDNA sequence (33). The rat VEGF primers corresponded to nucleotides 1–21 (5'-CGGATCCAAACCATGAGAC-TTCTGCTCTC-3') and 404–424 (5'-GGAATTCATTITT-CTGGCTTGTTCTCATC-3'). This 424-bp fragment lies in the region of VEGF-A common to all four splice variants (10). Fragments were cloned into pGEM-4Z (Promega Biotech, Madison, WI) and linearized, and riboprobes were generated using \([\text{33P}]\) UTP (2,000 Ci/mmol; NEN) and a commercially available kit (Promega). The Fli-1 and Flk-1 transcripts were hydrolyzed in 80 mM NaHCO\(_3\) and 120 mM Na\(_2\)CO\(_3\) to yield fragments of \(\sim 300–400\) bp. In situ hybridization was performed as previously described (14, 18), with the exception that the riboprobes were labeled with \([\text{38P}]\) UTP. After hybridization and high-stringency washes, slides were dipped in NTB-2 nuclear track emulsion (Eastman Kodak) and developed after 4–7 days and lightly counterstained with hematoxylin.

**Ribonuclease protection assay.** Tissue for ribonuclease protection assay (RPA) was harvested and homogenized in 4 M guanidinium isothiocyanate and then stored at \(-70^\circ\)C until RNA isolation. Total RNA was isolated using a commercially available kit (5 Prime → 3 Prime, Boulder, CO). The cloning and sequencing of the cDNAs for rat SP-A, SP-B, SP-C, and SP-D have been described previously (15, 17, 44, 46). With use of full-length rat cDNAs as templates, fragments of varying sizes for the four surfactant proteins were isolated by PCR. The fragments were directionally cloned into pGEM-4Z (Promega), sequenced to verify their identity, and then used
as templates to generate single-strand riboprobes. A BamH I site was placed at the 5’ end of the forward primer and an EcoR I site was placed at the 5’ end of the backward primer to facilitate directional cloning. The primers for SP-A were 5’-CGGATCCAGTCCTCAGCTTGCAAGGATC-3’ coding sense, corresponding to nucleotides 424–444, and 5’-GGAATTCCGTTCTCCTCAGGAGTCCTCG-3’ coding antisense, corresponding to nucleotides 549–569; a probe 200 bp in length was transcribed from this clone. The primers for SP-B were 5’-CGGATCCGAGCAGTTTGTGGAACAGCAC-3’ coding sense, corresponding to nucleotides 381–401; a probe 200 bp in length was transcribed from this clone. The primers for SP-C were 5’-CGGATCCCATCTGAGATGGTCCTTGAG-3’ coding sense, corresponding to nucleotides 1152–1172; a probe 176 bp in length was transcribed from this clone. The primers for SP-D were 5’-GGAATTCTCTGGAGCCATCTTCATGATG-3’ coding sense, corresponding to nucleotides 997–1017, and 5’-GGAATTCTGGTCCTTTGGTACAGGTTGC-3’ coding antisense, corresponding to nucleotides 1152–1172; a probe 176 bp in length was transcribed from this clone.

The constructs were linearized with BamH I and were labeled to high-specific activity with [32P]CTP (800 Ci/mmol; ICN, Irvine, CA) using a commercially available kit (Promega) and purified on an 8% polyacrylamide-7 M urea gel. Purified riboprobes were hybridized with 10 μg of fetal lung total RNA for 18 h at 45°C and then digested with ribonucleases A and T1 to remove unhybridized probe and RNA. The protected RNA duplexes were precipitated, resuspended in sample buffer, and electrophoresed on an 8% polyacrylamide-7 M urea gel. The gel was dried and exposed to Hyperfilm (Amersham Life Science Products, Arlington Heights, IL) at −70°C. The mRNA bands for SP-A, SP-B, SP-C, and SP-D were quantified using a PhosphorImager and ImageQuant software (Molecular Dynamics, Sunnyvale, CA). The relative amounts of surfactant protein mRNAs were normalized to an 18S rRNA with a riboprobe that was generated according to the protocol provided by the vendor (Ambion T7 MEGAshort script) and added to the same hybridization reaction. This surfactant protein RPA provides linear results over a range of 1 to at least 20 μg of total lung RNA.

The relative quantity of surfactant protein mRNA normalized to 18S for the two treatments was compared within each age group using a two-way ANOVA, with condition (control, nitrofen-treated lungs ipsilateral to CDH, or nitrofen-treated lungs contralateral to CDH), gestational age, and their interaction as predictors. Contrasts were used to test for treatment differences within each gestational age group. The average body weight was significantly decreased (*P < 0.05 and †P < 0.02) in nitrofen-treated fetuses on gestational days 19 (n = 20) and 21 (n = 9). Fetal weights were not significantly different in litters on gestational days 13 through 17.

Gross morphology. Eighty-four timed-pregnant Sprague-Dawley rats were treated with either olive oil (n = 37) or olive oil plus 100 mg of nitrofen (n = 47). To determine whether differences in fetal body weight existed, the fetuses were first weighed and then dissected under a microscope to document the presence of a hernia. Average fetal body weights were significantly decreased in late-gestation (days 19 and 21) nitrofen-treated fetuses compared with controls (Fig. 1) but were not significantly different in litters on gestational days 13 through 17. The occurrence of diaphragmatic hernias was 56–76%, which is similar to that reported by other investigators, and the majority of hernias were left-sided (8, 23, 28). Day 13 and 15 litters had no visible diaphragm closure; however, the lungs of nitrofen-treated fetuses at these ages appeared more posterior, inferior, and closer to the liver compared with controls.

The nitrofen-treated pups also had lungs that were smaller and more friable than control animals at all gestational ages studied. Furthermore, on day 17, but not at any other stage, subcutaneous edema was noted along the posterior body wall of the nitrofen fetuses (Fig. 2); subcutaneous edema was not observed in control animals at any gestational age. The friability noted in the lungs was seen at all gestational ages and appeared to affect the entire fetus.

Induction of lung buds and branching morphogenesis. Lung growth and patterning were documented by examining the lungs of 245 fetuses (108 control and 137 nitrofen) from 21 litters; eight litters were independently examined by a second observer. Lungs were
examined on gestational day 11, shortly after the lung primordium evaginates from the rat foregut endoderm, as well as on days 12 and 13. Gross lung size was examined, and terminal buds, which eventually become lobes of the lung, were counted in the day 13 lungs. No differences were noted in either the timing of lung bud outgrowth on gestational day 11 or lung size or branching on gestational day 12 between the treated and control groups. However, a significant ($P < 0.001$) decrease in lung branching was seen in the nitrofen-treated group on gestational day 13 (Fig. 3); whereas control lungs had an average of 6.2 terminal buds, lungs from nitrofen-treated fetuses had only 4.2 buds. No significant difference in results was found when comparing the numbers generated by each observer (intraclass correlation coefficient 0.77, $P = 0.850$). Lungs from nitrofen-treated fetuses on day 15 also appeared to have a reduced number of terminal buds, but this could not be quantitated because of the complexity of lung branching at this stage of development.

**Morphometry.** The percentage of mesenchymal tissue, as identified by vimentin staining, compared with...
mesenchymal tissue in nitrofen-treated lungs on gestational days 13, 15, 17, and 21. The most dramatic difference was seen on day 21, when mesenchymal thinning is well under way in normal lung development. The mean percentage was not significantly different for the day 19 litters.

**Proliferation.** Fetal lungs from eight gestational day 13 litters were labeled with [³H]thymidine for 4 h after harvest and then either processed for analysis of thymidine incorporation or fixed and processed for determination of the tissue labeling index by autoradiography. Examination of 9,600 cells from six different litters demonstrated that nitrofen-treated tissues had a significant decrease of ~30% in labeled cells in the epithelial compartment (n = 4,800 cells, P = 0.001) and a significant decrease of ~10% in mesenchymal-labeled cells (n = 4,800 cells, P = 0.004; Fig. 5). There was, however, no significant difference (P = 0.217) in [³H]thymidine incorporation per microgram of DNA between control (mean 3,350 ± 34 cpm/µg DNA) and nitrofen groups (mean 4,450 ± 685 cpm/µg DNA).

**Morphology.** Sections examined by light microscopy revealed that nitrofen-treated fetuses had more immature lungs than controls at early gestational ages. This immaturity became more apparent as the fetal age advanced. Air space size was substantially reduced and septa were notably thicker in nitrofen-treated lungs on days 19 and 21 (Fig. 6, A and B), suggesting that lung development had been retarded. Whereas distal epithelial acini appeared less organized in nitrofen-treated lungs, the cells nonetheless appeared differentiated, containing abundant amounts of glycogen deposits and darkly stained inclusion bodies. Generally, the epithelial cells in nitrofen-treated lungs remained columnar at later gestational ages instead of transitioning to the more cuboidal appearance associated with maturation.

When examined by electron microscopy, nitrofen-treated lung tissue exhibited wide septa with disorganized, compacted tissue, particularly around the air spaces. However, tubular myelin figures and osmophilic lamellar bodies were seen in the air spaces of both nitrofen-treated and control lungs, suggesting that type II cell maturation had progressed (Fig. 6, C and D). No differences in type II cell maturation were noted between nitrofen-treated lungs on the ipsilateral and contralateral side of the herniated diaphragm.

**Surfactant protein gene expression.** The lungs from 15 litters (7 control and 8 nitrofen) with gestational ages of 19 (n = 8) and 21 (n = 7) days were examined by RPA to determine differences in the relative amounts of surfactant protein mRNAs normalized to 18S rRNA. Significant decreases in SP-B and SP-C mRNAs (P = 0.047 and P = 0.049, respectively) were found in day 21 nitrofen-treated fetuses compared with controls. Significant decreases (P = 0.05, 0.05, 0.005, and 0.08 for SP-A, SP-B, SP-C, and SP-D, respectively) also were seen in the mRNA levels for SP-A, SP-B, and SP-C in day 21 fetuses when comparing mRNA levels in the lungs ipsilateral to the defect (ipsilateral means = 0.655, 1.225, 2.505, and 0.2255 for SP-A,

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Fig. 4. Top: lungs from control (A, C, and E) and nitrofen-treated (B, D, and F) fetuses were immunostained with an antibody against vimentin. An increase in the percentage of mesenchymal tissue in nitrofen-treated vs. control lungs was apparent on days 15 (A and B) and 17 (C and D) but was particularly striking on day 21 (E and F). All panels are at the same magnification (original magnification, ×75). Bottom: ratio of mesenchyme to epithelium was increased in nitrofen-treated vs. control lungs throughout gestation. Sections stained as on top were analyzed using the National Institutes of Health Image software analysis program to determine the percentage of vimentin-positive area was compared between nitrofen-treated and control litters using a two-way ANOVA. Treatment and gestational age and their interaction were used as predictors, with contrasts to test for treatment differences within each group. The percentage of mesenchymal tissue was significantly (**P < 0.01 and *P < 0.05) increased in nitrofen-treated lungs compared with controls at all gestational ages except day 19.
SP-B, SP-C, and SP-D, respectively) with contralateral lungs (contralateral means = 0.771, 1.866, 4.121, and 0.338 for SP-A, SP-B, SP-C, and SP-D, respectively). No differences in expression of any surfactant protein mRNA were found between nitrofen-treated and control lungs on gestational day 19 and in SP-D mRNA expression on gestational day 21 (Fig. 7).

The spatial expression of the type II cell marker SP-C was examined by in situ hybridization in fetal lungs on gestational days 13 through 21. Across all ages, SP-C mRNA expression was seen in the distal lung parenchyma. No obvious differences were seen in the spatial expression of SP-C mRNA between nitrofen-treated and control litters or in the intensity of the hybridization signal (Fig. 8). Hybridization with radio-labeled sense SP-C riboprobe was uniformly negative (data not shown).

VEGF, Flk-1, and Flt-1 expression. Because human infants with CDH often have decreased vascular development and pulmonary hypertension, we also examined the spatial expression of the vascular markers Flk-1 and Flt-1 and their ligand VEGF on gestational days 11 through 21 by in situ hybridization. VEGF mRNA was diffusely expressed in both epithelial and mesenchymal cells in the proximal and distal lung, and the intensity of its expression per cell increased with gestational age (data not shown). As we have previously shown (18), Flk-1 and Flt-1 mRNAs were observed in mesenchymal cells subtending both the proximal and distal epithelium at every stage of development. Flk-1 expression was limited to small groups of cells in early gestation but became much more diffuse as development proceeded. The pattern of Flk-1 expression appeared more extensive in control vs. nitrofen-treated lungs, particularly at early points in gestation. The intensity of the Flk-1 hybridization signal per cell, however, appeared similar at all gestational ages examined. Sections hybridized with radio-labeled sense probes for VEGF, Flk-1, and Flt-1 showed no hybridization signal (data not shown).

DISCUSSION

Despite a significant amount of clinical and basic research, the pathogenesis of CDH remains unclear. The most obvious explanation, that compression of the...
developing lung caused by herniation of viscera into the thoracic cavity leads to hypoplasia, is supported by a large literature using the fetal lamb surgical model. The lamb surgical model, however, does not completely duplicate the observations made on infants with CDH. Specifically, although the lung ipsilateral to the defect is hypoplastic, these lambs typically do not have extensive bilateral hypoplasia or the other congenital defects observed in human infants with CDH. It also should be noted that the diaphragmatic defect in humans does not occur by trauma (56). Nevertheless, the fact that the physical intrusion of viscera into the chest cavity results in pulmonary hypoplasia cannot be disregarded.

Observations made in nitrofen-treated rodents have provided an alternative to the view that CDH results solely from a hole in the diaphragm. The possibility that nitrofen has direct teratogenic effects on the developing lung was first suggested by the ultrastructural studies of Iritani (23), who observed murine lung bud hypoplasia on gestational days 11 and 12, followed by hypoplasia of the PHMP on day 13. This led to the speculation that closure of the diaphragm required a normally developed lung and that pulmonary hypoplasia was the cause of rather than the result of CDH. Using rat fetuses, Kluth et al. (28) observed that the lungs of day 14 nitrofen-treated fetuses were smaller, depending on the amount of liver present in the thoracic cavity, but that histological evidence of compression was not present. In a scanning electron microscopic study, Kluth et al. (27) concluded that the pleuroperitoneal canals are never wide enough to allow herniation of gut loops and that the diaphragmatic defect is a result of alterations in development of the PHMP that occur much earlier than previously thought. A recent study (1) in which a number of hypotheses regarding the pathogenesis of CDH were examined also concluded that nitrofen affects the initial stages of diaphragm development. This study also questioned the identity of the PHMP, suggesting that it is likely a portion of the pleuroperitoneal fold. Our data suggest that nitrofen causes a primary pulmonary hypoplasia that is evident before formation of the diaphragm is complete. Nothing in our data, however, suggests that malformation of the diaphragm is necessarily linked to this pulmonary hypoplasia, as suggested by Iritani (23).

Our data are the first quantitative demonstrations that nitrofen-treated fetuses have an early abnormality in the extent of lung branching morphogenesis that is initiated before completion of the diaphragm, which occurs on gestational day 16 in the rat. We observed that the initial evagination of the lung rudiments from the foregut endoderm on day 11 occurs normally in nitrofen-treated fetuses, but then the effects of nitrofen treatment become apparent between gestational days 12 and 13. This decreased growth persists throughout gestation, as evidenced by an increased ratio of mesenchyme to epithelium in nitrofen-treated vs. control fetuses. The proportion of lung mesenchyme gradually decreases over the course of gestation in normal development, but this process is retarded in nitrofen-treated fetuses. This could be due to an increased mesenchymal growth rate, a decreased epithelial growth rate, or a combination of both processes. Our data show that proliferation in both the epithelial and mesenchymal compartments is reduced in nitrofen-treated vs. control fetuses. The morphology of the nitrofen-treated lung tissue as observed by light and electron microscopy also appeared disorganized, particularly around the air space lumens, which were fewer in number and surrounded by thickened septa. Despite the tissue disorganization, the epithelial cells appeared to mature in parallel with...
those of control fetuses as evidenced by the presence of lamellar bodies in both the epithelial cells and lumens, along with secreted tubular myelin figures. This lack of effect of nitrofen on type II cell maturation has also been reported by other investigators (6).

The effects of nitrofen on expression of SP-A have been mixed. Coleman et al. (9) observed significant decreases in SP-A mRNA and protein in nitrofen-treated mouse fetuses late in gestation. Similar to our observations, Brandsma et al. (6) reported seeing no differences in SP-A in bronchoalveolar lavage fluid from nitrofen-treated rat fetuses with and without CDH. Mysore et al. (37) also saw no differences in SP-A expression among day 19 rat fetuses that received vehicle only, that were exposed to nitrofen and exhibited a diaphragmatic hernia, or that were exposed to nitrofen but did not develop a hernia. Decreases in SP-A mRNA and protein became apparent, however, when they examined nitrofen-treated fetuses on day 21. Thus it appears that the effects of nitrofen on SP-A expression are greatly influenced by the gestational age at which fetuses are evaluated.

We also observed significant decreases in the relative amount of mRNAs for SP-B and SP-C in late-gestation fetuses. Our data are the first reported for SP-B mRNA expression in this model. If the differences we observed reflect a true decrease in SP-B expression, it might have potentially important implications given the importance of SP-B to neonatal survival (39). Our RPA data, however, must be viewed with caution. The decrease in the ratio of mesenchyme to epithelium in nitrofen-treated fetuses results in a higher proportion of total RNA coming from cells that do not express surfactant proteins. Thus while the relative amount of SP-B and SP-C mRNAs found in total lung RNA may be decreased, there may be no change in the amount of these mRNAs on a per cell basis. This possibility is supported by our in situ hybridization results for SP-C mRNA, which, while not quantitative, showed no obvious difference in the intensity of SP-C expression in the distal epithelium in control and nitrofen-treated fetuses. Consistent with our in situ hybridization data, Coleman et al. (9) also did not observe any difference in SP-C expression in nitrofen-treated mice by immunostaining or RT-PCR. It should be noted that the role of the surfactant system in CDH may not be of primary importance because infants with CDH do not appear to have a positive response to exogenous surfactant administered in the perinatal period.

Previous studies have demonstrated that all nitrofen-treated rat fetuses have pulmonary hypoplasia, but growth retardation is greater in those with hernias. Consistent with these observations are our data that show that surfactant protein mRNA expression is decreased in the more hypoplastic lungs (those ipsilateral to the hernia) compared with the contralateral lung on the nonherniated side. Thus hypoplastic lungs in nitrofen-induced CDH may be the result of two coinci-

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**Fig. 7.** Left: comparison of surfactant protein gene expression in lungs from control and nitrofen-treated fetuses. Total RNA from isolated lungs of control and nitrofen-treated fetuses was hybridized with radiolabeled riboprobes for surfactant protein (SP) A, SP-B, SP-C, and SP-D. Ribonuclease-protected duplexes were resolved on an 8% polyacrylamide gel. This panel shows a representative autoradiogram. Right: signal from the protected bands was quantified by phosphorimaging. The average quantity of surfactant protein mRNA was normalized to 18S for the 2 treatments and was compared within each age group using a two-way ANOVA with condition (control and nitrofen-treated), gestational age, and their interaction as predictors. Contrasts were used to test for treatment differences at each gestational age. Significant (*P < 0.05) decreases were found in the amount of SP-B and SP-C mRNAs in lungs ipsilateral to the hernia in day 21 nitrofen-treated fetuses compared with control lungs and to lungs contralateral to the hernia. No differences were found in the expression of surfactant protein mRNAs between nitrofen and control lungs on gestational day 19 and for SP-D mRNA expression on gestational day 21.
dent processes: a primary developmental defect that is further exacerbated by herniation of the viscera and its resultant compression of the lung. This explanation would reconcile the observations made in model systems using teratogens such as nitrofen with those made in the lamb model of surgically created CDH. This possibility is strengthened by the recent demonstration that nitrofen has direct effects on lung explants in vitro (24).

As noted previously, human infants and animals with CDH have severe pulmonary hypertension with thick muscular vessel walls. Such infants can be difficult to manage, and pulmonary hypertension is a frequent cause of mortality (16, 50, 52). Because the nitrofen model of CDH resembles the human condition (42), we studied the spatial expression of early vascular markers to see whether abnormalities in vasculogenic patterning could explain the vascular abnormalities of CDH. In examining lungs from fetuses on days 11 through 21, the hybridization signal of the early vascular markers Flk-1, Flt-1, or their ligand VEGF appeared unchanged when comparing nitrofen-treated lungs with controls. The complexity of the pulmonary vasculogenic network appeared retarded in nitrofen-treated fetuses, especially at the early (days 11 through 15) time points in development. These observations are in agreement with those of Coleman et al. (9), who observed a decrease in the number of platelet endothelial cell adhesion molecule-1 (PECAM-1)-positive capillaries in the lungs of nitrofen-treated mice. They also reported an increase in the distance between the PECAM-1-positive vasculature and the epithelium in nitrofen-treated fetuses. The limits of resolution of our in situ hybridization did not allow us to make these measurements. However, we previously have reported that Flk-1-positive cells become progressively more closely apposed to the pulmonary epithelium during development (18). Given the apparent retardation of lung development seen in nitrofen-treated rat fetuses, we would predict that there would be a similar delay in the development of air space and capillary apposition. If VEGF, Flk-1, or Flt-1 is directly involved in the development of vascular abnormalities in nitrofen-treated lungs, the abnormality in expression of these genes must occur before gestational day 11. A more likely explanation is that other aspects of vascular development are affected, such as abnormal communication between vasculogenic cells and epithelial cells or other mesenchymal cells.

Another possible explanation for the vascular abnormalities seen in CDH is that the expression of endothelin, a potent vasoconstrictor, is enhanced. A number of human and rat studies have demonstrated increased endothelin-1 mRNA expression and immunostaining in CDH; whether this is a true cause of the disease or a marker of other events leading to the disease is not yet known (11, 40). However, increased endothelin expression does not explain the decreased numbers of vessels or morphological changes, such as a thick muscular wall, seen in vessels in CDH lungs.

The severe, bilateral lung hypoplasia and the decreased number of terminal lung buds seen in nitrofen-treated fetuses suggest that an insult to lung growth occurs between gestational days 12 and 13 that leads to decreased cellular division. On gestational day 13, when a significant decrease in lung branching was noted in nitrofen-treated lungs, the labeling index was decreased by 17.6% in the epithelial compartment of nitrofen-treated lungs and by 8% in the mesenchymal compartment, suggesting a slowing in cellular division in both compartments due to a developmental abnormality. In addition, there was a marked decrease in the body weight of nitrofen-treated fetuses at later gestational ages, suggesting a retardation of overall fetal growth.
growth and development. This could be due to the loss or downregulation of a pleiotrophic growth regulatory pathway.

The loss or impedance of a general growth regulatory mechanism might also explain the variety of organ abnormalities seen in human infants with CDH and in rat fetuses exposed to nitrofen. One potential candidate growth factor is insulin-like growth factor (IGF). In a recent study using transgenic mice, Louvi et al. (32) described the effects of the loss of IGF-I and its receptors IGF receptor-I and insulin receptor on fetal development. In mice lacking both IGF receptor-I and insulin receptor, several intriguing similarities to nitrofen-exposed fetuses were noted, including fetal weight retardation, multiorgan hypoplasia (including lung hypoplasia), death from pulmonary insufficiency, and a transient subcutaneous body wall edema. Oue et al. (43) have reported that IGF-I and IGF-II are significantly increased in nitrofen-treated rat fetuses harvested just before term. Similarly, Miyazaki et al. (36) described an increased spatial expression of IGF-I mRNA in human newborns with CDH compared with controls. Normally, the expression of IGF-I decreases before birth (55), and its persistence may represent a delay in its expression during fetal life or a lack of a negative-feedback control to shut off the production of IGF-I.

IGF is regulated by growth hormone, which is, in turn, regulated by thyroid hormone (20). Studies by Brandsma et al. (5) have suggested that noncompetitive binding of T3 receptor by nitrofen causes the resulting congenital defects, and a differential display study of genes expressed in the murine nitrofen model identified reduced expression of cDNAs that may have homology to the thyroid hormone receptor gene and fibroblast growth factor receptor-3 (7). However, a recent study by Tovar et al. (54) found no decrease in the amount of T3 or T4 in fetal lung tissue exposed to nitrofen when corrected to DNA content. Furthermore, thyrotropin-releasing hormone given by itself to nitrofen-treated rats had minimal effects on lung morphology (30) or pulmonary compliance (31). Thus the role of thyroid hormone in nitrofen-induced CDH remains unclear.

Compounds that bind to the steroid-thyroid-retinoid superfamily of receptors, such as glucocorticoids and retinoic acid, have a clear impact on lung growth and differentiation in the nitrofen model of CDH. Dexamethasone administered antenatally to nitrofen-treated fetuses resulted in increased lung disaturated phosphatidylcholine, decreased lung glycogen, and reduced septal thickness, all indexes of distal lung maturation (47). Improvement in morphology (30) was accompanied by improved compliance (31). Antenatal dexamethasone also has been shown to prevent pulmonary vascular muscularization in nitrofen-treated rats (48), perhaps via suppression of angiotensin-converting enzyme (41).

Evidence also suggests that retinoids may have an important role in the pathogenesis of CDH. The offspring of pregnant rats maintained on a vitamin A-deficient diet have a high incidence of CDH (57). This correlates with the observation that infants with CDH have ~50% less plasma retinol and retinol-binding protein than age-matched controls (33). A recent study by Thebaud et al. (51) has shown that a single dose of vitamin A reduced both the incidence and severity of CDH in nitrofen-treated rats. From these data, they speculated that nitrofen may act by competing with retinoic acid for retinoic acid receptors. If this is true, it would provide an explanation for the occurrence of developmental abnormalities in other organs that occur concomitantly with CDH. For example, cardiac malformations are often found in conjunction with CDH (2), and normal cardiac development requires retinoids (13). Another possibility is that retinoic acid, which increases growth hormone secretion (4), indirectly affects growth by increasing IGF expression.

Lung branching and growth are absolutely dependent on epithelial-mesenchymal tissue interactions that are operative from the initiation of lung development (for review see Ref. 45). Our data demonstrate that there is an early developmental defect in lung patterning and growth in rat fetuses exposed to nitrofen that occurs before diaphragm development. Our data demonstrate that there is a decrease in the proliferative capacity of epithelial and mesenchymal tissues in nitrofen-treated pups, which suggests the possibility that the observed pulmonary hypoplasia is the result of disruption of the normal tissue interactions that drive normal lung growth and morphogenesis. This possibility is currently under investigation.

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