Pathogenesis of nitrofen-induced congenital diaphragmatic hernia in fetal rats

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Allan, Douglas W., and John J. Greer. Pathogenesis of nitrofen-induced congenital diaphragmatic hernia in fetal rats. J. Appl. Physiol. 83(2): 338–347, 1997.—Congenital diaphragmatic hernia (CDH) is a developmental anomaly characterized by the malformation of the diaphragm and impaired lung development. In the present study, we tested several hypotheses regarding the pathogenesis of CDH, including those suggesting that the primary defect is due to abnormal 1) lung development, 2) phrenic nerve formation, 3) developmental processes underlying diaphragmatic myotube formation, 4) pleuroperitoneal canal closure, or 5) formation of the primordial diaphragm within the pleuroperitoneal fold. The 2,4-dichloro-phenyl-nitrophenyl ether (nitrofen)-induced CDH rat model was used for this study. The following parameters were compared between normal and herniated fetal rats at various stages of development: 1) weight, protein, and DNA content of lungs; 2) phrenic nerve diameter, axonal number, and motoneuron distribution; 3) formation of the phrenic nerve intramuscular branching pattern and diaphragmatic myotube formation; and 4) formation of the precursor of the diaphragmatic musculature, the pleuroperitoneal fold. We demonstrated that previously proposed theories regarding the primary role of the lung, phrenic nerve, myotube formation, and the closure of pleuroperitoneal canal in the pathogenesis of CDH are incorrect. Rather, the primary defect associated with CDH, at least in the nitrofen rat model, occurs at the earliest stage of diaphragm development, the formation of the pleuroperitoneal fold.

breathing; lung; phrenic

CONGENITAL DIAPHRAGMATIC HERNIA (CDH) is a developmental anomaly characterized by the malformation of the diaphragm. Normally, during in utero development, the diaphragm develops to form a continuous sheet that completely separates the thoracic and abdominal cavities. However, in the instance of CDH, regions of the diaphragm are missing. Consequently, the developing viscera have access through an opening in the diaphragm to the thoracic cavity, which they invade, occupying space normally reserved to accommodate the growing lungs. As a result, newborns with CDH (~1:3,000 births) suffer from a combination of pulmonary hypoplasia, pulmonary hypertension, and surfactant deficiency (19, 28). The mortality rate of infants suffering from CDH is ~50% (17). Moreover, it has been estimated that an additional 1 in 2,000 conceptions fails to reach term because of complications associated with CDH (15).

Although the mechanisms underlying the etiology of CDH remain obscure, there are a number of theories pertaining to the pathogenesis of the condition. First, a persistent theory states that the diaphragmatic malformation is merely a secondary defect caused by the maldevelopment of the adjacent lung tissue (18). Second, Iritani (18) has suggested that a perturbation of the normal innervation of the diaphragm by the phrenic nerve may subsequently arrest proper diaphragmatic muscle development. A third explanation for CDH, the most commonly cited, states that there is an abnormality with the closure of the pleuroperitoneal canal (27). Fourth, it has been suggested that the developing muscle fibers (myotubes) within the region of herniation may fail to form or are weak and prone to rupture in the presence of underlying forces produced by the expanding abdominal contents (27). However, very little scientific data support any of these theories, and a basic understanding is lacking of how the normal, let alone the pathological, diaphragm develops (20, 27). To increase such an understanding, we have examined the normal ontogenesis of the phrenic nerve and diaphragm in fetal rats (2) and, in the present study, we systematically test each of the above-mentioned hypotheses regarding the pathogenesis of CDH.

We have utilized the 2,4-dichloro-phenyl-p-nitrophenyl ether (nitrofen)-induced CDH rat model to examine lung growth, phrenic nerve innervation, and the formation of the diaphragmatic musculature in instances of CDH. We determined whether abnormalities in their development were either primary or secondary to herniation of the diaphragm. Nitrofen is a toxic herbicide that, when given as a single oral dose to a pregnant rat, produces a condition in fetal rats remarkably similar to that associated with CDH observed in human infants (5, 20–22, 26, 30). The weight, protein content, and DNA content of lungs (31) were compared in CDH and control animals before and after invasion of visceral organs into the thoracic cavity. Immunohistochemical labeling for growth-associated protein (GAP-43) and the polysialylated form of the neural cell adhesion molecule (PSA-NCAM), both expressed within growing phrenic axons (3), was performed to determine the pattern of phrenic axon intramuscular branching from its onset at embryonic day 14.5 (E14.5) up until near its completion at E18. In conjunction with GAP-43 and PSA-NCAM immunolabeling, electron microscopy of phrenic nerve cross sections provided information regarding nerve diameter and axonal number before and after programmed neuronal cell death in instances of CDH. Furthermore, we retrogradely labeled phrenic motoneuron cell bodies with the lipophilic dye 1′,1′-dioctadecyl-3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI) to examine the distribution of motoneurons in normal vs. CDH fetuses. Finally, the formation of the primordial diaphragmatic anlage, the pleuroperitoneal fold, and the onset of myotube formation within
the diaphragm was examined via immunolabeling for low-affinity nerve growth factor receptor (p75) and PSA-NCAM. As shown in the present study, the primordial diaphragm expresses the p75 receptor, whereas, as previously reported, developing diaphragmatic myotubes express PSA-NCAM (3).

METHODS

Delivery of Nitrofen

It was noted, during initial routine toxicological studies of the herbicide nitrofen, that, although it was relatively non-toxic to adult animals, it produced a number of lethal abnormalities associated with pulmonary and cardiovascular malfunction in fetuses exposed prenatally (5, 9, 13). Furthermore, when given as a single dose on gestation day E9 or E11, the major abnormality produced by nitrofen was a maldevelopment of the diaphragm that was strikingly similar to the developmental anomaly CDH. To produce diaphragmatic malformations specifically, the timing of the nitrofen delivery to the dam is critical. Administration of a single dose on day E9 produces hernias on the left and/or right side of the diaphragm. Delivery of nitrofen on day E11 produces solely right-sided hernias. The number of fetuses within a dam affected by the nitrofen is dose dependent and may also vary with the efficacy of teratogen transfer to each of the respective fetuses in a litter.

In the present study, nitrofen was obtained from the United States Environmental Protection Agency and prepared as a solution of 100 mg/ml in olive oil. The timing of pregnancies was determined from the appearance of sperm plugs in the breeding cages (time designated as E0). Pregnant dams on the ninth day of gestation (E9) were temporarily (−10 min) anesthetized with halothane (1.25% in 95% O2-5% CO2) and given 100 mg of nitrofen via a gavage tube to produce either left, right, or bilateral hernias (Table 1). In two animals, nitrofen was administered on E11 to produce solely right-sided hernias.

Cesarian Section and Tissue Preparation

Control and nitrofen-exposed fetal rats (E13.5-E18) were delivered by cesarean section from timed-pregnant Sprague-Dawley rats anesthetized with halothane (1.25% in 95% O2-5% CO2) and maintained at −37°C by radiant heat. The ages of fetuses were confirmed by comparison of their crown-rump length measurements with those published by Angulo y Gonzalez (6). This measurement is important for confirming the precise age of individual fetuses. Embryos were decapitated, and, while they were immersed in 4% paraformaldehyde in a 0.2 M sodium phosphate buffer (pH 7.4), the thoracic and abdominal cavities were exposed. The tissue was then postfixed at 4°C for between 4 and 20 h.

Table 1. Incidence of diaphragmatic hernias induced by nitrofen (administered E9)

<table>
<thead>
<tr>
<th>Total No. of Hernias</th>
<th>Normal</th>
<th>Left Hernia</th>
<th>Right Hernia</th>
<th>Bilateral Hernia</th>
</tr>
</thead>
<tbody>
<tr>
<td>181 (17 dams)</td>
<td>86 (48%)</td>
<td>39 (21%)</td>
<td>45 (25%)</td>
<td>11 (6%)</td>
</tr>
</tbody>
</table>

E9, day 9 of gestation; nitrofen, 2,4-dichloro-phenyl-p-nitrophenyl ether. Note that fetal reabsorptions had occurred on several occasions. Also, in 3 experiments, all fetuses present at time of inspection were normal despite administration of nitrofen. On 2 further occasions, nitrofen was administered on E11; a total of 0 left and 9 right hernias was produced compared with 21 normals. None of these data are included in the table.

Whole Embryo Sections

After fixation, fetuses were washed in phosphate-buffered saline (PBS) and then placed for 1 hr in a 7.5% gelatin solution maintained at 30°C. They were then embedded in a 20% gelatin-glycerol solution that was hardened by cooling. A small block containing the fetus was excised and fixed overnight. The block was appropriately oriented for transverse sectioning and was then vibratome sectioned at 50 µm. Serial sections were washed in PBS, immunostained while free floating, and then mounted onto gelatin-coated slides, dried, covered slipped, examined, and photographed by standard light microscopy. Photographs were digitally scanned (Scanjet et, Hewlett-Packard, Palo Alto, CA) into a computer, labeled by using CorelDraw software (Microsoft), and printed with a dye sublimation printer (Codonics NP600, Mitsubishi).

Diaphragm Whole Mounts

Whole diaphragms were isolated immediately from fetal rats, pinned flat, and postfixed for 4–20 h. They were then washed in PBS, immunostained while free floating, and mounted on gelatin-coated slides.

Immunolabeling

Antibodies. All primary antibodies used were diluted in PBS (pH 7.4) with 1% goat serum and 0.1% NaN3. We further added 0.4% Triton X-100 with the anti-p75 monoclonal antibody (MAb). Detection of GAP-43 was performed by using the mouse anti-GAP-43 immunoglobulin G (IgG) MAb (Sigma Chemical, St. Louis, MO) at a dilution of 1:500. PSA-NCAM was detected by using the mouse anti-PSA IgM MAb 12E3 (a generous gift from T. Seki, Juntendo University, Tokyo, Japan). 12E3-containing ascites fluid was diluted to 1:5,000–1:10,000. We detected p75 by using a mouse anti-p75 IgG MAb at a dilution of 1:75 (Boehringer Mannheim, Laval, Quebec). All secondary antibodies were diluted to 1:200 in PBS containing 1% goat serum. Secondary antibodies used for GAP-43, PSA-NCAM, and p75 receptor labeling were peroxidase-conjugated goat anti-mouse IgG (whole molecule; Sigma Chemical), biotinylated goat anti-mouse IgM (μ-chain specific; Sigma Chemical), and biotinylated goat anti-mouse IgG (whole molecule; Sigma Chemical), respectively.

Immunohistoch exy. Tissues for all immunolabeling, except for anti-p75, were immersed in methanol containing 3% H2O2 for 20–45 min, followed by incubation in 1:20 goat serum (Sigma) in PBS for 1 h. For anti-p75 immunolabeling, the tissues were immersed in PBS containing 0.4% Triton X-100 and 0.3% H2O2, followed by 1:20 goat serum in PBS with 0.4% Triton X-100. After three 10-min PBS washes, all tissues were incubated in diluted primary antibody overnight and examined as above. Controls were provided by primary antibody omission or by use of an inappropriate secondary antibody.

Dil labeling of phrenic motoneurons. Fetal rats at age E18 were removed as described above and immediately placed in...
4% paraformaldehyde; the head and abdomen were removed. Tissue covering the neuraxis and thorax was opened to allow rapid fixation. The tissue was then dissected to isolate the cervical neuraxis and the phrenic and brachial nerves. After the tissue was left in fixative on a shaker at 4°C for 2–4 days, the phrenic nerve was cut at the level of the thymus. The distal end of the nerve was striped of connective tissue and cuffed in a small crystal of fast DiI (Molecular Probes). The distal nerve and DiI crystal were sealed in a strip of Parafilm to prevent DiI diffusion into other tissues. Tissues were placed in 1.2% paraformaldehyde, pH 9.5, for 2 mo at 35°C to allow time for complete neuronal labeling.

The tissue was removed, and the cervical spinal cord, with lower brain stem, dorsal root ganglia, and ventral roots, was isolated. Tissues were placed in 4% paraformaldehyde on a shaker at 4°C for up to 7 days. They were then washed in PBS and embedded in 3.5% agar. A block containing the tissue was excised and fixed in 4% paraformaldehyde on a shaker at 4°C for up to 3 days. Vibratome serial sections of 70 µm in the horizontal plane were collected and washed in PBS containing 0.1% NaN₃ at pH 7.4. Sections were mounted onto microscope slides coated with gelatin and coverslipped to keep the sections moist. Slides were stored at 4°C in a humid chamber. Photographs of labeled neurons were taken by using a camera attached to a Leitz Diaplan epifluorescent microscope.

Electron Microscopy of Phrenic Axons

Axons from control and nitrofen-exposed rats were examined in phrenic nerve sections taken ~500 µm rostral to the point of innervation of the diaphragm. Nerves were immersed in a mixture of 2% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) for 2–4 h. After three washes of 5 min each in 0.1 M cacodylate (pH 7.4), the nerves were postfixed for 15 min in a mixture of 1% OsO₄ and 0.1 M phosphate buffer (pH 7.4). The tissue was then washed three times in distilled water, dehydrated in graded ethanol, and embedded in resin. Sections were cut with a microtome, stained with uranyl acetate and lead citrate, and observed with a Philips 410 electron microscope. Photomicrographs (>4,300) were taken, and measurements of nerve area were made by using an image-analysis system (Java; Jandel). Axon numbers were counted by viewing the photograph negatives with a stereomicroscope at 340x magnification.

Measurements of Lung Weight, Protein, and DNA Content

Preparation of the lungs. Lungs were removed, weighed, quickly frozen in liquid nitrogen, and stored at ~80°C until analyzed, when they were defrosted and kept on ice. Each lung was homogenized in 1 ml distilled water in an ice bath with a polytron homogenizer at setting number 5 for 30 s (Brinkman Instruments, Westbury, NY). The homogenate was divided into two 500-ml aliquots for measuring protein and DNA content.

Protein assay. Protein was determined by using an array spectrophotometer (model 8452A, Hewlett-Packard) with a Bradford microassay (Bio-Rad Laboratories, Hercules, CA; see Ref. 6). Bovine serum albumin (Sigma Chemical) was used for the standard.

DNA assay. The DNA quantitation technique was adapted from Downs and Wilfingher (11). Homogenate and DNA extraction solution (1 M NH₄OH, 10 mM EDTA) were incubated at 37°C in a shaking water bath for 30 min. After centrifugation at 2,500 revolutions/min for 3 min, 25 µl of supernatant was added to 35 µl of distilled water and further centrifuged in an evaporator centrifuge for 10 min. Distilled water was added to make a final volume of 500 µl. Then, 50 µl were removed and added to 1.5 ml dye solution, prepared by mixing 50 µl Hoechst 33538 and 100 ml DNA assay buffer (in mM: 100 NaCl, 10 EDTA, 10 Tris, pH 7.0). Samples to be measured were then vortexed and left at room temperature for 30 min before DNA concentration was determined by using a fluorescence spectrophotometer (Hitachi F-2000). Graded known concentrations of calf thymus DNA (Sigma Chemical) were used as a reference.

RESULTS

Hernias Induced by Nitrofen

Administration of a single dose of nitrofen on E9 caused right, left, and bilateral hernias (Table 1). Administration of nitrofen on E11 resulted in the formation of right-sided hernias only. The range of sizes and locations of diaphragm malformations are illustrated in Fig. 1. The range of defects included mild to very severe left-sided malformations with ~25–90% of the muscle missing, mild to medium right-sided malformations with 10–50% of the muscle missing, and bilateral malformations. All malformations included the posterolateral area referred to as the region of Bochdalek (27).

Lung Growth

Measurements of lung growth were made to determine whether the lung hypoplasia associated with CDH occurred pre- or postinvasion of the abdominal contents into the thoracic space (E15 or E18, respectively). For the lung measurements, only medium to severe defects were used. With these diaphragmatic malformations at E18, there was significant invasion of the thoracic cavity by abdominal contents. At E15, however, the abdominal contents had not grown sufficiently to herniate into the thoracic cavity. Body weight, lung weight, lung protein, and DNA content were measured in control and CDH rats at ages E15 and E18.

As illustrated in Table 2, there were no significant differences in the body weight, lung weight, lung protein, and lung DNA content between controls and animals with extensive diaphragmatic malformations at age E15. At age E18, there was a significant reduction in overall lung weight, as well as protein and DNA contents of the lungs, in herniated animals compared with control, all suggesting a decreased cell content within the lung. Thus, collectively, these data illustrate that lung hypoplasia is a secondary effect, resulting from the restricted thoracic space associated with the misplacement of abdominal contents, rather than a primary defect that precedes diaphragm malformation.

Phrenic Nerve

These measurements were made to assay whether or not the phrenic nerve was malformed with CDH and, if so, to determine whether or not phrenic nerve atrophy occurred before or secondarily to the diaphragmatic malformations. To quantify the diameter of phrenic...
nerves in normal and herniated animals, transmission electron micrographs of nerve cross sections were examined. Figure 2 shows the left and right nerves from three animals, one at age E15 (left defect) and two at age E18 (left and right defects). There was no significant difference in the areas of the nerve ipsilateral (589 ± 31 µm²; n = 3) and contralateral (550 ± 41 µm²; n = 3) to the diaphragmatic defect at E15. However, at E18, the phrenic nerve ipsilateral to the abnormality had atrophied. Measurements of nerve area confirmed that the nerve ipsilateral to the diaphragmatic defect was significantly smaller (814 ± 6 µm²; n = 6) than the contralateral nerve (1,348 ± 8 µm²; n = 9). Counts of axon numbers in phrenic nerves from two fetal rats at age E18 with large left-sided diaphragmatic defects demonstrated that the reduced nerve area resulted from a decrease in the total number of axons: 582 and 695 axons in the ipsilateral vs. 1,078 and 1,061 axons in the contralateral phrenic nerves were counted in the two animals, respectively. These observations can be explained by the fact that the majority of phrenic motoneuron cell death occurs between E15 and E16.

Thus the degree of motoneuron survival within the ipsilateral motoneuron pool would be compromised by the reduced musculature and its associated target-derived neurotrophic factors (16). The decrease in the normal number of phrenic motoneurons ipsilateral to the hernia after naturally occurring cell death was also evident from retrograde fills with Dil at age E18 (Fig. 3). The number of motoneur-

![Fig. 1. Range of diaphragmatic malformations induced by administration of 2,4-dichloro-phenyl-p-nitrophenyl ether (nitrofen) on gestational day 9 (E9). Diaphragms were removed on E15.5 (B), E16 (D), E17 (A, E), E17.5 (C), or E19 (F) and immunolabeled for the polysialylated form of the neural cell adhesion molecule (PSA-NCAM). Left-sided hernias of varying degrees are shown in A–C; %diaphragm missing varied from ~25 to 90%. Typical right-sided hernias are shown in D and E. Right-sided hernias usually involved between 15 and 50% of diaphragm surface area. F: example of bilateral hernia. As discussed in text, location and range of severity of nitrofen-induced hernias in rat parallel that observed in human infants with congenital diaphragmatic hernia (CDH). Scale bars, 500 µm.]

### Table 2. Effects of diaphragmatic hernias on lung growth at ages E15 and E18

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Body Wt, g</th>
<th>Lung Wt, mg</th>
<th>Lung/Body Wt, mg/g</th>
<th>Total Lung Protein, mg</th>
<th>Protein/Lung Wt, mg/g</th>
<th>Total Lung DNA, µg</th>
<th>DNA/Lung Wt, mg/g</th>
</tr>
</thead>
<tbody>
<tr>
<td>E15 control</td>
<td>12</td>
<td>0.36 ± 0.01</td>
<td>3.3 ± 0.2</td>
<td>9.2 ± 0.4</td>
<td>0.08 ± 0.01</td>
<td>26.0 ± 2.6</td>
<td>10.2 ± 1.2</td>
<td>3.3 ± 0.3</td>
</tr>
<tr>
<td>E15 hernia</td>
<td>11</td>
<td>0.35 ± 0.01</td>
<td>2.6 ± 0.3</td>
<td>7.4 ± 0.9</td>
<td>0.08 ± 0.01</td>
<td>33.3 ± 3.0</td>
<td>9.1 ± 1.0</td>
<td>3.5 ± 0.4</td>
</tr>
<tr>
<td>E18 control</td>
<td>14</td>
<td>1.4 ± 0.04</td>
<td>41.6 ± 1.3</td>
<td>29.2 ± 1.0</td>
<td>1.6 ± 0.06</td>
<td>38.3 ± 1.7</td>
<td>215.3 ± 12.7</td>
<td>5.2 ± 0.3</td>
</tr>
<tr>
<td>E18 hernia</td>
<td>13</td>
<td>1.4 ± 0.03</td>
<td>33.2 ± 1.3*</td>
<td>24.9 ± 0.8*</td>
<td>0.9 ± 0.1*</td>
<td>29.3 ± 3.1*</td>
<td>136.7 ± 13.9*</td>
<td>4.1 ± 0.5</td>
</tr>
</tbody>
</table>

Values are means ± SD. n, No. of rats; E15 and E18, day 15 and day 18 of gestation, respectively. *Significant difference from control value, P < 0.05.
Fig. 2. Assessment of phrenic nerve size in animals with CDH. Photos are series from transmission electron microscopic images of phrenic nerve cross sections taken from animals with either left- or right-sided hernias at ages E15 (top) or E18 (middle and bottom). Top shows contrast in size of phrenic nerve area (demarcated by dashed line) on left and right side from animal at E15 with large left-sided hernia. Nerve ipsilateral to the diaphragmatic malformation is similar in size to contralateral side. In contrast, at age E18 (middle and bottom), phrenic nerve ipsilateral to diaphragmatic malformation is smaller than contralateral nerve in large left- and right-sided hernias. This atrophy of phrenic nerve at older age can be explained by increased apoptosis that occurs secondarily to removal of a portion of muscle target during period of naturally occurring cell death. See text for further discussion. Magnification, ×4,300.

Fig. 3. 1,1'-Diocadecyl-3,3',3'-tetramethylindocarbocyanine perchlorate (DiI) retrograde fills of phrenic motoneurons at E18 demonstrate that fewer motoneurons survive within phrenic motoneuron pool on side ipsilateral to diaphragmatic malformation. A–D: ventrodorsal progression of motoneuron distribution within phrenic motor pool at cervical segments, levels C7–C8, respectively. Sections were cut at 70 μm. Note that fewer motoneurons are labeled throughout rostrocaudal distribution of phrenic motor pool on side ipsilateral (left) to hernia (3/4 of diaphragm was missing from left) compared with contralateral (right) side. Scale bar, 200 μm.

neurons counted within the phrenic motoneuron pool ipsilateral to large left-sided hernias was 67 ± 8% of the contralateral side (n = 6; P < 0.05). Note that there is normally a well-defined topographical innervation of the diaphragm, with the dorsal regions of the musculature innervated primarily by phrenic motoneurons within the C5–C6 cervical segments of the motoneuron pool (23). The sternal region is primarily innervated by motoneurons located at the level of the third and fourth cervical segments. Thus, without a compensatory mechanism whereby phrenic axons reestablish an essentially normal topographic projection within the remaining musculature, one would expect a massive die off of
neurons caudal to C4 regions. Clearly, this is not the case, as decreased access of phrenic axons to the normal amount of musculature results in a loss of survival that, while slightly heavier within caudal regions, is shared by the remainder of the phrenic motoneuron column. The phrenic nerve appears capable of compensation, in that, although translocated ventrally, the characteristic trifurcation at the primary branching site is retained (Fig. 4A). Furthermore, axons were often observed projecting around the herniated region, being diverted laterally or medially in search of their normal but displaced target (Figs. 1, 4, and 5, B and C).

Myotube Formation
To determine whether myotube formation was abnormal in the region of the herniation, we labeled for PSA-NCAM, a molecule that is expressed on the membranes of myotubes during myogenesis (3). Examples are shown of whole diaphragms taken from two animals at age E17, one with a large left diaphragmatic defect labeled for PSA-NCAM (Fig. 4A) and the other with a medium-sized defect labeled for GAP-43 (Fig. 4B). Cross sections, taken from diaphragms identical to those presented as whole mounts and immunolabeled...
for PSA-NCAM, show that the density of labeling, reflecting myotube number, was highest in regions adjacent to the hernia. Thus, myotube formation within the herniated diaphragms is only abnormal in that myotubes normally destined to span the region of herniation have accumulated adjacent to it. We did not, however, count the number of myotubes in control vs. CDH diaphragms. Thus, the possibility of the formation of fewer myotubes has not been addressed. Close examination of PSA-NCAM labeling demonstrates that the usual mediolateral extension of myotubes is often displaced as a result of the diaphragmatic malformation, with myotubes often extending more ventrodorsally around the lateral edge of the defective area (as in Fig. 1, B and E). The extension of myotubes along the lateral extent of the more severe defects (as in Fig. 1B) likely induced the often-observed abnormal projection of axons.

Closure of the Pleuroperitoneal Canal

We exposed pregnant rats to nitrofen at E9 and examined the fetal diaphragms for defects at E14.5, before pleuroperitoneal canal closure. Our rationale was that if the hernia occurred as a result of the failure of pleuroperitoneal canal closure, then no defects would be observed before E15. The left and right pleuroperitoneal canals in fetal rats typically close at ages E14.75 and E15.25, respectively (12). The location of the pleuroperitoneal canals in a normal diaphragm from an animal aged E14.5 is illustrated in Fig. 5A. Two diaphragms from animals aged E14.5 that have been exposed to nitrofen on E9 are shown beside the control diaphragm (Fig. 5, B and C). There are clear defects that appear before canal closure on both left (Fig. 5, B and C) and right sides (Fig. 5C). Furthermore, the region of diaphragmatic malformation clearly occurs medially of the canal. Thus the abnormality must occur independently from a simple failure of pleuroperitoneal canal closure.

Formation of the Primordial Diaphragm: the Pleuroperitoneal Fold

The primordial diaphragmatic muscle tissue is located within the pleuroperitoneal fold. This fold of tissue in a control animal is demonstrated in Fig. 6B. We knew from earlier observations that a track of cells, preceding phrenic nerve outgrowth and cells within the pleuroperitoneal fold, expressed NCAM and the low-affinity nerve growth factor (p75) receptor (2). The cells expressing these two molecules are likely Schwann and/or muscle cell precursors (29). Regardless, we took advantage of the characteristic immunolabeling for p75 receptors in control and nitrofen-exposed animals aged E13.5 to determine whether the pleuroperitoneal fold was malformed in the instance of CDH. We observed defects of the left and right pleuroperitoneal fold among nitrofen-exposed animals. Figure 6A shows a large defect within the left pleuroperitoneal fold of one such fetus at age E13.5. More rostral sections demonstrate that the nerve reached the displaced pleuroperitoneal fold by its projection through the pleuropericardial membrane (as in normal fetuses), which is fused with the diminished pleuroperitoneal fold.

DISCUSSION

This systematic study evaluated the status of the lung, the phrenic nerve, and diaphragmatic muscle formation at various stages during the course of the abnormal development of the diaphragm associated with nitrofen-induced CDH. We demonstrated that previously proposed theories regarding the primary role of the lung, phrenic nerve, myotube formation, and
the closure of pleuroperitoneal canal in the pathogenesis of CDH are incorrect. Rather, the primary defect associated with CDH occurs, at an early stage, with the formation of the primordial diaphragm within the pleuroperitoneal fold.

General Comments on the Nitrofen Model for CDH

Nitrofen is a diphenyl ether herbicide that acts by an unknown mechanism to control broad-leaved weeds. Initial toxicological studies revealed that, whereas nitrofen was relatively nontoxic to adult animals, it did produce a number of lethal abnormalities associated with pulmonary and cardiovascular malfunction in fetuses exposed prenatally (5, 9, 13, 24). When given as a single dose on gestation day E9 or E11, the major abnormality produced by nitrofen was the maldevelopment of the diaphragm. As illustrated in Fig. 1, the range and location of hernias produced by nitrofen in the rat are remarkably similar to that observed in the human congenital anomaly. In the rat CDH model, the extent of the invasion of abdominal contents into the thoracic cavity varied according to the size of the defect within the pleuroperitoneal fold. In humans, the extent of herniation also varies and is a critical determinant of the subsequent degree for pulmonary hypoplasia and the eventual prognosis (1). Furthermore, it is interesting to note that the rate of fetal reabsorptions increases in rats after administration of nitrofen at gestational age E9. In this context, it has been estimated that ~1,000–2,000 human fetuses do not survive to term because of complications associated with CDH and accompanying defects (15). Although there is no concrete data associating environmental toxins with the etiology of CDH, there does seem to be a remarkable similarity between the nitrofen model and the majority of human CDH anomalies. However, differences between the rat nitrofen model and the human condition include the fact that multiple congenital anomalies are associated with CDH in ~30% of infants (10, 25). Typically, associated anomalies (e.g., cardiac malformations, hydrocephalus, skeletal malformations) occur in the rat model only when the dam has been chronically exposed to high levels of nitrofen. Furthermore, ~10% of the cases in which CDH is associated with multiple congenital anomalies in infants can be linked to specific genetic abnormalities (i.e., trisomy 13 and 18 as well as Fryn’s syndrome; see Refs. 10, 25).

Lung Development

Previous reports have suggested that the lung is malformed independently, and perhaps as a cause, of the diaphragmatic anomalies. Two sources for this thinking have persisted in the literature. First, Iritani (18) showed a photograph of an abnormal lung and adjacent tissue that was referred to as the posthepatic mesenchymal plate (PHMP). In that experimental paradigm, nitrofen was fed ad libitum to pregnant rats from E5 to E11, and a number of developmental anomalies arose as a result. It may have been that massive doses of nitrofen administered at very early stages for long periods disrupted a number of tissues, including the lungs. However, Kluth et al. (22) reexamined the issue of whether the lung was atrophied at an early stage (E14) of diaphragmatic malformation in rats. They concluded, also, from visual inspection with scanning electron microscopy, that the lungs were normal before the invasion of the abdominal contents into the thoracic cavity. The quantitative analysis of lung size and cell content in the present study demonstrates that lung hypoplasia is indeed a secondary effect rather than a primary cause of the diaphragm malformation. The second line of evidence that has been presented implicating the lung as a source of the anomaly has been the fact that the lung contralateral to the herniation is sometimes hypoplastic in newborns with CDH. However, the contralateral hypoplasia, which is often minor compared with the ipsilateral defect, can be explained.

Fig. 6. Transverse cross sections from 2 E13.5 fetal rats immunolabeled for p75 receptor: animal affected by exposure to nitrofen (A) and a control animal (B). *Primordial diaphragmatic tissue within pleuroperitoneal fold at E13.5. A: animal affected by nitrofen clearly has a large component of laterally located pleuroperitoneal fold missing (outlined by dashed line). Arrows (A and B), entry of phrenic nerve to control and abnormal pleuroperitoneal folds. We propose that pathogenesis of CDH is associated with malformation of early stages of primordial diaphragm development. Lu, lung; FL, forelimb; A, aorta; L, lateral; D, dorsal. Scale bars, 100 µm.
by the fact that the mediastinum, and subsequently the
contralateral lung, is compressed by the presence of the
invading viscera. Thus, considering the evidence pre-

tated when nitrofen is administered in a manner to
minimize associated anomalies, lung development is
normal at the early stages of CDH progression and is
only compromised as a secondary result of the sub-
sequent migration of viscera through the diaphragmatic
opening.

Status of the Phrenic Nerve

The phrenic nerve innervating the herniated side of a
diaphragm in older fetal rats (after E16) often appears
smaller in diameter compared with that of the unaf-
ected side. The electron micrographs of the phrenic
nerve (Fig. 2) and the retrograde fills of phrenic moto-
nurons (Fig. 3) in the present study provide quantita-
tive evidence for this observation. However, these abnor-
malities are also a result of, rather than a cause of,
CDH. Before naturally occurring neuronal cell death
(E15; see Ref. 16), the numbers of phrenic motoneurons
and axons in CDH fetuses were similar to those in
control animals on both ipsi- and contralateral sides to
the herniation. This apparent discrepancy can be ex-
plained by the fact that the number of neuronal cell
bodies and axons that remain after the period of
apoptosis is critically determined by the target tissue
(14). In severely herniated diaphragms, a significant
proportion of the target is missing, and the remaining
musculature is arranged abnormally, thereby explain-
ing the increased phrenic motoneuron cell death.

Diaphragm Formation

The notion that myotubes in the region of the hernia-
tion form in a weak fashion and then rupture due to the
pressure induced by the underlying viscera does not
hold up with the nitrofen model. Rather, it is clear that
myotube formation is enhanced adjacent to the hernia,
with some of the myotubes normally destined for the
herniated region aligning next to the diaphragmatic
opening.

Similarly, our data do not support the idea that the
herniation results from a failure of pleuroperitoneal
opening. First, herniation often occurs medial to
the pleuroperitoneal canals. Secondly, it was obvious
that well-defined holes in the diaphragm were present
on either the right or left side at E14.5, 0.25 and 0.75
days before the closure of the right and left pleuroperi-
toneal canals, respectively (12). Kluth et al. (22) also
noted malformations on the right side of diaphragms at
E14 in response to nitrofen administration at E11,
which is consistent with the defects in the diaphragm
occurring before closure of the pleuroperitoneal canal.
The malformation of the diaphragmatic musculature
clearly occurs at a stage of development earlier than
myotube formation and pleuroperitoneal canal closure.
In the present study, the primordial diaphragmatic
tissue, the pleuroperitoneal fold, was found to be grossly
malformed in the nitrofen model of CDH. As a result,
the nerve migrates past its normal point of innervation,
which is missing in left hernias, and innervates tissue
ventrally. It is interesting that the phrenic nerve
branches in a pattern similar to normal development,
even when its primordial target is either missing or
misplaced in the instances of CDH. It would be interest-
ing to study potential topographically organized cues in
normal and herniated diaphragms to better understand
this adjustment phenomenon. After E13.5, herni-
ated diaphragms then develop without the normal
substrate for the medial and dorsal regions.

The tissue classified by Iritani (18) as the PHMP is
likely a portion of the pleuroperitoneal fold. As far as we
have been able to discern, there has not been a single
anatomical description of a tissue analogous to the
PHMP described independently from that proposed by
Iritani (18). Moreover, others have raised doubts regard-
ing the identity of the PHMP, suggesting that it is a
part of the dorsal mesenchyme of the septum transver-
sum. With the clearer visualization of the tissue with
the presently available immunohistochemical markers,
it is obvious that the phrenic nerve enters the pleuro-
peritoneal fold, which is fused at its ventral extent with
the septum transversum (2).

The present data clearly show that the diaphrag-
monic defect associated with maternal nitrofen expo-
sure occurs with the initial rather than the latter
stages of diaphragm development. It will now be im-
portant to determine whether it is a matter of improper
muscle precursor cell migration from somites or a
malformation of the mesenchymal tissue that provides
the supporting structure for diaphragm formation. The
fact that left-sided hernias only occur when nitrofen
is administered at E9 suggests that the primary insult
must occur during a well-defined time frame. A past
study (8) of the distribution and metabolism of orally
administered nitrofen in rats has shown that nitrofen
reaches the fetus within 2 h after administration, with
the level peaking at 4–6 h and declining to half the
initial value by 24 h. One proposal regarding the
pathogenesis of CDH suggests that there is an in-
creased amount of cell death in the cervical somites
associated with nitrofen administration (4). However,
the correlation was not quite so clear, as the increased
cell death was bilateral in instances of presumptive
left-sided herniation and totally absent in instances of
presumptive right-sided herniation. A further complica-
tion arose in that study, because it was assumed that all
herniations would be left-sided in response to nitrofen
administration at E9, which has been illustrated in this
and past studies (21) to be an overgeneralization.
Clearly, further studies regarding the source of dia-
aphragmatic premuscle tissue in the normal and patho-
logical states are needed to address this issue. We are
now focusing on two potential features of pleuroperi-
toneal fold development that may be affected during CDH
development: the migration of muscle precursors from
somites and the deposition of the mesenchymal sub-
structure on which the diaphragmatic musculature
forms.

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REFERENCES


