Diaphragm defects occur in a CDH hernia model independently of myogenesis and lung formation

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Examinations of the muscle precursor migration to the PPF and subsequent proliferation and differentiation in the animal model of CDH did not reveal any obvious abnormalities (4). However, the underlying substratum of the PPF appeared abnormal. This has led to our current hypothesis stating that the amuscular mesenchymal component of the PPF, likely derived from the somatopleure (30), is defective and does not provide a full foundation for the formation of diaphragmatic musculature. In this study, we test this hypothesis using mice in which muscle precursors fail to migrate to peripheral muscle, including the diaphragm, because of homozygous mutation (−/−) of the c-met gene. The c-Met protein, a receptor tyrosine kinase that is present on myogenic precursors, binds its ligand hepatocyte growth factor/scatter factor, signaling migration of these cells (5). Although the diaphragmatic musculature fails to form in the null mutants, the underlying connective tissue that comprises the

CONGENITAL DIAPHRAGMATIC HERNIA (CDH) is a serious developmental anomaly characterized by large regions of the diaphragm failing to form. Consequently, the developing viscera invade the thoracic cavity and impair lung growth and development. As a result, newborns with CDH suffer from a combination of pulmonary hypoplasia, pulmonary hypertension, and surfactant deficiency (15, 19). CDH is associated with a 30–60% mortality rate and significant morbidity among survivors (15, 24). Moreover, it is estimated that 1,200 conceptions fail to reach term because of complications associated with CDH (14). Textbook explanations of the origins of CDH typically state that it is a defect in the fusion of the diaphragm muscle with the abdominal wall (i.e., the closure of the pleuroperitoneal canals) that results in the hernia. We have re-examined the pathogenesis of CDH using a well-established animal model in which diaphragm defects characteristic of those found in infants with CDH are induced by administering specific teratogens midway through gestation (6, 8, 9, 27–29, 32). Data from those studies refute the hypothesis of a primary defect in the closure of the pleuroperitoneal canal (2). Rather, defects can be traced back to much earlier stages of development during the formation of the primordial diaphragm, the pleuroperitoneal fold (PPF). The PPF is a wedge-shaped tissue that tapers medially from the lateral cervical wall to the esophageal mesentery and fuses ventrally with the septum transversum. Myogenic cells and axons destined to form the neuromuscular component of the diaphragm migrate to the PPF, and it is the expansion of these components that leads to the formation of the diaphragm (4). Three-dimensional reconstructions of the PPF have demonstrated that the malformed areas in the animal model of CDH are consistently located in the dorsolateral region (10, 11). Correspondingly, the dorsolateral region of the diaphragm musculature is precisely the area affected in CDH. Thus the embryogenesis of the PPF has become a focus for elucidating the pathogenesis of CDH. Examinations of the muscle precursor migration to the PPF and subsequent proliferation and differentiation in the animal model of CDH did not reveal any obvious abnormalities (4). However, the underlying substratum of the PPF appeared abnormal. This has led to our current hypothesis stating that the amuscular mesenchymal component of the PPF, likely derived from the somatopleure (30), is defective and does not provide a full foundation for the formation of diaphragmatic musculature. In this study, we test this hypothesis using mice in which muscle precursors fail to migrate to peripheral muscle, including the diaphragm, because of homozygous mutation (−/−) of the c-met gene. The c-Met protein, a receptor tyrosine kinase that is present on myogenic precursors, binds its ligand hepatocyte growth factor/scatter factor, signaling migration of these cells (5). Although the diaphragmatic musculature fails to form in the null mutants, the underlying connective tissue that comprises the

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amuscular substratum forms fully, thus offering the opportunity to clearly visualize the formation of the amuscular component of the diaphragm in normal and teratogen-exposed animals.

We also test a further hypothesis that states that the lung hypoplasia associated with CDH is the primary defect that in turn causes the secondary defect in the developing diaphragm because of the loss of critical signals emanating from lung tissue (6, 18). Central to developing diaphragm because of the loss of critical signals emanating from lung tissue (6, 18). Central to developing diaphragm embryogenesis is regulated or influenced directly by the development of the adjacent lung tissue. We utilized Fgf10(+/−) null mutant mice that do not develop lung tissue (26) to address this issue. Specifically, we tested whether or not lung tissue was necessary for normal diaphragm formation and we tested whether or not lung tissue was necessary for developing lung tissue (26) to address this issue. Specifically, we tested whether or not lung tissue was necessary for normal diaphragm formation and 2) the induction of diaphragmatic defects in an animal model of CDH.

MATERIALS AND METHODS

Mutant mice. Drs. M. Tessier-Lavigne (University of California, San Francisco) and C. Birchmeier (Berlin) provided heterozygous breeding pairs of c-met mice. For genotyping, genomic DNA was isolated from ear-notch biopsies or fetal tail tissue according to standard protocols. Oligonucleotide primers WMet8s (5′-CTTTCATAGGCGATTTGGCTGTG-3′) and WMet10 (5′-GTACACTGGTGTGAAATGTCAGTTG-3′) were used to amplify a 650-bp fragment specific to the c-met wild-type allele. A 300-bp fragment of the mutant c-met allele was generated using primers Neo1L (5′-CGACGGCTGCTGTTCTCTTGG-3′) and WMet5 (5′-CACTGACCCAGAAGAGTGG-3′). For the wild-type and mutant bands, the DNA underwent 43 cycles of amplification consisting of denaturing (94°C, 40 s), annealing (65°C, 30 s), and extension (72°C, 15 s + 1 s/cycle). Sample (10 μl) was run on a 12% agarose gel to analyze the results of the PCR reactions.

Dr. D. Ornitz (Washington University in St. Louis) provided breeding pairs of Fgf10(+/−) mice. Genotyping was performed by PCR using published primer sequences (26). A 383-bp fragment was generated from the wild-type Fgf10 locus by PCR using primers P1 (5′-CTTCCAGATGTCCTCTCAGAGAC-3′) and P2 (5′-GTACGGCACTCTCTGTCAATG-3′). The mutant Fgf10 locus was amplified using primers P3 (5′-AAGCAGGGCTTCTGTGAGCCACTG-3′) and P4 (5′-TCAGAGAAGCAGTCAAGGAGGCCATA-3′) to produce a 582-bp fragment. DNA was extracted following established methods from ear-notch biopsies and fetal tail samples and amplified for 30 cycles (15 s at 94°C, 60 s at 60°C, and 90 s at 75°C).

Drug delivery. Timed-pregnant animals were treated on embryonic day 8 of gestation via gavage feed with CDH-inducing compounds dissolved in 600 μl of olive oil. Pregnant mice were briefly anesthetized with halothane. In the initial experiments, 25 mg of nitrofen (2,4-dichloro-phenyl-p-nitrophenyl ether; China National Chemical Construction Jiangsu, Nanjing, China) were administered to dams on embryonic day 8 based on past literature reporting a 25–30% occurrence of CDH in mouse embryos (6, 31). However, we observed a very low incidence of hernias in embryos from c-met dams bred on an ICR background treated with 25 mg of nitrofen (Table 1). The yield was too low to assay efficiently for hernias in the 25% of embryos homozygous for the c-met deletion. Thus we modified our protocol based on the fact that three other teratogens have been identified that induce defects similar to nitrofen (12). Although we did not perform a systematic study of which of these compounds, or combination of, were most effective at inducing hernias, we did find administration regimes that sufficed for testing the hypotheses in question. We used the administration of a mixture of 14.5 mg nitrofen and 14.5 mg bisdiamine (Acros Organics) to induce hernias in Fgf10(+/−) animals. A mixture of 14.5 mg nitrofen, 14.5 mg bisdiamine, and 4.5 mg SB-210661 (provided by Dr. H. M. Solomon, SmithKline Beecham Pharmaceuticals, King of Prussia, PA) was effective for inducing hernias in Fgf10(+/−) mice bred on a C57BL6/6X CBA background. Treated animals were returned to the original cage and housed in the laboratory.

Caesarean section and tissue isolation. On embryonic day 14, mice were anesthetized with halothane and maintained at 37°C with radiant heat. The fetuses were delivered by caesarean section. Upon delivery of each fetus, a sample of tail tissue was collected and frozen for genotyping. Each fetus was photographed using a Nikon 990 digital camera mounted on a Leica research microscope. Fetuses were then decapitated, and the thoracic and abdominal cavities were opened to determine the presence of diaphragmatic hernia.

RESULTS

Untreated c-met(+/−) mutant embryos appeared normal except for underdeveloped limb musculature resulting from impaired muscle precursor migration to peripheral muscles (Fig. 1A). The mutants also had amuscular “diaphragms” that were thin and lacked any visible striations indicative of muscle fibers (Fig. 1B). Table 1 summarizes the incidence of hernias induced by the different treatment regimes used with the c-met mouse model. A combination of nitrofen and bisdiamine administered midway through gestation induced diaphragmatic hernias in ~40% of c-met mice, with the majoring being left sided. Similar to what has previously been reported in the nitrofen mouse model (6), treatment with teratogens produced a spectrum of other visible defects, including large facial clefts, exencephaly, polydactyly, and “loose skin” in some embryos. Eighteen fetuses were c-met(+/−) as determined by genotyping. Of these, four had clear defects in the amuscular diaphragmatic membrane. Figure 1C shows a photomicrograph of a defective diaphragm with the liver herniating into the thoracic cavity. The defective region was always located in the left dorsolateral region (Fig. 1D). In size, the hernias covered from 40 to 70% of one

<table>
<thead>
<tr>
<th>Mutant</th>
<th>Nitrofen</th>
<th>Nitrofen-Bisdiamine</th>
<th>Nitrofen-Bisdiamine-SB-210661</th>
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<tbody>
<tr>
<td>c-met</td>
<td>2/50</td>
<td>75/170</td>
<td>59 left</td>
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<tr>
<td>Fgf10</td>
<td>0/14</td>
<td>0/12</td>
<td>6/26 embryos (from 4 dams); 6 left</td>
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Table 1. Incidence of hernias found in the mouse models with different drug regimens

No. of hernias are shown. N/A, not applicable.
side of the diaphragm. These defects were identical with respect to location, extent, and visceral intrusion to those seen in nitrofen-treated rats and infants with CDH.

In Fgf10(+/−) mice, the trachea forms but the lungs do not develop (26). Furthermore, limb bud formation is initiated, but outgrowth and muscularization of the limbs do not occur (Fig. 2A). However, fully formed, well-muscularized diaphragms developed in all Fgf10(+/−) mice despite lung agenesis (Fig. 2, B and C). Furthermore, posterolateral diaphragmatic defects characteristic of CDH (Fig. 2, D and E) were present in animals treated with a combination of nitrofen, bisdiamine, and SB-210661 (Table 1).

**DISCUSSION**

The combination of the drug-induced models of CDH and mutant mice allowed us to test two fundamental hypotheses regarding the pathogenesis of this serious developmental anomaly.

**Consideration of the animal model.** The nitrofen rodent model has been used widely to examine lung and muscle malformations associated with CDH. The use of this model arose from routine toxicological studies demonstrating that, although nitrofen was relatively nontoxic to adult animals, administration in utero resulted in ~50% of the fetuses developing diaphragm malformations that were remarkably similar to those seen in infants with CDH (3, 7, 21, 22). The similarities hold true with regard to the specific location and extent of diaphragmatic defects as well as the periodic occurrence of associated anomalies affecting cardiac, pulmonary, and skeletal tissues. More recently, three additional compounds that cause diaphragmatic defects in rats have been characterized (12). Biphenyl carboxylic acid (BPCA), bisdiamine [N,N′-octamethylenebis(di-chloroacetamide)], and SB-210661 all induce diaphragmatic defects in the fetuses isolated from treated pregnant rats. BPCA is a breakdown product of a thromboxane A2 receptor antagonist, bisdiamine is a spermatogenesis inhibitor, and SB-210661 is a benzo-furanyl urea derivative developed for inhibiting 5-lipoxygenase. The timing of administration of all of the CDH-inducing teratogens is critical. Rodents are most susceptible between embryonic days 8 and 11, a developmental window corresponding to gestational weeks 4–6 in humans. We found a combination of teratogens

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*Fig. 1. Diaphragmatic hernias can be induced in amuscular diaphragms of c-met(−/−) mice. A: image of a c-met(−/−) mouse at embryonic day 18. The null mutants have characteristic underdeveloped limb musculature resulting from abnormal muscle precursor migration to peripheral muscles. B: amuscular diaphragm with body wall attached from a c-met(−/−) mouse. The mesenchymal sheet is attached to the body wall laterally and to the esophageal mesentery medially. C: photomicrograph of a diaphragm with a left-sided defect. The liver is protruding through the defect (arrow). Note that the remainder of the liver can be seen through transparent amuscular diaphragm. D: amuscular diaphragm with body wall attached from a c-met(−/−) mouse exposed to bisdiamine/nitrofen. There is a defect (∗) in the left dorsolateral area of the diaphragm characteristic of CDH. E: genotyping of fetuses from heterozygous matings of c-met mice was performed as described in MATERIALS AND METHODS. The three possible genotypes can be distinguished. +/+, Wild type; +/−, heterozygous; −/−, homozygous (null).*
to be more effective in inducing hernias in the mouse strains used in this study. The precise mechanism of action of the CDH-inducing teratogens has not been elucidated, but recent data demonstrate that they all interfere with the retinoid signaling pathway by inhibiting retinaldehyde dehydrogenase (12).

**Interpretation of data from experiments using c-met**

The data derived from c-met(-/-) mice demonstrate that diaphragmatic defects can be produced independent of myogenic processes. This supports the hypothesis that the origin of the defect lies in the amuscular mesenchymal component of the primordial diaphragm, the PPF. These data provide a perspective on the mechanisms underlying CDH pathogenesis that is entirely novel from past theories. The focus now shifts from the muscularization of the diaphragm and closure of pleuropertoneal canals to understanding the mesenchymal amuscular component of the diaphragm and how it is malformed in CDH. Furthermore, the PPF mesenchymal substratum forms during the first 4 wk of gestation, and thus a reconsideration of the developmental stage at which the anomaly occurs is warranted.

**Interpretation of data from experiments using Fgf10(-/-) mice.** Two primary conclusions arise from data derived from Fgf10(-/-) mice. First, the diaphragm can form normally in the absence of lung tissue and any putative associated growth-related signals. Second, defects in the diaphragm in an animal model of CDH occur in the absence of lung tissue. Thus the diaphragmatic defects associated with CDH are a primary defect and not a secondary result of lung malformation. The concept that the lung hypoplasia is in fact secondary to the diaphragmatic defect is supported by data from the surgically induced sheep model of CDH that clearly demonstrates that a hole in the posterolateral diaphragm results in marked lung underdevelopment because of the invasion of abdominal

**Fig. 2.** In the absence of lung tissue, Fgf10(-/-) mice can develop diaphragmatic hernias. A: wild-type and Fgf10(-/-) mice. The Fgf10(-/-) mice have characteristic abnormal limb development. B and C: rostral and caudal views, respectively, of a diaphragm muscle isolated from an Fgf10(-/-) mouse. The diaphragm is well developed without any noticeable abnormalities. D: diaphragm muscle with body wall attached from an Fgf10(-/-) mouse exposed to nitrofen, bisdiamine, and SB-210661. There is a clear defect (*) in the left posterolateral area that is characteristic of CDH. E: close-up of the defective region. E, esophagus; CT, central tendon.
contents and abnormal fetal breathing movements (16, 17, 23, 25). However, there is convincing evidence demonstrating that the teratogens used in the rodent CDH model can directly interfere with lung development (1, 6, 13, 20). Whether or not this is simply a reflection of the specific pathogenesis of teratogen-induced CDH remains unresolved. The possibility of there being a common mechanism underlying the pathogenesis of CDH that targets primordial diaphragm and lung development in parallel requires further investigation (20). Regardless, the pathogenesis of the lung associated with CDH, whether primary or secondary, must remain a focus of investigation if advances are to be made in the treatment of the disorder. We simply argue that these data demonstrate that an understanding of the pathogenesis of the diaphragm defect requires a focus elsewhere from the lung.

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REFERENCES