Structure of the primordial diaphragm and defects associated with nitrofen-induced CDH

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Greer, John J., David Cote, Douglas W. Allan, Wei Zhang, Randal P. Babiuk, Linh Ly, Robert P. Lemke, and Keith Bagnall. Structure of the primordial diaphragm and defects associated with nitrofen-induced CDH. J Appl Physiol 89: 2123–2129, 2000.—The goals of this study were to further our understanding of diaphragm embryogenesis and the pathogenesis of congenital diaphragmatic hernia (CDH). Past work suggests that the pleuropertoneal fold (PPF) is the primary source of diaphragmatic musculature. Furthermore, defects associated with an animal model of CDH can be traced back to the formation of the PPF. This study was designed to elucidate the anatomic structure of the PPF and to determine which regions of the PPF malform in the well-established nitrofen model of CDH. This was achieved by producing three-dimensional renderings constructed from serial transverse sections of control and nitrofen-exposed rats at embryonic day 13.5. Renderings of left-and right-sided defects demonstrated that the malformations were always limited to the dorsolateral portions of the caudal regions of the PPF. These data provide an explanation of why the holes in diaphragmatic musculature associated with CDH are characteristically located in dorsolateral regions. Moreover, these data provide further evidence against the widely stated hypothesis that a failure of pleuropertoneal canal closure underlies the pathogenesis of nitrofen-induced CDH.

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RECENT EMBRYOLOGICAL STUDIES have identified the pleuropertoneal fold (PPF) as a primary source of diaphragmatic musculature (1). The PPF is a pyramid-shaped tissue that extends medially from the lateral cervical wall to the esophageal mesentery and fuses ventrally with the septum transversum. The early stages of PPF formation have yet to be systematically examined. However, we hypothesize that the embryogenesis of the PPF parallels that of the forelimb bud. Specifically, the substructure of the PPF is derived from mesenchymal cells migrating from the somatic mesoderm. Subsequently, muscle precursors migrating from the dermomyotome of cervical somites follow guidance cues provided by the somatopleure substructure and become localized in the PPF (www.med.unc.edu/embryo_images provides a clear pictorial review of early embryological events). The PPF becomes a well-recognized structure between embryonic days (E) 12.5 and 13.5 in the rat (gestational period is 21 days). The key development stages of phrenic nerve and diaphragm development after the formation of the PPF have already been elucidated (see Ref. 8 for review). Briefly, the phrenic nerve makes contact with the PPF during E13. Between E14 and E17, the phrenic nerve trifurcates and extends intramuscular branches to the ventral, dorsolateral, and crural areas of the developing diaphragm. Concomitant with phrenic branch outgrowth, diaphragmatic myoblasts migrate from the PPF and fuse to form myotubes that extend from the lateral aspect of the septum transversum to the body wall in the costal areas and from the esophageal mesentery to the dorsal aspects of the crural region. The tissue derived from the septum transversum eventually becomes surrounded by diaphragmatic muscle fibers and forms the central tendon.

A clear understanding of PPF structure is fundamental for an understanding of the early stages of diaphragm embryogenesis. Furthermore, the PPF has become a focus of attention in studies of the pathogenesis of an animal model of congenital diaphragmatic hernia (CDH) (2). This often-lethal developmental anomaly (~50% mortality) is estimated to occur in 1:3,000 births (6, 9, 16). The hallmark of the disorder is a hole in the dorsolateral region of the diaphragmatic musculature [with 85–90% of the defects occurring on the left side (16)]. The presence of a hole in the diaphragm allows for the invasion of the thoracic cavity by the growing abdominal contents and the subsequent impairment of normal lung growth. There is a well-established animal model of CDH in which defects very similar to those observed in the human condition are produced in rodent embryos by administering a single dose of the herbicide nitrofen to the dam on E8–9 (3, 5, 15). The biochemical mechanism by which nitrofen produces the defects is, at present, unknown. Nevertheless, the model has proven useful for testing several
hypotheses regarding the stage at which the initial malformation of the diaphragm occurs (see Ref. 7 for review). Studies using the nitrofen model have demonstrated that the initial defect can be traced back to the formation of the PPF.

The present study focuses on two main objectives: 1) to elucidate the anatomic structure of the primordial diaphragm and how these early defects could translate into the characteristic holes in the dorsolateral quadrant of the defective newborn diaphragm. These aims were achieved by generating three-dimensional (3D) reconstructions of normal and defective PPFs from serial transverse sections of control and nitrofen-exposed rat embryos. The reconstructions were generated from tissue isolated at E13.5, when the PPF has formed into a well-defined structure and defects are clearly discernible in the nitrofen model (1, 2).

METHODS

**Administration of nitrofen.** Nitrofen was obtained from the US 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 (designated as E0). Pregnant dams on the evening of the eighth day of gestation (E8) were temporarily anesthetized with halothane (1.25% in 95% O2-5% CO2) and given 100 mg of nitrofen via a gavage tube. Past work has demonstrated that nitrofen produces diaphragmatic defects in 30–90% of the embryos in a given dam, with these early defects could translate into the characteristic holes in the dorsolateral quadrant of the defective newborn diaphragm. Therefore, the nitrofen model (1, 2).

**Delivery of embryos via caesarean section and tissue preparation.** Control and nitrofen-exposed fetal rats (E13.5) were delivered from timed-pregnant Sprague-Dawley rats anesthetized with halothane (1.25% in 95% O2-5% CO2) and given 100 mg of nitrofen via a gavage tube. Past work has demonstrated that nitrofen produces diaphragmatic defects in 30–90% of the embryos in a given dam, with the majority being left-sided defects (2, 15).

**Immunolabeling for the low-affinity nerve growth factor (p75) receptor and polysialylated neural cell adhesion molecule (p75)** receptor and polysialylated neural cell adhesion molecule, desmin) that labels the primordium of diaphragmatic muscle mass (1, 2). We used the combination of differential H & E staining and immunolabeling profiles of developmentally regulated molecules, from previous studies, to establish the boundaries for delineating the PPF. The rostral border was

**RESULTS**

**Two-dimensional PPF structure.** The location of the PPF within a cervical section of an E13.5 rat is illustrated in Fig. 1A. PPF tissue is clearly discernable from surrounding structures (e.g., lung, liver, and body wall), on the basis of differential cell density and size revealed by H & E staining. The adjacent photomicrograph (Fig. 1B) shows a close-up image of the PPF tissue labeled for the low-affinity nerve growth factor (p75) receptor, one of several molecules (e.g., neural cell adhesion molecule, desmin) that labels the primordial diaphragmatic muscle mass (1, 2).
estimated to be the first serial transverse section in which the triangle-shaped PPF tissue could clearly be seen protruding medially from the cervical lateral wall. The phrenic nerve could be distinguished from the surrounding tissue within the medial aspect of rostrally located sections of the PPF. The PPF thickens and extends caudally while maintaining close contact laterally with the body wall and medially with the primary esophageal mesentery. The limit of the caudal boundary was estimated to be the point at which the PPF was no longer clearly discernable from the underlying liver tissue. Note that the liver extends to underlie both the left and right PPFs at this stage of embryonic development (Fig. 2). The extents of the ventrodorsal and mediolateral boundaries of the PPF used for the 3D reconstructions are outlined in Fig. 2. On the basis of the defined boundaries chosen, the average rostrocaudal extents of normal PPFs were...
206 ± 23 (SD) and 229 ± 16 μm (n = 18 for both) for the left and right sides, respectively. All dimensions are derived from fixed tissue and thus may be underestimated by ~15% due to tissue shrinkage.

Figure 2 illustrates cross sections of the PPF at E13.5 in embryos with normal and malformed PPFs. Sections are shown from the rostral, middle, and caudal regions of the PPF. The nitrofen-induced defects of the PPF, whether on the right or left side, were always found in sections taken from the middle and caudal regions of the PPF. The single right-sided defect measured was first noticeable at 110 μm from the rostral border of the PPF. The left-sided defects started at an average of 143 ± 24 μm (n = 11) from the rostral border of the PPF. Furthermore, the areas of PPF missing in the caudal sections were consistently located dorsolaterally. The extent to which the defect encompassed more medial areas of the PPF varied.

3D PPF structure. Figure 3 shows 3D reconstructions from embryos with and without defective left-sided PPFs. The 3D reconstructions are oriented to provide views from both the left and right lateral walls. This allows for a clear visualization of the medial and lateral perspectives of the bilateral PPFs. A comparison of the left control PPF (Fig. 3A) and the defective PPFs (Figs. 3, B-D) illustrates that the malformed regions are always located caudally and dorsolaterally. In contrast, the rostral, ventral, and most medial regions of the defective and normal PPFs are similar. Furthermore, PPF defects of varying magnitudes, ranging from mild (Fig. 3B) to moderate (Fig. 3C) to severe (Fig. 3D), were detected.

Figure 4 shows a rendering of a normal PPF and a PPF with a right-sided defect. As was the case for left-sided defects, the malformed PPF regions are those located caudally and dorsolaterally. Only the right PPF is shown from this embryo because the corresponding left PPF tissue sections were not of suitable quality for reconstruction.

Fig. 3. Representative 3D reconstructions of a normal PPF and PPFs with left-sided defects. Renderings are shown from the perspectives of looking through the right and left lateral cervical walls of E13.5 rat embryos. A: normal left and right PPFs. B–D: 3D reconstructions of left-sided defects of varying degrees (arrows point to defective regions). In all cases, the defects are in the dorsolaterally located regions of the caudal PPF. R, right PPF; L, left PPF. Scale bars = 100 μm.
Correlation between PPF and diaphragm muscle defects. Figure 5 was constructed to assist the reader in visualizing how the regional defects of the PPF could translate into the lack of dorsolateral diaphragmatic musculature at later stages of development. The defect in the PPF at E13.5 (Fig. 5A) is located in the typical dorsolateral portion. A representative, moderate-sized, nitrofen-induced defect of diaphragm musculature at E15.5 is shown in Fig. 5B. Note that the area of diaphragm musculature missing is also located in the dorsolateral portions. Therefore, although we do not, as of yet, have a complete mechanistic understanding of how muscle precursors in the PPF contribute to the various regions of the diaphragm musculature, the anatomic profiles of regional defects in the PPF and diaphragm correlate well.

Fig. 4. Representative 3D reconstructions of a normal PPF and a PPF with a right-sided defect. Renderings are shown from the perspectives of looking through the right and left lateral cervical walls of E13.5 rat embryos. A: normal bilateral PPF from a control animal not exposed to nitrofen. B: 3D reconstruction of a right-sided defect (arrows point to defective regions). As was the case for left-sided defects (Fig. 3), the malformation is located in the dorsolaterally located regions of the caudal PPF. Scale bars = 100 μm.

Fig. 5. Juxtaposed photomicrographs of a defective PPF (E13.5; A) and diaphragm (E15.5; B) illustrating the correlation between regional defects at the 2 stages of development. A: close-up of the PPF (*) in a transverse section from a nitrofen-exposed E13.5 embryo (without underlying liver tissue). There is a pronounced malformation (arrow) of the left PPF in the characteristic dorsolateral region. B: diaphragm from an E15.5 embryo with a classical nitrofen-induced, left-sided defect (arrow). The tissue has been immunolabeled for polysialylated neural cell adhesion molecule, which is expressed by the phrenic nerve and primary myotubes. Comparing the two images shows that the defect in the dorsolateral PPF could translate into a hole in the dorsolateral region of the diaphragm musculature at later stages of development. Furthermore, the size of the defect in the diaphragm musculature could be dictated by the magnitude of the initial PPF malformation.
DISCUSSION

The 3D images provide the first clear visualization of PPF morphology and dimensions in an embryo. Furthermore, the images of nitrofen-induced PPF defects allow for an appreciation of the precise areas that are malformed. The realization that the dorsolateral region is the defective area in all cases provides a plausible explanation of why the holes in diaphragmatic muscle are consistently found in the corresponding dorsolateral regions. Moreover, these data provide further support against the widely stated hypothesis that a failure of pleuroperitoneal canal closure underlies the pathogenesis of nitrofen-induced CDH.

Structure of the primordial diaphragm and PPF. Textbooks typically provide a brief description of diaphragm embryogenesis that states that the musculature is derived from multiple sources, including the esophageal mesentery, the septum transversum, and the body wall musculature (16). However, those descriptions are largely based on speculations derived from examining the arrangement of muscle fibers in fully formed embryonic diaphragms (14, 18). Although issues pertaining to the formation of the diaphragm remain unresolved, recent data strongly suggest that the PPF is a major component of the primordial diaphragmatic musculature (1). The septum transversum is a distinct structure that fuses with the PPF and forms the central tendon of the diaphragm. The 3D reconstructions provided in this study allow for a clear visualization of PPF morphology before the onset of diaphragm myotube formation. It could be argued that the rendered 3D image of the PPF would differ if the boundaries chosen in the tissue sections were varied. However, unless the boundaries were chosen well outside those suggested by the staining profiles described, the general shape of the PPF and the identification of the defective regions in the nitrofen model would not differ significantly from that presented.

Defects of the PPF associated with the nitrofen model of CDH. The first sign of a nitrofen-induced defect in diaphragm tissue can be traced back to the PPF. Studies by Iritani (10) and Kluth et al. (11–13) demonstrated nitrofen-induced defects in the region of the PPF as early as E13–14. They referred to the malformed tissue as the posthepatic mesenchymal plate or the dorsal plate of the septum transversum. However, when comparing the data, it appears that the tissue being described in all of the studies is the PPF. The 3D reconstructions provided in the present study allow for a clear visualization of the regional defects within the PPF. The malformed areas are consistently located in the dorsolateral region. Correspondingly, the dorsolateral region of the diaphragm musculature is precisely the area affected in CDH. Thus the data are consistent with a causal relationship between a defect in the initial PPF and later diaphragmatic musculature malformations. Furthermore, these data provide the focus for future studies that may examine potential mechanisms underlying the normal and pathological formation of the dorsolateral PPF.

It should be stressed that the regions of the PPF that are missing are not merely the somatic mesodermal tissues that expand to close the pleuroperitoneal canal (PPC). PPCs form dorsolaterally located channels that link the future abdominal and thoracic cavities. The failure of the PPC to close adequately is often cited as the key pathogenic factor associated with CDH (16). However, the defects are obvious before PPC closure, and the regions of missing musculature extend well beyond the PPC (2). It is apparent from the 3D reconstructions of defective PPFs that the degree to which more medially and ventrally located tissues are affected varies. It is reasonable to hypothesize that the variability in the size of the holes in the diaphragmatic musculature with CDH is dictated by the extent of the initial defect within the PPF.

There are a number of fundamental issues pertaining to the relationship between the PPF and CDH that remained unresolved. First, do PPF defects result from a problem with muscle precursor migration, proliferation, or differentiation? Alternatively, is there a problem with the formation of the underlying somatic mesodermal-derived connective tissue, on which the muscle precursors develop? The fact that there is a thickening of muscle fibers around the hole in the diaphragm (2) is consistent with the hypothesis that at least some of the muscle precursors normally destined for the defective region of the primordial diaphragm migrate around the missing substratum to occupy adjacent PPF tissue. Furthermore, the normal insertion point for the phrenic nerve is often missing in defects associated with the animal model of CDH. However, the phrenic nerve is not absent, but, rather, its insertion point is translocated to a more ventrally located region in the diaphragm musculature (2). This suggests that the PPF may be defective before the arrival of the phrenic nerve (before E13.25) and that there is a subsequent compensatory realignment of the nerve contact point within the remaining PPF tissue. Studies examining the earlier stages of normal and pathological PPF formation are underway to test these hypotheses.

A second issue of uncertainty is the potential role of the liver in the pathogenesis of CDH. It is clear that the liver occupies space normally reserved for the PPF when there is a defect present. Whether the presence of the liver tissue in this area is causal of the defect or merely represents an early stage of liver displacement cannot be clearly elucidated with the present data.

Third, there is predominance of left-sided defects in infants with CDH that remains unexplained (16). The ratio of left- vs. right-sided defects in the animal model varies depending on which embryonic day the nitrofen is administered to the dam (3, 5, 15). These data suggest an age-dependent variation in the susceptibility of left and right PPFs to malformations. However, a clear explanation for the laterality of the defect will require further understanding of the etiology of CDH or the mechanism of nitrofen teratogenicity.

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