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Congenital diaphragmatic hernia: focus on abnormal muscle formation

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ABSTRACT

Background: CDH is a major birth defect, characterized by high mortality. How the initial defective mesenchymal substructures affects muscle malformation is unclear. Defects of genes involved in diaphragmatic development, such as friend-of-GATA2 (Fog2), may play an important role in its pathogenesis. We investigated the expression of Fog2 and proteins of myogenesis in a series of CDH and in diaphragms at different fetal ages, in order to clarify the role of muscular components during diaphragmatic development in cases with CDH.

Material and methods: Specimen were obtained from seven diaphragms of CDH cases undergoing surgery, 3 entire diaphragms from non repaired CDH, 5 control diaphragms at different gestational ages (16, 17, 22, 32, and 40 g.w.), and 3 biopsy samples of normal voluntary muscle. The thickness of diaphragms at the edge of the defect in CDH and in developing diaphragms was measured. All samples were processed for HE staining and immunohistochemistry. Immunohistochemical expression of MyoD, Myf4, Pax7, Mib1 and Fog2 was evaluated.

Results: Mean thickness at the edge of the defect was 4.14 mm. Contralateral hemi-diaphragm in 3 autopsies and in controls at 32 and 40 weeks measured 2.25 mm; histology showed a higher density of desmin-positive muscular cells at the edge of defect. CDH displayed scattered Myf4-positive cells (range 0%–10%, mean 2.4%), numerous Pax7-positive cells (range 0%–24%, mean 12.1%) and less than 1% Mib1-positive cells. Controls showed a reduction of positive cell with the progression of gestational age for Myf4 (30% at 16 weeks, 20% at 17 weeks, 5% at 22 weeks, 1% at 32 and 40 weeks), Pax7 (85% at 16 weeks and 17 weeks, 35% at 22 weeks, 11% at 32 weeks) and Mib1 (20% at 16 weeks, 8% at 17 weeks, 7% at 22 weeks, 2% at 32 weeks). Fog-2 was diffusely positive in mesenchymal, mesothelial and muscular cells, in diaphragms from 16 to 22 weeks, decreasing to 20% of positive muscular cells in 32-week diaphragm. In CDH only mesothelial and mesenchymal cells were positive. Stem cell markers were negative in cases and controls.

Comment: CDH shows a thick muscular border, with high number of mature muscle cells and significant increase of quiescent satellite cells (PAX7 +, Mib1 −). Abnormal architecture may affect the normal process of myogenesis and thus signaling and cell-cell interactions of myocytes. The expression of Fog2 in mesothelial and mesenchymal cells in CDH demonstrates the absence of a genetic defect involving Fog2 in our cases. Being Fog2 expressed in muscle cells at early stage supports the hypothesis that the altered diaphragmatic genesis may undermine also the muscular component instead of the only mesenchymal one.

Congenital diaphragmatic hernia (CDH) is a severe developmental defect occurring in approximately 1:2500–1:3000 live births [1,2]. Although implementation of rational treatment strategies has resulted in reduced mortality trends in individual centers, there continues to be a great variability in overall CDH mortality with rates ranging between 20% and 60% [3] with significant long-term morbidity among survivors. The majority of CDH cases involve incomplete formation of the posterolateral portion of the diaphragm commonly occurring on the left side (about 85%) [4]. Most cases are isolated but associated organ malformations are often present, especially cardiac. CDH can also occur as part of genetic syndromes [5].

In both sporadic and syndromic CDH there is evidence for a potential role of genetic factors in pathogenesis and for genetic heterogeneity, although the genes involved are so far largely unknown. A proper development of the diaphragm requires the concerted action of GATA-4, Fog2, COUP-TFII and WT-1. The nuclear protein Friend of Gata-2 (Fog2; also known as Zpfm2; located on 8q23.1, OMIM 603693) is a transcriptional repressor gene, characterized by specific zinc fingers domains binding to members of the GATA family of transcription factors and regulating directly or indirectly, via COUP-TFII the expression of GATA-4 [6].

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Fog2 is expressed in the early development of septum transversum of the diaphragm in both humans and rodents. It may play a role during the genesis of diaphragmatic muscular component which starts in the pleuro-peritoneal folds (PPF). In fact, the so-called lil-mice, which carry a hypomorphic Fog2 allele determining a severely truncated Fog2 protein, have non-Bochdalek type diaphragmatic defects and pulmonary hypoplasia. In these mice, diaphragmatic myotubules show an abnormal distribution and aggregation, radiating in a dorsal ventral pattern rather than the medio-lateral pattern of wild-type mice. Abnormal regulation of GATA-4 by Fog2 might also be important for CDH development as demonstrated by midline defects of the non-muscle connective tissue of the diapragm in mice models heterozygous for a deletion mutation of the GATA-4 gene. COUP-TFII is located on human chromosome 15q26.2, a genomic region that has been deleted in CDH patients. It has been demonstrated to be necessary for Fog2 to repress the transcription of a GATA-4. Immunohistochemical expression of Fog2 only in pleuro-pulmonary folders, and not in muscular component of diaphragm during the embryogenesis in murine models, supports the hypothesis of a mesenchymal damage as the primary event in pathogenesis of CDH. In all models studied to date the initial defect occurs prior to muscle cell migration to the PPF and the muscle defects are secondary due to abnormal myocyte alignment and intercellular signaling. So far the role of Fog2 in human diaphragm development and in pathogenesis of CDH has been only partially investigated. Holder et al. reviewed literature for human genetic factors in CDH and reported only three CDH patients with 8q deletion [7]. Ackerman et al. described a Fog2 gene's mutation occurring in one CDH patient out of 30 investigated [6].

Differentiation of muscular components of diaphragm during fetal development is related to the coordinated expression of Pax7 and myogenic transcription factors (Myo-D, Myf-4) [8]. Their expression in CDH has been poorly investigated, especially in humans.

Fog 2, with its possible influence on development of muscular component, and specific muscular proteins, such as MyoD, Mib1, Pax 7 and Myf4, involved in the myogenesis, were chosen for the purposes of this study in order to provide information on how the muscle tissue in CDH human diaphragms shows partially altered organization and protein expression.

1. Matherials and methods

1.1. Case selection

1.1.1. Surgical biopsies and autopsy specimen

The prospectic study included: 1) biopsy samples from anterior and posterior border of diaphragmatic defect collected from all newborns who underwent surgery for correction of CDH at Pediatric Surgery Department from December 2007 to July 2009; 2) whole diaphragms removed during autopsy from those newborns who died before surgery (Fig. 1). Samples were formalin-fixed, paraffin-embedded and examined according to sampling protocol reported in Fig. 2.

1.1.2. Controls

Postmortem diaphragms from fetuses at 16, 17, 22, 32, and 40 gestational weeks, who had died for other causes and were unaffected by malformations, were removed at autopsy and examined according to the same sampling protocol as CDH autopsy diaphragms. All controls samples were formalin-fixed, paraffin-embedded and stained for Hematoxylin and Eosin (HE) and immunohistochemistry.

1.1.3. Ethics

Approval by Ethics Board and by parents was obtained before surgical sampling and autoptical examination.

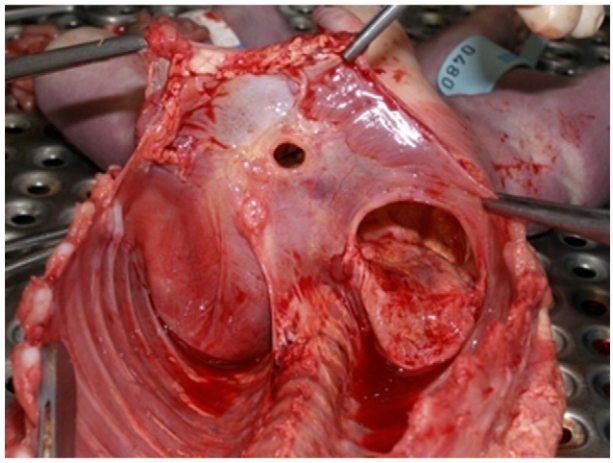

Fig. 1. Diaphragm removed during regular autopsy in CDH.

1.2. Diaphragm thicknesses

Thickness of diaphragms was measured during surgery at the edge of the diaphragmatic defect using a ruler (0.5 mm sensitivity); thicknesses of post-mortem diaphragms were measured both at the edge of the hole and at the contralateral cupola.

1.3. Histology

H&E stained sections from different sites of diaphragm were examined and the analysis of cellularity in different areas was performed, counting the average number of cells in at least 4 HPFs.

1.4. Immunohistochemistry

Immunohistochemical staining was performed on formalin-fixed, paraffin-embedded tissue sections using a fully automated system (Bond-maX; Leica, Newcastle Upon Tyne,UK). In brief, one 4-micron-thick section from each paraffin-embedded block was cut. The sections were deparaffinized in Bond Dewax Solution (Leica) at 72 °C, rinsed in ethanol, and rehydrated in distilled water. Antigen retrieval was performed by heating sections for 30 min at 99 °C in Bond Epitope Retrieval Solution 1 (Leica). Endogenous peroxidase was blocked by 3% hydrogen peroxide before 30 min of incubation with mouse monoclonal anti-MyoD1 (clone 5.8A, Dako, Glostrup, Denmark; 1:50), mouse monoclonal anti-Myf4 (clone LO26, Leica; 1:20), mouse monoclonal anti-Desmin (clone D33, Dako; 1:100), mouse monoclonal anti-Ki-67 (clone MIB-1, Dako; 1:100), rabbit polyclonal anti-Fog2 (sc-10755, Santa Cruz Biotechnology, Santa Cruz, CA; 1:50) and mouse monoclonal anti-Pax7 IgG (R&D Systems, 1:50) respectively. Specimens were then washed with phosphate-buffered saline (pH 7.0) and incubated with Bond Polymer Refine Detection Kit (Leica) according with the manufacturer's protocols. The staining was visualized with 3,3'-diaminobenzidine (DAB) and the slides were counterstained with Mayer's hematoxylin. The sections were then dehydrated, cleared, and mounted. Formalin-fixed, paraffin-embedded positive and negative controls were included in each run.

1.5. Interpretation of immunohistochemical results

Miogenin, Mib1, Pax7 and MyoD were considered positive when showing a nuclear staining. The proliferation index was evaluated counting the percentage of Mib1 positive cells in at least 4 fields at 40×.