Anatomy

Early development of pleuroperitoneal fold of the diaphragm in the rat fetus

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ABSTRACT. The embryonic diaphragm comprises four major structural components derived from the transverse septum, the dorsal foregut mesentery, the pleuroperitoneal folds (PPFs), and the body wall. In this study, the appearance of PPFs and related factors were investigated using light microscopy of horizontal sections of rat fetuses from embryonic day 12 to 13. In rat fetuses, the sign of PPF projection was noted in the sidewall of the pericardioperitoneal canal at embryonic day 12, and was confirmed as folds at embryonic day 12.25. Expressions of GATA4, COUP-TF2, and FOG2 were detected in PPF at the early stage of formation. Localizations of these factors suggested that COUP-TF2 and FOG2 are the main factors in PPF appearance and that GATA4 is unlikely to be a main factor, although it is necessary for PPF formation.

KEY WORDS: diaphragm, fetus, pleuroperitoneal folds, rat

The embryonic diaphragm comprises four major structural components: the central tendon, derived from the transverse septum; a dorsal midline portion, derived from the foregut mesentery; two dorsolateral shelves of tissues, derived from the pleuroperitoneal membranes (folds); and peripheral components, derived from the body wall [13].

The diaphragm is an organ dividing the thoracic cavity and abdominal border and is the mammalian structure, including the skeletal muscle, that induces breathing. Congenital diaphragmatic hernia (CDH) is defined as a projection of abdominal viscera into the thorax through an abnormal opening or defect that is present at birth. CDH is often complicated by hypoplasia of other structures such as pleural hypoplasia in Fryns syndrome or Donnai-Barrow syndrome [10, 12, 18, 22]. As most human CDHs result in undeveloped PPFs [1, 10, 18], PPFs play a key role in studies about the development of the diaphragm. However, the projection mechanism of PPFs is still unknown, and there are few detailed studies of the early process of diaphragm formation.

It is reported that the DNA of patients with CDH has various mutated domains. Of these domains, chromosomes 8p23.1 (GATA4 gene), 8q22-23 (FOG2 gene), and 15p26 (COUP-TF2 gene) are frequently mutated [10].

GATA4 is known as a transcriptional factor with zinc finger and is a member of a family of DNA-binding proteins. Its expression has been confirmed in the transverse septum, PPFs [4], and various structures including the heart, lungs, and stomach [1, 3, 14, 19, 21, 26]. As GATA4 knockout mice die due to growth delay and cardiac ateliosis from embryonic day 8.5 (E8.5) to 9.5 [14, 16], the function of GATA4 in diaphragm formation could not be investigated using these knockout mice.

FOG2 (Friend of Gata 2) is a co-factor of the GATA family and is concerned with development of the heart and the lungs with GATA4. It is reported that FOG2 knockout mice die due to poor development of the heart, lungs, and liver, and edema from E12.5 to E15.5 [25, 29]; thus, it is not known whether the diaphragm develops in these knockout mice. It has been reported that incomplete lung formation associated with CDH in humans was caused by pleuroperitoneal hypoplasia when FOG2 mutates, but on the other hand FOG2-mutated mice did not develop lung and skeletal muscles in the diaphragm [2]. In rats, FOG2 and MyoD are expressed in different cells in PPFs at E13, but these factors often exist in the same cells in the diaphragm at E16.5; therefore, it is suggested that FOG2 is responsible for forming PPFs before the skeletal muscles develop [6].

COUP-TF2 (Chicken Ovalbumin Upstream Promoter-Transcription Factor 2) is structurally a nuclear receptor with unknown ligand(s). COUP-TF2 is involved in heart development as GATA4 and FOG2, and COUP-TF2 knockout mice die because of defects in angiogenesis and heart development [17]. Tissue-specific null mutant mice using the Cre/loxP system from E9.5 do not die, but they have CDH caused by pleuroperitoneal hypoplasia [27]. Since COUP-TF2 exists in cells comprising PPF at E13 [5], COUP-TF2 may be closely related to the development of PPF.

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There are many reports on the development of PPF conducted using fetuses sectioned in transversal or sagittal planes. However, microscopic images of these sections cannot show from the root to the top of PPF projecting from a body wall. As such, the present study planned to clarify the process of diaphragm formation in rat fetuses (from E12 to E13) by observing the first appearance of PPF using fetal horizontal sections and by detecting factors involved in the formation of the diaphragm, to focus on the PPF.

MATERIALS AND METHODS

Animals

Wistar rats (Japan CLEA, Tokyo, Japan) were used. They were given a commercial diet (Labo MR Breeder, Nosan Corp., Yokohama, Japan) and water, both ad libitum. Females were placed with males overnight and were examined the next morning for the presence of sperm in the vaginal smear. The day of observation of sperm was counted as Day 0 of gestation. The experiments described in this article were carried out in accordance with the Azabu University Animal Experiment Guidelines (No.050208-5).

Fetuses from E12, 12.25, 12.5 and 13 were examined. To determine the proliferative activities of the PPF, the pregnant rats were injected with 5-bromo-2’-deoxyuridine (BrdU; Sigma-Aldrich, Poole, U.K.) at a dose of 100 mg/kg of maternal body weight intraperitoneally 2 hr before each sampling time.

Fetuses were harvested by cesarean section under anesthesia with isoflurane and fixed in Bouin’s fluid. The Bouin-fixed fetuses were routinely dehydrated, embedded in Paraplast (Sakura Finetek, Tokyo, Japan), and horizontally (coronally) sectioned serially at 5 µm.

Immunohistochemistry

Sectioned slides were immunohistochemically stained with the following antisera: GATA4 (Abcam, Cambridge, MA, U.S.A.), FOG2 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, U.S.A.), COUP-TF2 (Perseus Proteomics Inc., Tokyo, Japan), and BrdU (Dakocytomation, Glostrup, Denmark), using polymer methods (Histofine Simple Stain MAX PO kit, Nichirei Bioscience Inc., Tokyo, Japan). The reactivity of FOG2-antibody was confirmed by its localization in the ventricles, in accordance with a previous study [23].

RESULTS

Projection of PPF

Using serial horizontal sections, the development of PPF was observed.

At E12, PPFs were not observed (Fig. 1); however, a region that would develop into PPF protruded from the lateral body wall of the pericardioperitoneal canal to the body cavity (Fig.1B–D). The long axes of nuclei in the mesothelium cells in this region were perpendicular to the body cavity surface, whereas they are usually parallel to the surface. The PPF structure appeared for the first time at E12.25 (Fig. 1E). At E12.5, the PPF had grown and contacted the transverse septum in which liver buds were developing to branches in the ventral section (Fig. 2: asterisk). At E13, PPFs were more developed and contacted the dorsal mesentery of the foregut (Fig. 3: m).

Expressions of various factors

E12: GATA4 were expressed in lung epithelial cells and many mesenchymal cells, but were weakly expressed in the body wall including the region in which PPF would project (rPPF, Fig. 4: arrowhead). COUP-TF2 and FOG2 were expressed in almost all mesothelial cells of body wall and the underlying mesenchymal cells in rPPF. Many mesothelial cells of the body wall incorporated BrdU in the areas containing rPPF; however, BrdU-incorporated mesenchymal cells in this area were fewer than in other areas (Fig. 5).

E12.25: GATA4 was present in almost all mesenchymal cells and mesothelial cells that constituted a wide range of the body wall including the area in which PPF began to project (Fig. 4: arrow). COUP-TF2 was strongly expressed in all mesothelial cells in the body wall, and in almost all mesenchymal cells in the area of PPF. Similarly, FOG2 was strongly expressed in all mesothelial cells in the body wall, and in almost all mesenchymal cells in the area of PPF.

E12.5: GATA4 was expressed in many mesenchymal cells and mesothelial cells of the body wall including the area of PPF. COUP-TF2 was strongly expressed in all mesenchymal cells and mesothelial cells of the body wall containing PPF. FOG2 was also strongly expressed in COUP-TF2-positive areas. However, whereas many superficial cells of PPF incorporated BrdU, only a few mesenchymal cells of PPF did so (Fig. 5).

DISCUSSION

Previous studies on the diaphragm using rats were investigated mainly at E13-E13.5 [6, 7] when the PPF had already developed. In contrast to these studies, we observed the morphology at the moment of PPF appearance in horizontal sections of rat fetuses at E12.25 and clarified the PPF formation. Greer [8] observed PPF appearance in cross-sections of rat fetuses at E12-E14, but the descriptions concerning PPF at E12 and E13 were unclear. Mayer et al. [15] observed the rat diaphragm under a scanning electron microscope and stated that PPF initially appeared at E12.5, but we noted the sign of PPF appearance at E12 and confirmed its presence at E12.25 by observing horizontal sections of rat fetuses.
Intense GATA4 immunoreactivity was evident in many cells of PPF, the transverse septum (and its derivative the central tendon), and the esophageal mesenchyme at E12.5 in mouse fetuses [11]. FOG2 has been immunohistochemically confirmed to be expressed in PPF in the early fetal diaphragm of E13 rat fetuses [6], and COUP-TF2 and FOG2 in mouse fetuses at E12.5 (corresponding to E13.5 in rats) [28]. GATA4 is expressed in many mesenchymal cells of PPF with FOG2 at E13.5, and FOG2 and MyoD are expressed in different cells of PPF at E13, but these factors often exist in the same cells in the rat diaphragm at E16.5 [6]. Moreover, MyoD is expressed in mesenchymal cells of PPF at E13.25, while E13.5 is the stage at which the PPF is fully formed and immediately precedes the start of myotube formation in the rat [4, 6]. Three factors, GATA4, COUP-TF2, and FOG2, were proposed following an analysis of mutation sites in human congenital CDH patients [10]. We investigated the involvement of these factors in the timing of PPF appearance by immunohistochemically localizing them (Fig. 4). In E12 rat fetuses, all the factors investigated (GATA4, COUP-TF2, and FOG2) were expressed in mesothelial cells of the body wall including the region in which PPF would project (rPPF), but GATA4 were weakly expressed. In contrast, in the mesenchymal cells under the rPPF only COUP-TF2 and FOG2 were localized. These observations suggest that COUP-TF2 and FOG2 are mainly involved in the appearance of PPF. At E12.25 and 12.5, COUP-TF2 and FOG2 were localized in almost all mesothelial cells and mesenchymal cells of PPF, and GATA4 was expressed in many of these cells, suggesting that these three factors are required for the development of PPF. At E12, BrdU-positive cells appeared in the mesothelial cells in rPPF, suggesting that the appearance of PPF requires cell division of mesothelial cells. However, the relationships among cell division and other factors in PPF development remain unclear from our
To investigate the development of the diaphragm, generally, diaphragmatic hernia is experimentally induced by administration of nitrofen, and then morphological abnormality of the diaphragm formation, including low formation of PPFs, and involvement of gene expression or transcription factors are investigated using mice or rats. Nitrofen has been administered at E7 or E8 and E9, and the diaphragm has been investigated at E11.5 and E13 or later in mice and rats, respectively, showing that the timing of studies on the development of the diaphragm or developmental mechanism of CDH has been concentrated on the period after PPF appearance. Nitrofen inhibits retinaldehyde dehydrogenase 2 from converting retinol to retinoic acid (RA), which results in RA deficiency inducing diaphragmatic hernia. Several pathways have been proposed through RA investigated by analyzing diaphragmatic hernia induced by nitrofen or a retinoic acid receptor antagonist [7, 10, 20]. The feature common to these reports was that RA was considered to affect several steps of diaphragm development. Holder [10] reported that RA induces COUP-TF2, and FOG2 is involved in this event as a cofactor and induces GATA4 expression. However, in our study, COUP-TE2, FOG2 and GATA4 were expressed in PPF when PPF appeared. Nitrofen does not completely inhibit the formation of PPF; PPF development often continues until around the time of its connection to the other components [4, 5, 7, 9, 11, 25], so the RA signaling pathway may not be closely involved in PPF appearance. Our conclusion is supported in the following notion: nitrofen induces diaphragmatic hernia, but does not interfere with early development of the four components of the diaphragm, and it is considered to interfere with the development thereafter, for example, inhibiting completion of joining of PPF or other regions to the transverse septum and interfering with migration of myotome-derived myoblasts to the diaphragm [5, 7, 24, 28].

In this study, the sign of PPF projection was noted in the sidewall of the pericardioperitoneal canal at E12, and it was confirmed
as folds at E12.25. Expressions of GATA4, COUP-TF2, and FOG2 suggested that COUP-TF2 and FOG2 are the main factors in PPF appearance and that GATA4 is unlikely to be a main factor, although it is necessary for PPF formation.

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Fig. 4. Immunohistochemical expression of GATA4, COUP-TF2, and FOG2 in PPFs of fetuses from E12 to 12.5. Arrowheads show the region in which PPFs would project (rPPF). Arrows show PPFs. Bar, 50 µm.

Fig. 5. Immunohistochemical expression of BrdU in PPFs of fetuses from E12 to 12.5. Arrowhead shows the region in which PPF would project (rPPF). Arrows show PPFs. Bar, 50 µm.
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