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Does the developing liver inhibit early lung growth in congenital diaphragmatic hernia?

Abstract It has been hypothesised that the liver induces lung hypoplasia in congenital diaphragmatic hernia (CDH) by non-compressive intrathoracic growth rather than traditional mass herniation. Utilising a co-culture system, we tested the capacity of liver cells to inhibit lung growth by contact rather than compression. Heart, liver, and lungs were microdissected from normal rat embryos (n > 20 from at least three litters) on day 13.5 of gestation. Monolayer cultures of enzymatically dispersed livers and hearts were established at the same cell density. Lung primordia were cultured in direct contact with hepatic cells or partitioned from them by a permeable polytetrafluoroethylene membrane. This permits the contributions of diffusible factors and cell contact to be distinguished. Lungs were similarly cultured in direct contact with or partitioned from cardiac cells. Lungs cultured in isolation served as further controls. Daily inspection permitted assessment of in-vitro lung growth. Growth of lungs in direct contact with hepatic cells was equivalent to that of lungs partitioned from liver cells. Lungs in direct contact with cardiac cells and lungs partitioned from cardiac cells were also not inhibited compared to lungs cultured in isolation. Early lung development is thus not inhibited by humoral or contact-mediated interactions with embryonic liver cells. Lung hypoplasia in CDH is therefore unlikely to originate from contact inhibition with the developing liver. An intrinsic pulmonary defect may better explain hypoplastic lung development in CDH.

Keywords Congenital diaphragmatic hernia · Pulmonary hypoplasia · Organ culture · Cell culture · Embryology

Introduction

Congenital diaphragmatic hernia (CDH) is a major unsolved problem confronting modern paediatric surgery [1–3]. Current treatments have yet to improve the 40%–50% mortality of afflicted individuals [4, 5]. The devastating impact of lethal lung hypoplasia (PH) on the outcome of CDH is well-documented [6]. Nevertheless, the cause of such pulmonary maldevelopment remains unclear [7]. Scrutinising the developmental biology of PH may permit investigators to identify novel targets for innovative therapies in CDH.

In classic studies of experimental CDH, Kluth et al. hypothesised that the liver induces PH by non-compressive intrathoracic growth rather than traditional mass herniation [8]. Certain observations support this original proposal: the liver in human CDH may appear malformed rather than simply prolapsed. It faithfully moulds to its thoracic surroundings, and abnormal vasculature may accompany this morphogenesis [9, 10]. Ectopic hepatic tissue has been demonstrated in the thorax and even sequestered within the lung of CDH cases [11, 12].

These intriguing findings indicate ‘opportunistic’ hepatic growth rather than simple liver herniation in CDH. The impaired prognosis of ‘liver-up’ CDH supports a link between intrathoracic liver growth and the extent of PH [13, 14]. It can therefore be hypothesised that diaphragmatic non-closure in CDH permits proliferating liver cells to impede lung growth by protracted contact and not compression. Demonstrating such ‘growth competition’ would indicate that PH is a consequence of the diaphragmatic malformation and not an intrinsic pulmonary pathology. Using a novel co-culture system, we tested the capacity of normal rat liver cells to inhibit antenatal lung growth by contact rather than compression.
Materials and methods

Embryonic rat lungs were cultured on a monolayer of hepatic cells to mimic the prolonged contact of lung and liver permitted by the diaphragmatic defect in CDH. Polytetrafluorethylene (PTFE) membrane culture-dish inserts were used as an artificial in-vitro ’diaphragm’. These membranes are permeable to soluble factors whilst not allowing cell-cell contact across them. To ensure that effects on lung growth were due to cell contact rather than soluble factors, co-cultures were also performed with a membrane separating the lungs and liver cells like an artificial ‘diaphragm’. To establish that effects on lung growth were due to liver cells specifically, lungs were co-cultured in contact with, and also partitioned from, cardiac cells. Further control lungs were cultured without any cell monolayers. Five numbered experimental groups were utilised (Fig. 1): (1) Lungs and liver cells cultured together upon the PTFE membrane (Fig. 1A); (2) Lungs cultured upon and liver cells grown beneath the PTFE membrane (Fig. 1B); (3) Lungs and cardiac cells cultured together upon the PTFE membrane (Fig. 1C); (4) Lungs cultured upon and cardiac cells grown below the PTFE membrane (Fig. 1D); and (5) Lungs cultured alone upon the PTFE membrane (Fig. 1E).

Timed-pregnant Sprague-Dawley rats (Charles River UK) were utilised. Using aseptic technique, rat embryos were harvested by caesarean section under terminal anaesthesia (intraperitoneal so-

Fig. 1A–E Diagramatic representation of lung/cell monolayer coculture system: membrane culture-dish inserts with surrounding dish and float on culture medium. A Lungs and liver cells cultured together on PTFE membrane. B Lungs cultured on and liver cells beneath PTFE membrane. C Lungs and cardiac cells cultured together on PTFE membrane. D Lungs cultured on and cardiac cells below PTFE membrane. E Lungs cultured alone on PTFE membrane.

medium pentobarbitone) on day 13.5 of gestation. Developmental studies in the rat confirm that the diaphragm normally closes to separate the lungs and liver within the next 72 h [8]. Upon retrieval, embryos were transferred to an isotonic saline bath cooled on ice, microdissected from their extra-embryonic membranes, and secured in a lateral position using cranial and caudal entomology pins. Using a stereomicroscope and microsurgical instruments, a thoraco-abdominal incision was performed to expose the embryonic heart, lungs, and liver. The heart was teased from the lung rudiments before the oesophagus was carefully freed from the primitive carina. The trachea was then sectioned and the lungs, liver, and heart separated before individual transfer into serum-free culture medium (DMEM/F12 1:1, GibcoBRL, Life Technologies, UK) incorporating penicillin (100 IU/ml) and streptomycin (100 μg/ml; GibcoBRL, Life Technologies, UK).

Pooled livers were tryspinised (0.1 mg/ml for 4 min at 37 °C) and then agitated by pipetting to facilitate cell dispersion [15]. Adequate cell separation was confirmed by microscopy. Digestion was terminated by gentle centrifugation (<800 rpm for 1 min), which allowed removal of trypsin in the supernatant. Collected cells were resuspended in medium with 5% fetal bovine serum (FBS) added to quench residual trypsin activity. An aliquot of the resulting hepatic-cell suspension was removed for passage through a cell counter. This enabled hepatic cells to be plated at the same cell density (4.2 × 10^5/ml) either on, or in the dish below, a PTFE membrane culture-dish insert (Millicell, Millipore, UK). These membranes (pore size = 0.4 μm) permit diffusion of soluble factors whilst not allowing cell-cell contact across them. Pooled hearts were treated in an identical fashion to allow plating as described above for the liver cells. Plated cells were incubated at 37 °C in 5% CO2 for 24 h to allow cell adhesion to their support. During this time, lung rudiments were cultured separately with standard medium plus 5% FBS at 37 °C in 5% CO2.

At 24 h post-retrieval, lung primordia were transferred to the PTFE membranes that had monolayers of liver or cardiac cells on