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Lung and liver growth and retinoic acid status in human fetuses with congenital diaphragmatic hernia

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ABSTRACT

Background: Abnormal retinoic acid (RA) signalling is considered a major cause of congenital diaphragmatic hernia (CDH). Pulmonary hypoplasia and pulmonary hypertension are the major causes of morbidity and mortality in infants born with CDH. Experimental studies in animals have found that RA signalling is involved in lung and liver development, but animal models of CDH do not directly correlate with CDH in human fetuses. This study investigated if RA status is also linked to lung and liver growth in human fetuses with CDH.

Study design and patients: Hepatic stellate cells (HSC) in autopsy human fetal liver tissue were identified using cRBP-1 immunohistochemistry and the numbers of HSC manually counted. In mammals, RA is principally stored in HSC complexed to cRBP-1 and therefore cRBP-1$^+$ HSC numbers were used as an indicator of fetal RA status. The number of HSCs was correlated with liver and lung weights, calculated relative to either normal biometric values or fetal body weight.

Results: The number of cRBP-1$^+$ HSCs correlated with lung weight contralateral to the side of the diaphragmatic hernia ($r = 0.82$, $p = 0.025$) and combined lung weight ($r = 0.78$, $p = 0.039$) but not with ipsilateral lung weight ($r = 0.43$, $p = 0.33$), in fetuses with right and left CDH and a case of giant omphalocoele. Liver growth was influenced by contact with diaphragm but not significantly correlated with cRBP-1 expression ($r = 0.52$, $p = 0.056$).

Conclusion: Fetal RA stores, reflected in the number of cRBP-1$^+$ HSCs, influence lung growth as well as diaphragm development in human fetuses with CDH. Contact with diaphragm influenced liver growth.

1. Introduction

Congenital diaphragmatic hernia (CDH) is a significant clinical problem, with pulmonary hypoplasia (PH) and pulmonary hypertension the major causes of morbidity and mortality in isolated CDH [1]. Most information on the mechanisms responsible for diaphragm and lung malformations has been obtained from animal studies and it is difficult to obtain similar data in human fetuses, but the findings in animal studies may not be directly applicable to human patients [1]. We have sought to address this issue by studying human fetuses with CDH and comparing the findings to those in animal experiments. Abnormalities in any part of the retinoic acid (RA) signalling pathways are suggested as a possible cause of CDH and probably also PH, as RA is involved in many phases of lung development [2]. RA is mainly stored bound to cellular retinol binding protein-1 (cRBP-1) in hepatic stellate cells (HSCs) and HSC expression of cRBP-1 is reflective of fetal RA status [3,4]. We had previously reported that cRBP-1 expression was reduced in CDH fetuses as a group [5]. The expression of GFAP (used as a RA-independent marker of HSCs) was normal in these fetuses, suggesting that the defect was in the RA signalling rather than HSC development [5]. In the current study, we examined the relationship between hepatic cRBP-1 expression and lung and liver development in fetuses with CDH. We compared our findings to control fetuses without malformations and diaphragm eventration fetuses with liver displacement but no diaphragmatic hernia.

2. Patients and methods

2.1. Fetal cases and controls

In this study, 9 fetuses with CDH including one with giant

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omphalocoele, plus two fetuses with diaphragmatic eventration due to skeletal myopathy, one with multiple anomalies including thin diaphragm and growth restriction (included for comparison), were available for study, identified from hospital archives over an 11 year period (2006–2016). Fetal age was from 13 to 38 weeks of gestation. Control fetuses without malformations and from 19–38 weeks of gestation were also included. There was incomplete data in two cases (no lung weights): a 13 week fetus with left CDH and a 27 week fetus with bilateral diaphragm agenesis, the subject of a previous case report [6]. This case and some of the other fetuses (cases 4–7 and 9, see Table 1) and two controls (cases 16 and 17) had been included in our recent investigation on the development of HSCs in fetal liver [5]. All clinical details were derived from autopsy records and are listed in Table 1. The extent of liver herniation was described but quantitative data was not available. Presence or absence of liver herniation, liver weight and lung weights for each fetus (relative to normal biometric values) are compared in Table 2. All autopsies were performed or supervised by specialist perinatal/paediatric pathologists in the Royal Brisbane and Women's Hospital (Queensland) and Prince of Wales Hospital (NSW), Australia. Ethics approval for our study was obtained from the Human Research Ethics Committees of the Royal Brisbane and Women's Hospital, Prince of Wales Hospital and QIMR Berghofer Medical Research Institute. Parental consent for fetal autopsy and the use of tissue for research and scientific purposes was obtained.

2.2. Immunohistochemistry for cRBP-1

Immunoperoxidase stains for cRBP-1 were performed as previously described [5,7]. HSC density in fetal liver was assessed by manually counting the numbers of HSCs expressing cRBP-1 antigen (by CL) in the 10 contiguous high power fields (hpf, × 40 objective) within the region of the slide containing most HSCs. Only fetuses with specific consent for research and adequately preserved liver tissue were examined so that relatively few cases were available, especially as these conditions are rare and pregnancy is not usually terminated for congenital diaphragmatic hernia. The results of cRBP-1 expression in CDH fetuses were then used to correlate with both liver and lung weight. Liver and lung weights (ipsilateral, contralateral and combined lung weights) were calculated as a ratio compared to normal values for fetuses of similar gestational age, from biometric tables [8], as well as relative to fetal body weight. For each case, the appropriate gestational control from biometric charts was selected by considering fetal gestational age, fetal weight, foot length and crown rump length, taking into account any confounding factors, for example generalised oedema which can increase fetal weight (e.g., case 5).

2.3. Statistical analysis

The association between cRBP-1$^+$ HSC numbers and either lung or liver weights was assessed in Graphpad Prism 7, using Pearson's correlation, with $p < 0.05$ considered statistically significant.

3. Results

3.1. cRBP-1 and liver weight

The clinical features, lung and liver weights are shown in Tables 1 and 2. Liver and lung weights (measured in grams) were compared to mean values (also in grams) for gestational age, obtained from biometric tables [8]. The ratio of the organ weight in the CDH fetus was calculated relative to that of the published mean value. For example, a value of 1.0 showed that the organ weight in the study/CDH fetus was the same as that in the biometric tables, while a value of 0.5 showed that the organ in the study fetus was half the mean weight of controls stated in the biometric table. Compared to normal biometric values, liver weight was reduced in all 6 fetuses with liver displacement and reduced contact with the diaphragm vs. Control fetuses (Median, IQR; 0.85, 0.81–0.89 vs. 1.13, 1.11–1.28; $p = 0.0022$), while liver weight was normal or slightly increased in 3 CDH fetuses without liver herniation and 2 fetuses with displaced liver due to thin elevated diaphragms without liver herniation or loss of contact with the diaphragm (Median, IQR; 1.11, 0.98–1.3, $p = 0.62$).

The exception was a growth restricted fetus with intact but thin diaphragm (no organ herniation) and multiple congenital anomalies, where liver weight was also reduced (Table 2, case 10). HSCs have a characteristic morphology, showing perisinusoidal location, long slender processes and cRBP-1 expression by the immunoperoxidase method (Fig. 1A). In this study, we found that while cRBP-1 expression (measured as number of cRBP-1 expressing HSC/hpf, mean of 10 hpf) was reduced in 5 of 6 CDH fetuses with decreased contact between liver and diaphragm (Table 2) (Median, IQR; 3.25, 0.78–5.1, $p = 0.024$) relative to control fetuses of similar gestational age (Median, IQR; 6.8, 5.4–9.4), cRBP-1 expression was not reduced in CDH fetuses without liver herniation (Median, IQR; 7.2, 6.2–12.4; $p = 0.73$).

The CDH fetus with liver herniation but normal density of stellate cells expressing cRBP-1 (case 6) was born to a mother with alcohol use and depression, treated with Antabuse and Effexor respectively, which may have contributed to various fetal malformations [9], so that in this case retinoic acid signalling might not have had such a significant role in causing the malformations. Liver weight and density of HSCs expressing cRBP-1 were not significantly correlated, whether liver weight was calculated as a ratio of fetal body weight ($r = 0.29$, $p = 0.45$) or as a ratio of expected normal weight from biometric charts [8] ($r = 0.43$, $p = 0.25$, Fig. 1B). There was a trend towards statistical significance for liver weight compared with cRBP-1 expression when including control fetuses along with CDH fetuses, although this did not reach statistical significance ($r = 0.52$, $p = 0.056$, Fig. 1C), but not when diaphragm eventration fetuses were also included, possibly because one of these three fetuses (case 10) showed growth restriction, which is usually associated with increased cRBP-1 expression [5] and decreased liver weight. Further studies are warranted on the role of RA in control vs. CDH fetal liver development given these data were derived from a relatively small number of cases.

3.2. cRBP-1 and lung weight

Lung weights were reduced bilaterally and to a larger extent on the side of the diaphragmatic hernia in agreement with previous studies [10] except in one case (case 2), where bilateral pleural effusions were present (Tables 1 and 2).

Contralateral lung weight correlated with density of HSCs expressing cRBP-1 in these fetuses where lung weight was calculated as a ratio of expected normal weight from biometric charts [8] ($r = 0.82$, $p = 0.025$, Fig. 1D). Contralateral lung weight calculated as a ratio of body weight was also correlated to cRBP-1$^+$ HSCs and showed a trend towards statistical significance ($r = 0.74$, $p = 0.059$), possibly because of the small numbers of cases and presence of confounding factors such as fetal oedema (see Table 1). These calculations included the fetus with giant omphalocoele, where the right lung was considered the 'contralateral' lung, as the chest was distorted by scoliosis with the curvature to the right and the heart was deviated to the right (see Table 1). The right lung was about 55% of normal from biometric charts and left lung, about 44% of normal weight. Combined lung weight (calculated as a ratio of normal biometric values) was also significantly correlated with HSC cRBP-1 expression in CDH fetuses ($r = 0.78$, $p = 0.039$, Fig. 1E). In these fetuses, ipsilateral lung weight was not correlated with liver cRBP-1 expression whether calculated as a ratio of expected normal lung weight ($r = 0.43$, $p = 0.33$) (Fig. 1F) or as a ratio of body weight ($r = 0.24$, $p = 0.6$). Combined lung weight did not appear to correlate with cRBP-1 expression in fetuses with thin, elevated diaphragm or acute chorioamnionitis (controls) but there were too few fetuses for statistical analysis. In our study, total lung weight was not