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Basic Science

Evaluation of alveolar epithelial cells in the sheep model of congenital diaphragmatic hernia: Type 1 alveolar epithelial cells and histopathological image analysis

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ABSTRACT

Background: There are few reports comparing type 1 alveolar epithelial cell development with histopathological image analysis. We investigated these as indicators of maturity in fetal lambs' lungs in a congenital diaphragmatic hernia (CDH) model.
Methods: We created left CDH in 4 fetal lambs at 75 or 76 days' gestation (Group A). Controls were 5 sham-operated lambs (Group B); both groups delivered at term. The right lower lung lobe (RLL) and left lower lobe (LLL) were sampled. Using histopathological image analysis, alveoli/air sacs count (AC), alveoli/air sacs area percentage (AP), average area (AA), total area (TA), and perimeter (PM) were determined. We also evaluated total lung volumes, radial alveolar count (RAC), and Type 1 alveolar epithelial cells ratio (AT1 ratio), which we previously reported. Regression analysis was performed, with $p < 0.05$ considered significant.
Results: RLL and LLL AT1 ratio and LLL RAC in Group A were lower than in Group B. There are no significant differences demonstrated by histopathological image analysis. In Group A, the AT1 ratio in the LLL was lower than in the RLL. There were no differences between LLL and RLL in Group B.
Conclusion: AT1 ratio was superior to the other indicators evaluating lung maturity.

Congenital diaphragmatic hernia (CDH) results in pulmonary hypoplasia [1], which is associated with a high mortality [2,3] which is still a significant problem [4]. In an effort to reduce this mortality, fetal surgery to repair the defect was developed. Harrison [5] and Pringle [6,7] developed a successful fetal lamb model of CDH to create and repair the defect, to demonstrate the effectiveness of fetal surgery for this defect. These studies led to fetal repairs in humans, firstly as open repairs [8,9] and then after a chance finding in fetal lambs [10] and confirmation of the concept in another series of fetal lambs [11] open tracheal occlusion was attempted in human fetuses [12]. The results from these procedures were disappointing so tracheal occlusion using a balloon placed during a fetoscopic procedure was attempted [13]. This study concluded that the tracheal occlusion did not improve survival or morbidity. In Europe, this approach is being rigorously tested by means of the "Tracheal Occlusion To Accelerate Lung Growth" trial (TOTAL trial) [14], an international trial investigating the role of fetal therapy for severe and moderate pulmonary hypoplasia. In spite of these studies, as has been emphasized by Lally [4], fetal surgery for CDH can only be considered an experimental procedure, with any improvement in survival being counterbalanced by the tendency for the infants undergoing fetal repair to be delivered prematurely.

It is difficult to evaluate the extent of pulmonary hypoplasia by lung histology in humans. We hypothesized that pulmonary hypoplasia might be improved by fetal surgery even in fatal cases. Accordingly, we have attempted to assess pulmonary hypoplasia and maturity in a fetal lamb model of CDH using a basic science approach.

We have extensive experience with fetal lamb models. Recently, we evaluated pulmonary maturity in fetal lamb models of obstructive uropathy [15] and gastroschisis [16] using the Type 1 alveolar epithelial cell ratio (AT1 ratio). The vast majority of the gas exchange in the lung occurs across the incredibly thin membrane composed of the juxtaposed Type 1 alveolar epithelial cells (AT1) and capillary endothelial cells in the alveolar walls. We examined the AT1 ratio to evaluate the degree of pulmonary maturity, and there was a low AT1 ratio in both obstructive uropathy [15] and gastroschisis [16]. The AT 1 ratio was lower in gastroschisis lamb lungs than in control lamb lungs, although lung volume and Radial alveolar count (RAC) were not

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assessed [16]. Therefore, the AT1 ratio seems to be superior to the other commonly-used indicators for evaluating lung maturity.

There are multiple pathological techniques for evaluating pulmonary maturity in animal and human studies. Several researchers have applied pathological image analysis; a quantitative analysis of the air sacs or parenchyma calculated using image analysis software [17,18]. Image analysis techniques continue to evolve, allowing detailed quantitative analysis of histological specimens.

In this study, we evaluated pulmonary development using the AT1 ratio, image analysis and RAC in a fetal lamb model of CDH in an attempt to determine the best indicator of pulmonary maturity.

1. Material and methods

After approval was obtained from the Animal Ethics Committee of the Wellington School of Medicine and Health Sciences, University of Otago, Wellington (Wellington, New Zealand, approval numbers AEC 2-12, AEC 1-16) pregnant ewes at about 70 days' gestation were examined by ultrasound to confirm the pregnancy and to avoid unnecessary operations and then transported from the farm to the laboratory 24 to 48 h before the planned operation. Our perioperative and anesthetic management has been reported previously [6,15].

We created a left diaphragmatic hernia in 7 fetuses at 75 or 76 days' gestation (Group A). Under general anesthesia, the uterus was exposed through a left flank incision. The lamb's head and thorax were delivered through a transverse hysterotomy. A left thoracotomy was performed on the lamb, through which an incision was made in the lamb's diaphragm. Part of the stomach was gently pulled through from the diaphragmatic defect and brought up into the chest. The thoracotomy was closed. The fetus was then returned to the uterus, and the uterus and ewe's abdomen were closed as previously reported [6,15]. We exposed 5 fetuses through a transverse hysterotomy for 12 min as a sham-operation group (Group B). The pregnant ewes were returned to the farm, and returned to the laboratory shortly before term. The fetuses were delivered by cesarean section at term (145 days) and the lambs were sacrificed using the same techniques as described previously [6,15].

The body weight and crown-to-rump length were measured and the lamb's respiratory system was then removed en bloc. The lungs were fixed by intratrachael instillation of 10% formalin at 30 cm H_2O pressure as previously described in our studies [15,16]. Lung volumes were measured by water displacement.

Random samples were taken from the right lower lobe (RLL) and the left lower lobe (LLL) of each lung. They were processed for light microscopy in paraffin blocks and sliced at 3 μm. Histologic sections of the lung were stained with hematoxylin and eosin (HE) and Elastica–Masson (E-M). In addition to HE and E-M, we used immune histochemical staining for keratins (clone: AE1, AE3, mouse monoclonal, Nichirei, Tokyo, Japan) and periodic acid–Schiff stain (PAS) as double stained sections [15,16].

We measured the RAC in 10 of the E-M-stained sections in each lobe as previously reported [16]. We measured the AT1 ratio: the AT1 count/AT1 count + AT2 count (AT1; Type 1 alveolar epithelial cells, AT2; Type 2 alveolar epithelial cells) using the keratins and PAS double stained sections (CK + PAS) [15,16]. Keratins were positive in the cytoplasm of alveolar epithelial cells and provided a reliable marker for distinguishing between the alveolar epithelial cells and the stromal cells of alveolar septae and endothelial cells. Morphologically, AT1 were flat and AT2 were round. AT2 had PAS positive granules but AT1 didn't (Fig. 1). We examined the AT1 ratio to evaluate the degree of differentiation from AT2 to AT1 [16]. We utilized 5 sections stained with CK + PAS from each lung lobe, measuring the AT1 ratio in 10 randomly-selected alveoli sampled from each section.

We selected 5 HE stained sections from each lung lobe. From each section, 3 images were captured from randomly-selected high-power fields (magnification, 400×) and saved in TIFF format (1600 × 1200 pixels) by the digital camera for microscopes (Olympus DP21) (Fig. 2A). The LED lamp voltage was kept consistent throughout the image capture. These TIFF format images were analyzed by Image J which is a computer program for image analysis [19]. The RGB images were converted to 8-bit, and threshold determinations were used to digitally highlight alveoli/air sacs (Fig. 2B) [17]. The following data were recorded. The alveoli/air sacs count (AC) was the number of highlighted pixels in the alveolar areas of the field. The alveoli/air sacs area percentage (AP; %) was the percent of highlighted pixels calculated relative to the total area of the field. The alveoli/air sacs average area (AA; $10^2\mu m^2$) was the average area of highlighted pixels in each alveolar/air sac. The alveoli/air sacs total area (TA; $10^2\mu m^2$) was the amount of highlighted pixels of the field. The alveoli/air sacs perimeter (PM; μm) was the amount of perimeter around the highlighted areas. These data were calculated in each field and averaged.

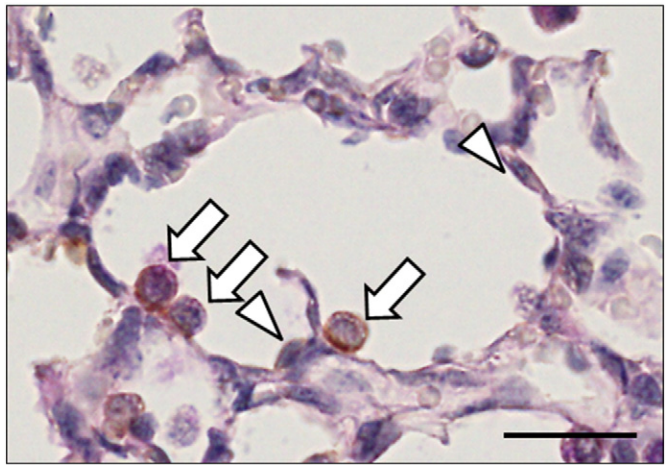

Fig. 1. Measuring Type 1 alveolar epithelial cell ratio. The Type 1 alveolar epithelial cell ratio = Type 1 alveolar epithelial cell count/(Type 1 alveolar epithelial cell count + Type 2 alveolar epithelial cell count). Type 1 alveolar epithelial cells were flat (arrow head) and Type 2 alveolar epithelial cells were round (arrow). Type 2 alveolar epithelial cells had PAS positive granules but Type 1 alveolar epithelial cells didn't (Keratins and PAS double stained sections. Scale bar is 25 μm).

Data were analyzed using Bell Curve for Excel (Social Survey Research Information Co., Ltd). Findings were expressed as mean ± SD. We initially compared the data from each lobe from Group A and Group B. We then compared RLL to LLL in Group A and in Group B. Statistical comparisons of the multiple data were evaluated by the Student t test. Regression analysis was performed, with $p < 0.05$ considered significant.

2. Results

We attempted to create a DH in 7 fetuses. All 7 survived. There were 2 fetuses without herniation and 1 fetus with what appeared to be spontaneous healing of the diaphragm. Four fetuses had CDH at term (Group A). None of these had a prolapsed liver. All 5 sham-operated fetuses survived. The total lung volume was 106 ± 43.2 ml in Group A vs. 276 ± 97.4 ml in Group B ($p = 0.01$). There were no differences for the values of body weight and crown-rump length between groups (Table 1).

When we compared the RLL in Group A and Group B, the AT1 ratio was 40.7 ± 1.22 (%) in Group A vs. 56.2 ± 1.73 (%) in Group B ($p < 0.01$). The RAC was 5.4 ± 2.2 in Group A vs. 7.6 ± 1.3 in Group B ($p = 0.08$). Using the image analysis, there were no significant differences for AC ($p = 0.63$), AP ($p = 0.06$), AA ($p = 0.16$), TA ($p = 0.06$) and PM ($p = 0.13$) (Table 2).

When we compared the LLL in Group A and Group B, the AT1 ratio was 30.3 ± 1.16 (%) in Group A vs. 55.9 ± 1.78 (%) in Group B ($p < 0.01$). The RAC was 3.8 ± 1.7 in Group A vs. 7.6 ± 1.9 in Group B ($p = 0.02$). Using the image analysis, there were no significant