Pulmonary Endocrine Cells in Hypoplastic Lungs Due to Foetal Urinary Tract Obstruction: A Microscopic Immunohistochemical Study

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METHODS: We performed a urinary tract obstruction (UTO) surgical procedure at 93–107 days’ gestation in lambs to investigate the relationship between pulmonary hypoplasia and the appearance of pulmonary endocrine cells by quantitative analysis of respiratory tract cells using light microscopic immunohistochemistry.

RESULTS: UTO produced a significant reduction in lung weight, lung/body weight ratio, air capacity, air capacity/body weight ratio (p < 0.01) and radial alveolar count (p < 0.05), which indicated the presence of lung hypoplasia. These foetuses also showed a significant increase in the number of neuron-specific enolase (NSE)-positive pulmonary endocrine cells, expressed as the number of NSE-positive cells per bronchus (p < 0.01) or bronchiole (p < 0.05), the number of NSE-positive cells per unit perimeter of bronchus or bronchiole (p < 0.01), and the number of NSE-positive cells per unit bronchial or bronchiolar surface area (p < 0.01).

CONCLUSION: These results suggest that UTO significantly retards and modifies the structural growth and functional development of pulmonary endocrine cells in NSE expression. We speculate that pulmonary endocrine cells and their mediators may play a role in the problems associated with UTO during intrauterine life. [Asian J Surg 2006;29(1):31–5]

Key Words: hypoplastic lung, immunohistochemistry, neuron-specific enolase, NSE, pulmonary endocrine cell, urinary tract obstruction

Introduction

Congenital obstructive nephropathy is a primary cause of renal failure in infants and children.1 Obstruction of urinary flow produces hydronephrosis and progressive renal damage.1–7 Without adequate foetal urinary output, the amniotic fluid decreases, resulting in foetal growth constraint, including the development of pulmonary hypoplasia.5

Neuron-specific enolase (NSE) is one of the isoenzymes produced by pulmonary endocrine cells. These cells are widely distributed throughout the airway mucosa and are thought to play an important role in lung development and maturation from intrauterine life to the postnatal period.1,8–15 There have so far been a few studies in human9 or rat16,17 lungs of hypoplasia due to congenital diaphragmatic hernia showing the distribution of pulmonary endocrine cells. To the best of our knowledge, there have been no immunohistochemical reports on the expression and localization of pulmonary endocrine cells in lamb lung tissue associated with urinary tract obstruction (UTO).

Previously, we quantitatively analysed NSE-positive endocrine cells in the lamb lung during different stages of develop-
ment using light microscopic immunohistochemistry. The purpose of our present immunohistochemical study was to determine whether or not the number of pulmonary endocrine cells increases in association with UTO during the intrauterine life of the lamb.

**Methods**

**Animals**

Sheep (mixed western breed) were obtained from sheep laboratories of both the University of California, San Francisco, and the University of California, Davis. The animals were mated only once and the mating day was taken to indicate 0 day gestation. The presence of a foetus was confirmed in the pregnant ewe by real-time ultrasonography before surgery. The animals were handled in accordance with permission and protocol for research from the Animal Use and Care Administrative Advisory Committee of the University of California, San Francisco.

The production of bilateral hydronephrosis and pulmonary hypoplasia by obstruction of the foetal urethra and ligation of the urachus has previously been described in detail. The uterus was opened using a GIA™ Stapler (US Surgical Corp, Norwalk, CT, USA). The urachus was ligated at the base of the umbilical cord. The bladder was obstructed by placing an ameroid constrictor on the perineal urethra or by ligation of the urethra through a transverse suprapubic incision. In this study, time-dated foetal lambs underwent urachal ligation and urethral obstruction at 93–107 days’ gestation (median, 100 days). Minimal amniotic fluid was lost during the procedures, which was not routinely replaced. Eight lambs (3 foetuses, ligation of the perineal urethra; 5 foetuses, ameroid constrictor placed on the perineal urethra) were available for the study (UTO group). The lambs were delivered by caesarean section at 136–145 days (median, 142 days).

The foetuses were weighed and measured. The bilateral main bronchi were cannulated with an appropriately sized catheter. All lambs were infused with 10% neutral buffered formalin at 20-cm water pressure before the first breath to prevent any consumption of lung fluid contents. The whole lobes from bilateral lungs of all sheep were randomly sectioned and embedded in paraffin. Pulmonary development was assessed by lung weight (LW), LW to body weight (BW) ratio (LW/BW), air capacity (AC; the volume of the lung determined by 10% neutral formalin displacement when inflated to 20 cm of water pressure), AC to BW ratio (AC/BW) and microscopic examination of lung sections. Eight untreated lambs from nonoperative ewes served as controls.

**Immunohistochemistry**

Lung paraffin blocks were cut into 5-μm sections, which were examined immunohistochemically using the avidin-biotin peroxide complex technique with rabbit polyclonal antibody against human NSE (Dako, Santa Barbara, CA, USA). Deparaffinized sections were washed three times with 0.1 M phosphate-buffered saline (PBS), pH 7.4. Endogenous peroxidase was blocked using 3.0% (W/V) hydrogen peroxide in absolute methanol for 30 minutes. Casein solution, used according to the method of Tacha and McKinney, and the Avidin/Biotin Blocking Kit® (Vector Laboratories Inc, Burlingame, CA, USA) were also used to block nonspecific background staining. After preincubation with 10% normal goat serum, each section was incubated in four stages: with rabbit polyclonal antibody against human NSE at a dilution of 1:1000 for 45 minutes at room temperature and washed with PBS; with biotinylated goat anti-rabbit immunoglobulin G (Sigma, St. Louis, MO, USA) for 45 minutes at room temperature; with Vectastain® Elite ABC kit (Vector Laboratories Inc) for 45 minutes and washed with PBS; and with a peroxidase substrate solution containing 0.02% (W/V) hydrogen peroxidase and 0.1% (W/V) diaminobenzidine tetrahydrochloride in PBS, pH 7.4. After washing, the sections were counterstained with haematoxylin. Non-immune rabbit serum was used instead of the primary antibody to NSE as a negative control.

**Morphometric study**

The radial alveolar count (RAC) was measured on sections stained with haematoxylin and eosin according to the method of Askenazi and Perlman. Fifty sites were examined for each case.

Morphometric analysis was performed using a Power Macintosh 8100/80AV (Apple Corp, Cupertino, CA, USA) and the National Institute of Health Image 1.61 image analysis processor (National Institute of Health, Bethesda, MD, USA) according to the modified morphometric analysis of Xu et al. Briefly, we measured the length of the subepithelial basement membrane of the bronchus or bronchiole, to give the perimeter of the bronchi or bronchioles, the area enclosed by the subepithelial basement membrane of the bronchus or bronchiole, as the bronchial or bronchiolar surface area, and the number of NSE-positive cells within the respective bronchus or bronchiole. The following morphometric parameters.
were then calculated: the number of NSE-positive cells per bronchus or bronchiole, the number of NSE-positive cells per unit perimeter of a bronchus or bronchiole (/mm), and the number of NSE-positive cells per unit bronchial or bronchiolar surface area (/mm²). It is generally considered that bronchi become bronchioles when the cartilage completely disappears from those walls and at about the same point the bronchial glands disappear. These findings allowed us to distinguish bronchi and bronchioles. At least 36 sites were examined for each case.

Statistical analysis
Results are shown as mean ± standard deviation (SD). Significant differences between UTO and control foetuses were determined using either unpaired Student’s t test for equal or unequal variances in comparisons of the gestational day, BW, LW, LW/BW, AC and AC/BW, and analysis of variance to compare the immunohistochemical results. A p value of less than 0.05 was considered to be statistically significant.

Results
There was no difference in the gestational age between UTO and control foetuses (Table). UTO foetuses weighed less than controls but the difference was not statistically significant. Lungs were significantly smaller in UTO foetuses than in controls and, therefore, the LW, LW/BW, AC, AC/BW were all significantly decreased in the UTO foetuses (p < 0.01).

The RAC of the UTO foetuses (2.29 ± 0.70) was consistently and significantly less than that in controls (3.01 ± 0.56; p < 0.05).

The epithelial lining of the airways in all lungs of both the UTO and control foetuses contained pulmonary endocrine cells positively stained for NSE. The most frequent staining pattern consisted of single or small groups of pulmonary endocrine cells forming lateral cytoplasmic processes extending either along the basement membrane or toward the airway lumen.

The immunohistochemical results of the morphometric study are shown in Figures 1 and 2. The number of NSE-positive cells per bronchus or bronchiole, the number of NSE-positive cells per unit perimeter of bronchus or bronchiole, and the number of NSE-positive cells per unit bronchial or bronchiolar surface area were all significantly higher in the UTO group than in the control group.

Discussion
The diagnostic criteria for pulmonary hypoplasia in previously published studies included LW/BW, RAC, tissue maturity, quantitative biochemical assays and DNA estimation. It is generally thought that low values of LW/BW, AC/BW and RAC indicate the presence of lung hypoplasia. Therefore, this study used a UTO procedure that effectively induced the development of lung hypoplasia. Our results suggest that UTO is a high-risk factor for pulmonary hypoplasia in lambs, as in human foetuses.

Pulmonary endocrine cells are found either as solitary cells or as clusters in the epithelial lining of the conducting airways in various mammals. The availability of a specific and reliable method to demonstrate endocrine cells is a prerequisite for obtaining a detailed quantitative assessment of their distribution and frequency. We recently described a new method for quantitative analysis of respiratory tract cells (Clara cells, pulmonary endocrine cells, surfactant apoprotein, etc.) using light microscopic immunohistochemistry. To the best of our knowledge, no study has previously analysed the intraepithelial endocrine cells of hypoplastic lung due to UTO in the lower respiratory tract of foetal sheep. Therefore, our present study is the first to precisely document changes in pulmonary endocrine cells in hypoplastic lamb lungs using immunohistochemical quantitative analysis.

Table. Gestational age, body weight (BW), lung weight (LW), lung/body weight ratio (LW/BW), air capacity (AC) and air capacity/body weight ratio (AC/BW)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Urinary tract obstruction (n = 8)</th>
<th>Control (n = 8)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gestational age (d)</td>
<td>142.0 ± 2.1</td>
<td>141.0 ± 1.4</td>
<td>-</td>
</tr>
<tr>
<td>BW (× 10^2 g)</td>
<td>30.5 ± 5.7</td>
<td>36.9 ± 7.6</td>
<td>-</td>
</tr>
<tr>
<td>LW (g)</td>
<td>60.3 ± 20.7</td>
<td>115.0 ± 22.8</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>LW/BW (× 10^{-3})</td>
<td>19.6 ± 4.7</td>
<td>30.3 ± 9.0</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>AC (× 10 mL)</td>
<td>8.4 ± 1.9</td>
<td>21.0 ± 2.9</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>AC/BW (× 10^{-3})</td>
<td>25.9 ± 6.6</td>
<td>56.4 ± 6.1</td>
<td>&lt; 0.01</td>
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Cutz pointed out that the functional considerations of pulmonary endocrine cells focus on three main areas: during intrauterine life, when the lungs are not used for gas exchange; at the time of birth and postnatally; and during adult life.28 He postulated that the principal role of pulmonary endocrine cells during intrauterine life is maintenance of active pulmonary vasoconstriction in the relatively hypoxic lung.28 This study focused on the first area, during intrauterine life, but did not look at the second or third areas. In other species such as humans29 and rabbits,12 pulmonary endocrine cells increase with advancing gestation from the early foetal period to term. In contrast, in the sheep lung, NSE-immunoreactive endocrine cells decrease with advancing gestation during the foetal period.18

We demonstrated that, compared with control lungs, hypoplastic lungs after UTO showed a significant increase in the number of pulmonary endocrine cells, i.e. the number of NSE-positive cells per bronchus or bronchiole, the number of NSE-positive cells per unit perimeter of bronchus or bronchiole, and the number of NSE-positive cells per unit bronchial or bronchiolar surface area. During the foetal period of sheep, the appearance of pulmonary endocrine cells in the UTO group is similar to that in the late canalicular period of pulmonary development (a few days before 120 days’ gestation), according to our previous study.18 These results suggest that UTO significantly retards and modifies the structural growth and functional development of pulmonary endocrine cells in NSE expression.

The overall pattern of growth and the differentiation of pulmonary endocrine cells is probably species-related and depends on the state of airway development.9,12 This study also suggests that hyperplasia of the pulmonary endocrine cell system in hypoplastic lungs following UTO already exists in lamb foetuses. On the other hand, the increase in pulmonary

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**Figure 1.** Immunohistochemical results of the morphometric study of bronchus are shown as mean ± standard deviation. The number of neuron-specific enolase (NSE)-positive cells per bronchus, the number of NSE-positive cells per unit perimeter of bronchus, and the number of NSE-positive cells per unit bronchial surface area are all significantly higher in the urinary tract obstruction (UTO) group than in the control group. *p < 0.01.

**Figure 2.** Immunohistochemical results of the morphometric study of bronchiole are shown as mean ± standard deviation. The number of neuron-specific enolase (NSE)-positive cells per bronchiole, the number of NSE-positive cells per unit perimeter of bronchiole, and the number of NSE-positive cells per unit bronchiolar surface area are all significantly higher in the urinary tract obstruction (UTO) group than in the control group. *p < 0.01; †p < 0.05.
endocrine cells may be only a secondary effect because the airways of hypoplastic lungs due to UTO are small and less mature.

Although the exact mechanism of hyperplasia of pulmonary endocrine cells in UTO lungs is still unclear, we speculate that pulmonary endocrine cells and their mediator may play a role in the problems associated with UTO during intrauterine life. Further studies using other mediators are now being performed in our department to answer these questions.

References