Prenatal Vitamin E Treatment Improves Lung Growth in Fetal Rats with Congenital Diaphragmatic Hernia

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The aim of this study was to test the effects of vitamin E on fetal lung growth in rats with congenital diaphragmatic hernia (CDH). Experimental congenital diaphragmatic hernia (CDH) was induced in rat fetuses by maternal administration of 100 mg nitrofen by gastric gavage on day 9.5 of gestation. Vitamin E was provided at days 16-20 of gestation, at 30 IU/day. Cesarean section was performed at day 21 of gestation. Immunohistochemistry was performed using anti-surfactant protein A (SP-A) and anti-SP-B polyclonal antibodies. RT-PCR evaluated SP-A and SP-B mRNA expressions. The lung weight/body weight ratio in rats with CDH was lower than the control (p<0.01). The number of type II pneumocytes positive for SP-A in untreated CDH rats (n=20) was lower than the control (n=20). The relative amounts of SP-A and SP-B were significantly higher in vitamin E-treated CDH rats (n=20) than untreated CDH rats (p<0.05). Our results suggest that antenatal vitamin E treatment increases the production of surfactant proteins in hypoplastic lung of rats with the CDH.

Key Words: congenital diaphragmatic hernia, fetal pulmonary hypoplasia, surfactant proteins, immunohistochemistry, reverse transcription polymerase chain reaction.

Introduction

Recent reports on congenital diaphragmatic hernia (CDH) show a variable mortality rate ranging from 8% to 79%\(^\text{1,3}\). The most common cause of death in severely affected infants is pulmonary hypoplasia. Studies in animal models and clinical reports have indicated that lung compliance, pressure-volume curve, and hyaline membrane formation of newborns with CDH resemble those seen in premature newborns with surfactant deficiency\(^\text{2}\). Recent studies have reported low levels of the surfactant protein A (SP-A) in human CDH case\(^\text{1}\) as well as in rats with experimentally-induced CDH\(^\text{5}\). SP-A levels are also reduced in the amniotic fluid of pregnancies complicated by CDH, showing a close correlation between SP-A amniotic concentration and prognosis\(^\text{6}\).

Our research strategy has been to identify possible therapeutic interventions in utero that may improve lung growth and maturity. Previous reports have shown that prenatal glucocorticoids improve pulmonary maturity in fetal rat and sheep models of CDH based on biochemical, histological, and physiological changes\(^\text{7,9}\). However, prenatal glucocorticoid therapy does not seem to alter fetal lung growth\(^\text{10}\). Recent studies have shown that vitamin E accelerates the in vitro growth and complexity of the hypoplastic rat fetal lung and that prenatal vitamin E treatment in vivo improves pulmonary hypoplasia in fetal rats with CDH\(^\text{11}\). The observation that vitamin E can enhance fetal lung growth suggests a role for oxidative processes in altered lung development and a possible therapeutic strategy for the treatment of lung hypoplasia associated with CDH.

The aim of the present study was to investigate whether maternal administration of vitamin E has any effect on the expression of SP-A and SP-B by immunohistochemistry and reverse transcription-polymerase chain reaction (RT-PCR) in lungs of rats with nitrofen-induced CDH.

Materials and Methods

Animals

Adult Sprague-Dawley rats were bred after overnight controlled mating. Observation of a positive smear was considered a proof of pregnancy; the day of observation was determined as day 0. At 9.5 days
of pregnancy (term, 22 days) 100 mg of nitrofen (Wako Chemical, Osaka, Japan) dissolved in olive oil was administered as a single dose by gastric gavage under a brief anesthesia. Control rats were treated with the same dose of olive oil but without nitrofen. This was followed by maternal administration of 30 IU of vitamin E at 16-20 days of gestation to a group of nitrofen-treated rats.

The dose of 150 IU was chosen according to Islam et al.\textsuperscript{11}.

Cesarean section was performed on day 21 of gestation. Only fetuses with left-side CDH were entered in this study. The fetuses were divided into 3 groups, each consisting of 20 rats: the first group represented the control, the second represented rats with nitrofen-induced left side CDH, and the third group represented newborns with nitrofen-induced left side CDH treated with vitamin E. Left lungs were removed from the chest, and half of the sample from each group (n=10) was processed for RNA extraction, while the other half was processed for histological examination. Left lung growth and development was assessed quantitatively according to the method of Saitoh and Saitoh\textsuperscript{12} using three histopathological parameters. These included the size of lung acini, number of generations of the terminal airspaces and the mean alveolar diameter. The three parameters were measured in 30 sections in each case\textsuperscript{13}.

**Immunohistochemistry**

The left lung tissue was fixed with 4% formaldehyde for 24 hours at 4°C. The samples were embedded in paraffin and stored at -80°C. Frozen tissue blocks were cut into 5-μm thick sections and mounted on polylysine-coated glass slides.

Immunohistochemistry was performed using the labeled streptavidin biotin (LSAB) method (Dako, Japan). A rabbit antihuman SP-A polyclonal antibody (Chemicon, Temecula, CA) at 1:100 dilution and a rabbit anti-rat polyclonal SP-B antibody (Chemicon) at 1:200 dilution, were used as primary antibodies. Biotin-labeled goat anti-rabbit (Dako) was used as secondary antibody. All sections were incubated with a solution of horseradish peroxidase-conjugated streptavidin (Dako). Peroxidase activity was determined using 3,3’-diaminobenzidine tetrahydrochloride (Dojindo Chemicals Co., Osaka, Japan) in PBS, containing 0.01% H₂O₂. The sections were counterstained with hematoxylin. For semiquantitative analysis, immunohistochemically-stained sections were scored into one of four grades ( −, ±, +, ++) based on the frequency and intensity of positively-stained epithelial cells. The above analysis was conducted by three investigators and the final score of each area analyzed was determined by consensus.

**Reverse transcription polymerase chain reaction**\textsuperscript{10}

Total RNA was extracted from each left lung tissue using RNAgents reagent (Promega, Madison, WI), according to the protocol recommended by the manufacturer. Total RNA was redissolved in diethyl pyrocarbonate (DEPC)-treated water and mRNA was isolated using streptavidin magnet particles and biotin labeled oligo (dT) probe (Promega). Polymerase chain reaction (PCR) was used as described recently\textsuperscript{16}. The specific primer sets used to amplify SP-A, SP-B, and β-actin cDNA and estimated size of PCR products are listed in Table 1. PCRs for SP-A (at 94°C for 30 seconds, at 56°C for 30 seconds, at 72°C for 60 seconds; 22 cycles), SP-B (at 94°C for 30 seconds, at 60°C for 0 seconds, at 72°C for 60 seconds, and at 72°C for 60 seconds; 26 cycles), and β-actin (at 94°C for 40 seconds, at 68°C for 40 seconds, and at 72°C for 90 seconds; 27 cycles) were performed on each sample. The PCR conditions used were confirmed in a series of preliminary studies to be within the exponential phase. The PCR products were electrophoresed on 2.0% agarose gel and stained by ethidium bromide to visualize DNA bands. The image of each band was quantitated by an image analysis software (IP Lab Gel, Signal Analytic Co.). The relative amount of mRNA, and SP-A and SP-B levels in each case were expressed as a ratio of the band intensity divided by that of β-actin.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence 5’ → 3’</th>
<th>Product size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SP-A Sense</td>
<td>GGA AGC CCT GGG ATC CCT GGA</td>
<td>557</td>
</tr>
<tr>
<td>Antisense</td>
<td>TGG GTA CCA GTT GGT GTA GT</td>
<td></td>
</tr>
<tr>
<td>SP-B Sense</td>
<td>GAG GAT ATT GTC CAC GTG CT</td>
<td>725</td>
</tr>
<tr>
<td>Antisense</td>
<td>ATA GCC TGT TCA CTC GTO T</td>
<td></td>
</tr>
<tr>
<td>β-actin Sense</td>
<td>CGT CAT ACT CCT GCT TCG TGA ATG GCC TCG G</td>
<td>838</td>
</tr>
<tr>
<td>Antisense</td>
<td>CGT CAT ACT CCT GCT TCG TGA TCC ACA TCT GC</td>
<td></td>
</tr>
</tbody>
</table>

**Statistical analysis**

All data were expressed as mean ± SD. Differences between groups were examined for statistical significance using the Student’s t-test. A P value less than 0.05 denoted the presence of a statistically significant difference. All statistical analyses were performed using
Results

Table 2 shows the fetal wet lung/body weight ratios of the three groups. The ratios of rats with CDH treated or untreated with vitamin E were significantly lower than that of the control group (p<0.01, each). Histopathological evaluation of the lungs showed significant reductions of all three parameters (size of lung acini, number of generations of the terminal airspaces and the mean diameter of alveoli) in rats with CDH compared with the control group (Table 2). However, administration of vitamin E significantly improved histological parameters compared with the untreated group.

Table 2. Effects of vitamin E on fetal lung/body weight and lung growth in rats with nitrofen-induced congenital diaphragmatic hernia (CDH).

<table>
<thead>
<tr>
<th></th>
<th>Untreated rats</th>
<th>Vitamin E-treated</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(n=10)</td>
<td>(n=10)</td>
</tr>
<tr>
<td>Lung/Body weight ratio (%)</td>
<td>2.35±0.23</td>
<td>1.69±0.38*</td>
</tr>
<tr>
<td>Histopathological parameters</td>
<td></td>
<td></td>
</tr>
<tr>
<td>size of lung acini (µm)</td>
<td>33±6</td>
<td>233±36*</td>
</tr>
<tr>
<td>number of generations of terminal airspace</td>
<td>3.33±0.48</td>
<td>2.66±0.48*</td>
</tr>
<tr>
<td>mean diameter of alveoli (µm)</td>
<td>40.6±5.1</td>
<td>36.6±7.8*</td>
</tr>
</tbody>
</table>

* p<0.01, compared with the control, †p<0.05, compared with untreated CDH group

SP-A and SP-B Immunohistochemistry

Many SP-A positive type II alveolar epithelial cells were noted in the lungs of control newborn rats (Fig. 1A), whereas only few cells were SP-A immunoreactive in rats with CDH (Fig. 1B). Vitamin E-treated CDH rats showed a significantly higher number of SP-A positive type II pneumocytes compared with untreated newborn rats (Fig. 1C). SP-B was not expressed in control rats (group 1, Fig. 2A) compared with few immunoreactive cells in lungs of rats with CDH (group 2). The number of SP-B-positive type 2 cells was significantly higher in vitamin E-treated newborn rats with CDH than in the untreated group (group 3, Fig. 2C).

Reverse transcription polymerase chain reaction

The PCR conditions described above successfully yielded amplified fragments of expected sizes for SP-A, SP-B and b-actin. The intensity of the band corresponding to b-actin mRNA was similar among the groups (Fig. 1). The intensity of SP-A bands was slightly stronger in the control than untreated CDH rats, suggesting reduced levels of SP-A in CDH lung.
Table 3. Expression of SP-A and SP-B in control rats and rats with nitrofen-induced congenital diaphragmatic hernia (CDH).

<table>
<thead>
<tr>
<th></th>
<th>Control rats</th>
<th>Untreated rats with CDH</th>
<th>Vitamin E-treated rats with CDH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Immunoreactivity to SP-A §</td>
<td>+</td>
<td>±</td>
<td>++</td>
</tr>
<tr>
<td>Immunoreactivity to SP-B §</td>
<td>+</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>Relative amount of SP-A mRNA (±SD)</td>
<td>1.34±0.06</td>
<td>1.23±0.20</td>
<td>1.43±0.24§</td>
</tr>
<tr>
<td>Relative amount of SP-B mRNA (±SD)</td>
<td>1.19±0.10</td>
<td>1.33±0.25*</td>
<td>1.53±0.21*§</td>
</tr>
</tbody>
</table>

§ Positive cells in alveoli: ++ many; + some; ± few; - almost none.
* p<0.01, compared with the control, †p<0.05, compared with untreated CDH group.

Discussion

Pulmonary surfactant, a complex mixture of lipid and proteins, provides phospholipid for the formation of the surface active film that lines air spaces and prevents alveolar collapse. The fetal lung acquires the capacity for surfactant synthesis relatively late in gestation. Augmented surfactant synthesis and secretion are initiated after completion of 85 to 90% of gestation in all mammalian species thus far studied (15, 16). In the human fetus, type II cells are first identified in the terminal sacs at 20 to 22 weeks of gestation, however, secretion of surfactant into the amniotic fluid is detectable only after 30 to 32 weeks of gestation (15). In the rat, levels of SP-A mRNA and protein, which are first detected on day 18 of gestation, increase markedly through day 21 to approximately 50% of the adult levels (17). SP-B is first detected in fetal lung tissue on day 18 of gestation and attains adult level by day 20 of gestation (17).

Vitamin E is the genetic term used for a group of at least eight compounds exhibiting biological activity of α-tocopherol, which has the highest antioxidant activity (18). Vitamin E is a potent intracellular chain-breaking antioxidant and is associated with glutathione, selenium, and vitamin C for regeneration of the tocopheryl radical (18). Current evidence strongly points to the role for vitamin E in prevention of cardiovascular disease (17). Previous observations that vitamin E can enhance fetal lung growth may suggest the involvement of oxidative processes in altered lung development and a possible therapeutic strategy for the treatment of lung hypoplasia associated with CDH. Studies in neonatal models have shown that increased oxygen concentrations lead to dysplastic and altered lung cell growth patterns as well as changes in insulin-like growth...
factor gene expression, which may be caused by oxygen-derived free radicals, suggesting that antioxidants may prevent growth alterations. The mechanisms through which fetal lung growth is induced by vitamin E remain unclear and are currently under study in our laboratory.

In conclusion, we analyzed the expression of SP-A and SP-B at both transcriptional and translational level after prenatal vitamin E treatment. Vitamin E increases the rate of transcription of surfactant proteins acting directly on type II cells. It exhibits a dose-dependent biphasic effect on the levels of SP-A, acting on SP-A mRNA; at low concentration, vitamin E increases SP-A gene transcription whereas at high concentration the upregulation of transcription is modulated by a reduction on mRNA stability resulting in a reduction of SP-A synthesis. The combined use of semiquantitative RT-PCR and immunohistochemistry in the present study provided the opportunity to evaluate the increased transcription and translation of in vivo antenatal vitamin E treatment at a dosage that does not interfaces with somatic growth. In our study, there was a trend, but not statistically significant, in spite of the treatments of vitamin E. This could be due to three reasons. Firstly, our numbers were probably too small to demonstrate a significant difference. Secondly, we used smaller doses of vitamins for short duration. It remains to be seen if maternal supplementation of large doses of antioxidants would reduce the oxidative stress in infants. Third reason could be both low maternal placental transfer and fetal lipid transport peculiarities. An attractive scheme would be to combine the early growth effects of antioxidants with later maturation and differentiation effects on surfactant production and alveolar formation by glucocorticoids. We are currently studying the effects of this combination therapy in rats with experimentally-induced CDH.

Acknowledgment

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References