Levels of Pulmonary Surfactant Protein A in Fetal Lung and Amniotic Fluid from Protein-malnourished Pregnant Rats

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Summary Surfactant protein A (SP-A) is a major apo-protein of pulmonary surfactant, which lines the alveolar walls, lowering the surface tension to prevent lung collapse. Pregnant rats were divided into two groups which received a diet with either 5% or 20% protein from gestational day 9. By a sensitive immunoassay, SP-A levels in the fetal lungs and the amniotic fluid showed a dramatic increase with advancing gestation after the initial appearance on gestational day 18 in both diet groups. Significantly lower levels of SP-A in pregnant rats fed 5% protein diet than those in pregnant rats fed 20% protein diet were observed in the fetal lungs on gestational day 21 and in the amniotic fluid on gestational days 20 and 21. The profiles of increased SP-A levels in the amniotic fluid reflected those in the fetal lungs during gestation. Immunohistochemical examination with anti-rat SP-A antibody at 21 days of gestation showed that the immunoreactive staining of bronchiolar epithelial Clara cells and alveolar type II cells were weaker in the fetal lung sections from pregnant rats fed 5% protein diet than in those from pregnant rats fed 20% protein diet. It is concluded that protein malnutrition in pregnant rats affects the biosynthesis of SP-A in the fetal lungs, which may have important consequences for prematurity and decreased respiratory functions in the neonatal lungs at birth.

Key Words protein malnutrition, surfactant protein A, pregnancy, fetal lungs, amniotic fluid, immunohistochemistry, rats

Pulmonary surfactant, a complex of lipids and surfactant specific apo-proteins, is synthesized and secreted by alveolar type II cells and bronchiolar Clara cells to coat the alveolar epithelium and to lower the surface tension at the air-liquid interface (1,2). Adequate production of pulmonary surfactant in the alveolar and

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bronchiolar fluid is important for newborn infants' survival (3). In prematurely developed newborn lungs, deficient pulmonary surfactant in the lung fluids is believed to cause respiratory distress syndrome. Surfactant protein A (SP-A) is the most abundant protein associated with pulmonary surfactant (4), enhancing the surface tension-lowering properties of phospholipid mixtures (5). Components of pulmonary surfactant appear in the amniotic fluid after passing through the respiratory tracts of the fetal lungs during pregnancy, and are correlated with the amount of pulmonary surfactant in the fetal lung fluids. SP-A and phospholipids in the amniotic fluid have therefore been used to estimate fetal lung maturation, and also as predictors of respiratory distress syndrome (6–8).

There is much evidence for the expression profile of pulmonary surfactant proteins during fetal lung development in humans (9–11) and rodents (12–14). However, there are few or no reports regarding the effects of nutritional states of pregnant mothers on surfactant protein levels in the fetal lungs. Malnutrition during pregnancy causes a delay in fetal lung maturation, and decreased respiratory functions in newborn infants (15,16). The purpose of the present study was to investigate the effects of protein malnutrition on the levels of SP-A in the fetal lungs and the amniotic fluid in pregnant rats. We report here that the levels of SP-A in the fetal lungs and the amniotic fluid were significantly lower in protein-malnourished pregnant rats than the levels in normally nutritional pregnant rats. These findings add to our understanding of prematurity and decreased respiratory functions in newborn infants delivered from malnourished mothers.

MATERIALS AND METHODS

Experimental animals. Virgin and specific pathogen-free Fisher strain rats at 11 weeks of age were obtained from Japan SLC Inc. (Shizuoka, Japan). They were mated and the gestational age was initially determined by designating the presence of a sperm-positive vaginal smear as day 0. The pregnant rats were divided into two dietary groups; one group was given a 20% protein diet through the gestational period and the other group received a 20% protein diet for the first 8 days of gestation and then a 5% protein diet from the 9th day of gestation to the final gestational day (day 21). The 20% protein diet contained 200 g casein-based protein plus 570 g cornstarch/kg, and the 5% protein diet contained 50 g casein-based protein plus 720 g cornstarch/kg. Methionine (3 g/kg) was supplemented in the latter diet. Both diets contained 100 g of sucrose/kg, 16 g of cod liver oil/kg, 64 g of soy bean oil/kg, 72 g of mineral mixture (Oriental Yeast Co., Ltd., Tokyo)/kg and 10 g of vitamin mixture (Oriental Yeast)/kg. The 20% and 5% protein diets were approximately isocaloric. Food was provided in powdered form. All rats were given water ad libitum and housed in an air-conditioned room at 22±2°C. Food intakes and body weight of the pregnant rats were measured daily. Fetuses at 18, 19, 20 and 21 days of gestation were delivered individually through a hysterotomy after anesthetizing pregnant rats with an intraperitoneal injection of methanol.
sodium pentobarbital. The amniotic fluid at each gestational day was collected by inserting a needle into the amnion cavity as described previously (17). The fetal lungs were excised and homogenized in four volumes of 50 mM Tris-HCl, pH 6.7, containing 0.5% Nonidet P-40, 50 μM Chymostatin, 20 μM leupeptin, 100 μM E-64 and 100 μM benzamidine, in a Polytron homogenizer (Kinematica, Lucerne, Switzerland) for 20 s at 0°C. The homogenate was sonicated on ice and then centrifuged at 25,000 × g for 20 min at 4°C. The resulting supernatants were stored at −20°C until use.

**Immunoassay for SP-A in the fetus lungs and the amniotic fluids.** The polyclonal antibody against rat SP-A was raised in rabbits and purified as described previously (18). Antibody to be biotinylated was introduced by Sulfo-Succinimidyl-6-(biotinamide)hexanoate (NHS-LC-biotin, Pierce Chemical Co., Rockford, IL). Next, 100 μL of the IgG fraction (10 mg/ml) dissolved in 0.1 M carbonate buffer (pH 8.5) was mixed with 5 μL of NHS-LC-biotin (4 mg/ml in the same buffer) and incubated at 4°C for 2 h. Non-reacted NHS-LC-biotin was removed by passage through an NAP-10 column containing Sephadex G-25 Medium (Pharmacia LKB Biotechnology AB, Uppsala, Sweden). The immunoassay using the anti-rat SP-A rabbit IgGs labeled with and without biotin was performed as described in detail (17). SP-A, purified to homogeneity from rat bronchoalveolar lavage fluid (18) and measured by the bicinchoninic acid-modified Lowry assay for protein (19), was used as the SP-A standard at the range of 0.1–5 ng/ml.

**Immunohistochemistry.** Paraffin sections of fetal rat lung were subjected to immunohistochemical staining for determination of SP-A positive cells as described previously (17,20), using the avidin-biotin-peroxidase complex method. Briefly, the sections were deparaffinized and soaked in 0.3% hydrogen peroxide in absolute methanol for 30 min at room temperature. After hydration, the specimens were treated with normal goat serum at a 1:60 dilution for 20 min at room temperature, then incubated at 4°C overnight with 4 μg/ml of antibody against SP-A in phosphate-buffered saline (PBS; 10 mM phosphate buffer, pH 7.2, containing 0.85% NaCl) containing 0.1% bovine serum albumin in a moist chamber. The sections were rinsed in PBS and incubated for 50 min at room temperature in a 1:200 dilution of biotinylated goat anti-rabbit IgG (Vectastain ABC Kit, Vector Laboratories, Burlingame, CA). Following a rinse in PBS, the sections were incubated for 60 min at room temperature in the avidin-biotin complex, rinsed in PBS, stained for 5 min with 50 mM Tris-HCl, pH 7.6, containing 0.1% 3,3′-diaminobenzidine tetrahydrochloride, 0.02% hydrogen peroxide and 0.65 mg/ml sodium azide. After washing with PBS, the sections were counterstained for 10 min with 1% methyl green, dehydrated and mounted.

Controls were prepared by using the conditions described above except that nonimmunized rabbit IgG was used instead of the primary antibody.

**Statistical analysis.** Data are expressed as M±SEM. Student’s t-test was performed to compare means between the two groups.
RESULTS

Pregnant rats receiving 20% or 5% protein diet ate a total of 258 g and 221 g of their particular diet during the pregnancy, respectively. The changes in body weight of the pregnant rats during the gestational period are shown in Table 1. From gestational day 16, the body weight of pregnant rats fed the 5% protein diet was significantly lower than that of pregnant rats fed the 20% protein diet.

Figure 1 shows the body (A) and lung (B) weights of the fetus from gestational day 18, which were significantly lower in the fetus of pregnant rats fed the 5% protein diet than in the fetuses of pregnant rats fed the 20% protein diet.

The increasing presence of SP-A in the fetal lungs over the gestational period and in neonatal rat lungs immediately after birth was assessed by a sensitive immunoassay (Fig. 2). The levels of SP-A were normalized by fetal lung protein content (Fig. 2A) or tissue weight of the fetal lungs (Fig. 2B). The presence of SP-A was first detectable in the fetal lungs at 18 days of gestation in the 5% protein diet group (2.1 ng/mg of lung weight and 33.2 ng/mg of lung protein) and in the

Table 1. Body weights (g) of pregnant rats.

<table>
<thead>
<tr>
<th>Day</th>
<th>5% protein diet</th>
<th>20% protein diet</th>
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<tbody>
<tr>
<td>Day 9</td>
<td>174 ± 3</td>
<td>175 ± 2</td>
</tr>
<tr>
<td>Day 14</td>
<td>177 ± 3</td>
<td>187 ± 3</td>
</tr>
<tr>
<td>Day 16</td>
<td>183 ± 4*</td>
<td>195 ± 3</td>
</tr>
<tr>
<td>Day 18</td>
<td>188 ± 5**</td>
<td>209 ± 4</td>
</tr>
<tr>
<td>Day 20</td>
<td>184 ± 8**</td>
<td>221 ± 6</td>
</tr>
<tr>
<td>Day 21</td>
<td>193 ± 7**</td>
<td>228 ± 1</td>
</tr>
</tbody>
</table>

The data represent M ± SEM.

* Significant difference between 5% and 20% protein diet groups (p < 0.1).
** Significant difference between 5% and 20% protein diet groups (p < 0.01).

Fig. 1. Changes of body (A) and lung weight (B) in fetal rats at late gestation (days 18–21) from pregnant rats fed 20% (open circle) and 5% protein diet (closed circle). The data represent M ± SEM from 3 to 10 fetuses in each gestational day. Significant differences from 20% protein group in each gestational day were determined by Student’s t-test, *p < 0.1, **p < 0.01.
Fig. 2. The levels of SP-A in the fetal lungs at late gestation (days 18–21) and immediately after birth in neonatal rat lungs from pregnant rats fed 20% (open circles) and 5% protein diet (closed circles). The contents of SP-A in the lungs were determined by an immunoassay as described in the "Materials and Methods" section. The data were normalized by lung protein contents (A) and lung weights (B) and represent M±SEM. Significant differences from the 20% protein group at each gestational day and birth were determined by Student's t-test, *p<0.1, **p<0.01.

20% protein diet group (3.0 ng/mg of lung weight and 50.0 ng/mg of lung protein), after which the levels of SP-A increased dramatically and reached 122 ng/mg of lung weight and 1,844 ng/mg of lung protein in the 20% protein diet group and 75.1 ng/mg of lung weight and 1,346 ng/mg of lung protein in the 5% protein diet group at the final gestational day (day 21). The levels of SP-A in the fetal lungs of each group were not significantly different until the gestation progressed to day 20. Significantly lower levels of SP-A in fetal lungs at the final gestational day (day 21) and neonatal rat lungs immediately after birth were observed in pregnant rats fed the 5% protein diet than in pregnant rats fed the 20% protein diet.

Immunohistochemically, the fetal lungs from pregnant rats fed the 20% protein diet at gestational day 21 contained many positive cells against the anti-SP-A antibody (Fig. 3A). Almost all alveolar spaces, although narrow, were open, where alveolar type II cells were stained with the anti-SP-A antibody. The respiratory bronchioles showed an intense staining with the antibody in nonciliated epithelial cells, identified as Clara cells. Large and amorphous materials labeled with the anti-SP-A antibody in bronchial spaces were frequently observed. In the fetal lungs from pregnant rats fed the 5% protein diet (Fig. 3B), the intensity of the cells stained positively for the anti-SP-A antibody was weaker than that from pregnant rats fed the 20% protein diet. The amorphous materials stained with the anti-SP-A antibody in the bronchial space appeared less frequently in the 5% protein diet group than in the 20% protein diet group.

By the immunoassay shown in Fig. 4, the presence of SP-A was first detectable at gestational day 18 in the amniotic fluid of pregnant rats fed 5% (12.4 ng/ml) and 20% protein diets (20.0 ng/ml), after which it dramatically increased and reached
DISCUSSION

There is a maturational delay in the lungs of fetuses of malnourished mothers, including decreased lung glycogen and the size of the lungs (16). Guarner et al. reported that phosphatidyl choline, a surfactant lipid component, is decreased in the fetal lungs derived from malnourished pregnant rats (15). The present study showed that the levels of SP-A in the fetal lungs and the amniotic fluid from protein-malnourished pregnant rats were significantly lower than those from pregnant rats receiving a normal protein diet. SP-A plays an important role in enhancing the surface tension-lowering properties of the surfactant monolayer on
Fig. 4. The levels of SP-A in the amniotic fluid of pregnant rats fed 20% (open circles) and 5% protein diet (close circles) at late gestation (days 18–21). The amniotic fluid was collected by insertion of a needle into the amnion cavity. The contents of SP-A in the amniotic fluid were determined by an immunoassay as described in the “Materials and Methods” section. The data represent M±SEM from 5 to 12 separate samples at each gestational day. Significant differences from the 20% protein group were determined by Student’s t-test, *p<0.1, **p<0.01.

the alveolar walls to prevent the lung collapse in collaboration with other hydrophobic surfactant proteins (5). Timely, adequate production of pulmonary surfactant in the fetal lungs is important for newborn infants to survive at birth. The present results suggest that malnourished pregnant mothers have a greater risk of producing a newborn with premature lungs and decreased pulmonary surfactant-mediated respiratory functions. The decreased SP-A levels in the fetal lungs from protein-malnourished rats were confirmed by immunohistochemistry, which showed weak stainings for the anti-SP-A antibody in both alveolar type II cells and bronchiolar Clara cells in the fetal lung sections from pregnant rats fed the 5% protein diet as compared with those from pregnant rats fed the 20% protein diet. The staining of large and amorphous materials with the anti-SP-A antibody in the bronchial spaces, indicating the presence of a large amount of pulmonary surfactant, was weaker and less prevalent in the fetal lungs from pregnant rats fed the 5% protein diet than those from pregnant rats fed the 20% protein diet. The synthesis of pulmonary surfactant proteins is regulated by a number of hormones and factors, including glucocorticoids, prolactin, insulins, growth factors, estrogens, androgens, thyroid hormones and cAMP (21). On the other hand, little is known about whether nutritional states affect the synthesis of pulmonary surfactant proteins in the lungs. Only our previous report showed that pulmonary surfactant obtained from adult rats at an early phase of starvation had a high content of SP-A (18).

SP-A is also involved in regulating surfactant homeostasis (22) and alveolar macrophage functions, including increased phagocytic activity (18,23) and chemotaxis (24). We also reported that pulmonary surfactant suppresses the infections of
the influenza virus and Sendai virus in rat lungs in vitro and in vivo (25). The present finding of the decreased levels of SP-A in the fetal lungs from protein-malnourished rats suggests a decreased SP-A-mediated immune response in newborn infants. It is debatable whether SP-A is important in the immunological defense system in the neonatal lungs.

SP-A is produced and secreted into the airway lumen by alveolar type II cells and bronchial and bronchiolar Clara cells in humans and rodents (4). The components of pulmonary surfactant appear in the amniotic fluid after passing through the respiratory tracts. Pulmonary surfactant lipids in human amniotic fluid are useful for estimating fetal lung maturity, because the phospholipids, especially saturated phosphatidylcholine, phosphatidylglycerol and phosphatidylinositol, contribute to the amount of pulmonary surfactant in the lung fluid (6). SP-A in human amniotic fluid has also been assessed to estimate fetal lung maturation (7,8). In the present study, the SP-A in the amniotic fluid of pregnant rats were first detectable at gestational day 18 in both diet groups, and then dramatically increased in both diet groups. After the first detectable levels of SP-A, the SP-A in the amniotic fluid at gestational days 20 and 21 was significantly lower in pregnant rats fed the 5% protein diet than in pregnant rats fed the 20% protein diet. The profile of the SP-A levels in the amniotic fluid in each group was correlated with that of SP-A levels in the fetal lungs (Figs. 2 and 4). These results indicate that protein malnutrition affects the amount of SP-A in the amniotic fluid, and suggests that measurement of SP-A in the amniotic fluid of malnourished mothers is meaningful for estimating fetal lung maturity in malnourished as well as nutritionally normal pregnant mothers.

In conclusion, this is the first report of decreased levels of SP-A in the fetal lungs and the amniotic fluid from pregnant rats maintained in a protein-malnourished state. We believe that protein malnutrition in pregnant rats affects the biosynthesis of SP-A in the fetal lungs, which may have important consequences for prematurity and decreased respiratory functions in the neonatal lungs at birth.

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