Fetal Pulmonary Surfactant in Amniotic Fluid of Ewes

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OGAWA, Y. Fetal Pulmonary Surfactant in Amniotic Fluid of Ewes. Tohoku J. exp. Med., 1972, 108 (4), 307-315 — The phospholipids in the amniotic fluid from healthy pregnant ewes at various stages of gestation were analyzed in relation to the fetal pulmonary surfactant. The most abundant phospholipid was phosphatidylcholine which increased as the term approached. Analysis on the positional specificity of amniotic phosphatidylcholine revealed a similar fatty acid composition to the surfactant phosphatidylcholine of the fetal lung. The increase in disaturated phosphatidylcholine concentration of amniotic fluid during gestation correlated well with the increase in the surfactant phospholipid of fetal lungs. These results indicate the presence of pulmonary surfactant of the fetal origin in the amniotic fluid, and serve as a basic value for the antenatal diagnosis of idiopathic respiratory distress syndrome.

Pulmonary alveoli are lined with a surface active material which is thought responsible for maintaining alveolar stability (Clements et al. 1961, Gruenwald 1964, Bolande and Klaus 1964). This surface active material, or surfactant, is a lipoprotein with disaturated phosphatidylcholine as a major component (Brown 1964, Fujiwara et al. 1965, Morgan et al. 1965, Pattle 1965). It has been demonstrated that disaturated phosphatidylcholine, particularly dipalmitoyl phosphatidylcholine, of the fetal lung increases with gestational age (Weinhold and Villee 1965, Chida et al. 1966, Brumley et al. 1967, Gluck et al. 1970). This increase correlates well with the development of surface activity of the lung (Brumley et al. 1967, Fujiwara et al. 1968, Adams et al. 1970).

Idiopathic respiratory distress syndrome (IRDS), or hyaline membrane disease, which is the major cause of mortality and morbidity among premature infants, is the only known condition for surfactant deficiency in humans (Avery and Mead 1959, Adams et al. 1965, Brumley et al. 1967, Adams et al. 1970).

Mammalian fetal lung produces the fluid which fills alveolar spaces in utero (Carmel et al. 1965, Adams et al. 1969). This lung fluid, rich in surface active phospholipid of the alveolar lining layer, drains periodically to the amniotic cavity via trachea (Adams and Fujiwara 1963, Enhörning and Adams 1965, Adams et al. 1967). This evidence suggests the possibility of the presence of fetal
pulmonary surfactant in the amniotic fluid.

The purpose of this study was to compare qualitatively and quantitatively phospholipids in amniotic fluid from healthy ewes of various stages of gestation. These normal biochemical data in animals are necessary to establish the means of assessing the fetal lung maturation and of antenatal diagnosis of hyaline membrane disease.

**MATERIALS AND METHODS**

Thirteen healthy pregnant ewes were used. All ewes used in this experiment were mixed-breed. They were mated under observation and test mated 18 to 20 days later; refusal to mate at this anticipated time of heat was taken to indicate pregnancy.

Between 113 to 148 days of gestation, a total of 21 samples of amniotic fluid were obtained by laparotomy. Direct amniocentesis through abdominal wall was avoided in order to eliminate the contamination with allantoic fluid. Ewes were anesthetized locally with 1% xylocaine infiltration in the abdominal wall. An incision of approximately 10 cm long was made in the lower abdomen to expose the uterine wall, and 20 to 30 ml of amniotic fluid were aspirated by a needle puncture. Pregnancy was not interrupted by this procedure but continued to the term. Healthy mature lambs were born to those ewes either by Cesarean section or vaginally between 145 and 150 days of gestation.

The samples were frozen until assay which was done within one week of collection. Any specimen which showed contamination with blood or meconium was discarded. Total lipids were extracted from amniotic fluid specimens using a mixture of chloroform: methanol (2:1, v/v), followed by aqueous washing of the extract as described by Folch et al. (1957). The chloroform extract was taken to dryness in vacuo and reconstituted to a known volume. Aliquots of the extract were analyzed in triplicate for phosphorus content according to the colorimetric method of Bartlett (1959).

Paired samples of the extract were further analyzed by thin layer chromatography to estimate the percent composition of phosphatidylcholine. Plates coated with silica gel H (Merck) in 0.25 mm thick were prepared according to the procedure of Skipski et al. (1963). Paired samples spotted on the plate with known phospholipid standards were developed with use of a solvent system of chloroform : methanol : water (65:35:4 by volume) at 25°C. Seven fractions were visualized after exposure to iodine vapor—the origin, lysophosphatidylcholine, sphingomyelin, phosphatidylcholine, phosphatidyl-ethanolamine, the unknown fraction and the solvent front. No further efforts were made to identify the unknown fraction, since the major purpose of this study was to separate the phosphatidylcholine fraction. Each fraction was marked, scraped into a tube and eluted 5 times with 10 ml of 1 N HCl in methanol. The lipid phosphorus was measured according to a modified procedure of Bartlett (1959) as described by Marinetti (1962) to calculate the percent composition of phospholipid classes.

Rest of the lipid extract was subjected to preparative thin layer chromatography for the separation of phosphatidylcholine to obtain a sufficient amount for its fatty acid analysis. Phosphatidylcholine on the preparative thin layer chromatogram was identified with Rhodamine 6G under ultraviolet light. The fraction was scraped off, eluted with chloroform-methanol and evaporated under nitrogen. The transmethylation was performed with super dry 5% HCl-methanol by the modified method of Stoffel et al. (1959).

The positional specificity of the phosphatidylcholine molecule was determined by analyzing the fatty acids of the whole molecule and the beta position. *Crotalus adamanteus* lyophilized venom (Ross Allen Reptile Institute) was employed as phospholipase A. Two mg of the venom in 0.2 ml of Tris buffer with 0.002 M CaCl₂ were added to each sample with 5 ml of diethyl ether, and incubated at room temperature for 3 hours while mixing the tube with an agitator (Brumley et al. 1967). The diethyl ether layer was removed and dried under nitrogen, then methylated with 5% HCl-methanol as described
above. The completeness of cleavage of phosphatidylcholine into lysophosphatidylcholine and free fatty acid after snake venom hydrolysis was confirmed by thin layer chromatography.

Fatty acid methyl esters were analyzed in a hydrogen flame gas-liquid chromatograph with a column packed with 12% diethylene glycol succinate polyester on 60-80 mesh celite 545. Standard fatty acid methyl esters mixture (F.A.M.E. series, Applied Science) was used to identify the constituent fatty acids. In most of the samples 6 peaks were obtained and they were identified as myristate, palmitate, palmitoleate, stearate, oleate and linolate. Some cases showed very small peaks of longer chain fatty acids only when the sensitivity was increased. Peak areas were calculated by multiplying peak height by peak width at a half-height. The total percent of saturated fatty acids was determined on each sample for the whole molecule and for the beta position. The value for the alpha position was calculated from these results. As there were no definite methods to estimate the absolute amount of disaturated phosphatidylcholine, the minimum concentration of disaturated phosphatidylcholine was calculated as stated by Brumley and co-workers (1967), and expressed as µmoles per 100 ml of amniotic fluid.

RESULTS

Fig. 1 shows the total phospholipid content of the amniotic fluid obtained at different stages of gestation. The total phospholipid content remained almost unchanged till 130 days of gestation and rapidly increased thereafter. Fig. 2 illustrates the change in amniotic phosphatidylcholine content during gestation. The concentration of phosphatidylcholine also showed a similar rise after 130 days of gestation. Table 1 shows the amniotic fluid phospholipids contents obtained from 13 healthy pregnant ewes dividing into 3 stages according to the gestational age: immature (before 130 days), transitional (between 130-140 days) and term (after 140 days gestation). In the immature stage, the mean total phospholipid content was 1.33 µmoles/100 ml of which phosphatidylcholine comprised 37.4%. In the transitional stage, the concentration of total phospholipid
Fig. 2. Changes in phosphatidylcholine of amniotic fluid from pregnant ewes during gestation.

Table 1. Amniotic phospholipid contents obtained from healthy ewes at various stages of gestation

<table>
<thead>
<tr>
<th>Gestation (Days)</th>
<th>Immature (7)</th>
<th>Transitional (8)</th>
<th>Term (6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean phospholipid (µmoles/100 ml)</td>
<td>120.0 ±5.2</td>
<td>132.9 ±2.8</td>
<td>145.7 ±1.2</td>
</tr>
<tr>
<td>% Distribution of phospholipids</td>
<td>37.4 ±8.0</td>
<td>45.5 ±5.7</td>
<td>69.4 ±1.6</td>
</tr>
<tr>
<td>%PC</td>
<td>22.3 ±2.8</td>
<td>19.3 ±2.7</td>
<td>11.2 ±0.6</td>
</tr>
<tr>
<td>%PE</td>
<td>23.1 ±3.1</td>
<td>21.4 ±2.3</td>
<td>14.1 ±0.4</td>
</tr>
<tr>
<td>%SP</td>
<td>17.2 ±4.9</td>
<td>13.8 ±4.9</td>
<td>5.3 ±1.4</td>
</tr>
<tr>
<td>%Others</td>
<td>0.50±0.20</td>
<td>1.41±1.07</td>
<td>4.78±1.77</td>
</tr>
</tbody>
</table>

Values are expressed as means ±SD. Numerals in parentheses indicate the number of samples studied. Abbreviations used: PC, phosphatidylcholine; PE, phosphatidylethanolamine; SP, sphingomyelin; Others, origin+lyso-phosphatidylcholine+unknown fraction+solvent front.

increased to approximately 3 µmoles/100 ml, and the percent distribution of phosphatidylcholine also showed some increase. At term the mean concentration of total phospholipid reached 6.86 µmoles/100 ml, and the percent composition of phosphatidylcholine increased to 69.4%. The mean phosphatidylcholine content showed three-fold increase from the immature to the transitional, and again three-fold increase from the transitional to the term.

Table 2 summarizes results of fatty acid analyses on the phosphatidylcholine fraction. Unfortunately positional specificity of the fatty acids in phosphatidylcholine from the immature group could not be determined, since the volume of amniotic fluid sample was too small to permit the analysis. The percentage
TABLE 2. Amniotic phosphatidylcholine fatty acid composition, percent saturated fatty acids, and calculated minimum disaturates in healthy ewes at different stages of gestation.

<table>
<thead>
<tr>
<th></th>
<th>Immature (7)</th>
<th>Transitional (6)</th>
<th>Term (6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole molecule</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>% C_{14:0}</td>
<td>7.6 ± 1.6</td>
<td>6.6 ± 1.4</td>
<td>7.5 ± 3.4</td>
</tr>
<tr>
<td>% C_{16:0}</td>
<td>50.3 ± 1.9</td>
<td>58.4 ± 7.2</td>
<td>68.4 ± 13.9</td>
</tr>
<tr>
<td>% C_{18:1}</td>
<td>4.5 ± 1.1</td>
<td>8.5 ± 1.3</td>
<td>6.6 ± 3.2</td>
</tr>
<tr>
<td>% C_{18:2}</td>
<td>8.8 ± 0.8</td>
<td>6.1 ± 2.4</td>
<td>4.9 ± 1.0</td>
</tr>
<tr>
<td>% C_{18:3}</td>
<td>12.3 ± 1.6</td>
<td>11.6 ± 1.1</td>
<td>5.9 ± 1.1</td>
</tr>
<tr>
<td>% Others*</td>
<td>16.6 ± 2.1</td>
<td>8.8 ± 4.4</td>
<td>6.7 ± 7.4</td>
</tr>
<tr>
<td>% Saturated</td>
<td>66.7 ± 2.8</td>
<td>71.2 ± 5.0</td>
<td>80.8 ± 9.9</td>
</tr>
<tr>
<td>Beta position</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>% C_{14:0}</td>
<td>5.9 ± 1.6</td>
<td>8.3 ± 0.8</td>
<td></td>
</tr>
<tr>
<td>% C_{16:0}</td>
<td>54.0 ± 6.4</td>
<td>55.0 ± 8.5</td>
<td></td>
</tr>
<tr>
<td>% C_{18:1}</td>
<td>10.6 ± 2.8</td>
<td>11.7 ± 6.1</td>
<td></td>
</tr>
<tr>
<td>% C_{18:2}</td>
<td>7.8 ± 3.3</td>
<td>4.5 ± 0.7</td>
<td></td>
</tr>
<tr>
<td>% C_{18:3}</td>
<td>13.5 ± 2.5</td>
<td>8.6 ± 4.2</td>
<td></td>
</tr>
<tr>
<td>% Others*</td>
<td>8.3 ± 2.2</td>
<td>8.9 ± 2.1</td>
<td></td>
</tr>
<tr>
<td>% Saturated</td>
<td>67.7 ± 6.1</td>
<td>70.8 ± 7.5</td>
<td></td>
</tr>
<tr>
<td>Minimum</td>
<td>74.7 ± 7.1</td>
<td>85.6 ± 10.0</td>
<td></td>
</tr>
<tr>
<td>% Minimum</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Minimum disaturates†</td>
<td>41.9 ± 9.5</td>
<td>58.3 ± 16.3</td>
<td></td>
</tr>
<tr>
<td>Minimum disaturated phosphatidylcholine (μmoles/100 ml)</td>
<td>0.69 ± 0.39</td>
<td>2.74 ± 1.43</td>
<td></td>
</tr>
</tbody>
</table>

Values are means ± S.D. Numerals in parentheses indicate the number of samples analyzed. *Others = fatty acids other than specified — mostly C_{16:2}. †Minimum disaturates = minimal percentage of alpha saturated and beta saturated phosphatidylcholine.

of saturated fatty acids of the whole molecule showed a gradual increase from the immature to the transitional stage, and to the term, reflecting the increase in palmitate.

The calculated minimum concentration of disaturated phosphatidylcholine showed a sharp increase from the transitional stage to the term. In the transitional stage, the minimum concentration of disaturated phosphatidylcholine remained below the level of 1 μmoles/100 ml, but the mean value of the 6 samples at the term increased to almost 3 μmoles/100 ml. In the immature stage the concentration of disaturated phosphatidylcholine should be less than 0.35 μmoles/100 ml, because the mean concentration of phosphatidylcholine was 0.50 μmoles/100 ml and the total percent of saturated fatty acids for the whole molecule was at most 70%.

Fig. 3 illustrates the change of minimum disaturated phosphatidylcholine after 130 days of gestation. The most abundant saturated fatty acid in the phosphatidylcholine molecule was palmitic acid as shown in Table 2; therefore the disaturated phosphatidylcholine in the transitional and term amniotic fluids is predominantly dipalmitoyl-phosphatidylcholine, indicating an increase of the surfactant in the amniotic fluid with the progress of gestation.
DISCUSSION

In the fetal lamb the lung secretes a fluid into the alveolar spaces which flows via pharynx either to the amniotic cavity or to the alimentary tracts, maintaining the alveolar spaces to a volume similar to the functional residual capacity of the postnatal lung (Adams et al. 1969). This has been evidenced by the experiment of nature observed by Potter and Bohlender (1941), and by Adams et al. (1963). The author also confirmed this finding by the experiment of tracheal ligation on the fetal lamb in utero (Lanman et al. 1971). According to the cineangiographic observation of Adams and his associates, the lung fluid was held in the trachea by the laryngeal sphincter which opened periodically to permit the fluid flow mostly into the esophagus, and to a lesser extent, into the amniotic cavity (Adams et al. 1967). Our observation in the previous experiment on the tracheal fluid flow showed the rate of 0.013 to 0.055 ml per kg of fetal weight per minute in the fetal lamb (Lanman et al. 1971). Reports from other laboratories indicated the similar flow rates (Enhörning and Adams 1965, Ross 1963).

In 1962, Helmy and Hack (1962) reported the presence of hydrolecithin, possibly disaturated phosphatidylcholine, in the human amniotic fluid. Nelson (1969) analyzed lipids in human amniotic fluids from normal and abnormal pregnancies. His data indicated the most abundant phospholipid to be phosphatidylcholine. Human amniotic fluid at the term contains approximately twice as much total phospholipid and phosphatidylcholine as in the ewe’s amniotic fluid at the term observed in the present study. The percent composition of phosphatidylcholine in both species is almost identical. In these reports of human amniotic fluid, however, any comments were not given to correlate the pulmonary surfactant.

The first document on the amniotic phospholipid in relation to surfactant
was the experiment with ewes performed by Fujiwara and co-workers (1964). They fractionated the lipids of the pooled amniotic fluids and revealed the presence of surface activity in the phospholipid fraction. Scarpelli (1967) also performed a comparative study on the composition of protein and phospholipid in the amniotic and the lung fluids of sheep by gel filtration. His experiment showed close similarities in the composition of both fluids, suggesting that the amniotic phospholipid originated from the fetal lung fluid.

The present study revealed the presence of disaturated phosphatidylcholine in appreciable amounts in the transitional and term amniotic fluid. It was also found that the amount of phosphatidylcholine increased as the term approached. The phospholipid of the term amniotic fluid was very similar to the one of the lung fluid in its fatty acid composition.

The time of appearance of surface activity in the fetal lung, measured as minimum surface tension and stability index on the surface balance, is reported to be between 120 and 130 days in lambs (Adams and Fujiwara 1963, Orzalesi et al. 1965). Biochemically appreciable amounts of disaturated phosphatidylcholine are detected in the alveolar washing of the fetal lamb in 120 to 134 days of gestation (Fujiwara et al. 1968). At the term the minimum concentration of alveolar disaturated phosphatidylcholine increases remarkably to the amount sufficient to cover the entire alveolar surfaces (Fujiwara et al. 1968).

The changing phospholipid content in the amniotic fluid observed in the present study correlates well with that of the alveolar phospholipid content of the developing fetal lamb found by Fujiwara and co-workers (1968). This correlation evidences the origin of amniotic disaturated phosphatidylcholine in the fetal alveolar surfactant.

Fetal pulmonary surfactant is essential for the maintenance of alveolar stability in the postnatal life. In the absence of surfactant, the alveolus collapses at each expiration. This phenomenon is characteristic of IRDS. Recent studies on fetal and newborn lambs and humans have indicated that those with biochemically immature lungs, i.e. those lungs contain insufficient amount of disaturated phosphatidylcholine, all developed IRDS (Fujiwara et al. 1968, Adams et al. 1970). Thus IRDS is believed to be caused by the immaturity of lungs or by the antenatal surfactant deficiency.

Gluck and his co-workers (1971) recently reported an antenatal diagnostic procedure for IRDS by phosphatidylcholine : sphingomyelin ratio in amniotic fluid. They stated that the absolute amount of phosphatidylcholine increased with the progress of gestation, while the content of sphingomyelin relatively unchanged. Their data on the fatty acid composition, however, were limited and no definite statements were made whether the changing phosphatidylcholine was predominantly comprised disaturated fatty acids. In the present study, fatty acids of phosphatidylcholine in each amniotic fluid samples were analyzed except for the one from very immature stage, confirming that the disaturated phosphatidylcholine increased as the term approached. Thus the data presented here are
a first evidence of the presence of fetal pulmonary surfactant in the amniotic fluid.

The basic data of this kind will serve as a base line value for the antenatal diagnosis of IRDS, though further studies on the amniotic fluid from the animal model of IRDS are necessary to establish the definite procedure.

Acknowledgment

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References

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