Surfactant phospholipids and proteins are increased in fetal sheep with pulmonary hypertension secondary to fetal systemic arteriovenous fistula

Alexandra Benachi,1,2 Jean-Marie Jouannic,3 Anne-Marie Barlier-Mur,1 Bernadette Chaillley-Heu,1 and Jacques R. Bourbon1

1Institut National de la Santé et de la Recherche Médicale U492, Faculté de Médecine, Créteil; 2Maternité, Hôpital Necker-Enfants Malades, Assistance Publique-Hôpitaux de Paris-Université Paris V; and 3Service de Gynécologie-Obstétrique, Hôpital Rothschild, Assistance Publique-Hôpitaux de Paris-Université Paris VI, Paris, France

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First published November 19, 2004; doi:10.1152/ajplung.00220.2004.—To determine whether prenatal surfactant storage was altered in a model of systemic arteriovenous fistula (SAVF) with pulmonary hypertension, a fistula was created between the internal jugular vein and the carotid artery in 120-day fetal lambs, and surfactant material was explored at 134 days. Total phospholipids (TPL) and disaturated phosphatidylcholine (DSPC) were increased in whole lung tissue. Phospholipid analysis of isolated lamellar body fraction evidenced a specific increase of surfactant pool size: TPL and DSPC in this fraction were enhanced 1.9 and 2.9 times, respectively, when referred to DNA. Although the steady-state level of transcripts of surfactant protein (SP)-A and SP-B was not found to be changed at the time of death, semiquantitative Western blot analysis revealed elevated SP-A and SP-B protein contents three- and twofold, respectively. These findings indicate markedly enhanced accumulation of surfactant material in the presence of surgically induced prenatal pulmonary hypertension. Although total lung cell number was increased by 26%, SP-B immunolabeling indicated that increased surfactant amount did not result from an increased alveolar type II cell proportion, but rather from an increased rate of storage. Whether similar changes in surfactant are encountered in human neonates with persistent pulmonary hypertension is worthy of investigation.

fetus; lung surfactant; alveolar type II cells; disaturated phosphatidylcholine

SYSTEMIC ARTERIOVENOUS FISTULA (SAVF) is a rare condition in the fetus that is known to be associated with persistent pulmonary hypertension (PPH) at birth. The newborn displays elevated pulmonary arterial pressure, abnormal pulmonary vascular reactivity, and high pulmonary vascular resistance leading to severe hypoxemia. Fetuses with vein of Galen arteriovenous malformation (14), highly vascularized sacrococcygeal teratoma, or chorioangioma are at risk of developing severe cardiac failure and PPH. To our knowledge, no studies have paid attention to the surfactant components in those neonates, probably because most of them were delivered prematurely due to severe heart failure and also because PPH is the primary concern. Because such studies can not readily be performed in humans, animal models of PPH may help in exploring surfactant material in this condition. Several experimental models of fetal pulmonary hypertension have been described over the past 15 years. Chronic intrauterine hypoxia (1), constriction (2), or ligation (31) of the ductus arteriosus induced pulmonary hypertension in the fetal lamb. In utero placement of aortopulmonary shunts in fetal lambs has also been shown to cause pulmonary hypertension at birth (34). More recently Jouannic et al. (27) have designed an experimental model of surgically created SAVF in the fetal lamb. They found that, at birth, there was an increase in pulmonary arterial pressure and resistance with no increase in pulmonary arterial blood flow (28). This was associated with striking structural remodeling of the pulmonary vascular bed similar to the pulmonary structural abnormalities found in neonates who died with PPH (23, 31).

The lung parenchyma is submitted to mechanical forces during fetal development (39). Those mechanical forces play a major role in lung growth and alveolar cell differentiation during intrauterine life. Thus forced lung expansion results in an overdifferentiation of type II cells into type I cells in vitro (19) and in vivo (9, 17), which results in a decrease in surfactant components (8, 19, 25, 30, 33). On the other hand, mechanical retraction favors expression of the type II cell phenotype and inhibits expression of the type I cell phenotype in a time- and amplitude-dependent manner (21). Using the model of aortopulmonary shunt that is associated with chronic pulmonary high blood flow (34), Gutierrez et al. (20) found a decrease in the lung content of two surfactant proteins, SP-A and SP-B, in 4-wk-old lambs, which suggests that alterations in pulmonary blood flow and/or pressure may also influence phenotypic expression of alveolar epithelial cells. More recently, the same group of investigators reported that these changes are not observed during the first week of life (29). However, the surfactant compartment was not thoroughly explored, since the phospholipid moiety was examined during the first week only. Moreover, lamb lungs were subjected to the hemodynamic challenge when already fully mature. It therefore appeared of interest to investigate the consequences of SAVF when it is performed before the end of prenatal alveolar cell maturation, i.e., when surfactant material is stored in preparation to birth. In the present study, we therefore used the fetal lamb model of SAVF to evaluate the consequence for surfactant component storage close to full term.

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**MATERIALS AND METHODS**

**Surgical preparation and physiological measurements.** Eleven fetal lambs were studied. In the fistula group (n = 6), an SAVF was created as previously described (27) at 120 days of gestation (full term 145 days). The control group (n = 5) consisted of unoperated fetuses whose twins had been operated on (n = 3) and of fetuses from unoperated ewes (n = 2). No pregnancy had more than two fetuses. Co-twins were considered to be control animals, because previous reports have shown that when the amniotic cavity of the second twin is not opened, hemodynamic and histological data are not different from those of fetuses from unoperated ewes (6, 7, 27). Briefly, under sterile conditions, the fetal neck was exposed through a 5-cm uterine incision while the fetal head was kept in the amniotic cavity. The internal jugular vein and the carotid artery were exposed, and an anastomosis was performed between a jugular venotomy and a carotid arteriotomy of 10–12 mm length. Part of the amniotic fluid was removed during surgery, the volume was determined, and immediately after, an equal amount of saline at 37°C was infused in the amniotic sac to restore initial volume. No oligohydramnios was observed at delivery. Animals received care in accordance with the “Principles of Laboratory Animal Care” and the “Guide for the Care and Use of Laboratory Animals” (5) and under the authority of the French Ministry of Agriculture. Physiological measurements including flow and pressure measurements and blood gas and pH determination were as described previously in detail (28).

**Lung tissue preparation.** At 134–139 days the ewes underwent a second laparotomy. Measurement of pressure and flow in the left pulmonary artery (LPA) have previously been described elsewhere (27, 28). Briefly, the fetal heart and great vessels were exposed through a left thoracotomy. Catheters were inserted into the atria, ventricles, aorta, and LPA by direct puncture. Blood flow was measured with an ultrasonic flow transducer size 6 (Transonic Systems, Ithaca, NY) placed around the LPA. Lambs were then killed by quick exsanguination. Tissue samples from the right lobe of each lung were immediately dropped into liquid nitrogen and kept frozen at −80°C until biochemical analyses. We prepared the left lobe for light microscopy by cannulating the main bronchus. The airways were tendined with 10% formaldehyde at 35 cmH2O pressure for 2 min, then the left main bronchus was ligated, and the fully tendsted lung was immersed in fixative for 48 h.

**DNA determination.** DNA was determined on tissue homogenates by the colorimetric diphenylamine method (12).

**Lamellar body isolation and phospholipid determination.** Surfactant [subcellular lamellar body fraction (LBF)] was extracted from homogenized tissue by a gradient ultracentrifugation method as described previously (8). Lipids were extracted from lung tissue samples and LBF by chloroform-methanol-water 1:2:0.8 (vol/vol/vol). Disaturated phosphatidylycholine (DSPC), the major surfactant component, was isolated from OsO4-treated lipid samples by thin-layer chromatography according to Patterson et al. (32). A trace amount of [14C]-labeled with [α-32P]deoxycytidine triphosphate (α-32P)dCTP; NEN, Perkin Elmer) using the Rediprime DNA labeling system from Amersham and purified on G-50 probe purification columns (Amersham). The blots were exposed to X-Omat Kodak Scientific imaging films for a suitable exposure time at −80°C. Autoradiographic signal was quantified by densitometry using image analysis software (NIH Image) and normalized to the relative amount of 18S ribosomal RNA. One transcript only was detected for SP-A and SP-C, consistent with previous reports. For SP-B, three transcripts were detected, but only the major band corresponding to the 2.6-kb mRNA was quantified.

**Surfactant protein analysis by Western blot.** Lung sample homogenates were solubilized under reducing conditions for SP-A analysis and under nonreducing conditions for SP-B analysis. Proteins were separated by 12% (SP-A) or 15% (SP-B) SDS-PAGE. After transferring proteins onto nitrocellulose membranes, we detected proteins with rabbit antibodies directed against bovine SP-A (Chemicon International, Temecula, CA) or bovine SP-B (same as above). A secondary donkey anti-rabbit IgG antibody conjugated with horseradish peroxidase was applied, and the resulting complex was visualized by chemiluminescence (ECL, Western Blotting Analysis System, Amersham). To control for variations in protein loading, each membrane was labeled with a monoclonal anti-β-actin antibody (Sigma, L’Isle d’Abeau, France). Band densitometry analysis was performed with the NIH Image analysis software.

**Histological and immunochemical studies.** After fixation, the lung was cut into slices 3–5 mm thick. One block was selected from two animals in each group. Tissue blocks were processed through graded alcohols, embedded in paraffin, and sectioned at 4 μm. Tissue sections were stained with hematoxylin and eosin. The radial alveolar count (RAC) was estimated according to Emery and Mithal (16), and the architecture of the pulmonary acinus was examined. An antisurfactant bovine SP-B antibody raised in the rabbit (kindly provided by Dr. J. A. Whitsett, Children’s Hospital Medical Center, Cincinnati, OH) was used to label lung type II cells (8). Paraffin sections were dewaxed, rehydrated, treated with phosphate-buffered saline (PBS) containing 1% bovine serum albumin, and then incubated with the SP-B antibody (1:250) for 1 h. After three washes with PBS-Tween 0.05%, sections were incubated for 45 min with a FITC-conjugated anti-rabbit IgG antibody (Biomedica, Foster City, CA). The nuclei were labeled with bisbenzimide dye Hoechst 33258 (Sigma) at 0.25 μg/ml for 15 min. After being rinsed with buffer, the sections were mounted with Vectashield solution and examined under a Zeiss fluorescence microscope. Controls consisted of incubation with PBS-BSA buffer or with nonimmune rabbit serum instead of the primary antibody. For each sample, the count of as many as 2,000 cells was performed on photographs of different fields of lung parenchyma.

**Statistical analysis.** Data are presented as means ± SE. Mean comparisons were made by Student’s t-test and the nonparametric Mann-Whitney U-test with P = 0.05 considered as the limit of statistical significance. Both methods indicated same levels of significance.

**RESULTS**

**Physiological study.** Lambs were delivered 14–15 days postoperatively (134–139 days). The mean fetal weight was not different in the control and fistula groups (2,496 ± 188 and 2,485 ± 194 g, respectively). The lung weights were not different between the two groups (fistula group, 124 ± 8 g; control group, 124 ± 9 g), and the lung weight-to-body weight...
ratio was the same in both groups (0.05 g/g). Relatively high lung weights and lung-to-body weight ratios are accounted for by the fact that lungs were not drained before being weighed. Mean LPA pressure was 48 ± 2 mmHg in the fistula group, which was significantly higher than in the control group (40 ± 2 mmHg, P < 0.01). Mean aortic pressure was not different between the two groups. There was no difference between the two groups for LPA blood flow (fistula group, 131 ± 18 ml/min; control group, 146 ± 14 ml/min). The pulmonary vascular resistance was 0.36 mmHg·ml⁻¹·min⁻¹ in the fistula group, which was significantly higher than in the control group (0.26 mmHg·ml⁻¹·min⁻¹, P < 0.05). There was no difference for systemic blood gas analysis, and pH values were normal in the two groups.

Effect of fetal SAVF on lung DNA. Lung DNA concentration was increased from 5.32 ± 0.18 mg/g of wet lung weight (ww) in control lambs to 6.75 ± 0.29 mg/g ww in lambs with fistula (P < 0.01). Total lung DNA relative to body weight was increased from 264.7 ± 10.0 mg/kg to 334.3 ± 15.9 mg/kg (P < 0.05). This indicates that the number of lung cells was increased by 26% in the SAVF fetuses.

Effect of fetal SAVF on surfactant phospholipids. Quantitative analysis of phospholipids was performed both in whole lung tissue and in LBF isolated from the lung and considered to represent the surfactant pool. TPL were increased in lung tissue from 11.98 ± 0.81 μmol/g in controls to 19.63 ± 1.56 μmol/g in SAVF fetuses (Fig. 1A), but this was likely to be due to increased cell number since the difference was not significant when TPL were referred to DNA (Fig. 1B). By contrast, the major surfactant component, DSPC, was increased 3.6 times when referred to lung ww (from 2.23 ± 0.27 to 6.05 ± 0.72 μmol/g) and 1.9 times when referred to DNA (from 4.09 ± 0.53 to 7.85 ± 0.058 mmol/μg), suggesting that the increase was at least partly independent from increased cell number (Fig. 1A and B). Analysis of LBF indicated that this increase was mainly due to increased surfactant pool. In LBF, TPL and DSPC were increased 1.9 (from 0.65 ± 0.05 to 1.22 ± 0.47 mmol/μg DNA) and 2.9 times (from 0.18 ± 0.03 to 0.52 ± 0.10 mmol/μg DNA), respectively (Fig. 1B). TPL of LBF represented 29% of whole tissue TPL in controls but 42% of whole tissue TPL in the fistula group. DSPC was even proportionally more largely enhanced since DSPC of LBF represented 33% of whole tissue DSPC in controls and as much as 58% in the fistula group.

Effect of fetal SAVF on surfactant proteins. mRNA level of SP-A, -B, and -C was found to be unchanged on the average in the fistula group compared with the control group (Fig. 2B), although SP-A mRNA tended to be increased in some SAVF fetuses (Fig. 2A, 8th and 9th lanes). It should be stressed, however, that a large, unexplained variability in expression level was found among each experimental group (Fig. 2A), which corresponded to actual individual differences as shown by repeated Northern blotting experiments. By contrast with mRNA data, SP-A and SP-B protein amounts evaluated by Western blot analysis did not display in each group the individual variability observed for transcripts and were markedly increased in SAVF fetuses (Fig. 3). The reduced 28- and 32-kDa isoforms of SP-A were enhanced threefold on average in the SAVF group compared with control group (311.3 ± 29.2% vs. 100.0 ± 22.5%), whereas the unreduced 18-kDa band of SP-B was increased about twofold (220.5 ± 30.2% vs. 100.0 ± 20.1%).

Lung morphology and type II cell immunolabeling. The analysis of hematoxylin and eosin-stained sections showed that the normal lungs at 134–139 days of gestation were characterized by future air spaces lined by a thin epithelium and subdivided by wall projections. The morphological aspect was similar in SAVF and control fetuses (Fig. 4, A and B). All subjects exhibited an RAC of ~4 with no apparent difference between control and fistula lungs. No immaturity of the lung parenchyma that could have accounted for the increased lung cell number was apparent in SAVF fetuses.

SP-B-immunoreactive cells displayed a bright fluorescence of the cytoplasm after incubation with anti-SP-B antibody. The type II cell density was estimated by the proportion of SP-B-positive cells referred to the total parenchymal cell number. Analysis on two SAVF and two control lung tissue samples indicated a proportion of SP-B-positive cells of 9.1 ± 0.5% and 7.9 ± 0.5% in control and SAVF fetuses, respectively. This indicates that enhanced surfactant contents could not be accounted for by changes in type II cell number. Fig. 4, C–F, displays representative fields that illustrate this finding.

Fig. 1. Phospholipid composition of lung tissue (whole lung) and lamellar body fraction (LBF) in control and systemic arteriovenous fistula (SAVF) lamb fetuses. Lung contents of total phospholipids (TPL) and disaturated phosphatidylcholine (DSPC) are expressed on a tissue wet weight (ww) basis (A) or per μg DNA (B). Significantly different from controls for: *P < 0.05; **P < 0.01. The increase of TPL in whole lung when referred to tissue ww and the absence of significant increase when referred to DNA indicate that this increase resulted from enhanced cell number. Increases of both TPL and DSPC in LBF are indicative of increased surfactant pool size. The increase was particularly marked for DSPC (2.9× on a μg DNA basis).
DISCUSSION

Because surfactant protein content was affected by chronically increased lung blood flow in the postnatal lamb (20), we evaluated in this study the effects of SAVF occurring during the prenatal period when surfactant storage takes place in alveolar type II cells. These cells are responsible for synthesis and secretion of surfactant, which is a compound of \( \sim 90\% \) lipids, mainly phospholipids, and \( 10\% \) proteins that maintains alveolar stability and homeostasis (10).

Jouannic et al. (28) have shown that the prenatal creation of an SAVF not only increased pulmonary arterial blood pressure and resistance but also led to structural pulmonary arterial remodeling and altered pulmonary vascular reactivity. Those striking changes in the fetal vascular bed, including increased number of peripheral muscular arteries together with an increase in pulmonary arterial medial thickness, were not associated with pulmonary arterial high blood flow at birth. However, in an additional Doppler echographic study, pulmonary arterial blood flow was found to be twice as high on day 1 postsurgery, suggesting that the SAVF, by increasing the blood volume returning to the right heart, may have impeded the functioning of the physiological fetal shunt (i.e., foramen ovale and ductus arteriosus) (26). They hypothesized that this temporary increase in pulmonary arterial blood flow in the fistula model may reflect a temporary decrease in the pulmonary arterial resistance similar to that observed in the model of prenatal ductus arteriosus compression (2).

Using this model, we evaluated the total amount of surfactant phospholipids, the amount of the principal surface-active phospholipid component, namely DSPC, and surfactant proteins. Tissue samples were from the same individuals as those included in the previous study (28).

Our results show a quantitative increase of lung tissue TPL and DSPC content in the fistula group compared with the control group. Quantification of phospholipids of LBF indicates that this increase was mainly due to increased surfactant pool. This increase is considerable since the amount of surfact-
tant DSPC was enhanced nearly three times compared with control fetuses. Although mRNA levels of SP-A and SP-B were found to be unchanged in the fistula group compared with the control group, SP-A and SP-B protein amounts were increased in all individuals of the fistula group in similar proportion as surfactant DSPC. It should be stressed, however, that the steady-state level of mRNAs only reflects the amount of transcripts present at the moment of death, whereas protein amounts represent the result of storage of surfactant material on the whole period between surgery and death and are therefore more informative. The apparent discrepancy between transcripts and proteins is most likely explained by a transient augmentation of mRNAs that would have in turn led to an increase in protein storage but that was no longer detectable at the time of death. This assumption is supported by the high level of SP-A mRNA observed in some of the SAVF fetuses that tended to increase the average value in this group. Alternatively, posttranscriptional changes may have occurred that led in turn to increased surfactant protein synthesis. As a whole, both phospholipid and protein moieties of surfactant were markedly increased in the lung of the SAVF fetuses. It should be pointed out that this increase does not correspond to an accelerated maturation of alveolar cells, since fetal lambs were investigated close to full term, i.e., when they had already achieved almost complete surfactant storage. This enhanced surfactant content therefore represents excessive surfactant storage above normal levels. The threefold increase in surfactant content can be accounted for neither by a change in the proportion of type II cells that appeared to be the same in both experimental groups nor by the overall increase of lung cell number in SAVF that was only 26%. Increased surfactant storage on a per-cell basis is therefore likely.

To our knowledge, the effects of increased pulmonary blood flow and/or pulmonary hypertension on the surfactant compartment were investigated previously in one model only that consisted in aortopulmonary shunt established at term in lamb fetuses (20, 29), the lamb lungs being studied after spontaneous delivery, at 1 mo of life in a first step (20), then during the first postnatal week (29). Whereas no change was found in the first week, at 1 mo the authors reported a decrease in SP-A mRNA content to 61 ± 8% of control value with a consistent decrease of SP-A to 50 ± 12% of age-matched control value, and although SP-B mRNAs were not significantly changed, SP-B was found to be decreased to 74 ± 25% of control value (20).
Lung phospholipid content and percentage of DSPC were unchanged after 1 wk (29) but were not examined at 1 mo. In this model, it is therefore a diminution of certain surfactant components that was observed, at least after 1 mo of extrauterine life.

Two major differences exist between the aortopulmonary shunt and the SAVF model used herein that may account for differences in their effects upon surfactant. First, the fistula in SAVF model has been created at 0.80 gestation, i.e., when alveolar epithelium is not fully mature in terms of surfactant storage, whereas aortopulmonary shunt in the previous studies (20, 29) was placed close to term, when lungs were already fully mature. In this model, contrary to our model, lamb lungs therefore did not experience hypertension during the phase of prenatal surfactant storage. The changes in surfactant indeed occurred during this phase in the SAVF model, whereas in the aortopulmonary shunt model, they developed between the first week and the first month of postnatal life. Surfactant regulation and synthesis rate differ quite largely at these different lung developmental stages, which may explain the different lung responses between both models. As far as the human fetus with PPH is concerned, the model investigated herein appears as more relevant. Second, differences in hemodynamics exist between both models that may also account for the different findings with regard to surfactant. The aortopulmonary shunt model is associated with sustained pulmonary high blood flow that persists throughout the perinatal period. Conversely, the SAVF model has been found to increase both pulmonary arterial blood pressure and resistance, whereas no difference was found for the LPA blood flow at birth, suggesting that the fistula model is not associated with chronically elevated pulmonary blood flow in utero. Doppler study of arterial pulmonary blood flow indicated that the fistula was associated with temporary pulmonary high blood flow at day 1 postsurgery (with pulmonary blood flow that was two times higher in the fistula group compared with controls), with a secondary normalization of the pulmonary blood flow by day 6 (26).

With regard to mechanisms possibly involved in surfactant changes in the present SAVF model, and similar to previous studies (20, 29), only assumptions can be proposed at present. Mechanical forces are known to modify alveolar epithelial cell phenotype as well as expression of surfactant proteins in vitro (18, 19, 21) and in vivo in normal (17, 25, 30, 33) and hypoplastic lungs (8, 9). Although such effects were previously observed for forces directly applied to the future air spaces, mechanical forces exerted by the vascular pressure may have similar consequences.

This notwithstanding, the underlying mechanisms might be more indirect and multifactorial. Among the various humoral effectors known to influence surfactant storage, changes in glucocorticoids (4) likely to have been enhanced by fetal stress or in growth factors and neuropeptides, including epidermal growth factor (37), fibroblast growth factor 7 (13), vascular endothelial growth factor (11), or gastrin-releasing peptide (36), may be involved. A possible involvement of atrial natriuretic peptide (ANP) could be particularly suspected since J pulmonary ANP gene expression or ANP release is influenced by hemodynamic overload and glucocorticoids (22), pulmonary hypertension (3, 15), or vessel stretch (35), 2 functional ANP receptors are present in alveolar type II cells (38), and J ANP has been shown to stimulate surfactant accumulation when given to rats in vivo (24). By contrast, an involvement of nitric oxide production evoked by Gutierrez et al. (20) for explaining the decreased surfactant protein expression observed in their model appears unlikely in the present model, since endothelial nitric oxide synthase protein expression was unchanged (28).

In conclusion, the present study brings out that surgically induced pulmonary hypertension occurring during the prenatal period of surfactant storage leads to accumulation of surfactant material over normal values. Human fetuses with a similar condition (i.e., peripheric arteriovenous fistula) often display fetal distress (14), and premature delivery is induced to initiate a neonatal treatment (surgery and/or vascular embolization). As far as they could be extended to the relevant pathological conditions in human fetuses, our observations suggest that surfactant deficit in these conditions is not likely and, therefore, that respiratory distress is likely to result from PPH rather than from hyaline membrane disease. The use of exogenous surfactant might have a deleterious effect in this condition, even for premature neonates. Whether these patients actually present alterations in surfactant storage similar to those observed herein is worthy of investigation.

GRANTS

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REFERENCES

13. Chelly N, Mouhieddine-Gueddiche OB, Barlier-Mur AM, Chaillée-Heu B, and Bourbon JR. Keratinocyte growth factor enhances matura-


