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Surfactant Composition and Function in Patients with *ABCA3* Mutations

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ABSTRACT: Mutations in the gene encoding the ATP binding cassette transporter member A3 (ABCA3) are associated with fatal surfactant deficiency. ABCA3 lines the limiting membrane of lamellar bodies within alveolar type-II cells, suggesting a role in surfactant metabolism. The objective of this study was to determine the surfactant phospholipid composition and function in patients with mutations in the *ABCA3* gene. Bronchoalveolar lavage (BAL) fluid was analyzed from three groups of infants: 1) Infants with *ABCA3* mutations, 2) infants with inherited surfactant protein-B deficiency (SP-B), and 3) patients without parenchymal lung disease (CON). Surfactant phospholipid profile was determined using two-dimensional thin-layer chromatography, and surface tension was measured with a pulsating bubble surfactometer. Phosphatidylcholine comprised 41 ± 19% of the total phospholipid in the BAL fluid of the ABCA3 group compared with 78 ± 3% and 68 ± 18%, $p = 0.008$ and 0.05, of the CON and SP-B groups, respectively. Surface tension was 31.5 ± 9.3 mN/m and was significantly greater than CON but no different from SP-B. We conclude that mutations in *ABCA3* are associated with surfactant that is deficient in phosphatidylcholine and has decreased function, suggesting that ABCA3 plays an important role in pulmonary surfactant phospholipid homeostasis. **(*Pediatr Res* 59: 801–805, 2006)**

Pulmonary surfactant is a mixture of phospholipids and proteins synthesized in alveolar type-II cells that is necessary for maintaining alveolar expansion at end-expiration. Phospholipids comprise approximately 90% of pulmonary surfactant, of which 70–80% is phosphatidylcholine (PC), 10% is phosphatidylglycerol (PG), along with minor amounts of phosphatidylinositol (PI), phosphatidylethanolamine (PE), phosphatidylserine (PS), and sphingomyelin (SM) (1). Pulmonary surfactant is uniquely enriched in PC and in the abundance of the dipalmitoyl form (DPPC), which comprises approximately half of the PC. DPPC is the major surface-active phospholipid component of pulmonary surfactant.

Surfactant PC is synthesized primarily in the endoplasmic reticulum of the alveolar type-II cells (2–4) and is transferred *via* the Golgi system to the lamellar bodies. Within the type-II cell, the lamellar body is the storage compartment for pulmonary surfactant associated lipids and for surfactant proteins B and C (SP-B and SP-C). The surfactant complex is secreted into the alveolar airspace by exocytosis, whereby the contents of the lamellar body are extruded into the alveolar lumen to form a lipid film that reduces surface tension at the air-liquid interface.

Whereas the synthesis of pulmonary surfactant phospholipids, in particular PC, has been described in detail, the intracellular trafficking of surfactant phospholipids is less well characterized. It has been postulated that the movement of phospholipids between membranes may be facilitated by phospholipid transfer proteins, several of which have been localized to mammalian lung (5,6). However, the exact role of these transporters and their specificity for different phospholipids are unknown.

The ABC family of transporters consists of over 40 proteins that use energy to drive the transport of macromolecules across plasma and intracellular membranes (7), and the ABCA subfamily is involved in the transport of phospholipids and cholesterol within and between cells. The 1704 amino acid ATP binding cassette transporter member A3 (ABCA3), encoded by an 80 kB gene mapped to human chromosome 16p13.3, has been localized to the limiting membrane of the lamellar bodies in alveolar type-II cells (8,9). Although the

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Abbreviations: ABC, ATP binding cassette; **ABCA3,** ATP binding cassette A3; **BAL,** bronchoalveolar lavage; **DPPC,** dipalmitoylphosphatidylcholine; **DSPC,** disaturated phosphatidylcholine; **PC,** phosphatidylcholine; **PE,** phosphatidylethanolamine; **PS,** phosphatidylserine; **SM,** sphingomyelin; **SP,** surfactant protein

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function of ABCA3 is unknown, the recent association of mutations in the *ABCA3* gene (*ABCA3*) with abnormal lamellar bodies and fatal surfactant deficiency suggests that ABCA3 is involved in the movement of one or more components of surfactant into or out of the lamellar body (10). However, the effects of ABCA3 mutations on surfactant homeostasis and function remain poorly understood.

Clinical and pathologic findings in patients with *ABCA3* mutations include respiratory distress after birth associated with abnormally small lamellar body inclusions, supporting the concept that *ABCA3* mutations disrupt surfactant homeostasis. Therefore, we investigated surfactant phospholipid composition and function in BAL fluid from infants who underwent lung transplantation for persistent respiratory distress associated with *ABCA3* mutations.

METHODS

Population. From 1995 to 2003, 12 infants <20 mo of age underwent lung transplantation for progressive neonatal-onset lung disease of unknown etiology. BAL samples were obtained at the time of transplant in seven of these patients, and sequence analysis identified *ABCA3* mutations in eight of these infants. SP-B and SP-C gene mutations were excluded in these infants by DNA sequence analysis. BAL and lung tissue samples were obtained at the time of transplantation. In addition, two siblings previously shown to be homozygous for *ABCA3* W1142× (Patients 1 and 2 in Ref. 10) were included as postmortem BAL was available for analysis. Two groups of infants were used for comparison: 1) a nonparenchymal lung disease group (CON), which included three infants transplanted for pulmonary vascular disease and one lung transplant donor, all of whom had normal surfactant function and did not require chronic ventilation before transplant; and 2) a parenchymal lung disease group that was comprised of four infants with SP-B deficiency, previously reported (11,12), and ventilated from the birth until sample acquisition. Of the SP-B–deficient patients, three underwent lung transplantation and the fourth had BAL fluid collected for diagnostic purposes. Informed consent was obtained from the families under a protocol approved by the Washington University Human Studies Committee.

Tissue and lavage samples. At transplantation, sections of the explanted lung were frozen immediately upon removal. BAL with sterile saline was performed on a single lobe of the explanted lung within 1 h of removal as previously described (11). BAL was centrifuged at 500 × *g* to remove cells and frozen at –70°C until analysis.

Genetic analysis. Fifty to 100 mg of frozen lung tissue obtained at the time of transplantation was homogenized in Trizol Reagent (GIBCO-BRL, Gaithersburg, MD), RNA isolated, and 5 μg of total RNA reverse transcribed with SuperScript II using reagents supplied by the manufacturer (Invitrogen, Carlsbad, CA) and 0.5 μg oligo dT, as previously described (13). ABCA3-specific cDNA was generated using primers designed to amplify each of the 30 coding exons (10). Ten overlapping products spanned the entire cDNA sequence, and primers were located such that the fragments overlapped exons so as to detect mutations that might result in skipping of an entire exon. Amplicons were sequenced in both orientations through the DNA analysis facility of the Johns Hopkins University School of Medicine using an Applied Biosystems 3730 DNA Analyzer (Applied Biosystems, Foster City, CA). DNA sequencing chromatograms were analyzed with the aid of software (Sequencher v4.2, Gene Codes Corporation, Ann Arbor, MI) and compared with the reference ABCA3 cDNA sequence (NCBI NM_001089) (10,13,14). The *ABCA3* gene was not sequenced in either of the control groups, given that they had well-defined reasons for lung transplantation.

Surface tension, protein, and phospholipid analysis. BAL fluid was centrifuged at 48,000 × *g* for 1 h to obtain a crude surfactant pellet. The pellet was resuspended in 300 μL of normal saline/5 mM $CaCl_2$. Total protein (15), total phospholipid (16), and disaturated phospholipid (17,18) were determined as described previously. Individual phospholipids were separated by thin-layer chromatography (TLC) (19) and lipid phosphorus determined (20). Minimum surface tension was determined with a pulsating bubble surfactometer after adjusting phospholipid concentration to 1.5 mM (Electronetics, Buffalo, NY) (21). ELISA was used to quantify SP-A and SP-B in the lavage fluid as previously described (22). No analysis of SP-C was performed on these samples. Individual phospholipid composition is expressed as percentage of total phospholipid on the TLC plate; DSPC is expressed as a percentage of PC, and minimum surface tension is expressed as mN/m.

Statistical analysis. ANOVA was used to compare measurements of surfactant composition and function between groups using SPSS (SPSS Inc., Chicago, IL). Results are expressed as means and SD.

RESULTS

Genetic analysis. Seventeen novel *ABCA3* mutations were identified in the eight patients who underwent lung transplantation for progressive lung disease in infancy. Patient characteristics and *ABCA3* genotypes are listed in Table 1. All were born at term gestation and developed respiratory distress at birth that required mechanical ventilation until the time of lung transplantation at 2–15 mo of age (Table 1). Patients 3 and 8 had previous siblings who died with a similar clinical picture; material for genetic analysis was not available from those siblings. All SP-B–deficient infants and all but one control infant were born at term and underwent transplantation or sample acquisition at 2.6 ± 1.8 mo and 6.3 ± 4.0 mo, respectively.

Table 1. *ABCA3 patient data*

Patient	Sex	Age at sample acquisition (mo)	Allele 1	Allele 2	Histopathologic diagnosis	PC/lipid (%)	Surface tension (mN/m)	Lipid/ protein	SP-A (μg)*	SP-B (ng)*
1	M	5	R194G	delF1203	Chronic pneumonitis of infancy	59.6	28	0.76	533	448
2	F	3	L1595P	D253Y	Interstitial pneumonitis/alveolar hypoplasia	NA	NA	NA	21.3	212
3	F	5	1112-20 G>A	3163del10	Pulmonary alveolar proteinosis/interstitial pneumonitis	25.4	37.8	0.33	60.8	66.5
4	F	2	M1I	delE203	Interstitial pneumonitis	31.2	34.6	0.39	68	24
5	M	15	1126ins15	1866del25	Interstitial fibrosis/interstitial pneumonitis	23.8	28.5	0.43	NA	18
6	F	3	P766S-L960F	1729delTC	Pulmonary alveolar proteinosis	NA	NA	NA	135	193
7	F	4	W179C	1382delTG	Interstitial pneumonitis/dysmature alveolar segments	78.1	11.1	0.48	51.9	59.1
8	F	4	R43L	P264R	Chronic interstitial pneumonitis	39.8	34.3	0.25	22.7	40

NA, not available.

* SP-A and SP-B determined by ELISA in tracheal aspirates obtained as part of initial evaluation studies in these patients and not at the time of transplant.

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Surface tension, protein, and phospholipid analysis. Surfactant extracts were available for analysis from six of the eight patients with *ABCA3* mutations and from two siblings reported previously. SP-A and SP-B were present in all samples tested. The proportion of PC in the surfactant extract of the ABCA3 patients was 41 ± 19% of the total phospholipid, which was significantly less than that of SP-B–deficient infants ($p = 0.05$) and the control infants ($p = 0.008$) (Fig. 1). DSPC comprised 47 ± 18% of the total PC, which was not significantly different from that of the other two groups ($p = 0.6$ and $p = 0.5$, SP-B deficient and control, respectively). The phospholipid to protein ratio in the ABCA3 group was 0.4 ± 0.2, which was significantly lower than both the SP-B–deficient and control groups (1.2 ± 0.4, $p = 0.04$ and 3.9 ± 0.7, $p < 0.001$ SP-B deficient and control, respectively) (Fig. 2*a*). Minimum surface tension in the surfactant extracts was 31.5 ± 9.3 mN/m for the ABCA3 group, which was significantly greater than the control group ($p < 0.001$) but not different from the SP-B–deficient group ($p = 0.3$) (Fig. 2*b*). Patient 7 had a normal surfactant profile and function but had received several doses of bovine surfactant replacement in the 24 h preceding lung transplantation. Excluding the data from this patient, PC comprised 36 ± 12% of the total phospholipid in the ABCA3 patients ($p = 0.004$ and $p = 0.001$, compared with SP-B deficient and control, respectively), and DSPC comprised 43 ± 13% of the total PC ($p = 0.1$ and $p = 0.1$, SP-B deficient and control, respectively). Minimum surface tension was 34.4 ± 4.6 mN/m, significantly increased compared with SP-B deficient and control groups ($p = 0.003$ and $p < 0.001$, respectively).

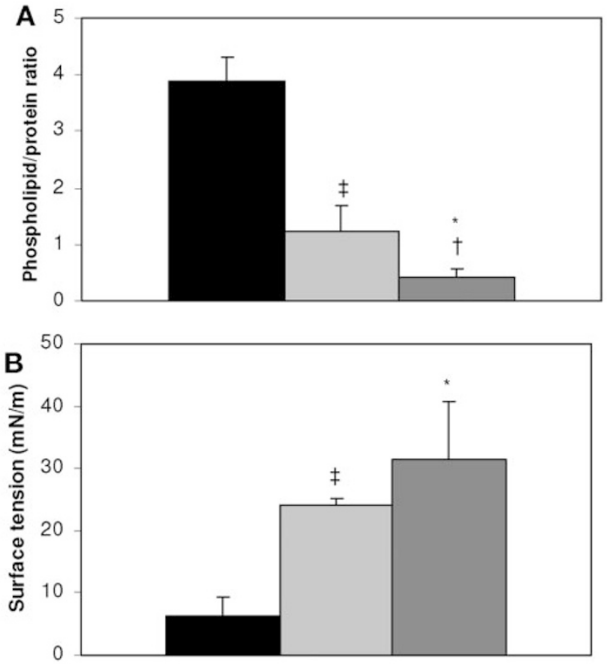

Figure 2. (*a*) Phospholipid to protein ratio in BAL fluid (mg phospholipid/mg protein, mean ± 1 SD). The phospholipid to protein ratio in the ABCA3 group (*dark gray bars*) was significantly lower than both the SP-B–deficient (*light gray bars*) and control groups (*black bars*) ($p = 0.04$ and $p < 0.001$ SP-B deficient and control, respectively). *ABCA3 significantly different from control. †ABCA3 significantly different from SP-B deficient. ‡SP-B deficient significantly different from control. (*b*) Surface tension in BAL fluid (mN/m, mean ± 1SD). Minimum surface tension was determined using a pulsating bubble surfactometer and was significantly elevated in the ABCA3 patients (*dark gray bars*), compared with controls (*black bars*) ($p < 0.001$) but was not different from that of SP-B deficient patients (*light gray bars*) ($p = 0.3$). *ABCA3 significantly different from control. ‡SP-B deficient significantly different from control.

DISCUSSION

The association between *ABCA3* mutations and fatal surfactant deficiency in newborns strongly supports the concept that ABCA3 is critical for normal surfactant function (10). Although clinical and pathologic findings suggest that ABCA3 plays an important role in surfactant lipid homeostasis, the underlying mechanism for lung dysfunction in these patients was unknown. The availability of BAL fluid for analysis in the lung transplant patients in this study offered the unique opportunity to determine whether *ABCA3* mutations cause surfactant dysfunction. The present study demonstrates altered surfactant lipid composition and decreased surfactant activity in patients with *ABCA3* mutations.

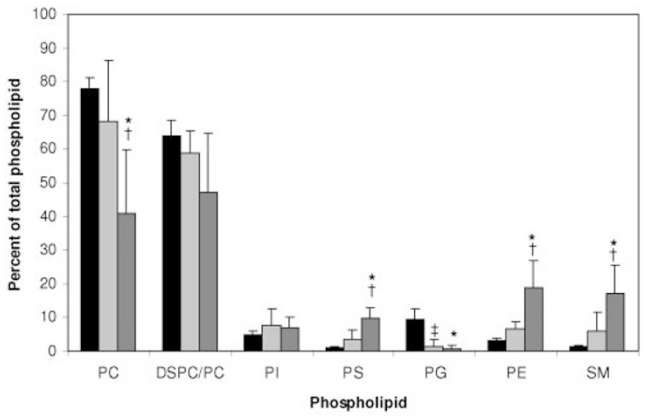

Figure 1. The percentage (mean ± 1 SD) of each phospholipid relative to total phospholipid in BAL fluid for each group. PC in the ABCA3 patients (*dark gray bars*) comprised 41 ± 19% of the total phospholipid content, which was significantly lower than that of SP-B–deficient patients (*light gray bars*) ($p = 0.05$) and control patients (*black bars*) ($p = 0.008$). Reciprocal increases in PS, PE, and SM were also present. *ABCA3 significantly different from control. †ABCA3 significantly different from SP-B deficient. ‡SP-B deficient significantly different from control.

Infants with *ABCA3* mutations had a significantly reduced proportion of PC in their BAL compared with SP-B–deficient and control patients. While the proportion of DSPC to PC remained unchanged in patients with *ABCA3* mutations, the decreased content of PC also indicates low absolute amounts of DSPC, findings that likely contribute to the observed surfactant dysfunction (despite the presence of SP-B). The significantly decreased phospholipid-to-protein ratio in the patients with *ABCA3* mutations also suggests either an absolute decrease in the amount of phospholipid or a nonspecific increase in protein in the alveolar space caused by chronic ventilation and lung injury. The finding that a decrease in the lipid-to-protein ratio was more severe in ABCA3 patients than

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in the SP-B–deficient infants, who required a similar degree and duration of mechanical ventilation, suggests that the absolute amount of phospholipid was decreased to a greater extent in the ABCA3 patients.

The majority of surfactant PC is synthesized in the endoplasmic reticulum of the type-II cells and then transferred *via* the Golgi system to the lamellar bodies. Although there is evidence for the presence of phospholipid transfer proteins within the alveolar type-II cells (5,6), their exact role in the trafficking of phospholipids is unknown. The predicted structure of the ABCA3 protein is similar to that of other ABC proteins (7,23,24). Other members of the ABCA family (ABCA1, ABCA4, ABCA7, and ABCA12) are known to transport endogenous lipids across membranes. ABCA1 transports cholesterol and phospholipids, with growing evidence that its primary substrate is PC (25), ABCA4 transports PE (26), ABCA7 transports PC and SM but not cholesterol (27), and ABCA12 plays a role in lipid efflux (28). These studies suggest that the ABCA family of transporters has specificity for lipid substrates. The localization of the ABCA3 protein in the lamellar body surface, the known roles of other ABCA proteins, and the low proportion of PC in pulmonary surfactant from patients with *ABCA3* mutations supports the concept that ABCA3 is a PC transporter. This concept was recently corroborated in pulmonary epithelial cells transfected with *ABCA3* mutations (29). The preserved DSPC/PC ratio seen in these patients suggests that PC transport is mediated through interaction of ABCA3 with the polar choline moiety. However, it does not rule out an interaction with the acyl groups.

An alternative explanation for the decreased proportion of PC in the surfactant of these patients is that the ABCA3 protein functions to transport the minor surfactant lipids (PS, PE, and SM) out of the lamellar body. Loss of function mutations in *ABCA3* might lead to increased proportions of the minor surfactant lipids in the pulmonary surfactant of these patients. However, this alternative seems less likely given the known specificity of the other ABCA proteins. Furthermore, most ABC transporters are oriented such that flux occurs away from the cytoplasm, either into organelles or out of the cell, and not the reverse, as would be case if ABCA3 were transporting phospholipids out of the lamellar body (25–27).

None of the *ABCA3* mutations in these patients have been previously identified, and the variety in both nature and location of the mutations further indicates the extent of allelic heterogeneity in this disorder. The ABCA3 mutations identified in the present patients are unlikely to represent benign polymorphisms. All were in coding regions of the gene and mutations were identified on both alleles in all eight patients. Four of the mutations were frameshift mutations, four mutations resulted in in-frame deletions or insertions, one eliminated the initiator methionine, and the remaining missense variants have not been identified in controls without lung disease and occurred in regions of the gene that are relatively conserved across species (10). This indirect evidence along with the identified specific alterations in surfactant phospholipid composition suggests that these mutations caused surfactant dysfunction that would have otherwise been lethal without lung transplantation. For other ABC transporters, such as CFTR, different mutations are associated with variable phenotypes (30). We cannot exclude the possibility that different *ABCA3* mutations may lead to different functional abnormalities within the protein and thus different biochemical phenotypes (31). One patient (#7) in this study had normal surfactant composition and function, despite two mutations that had a high probability of significantly disrupting protein expression and/or function. However, this patient received exogenous surfactant administration shortly before lung transplantation, which may have altered the composition and function of the surfactant recovered in the BAL. Unfortunately, there was not enough BAL remaining from these patients to examine this possibility or to investigate the role ABCA3 may play in the transport of other lipids such as cholesterol.

The recognition of *ABCA3* mutations as a cause of severe respiratory disease will allow us to prospectively identify these patients and perform more focused analyses of surfactant metabolism, composition, and function. In summary, patients with *ABCA3* mutations develop severe respiratory distress after birth in association with altered surfactant function and composition. Clinical, pathologic, and biochemical findings support the concept that ABCA3 plays a central role in surfactant lipid transport into the lamellar bodies of type-II cells.

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