Alveolar lipoproteinosis in an acid sphingomyelinase-deficient mouse model of Niemann-Pick disease

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Ikegami, Machiko, Rajwinder Dhami, and Edward H. Schuchman. Alveolar lipoproteinosis in an acid sphingomyelinase-deficient mouse model of Niemann-Pick disease. Am J Physiol Lung Cell Mol Physiol 284: L518–L525, 2003.—Types A and B Niemann-Pick disease (NPD) are lipid storage disorders caused by the deficient activity of acid sphingomyelinase (ASM). In humans, NPD is associated with the dysfunction of numerous organs including the lung. Gene targeting of the ASM gene in transgenic mice produced an animal model with features typical of NPD, including pulmonary inflammation. To assess mechanisms by which ASM perturbed lung function, we studied lung morphology, surfactant content, and metabolism in ASM-deficient mice in vivo. Pulmonary inflammation, with increased cellular infiltrates and the accumulation of alveolar material, was associated with alterations in surfactant content. Saturated phosphatidylcholine (SatPC) content was increased twofold, and sphingomyelin content was increased 5.5-fold in lungs of the ASM knockout (ASMKO) mice. Additional sphingomyelin enhanced the sensitivity of surfactant inhibition by plasma proteins. Clearance of SatPC from the lungs of ASMKO mice was decreased. Catabolism of SatPC by alveolar macrophages from the ASMKO mouse was significantly decreased, likely accounting for decreased pulmonary SatPC in vivo. In summary, ASM is required for normal surfactant catabolism by alveolar macrophages in vivo. Alterations in surfactant composition, including increased sphingomyelin content, contributed to the abnormal surfactant function observed in the ASM-deficient mouse.

METHODS

Mice. The ASMKO mouse (C57BL/6) was developed (12), and a colony was established from heterozygous breeding pairs in the vivarium at Mount Sinai School of Medicine, according to protocols approved by the Institutional Animal Care and Use Committee. After genotypes were identified by PCR (12), ASMKO mice and their wild-type littermates were

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sent to Cincinnati Children’s Hospital Medical Center for study. All studies were performed simultaneously with 8- to 10-wk-old mice. The studies were carried according to protocols approved by the Institutional Animal Care and Use Committee of Cincinnati Children’s Hospital Medical Center.

 Lung morphology and type II cell counts. Lungs were inflated fixed at 25 cmH2O with 4% paraformaldehyde, and type II cells were visualized using paraffin sections (5 μm) immunostained with rabbit polyclonal pro-surfactant protein (SP)-B antisera as described previously (26). Cells on four consecutive 2 × 104-μm2 fields were determined for wild-type (n = 3) and ASMKO mice (n = 3). Lung paraffin sections were stained with hematoxylin and eosin to visualize lung morphology. The rabbit anti-SP-D antibody was generated against purified mouse SP-D, and immunohistochemistry was performed.

 Saturated phosphatidylcholine pool size, phospholipid composition, and protein. Mice were deeply anesthetized with intraperitoneal pentobarbital sodium, and the distal aorta was cut to exsanguinate. A 20-gauge blunt needle was tied into the trachea, the chest was opened, and 0.9% NaCl was the airway during the time until the lungs were fully expanded (~1 ml). The fluid was then withdrawn by syringes three times for each aliquot, and the saline BAL was repeated five times. The samples were pooled, and the volume was measured. The lavaged lung tissue was homogenized in 0.9% NaCl. Aliquots of BALF and the lung homogenates were extracted with chloroform-methanol (2:1), and saturated phosphatidylcholine (SatPC) was isolated with 0.04% oxida tion using carbon tetrachloride and column chromatography (20). Under the conditions of our assay, isolated SatPC was confirmed to contain only saturated fatty acids, predominantly palmitate, on gas chromatography of the oxidation product (1). SatPC was quantified by measuring phosphorus with the Bartlett assay (2). For phospholipid composition, chloroform-methanol extracts of alveolar lavages, large aggregate surfactant, and lung tissue were used for two-dimen sional thin-layer chromatography (18). The spots were visual ized with iodine vapor, scraped, and assayed for phosphorus content (18). To isolate large aggregate surfactant, we centrifuged BALF at 40,000 g over 0.8 M sucrose in 0.9% NaCl cushion for 15 min. The large aggregate surfactant then was collected from the interface, diluted with 0.9% NaCl, and centrifuged again at 40,000 g for 15 min. The pellet was suspended in 0.9% NaCl and used as large aggregate surfactant (15, 16) for surface activity and phospholipid composition analyses. Total protein was determined for each sample of BALF (19).

 Surfactant protein pool sizes. The surfactant proteins in the BALF were analyzed by Western blot (17). Samples containing 1.35 nmol of SatPC were used for analysis of SP-A and SP-D, and samples containing 2.7 nmol of SatPC were used for analyses of SP-C. Proteins were separated by SDS-PAGE in the presence of β-mercaptoethanol. For SP-B analyses, aliquots containing 0.27 nmol of SatPC were electrophoresed under nonreducing conditions. SP-A and SP-D were separated on 5–16% acrylamide gels with Tris-glycine buffer. SP-B and SP-C samples were separated on 10–20% acrylamide gels with Tricine buffer (NoveX, San Diego, CA). After electrophoresis, proteins were transferred to nitrocellulose paper (Schleicher & Schuell, Keene, NH) for SP-A and SP-D or to nylon membranes into the autoradiography cassettes (Bio-Rad, Hercules, CA) for SP-B and SP-C. Immunoblot analyses were carried out with the following dilutions of antisera: 1:25,000 for guinea pig anti-rat SP-A (kind gift from Jeffrey Whitsett, Cincinnati Children’s Hospital), 1:10,000 for rabbit anti-bovine SP-B (Chemicon, Temecula, CA), 1:25,000 for rabbit anti-recombinant human SP-C (kind gift from Byk Gulden, Konstanz, Germany), and 1:10,000 rabbit anti-rat SP-D (Chemicon). Appropriate peroxidase-conjugated secondary antibodies were used at 1:10,000 dilution. Immunoreactive bands were detected with enhanced chemiluminescence reagents (Amer sham, Chicago, IL). Protein bands were quantitated by densitometric analyses with Alpha Imager 2000 documentation and ImageQuant analysis software.

 Precursor incorporation into SatPC. ASMKO and wild-type mice were given intraperitoneal injections of 10 μl saline/g body wt containing 0.8 μCi [3H]palmitic acid (16). The palmitic acid was stabilized in solution with 5% human serum albumin. Groups of five or six mice were killed at 5 and 48 h after radiolabeled precursor injection. BALF was recovered from each animal, and lung tissue was homogenized in saline. SatPC was isolated from the BALF and lung homogenate as described in Saturated phosphatidylcholine pool size, phospholipid composition, and protein, and radioactivity was measured. The percent secretion of labeled SatPC was calculated for the 5-h groups as the percentage of radioactivity in alveolar SatPC relative to the total radioactivity in the BALF plus lung tissue.

 1,3-Phosphatidyl-N-methyl-choline 1,2-dipalmitoyl degradation by alveolar macrophage. BALF was performed with PBS containing 0.5 mM EDTA and centrifuged at 1,000 g for 5 min. Recovered cells were resuspended in DMEM containing 0.1% BSA and were cultured at a density of 1 × 105 cells/well in flat-bottom, 96-well tissue culture plates. Cells were allowed to adhere for 1 h at 37°C. Nonadherent cells were removed, and alveolar macrophages were washed three times with DMEM containing 0.1% BSA. With this method, >90% of cells recovered were macrophages (31). Natural surfactant isolated from normal mouse BALF was labeled by mixing with 1,3-phosphatidyl-N-methyl-[3H]choline,1,2-dipalmitoyl (14) (13, 31).

 1-[3H]DPPC degradation by ASM. We estimated the degradation of [3H]DPPC by alveolar macrophage. The percentage of radioactivity in alveolar SatPC relative to the total radioactivity in the BALF plus lung tissue.
Measurement of granulocyte-monocyte colony-stimulating factor. Granulocyte-monocyte colony-stimulating factor (GM-CSF) is one of the factors known to regulate macrophage proliferation. In the GM-CSF-deficient transgenic mouse lung, surfactant accumulates due to the marked decrease in surfactant catabolism by macrophages (16, 31). To access the possible involvement of GM-CSF in surfactant catabolism in the ASMKO mouse lung, we measured GM-CSF levels. Lungs (four mice/group) were homogenized in saline containing a Complete Mini EDTA-free protease inhibitor mixture tablet (Roche Molecular Biochemicals, Indianapolis, IN), and lysates were recovered after low-speed centrifugation. Samples were concentrated 10-fold by ultrafiltration (Centricon 10; Amicon, Bedford, MA). GM-CSF was quantified by mouse ELISA assay kit (Pierce Endogen, Rockford, IL).

Surface activity. Surface activity of large aggregate surfactant was measured with the captive bubble surfactometer (27). The concentration of each sample was adjusted to 3 mmol SatPC/µl. Three microliters of surfactant samples were applied to the air-water interface of the 25-µl bubble by microsyringe. Sensitivity to protein inhibition was measured in the presence of 2 or 10% (vol/vol) sheep plasma protein. Surface tension was measured every 10 s for 300 s, equilibrium surface tension was measured, then bubble pulsation was started. The minimum surface tension after 65% bubble volume reduction was measured at the fifth pulsation.

Statistics. Values are means ± SE. Two-group comparisons were carried out by unpaired two-tailed t-test. Significance was accepted at P < 0.05.

RESULTS

Lung morphology and type II cell count. To determine whether the absence of ASM caused abnormalities in lung structure or in type II cell numbers, we inflation-fixed lungs and assessed lung morphology by light microscopy. Hematoxylin- and eosin-stained sections from ASMKO mice showed no air space changes compared with wild-type mice (Fig. 1, A and B). Foamy enlarged alveolar macrophages were observed throughout the lungs and filling air spaces (Fig. 1, C and D). Increased intensity of SP-D staining was observed in alveolar space, consistent with finding of pulmonary alveolar proteinosis (Fig. 1, E and F). SP-D staining was detected in alveolar type II cells and in alveolar macrophages.

Type II cells were stained for pro-SP-B. Type II cell numbers were not significantly different in ASMKO control mice. The numbers of type II cells counted per field were 35.8 ± 1.7 for wild-type mice and 35.3 ± 1.9 for ASMKO mice.

Fig. 1. Hematoxylin and eosin (H&E) staining of lung sections from wild-type (A, C) (original magnification ×20) and acid sphingomyelinase knockout (ASMKO) mice (B, D) (original magnification ×40). Representative lung sections from wild-type and ASMKO mice are shown. Large-size macrophages are seen in sections from ASMKO mice, but there are no obvious air space changes in ASMKO mice compared with wild-type mice. Immunohistochemistry was performed with rabbit anti-mouse surfactant protein (SP)-D (E, F). Increased intensity of SP-D staining is seen in the ASMKO mice lung.
SatPC, phospholipid composition, and total protein. All studies of the two genotypes were performed simultaneously with 8- to 10-wk-old mice. Surfactant SatPC pool size (μmol/kg) was measured in BALF, lung tissue after lavage, and total lung (Fig. 2). SatPC was twofold higher in BALF, tissue, and total lung of ASMKO mice than in wild-type mice (P < 0.01). A striking difference in phospholipid composition was observed in BALF. Sphingomyelin was increased 5.5-fold in ASMKO mice (P < 0.01) (Fig. 3A). Phospholipid compositions were determined in isolated large aggregate surfactant (Fig. 3B) and homogenized lung tissues (Fig. 3C). The percentage of sphingomyelin was increased fivefold in large aggregate surfactant (P < 0.01) and threefold in lung homogenates (P < 0.01) in ASMKO mice. The percent increase of sphingomyelin in lung homogenates from ASMKO mice was associated with a significantly lower percentage of phosphatidylcholine than in wild-type mice. Total protein in BALF was relatively low in both groups of animals but was significantly higher in ASMKO (22 ± 1 mg/kg) than in wild-type mice (18 ± 1 mg/kg) (P < 0.01).

Surfactant protein pool size. Surfactant proteins in BALF relative to SatPC are shown in Fig. 4A. Alveolar SatPC increased twofold in ASMKO mice, and the net amount of surfactant proteins relative to wild-type mice was calculated (Fig. 4B). The SP-A pool size was not different in ASMKO mice and wild-type mice. There are large variations in SP-B levels between the animals, and SP-B in ASMKO mice was not statistically different (P = 0.07) from wild-type mice. In ASMKO mice, SP-C was threefold higher, and SP-D was 4.5-fold higher relative to SatPC (Fig. 4A). Total SP-C content was sixfold higher, and SP-D was eightfold higher in ASMKO mice compared with wild-type mice (Fig. 4B).

Precursor incorporation. Mice were given body weight-adjusted doses of [3H]palmitic acid, and the amount of labeled SatPC was measured at 5 h. This time point best indicates net incorporation of the precursor into SatPC. Secretion of labeled SatPC into the alveoli was assessed after 5 h. The catabolism of labeled SatPC synthesized by the lung could be evaluated 48 h after precursor injection (Fig. 5). Five hours after precursor injection, no significant differences in incorporation of precursor into SatPC were observed between the two groups. Likewise, percent secretion in alveolar lavage fluid, calculated at 5 h as the percentage of radioactivity in alveolar lavage SatPC relative to the radioactivity in the total lung, was similar in both groups of mice: 9.1 ± 0.7% secretion in wild-type and 7.2 ± 0.8% in ASMKO mice. In contrast, 48 h after precursor injection, the amount of labeled SatPC in total lung and alveolar was 2.4-fold higher in ASMKO mice than in the wild-type mice, consistent with increased SatPC pool sizes and a decreased clearance of the surfactant lipid from the lung of ASMKO mice.

Decreased DPPC degradation by alveolar macrophages. Surfactant lipids are catabolized by both alveolar macrophage and type II cells in approximately equal proportions (10). Because loss of endogenously labeled surfactant evaluated 48 h after radiolabeled palmitic acid injection was significantly slower in ASMKO mice, catabolism of DPPC by isolated alveolar macrophages was studied in vitro. There was a marked decrease in [3H]DPPC catabolism by alveolar macro-
phages from ASMKO mice in vitro (Fig. 6). The reduction of DPPC degradation by alveolar macrophages seen in ASMKO mice was similar to that previously shown in GM-CSF-deficient mice (31).

**Pulmonary GM-CSF concentrations.** To assess whether the defect in DPPC degradation by macrophages was associated with low or deficient GM-CSF, we measured GM-CSF in lung homogenates from ASMKO mice. Lung GM-CSF levels were 10.4 ± 2.4 pg/g body wt for ASMKO mice and 6.8 ± 1.4 pg/g body wt for wild-type mice. There were no significant differences in lung GM-CSF concentrations between the two groups. These results suggest that GM-CSF deficiency does not mediate decreased surfactant catabolism by alveolar macrophages in the ASMKO mice.

**ASM does not degrade DPPC.** To assess whether the ASM was directly involved in the degradation of SatPC, we incubated [3H]DPPC with three different concentrations of recombinant ASM. After the incubation, 96% of radioactivity was recovered in the chloroform phase for all the ASM concentrations studied (three experiments for each concentration). DPPC degradation by ASM was not detected. Thus altered catabolism of surfactant SatPC by macrophage in ASMKO mice does not directly contribute to surfactant abnormalities in the ASMKO mice.

**Altered surface activity caused by increased sphingomyelin.** To evaluate the function of surfactant isolated from the ASMKO mice, we measured equilibrium surface tensions and minimum surface tensions using large aggregate surfactant in the presence and absence of plasma as an inhibitor of surfactant function. Equilibrium surface tensions and minimum surface tensions for surfactant from ASMKO mice were similar to surfactant from wild-type mice (Fig. 7). Surfactant from both wild-type mice and ASMKO mice was inhibited by 10% plasma protein as indicated by the increased equilibrium surface tensions and increased minimum surface tensions. The ASMKO surfactant was significantly more sensitive to plasma inhibition compared with surfactant from wild-type mice.

To test whether increased sphingomyelin levels inhibited surface activity, we added sphingomyelin (Sigma, St. Louis, MO) to wild-type large aggregate surfactant from normal mice and measured the minimum surface tension.
Lipid-in surfactant function likely contribute to pulmonary metabolism by alveolar macrophages. Alterations in surfactant lipid and proteins, and abnormal surface activity in surfactant from ASMKO mice. Surfactant catabolism was decreased, mediated at least in part by decreased surfactant phospholipid catabolism by the alveolar macrophages. Sphingomyelin content was increased in large aggregate surfactant and lung tissue homogenates from ASMKO mice, which accounts for the decreased surfactant surface activity in the presence of plasma.

Respiratory infections and progressive pulmonary dysfunction are a common cause of morbidity for type B NPD patients (28). The presence of foamy cells in the BALF (30), chest radiological evidence of reticuloendothelial system and neurons in this mouse model of NPD.

DISCUSSION

Deficiency of ASMKO causes a pulmonary alveolar proteinosis-like syndrome caused by defects in surfactant metabolism by alveolar macrophages. Alterations in surfactant function likely contribute to pulmonary abnormalities associated with NPD. Lipid-filled alveolar macrophages accumulated in lungs of ASMKO mice and NPD patients. The present studies demonstrate decreased surfactant catabolism, alterations in surfactant lipid and proteins, and abnormal surface activity in surfactant from ASMKO mice. Surfactant catabolism was decreased, mediated at least in part by decreased surfactant phospholipid catabolism by the alveolar macrophages. Sphingomyelin content was increased in large aggregate surfactant and lung tissue homogenates from ASMKO mice, which accounts for the decreased surfactant surface activity in the presence of plasma.

Respiratory infections and progressive pulmonary dysfunction are a common cause of morbidity for type B NPD patients (28). The presence of foamy cells in the BALF (30), chest radiological evidence of reticuloendothelial system and neurons in this mouse model of NPD.
The frequency and severity of pulmonary disease in NPD may be influenced by altered host defense due to a combination of altered macrophage function (4) and abnormal surfactant function. As a result of altered host defense in the lung, type B NPD patients have frequent respiratory infections (21). In infected and/or injured lungs, protein leak occurs from the vascular space to the alveolus. Because the function of surfactant with increased sphingomyelin content is easily inhibited, these abnormalities may enhance susceptibility of NPD patients to respiratory abnormalities following lung infection. The present study showed altered surfactant function by addition of sphingomyelin in surfactants both with and without SP-A in vitro.

Although surfactant protein levels in NPD surfactant were similar or higher than wild-type surfactant, resistance of NPD surfactant against protein inhibition was weak and related to enhanced sphingomyelin content. Susceptibility to infection may be enhanced by the abnormal surfactant composition in NPD patients and ASMKO mice. In addition, surfactant pool size is higher than in normal individuals, and pulmonary alveolar lipoidosis and proteinosis occur in NPD patients and ASMKO mice. Chest X-rays with diffuse alveolar lipidosis and proteinosis occur in NPD patients and ASMKO mice. In addition, surfactant pool size is higher than in normal individuals, and pulmonary alveolar lipoidosis and proteinosis occur in NPD patients and ASMKO mice. Chest X-rays with diffuse alveolar lipidosis and proteinosis occur in NPD patients and ASMKO mice.

The alveolar macrophages in ASMKO mice were impaired, these abnormalities may enhance susceptibility of NPD patients to respiratory abnormalities following lung infection. The present study showed altered surfactant function by addition of sphingomyelin in surfactants both with and without SP-A in vitro.

In summary, ASM deficiency caused a pulmonary alveolar proteinosis-like syndrome. Surfactant catabolism by alveolar macrophage was decreased, and surfactant function was diminished. Surfactant from ASM mice contained excessive concentrations of sphingomyelin and exhibited decreased resistance against inhibition of surface tension-lowering properties by plasma protein. Because pulmonary complications are a common clinical problem in NPD patients, it is likely that these results explain, at least in part, the mechanism(s) underlying lung disease in individuals with NPD.

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REFERENCES