Reversibility of lung inflammation caused by SP-B deficiency

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PULMONARY SURFACANT PROTEIN B (SP-B) is 79-amino acid polypeptide synthesized, processed, and secreted from alveolar type II cells. SP-B interacts with surfactant phospholipids, enhancing spreading and stability of surfactant films formed at the air-liquid interface and reducing surface tension to near 0 mN/m as alveolar surface area decreases during respiration (32, 43, 44). The importance of SP-B in lung physiology was previously shown that signal transducer and activator of transcription (STAT)-3 expression in respiratory epithelial cells plays a critical role in maintenance of SP-B homeostasis during lung injury and recovery (17). Acute respiratory distress syndrome, a frequent life-threatening pulmonary disease, is associated with decreased content of SP-B in bronchoalveolar lavage (BAL) fluid (BALF) (13, 14, 35).

The nature of the interrelationships between SP-B deficiency and lung inflammation is not well understood. We have used a transgenic mouse model in which the human SP-B cDNA was expressed in the alveolar epithelium under conditional control of a doxycycline-inducible transgene (28) to determine whether SP-B deficiency causes lung inflammation in the absence of injury or infection.

METHODS

Transgenic mice. The Animal Care and Use Committee at the Cincinnati Children’s Hospital Research Foundation, Cincinnati, OH, approved all animal procedures and protocols. A conditional doxycycline regulatable transgenic system was used to induce SP-B expression in the lung (26, 28). The conditional transgenic mice [CCSP-rTα, (tetO)7 SFTPBSftpb−/−] were generated from two transgenic mouse lines (31, 37): 1) “activator” mice (CCSP-rTα expressing reverse tetracycline transactivator transcription factor (rTα) under the control of a 2.3-kb element from the rat Clara cell secretory protein (CCSP) gene promoter that confers expression in both conducting and alveolar respiratory epithelial cells, and 2) “responder” transgenic mice [(tetO)7 SFTPB], in which the human SP-B cDNA was expressed under the control of the tetracycline response elements. CCSP-rTα/(tetO7) SFTPBSftpb−/− mice were bred with Sftpb−/− mice to generate CCSP-rTα, (tetO7) SFTPBSftpb−/− mice (28), herein termed “conditional transgenic mice.” Because Sftpb−/− mice die perinatally unless the SP-B transgene is expressed, conditional transgenic mice were maintained on doxycycline-containing food from embryonic day (E)1 until 7 wk of age. Doxycycline was removed (Off Dox) for 4 days (or 7 days for mortality studies) and doxycycline resumed (On Dox). Lung mechanics, surfactant function, surfactant composition, morphology, and inflammation were studied during lung

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injury (Off Dox) and during lung recovery (On Dox). Study groups included conditional transgenic mice that were 1) continuously treated with doxycycline (control); 2) removed from doxycycline for 1, 2, 3, and 4 days; and 2) those removed from doxycycline for 4 days then retreated with doxycycline for 1, 4, 7, 13, 20, 32, and 41 days. Male and female mice were equally distributed to each group.

**BAL and lung homogenate.** Mice were deeply anesthetized with intraperitoneal pentobarbital sodium, and the distal aorta was cut. A 20-gauge blunt needle was tied into the trachea, the chest was intraperitoneal pentobarbital sodium, and the distal aorta was cut. BALF was centrifuged at 284 g for 10 min to separate cells from supernatant. In the cell pellet, saturated phosphatidylcholine (Sat PC) was not detectable, and the number of cells was the same as that recovered by high-speed centrifugation. The supernatant was used to quantitate surfactant lipids, surfactant proteins, cytokines, and total protein in BALF. Lung was homogenized in 0.9 M NaCl, and total volume was adjusted to 2 ml. IL-6, IL-1β, TNF-α, and macrophage inflammatory protein-2 (MIP-2) were quantitated in BALF and in supernatants prepared from lung homogenates after centrifugation at 1,000 g for 10 min with murine sandwich ELISA kits (R&D Systems, Minneapolis, MN). Total protein in aliquots of BALF from each mouse was measured by the method of Lowry et al. (24) and reported as milligram per kilogram body weight. Cells isolated from BALF were stained with trypan blue and counted on a hemocytometer. Differential cell counts were performed on cytospin preparations after staining with Diff-Quik (Scientific Products, McGaw Park, IL).

**SP-B, SP-C, surfactant phospholipid, and lung histology.** The content of SP-B and SP-C in the same volume of BALF was analyzed in four mice from each group by Western blot analysis. Total BALF volume was similar for all the groups. Separation of surfactant proteins was carried out under nonreducing electrophoretic conditions followed by Western blotting with 1:15,000 dilution rabbit anti-bovine SP-B (Chemicon International, Temecula, CA) or under reducing electrophoretic conditions followed by Western blotting with 1:10,000 dilution of rabbit anti-recombinant human SP-C (19, 26). Appropriate peroxidase-conjugated secondary antibodies were used at 1:10,000 dilution. Immunoreactive bands were detected with ECL reagents (Amersham Health, Chicago, IL). Protein bands were quantitated by densitometric analyses with Alpha Imager 2000 documentation and ImageQuant analysis software.

Aliquots of BALF were extracted with chloroform-methanol (2:1), and Sat PC was isolated with osmium tetroxide (25) followed by phosphorus measurement (2). For phospholipid composition, chloroform-methanol extracts of BALF from two mice were pooled and used for two-dimensional thin-layer chromatography. The spots were visualized with iodine vapor, scraped, and assayed for phosphorus content (19).

For histology, mice were killed by an injection of pentobarbital. Lungs were inflation fixed with 4% paraformaldehyde in PBS at 25 cmH$_2$O and immersed in the same fixative. Tissue was fixed overnight, washed with PBS, dehydrated in the series of alcohols, and embedded in paraffin. Tissue sections were stained with hematoxylin-eosin and trichrome and Gomori’s iron stain (23).

**Soluble L-selectin, CD-11b, and CD-18.** Soluble L-selectin (sL-selectin), CD-11b, and CD-18 were assessed before and after withdrawal of doxycycline for 1–4 days. sL-selectin was quantitated in BALF and in supernatants from lung homogenates after centrifugation with murine sandwich ELISA kits (R&D Systems, Minneapolis, MN). Aliquots of alveolar cells and blood after erythrocyte lysis were incubated on ice with monoclonal antibodies conjugated to fluorescein isothiocyanate isomer 1 against mouse CD-11b (murine CR3) or mouse CD-18 (cell surface glycoprotein and selectin), CD-11b, and CD-18 were assessed before and after with-
Surfactant function. Large-aggregate surfactant was isolated from BALF from transgenic mice by centrifugation at 40,000 g for 15 min over a 0.8 M sucrose in 0.9% NaCl cushion. Surface activity was measured on the isolated large-aggregate surfactant with a captive bubble surfactometer (33). The concentration of each sample was adjusted to 7 nmol phospholipid/μl, and 3 μl of the sample was applied to the air-water interface of a 25-μl volume bubble by microsyringe (n = 3). Surface tension was measured every 10 s for 300 s to establish equilibrium surface tension, and then bubble pulsation was started. The minimum surface tension after 80% bubble volume reduction at rate of 12 cycle/min was measured, which did not change significantly after the third pulsation. The minimum surface tension at the fifth pulsation was reported.

Lung mechanics. Lung mechanics were studied in tracheostomized mice under anesthesia by intraperitoneal injection of ketamine and xylazine. Mice were ventilated with a tidal volume of 8 ml/kg at a rate of 450 breaths/min and a positive end-expiratory pressure of 2 cmH2O by a computerized Flexivent System (SCIREQ Scientific Respiratory Equipment, Montreal, Quebec, Canada) (23, 34). This machine allows accurate measurement of volume by using the position of ventilator piston and pressure in the cylinder. After mechanical ventilation for 2 min, two measurement maneuvers were performed. A sinusoidal 1-Hz oscillation was applied to the tracheal tube. The single compartment model was fit to these data by multiple linear regression to calculate dynamic resistance, elastance, and compliance of the airway. For the second maneuver, an 8-s forced oscillatory signal containing frequencies between 0.5 and 19.6 Hz was applied to the tracheal tube. Mechanical input impedance was calculated, and a model containing a constant-phase tissue compartment was fit to input impedance to evaluate tissue damping, tissue elastance, and tissue hysteresivity (16).

STAT-3. STAT-3 concentration in lung tissue was assessed by Western blot. Homogenized lung tissue was sonicated in chelation buffer [5% 1 M Tris·HCl, pH 7.5; 1% 100 mM EGTA; 0.2% 500 mM EDTA; 1 complete mini-protease inhibitor cocktail tablet (Roche Diagnostics, Mannheim, Germany); and 1% PMSF] and centrifuged at 20,000 g for 15 min. Supernatant containing 50 μg of protein was electrophoresed followed by Western blotting using primary antibody (1:1,000) rabbit polyclonal against STAT-3 (Santa Cruz Biotechnology, Santa Cruz, CA) and secondary antibody (1:5,000) goat anti-rabbit (Calbiochem, La Jolla, CA). Immunohistochemistry for STAT-3 and phosphorylated (p) STAT-3 was performed as described previously (17) except that the antibody was 1:400 rabbit polyclonal against STAT-3 (Cell Signaling, Beverly, MA) and 1:50 rabbit polyclonal against pSTAT-3 (Cell Signaling).

Statistical analysis. Each group was compared with control conditional transgenic mice (continuously treated with doxycycline) by unpaired Student’s t-tests. Results were expressed as means ± SE. Significance was accepted at the 5% level.

RESULTS

Twelve groups (n = 6 mice per group) were studied. Body weight (25.4 ± 0.5 g, n = 72) and recovered BALF volume (4.80 ± 0.03 ml, n = 72) were similar for all the groups. The control group comprised conditional transgenic mice that were continuously treated with doxycycline from E1.

Reversibility of respiratory distress caused by SP-B deficiency. Conditional transgenic mice maintained on doxycycline survived normally following birth. Respiratory distress, evidenced by cyanosis and abnormal breathing, was observed on day 4 after removal from doxycycline. Mortality was 40% on day 4 and 70% on day 6 (n = 24). To assess reversibility of pulmonary abnormalities, we performed further studies on mice in which doxycycline was removed for 4 days before replacement of doxycycline. Withdrawal of doxycycline resulted in a dramatic decrease in SP-B content in BALF from day 1 (Fig. 1, A and B). Twenty-four hours following readministration of doxycycline, SP-B content in BALF was increased to levels similar to that in mice without doxycycline for 24 h (P = 0.08). Four to forty-one days after doxycycline was resumed, SP-B levels were similar to those of control mice. SP-C content did not change when mice were removed from doxycycline for 4 days (Fig. 1, A and C). Furthermore, Sat PC pool size (8.11 ± 0.21 μmol/kg body wt, n = 72, P = 0.8) and phospholipid composition were also similar in all groups studied (n = 3 pool samples for each group, 2 mice/pool, P = 0.7): phos-
Phosphatidylcholine was 76.5 ± 0.8%, phosphatidylglycerol was 8.2 ± 0.5%, and phosphatidylinositol was 2.5 ± 0.1%. Thus acute SP-B deficiency did not influence surfactant lipid or SP-C content.

Removal of the adult mice from doxycycline resulted in altered surfactant function within 24 h. Equilibrium surface tension (Fig. 2A) was not altered on day 1 but was significantly increased 2 and 4 days after removal of doxycycline. Minimum surface tension (Fig. 2B) of isolated surfactant (adjusted to the same concentration of phospholipid) was higher on the first day after removal of doxycycline. Equilibrium and minimum surface tension were completely corrected after replacement of doxycycline for 7 days (26).

SP-B reversed abnormalities in lung mechanics and morphology. Four days after removal of doxycycline, lung compliance was significantly decreased (Fig. 3E). Resumption of doxycycline corrected lung compliance within 24 h. Lung hysteresivity, calculated from tissue damping divided by tissue elastance, decreased rapidly following doxycycline removal and was gradually restored when doxycycline was resumed (Fig. 4). Airway elastance, airway resistance, tissue elastance, and tissue damping were not significantly altered by SP-B deficiency (Fig. 3, A–D). Cellular infiltration and interstitial edema were observed 4 days after removal from doxycycline (Fig. 5B). Lung hemorrhage and erythrocyte uptake by alveolar macrophages were detected (Fig. 5B, inset). Lung architecture was normalized after doxycycline replacement (4–41 days) (Fig. 5D). There was no evidence of pulmonary fibrosis as
assessed by trichrome staining 41 days after replacement of doxycycline (data not shown).

Reversibility of lung inflammation. Total protein in BALF was increased 3 days after removal from doxycycline (Fig. 6A), suggesting increased protein permeability. BALF protein remained increased 1 day after replacement of doxycycline but was restored to normal levels at 4 days. Three days after removal from doxycycline, total cell numbers in BALF were increased sixfold and gradually declined after treatment was resumed (Fig. 6B). The predominant cells in BALF were macrophages (Fig. 6C) and neutrophils (Fig. 6D). Neutrophils were still detected in BALF 7 days after administration of doxycycline but were not detected on day 13. Concentrations of IL-6, IL-1β, and MIP-2 in lung homogenates and BALF were increased 2–3 days after removal of doxycycline (Fig. 7). TNF-α levels were low or undetectable in all samples (data not shown). Concentrations of IL-6, IL-1β, and MIP-2 were rapidly restored 4 days after retreatment with doxycycline.

Increased sL-selectin but unchanged CD-11b and CD-18 in the acute phase of lung inflammation. Migration of leukocytes into the sites of inflammation in the lung is known to be influenced by L-selectin and/or CD-11b/CD-18 (7). sL-selectin was measured in plasma, BALF, and lung homogenates 0–4 days after removal from doxycycline (Fig. 8, A and B). sL-selectin levels were significantly increased 3–4 days after removal of doxycycline consistent with the observation that neutrophils were increased in BALF at this time. sL-selectin levels in plasma were similar in all groups of mice studied (not shown). FACS analysis was used to quantitate the percentages of CD-11b- or CD-18-positive cells in BALF and blood. Four days of withdrawal of doxycycline did not influence CD-11b
and CD-18 (data not shown). Together, these results indicate that decreased SP-B induced sL-selectin that may contribute to increased inflammation in the lung.

**Increased STAT-3 and phosphorylated STAT-3 during lung injury.** STAT-3 and pSTAT-3 were increased in the lung tissue during lung injury induced by SP-B deficiency as assessed by Western blot (Fig. 8C) and by immunohistochemistry (Fig. 9). Increased STAT-3 and pSTAT-3 were detected in both alveolar macrophages and alveolar epithelial cells. STAT-3 activates Sftpb expression in vivo and in vitro (45); however, in mice used in this study, SP-B was decreased when doxycycline was withdrawn. The findings that STAT-3 is induced during SP-B deficiency suggest a compensatory mechanism to maintain SP-B expression. Despite increased STAT-3 and pSTAT-3 in alveolar epithelial cells, SP-B deficiency induced pulmonary failure, demonstrating the primary role of SP-B in the maintenance of lung function.

**DISCUSSION**

Selective reduction of SP-B in the adult mouse lung caused reversible pulmonary inflammation, surfactant dysfunction, and disrupted pulmonary mechanics. Pulmonary inflammation and infection are known to inhibit SP-B production and function. Although it is generally assumed that epithelial cell injury disrupts surfactant function, at least in part by altering SP-B content or activity, the present study demonstrates that the loss of SP-B, per se, is sufficient to perturb surfactant function and initiates both cytokine expression and inflammation in the absence of infection or toxic stimuli in the adult lung.

Sftpbl−/− mice and human infants die of acute respiratory failure in the neonatal period (6, 29). Physiological and biochemical findings in mice and infants are consistent with surfactant deficiency. Decreased surfactant activity, lack of lamellar bodies and tubular myelin, abnormalities in surfactant composition, misprocessing of pro-SP-C, and lack of SP-C were observed in the lungs of Sftpbl−/− mice (40) and infants bearing mutations in the Sftpbl gene (3, 9). In the present study, neither SP-C nor phospholipid content and composition were perturbed by the loss of SP-B in the adult mice. The period of SP-B deficiency may not have been sufficient to perturb SP-C processing or phospholipid content. Residual pro-SP-B may maintain SP-C processing. Alternatively, compensatory pathways not active in the neonate may serve to maintain SP-C levels and phospholipid composition in adult mice following loss of SP-B. The loss of SP-B in the present conditional model results in surfactant dysfunction and respiratory failure despite maintenance of SP-C and surfactant lipid composition, demonstrating the critical role of SP-B in lung function in the adult. Normal levels of SP-C did not protect the lung from injury in this model.

SP-B deficiency causes pulmonary inflammation, supporting a model in which disruption of surface forces can induce the expression of proinflammatory signals. In the present study, L-selectin was rapidly induced following loss of SP-B. L-selectin is known to mediate pulmonary inflammation by enhancing the migration of leukocytes into the lung (5, 8). The increase in sL-selectin, but not CD-11b/CD-18, accompanied respiratory dysfunction associated with loss of SP-B. Ventilator- or stretch-induced lung injury causes enhancement of L-selectin level but did not perturb CD-18 (5). In the present study, CD-11b and CD-18 were unaltered after removal of the mice from doxycycline treatment. L-selectin is a member of the adhesion molecule family, expressed on the surface of most leukocytes. L-selectin receptors are cleaved to soluble forms (sL-selectin) in the lung following inflammation (8). The present study supports a role in which L-selectin may initiate neutrophil immigration during SP-B deficiency.
Increased minimum surface tension of isolated surfactant and decreased lung hysteresivity were demonstrated within 24 h after removal from doxycycline, demonstrating the critical role of SP-B in surfactant function. Lung hysteresivity is an indicator of the viscoelastic properties of the pulmonary parenchyma and is determined by both tissue and surfactant (11, 21). In conditional transgenic mice, morphological changes in the lung were first observed 3 days after doxycycline removal. Thus decreased hysteresivity likely reflected changes in surfactant function caused by decreased SP-B, rather than changes in lung structure. In addition to the lack of SP-B, leakage of proteins into the alveoli may have contributed to surfactant dysfunction during SP-B deficiency. Mechanisms by which the lack of SP-B causes alveolar protein accumulation, whether from cellular sources or from alveolar-vascular permeability, are unclear in the present study. The increase in nonsurfactant proteins in the alveolus may provide inhibitory proteins that can inactivate surfactant (4, 18). In vivo, lung injury seen in the SP-B deficiency is likely related to both surfactant dysfunction associated with decreased SP-B concentration and the presence of inhibitory proteins related to changes in alveolar capillary permeability or cell injury. The effects of SP-B deficiency on both surfactant function and lung histology were readily restored by resumption of doxycycline and were accompanied by inhibition of production of proinflammatory cytokines.

Acute respiratory distress syndrome is a frequent life-threatening disease that is associated with loss of SP-B (14, 35). In the normal lung, SP-B mRNA and SP-B protein are maintained following lung injury and recovery (30) through activated STAT-3 signaling pathways (1, 36). We recently showed that selective deletion of STAT-3 in respiratory epithelial cells of transgenic mice resulted in loss of SP-B in the lung and accelerated death following exposure to hyperoxia (17). STAT-3 activates multiple genes related to lung protection. STAT-3 was phosphorylated and increased in respiratory epithelial cells after doxycycline withdrawal, likely indicating a compensatory response to the lack of SP-B. Because SftpB is deleted in this model, STAT-3 is unable to enhance SP-B expression. The present study demonstrated the critical importance of SP-B homeostasis during lung injury and recovery from injury.

The transgenic mice used in this study were generated from mice expressing rtTA under control of the CCSP promoter that drives expression in alveolar and bronchiolar cells in vivo (28). In previous work from this laboratory, compound transgenic mice were generated in which SP-B expression was regulated by rtTA under control of the 3.7-kDa human SP-C promoter (26). In both cases, doxycycline-regulated expression of the human SP-B transgene rescued the animals from lethal effects of SP-B gene targeting. In the present study, we observed modest changes in air space structure that we did not see with the SP-C promoter. Whereas lung compliance of normal, nontransgenic adult mice is generally 1.2–1.5 ml/cmH2O/kg (10, 12), in the present study, increased compliance (2.1 ± 0.2 ml/cmH2O/kg) was observed in control mice maintained on doxycycline. Mild peripheral air space enlargement was observed in this mouse line that may be related to the presence of multiple transgenes or to heterogeneity in the sites and levels of expression of SP-B using the CCSP-rtTA system. The lack of nondoxycycline-dependent SP-B expression, however, makes this line very useful for the precise regulation of SP-B expression in vivo.

SP-B homeostasis is required for lung function, for host defense, and for recovery following lung injury. In the present study, the loss of SP-B was sufficient to induce pulmonary inflammation and lung dysfunction. Activation of pathways that maintain or increase SP-B expression in respiratory epithelial cells or maintain alveolar SP-B content and function may provide protection of the lung during lung injury.

**GRANTS**

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REFERENCES


Fig. 9. Immunohistochemistry for STAT-3 (A–C) and phosphorylated (p) STAT-3 (D–F). STAT-3 was increased after removal from Dox. As shown for B, C, E and F, STAT-3 and pSTAT-3 were increased in both alveolar macrophages and alveolar epithelial cells. Scale bar = 50 μm.


