Targeted disruption of the surfactant protein B gene disrupts surfactant homeostasis, causing respiratory failure in newborn mice

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Communicated by John A. Clements, University of California, San Francisco, CA, May 8, 1995 (received for review April 3, 1995)

ABSTRACT Surfactant protein B (SP-B) is an 8.7-kDa, hydrophobic protein that enhances the spreading and stability of surfactant phospholipids in the alveoli. To further assess the role of SP-B in lung function, the SP-B gene was disrupted by homologous recombination in murine mouse embryonic stem cells. Mice with a single mutated SP-B allele (+/-) were unaffected, whereas homozygous SP-B -/- offspring died of respiratory failure immediately after birth. Lungs of SP-B -/- mice developed normally but remained atelectatic in spite of postnatal respiratory efforts. SP-B protein and mRNA were undetectable and tubular myelin figures were lacking in SP-B -/- mice. Type II cells of SP-B -/- mice contained no fully formed lamellar bodies. While the abundance of SP-A and SP-C mRNAs was not altered, an aberrant form of pro-SP-C, 8.5 kDa, was detected, and fully processed SP-C peptide was markedly decreased in lung homogenates of SP-B -/- mice. Ablation of the SP-B gene disrupts the routing, storage, and function of surfactant phospholipids and proteins, causing respiratory failure at birth.

Surfactant protein B (SP-B) is a 79-aa amphipathic peptide closely associated with surfactant phospholipids in the alveoli of the lung (1, 2). SP-B enhances the rate of spreading and increases the stability of monolayers formed from surfactant phospholipid mixtures in vitro, improves respiratory function in surfactant-deficient lungs in vivo, and contributes to tubular myelin formation in vitro (3–6). Deficiency of SP-B and other surfactant components, including surfactant phospholipids and SP-A, is associated with respiratory distress syndrome in premature infants and adults with respiratory distress syndrome (ARDS) (7, 8). The potential role of SP-B in lung function was recognized in an infant who died from respiratory failure in the postnatal period in association with a lack of SP-B protein or SP-B mRNA in airway secretions or lung tissue (9). This infant was homozygous for a mutation in the fourth exon of the human SP-B gene (10). Human SP-B mRNA is produced from a single gene located on chromosome 2 at a site syntenic with the murine SP-B gene located on chromosome 6 (11, 12). To further clarify the role of SP-B in lung function, we produced an animal model of hereditary SP-B deficiency by creating a mutation of the murine SP-B gene in embryonic stem (ES) cells.

MATERIALS AND METHODS

Identification of Genomic Clones. A 129J mouse genomic library made in λ DASH II (Promega) was kindly provided by M. Shull (University of Cincinnati) and was screened for SP-B by using ~300 bp of the 5’ end of the mouse SP-B cDNA as probe (13). Seven genomic equivalents were screened in duplicate, and eight positive clones were identified.

Targeting Construct. A 4-κb Xba I fragment containing exons 1–6 of the mouse SP-B gene was subcloned into pGEM-7Z (Fig. 14). The Neo' gene expressed under direction of the constitutive eukaryotic phosphoglycerate kinase gene promoter (14) was inserted into a unique EcoRV site in exon 4. This insertion was designed to disrupt the SP-B gene and provided a positive selection for targeted cells when grown in the presence of the neomycin analogue G418. The HSV tk gene was inserted into the Cla I site of the targeting vector for selection against randomly integrated DNA in the ES cell clones by culture with ganciclovir. The targeting DNA was linearized at a unique Nor I site prior to electroporation into ES cells.

ES Cell Clones. ES cells (D3) from 129J mice were provided by Thomas Doetschman (University of Cincinnati) and were grown on neomycin-resistant mouse embryonic fibroblasts and electroporated with the SP-B targeting construct (20 μg of DNA for 8 × 10⁷ cells). Cells were cultured with G418 (150 μg/ml) and ganciclovir (2 μM), and surviving colonies subjected to Southern blot analysis after BanHI digestion of cell DNA. Southern analysis was performed with a SP-B gene fragment spanning exon 7, intron 7, and exon 8 as probe. Cells from two clones were microinjected into C57BL host blastulae, and chimeric males were bred to Swiss Black females. Resulting agouti offspring were screened for germline transmission of the targeted mutation by Southern blot analysis of tail DNA. These mice were crossed with Swiss Black mice to establish a colony of heterozygous SP-B +/- mice before breeding to homozygosity.

Immunohistochemistry and Western Blot. Lungs from newborn or fetal day 17–18 mice (delivered by hysterotomy) were immunostained for SP-B and SP-C with rabbit polyclonal antibodies generated against recombinant human pro-SP-B (15, 16), pro-SP-C (17), and the active airway SP-B peptide (16). Western blot analysis of lung homogenates used rabbit polyclonal antibodies generated against SP-A, SP-B peptide, SP-B-C, and pro-SP-C (15–18).

RNA Analysis. RNA was extracted from the lungs and liver of newborn mice. Mouse SP-A, SP-B, and SP-C cDNAs, Neo' cDNA, and an oligonucleotide complementary to 18S rRNA were used to probe Northern blots. SI nuclease assays were performed with probes for SP-A, -B, and -C mRNA as described (19).

RESULTS

Southern blot and restriction analysis of the locus demonstrated that SP-B was encoded by a single gene that shared

Abbreviations: SP, surfactant protein; ES cell, embryonic stem cell; Neo', neomycin resistance; HSV, herpes simplex virus; tk, thymidine kinase.
close identity with the mouse SP-B gene isolated from C57/BL6 genomic DNA (20) (Fig. 1A). The mouse SP-B gene consisted of 11 exons and 10 introns, spanning ~9.5 kb. A DNA fragment flanking exon 4 was produced with appropriate restriction enzymes to generate a targeting construct consisting of the 5' region of the SP-B gene interrupted by the Neo' gene and flanked by the HSV tk gene (Fig. 1A). Appropriately targeted ES cells represented 6 of 280 colonies screened. Two targeted clonal ES cells were chosen for microinjection into mouse blastocysts and were transferred to surrogate dams to produce chimeric animals that were subsequently bred to produce heterozygous offspring bearing the SP-B gene mutation. Germline-heterozygous animals were crossed to produce homozygous SP-B gene-targeted mice that were identified by restriction mapping and Southern blot analysis with both SP-B and Neo' cDNA probes (Fig. 1B). Litters from 14 separate matings of SP-B +/- mice, representing 125 pups, have been observed to date.

Heterozygous SP-B +/- mice were indistinguishable from normal littermates and had normal growth, development, and reproduction. In contrast, no homozygous SP-B +/- mice survived the neonatal period, the pups dying from respiratory failure immediately after birth. SP-B-deficient pups were of normal size and weight at birth. When delivered by hysterotomy on days 17–18, the numbers of heterozygous and homozygous offspring were consistent with Mendelian inheritance of a recessive gene. Because of the respiratory failure in SP-B +/- mice, the dams occasionally cannibalized affected pups in the immediate postnatal period. Thus the precise numbers of SP-B +/- pups from all of the SP-B +/- crosses cannot be accurately assessed. However, of 125 pups born, 33 died soon after birth. While lethal postnatally, SP-B deficiency did not alter prenatal survival or development. Birth weights and gross anatomy of organs other than the lungs were not affected in the SP-B +/- mice.

Postnatal Respiratory Failure in SP-B +/- Mice. At birth SP-B +/- mice initiated normal respiratory efforts but failed to inflate their lungs, remained cyanotic, and generally succumbed within 20 min. At term gestation, SP-B +/- and SP-B +/- mice initiated normal respiration, became pink, and rapidly inflated their lungs as assessed by the appearance of a white patch on the thorax representing the inflated lung. At autopsy, lungs of SP-B-deficient mice were poorly inflated and pathological findings were not observed in other organs. Histologic examination of lungs from SP-B +/- mice demonstrated normal morphogenesis of the conducting and peripheral airways without evidence of inflammation or desquamation of the epithelium (Fig. 2). When sacrificed prior to birth, the morphology and branching of the conducting and peripheral airways were indistinguishable in SP-B +/- or SP-B +/- mice, consistent with normal morphologic development of the lungs in the SP-B +/- mice.

Pro-SP-B and the active SP-B peptide were not detected in the lungs from the SP-B +/- mice by immunohistochemistry or Western blot confirming the functional ablation of the SP-B gene (Fig. 2). In contrast, pro-SP-B was readily detected in the type II cells and in bronchioles of SP-B +/+ and +/- mice, which were indistinguishable. Northern blot and N1ase analysis of SP-A, SP-B, and SP-C mRNA confirmed the complete lack of SP-B mRNA in the SP-B +/- mice; the abundance of SP-A and SP-C mRNA was similar in all groups of mice (Fig. 3). The finding that SP-B mRNA was absent in the SP-B +/- mice is similar to that observed in a human infant bearing a missense mutation in the exon 4 (9) and most likely represents the synthesis of an unstable mRNA. Immunohistochemistry utilizing antiserum generated against the amino-terminal portion of recombinant human pro-SP-C detected pro-SP-C in the lungs of SP-B +/- mice (data not shown). However, by Western blot analysis a pro-SP-C fragment (8–9 kDa) was detected in homogenates prepared from all SP-B +/- mice, but not SP-B +/+ or SP-B +/- mice (Fig. 4). Pro-SP-B and the active SP-B peptide were absent and the abundance of SP-C (active peptide, 3–4 kDa) was markedly reduced in SP-B +/- mice (Fig. 4).

Ultrastructural abnormalities in the lungs of SP-B +/- mice were confined to the respiratory epithelium. Fully formed lamellar bodies were not detected in type II cells of the SP-B +/- mice (Fig. 5). Abnormal Golgi apparatus and large, atypical, intracellular membranous inclusions were noted in SP-B +/- mice but not SP-B +/+ or SP-B +/- mice. Alveolar lipid aggregates consisting of small vesicles and electron-dense proteinaceous material were seen in the airways of SP-B +/- mice, most likely representing secretion of the abnormal lipids and proteins seen intracellularly. Tubular myelin was not detected in the alveolar space of SP-B +/- mice. Lamellar bodies and tubular myelin were readily apparent in the lungs of SP-B +/+ and +/- mice. Ultrastructural characteristics of
bronchiolar epithelial cells were not altered in the SP-B \(-/-\) mice.

**DISCUSSION**

Genetic ablation of the mouse SP-B gene caused lethal postnatal respiratory failure associated with pulmonary atelectasis. Immunostaining and mRNA analysis confirmed the lack of expression of the SP-B gene without detectable changes in mRNAs encoding SP-A or -C. Fully formed lamellar bodies and tubular myelin—the intracellular and extracellular forms of surfactant, respectively—were not detected in SP-B \(-/-\) mice. These findings demonstrate the critical role of SP-B in lung inflation following birth and in the intracellular routing and/or storage of surfactant lipids and proteins by type II epithelial cells, creating an animal model for the study of the pathogenesis and treatment of hereditary SP-B deficiency.

**Fig. 3.** Northern blot analysis of RNA from lungs and liver of heterozygous SP-B \(+/-\) mice (lanes 1–3) and lungs of SP-B \(-/-\) mice (lanes 4–6). RNA (20 \(\mu\)g per lane) was probed with labeled cDNAs for mouse SP-A, SP-B, and SP-C mRNA and Neo' mRNA and with an 18S rRNA-specific oligonucleotide.

**Fig. 4.** Expression of SP-B and SP-C protein in SP-B gene-targeted mice. Lung homogenates were prepared from newborn mice and equal amounts of protein from homozygous SP-B \(-/-\) (lanes 1) and wild-type (lanes 2) mice were analyzed by SDS/PAGE and immunoblotting. (A) Monospecific antiserum to SP-B detects mature peptide (arrow) only in \(+/-\) mice. (B) Antiserum directed against both SP-B and SP-C peptides demonstrates dramatic reduction in mature SP-C in SP-B \(-/-\) mice (arrowhead). (C) Antiserum directed against the amino-terminal propeptide of SP-C detects the proprotein (arrow) in SP-B \(+/+\) mice; aberrantly processed SP-C at 8.5 kDa (arrowhead) is detected only in SP-B \(-/-\) mice.
FIG. 5. Ultrastructure of the respiratory epithelium and alveolar surfactant from SP-B-deficient mice. Lung tissue was obtained from a litter of SP-B gene-targeted mice on day 17.5 of gestation and prepared for electron microscopy. Intracellular forms of surfactant in SP-B +/− and SP-B −/− mice are shown in A and B, respectively. Alveolar forms of secreted surfactant from SP-B +/+ and SP-B −/− mice are shown in C and D, respectively. [Bars = 500 nm (A and B) or 1000 nm (C and D).]
Hereditary SP-B deficiency was recently recognized as a cause of postnatal respiratory failure in human infants, causing neonatal respiratory distress in three siblings homozygous for a frameshift mutation in exon 4 of the human SP-B gene (9, 10). As in the present mouse study, heterozygous siblings and parents in this family were not affected clinically. The SP-B-deficient infants died of chronic respiratory insufficiency at several months of age after intensive therapy with oxygen, positive pressure ventilation, and/or extracorporeal membrane oxygenation. In the present studies and in the reported human infants with hereditary SP-B deficiency, abnormalities were confined to the respiratory tract, consistent with the lung epithelial cell-specific expression of SP-B in humans and mice. Pathologic findings in infants with hereditary SP-B deficiency demonstrated bronchopulmonary dysplasia, variable alveolar proteinosis, and desquamation of type II epithelial cells, as well as a lack of tubular myelin (21). In the present study, the lungs of SP-B-deficient mice were atelecatic but lacked the proteinosis and disruption of the respiratory epithelium seen in infants with hereditary SP-B deficiency (22), supporting the concept that some of the histopathological features of SP-B-deficient infants are related to lung injury associated with postnatal intensive care. In the SP-B --/-- mice, apical secretion of the aberrant lipid vesicles was apparent, and there was no evidence of aberrant basalar or lateral trafficking of lipid vesicles.

The lack of SP-B and presence of pro-SP-C in the lungs of the SP-B-deficient mice are consistent with recent findings in alveolar lavage from human infants with SP-B deficiency in which an amino-terminal fragment of pro-SP-C (12 kDa) peptide was identified (17). Since pro-SP-C is not present in the alveoli of laboratory animals or humans, the identification of the pro-SP-C fragment by Western blot analysis or ELISA may be useful in the clinical diagnosis of this genetic disease. Absence of the active SP-B peptide and the presence of the aberrantly processed pro-SP-C in either lung lavage fluid or in lung sections, assessed immunochromically, is presently used in our laboratory for the clinical diagnosis of SP-B deficiency in human neonates. The finding that surfactant phospholipids were also altered in the amniotic fluid from an infant with hereditary SP-B deficiency is consistent with the presently observed disruption of lamellar bodies in type II cells of the SP-B --/-- mice (23). As in the SP-B --/-- mice, ultrastructural findings showed that a patient with hereditary SP-B deficiency also lacked tubular myelin.

The failure of exogenous surfactant replacement to correct respiratory function in a patient with hereditary SP-B deficiency (23) may be related to alterations in type II cell function that are more profound than that caused by the lack of a functional SP-B peptide in the alveolus. Aberrant processing and secretion of both phospholipids and surfactant proteins may contribute to surfactant dysfunction in SP-B deficiency. Accumulation of alveolar proteins, including the secretion of pro-SP-C fragments into the alveolus, may inhibit surfactant function in SP-B deficiency. Deficiency of the active SP-C peptide seen in the SP-B --/-- mice suggests that disruption of the SP-B gene causes aberrant processing of pro-SP-C, creating a deficiency of both SP-B and SP-C in the alveolus. Although clinical samples have been limited, we were also unable to identify mature SP-C in surfactant from a human infant with hereditary SP-B deficiency (17). This profound disruption of intracellular and extracellular surfactant homeostasis in the SP-B --/-- mice supports the findings that therapy with exogenous surfactant preparations containing SP-B is not sufficient to treat hereditary SP-B deficiency.

To date, human hereditary SP-B deficiency has been reported in only a few patients, and the incidence of SP-B deficiency in the general population is not known. In contrast, non-genetic SP-B deficiency is associated with and perhaps critical to the pathogenesis of lung dysfunction associated with respiratory distress syndrome in premature infants and with adult respiratory distress syndromes in older individuals (7, 8).

In summary, ablation of the SP-B gene in transgenic mice demonstrates the critical role of SP-B in surfactant homeostasis and in the initiation of lung inflation at birth. SP-B --/-- mice will be useful in developing strategies for diagnosis and therapy of this lethal neonatal lung disease and will provide insights into the important role of SP-B in surfactant biology.

We acknowledge the expert assistance of John Duffy and Thomas Doetschman in the Transgenic Core; Sherri Profitt; and Melanie Bruno, Ph.D. This work was supported by the National Institutes of Health (Center for Gene Therapy Grants HL51835 and HL38859; Program of Excellence Grant HL41496) and the Cystic Fibrosis Foundation. M.T.S. is funded by National Institutes of Health Grant HL14214.