Surfactant protein D (SP-D) gene-targeted mice develop severe pulmonary disease associated with emphysema, pulmonary lipidosis, and foamy macrophage infiltrations. To determine the potential reversibility of these abnormalities, transgenic mice were developed in which SP-D was conditionally replaced in the respiratory epithelium of SP-D−/− mice. SP-D was not detected in the absence of doxycycline. Treatment with doxycycline after birth restored pulmonary SP-D concentrations and corrected pulmonary pathology at adulthood. When SP-D was replaced in adult SP-D−/− mice, alveolar SP-D was restored within 3 days, pulmonary lipid abnormalities were corrected, but emphysema persisted. In corrected adult SP-D−/+ mice, loss of SP-D caused focal emphysema and pulmonary inflammation but did not cause phospholipid abnormalities characteristic of SP-D−/− mice. Thus, abnormalities in surfactant phospholipid homeostasis and alveolar macrophage abnormalities were readily corrected by restoration of SP-D. However, once established, emphysema was not reversed by SP-D. SP-D-dependent processes regulating surfactant lipid homeostasis were disassociated from those mediating emphysema.

Reversibility of Pulmonary Abnormalities by Conditional Replacement of Surfactant Protein D (SP-D) in Vivo*

Liqian Zhang, Machiko Ikegami, Chitta R. Dey, Thomas R. Korfhagen, and Jeffrey A. Whitsett‡

From the Division of Pulmonary Biology, Cincinnati Children’s Hospital Medical Center, Cincinnati, Ohio 45229-3039

Translated into English as per the guidelines provided.

This work was supported by National Institutes of Health Grants PPG HL61646 and HL38965 (to J. A. W.), Grant HL63329 (to M. I.), and Grant SCOR HL56387 (to J. A. W. and T. R. K.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† To whom correspondence should be addressed: Cincinnati Children’s Hospital Medical Center, Divisions of Neonatology and Pulmonary Biology, 3333 Burnet Ave., Cincinnati, OH 45229-3039. Tel.: 513-636-4830; Fax: 513-636-7868; E-mail: jeff.whitelett@cchmc.org.

‡ The abbreviations used are: SP-D, surfactant protein D; CCSP, Clara cell secretory protein; rtTA, reverse tetracycline transactivator; BALF, bronchoalveolar lavage fluid; satPC, saturated phosphatidylcholine; r; rat; m, mouse.

Surfactant protein D (SP-D) is a 43-kDa member of the collectin family of mammalian lectins (for review, see Refs. 1 and 2). SP-D is synthesized in many organs including the lung where it is expressed primarily in type II epithelial cells in alveoli and in non-ciliated bronchial cells in conducting airways (3). Studies in SP-D gene targeted animals demonstrated its unexpected role in surfactant phospholipid homeostasis. SP-D−/− mice developed increased tissue and alveolar phospholipid pool sizes that were established soon after birth (4, 5). Abnormalities in alveolar macrophage morphology and emphysema were also apparent before 1 month of age and increased with age (6). Increased oxidant production, activation of NFkB, and enhanced metalloproteinase expression by alveolar macrophages were observed, perhaps contributing to the alveolar remodeling seen in SP-D−/− mice (6, 7). In addition to abnormalities in lung structure and phospholipid metabolism, SP-D−/− mice are highly susceptible to pulmonary infections by respiratory syncytial and influenza A viruses. SP-D−/− mice failed to clear either virus effectively and developed exaggerated inflammatory responses following infection (8). Since SP-D-dependent abnormalities in lipid metabolism were established immediately following birth and continued postnatally, it has been unclear whether SP-D is required during lung morphogenesis or whether there is an ongoing requirement for SP-D for regulation of pulmonary surfactant metabolism in the adult lung. To further clarify the role of SP-D in pulmonary homeostasis, recombinant SP-D was conditionally expressed in respiratory epithelial cells under control of the Clara cell secretory protein (CCSP) promoter in SP-D−/- gene targeted mice in vivo. Abnormalities in surfactant lipid homeostasis and monocytic infiltrates were reversed by expression of SP-D in adult SP-D−/− mice. Reversal of complementation in adult SP-D−/− mice caused emphysema and alveolar infiltrates but did not perturb phospholipid homeostasis.

Materials and Methods

Animal Husbandry—Mice used in the experimental procedures were handled in accordance with approved protocols through the Institutional Animal Care and Use Committee at Cincinnati Children’s Hospital. All mice had been maintained in containment facilities. At the time of study, all mice appeared healthy and without evidence of infection. Sentinel mice in the colony were serologically negative for common murine pathogens.

Generation of Transgenic Mice—The 1.3-kb cDNA encoding recombinant rat SP-D (rSP-D), a generous gift from Dr. E. C. Crouch, Washington University, was inserted into the NoI site of (tetO)−CMV plasmid (9). Restriction enzyme digestion and 5’ end sequencing confirmed the orientation of the insert. The transgene was microinjected into fertilized FVB/N oocytes by the Cincinnati Children’s Hospital Transgenic Core facility. Founders were identified by two sets of transgene-specific PCR using upstream primer 5’-ATA GGA CCC CAA GGC AAA CCAG-3’ and downstream primer 5’-TGT GCT-TGT ACC TCT AGG-3’ in rSP-D and upstream primer 5’-CAC CGG CGC GCA TCC AGC-3’ in cytomegalovirus (CMV) promoter and downstream primer 5’-CAC CCC CCA GAA TAG AAT GAC AAC AAA CCAG-3’ in cytomegalovirus (CMV) promoter and downstream primer 5’-CAC CCC CCA GAA TAG AAT GAC AAC AAA CCAG-3’ in CMV promoter. Transgenic animals were crossed with CCSP-rtTA mice (9, 15) and SP-D−/− mice (4) to generate triple transgenic mice (CCSP-rtTA +, (tetO)−/− rSP-D+, mSP-D−/−). Triple transgenic mice were treated with doxycycline containing feed pellets (25 mg/g; Harlan Tekland, Madison, WI) to induce the expression of rSP-D protein (Fig. 1). Several (tetO)−/− rSP-D transgenic mouse lines were generated. Founder line 10 expressed rSP-D at levels similar to those in wild type mice after treatment with doxycycline and was therefore utilized for this study.

Western Blot Analysis—Mice were weighed, anesthetized by intraperitoneal injection of pentobarbital, and exsanguinated by severing the distal aorta. Bronchoalveolar lavage was performed five times with 1 ml of normal saline, and the collected volume was measured (10). Bronchoalveolar lavage fluid (BALF) (25 μl) from each mouse was dried and reconstituted in 15 μl of Laemmli sample buffer (Bio-Rad) containing β-mercaptoethanol. Proteins were resolved on 10–20% SDS-Tris-glycine-polyacrylamide gels (NOVEX, San Diego, CA) and transferred to nitrocellulose membranes. Blots were blocked with 5% nonfat milk and incubated overnight at room temperature with rabbit anti-mouse...
Conditional Expression of Recombinant SP-D in Vivo—SP-D mRNA and protein were undetectable in SP-D–/– mice or in triple transgenic (CCSP-rTA, tetOγ-rSP-D, SP-D–/−) mice in the absence of doxycycline (Fig. 2A, lane 1). Administration of doxycycline to adult mice for 2 weeks resulted in production of physiologic levels of the rat SP-D in lungs of triple transgenic mice (Fig. 2A, compare lanes 3 and 6). Thus, in triple transgenic (CCSP-rTA, tetOγ-rSP-D, SP-D–/−) mice, the only detectable SP-D was derived from the rat SP-D transgene. When maintained on doxycycline from birth, airspace size, alveolar macrophage morphology, and pulmonary SatPC pool sizes were not different from control SP-D–/– mice (Figs. 3 and Fig. 4B). In contrast, in the absence of doxycycline, pulmonary abnormalities characteristic of SP-D–/– mice were readily apparent in the triple transgenic mice (Fig. 3 and Fig. 4A). In the absence of doxycycline, the severity of emphysema, macrophage abnormalities, and lipid accumulations was not distinguishable from that in SP-D–/– mice. Thus, continuous expression of rSP-D from birth completely corrected abnormalities in lung morphology and surfactant phospholipid metabolism in SP-D–/– mice.

**RESULTS**

**Conditional Expression of SP-D in Vivo**—Pulmonary abnormalities were established in SP-D–/– mice by maintaining the triple transgenic mice without doxycycline until 2 months of age, at which time SP-D remained undetectable. Increased SatPC, foamy macrophage infiltrates, and emphysema characteristic of SP-D deficiency were observed. Pulmonary phospholipid content was decreased within 3 days and...
Normalized 7–14 days after treatment with doxycycline (Fig. 5). Again, rSP-D was undetectable in the absence of doxycycline and was rapidly induced, reaching physiologic levels 3 days after administration of doxycycline (Fig. 2B). Abnormalities in alveolar macrophage morphology were also readily reversed by restoration of SP-D in the adult mice (Fig. 6C). To further assess the reversibility of airspace remodeling, triple transgenic mice were treated with doxycycline for 2 weeks after 10 weeks of treatment (D and G), or removed from doxycycline for 4 weeks after 8 weeks of treatment (D and H). Lung morphology is shown after staining with hematoxylin-eosin (A–D). Immunohistochemistry was performed with rabbit anti-mouse SP-D (E–H). Thick arrows point to staining in bronchiolar cells, and thin arrows point to staining in Type II cells.

DISCUSSION

In the present study, rat recombinant SP-D was expressed at physiological levels. Conditional expression of recombinant rat SP-D completely corrected pulmonary abnormalities in SP-D−/− mice when expressed from birth. In adult SP-D−/− mice, extinction of the transgene expression resulted in a rapid decrease in alveolar SP-D, consistent with the known clearance rate of SP-D (14) and the pharmacodynamics of doxycycline on which reversal of transgene expression was dependent (15). In complemented adult SP-D−/− mice treated with doxycycline from birth, removal from doxycycline resulted in decreased tissue SP-D, reappearance of abnormal alveolar macrophage infiltrates, and airspace remodeling that were detected within 7–14 days. In contrast, SatPC was not reversed by loss of SP-D in adult mice. Thus, SP-D-dependent lung remodeling was neither dependent on lipid accumulation nor on processes that affect lung morphogenesis. Although surfactant pool size and alveolar macrophage abnormalities in SP-D−/− mice were readily reversed by restoration of SP-D, emphysema persisted, demonstrating that irreversible changes in lung parenchyma had occurred in the absence of SP-D. Lack of SP-D results in rapid onset of alveolar macrophage activation and initiation of
tissue remodeling in adult animals. Thus, SP-D is required for maintenance of pulmonary homeostasis in the mature and developing mouse lung.

The finding that replacement of SP-D corrected phospholipid and alveolar macrophage abnormalities but did not correct emphysema is of considerable clinical interest. Emphysema can be caused by both developmental and inflammatory processes, leading to the loss of lung parenchyma reflected by airspace enlargement. D. Massaro and G. D. Massaro (16) recently summarized findings in rodent models wherein emphysema was partially reversible. In the present study, replacement of SP-D for 1–3 months did not correct established emphysema in the SP-D/−− mice. Such findings are consistent with clinical observations demonstrating that various forms of pulmonary hypoplasia and emphysema do not resolve. Therefore, despite the rapid correction of lipid surfactant and macrophage abnormalities by expression of SP-D, emphysema persisted. This conditional system will be of use in identifying agents that may enhance alveolarization.

It has been unclear whether alveolar macrophage infiltration and emphysema in SP-D/−− mice are secondary to lipid accumulation or are mediated by distinct pathological mechanisms. In the present study, macrophage infiltrates and remodeling were observed after removal of the corrected mice from doxycycline. In sharp contrast, lung SatPC remained at normal wild type levels after prolonged removal from doxycycline and absence of SP-D. Thus, these experiments dissociate abnormalities in surfactant homeostasis from the airway remodeling. A similar dissociation of these two processes was observed in studies in which the SP-D mutant protein (Rr-SP-Dser-15/ser-20) caused a dose-dependent inhibitory effect on SP-D function, producing airspace but not lipid abnormalities in SP-D/−− mice (17). Likewise, expression of a chimeric protein consisting of the neck and carbohydrate recognition domain of conglutinin linked to the SP-D amino-terminal and collagenous domain corrected surfactant phospholipid homeostasis but did not improve emphysema or macrophage activation in the SP-D/−− mice (18). Taken together, both structural and temporal requirements for the correction of pulmonary surfactant abnormalities are distinct from those mediating alveolar macrophage activation and tissue remodeling, suggesting that these two processes are mediated by distinct mechanisms in vivo. Furthermore, the finding that surfactant SatPC remained normalized in adult mice despite a prolonged absence of SP-D suggests that SP-D plays a critical role in establishing SatPC concentrations during postnatal development. Since lung SatPC pools are markedly higher in neonatal mice than in adults (6, 14), SP-D appears to be required for the establishment of the low SatPC pool size characteristic of mature mice. Once normal SatPC concentrations were established in the complemented SP-D/−− mice, loss of SP-D in adulthood did not cause the abnormal accumulation of surfactant lipid. Therefore, SP-D is not required for maintenance of steady state SatPC pools in adult mice.

The sites and level of expression of rSP-D in the present studies were virtually identical to those of the endogenous mouse and human SP-D protein (3, 19), the transgene being expressed in type II epithelial cells and bronchiolar epithelial cells in the lung. As demonstrated previously by Fisher et al. (20), rat recombinant SP-D fully corrected pulmonary abnormalities in the SP-D/−− mice and did not cause observable pulmonary abnormalities in wild type mice. Thus, the effects of SP-D deficiency on lung physiology are not related to the effects of SP-D in other organs but are dependent on local pulmonary requirements for SP-D. In the present study, the rat CCSP promoter was utilized to express the transgene. Although CCSP is normally expressed only in conducting airways in the mouse, this rat CCSP promoter element expresses transgenes in both bronchiolar and alveolar epithelial cells, sites consistent with the known expression pattern of SP-D in the mouse (21). The sites and extent of expression of SP-D observed in the present study are also consistent with previous studies with the CCSP-rtTA mice from this laboratory (15). The lack of detectable expression in the absence of doxycycline has made the CCSP-rtTA activator line highly useful for conditional expression of genes in vivo (9, 15). The time course of SP-D expression following administration or removal from doxycycline is consistent with previous studies in which luciferase activity was used to monitor of gene expression in SP-C-rtTA and CCSP-rtTA transgenic mice. Gene expression was induced within 6–12 h following addition of doxycycline to drinking water or food, and activity was generally decreased within 12–24 h after removal of doxycycline (15). Thus, the rapid loss of SP-D protein from BALF following removal of doxycycline is consistent with the known pharmacodynamics of doxycycline, the stability of rtTA, and the half-life of SP-D protein in the lung, the latter being approximately 8 h in the mouse (14). The distinct temporal differences for reversibility of macrophage abnormalities, as compared with surfactant phospholipid concentrations, suggest that these aspects of pulmonary homeostasis are influenced by distinct pathways.

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J. Biol. Chem. 2002, 277:38709-38713.
doi: 10.1074/jbc.M206200200 originally published online August 5, 2002

Access the most updated version of this article at doi: 10.1074/jbc.M206200200

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