Airway injury in lung disease pathophysiology: selective depletion of airway stem and progenitor cell pools potentiates lung inflammation and alveolar dysfunction

Susan D. Reynolds, Adam Giangreco, Kyung U. Hong, Kathleen E. McGrath, Luis A. Ortiz, and Barry R. Stripp

1Department of Environmental and Occupational Health, University of Pittsburgh, Pittsburgh, Pennsylvania 15260; and 2Department of Pediatrics, University of Rochester, Rochester, New York 14620

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Reynolds, Susan D., Adam Giangreco, Kyung U. Hong, Kathleen E. McGrath, Luis A. Ortiz, and Barry R. Stripp. Airway injury in lung disease pathophysiology: selective depletion of airway stem and progenitor cell pools potentiates lung inflammation and alveolar dysfunction. Am J Physiol Lung Cell Mol Physiol 287: L1256–L1265, 2004. First published August 6, 2004; doi:10.1152/ajplung.00203.2004.—Identification of early events that contribute to the establishment of chronic lung disease has been complicated by the variable involvement of the airway and alveolar compartments in the complex physiology of end-stage disease. In particular, the impact of airway injury on alveolar integrity and function has not been addressed and would be facilitated by development of animal models of lung disease that specifically target a single cell type within the airway epithelium. We have previously demonstrated that ganciclovir treatment of Cc17 transgenic mice, which express the herpes simplex thymidine kinase gene under regulation of the mouse Clara cell secretory protein (CCSP) promoter, results in elimination of the airway progenitor and stem cell pools and a consequent failure of airway regeneration that is associated with rapid morbidity and mortality. In this study, we used the CCSP model to test the hypothesis that selective airway injury initiates profound lung dysfunction through mechanisms that compromise alveolar integrity. Results demonstrate that elimination of the CCSP-expressing cell population results in secondary alveolar inflammation, edema, and depletion of the alveolar type II cell population. On the basis of these data we conclude that selective airway injury can serve as the inciting injury in diseases characterized by severely compromised alveolar function.

Clara cell secretory protein; herpes simplex thymidine kinase gene; transgenic mice; surfactant protein C; ganciclovir; lung injury; repair; chronic lung disease; asthma

MECHANISMS UNDERLYING the development, perpetuation, and exacerbation of chronic lung disease have been difficult to define due in part to the variable contribution of airway and alveolar compartments to pathophysiological outcomes. Typically mucosecretory diseases including chronic obstructive pulmonary disease (COPD), chronic bronchitis, cystic fibrosis, and asthma are classified as airway disorders and include metaplastic conversion of the airway secretory cell population to a goblet cell phenotype. This pathology is associated with hypersecretion and accumulation of mucus within the airway lumen, a decrease in mucociliary clearance, and sporadic or chronic infection. Although these diseases are characterized by cycles of airway epithelial injury and incomplete or inappropriate repair, involvement of the alveolar epithelium in disease progression varies (18). Parenchymal inflammation and air space enlargement are commonly associated with secretory cell metaplasia in COPD (18). This syndrome is strikingly mimicked by constitutive or regulated overexpression of IL-13 (33), as well as some T helper (Th) 1 cytokines in airway secretory cells (20). Although Th2 cytokines are not directly implicated in COPD, these studies suggest that subtle alterations in the functional properties of the airway secretory cell population impact alveolar homeostasis and function. Dynamic interactions between the airway and alveolar compartments are likely to play a critical role in lung homeostasis and processes leading to alterations in airway function may interrupt this relationship. However, the precise role of airway injury/remodeling in regulation of alveolar and function are unknown.

The linkage between airway and alveolar pathology in acute lung injury is similarly difficult to dissect. In the case of acute respiratory distress syndrome (ARDS) and acute lung injury (ALI), acute respiratory failure is associated with multiple but distinct stimuli that can have either local or systemic origins (24). Sepsis is the most common cause of these syndromes and has a direct impact on secretory cell function, although a correlation between airway trauma (initiated by aspiration of gastric contents, near drowning, inhalation of toxic compounds, or ventilator-induced barotrauma and hyperoxia) and the development of ARDS indicates a potential connection between direct airway injury and alveolar dysfunction. Although the inciting events leading to the development of ARDS are disparate, this disease is consistently characterized by rapid and widespread damage to the alveolar epithelium and vasculature that is associated with accumulation of a protein-rich exudate, neutrophilic inflammation, and surfactant insufficiency. Improvements in lung ventilation, commonly termed lung-protective ventilatory strategies, have improved survival of ARDS patients; however, mortality remains high (30%) (26).

The conducting airway epithelium serves a number of critical functions including regulation of lung fluid balance, secretion of effectors of the innate immune response, regulation of the adaptive immune response, clearance and in some cases metabolism of inhaled environmental agents, and renewal of the epithelium following injury (19). Each of these activities is either directly or indirectly associated with integrity of the Clara cell secretory protein (CCSP)-expressing cell, which...
functions as the predominant secretory cell of the airway epithelium as well as the preferred progenitor cell for renewal of the epithelium following injury (23). Previous studies from others as well as our own analysis indicate that the mode of epithelial regeneration is dependent on the cell targeted during the injury process. Acute airway injury that targets the terminally differentiated ciliated cell population is rapidly resolved through proliferation and differentiation of secretory cells distributed throughout the conducting airway epithelium (9, 11, 12). In contrast, targeted depletion of the secretory cell population through parenteral exposure to lipophilic agents such as naphthalene results in activation of a tissue-specific airway stem cell population sequestered within the neuroepithelial body and bronchoalveolar duct junction microenvironments (13, 28).

The goals of the present study were to define roles for the conducting airway epithelium in establishment and progression of lung disease through investigation of the hypothesis that selective airway injury initiates profound lung dysfunction via mechanisms that compromise alveolar integrity. Use was made of a novel transgenic mouse model allowing for generation of a precise airway lesion through conditional ablation of epithelial stem and progenitor cell pools of the conducting airway (15, 29). Injury of conducting airways and the ensuing failure of repair resulted in progressive deterioration of lung function that included loss of alveolar barrier function, recruitment of inflammatory cells, and alterations to alveolar type II cells that included reduced expression of surfactant protein (SP)-C. We conclude that airway integrity is a critical determinant of more complex pathophysiological changes to the lungs in this mouse model. These data suggest that the changes in the composition and function of the conducting airway epithelium has potential to serve as an initiating event in the progression of chronic lung disease in humans.

MATERIALS AND METHODS

Animals

Male FVB/n and Clara cell secretory protein-herpes simplex thymidine kinase (CCtk) transgenic mice were maintained as a specific pathogen-free in-house colony, and representative animals were screened quarterly using a comprehensive 16-agent serologic panel (Microbiological Associates, Rockville, MD). Mice were allowed food and water ad libitum and maintained on a 12:12-h light-dark cycle. Animals used in this study were 2–4 mo old. All procedures used in this study were approved by the Institutional Animal Care and Use Committee of the University of Pittsburgh.

Naphthalene Treatment

Naphthalene treatment was carried out as previously described (30). Each animal received 275 mg naphthalene/kg body wt ip and was recovered in filtered air for 2, 3, 5, or 10 days. At least four mice were used for each recovery time point.

CCtk Transgenic Mice and Ganciclovir Treatments

Generation and characterization of CCtk transgenic mice have been previously described (29). Transgenic mice (n = 4) were acutely exposed to ganciclovir (GCV, Cytovene IV; Roche Applied Science, Indianapolis, IN) over a 24-h period or chronically exposed to GCV (GCV, Cytovene IV; Roche Applied Science, Indianapolis, IN) over a 24-h period or chronically exposed to GCV. GCV treatment of nontransgenic mice has no impact on lung gene expression (15, 29).

Gene Expression Analysis

Total lung RNA was isolated by as previously described (3). mRNA abundance was determined by S1 nuclease protection analysis with previously described methods and templates (30). Bands intensities were analyzed with a phosphorimager and ImageQuant software (Molecular Dynamics, Sunnyvale, CA) and were normalized to L32. Results are reported as means ± SE. Significance was determined by two-way ANOVA with challenge and time of recovery as the two factors analyzed.

Tissue Fixation

Mice were killed by intraperitoneal injection of 100 mg/kg pentobarbital sodium. Lung tissue was inflation fixed with neutral buffered formalin (NBF) at 10 cmH2O pressure for 10 min, immersed in NBF overnight at 4°C, immersed in phosphate-buffered saline (PBS) overnight at 4°C, dehydrated through graded ethanol, and embedded in paraffin.

Histology

Paraffin-embedded lung tissue was sectioned at 5 μm and adhered to glass microscope slides. Sections were dewaxed with xylene and stained with hematoxylin and eosin for histological assessment. Images of representative fields were acquired under an Olympus Provis AX70 microscope (Olympus, Lake Success, NY) equipped with a Spot RT color digital camera (Diagnostic Instruments, Sterling Heights, MI) linked to a personal computer running Image-Pro Plus software (Media Cybernetics, Silver Spring, MD).

Dual Immunofluorescence

Cells coexpressing CCSP and thymidine kinase were identified using dual immunofluorescence techniques. Primary antibodies were goat anti-rat CCSP and rabbit anti-thymidine kinase and have been used as previously to assess expression of these markers in CCtk mice (29). Secondary antibodies were purchased from Molecular Probes (Eugene, OR) and were used in the following combinations: Alexa Fluor 488 donkey anti-goat IgG for CCSP and Alexa Fluor 594 donkey anti-rabbit IgG for thymidine kinase. All secondary antibodies were used at a dilution of 1:500. Stained sections were mounted with Fluormount-G containing 2 μg/ml 4,6-diamidino-2-phenylindole (DAPI, Sigma). Images of representative fields were acquired under an Olympus Provis AX70 microscope (Olympus) equipped with a DAPI/Texas red dual optical excitation filter cube and an FITC optical excitation filter cube (Olympus) and a Spot RT color digital camera (Diagnostic Instruments). Images were processed with Image-Pro Plus software (Media Cybernetics) and Adobe Photoshop.

In Situ Hybridization

Templates for generation of antisense and sense SP-C cRNA probes were generated as previously described (31). Probes were labeled with digoxigenin, quantified, and hybridized according to the manufacturer’s directions (Roche Applied Science). Hybridized probe was detected via overnight incubation with a 1:500 dilution of alkaline phosphatase-conjugated sheep antidigoxigenin antibody and antigen-antibody complexes detected with nitroblue tetrazolium salt (NBT) and 5-bromo-4-chloro-3-indolyl phosphate according to the manufacturer’s directions (Roche Applied Science). The number of SP-C mRNA-expressing cells in 10 random high-power fields that lacked airway was determined for each of three control and three GCV-exposed mice on days 7 and 14. The means ± SE were reported. Significance was assessed by Student’s t-test.
Proliferation

For analysis of alveolar proliferation, mice were treated with vehicle or GCV according to the acute protocol and injected with 2.5 μCi [3H]thymidine (ip) 1 h before death. Three mice were analyzed per treatment group. Tissue sections were dewaxed with xylene, dried, and coated with NBT2 emulsion according to the manufacturer’s directions (Kodak, Rochester, NY). Emulsion was exposed for 30 days and developed as previously described and coated sections counterstained with hematoxylin. Proliferative cells were defined as those having five or more grains over the nucleus. Ten random high-power fields lacking airway were counted for each of three or four animals in each group, and the mean was determined. Results were reported as the mean and SE for each experimental group. Significance was assessed by the Student’s t-test.

Body Weight Determination

Body weight was determined before treatment and on a daily basis for a group of five control animals, five naphthalene-treated animals, and 10 animals acutely or chronically exposed to GCV. The weight of the miniosmotic pump was determined at necropsy and subtracted from the daily body weight. Weights are reported as a percentage of the initial weight.

Bronchoalveolar Lavage

Control and treated animals were anesthetized with 15 μl/g body wt 2% avertin and exsanguinated. The trachea was cannulated, and two 1-ml aliquots of 1× PBS were instilled and pooled (pool 1). The lungs were then lavaged an additional six times with 1 ml of 1× PBS and the recovered fluid pooled (pool 2). Cells from the two pools were recovered through centrifugation at 300 g, collected, counted, and cytopsin onto glass microscope slides. Cells were categorized according to morphology following Diff-Quick staining. Protein concentration in pool 1 was determined by BCA assay using bovine serum albumin standards (Pierce, Rockford, IL).

RESULTS

Decreased SP-C Gene Expression in GCV-Treated CCtk Mice

We have previously reported that GCV treatment of CCtk transgenic mice resulted in rapid and irreversible ablation of the CCSP-expressing cell population, which includes both the transit amplifying and stem cell pools (15, 29). Within 10 days of GCV exposure, transgenic mice in either the chronic or acute exposure protocols were tachypnic, hypothermic, lethargic, and cyanotic and the lungs of these mice were fluid filled and occasionally hemorrhagic. In contrast, naphthalene-mediated Clara cell depletion in strain-matched FVB/n mice was reversed by day 5, and the airway was repaired within 20 days of treatment (30). Comparison of these two models of Clara cell injury suggests that the morbidity and mortality noted in GCV-exposed CCtk transgenic mice was the result of secondary complications stemming from unresolved airway damage.

To determine the impact of Clara cell depletion on other lung compartments, airway and alveolar gene expression was compared in wild-type mice treated with 275 mg naphthalene/kg body wt and recovered 1–14 days and in CCtk transgenic mice continuously treated with 4.5 mg GCV/day for 6–14 days (chronic exposure). Animals were killed at the indicated times, and total lung RNA was assayed for expression of the Clara cell markers CCSP, CyP450–2F2 (2F2), and the alveolar type II cell marker (SP-C) by S1 nuclease protection assay.

As previously reported, naphthalene-mediated lung injury was characterized by a 95% decrease in the abundance of Clara cell-specific mRNAs on day 2 and a rapid repair process that resulted in re-expression of Clara cell-specific genes by days 5–10 (Fig. 1A). Levels of SP-C mRNA in naphthalene-treated mice did not differ significantly from control values at any time point after naphthalene exposure (Fig. 1A). Chronic GCV exposure of CCtk transgenic mice resulted in a 94% depletion of CCSP and 2F2 mRNA by day 6 and a 98% reduction in these markers by day 12 (29) (Fig. 1B). No additional changes in Clara cell-specific gene expression were noted between days 12 and 14. In these chronically exposed mice, alveolar type II cell gene expression was similar to control values on treatment day 6 but was significantly reduced on days 12 (28% of control, P < 0.05) and 14 (22% of control, P < 0.05, Fig. 1B). This analysis confirmed our previous reports that Clara cell ablation is completed within 6 days of GCV exposure in CCtk mice and demonstrated that expression of Clara cell-specific genes is not reactivated in an extended 14-day recovery period. Quantification of SP-C mRNA abundance indicated that ex-
pression of this gene was not altered in naphthalene-treated mice. However, decreased expression of SP-C followed the period of active Clara cell ablation in GCV-exposed CCtk mice. Distinctions in the profile of SP-C gene expression in naphthalene- and GCV-exposed CCtk mice indicate that a failure in airway secretory cell regeneration was associated with an alteration in the alveolar compartment manifested by a change in the alveolar type II cell population.

Transgene Expression Pattern Following Injury

Our previous studies demonstrate that the mouse CCSP promoter-regulated thymidine kinase transgene was expressed exclusively in CCSP-immunopositive cells of the steady-state airway epithelium (29). However, alveolar type II cell expression of transgenes regulated by the rat CCSP promoter has been reported (8, 21). This observation raised the possibility that expression of the CCtk transgene was induced in lung compartments other than airways following airway injury and that decreased SP-C mRNA abundance could be attributed to alterations in the expression pattern of the CCtk transgene. Two approaches were used to address this issue. First, mice were acutely exposed to 9.6 mg GCV/day for 1 day and recovered in the absence of drug for 1–14 days, and expression of Clara and alveolar type II cell markers was assessed by S1 nuclease protection assay. The course of Clara cell depletion in acutely exposed CCtk transgenic mice mirrored that observed in chronically exposed mice (compare Figs. 1B and 2). Expression of Clara cell markers was reduced 94% on recovery day 6, and only minimal changes in the level of these markers were noted at later time points (15) (Fig. 2). Depletion of SP-C in the acute exposure model was not as extensive as that noted in chronically exposed mice but was statistically different from controls on recovery days 5 (65% of control, \( P < 0.05 \)), 10 (53% of control, \( P < 0.005 \)), and 14 (47% of control, \( P < 0.005 \); Fig. 2). This study demonstrated that Clara cell-specific gene expression was not reactivated in CCtk mice acutely exposed to GCV and that reductions in SP-C gene expression are characteristic of CCtk mice exposed to GCV using either the chronic or acute exposure protocols. The observation that SP-C mRNA abundance was initially decreased 4 days after the cessation of GCV exposure suggests that the decline in expression of SP-C was secondary to the loss of Clara cells rather than a consequence of drug-mediated injury of cells in which the transgene was ectopically expressed.

As a second method for assessing focal alterations in transgene expression following GCV-mediated CCSP-expressing cell ablation, the distribution of thymidine kinase protein was determined by immunofluorescence at various times after acute GCV exposure. In agreement with previous results (15), this analysis demonstrated a decrease in the number of thymidine kinase-positive cells within the airway epithelium between recovery days 1 and 14 (Fig. 3, A–F). However, no thymidine kinase-expressing cells were observed within the nonairway portion of the lung including the alveolar epithelium, endothelium, and mesenchymal layers at any time point. The number of CCSP-expressing cells was similarly diminished following GCV treatment (Fig. 3, A–F, green signal), and expression of the endogenous protein, which would be expected to follow

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**Fig. 2.** Analysis of gene expression in CCtk mice acutely exposed to ganciclovir. CCtk transgenic mice were exposed to 9.6 mg ganciclovir over a 24-h period and recovered for 1–14 days. Gene expression was assayed as described in Fig. 1. CCSP (●), CyP450 –2F2 (■), SP-C (▲). Significance (*) was accepted at \( P < 0.005 \).

**Fig. 3.** Dual immunofluorescence analysis of CCSP and thymidine kinase protein. CCtk transgenic mice were acutely exposed to vehicle (A) or ganciclovir and recovered for 1.5 (B), 2 (C), 3 (D), 5 (E), or 10 (F) days. Lung tissue sections were stained simultaneously for CCSP (green) and thymidine kinase (red). Nuclei were counterstained with 4,6-diamidino-2-phenylindole (blue). Photomicrographs are representative of 3 animals from each time point. Original magnification \( \times 100 \).
that of the mouse CCSP promoter-regulated CCtk transgene, was also undetectable in the parenchyma. In agreement with the gene expression analysis presented in Fig. 2, these data demonstrate that thymidine kinase/CCSP-expressing cells were eliminated from the airways of CCtk transgenic animals acutely exposed to GCV and indicate that ectopic expression of the transgene in the alveolar epithelium was either absent or too low to be detected by the immunofluorescence method. Together, these two lines of evidence supported the conclusion that morbidity observed in GCV-treated CCtk mice was a consequence of irreparable airway epithelial injury.

Consequences of Airway Secretory Cell Ablation

Exposure of animals to environmental or pharmacological agents that injure the distal airway epithelium has been associated with decreased viability and a time-dependent increase in the number and type of inflammatory cells in the bronchoalveolar lavage (BAL) fluid, with the accumulation of protein in the alveolar compartment, and with an increase in parenchymal cell proliferation (25). To determine whether cell type-specific ablation of the CCSP-expressing cell population correlated alveolar injury, these hallmarks of parenchymal damage were assessed in CCtk transgenic mice chronically or acutely exposed to GCV. Naphthalene-treated wild-type mice were assessed as a control population.

Parenchymal pathology. Hematoxylin and eosin staining of lung tissue sections from chronically exposed mice showed infiltration of the lung parenchyma by inflammatory cells on day 7, whereas peribronchiolar and alveolar edema, regions of consolidation consistent with inflammatory cell influx, and loss of alveolar septation were observed in later stage mice (data not shown). Similar alterations in lung morphology were noted in acutely exposed mice, although they were first observed on day 10 (data not shown). Analysis of elastic fiber deposition using van Gieson elastica stain failed to detect fibrotic lesions in either the airway or alveolar space (data not shown). This analysis suggests that the chronic and acute GCV exposure regimes resulted in inflammation of the alveolar compartment and alterations in vascular permeability.

Inflammation. To assess the role of inflammation in naphthalene- and GCV-mediated disease progression, wild-type mice were treated with naphthalene, and CCtk transgenic mice were exposed to GCV either chronically or acutely. Lungs of control and treated mice were lavaged at the indicated time points, and the representation of various inflammatory cell populations was determined by differential cell counts. Results of this analysis indicate that lavagable cells were 98% macrophages, 1.5% neutrophils, and 0.5% lymphocytes in the steady state. Either chronic or acute GCV exposure resulted in accumulation of foamy macrophages within 3 days (Fig. 4A) and in neutrophil extravasation starting on days 6–7 (Fig. 4B). By day 14 neutrophils represented 33% of lavagable cells in chronically exposed mice and 38% of this population in acutely exposed mice (Fig. 4B). In contrast, few if any neutrophils were recovered from naphthalene-treated mice at any time point (Fig. 4B). Lymphocytes were a significant fraction of the lavagable cells recovered from chronically exposed CCtk mice on day 7 and day 14 and from acutely exposed CCtk mice on day 14. Lymphocytes were recovered from naphthalene-treated mice on day 3 only. These results indicate that the macrophage was the predominant inflammatory cell type throughout the injury and repair process in naphthalene-treated mice. In contrast, lung injury in GCV-exposed CCtk mice was associated with a shift in the inflammatory cell profile to one dominated by neutrophils during the midstage disease and neutrophils and lymphocytes during end-stage disease. Extravasation of neutrophils into the alveolar space correlated with the initiation of decreased SP-C gene expression.

Accumulation of a protein-rich exudate. The impact of airway injury on the integrity of the epithelial-endothelial boundary was assessed by analysis of protein concentration in BAL fluid recovered from control mice or those exposed to naphthalene or GCV. Naphthalene-treated mice experienced a transient increase in BAL protein concentration that returned to baseline by recovery day 5 (Fig. 5). Both chronic and acute GCV exposure was associated with an increase in BAL protein concentration by day 7 (Fig. 5). This increase was statistically different from control on day 14 in chronically exposed mice and on days 6–14 in acutely treated mice. The kinetics of protein accumulation suggests that vascular leakage is associ-
Alveolar proliferation. Proliferation in the alveolar compartment was assessed in CCtk transgenic mice exposed to GCV using the acute protocol. Mitotic cells were labeled in vivo with tritiated thymidine 1 h before death and detected by autoradiography of tissue sections. Mitotic cells were defined as those with five or more grains located over the nucleus and were quantified as the number of such cells/high-power field. Three sections from each of three or four animals in each recovery group were evaluated. Results of this analysis demonstrate a low level of proliferation in the steady-state alveolus and on days 1 and 3 of recovery (Fig. 6). Mitotic cells in these animals tended to be randomly distributed throughout the alveolar region. A 7.9-fold increase in proliferation was observed between days 3 and 6 (0.57 to 4.53), and the number of proliferative cells was significantly different from control values on day 6 ($P < 0.001$). The number of mitotic cells was further increased between days 6 and 10 (4.53 to 12.26), and the number of mitotic cells on day 10 was significantly different from both control ($P < 0.001$) and from day 6 values ($P < 0.001$). In contrast with control and early recovery time points, labeled cells in the day 6 and day 10 samples were nonuniformly distributed and tended to be clustered in regions of alveolar consolidation. The results of this assay indicate that enhanced alveolar proliferation was a consequence of cell type-specific airway injury. This finding, in combination with the previously described histopathological and pathophysiological alterations to the lungs of GCV-treated CCtk transgenic mice, indicates that alveolar injury was a consequence of cell type-specific injury to the airway epithelium.

Decreased Number of SP-C-Expressing Cells

Analysis of SP-C gene expression (Figs. 1B and 2) indicates that decreased abundance of this message was a characteristic of end-stage disease in CCtk transgenic mice. To distinguish between a generalized decrease in SP-C gene expression and overt loss of SP-C-expressing cells, nonradioactive in situ hybridization was used to compare the spatial distribution and number of SP-C-mRNA-expressing cells in control mice and those chronically treated with GCV for 7 or 14 days. Analysis of control tissue demonstrated a normal distribution of SP-C-expressing cells throughout the lung parenchyma (Fig. 7, A and B). In contrast, SP-C-expressing cells were less frequent in chronically exposed mice on treatment day 14 (Fig. 7, C and D), and quantitative assessment of SP-C mRNA-expressing cells demonstrated an 80% decrease in this cell type between days 7 and 14 (Fig. 7E). Residual SP-C-expressing cells tended to be centrally located, and a consistent decrease in the number of peribronchial SP-C-expressing cells was noted. Interestingly, intensity of the SP-C signal within the remaining message positive cells was qualitatively similar to that of control (compare Fig. 7, B and D), supporting the conclusion that this pathology is the result of loss of alveolar epithelial type II cells rather than a widespread decrease in SP-C gene expression. These data indicate that the specific airway injury mediated by metabolism of the procytotoxic drug GCV within in Clara cells resulted in a 30% decrease in SP-C mRNA abundance and in the number of SP-C-expressing cells in those chronically treated with GCV for 7 or 14 days. Analysis of acutely exposed mice demonstrated a similar pattern of weight loss that progressed to 25% of the initial body weight by days 13–14 and a moribund

Morbidity and Mortality of GCV-treated CCtk Mice

To correlate epithelial gene expression and alveolar pathology with morbidity and mortality, changes in body weight were assessed as a function of time in CCtk transgenic mice exposed to vehicle or to GCV and in mice treated with naphthalene. Vehicle exposure of CCtk mice was associated with a slight increase in body weight over the 2-wk analysis period (Fig. 8). In contrast, chronic GCV administration was associated with profound weight loss that began 3–7 days after initiation of the exposure and resulted in a 30% decrease in body weight by days 13–14 (Fig. 8). At this time, all remaining mice were moribund. Similar analysis of acutely exposed mice demonstrated a similar pattern of weight loss that progressed to 25% of the initial body weight by days 13–14 and a moribund
state (Fig. 8). No evidence of infection at the pump insertion site, pneumonia, or sepsis was noted in mice in either exposure protocol, at any time point. Both treatment regimens resulted in 80% lethality by day 10 and 100% lethality by day 15. In contrast, naphthalene-treated mice underwent a transient weight loss that was reversed on day 5 and resolved by day 14 (Fig. 8). These data indicate that airway injury and repair as modeled by naphthalene treatment was associated with a reversible decrease in body weight, whereas GCV-mediated Clara cell ablation resulted in profound weight loss and mortality.

**DISCUSSION**

Pathological assessment of acute and chronically diseased human lung has suggested that disease conditions that were previously thought to be confined to either the airway or alveolar space are in fact a composite of functional alterations in both compartments (24). Although development and adoption of treatment protocols that recognize the linkage between these two compartments might improve patient outcome, the lack of studies demonstrating causal relationship between airway injury and alveolar dysfunction has limited progress in this area. In this study we have utilized the CCtk transgenic model to determine the impact of cell type-specific airway injury on alveolar integrity. Results demonstrate that irreparable ablation of the airway secretory cell population, including both the conditionally differentiated transit amplifying cells and the variant CCSP-expressing stem cell, results in persistent macrophage activation and a transition to neutrophilic inflammation. Subsequent alveolar alterations include vascular leakage that results in accumulation of protein within the alveolar space and alveolar proliferation that is secondary to alterations in alveolar architecture. Finally a decrease in the number of SP-C-expressing alveolar type II cells is associated with profound weight loss and death. These results suggest a direct linkage between airway injury and alterations in alveolar homeostasis. The absence of such alveolar complications in the naphthalene model of secretory cell depletion and regeneration highlights the importance of rapid reconstitution of the airway epithelium following secretory cell depletion for maintenance...
of alveolar function. On the basis of these results we conclude that compromised alveolar integrity is a direct consequence of extensive airway injury, and we suggest that unresolved airway damage can act as an inciting event in acute lung diseases that are characterized by profound lung dysfunction.

Distinctions in the inflammatory cell profile of strain-matched naphthalene-exposed wild-type and GCV-treated CClk transgenic mice indicate that the secondary alveolar injury that characterizes the CClk model is a consequence of processes leading to the recruitment of neutrophils to the lung. In both models, treatment results in rapid depletion of the secretory cell population and macrophage activation. Injury and repair in the naphthalene model are associated with a transient decrease in body weight but do not result in neutrophilia or vascular or epithelial alterations within the alveolar compartment. In contrast, the inflammatory response in GCV-treated CClk transgenic mice is characterized by a transition from a macrophage- to a neutrophil-biased infiltrate on days 6–7 and a subsequent increase in the representation of lymphocytes on day 14. The kinetics of this response suggests that prolonged airway injury exacerbates the inflammatory process and alters the profile of cells participating in this response. Although this study did not identify the types or source of inflammatory mediators responsible for neutrophil recruitment, preliminary analysis of gene expression suggests that interferon (IFN)-γ and IFN-γ-regulated cytokines may be involved in this process (S. D. Reynolds, unpublished observations).

Numerous studies in both human and mouse indicate that the airway and alveolar epithelium are potent sources of chemoattractant cytokines as well as modulators of the inflammatory response (1, 4), and the present study suggests a potential role for airway-derived factors in the initiation and/or control of destructive alveolar inflammation. An alternative source of neutrophil chemokines could be the macrophage itself. IFN-γ, TNF-α sensitization, or Fas ligand (FasL)-mediated apoptosis of this population has been shown to initiate a profound inflammatory response that is the result of proinflammatory cytokine secretion by preapoptotic macrophages. This mechanism has been correlated with rejection of FasL-expressing tissue grafts (14) and with parenchymal inflammation in silica-treated mice (2) and may be the process responsible for alveolar injury and loss of alveolar type II cells in the CClk model. Finally, peptides derived from the denuded basement membrane may serve as chemoattractants in this model. Previous analysis demonstrated squamation of the terminal bronchiolar epithelium in GCV-treated CClk mice and the potential for exposure of the underlying matrix (29). Collagen-derived peptides as well as other matrix components are potent chemoattractants for inflammatory cells (5, 22, 32) and could be instrumental in conversion of the inflammatory cell profile from one that is predominately phagocytic to one that is granulocytic. Although the present study identifies a clear linkage between airway injury and alveolar dysfunction and a potential role for neutrophilic inflammation in this process, additional studies are needed to detail the factors that drive neutrophil recruitment in this model, their source, and the impact of this process on the epithelium and vasculature of the alveolus.

End-stage disease, which followed neutrophil extravasation into the bronchoalveolar space, was characterized by respiratory distress and weight loss that progressed to 25–30% of the initial body weight. Pathological assessment demonstrates that this morbidity was associated with compromised alveolar integrity indicated by structural alterations, an increase in lavage protein concentration on days 10–14, and a 20–60% decrease in SP-C mRNA abundance that paralleled an increase in mitotic index within the parenchyma. Downregulation of SP-C gene expression has been correlated with entry of alveolar type II cells into the cell cycle in some models of alveolar injury (R. Ryan, personal communication) and may be a component of the alveolar response to airway injury in the present study. Analysis of alveolar type II cell proliferation in response to hyperoxia-indexed alveolar damage has demonstrated the importance of checkpoint control in limiting the genotoxic impact of oxidative stress on the alveolar epithelium (27). The clear demonstration of alveolar proliferation on days 6 and 10 in this study and the subsequent ~80% decrease in the number of SP-C-expressing cells on day 14 suggest that this protective mechanism is uncoupled under conditions of profound inflammation and identify a potential point of intervention for protection of the alveolus from collateral injury.

Ectopic expression of the CClk transgene in alveolar type II cells is also a potential cause of alveolar injury in this model, and support for this mechanism of alveolar injury would have important implications for the impact of airway injury of alveolar type II cell phenotype. However, several lines of evidence argue against this possibility. First, dual immunofluorescence analysis of thymidine kinase and CCSP protein distribution failed to detect either thymidine kinase or CCSP in alveolar cells of the steady-state lung or following GCV-mediated injury. Although the sensitivity of this analysis is limited, this mechanism, if substantiated, would indicate that the threshold levels of cellular thymidine kinase required for cell killing in alveolar cells is substantially less than that necessary for ablation of airway secretory cells. Second, decreased expression of Clara cell markers is an early consequence of GCV exposure (60% depletion within 1 day of exposure), whereas decreases in levels of SP-C exhibited a more protracted time course, and frank loss of alveolar type II cells was observed only at the latest time points. A statistically significant decrease in SP-C mRNA abundance was first observed on day 5 in acutely exposed mice, 4 days after cessation of GCV administration. At this point, GCV, which is cleared with first-pass kinetics (7), would be present at extremely low concentrations that would likely be below the K_m of the thymidine kinase enzyme. Third, similar outcomes were observed for CClk transgenic mice exposed to GCV by the chronic or the acute exposure protocols, an indication that short-term exposure to drug is sufficient to initiate downstream alterations in alveolar homeostasis. Finally, alterations in SP-C gene expression paralleled rather than preceded changes in BAL parameters and the proliferative response within the alveolus. This observation suggests that type II cell injury is a consequence of the same process that leads to vascular leakage rather than a cause of this alteration in alveolar integrity.

The alveolar type II cell, like the airway secretory cell, is a multifunctional cell that is critical for secretion of surfactant and other components of the extracellular lining fluid and as a bipotential progenitor cell for maintenance of the alveolar epithelium (10). Decrements in either or both of these functions have been implicated in human lung diseases involving alveolar immaturity and injury (6). Surfactant insufficiency has
been demonstrated in acute lung injury and is the likely cause of atelectasis within the alveolar compartment and may also contribute to collapse of small airways. Although mechanisms leading to aberration of the surfactant layer have not been fully elucidated, a connection between altered Clara cell phenotype and alveolar proteinosis has been postulated (17). Although such a linkage is attractive, especially in light of the present study, analysis of CCSP knockout mice has failed to detect alterations in surfactant gene expression or alveolar integrity that are a consequence of CCSP deficiency or the associated perturbation in Clara cell secretory function (16). These observations further support the conclusion that unresolved airway injury rather than simple Clara cell deletion is the basis for alveolar complications in this model.

Previous analysis of bronchiolar injury and repair following secretory cell deletion has resulted in identification of a variant population of CCSP-expressing cells with characteristics of tissue-specific stem cells (13, 15, 28). These cells are restricted to the neuroepithelial body (NEB) and bronchoalveolar duct junction (BADJ) microenvironments, are relatively undifferentiated, and are quiescent in the steady state. After naphthalene-mediated progenitor cell deletion, these cells proliferate a limited number of times and give rise to a population of transit amplifying cells that in turn proliferate, migrate out of the microenvironment, and differentiate to regenerate the bronchiolar epithelium. Ablation of all CCSP-expressing cells through GCV treatment of CCtk mice inhibited the stem cell-mediated regenerative response and led to the identification of the NEB- or BADJ-sequestered variant CCSP-expressing cell as the bronchiolar stem cell. The present series of studies illustrates the importance of a rapid stem cell-mediated restoration of airway function for protection of the alveolus from secondary injury and indicates that direct airway injury including smoke or steam exposure, infection, aspiration, and near drowning may adversely impact alveolar homeostasis. As such, airway protective strategies have the potential to attenuate alveolar injury that is the cause of morbidity and mortality associated with ALI and ARDS.

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