Airway injury in the pathophysiology of lung disease: Selective depletion of airway stem and progenitor cell pools potentates lung inflammation and alveolar dysfunction.

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Abstract

Identification of early events that contribute to the establishment of chronic lung disease have 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 CCtk transgenic mice, which express the herpes simplex thymidine kinase gene under regulation of the mouse 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 have used the CCtk 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 2 cell population. Based on these data we conclude that selective airway injury can serve as the inciting injury in diseases characterized by severely compromised alveolar function.
Introduction

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. While 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 airspace enlargement are commonly associated with secretory cell metaplasia in COPD (18). This syndrome is strikingly mimicked by constitutive or regulated over-expression of IL-13 (33), as well as some Th1 cytokines in airway secretory cells (20). While 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. While 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 bronchioalveolar duct junction microenvironments (13, 28).

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 2 cells that included reduced expression of surfactant protein 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 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 hr/day light/dark cycle. Animals used in this study were 2-4 months old. All procedures used in this study were approved by the IACUC of the University of Pittsburgh.

Naphthalene Treatment. Naphthalene treatment was carried out as previously described (30). Each animal received 275 mg naphthalene / kg body weight intraperitoneally (i.p.) and was recovered in filtered air for 2, 3, 5, or 10 days. At least 4 mice were used for each recovery time point.

CCtk transgenic mice and ganciclovir (GCV) treatments. Generation and characterization of CCSP-HSVtk (CCtk) transgenic mice have been previously described (29). Transgenic mice (n = 4) were acutely exposed to ganciclovir (Cytovene IV, Roche Applied Science, Indianapolis, IN) over a 24 hour period or chronically exposed to GCV throughout the course of the experiment. Drug was administered using miniosmotic pumps (ALZET, Palo Alto, CA) as described previously (29). Control CCtk transgenic mice (n=4) were exposed to saline using a miniosmotic pump. GCV treatment of non-transgenic mice has no impact on lung gene expression (15, 29).
Gene expression analysis. Total lung RNA was isolated by as previously described (3). Messenger RNA abundance was determined by S1 nuclease protection analysis using previously described methods and templates (30). Bands intensities were analyzed using a phosphorimager and ImageQuant software (Molecular Dynamics, Sunnyvale, CA) and were normalized to L32. Results are reported as the mean ± standard error. Significance was determined by two-way ANOVA with challenge and time of recovery as the two factors analyzed. Significance was accepted at \( p \leq 0.05 \).

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

Histology. Paraffin-embedded lung tissue was sectioned at 5 microns and adhered to glass microscope slides. Sections were dewaxed with xylenes and stained with hematoxylin and eosin for histological assessment. Images of representative fields were acquired using 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 PC running Image-Pro Plus software (Media Cybernetics, Silver Spring, MD).
*Dual immunofluorescence.* Cells co-expressing 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 Fluoromount-G containing 2\(\mu\)g/mL 4,6-diamidino-2-phenylindole (DAPI, Sigma). Images of representative fields were acquired using an Olympus Provis AX70 microscope (Olympus, Lake Success, NY) equipped with a DAPI/Texas Red dual optical excitation filter cube and an FITC optical excitation filter cube (Olympus, Lake Success, NY) and a Spot RT color digital camera (Diagnostic Instruments, Sterling Heights, MI). Images were processed using Image-Pro Plus software (Media Cybernetics, Silver Spring, MD) and Adobe Photoshop.

*In situ hybridization.* Templates for generation of antisense and sense surfactant protein 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, Indianapolis, IN). 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 (BCIP) according to the manufacturer’s directions (Roche Applied Science, Indianapolis, IN). The number of
SP-C mRNA expressing cells in 10 random high power fields that lacked airway was determined for each of 3 control and 3 ganciclovir-exposed mice on days 7 and 14. The mean ± S.E.M. was 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 (i.p.) one hour prior to sacrifice. 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 5 or more grains over the nucleus. Ten random high power fields lacking airway were counted for each of 3 to 4 animals in each group and the mean determined. Results were reported as the mean and S.E.M. for each experimental group. Significance was assessed by the student’s t test.

Body weight determination. Body weight was determined prior to treatment and on a daily basis for a group of 5 control animals, 5 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 percent of the initial weight.

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

*Decreased surfactant protein C gene expression in ganciclovir-treated CCtk mice*

We have previously reported that ganciclovir (GCV) treatment of CCtk transgenic mice resulted in rapid and irreversible ablation of the Clara cell secretory protein (CCSP)-expressing cell population which includes both the transit amplifying and stem cell pools (15, 29). Within ten 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 suggested that the morbidity and mortality noted in GCV-exposed CCtk transgenic mice was the result of secondary complications stemming from unresolved airway damage.

In order to determine the impact of Clara cell depletion on other lung compartments, airway and alveolar gene expression were compared in wild type mice treated with 275 mg naphthalene / kg body weight 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 sacrificed at the indicated times and total lung RNA assayed for expression of the Clara cell markers CCSP, CyP4502F2 (2F2), and the alveolar type 2 cell marker surfactant protein C (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 message 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 2 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 was not reactivated in an extended 14 day recovery period. Quantification of SP-C mRNA abundance indicated that expression 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 indicated 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 2 cell population.

Transgene expression pattern following injury

Our previous studies demonstrated that the mouse CCSP promoter regulated thymidine kinase (TK) transgene was expressed exclusively in CCSP-immunopositive cells of the steady state airway epithelium (29). However, alveolar type 2 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, recovered in the absence of drug for 1-14 days, and expression of Clara and alveolar type 2 cell markers 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, suggested 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 TK positive cells within the airway epithelium between recovery days 1 and 14 (Figure 3 A-F, red signal). However, no TK-expressing cells were observed within the non-airway 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 (Figure 3 A-F, green signal) and expression of the endogenous protein, which would be expected to follow that of the mouse CCSP promoter-regulated CCtk transgene, was also undetectable in the parenchyma. In agreement with the gene expression analysis presented in Figure 2, these data demonstrated that TK / CCSP-expressing cells were eliminated from the airways of CCtk transgenic animals acutely exposed to GCV and indicated that ectopic expression of the transgene in the alveolar epithelium was either absent or too low to be detected by immunofluorescence method. Taken 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 fluid, with the accumulation of protein in the alveolar compartment, and with an increase in parenchymal cell proliferation (25). In order 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 treatment day 7 while 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 suggested that the chronic and acute GCV exposure regimes resulted in inflammation of the alveolar compartment and alterations in vascular permeability.

**Inflammation:** In order 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 determined by differential cell counts. Results of this analysis indicated 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 day 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 as 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 mid-stage 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 bronchoalveolar lavage 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, circles). Both chronic and acute GCV exposure were associated with an increase in bronchoalveolar lavage (BAL) protein concentration by day 7 (Fig. 5, squares and triangles). 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 suggest that vascular leakage is associated with lung inflammation (Fig 5) and the decrease in SP-C gene expression (Fig. 1A and Fig. 2).

**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 one hour prior to sacrifice and detected by autoradiography of tissue sections. Mitotic cells were defined as those with 5 or more grains located over the nucleus and were quantified as the number of such cells / high power field. Three sections from each of 3 to 4 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 non-uniformly 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 indicated that alveolar injury was a consequence of cell type specific injury to the airway epithelium.
**Decreased Number of SP-C Expressing Cells**

Analysis of surfactant protein C gene expression (Fig 1B and Fig. 2) indicated that decreased abundance of this message was a characteristic of end-stage disease in CCtk transgenic mice. In order to distinguish between a generalized decrease in SP-C gene expression and overt loss of SP-C expressing cells, non-radioactive 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 7A and B). In contrast, SP-C expressing cells were less frequent in chronically exposed mice on treatment day 14 (Fig 7C 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 7B and D) supporting the conclusion that this pathology is the result of loss of alveolar epithelial type 2 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 ganciclovir within in Clara cells results in secondary loss of alveolar type 2 cells. The observation that a change in SP-C mRNA abundance and in the number of SP-C expressing cells was significant immediately prior to death suggested that loss of this vital cell population contributed to the demise of these mice.
Morbidity and Mortality of GCV-treated CCtk mice

In order 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 week analysis period (Fig 8, diamonds). 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, squares). 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, triangles). 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, circles). These data indicated that airway injury and repair as modeled by naphthalene treatment was associated with a reversible decrease in body weight while 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). While 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 the number of surfactant protein C-expressing alveolar type 2 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 highlight the importance of rapid reconstitution of the airway epithelium following secretory cell depletion for maintenance of alveolar function. Based on 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 CCtk transgenic mice indicate that the secondary alveolar injury that characterizes the CCtk 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 is associated with a transient decrease in body weight but does not result in neutrophilia or vascular and epithelial alterations within the alveolar compartment. In contrast, the inflammatory response in GCV-treated CCtk 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. While this study did not identify the types or source of inflammatory mediators responsible for neutrophil recruitment, preliminary analysis of gene expression suggests that interferon gamma (IFN\(\gamma\)) and IFN\(\gamma\)-regulated cytokines may be involved in this process (SDR unpublished observations). Numerous studies in both human and mouse indicate that the airway and alveolar epithelium are potent sources of chemotactic 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\(\gamma\), TNF\(\alpha\) 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), with parenchymal inflammation in silica-treated mice (2), and may be the process responsible for alveolar injury and loss of alveolar type 2 cells in the CCtk model. Finally, peptides derived from the denuded basement membrane may serve as chemotactic agents in this model. Previous analysis demonstrated squamation of the terminal bronchiolar epithelium in GCV-treated CCtk 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. While 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 extrasivation into the bronchoalveolar space, was characterized by respiratory distress and weight loss that progressed to 25-30% of the initial body weight. Pathological assessment demonstrated 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. Down regulation of SP-C gene expression has been correlated
with entry of alveolar type 2 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 2 cell proliferation in response to hyperoxia-induced 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 suggests 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 CCtk transgene in alveolar type 2 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 2 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. While 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 a early consequence of GCV exposure (60% depletion within 1 day of exposure) while decreases in levels of SP-C exhibited a more protracted time course and frank loss of alveolar type 2 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, four 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 Km of the thymidine kinase enzyme. Third, similar outcomes were observed for CCtk transgenic mice exposed to GCV using 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 2 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 2 cell like the airway secretory cell is a multi functional 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. While 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 depletion is the basis for alveolar complications in this model.

Previous analysis of bronchiolar injury and repair following secretory cell depletion 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. Following naphthalene-mediated progenitor cell depletion, 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 indicate 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 acute lung injury (ALI) and acute respiratory distress syndrome (ARDS).
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Figure Legends

Figure 1. S1 nuclease protection analysis of gene expression. Total lung RNA from wild type mice treated with 275 mg naphthalene/ mg body weight (A) or CCtk transgenic mice treated with ganciclovir according to the chronic exposure protocol (B) was assayed for the Clara cell-specific markers CCSP(closed diamond), CyP450-2F2 (closed square), or the alveolar type 2 cell marker surfactant protein C (closed triangle). Expression levels for each message were normalized to the internal control L32 and are expressed as a percent of control. Three to 6 individuals were assayed for each experimental group and the mean ± S.E.M is reported for each time point. Significance (*) was accepted at p<0.005.

Figure 2. Analysis of gene expression in CCtk mice acutely exposed to ganciclovir. CCtk transgenic mice were exposed to 10 mg ganciclovir over a 24 hours period and recovered for 1-14 days. Gene expression was assayed as described in Figure 1. CCSP(closed diamond), CyP450-2F2 (closed square), SP-C (closed triangle). Significance (*) was accepted at p<0.005.

Figure 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 DAPI (blue). Photomicrographs are representative of 3 animals from each time point.
Original magnification 100X.

Figure 4. Differential cell analysis. Cells recovered from bronchioalveolar lavage of wild type mice treated with 275 mg naphthalene/ kg body weight (closed diamond) and CCtk mice exposed either chronically (closed square) or acutely (closed triangle) to ganciclovir were cytospun onto glass slides and representation of macrophages (A), neutrophils (B), and lymphocytes (C) determined following Diff Quick staining. A total of 400 cells were counted for each of 3-6 animals and results are reported as a percent of the total. The mean ± S.E.M for each group is presented. Significance (*) was accepted at p<0.05.

Figure 5. Analysis of protein concentration in bronchioalveolar lavage fluid. Mice were treated with naphthalene or ganciclovir and recovered as previously described. Bronchioalveolar lavage fluid recovered from the first 2 x 1 ml lavage was pooled, the cells removed by centrifugation, and protein concentration determined by BCA assay. Three to 6 animals were assayed in each treatment group: 275 mg naphthalene/ kg body weight (closed circle), CCtk mice exposed either chronically (closed square) or acutely (closed triangle) to ganciclovir. Results are reported as the mean ± S.E.M. Significance (*) was accepted at p<0.05.

Figure 6. Analysis of alveolar proliferation. CCtk transgenic mice were acutely exposed to ganciclovir and recovered the indicated period of time. One hour prior to sacrifice, 2.5 µCi [³H] thymidine was injected (ip) to mark proliferating cells. Lung tissue sections were prepared, coated with NBT2 photographic emulsion, exposed for 30 days, and
developed according to the manufacturer’s directions. Mitotic cells were defined as those having at least 5 autoradiographic grains within a nuclear profile. Ten high power fields were assayed for each of 3 animals at each time point. Results are reported as the mean ± S.E.M. for each group. Significance (*) was accepted at p<0.001.

Figure 7. In situ hybridization analysis of surfactant protein C mRNA. Non-radioactive in situ hybridization was used detect surfactant protein C (SP-C) message in untreated CCtk mice (A, B) and in CCtk mice chronically exposed to ganciclovir for 10 days (C, D). Examples of SP-C mRNA expressing cells are indicated by arrows. Original magnification in A and C was 100X and in B and D was 400X. The number of SP-C mRNA positive cells in 10 random high power fields that lacked airway was determined for each of 3 control (C) and 3 ganciclovir-exposed mice on days 7 and 14. The mean ± S.E.M. is reported. Significance (**) was accepted at p<0.005.

Figure 8. Analysis of body weight. The weight of untreated wild type mice (closed diamond), wild type mice exposed to 275 mg naphthalene/ mg body weight (closed circle), and CCtk mice exposed either chronically (closed square) or acutely (closed diamond) to ganciclovir was followed over a 2 week period. Values for 6-10 mice in each group were expressed as a percent of initial weight and the mean ± S.E.M. for each time point reported. Significance (*) was accepted as p<0.005.
References

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Figure 1.
Figure 2.
Figure 4.

A. Macrophages

B. Neutrophils

C. Lymphocytes
Figure 5.
Figure 6

Proliferation

Number Mitotic
(Cells/High Power Field)

Time (Days)

0 1 3 6 10
Figure 7.
Figure 8

Average Body Weight

- Control
- Naphthalene
- Chronic GCV
- Acute GCV

Body Weight (% Control)

Time (Days)