Targeted Type 2 Alveolar Cell Depletion

A Dynamic Functional Model for Lung Injury Repair

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Abstract

Type 2 alveolar epithelial cells (AEC2) are regarded as the progenitor population of the alveolus responsible for injury repair and homeostatic maintenance. Depletion of this population is hypothesized to underlie various lung pathologies. Current models of lung injury rely on either uncontrolled, nonspecific destruction of alveolar epithelia or on targeted, nontitratable levels of fixed AEC2 ablation. We hypothesized that discrete levels of AEC2 ablation would trigger stereotypical and informative patterns of repair. To this end, we created a transgenic mouse model in which the surfactant protein-C promoter drives expression of a mutant SR39TK herpes simplex virus-1 thymidine kinase specifically in AEC2. Because of the sensitivity of SR39TK, low doses of ganciclovir can be administered to these animals to induce dose-dependent AEC2 depletion ranging from mild (50%) to lethal (82%) levels. We demonstrate that specific levels of AEC2 depletion cause altered expression patterns of apoptosis and repair proteins in surviving AEC2 as well as distinct changes in distal lung morphology, pulmonary function, collagen deposition, and expression of remodeling proteins in whole lung that persist for up to 60 days. We believe SPCTK mice

demonstrate the utility of cell-specific expression of the SR39TK transgene for exerting fine control of target cell depletion. Our data demonstrate, for the first time, that specific levels of type 2 alveolar epithelial cell depletion produce characteristic injury repair outcomes. Most importantly, use of these mice will contribute to a better understanding of the role of AEC2 in the initiation of, and response to, lung injury.

Keywords: type 2 alveolar epithelial cells (AEC2); distal lung injury; injury-repair; lung disease

Clinical Relevance

Creation of a new model for alveolar epithelial type 2 cell (AEC2) ablation has revealed that divergent, clinically relevant outcomes can be elicited by specific and quantifiable destruction of the AEC2 population. These findings support the concepts that AEC2 play a critical role in maintaining lung homeostasis.

Chronic lung diseases are the third leading cause of death in the United States, accounting for 9% of all deaths and for 4 of the top 10 causes of infant mortality (1). As

of 2010, the economic burden of lung diseases and their treatment was estimated at \$106 billion annually (1). In light of the high levels of morbidity and mortality

for those afflicted by debilitating lung diseases, new tools are required to further elucidate the pathogenesis of these diseases and to provide model systems in

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which new therapies can be identified and tested.

A significant subset of emphysematous and fibrotic lung diseases are presumed to result from injury to the alveolar epithelium, including chronic obstructive pulmonary disease and idiopathic pulmonary fibrosis (2–7). Although two distinct epithelial cell types make up the alveolar epithelium, type 2 alveolar epithelial cells (AEC2) are critical for maintaining distal lung homeostasis after injury (8–11). Our lab has previously shown that injury-activated AEC2 are proliferative, are resistant to apoptosis, and exhibit increased levels of telomerase activity after injury (12–14), further supporting of the role of AEC2 as a progenitor population. These findings, along with those of other groups studying AEC2, support the hypothesis originally proposed by Haschek and Witschi that distal lung deficits, and fibrosis in particular, result from epithelial injury and dysregulated repair by AEC2 (15) (for review, see Ref. 16). A better understanding of how injury and disease specifically impact the homeostatic functions of AEC2 is vital to potentially improve their ability to repair and restore lung function.

To date, the role of AEC2 in the initiation of repair and regeneration has been explored in two broad categories of animal models. Exposure models use particulates, infectious agents, or noxious gasses to cause generalized alveolar or airway destruction. Genetic models use global or cell-specific gene knockdown or overexpression to drive injury progression. Many of these models induce widespread and nonspecific injury to multiple diverse cell types. Therefore, AEC2-specific injury models are required to specifically target and activate AEC2 so that their role in repair and regeneration can be fully characterized.

Two genetic models of AEC2-specific injury using related strategies have been reported with very different outcomes. The first model used expression of the diphtheria toxin receptor in AEC2 driven by the surfactant protein C (SPC) promoter (17). After intraperitoneal injection of diphtheria toxin, distal lung injury progression was observed, with a 40% reduction in SPC expression but no apparent AEC2 death. A second model used the tamoxifen-inducible SPC-creER to drive the expression of diphtheria toxin A (DTA) in SPC-expressing AEC2 (18). This model

estimated an AEC2 reduction of up to 52% but reported no substantial lung injury or fibrosis. These models, while extremely useful, did not fully address the role of specific levels of AEC2 depletion in the induction, repair, or regeneration of distal lung.

Herein, we present a new model of AEC2-specific injury using the SR39TK mutant of *Herpes simplex* viral thymidine kinase (HSV-1 TK) (19, 20) under the control of the human SPC promoter. In this model, intraperitoneally administered ganciclovir (GCV) is converted to a toxic metabolite in SPC-expressing AEC2, which induces targeted cell injury and death. We hypothesized that this model, termed SPCTK, could provide a wide dosable range for the inducing agent (GCV), thereby causing varying degrees of AEC2 depletion. In SPCTK mice, we found that both mild and severe levels AEC2 depletion can be precisely and reproducibly induced, each with a unique pattern of subsequent lung pathology and regeneration. We also observed changes in expression of apoptosis-related and damage repair proteins in surviving AEC2 and altered distal lung histology, whole lung remodeling protein expression, collagen levels, and lung mechanics that persist up to 60 days after specific levels of AEC2 ablation.

Materials and Methods

All animal studies were approved by the Institutional Animal Care and Use Committee at Children's Hospital Los Angeles.

Creation, Validation, and Dosing of SPCTK Transgenic Mice

The human SPC 3.7-kb promoter (21, 22) and mutant SR39TK *Herpes simplex* thymidine kinase were subcloned into a modified pCCL lentiviral expression vector (see Figure E1 in the online supplement) (19, 20, 23, 24, 25). For *in vitro* validation, 8-week-old SPCTK and wild-type mice were hyperoxia treated to stimulate modest AEC2 proliferation (14), and AEC2, fibroblasts, and CD16/32-positive immune cells were isolated. Primary cells at 30% confluency were treated with 0.05, 0.1, 0.5, or 5 μ M GCV 24 hours after plating and assessed for viability via Trypan Blue exclusion at 0, 48,

72, and 96 hours after plating. RNA was extracted using the RNeasy kit (Qiagen, Valencia, CA) and SR39TK expression analyzed via RT-PCR in triplicate. In a separate experiment, AEC2 isolated from WT and SPCTK mice were plated for 24 hours. At that point, samples were either harvested for RNA extraction and analysis of SPC, SR39TK, and actin expression; left untreated; or treated with 5 μ M GCV. Attached cells from GCV-treated and untreated samples were trypsinized and counted 48 hours later. This experiment was repeated for cells cultured for 48, 72, or 96 hours after isolation. For *in vivo* studies, 10, 50, or 100 mg/kg GCV was administered via intraperitoneal injection on 3 consecutive days to animals at 2 months of age. Lungs were harvested at Day 0 (the first day after treatment) or at 7, 14, 28, and 60 days after treatment.

Tissue Harvest and Processing

For histology, whole lung specimens were inflated at 20 cm H₂O pressure, fixed in 10% formalin, and sectioned. Carazzi Hematoxylin and Eosin, Sirius Red/Fast Green FCF (Sigma, St. Louis, MO) and Hart's Reagent/Tartrazine staining were performed to assess lung histology, collagen, and elastin, respectively. Mean linear intercept analysis was performed on photomicrographs of hematoxylin and eosin-stained sections. Fluorescent immunohistochemistry was performed as described previously (26). AEC2 were quantified by FACS after depletion of fibroblasts by differential adherence to plastic and subsequent removal of CD45⁺ cells via MACS column filtration (Miltenyi Biotech, San Diego, CA) and immunostaining with rabbit anti-SPC (Seven Hills) and FITC-labeled secondary antibody. Immunohistochemistry was used to determine the cellular composition of this fraction before FACS. For Western blotting, total protein from AEC2 and homogenized whole lung was determined before use via the Bradford method (Bio-Rad Laboratories, Hercules, CA). Primary antibodies and all HRP-labeled secondary antibodies (Sigma) were used according to the manufacturer's instructions.

Measurements of Lung Mechanics and Collagen Quantification

Pulmonary mechanics were measured as previously described (27). After analysis,

whole lungs were excised for hydroxyproline analysis (BioVision, Milpitas, CA) and soluble collagen analysis (Biocolor, County Aintree, UK).

Data Presentation and Statistical Analysis

Data are expressed as mean $\pm$ SD unless otherwise stated. Two group comparisons were determined using a two-tailed Student's *t* test. One-way ANOVA with post-hoc Tukey or Dunnett's testing was used for multiple comparisons. All statistical analyses were performed using SigmaPlot 12 (Systat Software Inc., San Jose, CA) or SPSS (IBM, Armonk, NY). *P* values ≤ 0.05 or 0.005 were considered significant.

Results

Specificity and Sensitivity of SPCTK-Derived Cells to GCV *In Vitro*

We first examined the sensitivity of AEC2 isolated from SPCTK mice to GCV to determine the impact of dosing on growth and survival. Isolated AEC2 cultured 24 hours and at approximately 30% confluency were treated with *in vitro* GCV doses consistent with previous reports (28) and harvested at 0, 48, 72, or 96 hours (Figure 1A). Wild-type and SPCTK-derived AEC2 treated with vehicle only demonstrated similar growth curves, showing increased cell numbers at 72 and 96 hours ($P < 0.05$) with 30 to 40% more cells per well after 96 hours. GCV-treated AEC2 isolated from SPCTK mice showed

significant growth inhibition at 48, 72, and 96 hours after treatment ($P < 0.05$). AEC2 isolated from wild-type mice were unaffected by high-dose GCV treatment ($5 \mu\text{M}$) *in vitro*. These experiments confirmed that the survival of AEC2 isolated from SPCTK, but not wild-type, mice are strongly and negatively affected by GCV treatment *in vitro*.

The specificity of SPC-SR39TK mRNA expression *in vitro* was determined in lung fibroblasts, CD16/CD32-positive immune cells, and isolated AEC2, analyzed via RT-PCR for SR39TK expression (Figure 1B). SR39TK expressing and nonexpressing plasmids were used as controls. All three wild-type cell populations and SPCTK fibroblasts were negative for SR39TK expression, whereas AEC2 isolated from

Figure 1. Validation of surfactant protein C (SPC)-SR39TK (SPCTK) sensitivity and expression. (A) Untreated SPCTK and wild-type type 2 alveolar epithelial cells (AEC2) proliferate over 96 hours of culture with significant increases in cell number at 72 and 96 hours. In ganciclovir (GCV)-treated AEC2 from SPCTK mice, cell growth was significantly reduced at 72 and 96 hours. (B) SR39TK mRNA was detected in AEC2 and immune cells from SPCTK, but not wild-type, mice. (C) AEC2 isolated from SPCTK mice exhibited reduced viability after GCV treatment, whereas the treated viability of fibroblasts and immune cells was unchanged. Data are presented as mean $\pm$ SD ($n = 3$). * $P \leq 0.05$ and $^{\dagger}P \leq 0.005$ versus untreated SPCTK and wild-type mice at the same time point. $^{\ddagger}P \leq 0.005$ versus 0-hour untreated SPCTK and wild-type mice. $^{\S}P \leq 0.005$ versus untreated AEC2. A, alveolar epithelial type 2 cells (AECs); F, fibroblasts; I, immune cells.

SPCTK mice were SR39TK positive. Surprisingly, SR39TK mRNA was also detected in the SPCTK immune cell population, although whether this is the result of SPC expression or phagocytosis of dying AEC2 is unknown. To determine if functional SR39TK protein was present within non-AEC2 populations, fibroblasts, immune cells, and AEC2 isolated from SPCTK mice were cultured for 24 hours and treated with high-dose GCV (5 μ M) or vehicle (Figure 1C). As expected, AEC2 cultured from SPCTK mice and treated at 24 hours with 5 μ M GCV showed significantly reduced viability at 48, 72, and 96 hours ($P < 0.05$). No significant change in cell viability was observed in either GCV- or vehicle-treated fibroblasts and immune cells. These results confirmed that active SR39TK was not present in fibroblasts or immune cells of SPCTK animals and that these populations were insensitive to GCV treatment. In addition, down-regulation of SPC expression in long-term AEC2 cultures correlated with down-regulation of SR39TK expression and resulted in loss of sensitivity to GCV (Figures 1D and 1E). Together these results indicated that GCV-directed SR39TK-induced killing in the lung is confined to SPC-expressing cells.

SPCTK Mice Exhibit GCV Dose-Dependent Survival *In Vivo*

The appropriate dosing range of GCV for ablating AEC2 in SPCTK mice *in vivo* was based on prior studies using the SR39TK mutant (19). HSV1-TK/GCV treatment strategies require high doses of up to 375 mg/kg GCV administered via implanted miniosmotic pump or by 5-day intraperitoneal injection (29). Due to the higher sensitivity of SR39TK, GCV could be administered to SPCTK mice at lower doses of 10, 50, and 100 mg/kg by 3-day intraperitoneal injection (Figure 2A). Using this dosing regime, 100 mg/kg GCV proved to be lethal by 13 days after treatment for SPCTK mice (Figure 2B). SPCTK mice exhibited 93 and 100% survival by 28 days after the 50 and 10 mg/kg GCV treatments, respectively. GCV was not lethal at any dose for wild-type mice.

GCV Treatment Causes Specific, Gross Changes in Alveolar Morphology in a Dose-Dependent Manner

Treatment of SPCTK mice with GCV caused notable alterations of alveolar

Figure 2. GCV treatment and SPCTK survival. (A) SPCTK mice at 2 months of age were dosed with GCV on 3 consecutive days and killed at 0 to 60 days after treatment. (B) SPCTK mice receiving 100 mg/kg GCV displayed 100% mortality by Day 12 after treatment, whereas 50 mg/kg GCV caused only 2% mortality by Day 28 after treatment. SPCTK mice treated with 10 mg/kg GCV exhibited no mortality over the 28-day post-treatment period (10 mg/kg GCV, $n = 25$; 50 mg/kg GCV, $n = 82$; and 100 mg/kg GCV, $n = 15$).

morphology, which corresponded to GCV dose and recovery time. In hematoxylin and eosin-stained lung sections of 50 mg/kg GCV-treated mice, notable septal hypercellularity, infiltration of cells into alveolar septal walls, and alveolar collapse were evident at 14 and 28 days after treatment (Figures 3, arrows and E2). In SPCTK mice that received 10 mg/kg GCV, alveolar structure was only mildly affected at 14 days and appeared restored by 28 days. By 60 days, normal alveolar architecture was apparently restored in both the 10 and 50 mg/kg GCV-treated cohorts, although lingering infiltrate could occasionally be observed in the latter. In 100 mg/kg GCV-treated mice, alveolar collapse, septal hypercellularity, and immune cell infiltration progressed very rapidly and reached levels that substantially compromised alveolar morphology by 10 days after GCV treatment.

AEC2 Depletion Can Be Precisely Controlled in SPCTK Mice

Fluorescent immunohistochemical staining was used to visualize alveolar integrity

(Figure 4). The distribution of AEC1 marker T1 α was not obviously altered after 10 mg/kg GCV treatment. Foci of alveolar collapse and disrupted T1 α -expressing AEC1 (Figure 4, arrows) were observed in SPCTK mice that received 50 mg/kg GCV, whereas 100 mg/kg GCV-treated lungs displayed widespread alveolar collapse and AEC1 disruption (14 and 10 d after treatment, respectively). No CC10-positive airway cells were observed within the alveolar compartment over the 60-day recovery period after injury and CC10-positive airway cells were not obviously altered (not shown). A reduction of SPC-positive AEC2 was apparent at 7 to 14 days for all doses of GCV. To specifically determine the impact of varying GCV treatments on AEC2 *in vivo*, fibroblast- and CD45⁺ immune cell-depleted AEC2 isolated from GCV-treated SPCTK mice and wild-type controls were quantitated by FACS. In untreated SPCTK mice, SPC-positive cells comprised 26.3% of the fibroblast-negative, CD45-negative population (Figure 5). Immunohistochemical analysis of cytoplasts

Figure 3. Gross changes in alveolar morphology in SPCTK mice. Hematoxylin and eosin-stained lung sections of GCV-treated mice demonstrate interstitial hypercellularity, thickened alveolar septae, and fibrotic foci (arrows), which is markedly increased in the 50 and 100 mg/kg cohorts (≥ 3 mice per group) at 14 and 28 days after treatment. By 60 days after treatment, lungs of mice that received 10 and 50 mg/kg GCV return to apparently normal architecture. Scale bar = 200 μ m.

from these isolates showed the SPC-negative population was mainly composed of platelet endothelial cell adhesion molecule (CD31⁺)-positive endothelial cells and cytokeratin-positive cells of unknown origin. CC10-positive club cells

were only occasionally observed, and T1 α -positive AEC1 were rare (Figure E3). The percentage of AEC2 in the fibroblast- and CD45-negative population is within the range established for these cells in distal lung by alternative sorting approaches (30).

After GCV treatment of SPCTK-derived AEC2, the abundance of survivors was dose and time dependent. By 7 days after GCV, SPCTK mice receiving all GCV doses displayed a 38 to 52% reduction of SPC-positive cells ($P \leq 0.05$). At Day 14, mice that received 10 mg/kg GCV showed a rebound of SPC-positive cells to 145% of the number recorded for untreated SPCTK mice before a return to baseline percentage by Day 28. In contrast, mice that received high doses of GCV showed an extended period of decline in SPC-positive cell number. In the 50 mg/kg GCV cohorts, AEC2 were maximally depleted by 80% at 14 days after treatment ($P \leq 0.05$), whereas 100 mg/kg treatment resulted in 82% reduction of AEC2 by Day 10 ($P \leq 0.05$). By Day 28 after treatment, the surviving 50 mg/kg GCV cohort exhibited a partial restoration of SPC-positive cell numbers to 62% of untreated SPCTK mice. Whether SPC-positive survivors of this treatment are capable of fully restoring AEC2 numbers to normal levels after additional recovery time is unknown. In wild-type mice, AEC2 percentages were not significantly altered by 10 or 50 mg/kg GCV across all time points ($n = 1$ for each time point with data pooled to establish mean). However, wild-type mice treated with 100 mg/kg GCV displayed mild toxicity with an approximately 29% reduction in AEC2 across all time points. These findings demonstrate that in SPCTK mice, AEC2

Figure 4. Cellular alterations in GCV-treated SPCTK mice. GCV-treated SPCTK mice exhibit regions of fibrotic foci of collapsed alveoli (arrows). T1 α expression in AEC1 (red) is reduced or lost in fibrotic foci of 50 mg/kg GCV at 14 days after treatment and in large areas of lung in the 100 mg/kg GCV cohort by 10 days after treatment. At both the 10 and 50 mg/kg treatment levels, reduced numbers of SPC-positive AEC2 (green) are apparent by 14 days after treatment. Scale bar = 200 μ m (≥ 3 mice per group).

Figure 5. AEC2 depletion in SPCTK mice. Treatment of SPCTK mice with GCV causes progressive time- and dose-dependent reduction in AEC2 abundance. In SPCTK mice that received 10 mg/kg GCV, AEC2 are significantly depleted by 50% by Day 7 after treatment before rebounding and returning to normal levels by Day 28. AEC2 abundance in SPCTK mice treated with 50 mg/kg GCV showed significant depletion of 52% at 7 days and 80% at 14 days after treatment and was partially restored to normal levels at Day 28. In the 100 mg/kg GCV cohort, AEC2 abundance is significantly depleted by 83% at 10 days after treatment. AEC2 abundance in pooled, treated, wild-type (WT) lungs did not significantly vary from untreated SPCTK controls at any dosage. Data are presented as mean $\pm$ SD, treated WT = n of 1 from each treatment group (pooled n = minimum of 4; n = minimum of 3 for all other groups). * $P \leq 0.05$. § $P \leq 0.005$.

depletion and recovery are GCV time- and dose-dependent and suggest that the 50 mg/kg dose invokes an AEC2 reduction close to the survivable maximum.

Administration of 10 versus 50 versus 100 mg/kg GCV in SPCTK Mice Drastically Alters Phenotypic Outcomes

To determine the acute changes in survivor AEC2 and whole lung that result from lethal AEC2 depletion after treatment with 100 mg/kg GCV, as well as the AEC2 survivor and whole lung changes that result from sublethal AEC2 depletion using 10 and 50 mg/kg doses, AEC2 and whole lungs were isolated from duplicate GCV-treated wild-type and SPCTK cohorts. As a first test, pulmonary mechanics were measured in 10 and 50 mg/kg GCV-treated mice before whole lung harvest. Data from 60 days after injury are presented in Figure 6. This time point for analysis was chosen to eliminate any confounding influence of infiltrate still present in lungs at earlier time points. Because 100 mg/kg GCV-treated animals did not survive beyond 13 days, they were not included in this analysis. Pressure-volume (PV) loops describe the mechanical behavior of the lungs and chest wall during inflation and deflation. A shift of the PV loop upward along the volume axis results indicates the development of a more compliant, less elastic lung (31–33).

Conversely, a shift of the PV loop downward along the volume axis indicates that more pressure is required to inflate the lungs to a given volume (32). At 60 days of recovery after 10 mg/kg GCV treatment, we recorded a marked upward shift in PV loops (Figure 6A). Additionally, quasistatic compliance, which describes the elastic recoil of the lungs at a given volume, was significantly increased ($P \leq 0.05$) (Figure 6B) in SPCTK mice versus wild-type controls, demonstrating the development of a more compliant phenotype. No differences in PV loops or quasistatic compliance were observed at 60 days after treatment with 50 mg/kg GCV. In addition, measurements of airway resistance demonstrated no significant changes after 10 or 50 mg/kg GCV treatment (Figure 6C), supporting the hypothesis that the SPCTK model is an efficient model for targeted alveolar injury with a minimal impact on the airway. The basis for the increase in quasistatic compliance and the upward shift in PV loops in lungs 60 days after 10 mg/kg GCV treatment was not due to the presence of more open air spaces as measured by mean linear intercept analysis (Figure E4) or altered levels of elastin in whole tissue (Figure E5).

GCV Treatment Alters Collagen Deposition in Lungs of SPCTK Mice

SPCTK mice display progressive accumulation of collagen in the distal lung

compartment after GCV treatment (Figures 7 and E6). In all cohorts of GCV-treated SPCTK mice, collagen accumulation in the alveolar septal walls was observed by 10 to 14 days after treatment. In the 50 mg/kg cohort, collagen accumulation was seen in alveolar septal walls surrounding foci of small fibrotic lesions (arrow) at 14 days, which became more pronounced at 28 days after treatment (arrow). Whereas fibrotic lesions in the 10 and 50 mg/kg GCV-treated cohorts resolved to near baseline levels by 60 days after treatment, small, abnormal foci persisted in the 50 mg/kg GCV-treated SPCTK mice (Figure E6). Collagen deposition was much more advanced in mice that received the 100 mg/kg GCV treatment, with substantial collagen deposition evident at 10 days after treatment. Although the identity of collagen-producing cells was not established, these data indicate a robust response of many distal lung cell types after AEC2 depletion that could include fibroblasts, myofibroblasts, injured or survivor AEC2, and/or infiltrating or resident macrophages.

Levels of whole lung soluble collagen, which represents the newly synthesized and/or recycled form, and deposited collagen, which represents the more stable, crosslinked form, were measured in untreated SPCTK and wild-type mice and in all GCV-treated SPCTK cohorts at

Figure 6. Measurements of pulmonary mechanics after mild versus severe AEC2 depletion. (A) Pressure-volume loops of SPCTK mice given 10 mg/kg GCV shifted upward versus wild-type at 60 days (top), whereas the 50 mg/kg GCV-treated cohort showed no change (bottom). (B) Measurement of quasistatic compliance demonstrates a significant increase 60 days after 10 mg/kg GCV treatment representing the development of an emphysematous phenotype, with no change observed after 50 mg/ml GCV treatment. (C) Measurements of resistance in the 10 and 50 mg/kg GCV-treated cohorts at 60 days demonstrated no difference between the wild-type and SPCTK cohorts. Data in A are presented as mean $\pm$ SEM; data in B and C are presented as mean $\pm$ SD ($n = 6$ per genotype and dose). * $P \leq 0.05$.

28 and 60 days after treatment (Figure 8). Deposited collagen (via hydroxyproline assay) was significantly increased at 60 days of recovery in 10 mg/kg GCV-treated SPCTK mice ($P \leq 0.05$) and at 28 and 60 days of recovery ($P \leq 0.05$) in 50 mg/kg GCV-treated SPCTK mice (Figure 8A). Conversely, soluble collagen was significantly decreased ($P \leq 0.05$) at 28 days of recovery in the 10 mg/kg GCV-treated mice and remained unchanged in 50 mg/kg GCV-treated SPCTK mice (Figure 8B). In the 100 mg/kg GCV-treated cohort, a significant increase in soluble collagen was noted at 10 days of recovery ($P \leq 0.05$) (Figure 8B). These data indicate distinct

repair responses depending on the level of AEC2 depletion.

GCV Treatment in SPCTK Mice Alters AEC2 Survivor and Whole Lung Protein Expression

To examine molecular changes in AEC2 that survived TK killing, cells were isolated from untreated (U) and GCV-treated WT (T) and SPCTK mice (Figures 9A–9C). Expression patterns from GCV-treated SPCTK AEC2 lysates pooled from three animals were analyzed for each time point. Untreated AEC2 were also pooled from three individual animals, and GCV-treated WT control AEC2 were pooled from a single sample from each time point (T). Western

blotting for proliferating cell nuclear antigen (PCNA) expression was used as a marker for a cell cycle entry by survivor AEC2. In the 10 mg/kg cohort, PCNA expression was prolonged but was strongest at Day 14, which correlates with the strong rebound shown in Figure 5. In contrast, the most significant increase in PCNA expression in the 50 mg/kg cohort occurred at Day 7, which correlates with the lowest point in AEC2 numbers after depletion but presumably indicates a survival response. Likewise, the peak of PCNA expression in AEC2 survivors of lethal depletion occurred at Day 3, even as overall numbers were steadily decreasing. AEC2 lysates were also Western blotted to analyze expression of proapoptotic marker Bax and antiapoptotic protein Bcl_{XL} (Figures 9A–9C) as well as the ratio of the two (quantitation of Bax/Bcl_{XL} ratio by densitometry) (Figures 9D–9F). These data showed dominant expression of Bcl_{XL} in the early AEC2 survivor response to mild AEC2 depletion (Figures 9A and 9D; Days 0 and 3). In contrast, Bax expression in this cohort increased over time, presumably as a mechanism to return AEC2 numbers to baseline (Day 28). Severe depletion of AEC2 stimulated some Bax expression in survivors (Figures 9B and 9E; Days 0 and 3), but this was notably counteracted by Bcl_{XL} expression, which increased with time (50 mg/kg on Day 28). These data may indicate an attempt by survivor AEC2 to maintain a minimum population for recovery. There was no increase in Bcl_{XL} expression in survivors after lethal levels of AEC2 depletion (Figure 9C; Days 0, 3, and 7), and the ratio of Bax to Bcl_{XL} was high as remaining AEC2 rapidly began dying at Day 3 (Figure 9F).

To analyze the injury repair response in surviving AEC2, expression of 8-oxoguanine glycosylase (Ogg1), the primary driver of base-excision repair, and phosphorylated H2AX (P-H2AX), a marker for double-stranded DNA breaks that can stimulate either a DNA repair response or trigger apoptosis was examined (Figures 9A and 9B). These assays showed disparate changes in expression after mild, severe, and lethal depletion. In both the 10 and 50 mg/kg cohorts, Ogg1 expression was negligible in SPCTK AEC2 survivors at acute time points but increased at later stages (10 mg/kg, Days 7–28 and 50 mg/kg, Days 14 and 28). Ogg1 was not expressed in AEC2 survivors after 100 mg/kg GCV

Figure 7. Changes in collagen distribution after GCV treatment. Histological analysis of adult mouse lung tissue embedded in paraffin, stained with Sirius Red/FCF Green, for collagen visualization examined. Mice that received 10 mg/kg showed mild collagen accumulation, whereas the 50 mg/kg cohort showed increased collagen and fibrotic foci (arrows). Treatment with 100 mg/kg GCV caused rapid and severe collagen deposition and became lethal by 14 days. All collagen types, red; noncollagenous tissue; green/blue. Scale bar = 200 μ m (n = minimum of 3).

treatment. P-H2AX expression in 10 mg/kg GCV survivors peaked at Day 3 and remained elevated throughout recovery, whereas in the 50 mg/kg cohort, evidence of double strand breaks appeared at Day 0, regressed at Days 3 and 7, and then rose sharply at Days 14 and 28. In 100 mg/kg GCV-treated samples, there was a modest rise in P-H2AX at Day 3 and then complete loss of this marker by Day 7. These patterns of DNA damage and repair responses indicate a significantly different status for AEC2 survivors depending on the level of AEC2 depletion.

As suspected from analysis of isolated AEC2 and whole lung collagen, treatment of SPCTK animals with GCV also alters the protein expression of tissue remodeling proteins in whole lung (Figures 10A and 10B). In low-dose cohorts, changes in expression of the matrix metalloproteinase (MMP) proteins MMP-2 and MMP-9 were not notable until late in recovery (Day 60), which could account for the shift in pulmonary mechanics at this time point (Figure 6). Activation of MMPs in the 50 mg/kg cohort showed increased expression of MMP-2 and MMP-9 much

earlier at Days 3 to 28. MMP expression and activation at the early stage after severe AEC2 depletion may be stimulated by injury-responsive cytokines and chemokines released from damaged vasculature, which may also be the source of the infiltrate observed at these same time points after severe AEC2 depletion (Figures 3 and 7) (27, 34, 35).

Discussion

Previous models of targeted AEC2 depletion have used the diphtheria toxin system to induce cell injury or death. Sisson and colleagues used the human SPC promoter to drive expression of the diphtheria toxin receptor not normally present in the mouse (17), resulting in significant increases in collagen levels by 14 days after treatment, and, ultimately, fibrosis. However, the authors reported no observable difference in numbers of SPC-positive AEC2 at any time point, and only a modest reduction of SPC gene expression was observed at 14 days after treatment. The use of the inducible SPC-creER system by Barkauskas

and colleagues to drive constitutive expression of the DTA subunit in SPC-expressing AEC2 after a single dose of tamoxifen (18) caused up to 20% lethality and activated DTA expression in up to an estimated 52% of SPC-expressing cells, although the exact percentage of SPC-positive cells depleted was unreported. This model demonstrated early apoptosis in AEC2 and qualitatively reported a return to normal AEC2 distribution by 21 days, with only minor histologic changes in lung structure and no fibrosis. In both cases, the DT system proves to have a limited dosing range between effective and lethal treatment levels. Comparison of these models raises the key question of why similar models of AEC2 depletion elicit such varied outcomes. We believe the data generated by the SPCTK model, where the severity of AEC2 depletion closely linked to outcomes within specific time frames, can explain these differences.

The SPCTK model has several unique features that make it an excellent tool for elucidating the contribution of AEC2 to repair, rehabilitation, and/or regeneration of injured lung. These features include (1) the

Figure 8. Assessment of whole lung hydroxyproline content and soluble collagen in wild-type versus SPCTK GCV-treated cohorts. (A) Hydroxyproline content analysis of 10 mg/kg GCV-treated cohorts indicated a significant increase in deposited collagen in SPCTK mice at 60 days after GCV treatment. The 50 mg/kg GCV-treated cohorts demonstrated significantly increased hydroxyproline content at both 28 and 60 days after GCV treatment, whereas the 100 mg/kg GCV-treated cohorts, which were killed at 10 days after GCV treatment, did not display a significantly increased hydroxyproline content. (B) Assessment of soluble collagen content in the 10 mg/kg cohorts demonstrates a significantly decreased level only at 28 days after GCV treatment. The 50 mg/kg cohorts demonstrated no difference in soluble collagen content at either 28 or 60 days, whereas significantly elevated levels of soluble collagen were recorded in the 100 mg/kg cohorts at 11 days after GCV treatment. Data are presented as mean $\pm$ SD (n = minimum of 3). * $P \leq 0.05$.

GCV/SR39TK-directed ablation approach that provides a wide dosable range to precisely control the severity of AEC2 injury or depletion and (2) the ability to link the severity of AEC2 injury or depletion with stereotypical AEC2 survivor and whole lung response and repair patterns.

Although the approach used by Barkauskas and colleagues, in which the DTA subunit knocked into the murine *Sftpc* locus no doubt provides highly specific targeting of SPC-expressing AEC2, the dosable range of all DT models is limiting (18). We chose to construct a model in which expression of SR39TK is driven by the well-characterized human SPC promoter, which has been shown to be specific for murine AEC2 in adult lung (21, 24, 36, 37, 38). As integration effects on expression pattern of the human SPC promoter have been noted (24), we validated the specificity of resulting

SR39TK expression *in vitro* and *in vivo* and showed that the most direct effects of SR39TK were confined to SPC-positive expressing AEC2. This was supported by histological observations and pulmonary mechanics, which showed no impact of GCV treatment on SPCTK airway structures or function.

The TK obliteration system uses expression of the HSV1-TK, which is reportedly not itself harmful to mammalian tissues (39–41). Unlike mammalian TK, viral TKs phosphorylate the nucleoside analogs acyclovir and GCV, which are further phosphorylated by cellular kinases and incorporated into DNA to cause inhibition of DNA synthesis and cell death (40, 41). Unfortunately, HSV1-TK requires high doses of GCV to initiate cell death and can have off-target effects (42). The SR39TK mutant, which exhibits substantially higher sensitivity, allows for

the use of GCV doses an order of magnitude lower than previously reported for HSV1-TK (20, 28). Thus, the SPCTK model demonstrates a wide dosable range with a 10-fold difference between the minimal effective dose and a lethal dose (10 and 100 mg/kg GCV, respectively) and induces specific and quantifiable levels of AEC2 depletion.

Using doses of 10, 50, and 100 mg/kg GCV, we observed a broadly similar reduction of AEC2 numbers over the first 7 days after treatment. Beyond this time point, AEC2 survivor and whole lung responses followed divergent paths. Treatment with the highest dose (100 mg/kg) proved lethal at 13 days due to pulmonary edema, with an 82% reduction of AEC2. Interestingly, although a 50 mg/kg GCV dose caused an 80% depletion of AEC2 over a slightly longer period (14 d), the majority of this cohort (93%) did not develop fatal pulmonary

Figure 9. Changes in survivor AEC2 apoptotic, DNA damage, and damage repair proteins. Lysates of AEC2 isolated from untreated SPCTK (U) or from treated wild-type (T) or from GCV-treated mice were probed for expression of proliferating cell nuclear antigen (PCNA), Bax, Bcl_{XL}, Ogg1, and P-H2AX by Western blotting. SPCTK samples for each time point after GCV treatment were pooled from three animals. Untreated SPCTK samples were also from three animals. Treated wild-type samples were an *n* of 1 for each time point and were pooled to generate the T sample. Actin expression was used as a loading control. (A) Protein expression patterns in AEC2 isolated from 10 mg/kg GCV-treated mice. (B) Protein expression patterns in AEC2 isolated from 50 mg/kg GCV-treated mice. (C) Protein expression patterns in AEC2 isolated from 100 mg/kg GCV-treated mice. Blots were scanned using densitometry, and the ratio of Bax/Bcl_{XL} expression was calculated. (D) Bax/Bcl_{XL} ratio

edema but progressed to fibrotic injury at 28 days after treatment. This difference suggests that the depletion achieved by the 50 mg/kg GCV treatment is very near the maximal survivable level, but the rate of killing may be slow enough that a viable survivor AEC2 population, or contributions from other distal lung epithelial progenitors can eventually drive recovery. At the other end of the spectrum of lung injury, treatment of SPCTK mice with 10 mg/kg GCV caused only a modest reduction in AEC2 and featured a significant rebound in AEC2 numbers over the course of recovery.

In the 10 mg/kg GCV-treated cohort, the AEC2 rebound resolved rapidly and was accompanied by increased expression of the apoptotic factor Bax in survivor AEC2 28 days after treatment, perhaps as a response to the rebound at Day 14. Increased whole lung MMP-2 and MMP-9 expression was also noted at Day 60, indicating late remodeling. Both of these changes are believed to contribute to the late change in mechanics at Day 60. In contrast, the 50 mg/kg cohort showed no late change in mechanics. Instead of late and predominant Bax expression, increased expression of the antiapoptotic protein Bcl_{XL} was observed in survivor AEC2 isolated at the 28-day time point, as was a modest increase in MMP-2 and MMP-9 expression that peaked at Days 14 to 28. We hypothesize that the combination of alveolar destruction and deposited collagen still present late in recovery, coupled with the inherent nature of the mechanical tests performed to measure pulmonary mechanics as an aggregate of the whole lung, resulted in the lack of a measured difference.

In the 10 mg/kg GCV-treated cohort, the late shift in mechanics, the increase in hydroxyproline content, and the increased AEC2 Bax and whole lung MMP-2 and MMP-9 expression at Day 60 after injury may represent long-term remodeling, although the trigger for this delayed response is unclear (43). Taken together with data from the 50 mg/kg GCV-treated SPCTK, with its increase in hydroxyproline

Figure 9. (Continued). after 10 mg/kg GCV treatment compared with control ratios. (E) Bax/Bcl_{XL} ratio after 50 mg/kg GCV treatment compared with control ratios. (F) Bax/Bcl_{XL} ratio after 100 mg/kg GCV treatment compared with control ratios.

Figure 10. Changes in whole lung tissue remodeling protein expression. Lysates from GCV-treated mice were probed for expression of matrix metalloproteinase (MMP)-2, MMP-9, and actin. SPCTK samples for each time point after GCV treatment were pooled from three animals. Treated wild-type samples were an *n* of 1 for each time point and were pooled to generate the T sample. Actin expression was used as a loading control. (A) Protein expression patterns in whole lung isolated from 10 mg/kg GCV-treated mice. (B) Protein expression patterns in whole lung isolated from 50 mg/kg GCV-treated mice. Asterisks demonstrate late rise in expression of MMP-2 and MMP-9, 60 days post-GCV treatment.

over a broad period (Days 28 and 60), characteristic of classical fibrotic remodeling (44), and slow restoration of AEC2 numbers, these analyses suggest that the injury-repair process after both 10 and 50 mg/kg treatments is not complete by 60 days. In contrast, although the 10-day survival period for the 100 mg/kg cohorts was too acute for significant changes in hydroxyproline to be recorded, a significant increase in soluble collagen was observed by Day 11, accompanied by the hypercellularity and edema that proved to be fatal in this SPCTK cohort. Presumably the whole lung response to AEC2 destruction at all levels in treated SPCTK mice is to increase both soluble and

crosslinked collagen in an attempt to repair the vascular leak (as evidenced by cellular infiltration) induced by AEC2 destruction.

Conclusions

The concept of lung disease as a disorder of epithelial death or disrepair, particularly in the case of pulmonary fibrosis as popularized by Haschek and Witshchi (15) (for review, see Ref. 16), has made a resurgence. We believe the SPCTK model supports this hypothesis. When AEC2 death is modest, transient, and readily resolved, as in SPCTK mice receiving 10 mg/kg GCV, the return to apparently normal lung architecture can still have

a long-term impact on lung function. When severe or prolonged AEC2 death or dysfunction occurs, unresolved injury to the alveolar epithelium causes increased collagen deposition and creates conditions in which very long periods of recovery are required. In contrast, rapid destruction of AEC2 at a high level causes significant, acute injury and death. The fact that these divergent, clinically relevant outcomes can be elicited by specific and quantifiable destruction of the AEC2 population supports the concept that AEC2 play a critical role in maintaining lung homeostasis. ■

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