Bronchiolitis Obliterans and Pulmonary Fibrosis after Sulfur Mustard Inhalation in Rats

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Abstract

Inhalation of powerful chemical agents, such as sulfur mustard (SM), can have debilitating pulmonary consequences, such as bronchiolitis obliterans (BO) and parenchymal fibrosis (PF). The underlying pathogenesis of disorders after SM inhalation is not clearly understood, resulting in a paucity of effective therapies. In this study, we evaluated the role of profibrotic pathways involving transforming growth factor- β (TGF- β) and platelet-derived growth factor (PDGF) in the development of BO and PF after SM inhalation injury using a rat model. Adult Sprague-Dawley rats were intubated and exposed to SM (1.0 mg/kg), then monitored daily for respiratory distress, oxygen saturation changes, and weight loss. Rats were killed at 7, 14, 21, or 28 days, and markers of injury were determined by histopathology, pulmonary function testing; and assessment of TGF- β , PDGF, and PAI-1 concentrations. Respiratory distress developed over time after SM inhalation, with progressive hypoxemia, respiratory distress, and weight loss. Histopathology confirmed the presence of both BO and PF, and both gradually worsened with time. Pulmonary function testing demonstrated a time-dependent increase in lung resistance, as well as a decrease in lung compliance. Concentrations of TGF- β , PDGF, and PAI-1 were elevated at 28 days in

lung, BAL fluid, and/or plasma. Time-dependent development of BO and PF occurs in lungs of rats exposed to SM inhalation, and the elevated concentrations of TGF- β , PDGF, and PAI-1 suggest involvement of these profibrotic pathways in the aberrant remodeling after injury.

Keywords: PAI-1; sulfur mustard; transforming growth factor- β ; lung fibrosis; bronchiolitis obliterans

Clinical Relevance

Sulfur mustard inhalation exposure causes bronchiolitis obliterans and pulmonary fibrosis in humans. Because no human studies can be performed with sulfur mustard, animal studies are done to find an antidote. To our knowledge, this paper describes the first animal model of bronchiolitis obliterans and pulmonary fibrosis late after sulfur mustard inhalation exposure. We found that transforming growth factor- β 1, platelet-derived growth factor, and PAI-1 are chronically elevated in rat lungs exposed to sulfur mustard.

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Sulfur mustard (SM) is a chemical warfare agent first introduced during World War I. In the past 100 years, SM has been used extensively in the Middle East, resulting in more than 100,000 chemical casualties. Unfortunately, SM has remained a great threat in modern times (1). Exposure to SM causes acute injuries to the eyes, skin, and respiratory tract (2, 3). Airway injury is the principal cause of early mortality and morbidity and is dose dependent. We have previously delineated the mechanism involved in acute airway obstruction after SM inhalation (4–6). We have shown that increased bronchial vascular permeability occurs, with procoagulant plasma fluid leaking into the airway lumen through a layer of damaged airway epithelium, forming airway casts. When the fibrinolytic tissue plasminogen activator (7, 8) or the anticoagulant heparin (9) is delivered directly into the lungs, morbidity and mortality are significantly improved in the acute setting.

Chronically, survivors of SM inhalation experience late pulmonary sequelae related to exposure, including chronic bronchitis, bronchiolitis obliterans (BO), pulmonary fibrosis (PF), emphysema, and bronchiectasis (1–3, 9–15). Indeed, 75% of survivors exposed to SM in the Iran–Iraq conflict were noted to develop lung fibrosis (3, 16). Furthermore, a number of SM-exposed patients with normal imaging or pulmonary function test results were shown to have BO on the basis of lung biopsy (17–19).

The two major cytokines and pathways implicated in the development of lung fibrosis after chemical inhalation involve transforming growth factor- β (TGF- β) (20–22) and platelet-derived growth factor (PDGF) (20, 21, 23–26). Activated TGF- β is a strong stimulus for fibroblast recruitment and proliferation (27, 28). It has been implicated in the development of idiopathic PF (29) and has been noted to be increased in some patients with BO after SM inhalation (30, 31). PDGF, a profibrotic mitogen, also plays an essential role in fibroblast proliferation, differentiation, migration, and survival (32–34). Many fibroproliferative diseases of the lung have been linked to elevated levels of PDGF signaling (29, 35–37), including lung transplant-associated BO (38, 39). In addition to these cytokines, the procoagulant protein PAI-1 has also been implicated as having a role in the development of some fibrotic diseases (40).

Currently, no effective therapy exists for SM-associated fibrotic lung diseases.

Animal models can be extremely useful in understanding the pathogenesis of disease progression. Until now, animal models of SM inhalation have been focused mainly on the acute and subacute phases of injury (41–43) and less on chronic disease progression (4, 44). The goals of the present study were to evaluate the involvement of TGF- β 1, PDGF, and PAI-1 pathways in the development of chronic lung fibrosis seen after SM inhalation exposure. In addition, we aimed to evaluate clinically relevant parameters associated with pulmonary disease progression in a rat model of SM inhalation. We hope that, taken together, our findings could assist in the future development of new antifibrotic therapies for SM-exposed individuals.

Methods

See the data supplement for a detailed description of the methods.

Animals

This study was approved by the Institutional Animal Care and Use Committee of the University of Colorado Denver (UCD) and the U.S. Army Medical Research Institute of Chemical Defense (USAMRICD). The investigators adhered to the National Institutes of Health Guide for the Care and Use of Laboratory Animals (1996), and this research was carried out in compliance with the Animal Welfare Act.

Chemicals and Reagents

The SM agent was provided by the USAMRICD (Aberdeen Proving Ground, MD) or the UCD facilities (Aurora, CO). All other chemicals used were from purchased from Sigma-Aldrich unless otherwise stated.

SM Inhalation Exposure

SM inhalation exposure was induced at sea level at the USAMRICD Aberdeen Proving Ground and at altitude at the University of Colorado Denver (5,280 ft elevation). Rats were exposed to SM inhalation (1.0 ± 0.05 mg/kg) under general anesthesia, using an intubated SM vapor model as previously described by Anderson and colleagues (41).

Euthanasia

Animals were killed as scheduled on Day 7, 14, 21, or 28, or if they had weight loss greater than 30%, or when published euthanasia criteria were met (8). Animals

were killed by terminal ketamine/xylazine/acepromazine cocktail intraperitoneal administration, followed by diaphragmatic puncture and exsanguination.

BAL Fluid and Lung Tissue

Acquisition

After the animals were killed, the trachea was cannulated; the left bronchus was clamped; and the right lung was lavaged with two 2.5-ml washes of saline solution and pooled. The BAL fluid (BALF) samples were centrifuged at $2,000 \times g$ (10 min, 4°C), and the resulting supernatants were frozen (-80°C). The right lung was tied off and frozen. The left lung was then unclamped and intratracheally fixed with paraformaldehyde (4% in PBS) at 20 cm H_2O for 30 minutes before removal by gross dissection.

Clinical Data and Supplemental Care

The animals were weighed and monitored for respiratory distress (8) once daily. The flexiVent system (SCIREQ) was used for pulmonary function testing in sedated rats after tracheotomy. Pulse oximetry (Starr Life Sciences pulse oximeter) was used to measure heart rate and oxygen saturation daily, as well as during flexiVent analysis. A subcutaneous fluid bolus was administered daily (10 ml/kg) if greater than 20% weight loss occurred.

Histology and Immunofluorescence

Histological slides were stained with hematoxylin and eosin or Masson's trichrome stain (kit number AR173; Dako), and a modified Ashcroft scoring technique was followed as previously described (45). For immunofluorescence, cells containing TGF- β 1 and platelet-derived growth factor receptor- α (PDGFR- α) were localized in sections with primary rabbit antirat TGF- β 1 or PDGFR- α (1:1,000; Abcam) or with IgG control (1:1,000; Dako), followed by secondary Alexa Fluor goat antirabbit antibody (1:1,000; Thermo Fisher Scientific) and DAPI Fluoromount-G mounting medium (SouthernBiotech).

Cytokine/Protein Quantification

ELISAs for TGF- β 1 and PDGF-A/B (R&D Systems), and PAI-1 (Molecular Innovations) were performed on BALF, plasma, and/or homogenized right lower lobes. Total protein was measured for cytokine normalization using the Pierce bicinchoninic acid assay kit (Thermo Fisher Scientific).

Statistical Analysis

Prism 6.0 software (GraphPad Software) was used for statistical analysis with one-way ANOVA followed by Tukey's *post hoc* analysis of multiple comparisons with naive controls. A log-rank (Mantel-Cox) test was performed for survival analysis. Mean values are reported with SEM. $P < 0.05$ was considered significant.

Results

Lethal Dose Evaluation

A Kaplan-Meier survival curve was generated for each SM exposure dose at sea level (see Figure 1A). At the highest SM dose of 3.8 mg/kg ($n = 12$), inhalation resulted in a median lethal dose (LD_{50}) at 8.4 hours and an absolute lethal dose (LD_{100}) at 12 hours. At a 1.4-mg/kg SM inhalation dose ($n = 9$), the LD_{50} was at 2.5 days and an LD_{100} at 20 days. Exposure to 1.0 mg/kg ($n = 20$) resulted in an LD_{50} at 4.8 days and a 70% LD at 28 days, whereas exposure to 0.75 mg/kg ($n = 12$) resulted in an LD_{50} at 26 days and an 83% LD at 28 days. The lowest inhaled dose (0.5 mg/kg; $n = 13$) was the least lethal, with an 8% LD at 28 days.

We then repeated select dosimetric studies at Denver altitude (5,280 ft) to validate the model we obtained at sea level (see Figure 1B). At a 1.0-mg/kg SM exposure, lethality was not different between altitude ($n = 29$) and sea level ($n = 14$) ($P = 0.98$). In both models, mortality occurred in a biphasic mode, with the initial onset of deaths occurring acutely within the first 7 days after exposure, followed by a secondary progressive decline beginning 14 days after exposure.

Oxygen Saturation

Oxygen saturation as measured by pulse oximetry (Sp_{O_2}) for rats exposed to 1.0 mg/kg SM was obtained at both at sea level ($n = 29$) and at high altitude (5,280 ft; $n = 14$) (see Figure E1 in the data supplement). At sea level, Sp_{O_2} fell to less than 90% on Day 1 after exposure, reaching the lowest level on Day 5 ($61.7 \pm 8.6\%$) and improving to greater than 90% after Day 7 ($91.5 \pm 2.0\%$). On Day 13, Sp_{O_2} fell again to less than 90%, with a drastic rate of decline, reaching less than 70% by Day 19 ($68.9 \pm 3.0\%$), where it stayed until Day 28. At high altitude, Sp_{O_2} fell to less than 90% on Day 1 ($85.6 \pm 1.2\%$) and remained below 90% for the entire study. Sp_{O_2} stabilized on Days

Figure 1. Kaplan-Meier survival curves after sulfur mustard (SM) exposure. (A) Various exposure doses of SM inhalation in rats at sea level have a dose-dependent lethality over 28 days. Black line = 0.5 mg/kg; purple line = 0.75 mg/kg; blue line = 1.0 mg/kg; green line = 1.4 mg/kg; red line = 3.8 mg/kg. (B) Kaplan-Meier survival curve at Denver altitude (5,280 ft) (blue line) and at the Aberdeen Proving Ground (sea level) (black line) with 1.0 mg/kg SM exposure. Lethality was not significantly different between altitude and sea level ($P = 0.98$). In both models, mortality occurred in a biphasic mode, with the initial onset of death occurring acutely within the first 7 days after exposure, followed by a secondary progressive decline beginning 14 days after exposure.

2–6 ($88.0 \pm 1.1\%$), but on Day 7 it began to decline progressively ($84.8 \pm 1.4\%$), reaching less than 70% by Day 19 ($66.8 \pm 2.5\%$), where it stayed until Day 28. At both locations, progressive Sp_{O_2} decline was noted between Days 7 and 28.

Weight Loss and Clinical Distress Scores

Weight change from baseline was measured daily after inhalation exposure to 1.0 mg/kg SM in rats, and results were compared between altitude ($n = 29$) and sea level ($n = 14$) (see Figure E2). At altitude, weight decreased immediately after exposure and

reached a nadir on Day 1 ($-9.5 \pm 0.9\%$ change from baseline). From Days 2 to 12, steady weight gain occurred ($2.1 \pm 0.5\%$ increase/d) with weight above baseline by Day 7. After Day 12, a steady weight decline was again noted until the end of the study. At sea level, weight also decreased immediately after exposure and reached a nadir on Day 2 ($-11.6 \pm 1.0\%$ change from baseline). From Days 2 to 14, steady weight gain occurred ($2.3 \pm 1.3\%$ increase/d), with weight above baseline by Day 7. After Day 14, a steady weight decline was also noted until the end of the study, and this was more drastic than at altitude, with

a maximum weight loss noted on Day 28 ($-15.5 \pm 2.7\%$).

Next, we evaluated clinical distress scores over 28 days after 1.0-mg/kg SM inhalation, and results were compared between altitude ($n = 29$) and sea level ($n = 14$) (see Figure E3). During the acute phase of injury (within the first 7 d of SM exposure), we found that the clinical distress scores were significantly higher at sea level than at altitude (peak clinical score, 3.59 ± 0.44 vs. 1.17 ± 0.22 , respectively; $P < 0.0001$), but this difference normalized by Day 11. After Day 11, clinical distress scores at both sites increased similarly and progressively until the end of the study at Day 28.

Pulmonary Function Testing

Pulmonary function testing via the flexiVent apparatus was performed before animals were killed at Days 7 ($n = 6$), 14 ($n = 4$), 21 ($n = 6$), and 28 ($n = 2$) after inhalation exposure to 1.0 mg/kg SM in rats (see Figures 2A–2D). Owing to low survival at 28 days, only a few animals were still alive for flexiVent analysis at that time point. We found that before Day 14 after exposure, total lung resistance of exposed animals did not statistically differ from that of naive control animals ($n = 9$) (see Figure 2A). However, by Day 21, SM-exposed animals had a 13-fold increase in total lung resistance relative to naive control animals (means, 1.16 ± 0.12 cm H₂O · s/ml vs. 0.09 ± 0.13 cm H₂O · s/ml; $P < 0.0001$). By 28 days, total lung resistance was even more elevated in exposed rats (1.30 ± 0.03 cm H₂O · s/ml). Newtonian resistance, a measurement representative of central airway resistance, was also found to be increased at Days 21 and 28 after SM inhalation exposure compared with that in naive control animals (see Figure 2B). By Day 21, we found that Newtonian resistance was elevated 8.5-fold from naive levels (mean, 0.25 ± 0.14 vs. 0.04 ± 0.03 cm H₂O · s/ml; $P < 0.05$) and remained elevated at Day 28. Similarly, tissue damping, a measurement representative of the parenchymal resistance component, was also found to be increased after SM compared with that in naive control animals (see Figure 2C). By Day 21, we found that tissue damping was elevated eightfold from naive levels (mean, 2.69 ± 0.31 vs. 0.33 ± 0.02 cm H₂O/ml; $P < 0.0001$) and remained elevated similarly at Day 28. Expectedly, total lung compliance declined in a temporal

Figure 2. Lung function testing in rats exposed to SM (1.0 mg/kg) compared with naive control rats at 7, 14, 21, and 28 days. (A) Mean total lung resistance (R ; cm H₂O · s/ml) increased over time after SM exposure. At Days 7 and 14, R was not different from that in naive control rats, but by Day 21, R was significantly elevated ($P < 0.0001$), and it remained elevated at Day 28 ($P < 0.0001$) compared with naive levels. (B) Mean Newtonian resistance (R_n ; cm H₂O · s/ml) increased over time after SM exposure. At Days 7 and 14, R_n was not different from that in naive control rats, but by Day 21, R_n was significantly elevated ($P < 0.05$), and it remained elevated at Day 28 ($P < 0.05$) compared with naive levels. (C) Mean tissue damping (G ; cm H₂O/ml) increased over time after SM exposure. At Days 7 and 14, G was not different from that of naive control rats, but by Day 21, G was significantly elevated ($P < 0.0001$), and it remained elevated at Day 28 ($P < 0.0001$) compared with naive levels. (D) Mean total lung compliance (C ; ml/cm H₂O) decreased over time after SM exposure. By Day 14, C was significantly decreased ($P < 0.0001$) compared with that of naive control rats, and it continued to decrease further at Day 21 ($P < 0.0001$) and Day 28 ($P < 0.0001$). Data are presented as mean $\pm$ SEM. P values were derived using ANOVA with Tukey's *post hoc* test. ** $P < 0.01$, **** $P < 0.0001$.

fashion (see Figure 2D) and was significantly decreased by 14 days with levels twofold lower than naive levels (mean, 0.27 ± 0.01 vs. 0.67 ± 0.05 ml/cm H₂O; $P < 0.0001$). By Day 21, a fourfold decrease was noted compared with naive levels (mean, 0.14 ± 0.01 ml/cm H₂O; $P < 0.0001$), which continued similarly at Day 28. These pulmonary function findings were consistent with a mixed obstructive and restrictive lung disease pattern.

Histopathological Evidence of BO

BO lesions were identified in distal bronchiolar airways by Day 21 postexposure

(see Figures 3B and 3D). Examination of histological lung sections of rats exposed to 1.0 mg/kg SM (Day 21; $n = 14$) revealed dense, consolidated collagen constricting the distal airways, whereas naive lungs were clear of these lesions (see Figures 3A and 3C). Findings of both constrictive (Figure 3B) and proliferative (Figure 3D) BO were present. Higher inhaled doses of SM showed worse obstruction by BO lesions (see Figure E4). Airway BO lesions were most pronounced in the dependent lobes, including the right lower lobe and lower portion of the left lobe. In addition, an increasing number of airways had

Figure 3. Histopathology of distal rat lung bronchioles after SM inhalation (1.0 mg/kg) exposure compared with that of naive control rats. (A) Naive rat bronchiole showing normal lung histology (Masson's trichrome stain; magnification, $\times 10$). (B) Representative rat bronchiole 28 days after SM inhalation, with significant collagen deposition (blue) and luminal narrowing, consistent with constrictive type bronchiolitis obliterans (Masson's trichrome stain; magnification, $\times 10$). (C) Higher-magnification view of naive control rat bronchiole showing normal histopathological pattern (Masson's trichrome stain; magnification, $\times 20$). (D) Higher-magnification view of a representative distal rat bronchiole 28 days after SM with extensive collagen deposition (blue), hypercellularity, and greater than 90% luminal narrowing, consistent with constrictive type bronchiolitis obliterans (Masson's trichrome stain; magnification, $\times 20$). Dot-dashed circle estimates the normal airway luminal circumference (red dashed line) (Masson's trichrome stain). Scale bars: 200 μm (A and B) and 100 μm (C and D).

abnormal collagen deposition over time, with greater than 75% of bronchioles affected by 28 days (data not shown). Thus, rats exposed to SM inhalation showed histological changes of both proliferative and constrictive BO by 21 days after the initial insult, and BO development was both time and dose dependent.

Histopathological Evidence of Parenchymal PF

Histological analysis of rat lungs after SM exposure (1.0 mg/kg; $n = 14$) revealed progressive interstitial fibrosis, or PF, over 28 days compared with naive rat lungs (see Figures 4A–4D). The distribution of these PF lesions was heterogeneous but present in all lung lobes, with a predilection for the subpleural regions as well as areas adjacent to BO lesions. PF was first noted on Day 14 after exposure (see Figure 4B), and

intensified by Days 21 and 28 (see Figures 4C and 4D). In addition, an increase in alveolar airspaces adjacent to fibrotic areas was noted at these later time points. Next, the severity of the PF lesions after SM exposure was quantitated using modified Ashcroft scoring (see Figure 5A and 5B). We found that all lung lobes were affected (see Figure 5A), with the right upper lobe and right lower lobe having the most severe lesions (scores of 3.50 ± 0.59 and 2.83 ± 0.75 , respectively; $P < 0.0001$). PF lesions were first noted by Day 17 ($n = 3$) (see Figure 5B), worsening in severity over time ($n = 3$; scores of 1.67 ± 0.33 at Days 17–18 vs. 4.00 ± 1.16 at Days 24–28; $P > 0.05$). Naive control animals had no PF lesions ($n = 12$; score of 0).

Cytokine/Protein Analysis

We next did an evaluation for alterations in concentrations of the profibrotic cytokines

TGF- $\beta 1$ and PDGF-A/B and the procoagulant PAI-1 in lung tissue, BALF, and/or plasma (see Figure 6). In lung tissue, TGF- $\beta 1$ concentrations increased 2.3-fold by 21 days after SM exposure compared with naive lung tissue (151 ± 21 vs. 65 ± 8 pg/mg/ml total lung homogenate protein, respectively; $n = 5$ /group; $P < 0.01$) (see Figure 6A), with sustained elevation to Day 28 ($n = 4$). Similarly, in BALF, TGF- $\beta 1$ concentrations increased 7.5-fold by Day 21 after SM exposure compared with naive concentrations (159 ± 74 vs. 22 ± 2 pg/mg/ml total protein, respectively; $n = 5$ /group; $P < 0.05$) (see Figure 6B) and remained elevated to Day 28. Regarding PDGF concentrations, in lung tissue, PDGF-A/B concentrations increased 7.5-fold by 21 days ($n = 5$) after SM exposure compared with naive concentrations ($n = 7$) (12 ± 1 vs. 1.7 ± 0.1 pg/mg/ml total lung homogenate protein; $P < 0.0001$; see Figure 6C). In BALF, PDGF-A/B concentrations increased 1.5-fold by Day 21 after SM exposure compared with naive concentrations (27 ± 6 vs. 18 ± 6 pg/mg/ml total protein, respectively; $n = 5$ /group; $P < 0.05$) (see Figure 6D) and remained elevated similarly at Day 28. Thus, whereas both TGF- $\beta 1$ and PDGF-A/B concentrations were elevated at Days 21 and 28 (but not before), TGF- $\beta 1$ concentrations were higher in BALF, and PDGF-A/B concentrations were higher in lung tissue. This may represent different locations of each cytokine within the pulmonary compartments.

PAI-1 concentrations were evaluated in plasma and BALF over time after SM exposure ($n = 3$ /group). In plasma, PAI-1 concentrations increased 10-fold acutely by Hour 7 after SM exposure compared with naive concentrations (0.44 ± 0.21 vs. 0.04 ± 0.01 ng/ml) (see Figure 6E), fell to naive concentrations at Day 1, then increased 40-fold from naive concentrations by Day 28 (1.71 ± 0.90 ng/ml; $P < 0.05$). Similarly, in BALF, PAI-1 concentrations increased threefold acutely by Hour 7 compared with naive concentrations (1.57 ± 0.11 vs. 0.54 ± 0.09 ng/mg) (see Figure 6F), remained elevated by Day 1, and drastically increased to 18-fold compared with naive concentrations by Day 28 (9.21 ± 3.87 ng/mg; $P < 0.001$).

Immunofluorescence Staining for TGF- $\beta 1$ and PDGFR- α

To help localize the increased TGF- $\beta 1$ and PDGF within the pulmonary bed after SM exposure, we employed immunofluorescence staining techniques

Figure 4. Histopathology of lung parenchyma after SM inhalation (1.0 mg/kg) exposure over time (14–28 d) compared with naive control animals. (A) Naive rat lung showing normal parenchymal structure. (B) Representative rat lung parenchymal section 14 days after SM exposure with moderate amounts of collagen deposition (blue), showing nearly 30% of parenchyma with collagen staining (inset). (C) Representative rat lung parenchymal section 21 days after SM exposure with progressive increase of interstitial infiltrates and collagen deposition, with nearly 50% of alveolar cross-sectional area occluded with fibrotic material (inset). (D) Representative rat lung parenchymal section 28 days after SM exposure showing diffuse parenchymal fibrosis. Greater than 50% collagen deposition (blue) is present within the subpleural regions (inset). All images show Masson's trichrome stain; magnification of main images, $\times 4$; magnification of insets, $\times 40$. Scale bars: 500 μm .

on left lobe lung sections (see Figure 7). We found increased TGF- $\beta 1$ staining after SM exposure by Day 21 ($n = 3$) (see Figures 7A and 7B), with a predilection for PF

interstitial regions, particularly subpleural areas. BO lesions also had sporadic TGF- $\beta 1$ staining (image not shown). Similarly, we found increased PDGFR- α staining after

Figure 5. Modified Ashcroft scoring for parenchymal fibrosis in rat lungs after SM (1.0 mg/kg) exposure compared with naive control animals. (A) All individual lung lobes were affected 17–28 days (pooled data) after SM inhalation exposure, with the highest Ashcroft scores, and thus the most severe fibrosis, in the RUL and RLL (**** $P < 0.0001$ compared with naive that had 0 scores). (B) RLL Ashcroft scores increased over time after SM exposure, with worse scores at 24–28 days over earlier time points. Data are presented as mean $\pm$ SEM; ANOVA with Tukey's *post hoc* test. LLL = left lower lobe; RA = right accessory; RLL = right lower lobe; RML = right middle lobe; RUL = right upper lobe.

SM exposure by Day 21 ($n = 3$) (see Figures 7C and 7D), but only within PF interstitial regions. No PDGFR- α staining was found within BO lesions, suggesting that although the TGF- β pathway may be involved in both PF and BO development, the PDGF pathway is likely involved only in the development of PF.

Discussion

To our knowledge, this is the first paper to describe BO and PF in an animal model 28 days after SM inhalation exposure. Histological findings of BO and PF were further supported by progressive clinical symptoms of hypoxemia and respiratory insufficiency. In addition, TGF- $\beta 1$ and PDGF-A/B were both elevated in rat lungs weeks after SM exposure and localized to regions of BO and PF on the basis of immunofluorescence analysis.

Prior animal models of SM inhalation have been focused on the acute and subacute injuries that occur within the first 7 days of exposure (41–43, 46). In acute/subacute injury (< 14 d after insult), cell necrosis and sloughing occur, as well as increased inflammation and airway wall edema resulting from increased bronchovascular permeability. Procoagulant plasma fluid leaks into the airway lumen through a layer of damaged airway epithelium, ultimately forming airway casts (4–6). When the fibrinolytic tissue plasminogen activator (7, 8) or the anticoagulant heparin (9) is delivered directly into the lungs, morbidity and mortality are significantly improved in the acute setting. Whereas previous animal models were focused on the acute/subacute phases of injury, the animal model in the present study was focused on the repair characteristics through the acute, subacute, and late stages of healing, the fibroproliferative phase. The advantage of this 28-day model is not only its delineation of the stages of aberrant repair and fibrosis over time past any inflammation phase but also its direct demonstration of pulmonary function, as well as mechanistic insight leading to lung fibrosis up to 28 days after acute SM inhalation.

Despite significant improvements in overall morbidity and mortality in the acute setting with these novel therapeutic agents, civilians and soldiers exposed to SM inhalation develop long-term morbidity and mortality months to years after the initial

Figure 6. Transforming growth factor- β 1 (TGF- β 1), platelet-derived growth factor A/B (PDGF-A/B), and PAI-1 in lung, BAL fluid (BALF) and plasma after SM inhalation exposure. (A) TGF- β 1 concentrations in lung homogenates were significantly elevated at 21 days ($P < 0.01$) and 28 days ($P < 0.05$) after SM exposure compared with naive control concentrations. (B) TGF- β 1 concentrations in BALF were nonsignificantly elevated at 21 and 28 days after SM exposure compared with naive control concentrations. (C) PDGF-A/B concentrations in total lung homogenates were significantly elevated at 21 days ($P < 0.0001$) compared with naive control concentrations. (D) PDGF-A/B concentrations in BALF were nonsignificantly elevated at 21 and 28 days after SM exposure compared with naive control concentrations. (E) PAI-1 concentrations in plasma were significantly elevated at 28 days ($P < 0.05$) after SM compared with naive control concentrations. (F) PAI-1 concentrations in BALF were significantly elevated at 28 days ($P < 0.001$) compared with naive control concentrations. Data are presented as mean $\pm$ SEM; ANOVA with Tukey's *post hoc* test. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

exposure (1–3, 9–15). In the present study, we observed rats for 1 month after SM exposure to assess for persistent symptoms of respiratory insufficiency consistent with those seen in humans. Weeks after SM exposure, rats demonstrated progressive deterioration with declining oxygen

saturation (Figure E1) and weight loss (Figure E2), as well as progression of mean clinical distress scores (Figure E3). This progressive decline in oxygen saturation and weight loss started at 2 weeks after SM exposure and progressed until the study's end at 28 days. Over the same period,

survival also declined. Dissimilar from prior animal models used to study PF, oxygen saturation and respiratory symptoms after SM exposure did not improve with time.

To explain the progressive decline in clinical parameters weeks after SM exposure, lung function testing and histological examination were performed on rats at Days 14, 21, and 28 after SM exposure. At Days 21 and 28, lung function testing revealed that total lung resistance and tissue damping increased, whereas lung compliance decreased (Figure 3).

Newtonian resistance, a marker of airway resistance, was also increased at Days 21 and 28. Collectively, these changes were most suggestive of both restrictive and obstructive lung disease affecting both the distal lung parenchyma and conducting airways. Long-term follow-up of civilians and soldiers exposed to SM demonstrated similar changes in lung function testing with reduced FVC, reduced FEV₁, and reduced DLCO (16). In SM-exposed individuals with reduced FEV₁ values, administration of a bronchodilator does not significantly improve FEV₁, consistent with a fixed obstruction. Similarly to lung function testing in humans, results of lung function testing in rats weeks after exposure mimicked pulmonary function testing and were suggestive of both restrictive and obstructive pathologies (17, 47–49).

Histological examination revealed that both BO (Figure 4) and PF (Figure 5) were present weeks after exposure. In rat lung sections, distal bronchial narrowing with subepithelial collagen deposition and luminal obstruction by granulation-type tissue was present at Days 21 and 28. In the more distal parenchyma, interstitial fibrosis was also present by Day 14 and persisted until study's end. The distribution of fibrosis was more appreciable in the dependent lobes, surrounding constricted bronchioles, and in subpleural regions. An increase in alveolar airspace adjacent to fibrotic areas was also seen. We are uncertain whether such changes represent contraction and/or torsion from adjacent fibrotic areas leading to "stretch" of neighboring parenchyma or possibly destruction or involution of alveolar septae. In civilians and soldiers who have undergone lung biopsy because of chronic respiratory symptoms, the majority of biopsies demonstrate abnormal lung pathology. The most common pathologies include constrictive

Figure 7. Immunofluorescence staining of lung sections for TGF- β 1 and platelet-derived growth factor receptor- α (PDGFR- α) after SM inhalation exposure (1.0 mg/kg) compared with that in naive control specimens. (A) Naive rat lung showing a minimal amount of TGF- β 1 (green) staining within the lung parenchyma. (B) Representative rat lung parenchymal section 21 days after SM exposure showing increased TGF- β 1 (green) staining in alveolar regions that correspond to fibrotic areas within the lung. (C) Naive rat lung showing a minimal amount of PDGFR- α (red) staining within the lung parenchyma. (D) Representative rat lung parenchymal section 21 days after SM exposure showing increased PDGFR- α (red) staining in alveolar regions that correspond to fibrotic areas within the lung. Magnification of main images, $\times 4$; magnification of insets, $\times 20$. Scale bars: 500 μ m.

bronchiolitis and BO (50, 51), but organizing pneumonia and usual interstitial pneumonia have also been reported (12, 51). Thus, the histopathological changes seen in rats in the present study parallel the chronic pathology seen in SM-exposed humans.

Two major cytokines implicated in the development of lung fibrosis after chemical inhalation are TGF- β (20–22) and PDGF (20, 21, 23–26). TGF- β 1 protein concentrations were chronically elevated in BALF and total lung homogenate at Days 21 and 28 after SM exposure compared with nonexposed control animals in the present experiments (Figure 6). In addition, PDGF-A/B was elevated in total lung homogenate 21 days after SM exposure. These profibrotic cytokines were elevated when fibrosis was most apparent in rat lungs weeks after the initial SM injury. Furthermore, TGF- β 1 and PDGFR- α signaling localized to areas of BO and PF on immunofluorescence

staining. Chronically elevated protein concentrations of TGF- β 1 from BALF and sputum aspirates have been reported in soldiers with BO or PF after SM exposure (31, 52). In addition, PDGF has been associated with declining lung function and the development of lung transplant-associated BO (38, 39). Considering that both TGF- β 1 and PDGF were elevated in rat lungs weeks after SM exposure, these profibrotic pathways likely contribute to long-term lung fibrosis after SM inhalation injury.

Both TGF- β 1 and PDGF activate multiple pathways that lead to fibrosis. One of the downstream pathways activated by TGF- β is the expression of the procoagulant protein PAI-1. Indeed, we found an elevated procoagulant factor PAI-1 early after SM exposure, but even more so at 28 days, both in BALF and in plasma. The elevation of PAI-1 was directly proportional to the SM dose received (data not shown). The high PAI-1 concentrations

at 28 days indicated that continued aberrant coagulation may impact the development of late disease after SM. Elevated PAI-1 concentrations have been found in several fibrotic diseases, including liver fibrosis, bleomycin-induced PF, and glomerulosclerosis (40). Increased PAI-1 will lead to continued suppression of fibrinolysis, and thus a slower degradation of the fibrin network, and stabilization of the extracellular matrix that may play a role in fibrosis. In addition, PAI-1 has been shown to regulate adhesion and migration of a variety of cells, including collagen-producing cells. Thus, PAI-1 could have contributed not only to the development of BO in our model but also to the parenchymal fibrosis.

The purpose of this study was to assess lung fibrosis in rats after SM inhalation and to help explain its development with two profibrotic pathways. Considering that SM is a chemical warfare agent, SM experiments are closely monitored by the federal government. Owing to these constraints, SM inhalation exposures were originally performed only on a military base at sea level (USMRICD Aberdeen Proving Ground) and only more recently were conducted at our UCD facility at higher altitude (5,280 ft). Survival, oxygen saturation, clinical distress scoring, and pulmonary function test results were not statistically different between the two locations. The fact that these parameters all proceeded in a similar manner in Denver and at sea level indicates that the chronic disease process ensued at both sites. Thus, the change in altitude did not affect long-term outcomes associated with SM exposure.

To our knowledge, the present study is the first to show the development of BO and PF 1 month after SM inhalation exposure in rats. TGF- β 1 and PDGF, two profibrotic cytokines and pathways, remained chronically elevated in rat lungs after SM inhalation exposure. PAI-1, a procoagulant protein, also remained elevated in both rat lung and plasma after SM exposure. Therapeutic inhibitors targeting these pathways may improve survival and decrease long-term morbidity associated with SM inhalation injury. ■

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