Alveolar Micromechanics in Bleomycin-induced Lung Injury

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Abstract

Lung injury results in intratidal alveolar recruitment and derecruitment and alveolar collapse, creating stress concentrators that increase strain and aggravate injury. In this work, we sought to describe alveolar micromechanics during mechanical ventilation in bleomycin-induced lung injury and surfactant replacement therapy. Structure and function were assessed in rats 1 day and 3 days after intratracheal bleomycin instillation and after surfactant replacement therapy. Pulmonary system mechanics were measured during ventilation with positive end-expiratory pressures (PEEPs) between 1 and 10 cm H₂O, followed by perfusion fixation at end-expiratory pressure at airway opening (Pao) values of 1, 5, 10, and 20 cm H₂O for quantitative analyses of lung structure. Lung structure and function were used to parameterize a physiologically based, multicompartiment computational model of alveolar micromechanics. In healthy controls, the numbers of open alveoli remained stable in a range of Pao = 1–20 cm H₂O,

whereas bleomycin-challenged lungs demonstrated progressive alveolar derecruitment with Pao < 10 cm H₂O. At Day 3, ~40% of the alveoli remained closed at high Pao, and alveolar size heterogeneity increased. Simulations of injured lungs predicted that alveolar recruitment pressures were much greater than the derecruitment pressures, so that minimal intratidal recruitment and derecruitment occurred during mechanical ventilation with a tidal volume of 10 ml/kg body weight over a range of PEEP. However, the simulations also predicted a dramatic increase in alveolar strain with injury that we attribute to alveolar interdependence. These findings suggest that in progressive lung injury, alveolar collapse with increased distension of patent (open) alveoli dominates alveolar micromechanics. PEEP and surfactant substitution reduce alveolar collapse and dynamic strain but increase static strain.

Keywords: acute lung injury; alveolar dynamics; modeling; stereology; surfactant

Acute lung injury (ALI) results in edema formation, inflammation, and surfactant dysfunction with high surface tension and alveolar instability (1, 2). Hence, ALI induces abnormal changes in alveolar micromechanics that lead to altered

alveolar dynamics, as reflected in the way in which alveolar dimensions vary throughout the respiratory cycle. Alveolar dynamics in healthy lungs can manifest in a variety of ways, including isotropic balloon-like changes in alveolar size, changes in alveolar

geometry and shape, and anisotropic unfolding of alveolar walls (3). Nevertheless, the existence of alveolar recruitment and derecruitment (R/D) during the respiratory cycle in healthy lung has been challenged in several studies

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Clinical Relevance

In lung injury, alterations of alveolar micromechanics, such as alveolar recruitment and derecruitment (R/D) and overdistension, affect lung macromechanical properties and contribute to disease progression. Detailed insights into this process are urgently needed. In this study, we used morphometric analysis to develop computational predictions of alveolar R/D and alveolar unit volumes. Our analysis indicates that very little intratidal R/D occurs during mechanical ventilation with normal tidal volume (10 ml/kg) after bleomycin-induced injury, as the pressures needed to recruit alveoli are much greater than those needed to prevent their derecruitment. Instead, alveolar collapse occurs through pressures up to 30 cm H₂O, and elevated static and dynamic alveolar distension dominates the pathophysiology. Increased positive end-expiratory pressure after a recruitment maneuver and preventive surfactant substitution reduces alveolar collapse and the dynamic strain of open alveoli but increases static strain.

(2, 4–9). By contrast, during mechanical ventilation of injured lungs, there is strong evidence for abnormal and heterogeneous alveolar dynamics, such as intratidal alveolar R/D (10, 11), and asynchronies that include alveolar pendelluft, inverse alveolar ventilation, and alveolar stunning (12, 13). Furthermore, abnormalities in alveolar micromechanics aggravate lung injury via the high surface forces generated during intratidal alveolar R/D (14, 15) and the resulting increased static and dynamic strains that are then imposed on the remaining open alveoli. These two mechanisms give rise to modes of injury known as atelectrauma and volutrauma, respectively, both of which can contribute significantly to ventilator-induced lung injury (VILI) (16–19).

Network models of the lung parenchyma suggest that the spatial distribution of alveolar strain is relatively homogeneous in a healthy lung. In an injured lung, however, significant regional

heterogeneities develop when the reduced volumes of collapsed or flooded alveoli increase the strains and stresses of neighboring open alveoli. Such derecruited alveoli have thus been designated as “stress concentrators” (20, 21). The phenomenon of stress concentration also suggests that atelectrauma and volutrauma have the potential to interact, rather than injuring the lung in a simply additive fashion. Indeed, mortality in patients suffering from acute respiratory distress syndrome (ARDS), although still unacceptably high, is reduced when they are mechanically ventilated with reduced tidal volumes and maximal inspiratory pressures (22), likely because this both limits the distension of open alveoli and decreases the likelihood that closed alveoli will be forced to open. On the other hand, adjustment of positive end-expiratory pressure (PEEP) to prevent alveolar collapse has not been shown to have a reproducible survival benefit (23, 24), even though maintaining PEEP above the atelectrauma threshold would seem to make perfect physiological sense (25, 26). Elucidating this confusing picture requires greater insight into the alveolar micromechanics of the injured lung, and could lead to much needed improvements in strategies for ventilating patients with ARDS.

The early stages of the bleomycin rat model of lung injury (i.e., before the fibroproliferative phase) fulfill the criteria established by the Acute Lung Injury in Animals Study Group (27). We have previously characterized the time course of pathological alterations after intratracheal bleomycin instillation. During the first week, in the absence of fibrotic remodeling, we observed histological evidence of lung injury (e.g., thickening of alveolar walls, swelling and fragmentation of alveolar epithelial cells, intra-alveolar edema, neutrophilic infiltrates, and microatelectasis), alterations of the alveolar capillary barrier (e.g., elevated protein and albumin levels in BAL, and swelling of the blood–gas barrier), inflammatory responses (e.g., increases in neutrophilic granulocytes and the inflammatory markers IL-1 β , IL-6, and monocyte chemoattractant protein in BAL), and physiological dysfunction (e.g., increases in pulmonary system elastance and impaired blood oxygenation) (28–30). Accordingly, our goal in the present study was to characterize alveolar micromechanics as a function of lung injury progression after bleomycin challenge. To that end, we

measured organ-scale macromechanics and design-based stereological quantifications of microarchitecture in the rat model of bleomycin-induced ALI. We linked the data from the macro- and microscales using a computational model of the alveolus that represents both the nonlinear viscoelastic properties of the parenchymal tissue and the dependence of R/D on pressure and time. Surfactant replacement therapy (SRT) has been shown to improve physiological dysfunction (28) as well as microatelectasis, intra-alveolar edema formation, and inflammatory responses (30). Surfactant dysfunction is one of the dominating pathologies in the bleomycin model during the early stage. Surfactant reduces end-expiratory interfacial surface tension within the alveoli, thereby preventing the collapse of distal airspaces (31). In addition, high surface tension has been linked to atelectrauma resulting in expiratory alveolar derecruitment if expiratory pressure at airway opening (Pao) values fall below certain limits (10, 32). With regard to the bleomycin model, we therefore hypothesized that the Pao values needed to prevent expiratory alveolar derecruitment could be shifted toward lower values by SRT. Therefore, we added a bleomycin-injured group that was treated with exogenous surfactant. Some of the results of these studies have been previously reported in the form of abstracts (33, 34).

Methods

A detailed description of the methods used in this work is provided in the data supplement.

Animal Model

Animal experiments were approved by the authorities of the Lower Saxony State Office for Consumer Protection and Food Safety in accordance with European Animal Welfare Regulations (approval number 12/1022). Lung injury was induced in rats by intratracheal bleomycin instillation (29) and the animals were analyzed by means of lung mechanical measurements followed by design-based stereology at Day 1 (D1) and D3 after bleomycin treatment. The SRT group was treated with surfactant (Curosurf, an in-kind donation from Chiesi Pharmaceuticals) on D1 and D2 (intratracheal application of 100 mg/kg body weight per day) and analyzed on D3.

Invasive Pulmonary Function Tests

Under deep narcosis, the animals were tracheostomized and connected to a FlexiVent ventilator. Derecruitment tests were performed at PEEP values of 1–10 cm H₂O. Tissue elastance (H) was repetitively determined using forced oscillations according to established methods (29). H describes pulmonary system elastance during tidal breathing at a prescribed PEEP. Quasistatic pressure-controlled pressure–volume (PV) loops were recorded. Starting with an onset Pao of 3 cm H₂O, a stepwise pressure increase of 3.86 cm H₂O every second was performed to a maximum of 30 cm H₂O within 7 seconds. After the Pao had been kept stable at 30 cm H₂O for 1 second, the pressure was again stepwise decreased to 3 cm H₂O. The covered range of PV relationships represents a partial PV loop above the functional residual capacity (FRC). For healthy rat lungs, it has been shown that the partial PV loop above the FRC can be superimposed onto a full PV loop if corrected by the FRC, meaning that the shapes and therefore information regarding the elastic properties of the respiratory system above the FRC are identical (35). The raw data for these partial, quasistatic PV loops were used during the computational model fitting

described below and in the data supplement.

Fixation, Tissue Processing, and Design-based Stereology

Quantitative evaluation of lung structure was performed according to American Thoracic Society/European Respiratory Society guidelines (36). Lungs were fixed *in situ* by vascular perfusion at Pao = 1, 5, 10, and 20 cm H₂O on expiration. Five lungs per Pao in each group were included in this study (80 in total), processed, and analyzed by design-based stereology according to a previously published methodology (37–41). Volume fractions of alveolar and ductal airspaces, as well as interalveolar septa and intra-alveolar edema, were calculated. The density of the air-covered surface area of alveoli ($S_V[\text{alvair}/\text{par}]$), and the density of open alveoli ($N_V[\text{alv}/\text{par}]$) were determined. Densities were converted to total volume, total surface area, or total number per lung. The mean linear intercept length was calculated as the volume-to-surface ratio of acinar airspaces ($L_m[\text{indir}]$) (5). The volume-weighted mean alveolar volume ($v_v[\text{alv}]$) was determined using the point-sampled intercepts method (42). Because intra-alveolar edema (fluid) could not be detected at the light-microscopic

level in D1 and healthy control animals due to limitations of the resolution, we re-used electron-microscopy sections from a previous study that evaluated the same animal model of bleomycin-induced lung injury (29). The goal was to determine the volume of intra-alveolar edema and the surface fraction of alveolar epithelium covered by alveolar fluid/edema ($S_S[\text{alved}, \text{alvepi}]$) in D1 and healthy control animals.

Modeling Alveolar R/D and Strain

We used structural and lung mechanical data to parameterize a physiology-based computational model (Figure 1) by iteratively minimizing the difference between measured and predicted elastance, lung volume, and alveolar recruitment using a parallel implementation of the pattern search algorithm (43–47), as we have previously described (48, 49). The model parameters are defined in Table 1. The model consists of a single alveolar duct compartment and 1,024 parallel alveolar units, and represents an extension of our previous simulation studies of alveolar dynamics (48–53). The alveolar ducts, or intra-acinar conducting airways, do not have a well-defined wall. Instead, the duct boundary is formed by the alveolar entrance rings (54). The structural basis of

Figure 1. Computational model. The FlexiVent drives gas into the alveolar duct compartment against an equipment flow resistance (R_{Eq}), airway resistance (R_{Aw}), and shunt gas elastance (E_{Gas}). The alveolar duct airspace is represented by a single duct compartment with volume V_D and pressure P_D . Vertical expansion of the duct compartment is opposed by a nonlinear spring with stiffness $E_D = \beta \times E_A$ and a viscous damper with resistance $R_D = 0.03 E_D$. The ratio of alveolar to duct gas volumes $\beta = V_A/V_D$ is shown in A and the parameter values for the best-fit lines are provided in Table E1. The effective combined stiffness of all patent alveoli $E_A = E_{Unit} \times (O_F N_{Units})^{-1}$, where O_F is the open fraction of alveoli determined via virtual trajectories, $N_{Units} = 1,024$ is the number of alveolar units in the simulation, and E_{Unit} is the nonlinear spring stiffness opposing distension of the alveolar unit. The form of E_{Unit} is depicted in inset B, which shows the low-volume nonlinear elastance threshold volume (V_{Low}) and increase rate (E_{Low}), the high-volume nonlinear elastance threshold elastance volume (V_{Crit}) and rate (E_{Fac}), and the intermediate-volume baseline elastance (E_{Base}). The expansion of the alveoli is also opposed by a viscous damper with resistance $R_{Unit} = 0.03 E_{Unit}$. The horizontal width of the duct compartment is altered in concert with O_F , so the duct volume is corrected by the O_F to represent the derecruitment of ducts. The alveoli are directly coupled to the duct compartment, so there is zero airflow resistance between the duct and the alveoli. D1 = Day 1; D3 = Day 3; Pao = pressure at airway opening; PEEP = positive end-expiratory pressure; SRT = surfactant replacement therapy.

Table 1. Computational Model Parameters and Variables

Parameter	Description
μ_C	Mean virtual trajectory closing pressure
E_A	Combined spring stiffness for all patent alveolar compartments
E_D	Duct compartment nonlinear spring stiffness
E_{Fac}	Rate of high-volume alveolar compartment elastance increase
E_{Gas}	Ventilator gas shunt elastance
E_{Low}	Rate of low-volume alveolar compartment elastance increase
E_{Unit}	Alveolar compartment spring stiffness
O_F	Open fraction of alveolar compartments
P_A	Alveolar pressure
P_{Ao}	Pressure at airway opening (tracheal)
P_C	Virtual trajectory closing pressure
P_{Cyl}	Ventilator cylinder pressure
P_D	Duct compartment pressure
P_O	Virtual trajectory opening pressure
R_A	Combined dashpot resistance for all patent alveolar compartments
R_{aw}	Airway resistance
R_D	Duct compartment dashpot resistance
R_{eq}	Ventilator circuit flow resistance
R_{Unit}	Alveolar compartment dashpot resistance
S_C	Virtual trajectory closing velocity constant
S_O	Virtual trajectory opening velocity constant
V_A	Alveolar volume that is equal to the sum of all alveolar compartment volumes
V_{ARV}	Alveolar residual volume
V_{Crit}	Alveolar compartment volume threshold for high-volume elastance
V_D	Duct compartment volume
V_{DRV}	Duct compartment residual volume
V_L	Lung volume
V_{Low}	Alveolar compartment volume threshold for low-volume elastance increase
α	Fraction of nonrecruitable alveoli
β	Ratio of alveolar to duct volume (V_A/V_D)
σ_C	SD of the virtual trajectory closing pressure

these entrance rings is formed by elastic and collagen fibers, which are part of the axial network of connective tissues that coil around the alveolar ducts (2). Based on these anatomical considerations, we assigned elastance and tissue resistance properties to the alveolar duct and alveolar compartments, and tracked volumes independently in these spaces (Figure 1). The alveolar (V_A) and ductal (V_D) air volume ratio (β) = V_A/V_D (Figure 1A) was calculated from the measured volumes and used to determine the fraction of air in each simulation compartment, and thus the ductal elastance may be calculated from the alveolar elastance. As in our previous models, the dynamics of alveolar R/D are represented with virtual trajectories to predict the lung open fraction (O_F). Derecruitment of the alveolar ducts is represented by varying the width of the duct compartment in proportion to O_F . The nonlinear elastance of the alveoli (Figure 1B) is determined in the fitting procedure, and the elastance of the duct compartment is prescribed to be the product of the predicted alveolar elastance and the ratio of the alveolar and duct

airspace volumes ($\beta = V_A/V_D$) determined using stereology (Figure 1A). Finally, mean alveolar tidal volumes and intratidal alveolar R/D were simulated during ventilation with a tidal volume of 10 ml/kg body weight and PEEPs ranging from 1 to 15 cm H₂O.

Statistics

Two-way ANOVA on ranks was used to test for Pao/PEEP effects and group effects on lung structure and function. Tukey's test for multiple comparisons was added. Correlation analyses were performed using a nonparametric Spearman test on pooled data. Statistical tests were performed using GraphPad Prism, version 7.02.

Results

Lung Mechanics

The average pulmonary system elastance (H) during ventilation at PEEP = 1–10 cm H₂O after a recruitment maneuver is shown in Figure 2A and detailed in the data supplement (Table E4). In all study groups, the minimum H was observed at PEEP =

5 cm H₂O ventilation. The D3 and SRT groups had significantly higher H values than the healthy control group across all PEEPs. Compared with the control group, the D1 group did not show significantly increased H except at PEEP = 10 cm H₂O. At PEEP = 1 and 10 cm H₂O, H was significantly higher in the D3 group than in the SRT group.

Lung Structure

Representative light micrographs are provided in Figure 3. Even at low Pao, microatelectasis was not observed in the healthy controls, and increases in Pao demonstrated homogeneous alveolar and ductal airspace expansion. The D1 group exhibited microatelectasis in some areas at Pao = 1 cm H₂O, and increasing Pao to 5 cm H₂O reopened these collapsed regions. Alveolar edema was sparse at the light-microscopy level at D1. Electron microscopy, however, revealed a very thin layer of alveolar edema (Figure E2). At D3, microatelectasis with interposed alveolar edema fluid was found throughout the range of Pao = 1–20 cm H₂O. We did not observe obvious differences in pathological alterations of lung structure between the SRT and D3 groups, so a quantification by means of design-based stereology was necessary to draw a conclusion as to whether or not surfactant substitution had beneficial effects on lung structure.

Stereological data are shown in Figure 4 and detailed in Table E5. The volume of alveolar V(alvair,lung) and ductal V(ductair,lung) airspaces increased nonlinearly with increasing Pao, whereas the volume fraction of septal walls $V_V(\text{sep}/\text{par})$ decreased and the absolute volume of septal wall remained roughly constant in all study groups (Figures 4A and 4B; Table E5). The mean linear intercept length of distal airspaces, which reflects the dimensions of the alveolar and ductal airspaces taken together, is illustrated in Figure 4C and shows a significant increase compared with healthy lungs in the D3 group at Pao = 5 and 20 cm H₂O. Parameters related to the alveolar airspaces demonstrated the most significant differences between the bleomycin-injured and healthy control groups (Figure 4A and Table E5). V(alvair,lung) was significantly lower in the D3 and SRT groups than in healthy controls for Pao = 1–10 cm H₂O, and the difference increased with Pao. At D1, V(alvair,lung) differed from controls at

Figure 2. Lung mechanical data. Tissue elastance H as a function of PEEP ventilations ranging from 1 to 10 cm H₂O are given for healthy controls and bleomycin-injured lungs at D1 and D3 after bleomycin instillation, as well as at D3 after bleomycin and SRT. Tissue elastance was determined by a forced oscillation technique and fitting of the impedance spectra to the constant phase model. The mean and SD of five measurements during PEEP 5 and 10 cm H₂O ventilation and 10 measurements during PEEP 1 and 3 cm H₂O is given (additional data for PEEP 1 and PEEP 3 cm H₂O ventilation were taken from Reference 29). A two-way ANOVA on ranks, considering group and PEEP, followed by Tukey's correction for multiple comparisons was used for statistical analyses. At a given PEEP ventilation, statistical significant differences are indicated for $P < 0.05$ as follows: *healthy control versus D1, †healthy control versus D3, †healthy control versus SRT, §SRT versus D3.

low and higher Pao (Figure 4A). The SRT group had significantly higher $V(\text{alvair, lung})$ than the D3 group for Pao = 1–10 cm H₂O. The behavior of $V(\text{ductair, lung})$ with increasing Pao in the D1, D3, and SRT groups was more similar to that observed in the healthy control group (Figure 4B).

The total number of open alveoli per lung ($N(\text{alv, lung})$) did not show any significant Pao effects in the control group, whereas bleomycin-injured lungs showed significant decreases in $N(\text{alv, lung})$ with Pao < 10 cm H₂O (Figure 4D). Increasing Pao normalized $N(\text{alv, lung})$ at D1, but not at D3 and SRT. Compared with D3, SRT demonstrated a significantly higher $N(\text{alv, lung})$ at Pao = 5 and 20 cm H₂O. The volume-weighted mean volume of alveoli ($v_v[\text{alv}]$) (Figure 4E) and the number-weighted mean volume of alveoli ($v_N[\text{alv}]$) (Table E5) were increased in D3 lungs compared with controls at Pao = 1–10 cm H₂O. The number-weighted mean volume is the ratio of the total alveolar airspace volume and the number of open alveoli per lung, and represents the mean of the alveolar size distribution. By contrast, the volume-weighted mean volume overrepresents larger alveoli and is also dependent on the coefficient of variation of the intraindividual size distribution. Hence, the increases in the volume-weighted mean volume over the number-weighted mean volume reflect alveolar size heterogeneity. In other words, the volume-weighted mean alveolar volume is the number-weighted mean alveolar volume amplified by the size

variation. SRT lungs demonstrated a significant reduction in $v_v[\text{alv}]$ at Pao = 10 cm H₂O compared with D3. The highest coefficient of variation of alveolar size $CV(v_X[\text{alv}])$, representing alveolar size heterogeneity, was found at D3 at Pao = 10 cm H₂O (Table E5). The control lungs provided a stable total surface area of alveolar walls ($S(\text{alvair, lung})$), even with low Pao (Figure 4F), whereas the D1, D3, and SRT groups demonstrated a significant reduction at Pao = 1 cm H₂O compared with higher Pao in the same groups. As with the alveolar number, $S(\text{alvair, lung})$ was normalized toward control values with increasing Pao at D1 (but not at D3 or SRT), and SRT had significantly higher values than D3. Of note, between end-expiratory Pao values of 1 cm H₂O and 20 cm H₂O, the mean $L_m(\text{indir})$ increased from 60.2 μm (SD = 4.0 μm) to 100.5 μm (SD = 7.1 μm) and therefore by a factor of 1.67 in a healthy rat lung, whereas $S(\text{alvair, lung})$ increased only by a factor of 1.39. In the same range of pressures, the D3 group demonstrated an increase of $L_m(\text{indir})$ from 68.8 μm (SD = 13.2 μm) to 123.4 μm (SD = 6.2 μm) by a factor of 1.79, whereas $S(\text{alvair, lung})$ was increased by a factor of 1.66. At the electron-microscopic level, D1 demonstrated a clear increase in alveolar fluid volume ($V(\text{alved, lung})$) compared with healthy controls (Figures 4G and E2), which was associated with an increase in the fraction of alveolar epithelium covered by edema fluid ($S_S[\text{alved/alvepi}]$; Figure 4H). At D3, alveolar edema was clearly visible at

the light-microscopic level, and $V(\text{alved, lung})$ demonstrated a significant difference between D3 and SRT (Figure 4I).

Structure–Function Relationships

Figures 5A–5C show the relationship between structural data obtained at Pao = 1, 5, and 10 cm H₂O and H measured at the corresponding PEEPs. Increased H at PEEP = 1 and 5 cm H₂O was highly correlated with a decrease in $V(\text{alvair, lung})$ ($r = -0.93$; Figure 5A). In healthy lungs, the decline in $V(\text{alvair, lung})$ from Pao = 5 cm H₂O to Pao = 1 cm H₂O was due to a significant decrease in mean alveolar size ($v_N[\text{alv}]$; Table E5) with stable $N(\text{alv, lung})$, whereas in bleomycin-injured lungs, $N(\text{alv, lung})$ decreased and $v_N[\text{alv}]$ remained roughly stable. Hence, in healthy controls, the increase in H between PEEP = 5 cm H₂O and PEEP = 1 cm H₂O showed a correlation with $v_N[\text{alv}]$ (Figure 5B), whereas in bleomycin-injured lungs, an inverse correlation was established between H and $N(\text{alv, lung})$ (Figure 5C). The reduction in alveolar size at Pao = 1 cm H₂O was paralleled by the occurrence of pleats in the septal wall (Figures 5D and E3) without microatelectasis in healthy lungs. Microatelectasis was found starting at D1 (Figure 5E). Hence, control lungs were characterized by smaller but stable alveoli, and after bleomycin challenge alveolar derecruitment was apparently responsible for the increase in H seen at low lung volumes. At PEEP = 5 and 10 cm H₂O, increased H was correlated with an increase in $v_v[\text{alv}]$ (Figure 5F) and $CV(v_X[\text{alv}])$ ($P < 0.01$, $r = 0.58$).

Modeling Alveolar Micromechanics

The parameterized model satisfactorily simulated lung mechanical and structural properties (Figures 6A and 6B, and E1; Tables E2 and E3). The increased elastance measured during the derecruitment tests (Figure 6A) at PEEP = 2 cm H₂O compared with 5 cm H₂O was predicted to occur due to reduced O_F in the D1, D3, and SRT groups. Measurements of structure indicated that alveolar derecruitment did not occur at positive Pao in the control group. Instead, the increase in elastance at PEEP = 2 cm H₂O was resolved in the simulations by applying a nonlinear increase in elastance at low volumes (Figure 1B) to represent the observations of septal pleating described above. The alveolar derecruitment reflected in

Figure 3. Light microscopy. Representative low-power light-microscopic images from lungs fixed at different end-expiratory Pao values in healthy controls (C) and D1, D3, and SRT groups. Microatelectasis (arrows) disappeared with increasing Pao in D1, and such lung regions were still found at Pao = 20 cm H₂O in the D3 and SRT groups. In healthy controls, microatelectasis was absent even at low Pao (1 cm H₂O). Scale bars: 500 μ m.

Figure 6B shows similar trends for D1, D3, and SRT. The mean (μ_C) and SD (σ_C) of alveolar closing pressures for these groups are quite similar (Table 2 and Figure 6C). Likewise, the recruitment pressures (Figure 6C) remain consistent in the D1, D3, and SRT groups. However, there is a fraction of nonrecruitable units (α) that increases from control to D1 and D3. Surfactant substitution is able to return ventilation to some of these units (Table 2, α). The model parameters listed in Table 2 were determined by iteratively adjusting the model parameters to minimize an objective function that describes the difference between the measured and predicted pressures, elastances, and alveolar recruitment (O_F of the alveoli) as detailed in Equation 7 in the data supplement.

A comparison of the PV relationship for a single alveolus (Figure 7A) between the control and D1 groups shows similar behavior except that the controls demonstrate nonlinear, low-volume behavior, and D1 has a moderate elevation in alveolar residual volume (V_{ARV} , Table 2) and rate of elastance increase at high volumes (E_{Fac} , Table 2). The D3 and SRT groups are characterized by a striking increase in V_{ARV} . The PV relationship for a single alveolar duct (Figure 7B) shows a trend similar to that observed for the alveoli. However, based on the slope of the simulated PV relationship, we do not predict an increase in the stiffness of the alveolar duct compartment at high volumes from control to D1. In addition, there is a decrease in the gas volume delivered to the

duct units from D3 to SRT. During ventilation with a tidal volume of 10 ml/kg body weight, the simulations demonstrated the greatest changes in mean alveolar unit volume (e.g., mean alveolar tidal volume) at D3 (Figure 7C) due to the reduction in patent alveoli. During mechanical ventilation, increased PEEP after a recruitment maneuver resulted in improved alveolar recruitment and therefore reduced mean alveolar tidal volumes in the injured lungs (Figure 7D). The application of SRT reduced mean alveolar tidal volumes when compared with D3 due to increased O_F . It is important to note that the simulations predict intratidal alveolar recruitment and derecruitment for <0.2% of alveoli for the analysed range of PEEP values (Figure 7D). During mechanical ventilation with

Figure 4. Acinar microarchitecture. Stereological data demonstrate the effects of end-expiratory Pao on alveolar microarchitecture in healthy controls and D1, D3, and SRT groups. Data are given as mean and SD of $n = 4-5$ lungs. Total volumes per lung of (A) alveolar airspaces and (B) ductal airspaces, as well as (C) the mean linear intercept length of the acinar airspaces ($L_m[\text{indir}]$), are illustrated. The alveolar microarchitecture was further quantified by means of the total number of (D) open alveoli per lung, (E) volume-weighted mean volume of alveoli, and (F) surface area of alveolar walls. Alveolar edema was visible at the ultrastructural level in the D1 group. The total volume of alveolar fluid/edema ($V(\text{alved, lung})$) is illustrated in G, and the surface fraction of alveolar epithelium covered by edema ($S_s(\text{alved/alvepi})$) is shown in H. In G and H, data were generated at electron-microscopic resolution. In the D3 and SRT groups, alveolar edema was visible at the light-microscopic level. The total amount of alveolar edema per lung was independent of Pao, and individual data are given in I, comparing D3 and SRT. (G–I) Student's t test was used for statistical analyses. For all other graphs, two-way ANOVA on ranks, taking group and Pao effects into account, followed by Tukey's correction for multiple comparisons was used. At a given Pao, statistical significant differences are indicated for $P < 0.05$ as follows: *healthy control versus D1, †healthy control versus D3, ‡healthy control versus SRT, §SRT versus D3.

tidal volume = 10 ml/kg body weight and PEEP = 1 cm H₂O at D1, the simulations predicted that 40–50% of the alveoli would be derecruited throughout the respiratory cycle. The existence of microatelectasis, observed after perfusion fixation at end-inspiratory Pao, supports this model prediction (Figure E4).

Discussion

The effects of ALI on alveolar micromechanics during the respiratory

cycle have sparked controversy in recent years (10, 13). Alveolar micromechanics influence macroscale lung mechanical measurements at the tracheal opening and are vitally important for not only the pathogenesis of VILI (16, 55) but also fibrotic remodeling after lung injury (56–60); therefore, it is of the utmost importance to understand alterations at the micromechanical level (61). Real-time imaging of alveoli would be optimal for this purpose, but this is only possible for subpleural alveoli (12, 62). Currently, no imaging technique is available for dynamic

in vivo visualization in a manner that allows unbiased inclusion of alveoli from different regions of the lung. We therefore used simulations based on organ-scale lung mechanical and design-based stereological data to predict alterations in alveolar micromechanics in progressive bleomycin-induced lung injury. We evaluated the effects of preventive SRT on micromechanics to examine the hypothesis that a reduction in surface tension results in a shift of alveolar closing pressures toward lower values. This is important because surfactant dysfunction has been shown to

Figure 5. Structure–function relationships. (A–C and F) Structural data from design-based stereology at a defined end-expiratory Pao were directly correlated with tissue elastance H measured during corresponding PEEP ventilation. (A) A high inverse correlation between H and the volume of alveolar airspaces per lung ($V[\text{alvair, lung}]$) could be established using pooled structural data from all four groups at Pao = 1 and 5 cm H₂O, and lung mechanical data at PEEP = 1 and 5 cm H₂O. (B and C) The increase in H measured between PEEP = 5 cm H₂O and PEEP = 1 cm H₂O correlated with a decrease in the number-weighted mean volume of alveoli in healthy lungs (B), whereas in bleomycin-injured lungs an inverse correlation with the number of open alveoli was established in this range of PEEP ventilation (C). (D) At higher light-microscopic or electron-microscopic magnification, the decrease of Pao from 5 to 1 cm H₂O was linked to the formation of pleats (arrows). The alveolar epithelial basal lamina could be followed, forming a folding, whereas corresponding alveolar epithelium could hardly be separated within the fold because opposed cell bodies were touching each other (arrowheads in D). (E) In the D1 group, Pao = 1 cm H₂O was associated with microatelectasis. (F) The increase in H between 5 and 10 cm H₂O correlated with an increase in the volume-weighted mean volume of alveoli and thus alveolar size, as well as the coefficient of variation of alveolar size, using pooled data from all study groups. For analyses of correlation, a nonparametric Spearman test was used in A–C and F. Scale bars in light-microscopic and electron-microscopic images: 50 μm and 2 μm, respectively. air = alveolar airspace; bgb = blood–gas barrier; cap = capillary lumen; col = collagen fibrils.

occur early in ventilation-induced (1) and bleomycin-induced (29, 63) lung injuries, and thus SRT may provide a means of reversing the injury mechanism based on altered alveolar micromechanics.

At the structural level, bleomycin-induced injury resulted in a progressive decrease in alveolar surface area and a loss of patent alveoli at low Pao. These structural changes were reversible at D1 as long as Pao remained above 5 cm H₂O during deflation from elevated airway pressures, whereas at D3, widespread alveolar derecruitment could still be found even at expiratory Pao values of up to 20 cm H₂O (Figure 4D). Simulations (Figure 7D) indicated that D1 was characterized by a population of alveoli

that opened at pressures between 10 and 30 cm H₂O (Figure 6C), which were much greater than the pressures needed to prevent their collapse during expiration (Figure 6C and Table 2). At D3, more than half of the alveoli (Table 2, α) were not recruitable at Pao $\leq$ 20 cm H₂O.

To investigate the mechanical forces potentially responsible for injury pathogenesis, we performed simulations of ventilation with PEEPs ranging from 0 to 15 cm H₂O and a tidal volume of 10 ml/kg body weight (Figures 7C and 7D). These simulations did not demonstrate cyclic R/D (Figure 7D); the maximum change in alveolar O_F during tidal ventilation predicted in the four experimental groups

was less than 0.2%. Instead, the alveoli remained collapsed throughout the ventilatory cycle in the D1, D3, and SRT groups. The predicted high opening pressures and large proportion of nonrecruitable alveoli reflect findings in patients with ARDS and, in some cases, little or no evidence of cyclic reopening of distal airspaces and a high proportion of nonrecruitable lung parenchyma (26, 64). In addition, the Pao needed to reinflate collapsed parenchyma in patients with mild ARDS were between 10 and 30 cm H₂O, which parallels our predictions of alveolar opening pressures for bleomycin-injured lungs (26) (Figure 6C). Hence, the simulations observed in the bleomycin

Figure 6. Modeling of micromechanics. (A) Measured (points) and predicted (lines) elastance during derecruitment tests at PEEP = 2, 5, and 10 cm H₂O. (B) The progressive increases in elastance with time after bleomycin were accompanied by alveolar derecruitment. SRT improved alveolar recruitment compared with D3. The estimates of alveolar number are normalized by the maximum alveolar number in the control group to provide the measured (points) and predicted (lines) alveolar O_F . (C) Virtual trajectory closing and opening pressure probability density functions show the probability density functions for the closing (P_C , lines) and opening (P_O , lines with symbols) pressures for the D1 (gray), D3 (black dashed), and SRT (gray dashed) groups. The integrated values are less than unity because the maximum O_F for D1 > SRT > D3. The mean and SD of the closing pressure distributions were determined as part of the solution process.

model regarding alveolar R/D properties mimic aspects of the recruitability of lung parenchyma in some patients with ARDS.

After bleomycin-induced injury, our simulations and morphometric measurements demonstrated increased mean alveolar residual volumes (Table 2, V_{ARV} ; Figure 7A), end-expiratory alveolar volumes during mechanical ventilation across a range of PEEPs (Figure 7C), and mean individual alveolar tidal volumes. These changes were accompanied by a population of alveoli that could not be recruited at pressures below 30 cm H₂O. These predictions are in agreement with the stereological data provided in Figure 4 and Table E5, and may be partially explained by a surface tension-induced alveolar collapse of predominantly smaller alveoli so that the mean alveolar volume is shifted toward larger alveoli. In light of these findings, it is likely that alveolar collapse acts as a stress concentrator so that tethering forces on the

adjacent alveoli that remain open are increased, causing these open alveoli to gain volume due to redistribution of acinar air (20, 21). A closer look at the slope of the alveolar PV relationship presented in Figure 7A reveals that although alveoli at low pressures had greatly increased sizes in the bleomycin groups, the individual alveolar elastances over this pressure range showed only minor differences (Table 2, E_{base}). This aspect can also most likely be explained by alveolar interdependence and outward tethering forces generated by stress concentrators such as collapsed alveoli (20). Numerical simulations of the parenchyma indicate that atelectasis causes a marked increase in septal strains in the adjacent patent alveoli (21), and the transmission of these forces potentiates the mechanical inhomogeneity (65) that characterizes the rat lung (66).

The increased end-expiratory alveolar unit volume, particularly in the D3 and SRT

groups, indicates a marked increase in static strain, and the increased alveolar tidal volume indicates increased dynamic strain during ventilation (Figure 7C). Both of these factors have been implicated as potentially injurious mechanisms in VILI (67, 68). Our predictions of the mean alveolar unit volume in the D3 animals at a static $P_{ao} = 5$ cm H₂O exceed values found in healthy controls at 30 cm H₂O (Figure 7A). In other words, the end-expiratory alveolar distention at PEEP = 5 cm H₂O in the injured rats is greater than the alveolar distention at total lung capacity in the controls. These predictions are supported by the stereological measurements showing $v_v(\text{alv})$ to be greater in the D3 animals at $P_{ao} = 5$ cm H₂O than in the controls at $P_{ao} = 20$ cm H₂O (Figure 4E). Isotropic stretching of alveolar walls, and therefore of the basal lamina and alveolar epithelium, has been suggested to occur in healthy lungs at lung volumes

Table 2. Best-Fit Model Parameters

	Control	D1	D3	SRT
μ_C (cm H ₂ O)	*	−0.7	0.7	−0.7
σ_C (cm H ₂ O)	*	4.1	3.5	4.6
E_{Base} (cm H ₂ O·ml ^{−1})	2.3	3.0	2.4	3.2
E_{Fac} (cm H ₂ O·ml ^{−3})	3.1	11.8	0.9	0.8
V_{Crit} (ml)	4.6	3.4	3.6	2.6
V_{ARV} (ml)	0.96 [†]	2.0	5.0	6.3
V_{Low} (ml)	1.2	0	0	0
α	0.11	0.14	0.53	0.34

Definition of abbreviations: D1 = Day 1; D3 = Day 3; SRT = surfactant replacement therapy.

The model parameters determined using the parallel pattern search algorithm are the mean virtual trajectory closing pressure (μ_C), the SD of the virtual trajectory closing pressure (σ_C), the baseline alveolar elastance (E_{Base}), the rate of high-volume alveolar elastance increase (E_{Fac}), the threshold volume for the high-volume alveolar elastance increase (V_{Crit}), the alveolar residual volume (V_{ARV}), the threshold volume for the low-volume alveolar elastance increase (V_{Low}), and the fraction of nonrecruitable alveoli (α).

*Parameters that were not determined for the control group because the numerical fit for these parameters was dependent on derecruitment, which was not observed in healthy rats.

[†]Predictions of V_{ARV} in the control group were unreliable owing to the negligible amount of derecruitment in those animals. As such, we partition the reported residual capacity (72) into V_{ARV} using the observed distribution of ductal and alveolar air (Figure 1A).

above 80% of total lung capacity (8), whereas at lower volumes, unfolding of pleats (Figures 5D and E3) or changes in alveolar shape represent mechanisms of volume changes involving minimum stretch. In view of the considerably increased residual alveolar volume after bleomycin-induced lung injury, it appears to be reasonable to conclude that mechanisms such as unfolding of pleats or changes in shape are not able to result in appropriate alveolar volume adaptations during tidal ventilation. Because of the heterogeneity induced by lung injury, it is also unlikely that isotropic stretching occurs after bleomycin challenge. Instead, anisotropic and heterogeneous stretching of septal walls after bleomycin challenge, to allow the alveoli to adapt to volume changes, can be predicted to occur even in the range of low airway pressures (Figures 7A and 7C).

There was a considerable loss of surface area of the alveoli ($S_{[alveolar, lung]}$) (Figure 4F), in particular from $P_{ao} = 5$ cm H₂O to $P_{ao} = 1$ cm H₂O, in the D3 group. This area loss coincides with a decrease in the number of open alveoli (Figure 4D) and comparably high changes in the mean linear intercept length of distal airspaces ($L_m[indir]$, Figure 4C). In cases where pure alveolar derecruitment or folding of septal pleats is responsible for the loss of surface area in this range of pressures, the changes in $L_m[indir]$ could be expected to be minimal. Therefore, these observations

suggest that distension of acinar airspaces occurs via stretching of the septal walls even at these low airway pressures.

In addition, local tidal lung strain has been shown to correlate with local inflammation and could be reduced by increasing PEEP (19). Either SRT or a recruitment maneuver with subsequent ventilation at elevated PEEPs improves recruitment (Figure 7D) and thus lowers the mean alveolar tidal volume. SRT has been shown to improve the parameters of ALI after bleomycin challenge in previous studies (28, 30). Although repetitive SRT starting at D1 after bleomycin challenge has been shown to dramatically improve alveolar surfactant function (30), there was still a cohort of unstable alveoli derecruiting at low pressures and a considerable fraction of alveoli that could not be recruited up to 30 cm H₂O (Table 2, α ; Figure 4D). However, compared with the D3 group, the SRT group demonstrated a slight shift of the distribution of alveolar closing pressures toward lower values (Table 2, μ_C) as well as a reduction of the fraction of nonrecruitable alveoli (Table 2, α). These observations were associated with a significant decrease in the volume of intra-alveolar edema due to SRT (Figure 4I), which reproduces previous findings in this model (30). Thus, it seems likely that the alveolar derecruitment in this model is dependent on both the volume and surface tension of the lining fluid, and the improved recruitability of the alveoli could

also be attributed to anti-edematous effects. Increased PEEP after a recruitment maneuver (similar to SRT) further increased the end-expiratory static alveolar distension (Figure 7C). According to *in vitro* models, cyclic stretching is much more harmful to lung epithelial cells than tonic stretching with the same amplitude (69), so it can be speculated that PEEP and preventive SRT might reduce VILI after bleomycin-induced lung injury. Taken together, our findings indicate that in this model, alveolar R/D appears to play a secondary role, and static and dynamic stretches of open alveoli represent the dominant mechanism of altered alveolar micromechanics. It is important to note that a pathologic increase in strain, and thus volutrauma, may occur both during mechanical ventilation and in free-breathing animals, as our predictions suggest large increases in strain at low airway pressures. It is thus plausible that similar transpulmonary pressure gradients are acting during spontaneous breathing.

The primary limitations of this study arise from linking lung mechanical data from living animals to structural data from fixed lungs. Nevertheless, a strong correlation ($P < 0.0001$, $r = 0.82$; Figure E5) was found between air volumes delivered to the lung by the ventilator before tracheal ligation and the total volume of the lung after tracheal ligation and vascular perfusion fixation. The air volume delivered by the respirator was smaller than the lung volume measured by the fluid displacement method (Figure E5) because the latter also included the residual volume, tissue components, and blood volume, which were not reflected in the volumes of displaced air measured by the ventilator. Furthermore, heart arrest and related stiffening of the respiratory system, fixation artifacts, and incomplete fixation of elastic fibers might limit the transferability of lung structural data determined by stereology and lung mechanical data measured in a living animal (70). For example, volume loss due to residual elastic recoil has been estimated to amount to 8–12% (8, 70). Perfusion of the lung with osmium tetroxide followed by dehydration with ethanol would have eliminated the elastic recoil (70), but this could not be justified due to its toxicity. Therefore, volumes measured by the FlexiVent system in a living animal were used for the simulations. The volume displaced by the ventilator was

Figure 7. Predicted alveolar dynamics. (A) The predicted alveolar unit pressure–volume relationship shows increased residual volume for D3 and SRT. Data from Lamm and colleagues (72) were used for the residual capacity in the control group because predictions of V_{ARV} and V_{DRV} were unreliable owing to the lack of derecruitment in that case. (B) The product of the alveolar duct unit volume and the total number of ducts provides a scaled representation of the pressure–volume relationship for a single duct. (C) Predictions of the alveolar unit volume range during low tidal volume ventilation ($V_T = 10 \text{ ml/kg}$) over a range of PEEPs show a striking increase for D3 and SRT. (D) The increased alveolar volumes are associated with a reduced fraction of open alveoli and less than 0.2% intratidal recruitment and derecruitment for all groups.

assigned to the alveolar or alveolar duct compartment according to the volume fractions measured by stereology (Figure 1A and Table E5). This approach provided a good model fit to the experimentally observed pressures, elastances, and alveolar recruitment (Figures 6A, 6B, and E1) with the exception of the PV relationship for the control group (Figure E1), where the simulations demonstrated substantially less hysteresis than the measurements. This discrepancy is a consequence of the modeling approach that was used to recapitulate the stiffening of the uninjured lungs at low volume, due to the septal folding observed in the control group (Figures 5D and E3). We elected to simulate folding/unfolding behavior by stiffening the alveolar springs below a threshold volume (V_{Low}) at a rate (E_{Low}) because a unique set of model parameters

could not be identified in simulations that incorporated both septal and alveolar recruitment. Also, from a structural point of view, we did not determine the fraction of the “unfolded” surface area (7), which in principle could be used to calculate an “ O_F ” of surface area in control lungs in analogy to the O_F of alveoli in the injured lungs. Inclusion of such an O_F in the model might have resulted in a hysteresis in the healthy controls as well. However, this would have required electron-microscopic resolution.

The bleomycin-induced lung injury model at D3 after instillation fulfills the four relevant criteria for ALI that were established by the Acute Lung Injury in Animals Study Group (27) and applied in previous preclinical ALI studies (71). However, the clinical relevance of the direct injury caused by airway instillation of bleomycin is questionable. In addition, the

development of ALI is much slower compared with other models, such as hydrochloric acid airway instillation, or the application of a harmful mechanical ventilation pattern. Accordingly, the transferability of our findings to other animal models or clinical ARDS may be limited. However, one interesting aspect resulting from the slower development of the typical features of ALI is that the progression of injury can be characterized, in contrast to rapid-onset animal models that may mask the micromechanical factors. These alterations in alveolar mechanics are accompanied by electron-microscopic evidence of injury of alveolar epithelial type I cells that precedes light-microscopic evidence of injury or physiological dysfunction (29), so it can be considered as a subclinical manifestation of ALI at D1. Also, we have documented an

increase of neutrophilic granulocytes in BAL fluid at D3 after bleomycin instillation (30). However, at the light-microscopic level, microatelectasis dominated the pathology and accompanying alveolar inflammatory infiltrates were relatively sparse compared with those seen at later time points in this model (29). Hence, inflammatory infiltrates as such were not directly taken into consideration in our modeling approach.

In summary, we have presented structural data demonstrating progressive alveolar derecruitment with decreasing Pao in injured lungs that was not present in control animals. The progressive

derecruitment at low Pao in the injured animals resulted in increased pulmonary elastance. Injury progression was associated with increased alveolar size, indicating that parenchymal tethering increases tissue strain in the acutely injured lung. Model simulations of control and bleomycin-injured lungs did not demonstrate evidence for cyclic alveolar R/D during tidal ventilations with 10 ml/kg body weight. Instead, widespread alveolar collapse without cyclic R/D was predicted for the D1, D3, and SRT groups, which led to a marked increase in static and intratidal dynamic stretch of open alveoli. Dynamic strain in the injured

animals could be moderated by recruitment maneuvers followed by ventilation at elevated PEEP, which, as a consequence, also increased static alveolar strain. The decisive effect of SRT after bleomycin challenge was a reduction of nonrecruitable alveoli (Figure 4D; α in Table 2) and alveolar edema (Figure 4I) leading to improved macromechanics, including decreased alveolar tidal volumes (Figure 7C) and thus less dynamic strain during mechanical ventilation. ■

Author disclosures are available with the text of this article at www.atsjournals.org.

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