The response to recruitment worsens with progression of lung injury and fibrin accumulation in a mouse model of acid aspiration

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Submitted 19 December 2006; accepted in final form 7 March 2007.

Am J Physiol Lung Cell Mol Physiol 292: L1580–L1589, 2007. First published March 9, 2007; doi:10.1152/ajplung.00483.2006.—Recruitment in acute lung injury (ALI) is characterized by hypoxemic respiratory failure secondary to noncardiogenic pulmonary edema. Although not a requisite component of the clinical definition (7), lung compliance is invariably reduced in ALI (45, 47). This reduction in compliance is thought to reflect both a reduction in resting lung volume from alveolar flooding or collapse, and an increase in alveolar surface forces in the remaining aerated lung (46, 47). In addition to the contribution from edema fluid, these increased surface forces are thought to result from inhibitory binding of surfactant by plasma proteins, particularly fibrin, a potent inhibitor of surfactant function in vivo (51, 52) and a chief component of the hyaline membranes that line injured alveoli (4).

The hypoxemia and low lung compliance in ALI can, in some patients, however, be reversed by a recruitment maneuver, delivered as either a brief or sustained deep inflation (DI) with the intention of reopening collapsed regions of the lung. Once open, the lung may be kept open by the application of sufficient positive end-expiratory pressure (PEEP) (23, 38). Regrettably, these maneuvers have met with variable success in clinical trials (10, 11), perhaps owing to a disparity in response to recruitment among patients with ALI of differing origin (17, 39, 44) or at varying stages of injury (21). Although not yet determined, this impaired response to recruitment may, in fact, be a marker of injury severity. We thus hypothesized that the lungs’ mechanical response to recruitment and PEEP would deteriorate as the severity of injury progresses over time. We tested this hypothesis by examining the response of the lung to DI and PEEP at various points during the pathogenesis of ALI in a clinically relevant mouse model of acid aspiration, while simultaneously measuring indexes of lung permeability, fibrin accumulation, and markers of depressed fibrinolysis (2).

METHODS

Female C57BL/6 mice, 8–10 wk old (18.2 ± 1.0 g), were anesthetized with 400 mg/kg intraperitoneal (IP) avertin (tribromo-ethyl alcohol, Aldrich, Milwaukee, WI) and positioned vertically upright, their tongues were retracted, and their deep oropharynx was instilled with 75 μl of either hydrochloric acid (HCl) (pH 1.8) or sterile phosphate-buffered normal saline (PBS) (pH 7.4) using a syringe and bulb-tipped steel feeding catheter. The tongue was held retracted until the mice fully aspirated the fluid. Then the mice were briefly rotated to the left and right lateral decubitus position, allowed to recover from anesthesia, and monitored periodically thereafter. Mice predetermined to have had a poor aspiration at the time of injury were automatically excluded from the study. Lung mechanics were later measured among subgroups at 4, 14, 24, or 48 h following acid (n = 8 per group) or saline (n = 5 per group) aspiration. At this point, the mice were anesthetized with IP pentobarbital (90 mg/kg), then underwent surgical tracheal intubation with an 18-gauge metal cannula, and were ventilated in a quasi-sinusoidal fashion at 180 breaths/min on a flexiVent (SCIREQ, Montreal, Canada) small-animal ventilator. The cylinder piston displacement was set at 0.25 ml, which resulted in tidal volumes of 0.20 ml (~10 ml/kg) when accounting for gas compression. PEEP was controlled by submerging the expiratory limb from the ventilator to the desired depth in a water trap. The mice were paralyzed with an IP injection of pancuronium bromide (0.5 ml/kg) and allowed 5 min to adjust to the ventilator at a PEEP of 3 cmH2O. To ensure adequate anesthesia, heart rate was monitored by continuous electrocardiogram (Silogic International), measured via transcutaneous needle electrodes. The entire protocol was reviewed and approved by the Institutional Animal Care and Use Committee at the University of Vermont.
Experimental protocol. Following a 5-min stabilization period, the level of PEEP was set at either 1 or 6 cmH2O, and two 1.0-ml DI s were sequentially delivered over 4 s (each lasting 2 s) at constant flow, with a pressure limit of 25 cmH2O. This pressure limit represents the typical pressure achieved during a stepwise pressure-volume (PV) curve and was chosen to limit volutrauma and risk of pneumothorax. The mice were then returned to quasi-sinusoidal ventilation at 180 breaths/min. Respiratory system input impedance (Zrs) was measured via a forced-oscillation technique (described later) immediately following the two DIs, then subsequently every 15 s for 5 min, and then every 30 s for an additional 2 min. Each post-DI measurement period was accompanied by PEEP, delivered in the order of 1, 3, and 6 cmH2O to one-half of the mice randomly in each group and in the reverse order in the other one-half. At the end of each post-DI measurement period, a quasi-static PV curve was obtained from functional residual capacity by dropping PEEP to 0 cmH2O and immediately delivering seven steps of inspiratory volume to a total volume of 0.8 ml, followed by seven equal expiratory steps, pausing at each step for 1 s. Plateau cylinder pressure was measured during each post-DI and plotted against piston displacement (corrected for gas compression and slow leak).

Specimen collection. Mice that died prematurely on the ventilator before the end of the protocol were excluded from the analysis. This number came to five, four, and six mice at the 14-, 24-, and 48-h time points, respectively. No mice died prematurely at the 4-h time point, and no mice died prematurely before mechanical ventilation at any time point. At the end of the timed ventilator protocol, the abdomen was opened, and the left ventricle was visualized and punctured through the diaphragm to obtain blood, which was spun down, and the serum was stored at −80°C. Immediately following thoracotomy, bronchoalveolar lavage fluid (BALF) was obtained by instilling 1 ml of PBS into the lungs via the tracheal cannula and slowly suctioned back for a return of −0.8 ml. The left atrium was then cut, and 10 ml of PBS were slowly perfused through the right ventricular outflow tract to Blanch the lungs of intravascular blood. Blanched lungs were then surgically removed. The left lung was tied off with suture, dissected away, flash frozen in liquid nitrogen, and stored at −80°C. The right lung was instilled with 10% buffered formalin to a pressure of 30 cmH2O, fixed with 70% ethanol, and later embedded in paraffin, cut, and mounted for staining.

BALF analysis. Immediately following collection, BALF was centrifuged, and the supernatant was stored at −80°C. The cell pellet was resuspended, and the total cell count was determined by an Advia 120 hematology analyzer (Bayer, Tarrytown, NY). Cytosop slides were stained with hematoxylin and eosin for differential count determination. Protein content was calculated using a colorimetric assay (Bio-Rad Laboratories, Hercules, CA), standardized to graded concentrations of BSA. ELISA kits for D-dimer (Diagnostic Stago), murine total plasminogen activator inhibitor-1 (PAI-1), and active murine PAI-1 (Molecular Innovations, Southfield, MI) were used according to the manufacturer’s protocol for BALF measurements.

Permeability index. Immediately before anesthesia, mice received a tail vein injection with 2.5 mg/ml solution of FITC-labeled dextran (4,000 Da) (Sigma Chemical, St. Louis, MO) at a dose of 25 mg/kg, according to a protocol previously described (24). Following BALF and serum collection, 50 µl of BALF and diluted serum were loaded on a microwell plate, excited at 485 nm, and read at 528 nm, and the relative fluorescence of BALF to serum (expressed as a percentage) was used as an index of combined endothelial and epithelial permeability.

Tissue fibrin immunohistochemical staining. Formalin-fixed, cut, and mounted slides were deparaffinized with xylene and graded ethanol series and rinsed with water. Antigen was unmasked with sodium citrate, and slides were treated with a M.O.M. immunodetection kit (Vector Laboratories, Burlingame, CA) to block unwanted background staining. Fibrin and fibrinogen were stained using a mouse anti-fibrinogen β-chain antibody (Accurate Chemical & Scientific, Westbury, NY), followed by a biotinylated anti-mouse antibody, a Vectastain avidin-based alkaline phosphatase solution, and a vector red alkaline phosphatase substrate. Following fibrin(ogen) staining, hematoxylin was used for background staining.

Quantitative RT-PCR. Snap-frozen lungs were pulverized using liquid nitrogen-chilled mortars and pestles. RNA was extracted using TRIzol, then DNase was treated using RNeasy columns (Qiagen), and 1.0 µg of total RNA was used as a template to synthesize the first-strand cDNA using random primers and Superscript II reverse transcriptase mix, according to instructions by the manufacturer (GIBCO-BRL). Real-time semiquantitative RT-PCR was performed using the Taqman Universal PCR Master Mix and the ABI PRISM 7700 Sequence Detection System. The Assay-On-Demand primers and probes used were mouse hypoxanthine guanine phosphoribosyltransferase 1 (Hprt1) ( assay Mm00446948_m1) and mouse serpine-1 (assay Mm00435860_m1) genes, both purchased from Applied Biosystems (Foster City, CA). cDNA levels were measured using the ΔΔthreshold cycle method and normalized to Hprt1, with the data presented as mean expression relative to the housekeeping gene, Hprt1, and then calculated as a quotiente relative to a randomly chosen naive control.

Fibrin Western blot. Frozen lung tissue specimens were individually crushed, weighed, and suspended in an extraction buffer composed of sodium phosphate (10 mM), EDTA (5 mM), β-aminocaproic acid (100 mM), aprotinin (10 µU/ml), heparin (10 µU/ml), and PMSF (2 mM). Then each sample was homogenized at 2,600 rpm (POLYTRON, Kinematica) for 1 min and spun at 10,000 g for 10 min at 4°C, after which the supernatant was removed, and the pellet was resuspended in sodium phosphate (10 mM) and EDTA (5 mM). Samples were spun again at 10,000 g for 10 min, the supernatant was removed, and the pellet was resuspended in urea and spun at 14,000 g, and the supernatant was again discarded. The pellet was then resuspended in an SDS buffer and incubated at 65°C for 1.5 h before Beta-mercaptoethanol (β-ME) was added and loaded into the gel. A fibrin standard was generated by mixing 5 µg of murine fibrinogen (Sigma Chemical, St. Louis, MO) with 5 units of bovine thrombin (Sigma Chemical), incubated at 37°C for 10 min, then solubilized in SDS buffer and β-ME, and diluted to a concentration such that the greatest total amount of fibrin loaded into the well was 2,000 ng. Following gel electrophoresis, each gel was first blocked with 1% BSA on Tris-buffered saline/Tween (TBST) and incubated overnight at 4°C with a 1:500 dilution of monoclonal mouse anti-β-chain fibrin antibody (MAB350, American Diagnostica, Stamford, CT), previously conjugated to biotin, according to manufacturer protocol (EZ-Link Sulfo-NHS-LC-Biotin, Pierce, Rockford, IL). The MAB350 antibody specifically binds to a peptidopeptide sequence on the β-chain of fibrin after cleavage of fibrinopeptide B by thrombin, thus binding specifically to previously polymerized fibrin monomers, and not to fibrinogen (54). The gel was then washed in TBST and incubated for 30 min with a 1:1,000 dilution of avidin-horseradish peroxidase (R&D), washed in TBST, and exposed with Amersham ECL reagent (GE, Healthcare) for 10 min before chemiluminescence measurements.

Impedance data analysis. Zrs was determined by measuring piston volume displacement and cylinder pressure while delivering 2- s oscillatory volume perturbations to the airway opening in a manner described previously (1). These perturbations were composed of 13 superimposed sine waves with frequencies ranging from 1.0 to 20.5 Hz, all mutually prime to reduce harmonic distortion that can occur in nonlinear systems (25). Initial dynamic calibration signals were obtained to correct for the physical characteristics of the ventilator and tubing in subsequent measurements of Zrs (26, 50). Zrs itself was determined via Fourier transform from the signals of ventilator piston volume and cylinder pressure, as described previously (20, 26). Zrs was interpreted by being fit with the model.
IMPAIRED RECRUITMENT DURING PROGRESSION OF LUNG INJURY

\[ Z_{rs} = R_N + i2\pi f iaw + \frac{G - iH}{(2\pi f)^2} \]  

where

\[ \alpha = \frac{2}{\pi} \arctan \left( \frac{H}{G} \right) \]

and \( i \) represents the square root of \(-1\) and \( f \) represents frequency. The parameters \( R_N \) and \( \alpha \) are thought to characterize the resistive and inertive properties, respectively, of the airways, while \( G \) and \( H \) characterize the dissipative and elastic properties of the lung tissues, respectively (25). In particular, the parameter \( H \) is equal to respiratory elastance at an oscillation frequency of \( 1/2\pi \) Hz. Hysteresivity (\( \eta \)) is the quotient \( G/H \). Increases in \( \eta \) are believed to reflect changes in intrinsic tissue properties and/or increased regional heterogeneity in lung function (34, 40). We invoked the normalization scheme of Ito et al. (32) to express \( G \) and \( H \) in the same units as \( R_N \) (cmH\textsubscript{2}O·s·m\textsuperscript{-1}) without changing their numerical values.

Computed tomography imaging and lung volume. Using the identical protocol described previously, a separate group of mice (\( n = 5 \) per time point) were exposed to 75 μL of HCl (pH 1.8) and placed on mechanical ventilation at either 4 or 48 h after injury. The mice were ventilated with a PEEP of 1 cmH\textsubscript{2}O for 7 min and then given a lethal dose of pentobarbital before being switched over to 100% inhaled nitrogen (N\textsubscript{2}) gas for 3 min (to prevent further resorption atelectasis). Once cardiac death was confirmed by electrocardiogram, the trachea was tied off at end-exhalation (PEEP 1 cmH\textsubscript{2}O). High-resolution computed tomography (CT) images (47 μm/voxel edge, 80 kVp, 450 mA, over 80 min) were obtained using a GE Medical Systems eXplore Locus laboratory volumetric cone-beam micro-CT scanner. Lung volumes [thoracic gas volume (VTG)] were calculated using Microview visualization software, version 2.0.29 (GE HealthCare, London, ON, Canada). Two-dimensional (2D) regions of interest (ROIs) were created on 10 cross-sectional images selected from a range of slices between the proximal trachea and lung bases. The 2D ROIs were defined by contours drawn freehand closely around the lungs and trachea to exclude all extrarotaxic air (bowel gas and air outside the body was not included). 2D ROIs were then automatically created for all cross sections by linear interpolation, and three-dimensional (3D) ROIs were then generated by compiling all 2D regions. Frequency histograms of Hounsfield units (HU) were calculated for the voxels contained within each 3D ROI. The frequencies of the HUs between \(-1,000\) and 0 (corresponding to N\textsubscript{2} and water, respectively) were then converted to fractions of gas by multiplying each HU value by its corresponding number of voxels and then dividing by \(-1,000\). These fractions were then summed and multiplied by the voxel volume of 1.038 × 10\textsuperscript{-7} ml to yield an estimate for VTG. Isosurface renderings were created using an algorithm in the Microview software package, wherein a 3D surface is drawn over all contiguous voxels (within the previously defined thoracic ROI), having a gray-scale value at or above a given threshold. Image data were inverted so that the high end of gray-scale values corresponded to voxels representing regions of low X-ray attenuation (low density). A threshold value corresponding to \(-500\) HUs in noninverted data was selected, so that the spaces enclosed by the surface were considered to be occupied by at least 50% air. The resulting isosurface rendering constitutes a “virtual casting” of the airways and parenchyma.

All graphing and statistical analyses were performed using Origin software (version 7.5, Northampton, MA). ANOVA was used to compare values among all groups, followed by post hoc Bonferroni tests for means comparison between groups. A graphic representation of the protocol design is outlined in Fig. 1.

RESULTS

Lung function and derecruitment. Mean values for \( G \) and \( H \) rose immediately following DI in all acid-injured mice, but \( G \) and \( H \) rose more by 24 and 48 h from injury compared with 4 and 14 h (Fig. 2). Immediate post-DI values for \( R_N \) (data not shown) were never significantly elevated compared with saline controls at any level of PEEP at any time point of injury, but did rise over time significantly following DI at all time points during PEEP of 1 cmH\textsubscript{2}O (\( P < 0.05 \)). Immediate post-DI measures of \( H \) were significantly greater than those of saline controls at PEEP of 1 cmH\textsubscript{2}O for all time points, and at PEEP of 3 and 6 cmH\textsubscript{2}O by 14, 24, and 48 h (\( P < 0.05 \)). Immediate post-DI measures of \( G \) were not significantly elevated by 4 h, but were significantly elevated at PEEP of 1 cmH\textsubscript{2}O by 14 h, and at PEEP of 1, 3, and 6 cmH\textsubscript{2}O by 24 and 48 h (\( P < 0.05 \)). Of particular interest to our hypothesis, the total rise in \( H \) following DI (Fig. 2A) was greater than that of saline controls in all groups except at PEEP of 6 cmH\textsubscript{2}O, 4 h from injury (\( P = 0.08 \)). The total rise in \( H \) following DI tended to increase as the
time following acid instillation progressed, while the response of $H$ to added PEEP decreased as time from injury progressed (Fig. 2). The total rise in $H$ at PEEP 3 cmH$_2$O was significantly greater at 48 h (32.2 ± 4.5 cmH$_2$O·s·ml$^{-1}$), compared with that at 4 h (15.4 ± 3.4 cmH$_2$O·s·ml$^{-1}$). The total rise in $H$ at PEEP 6 cmH$_2$O became significantly greater at 48 h (15.7 ± 3.3 cmH$_2$O·s·ml$^{-1}$), compared with both 4 h (5.9 ± 1.0 cmH$_2$O·s·ml$^{-1}$) and 14 h (6.4 ± 0.8 cmH$_2$O·s·ml$^{-1}$). A similar pattern in the response to PEEP by 48 h was observed for the total rise in $G$ following DI (Fig. 2B). By 4 and 14 h, the total rise in $G$ following DI was only significantly higher than that of saline controls during PEEP of 1 cmH$_2$O. By 24 and 48 h, the total rise in $G$ following DI became significantly higher than control during PEEP of 1, 3, and 6 cmH$_2$O. At 48 h, the total rise in $G$ during PEEP 3 cmH$_2$O (1.77 ± 0.34 cmH$_2$O·s·ml$^{-1}$) was greater than at 4 and 14 h (0.71 ± 0.30 and 0.66 ± 0.12 cmH$_2$O·s·ml$^{-1}$, respectively, ANOVA, $P < 0.05$).

Increasing the level of PEEP always had a significant effect on the post-DI rises in $G$ and $H$ at every time point studied (ANOVA, $P < 0.0001$). However, the magnitude of difference in the mean rise in $H$ between increasing levels of PEEP was less at 48 h than at all other time points, with the exception of the difference between PEEP of 3 and 6 cmH$_2$O at 4 h. This latter exception was likely due to the greater effect of PEEP 3 cmH$_2$O at 4 h (see Fig. 2).

The PV curves demonstrated a significant decline in hysteresis following ventilation at a PEEP of 6 cmH$_2$O compared with either 1 or 3 cmH$_2$O at all time points (Fig. 3, A and B). However, PEEP was less effective at reducing hysteresis at 24 and 48 h compared with the earlier time points (Fig. 3, C and D). The mean area enclosed by the PV loops obtained following ventilation at PEEP 3 cmH$_2$O was significantly different from that following ventilation at 1 cmH$_2$O at 4 h, but this comparison was not significantly different at the later time points.

**BALF.** Mean values for BALF total protein concentrations increased progressively as time advanced from injury (Fig. 4, left axis) and were significantly elevated by 24 and 48 h compared with values from 4 h (ANOVA, $P < 0.05$). Relative ratios of BALF to serum FITC-dextran concentrations were significantly elevated above saline controls at every time point, and there was a statistical trend for these values to increase.
over time (ANOVA, \( P = 0.092 \)) (Fig. 4, right axis). BALF demonstrated a significant rise in total cells by 4 h and a significant rise in neutrophils by 24 h, relative to saline controls (\( P < 0.05 \)) (Fig. 5), and both remained elevated through to 48 h.

**Fibrin accumulation.** The clearance of fibrin is chiefly governed by the relative quantity and activity of fibrinolysis promoters and fibrinolysis inhibitors; the latter of such is represented by PAI-1 (28). As an indirect measure of fibrinolysis inhibition, total PAI-1 antigen and active PAI-1 levels in the BALF were measured and were noted to be significantly elevated by 48 h (Fig. 6) compared with saline controls and all other time points following acid injury (ANOVA, \( P < 0.05 \)). Mean relative lung tissue mRNA levels for the PAI-1 gene, serpine-1 (Fig. 6), demonstrated a significant increase in transcription of mRNA for PAI-1 by 24 h (relative to saline controls) and particularly by 48 h (relative to all other groups) (ANOVA, \( P < 0.05 \)). Immunohistochemical staining signified greater levels of fibrinogen and fibrin at 24 and 48 h compared with 4 and 14 h (representative specimens from 4 and 48 h shown in Fig. 7). BALF levels of the fibrin split product, D-dimer, were significantly elevated at 14, 24, and 48 h (Fig. 8A) compared with both saline controls and specimens from the 4-h time point (ANOVA, \( P < 0.05 \)). Total lung fibrin levels also increased progressively over time and were significantly elevated at 48 h compared with 4 h (Fig. 8B). A representative image from the immunoblot gels is provided in Fig. 8C.

**CT lung volumes.** Mean \( V_{TG} \) from nitrogen-fixed lungs (Fig. 9A) following 7-min ventilation at a PEEP of 1 cmH\(_2\)O was significantly lower at 48 h following acid aspiration (0.085 ± 0.010 ml) compared with 4 h following aspiration (0.206 ± 0.043 ml, \( P = 0.017 \)). An inverse linear correlation was found between the post-DI rise in H (over 7-min ventilation at PEEP 1 cmH\(_2\)O) and the corresponding postventilation estimates of \( V_{TG} \) for the separate groups of mice imaged at 4 and 48 h (Fig. 9B, \( R = 0.90, P < 0.001 \)). Representative 3D isosurface renderings at 4 and 48 h are shown in Fig. 9C and illustrate the reduction in the amount of open lung at 48 h compared with 4 h following aspiration.

**DISCUSSION**

The results of this study demonstrate that acid aspiration in mice, as expected, leads to a substantial deterioration in lung function. However, contrary to previous studies in acid-injured rats demonstrating that indexes of lung permeability recover by 14 h (35), we found that injury worsened progressively over the 48 h following aspiration in our model. The primary mechanical derangement we observed was an increased tendency for derecruitment of the lung to occur during mechanical ventilation. This was evidenced by progressive and proportionate increases in the tissue parameters G and H following a DI (Fig. 2) and visualized loss of aerated lung units on micro-CT images (Fig. 9). Furthermore, the degree of post-DI lung derecruitment increased progressively with time after aspiration, while PEEP became less effective at both ameliorating this derecruitment (Fig. 2) and reducing PV hysteresis.
particularly by 48 h. Together, these findings demonstrate that acid aspiration in mice causes an alveolar instability, and that this instability continues to progress for at least 48 h. This suggests that alveolar surfactant function, which is crucial for alveolar stability, was being progressively disrupted in the mice. Plasma proteins, and fibrin in particular, are potent inhibitors of surfactant function (51). Hence it is noteworthy that we established that both permeability and alveolar fibrin increased in concert with the observed mechanical derangements over the 48 h following acid aspiration. While no single parameter of injury increased significantly at each successive time point, taken together the parameters demonstrate a consistent trend of injury progression to account for the worsening response to recruitment and PEEP over time.

Increased permeability from both epithelial and endothelial injury is a well-recognized feature of ALI (60), and one would expect this to lead to elevated levels of plasma proteins, including fibrin, in the alveolar space. However, alveolar fibrin may have been further elevated in our mice through the inhibition of fibrinolysis, as evidenced by increased lung transcription and alveolar elaboration of PAI-1. The increased levels of the fibrin split product D-dimer in BALF could be due to either increased levels of total fibrin or an increased breakdown of fibrin, and thus the lack of difference between 14, 24, and 48 h (Fig. 8A) does not lend as much insight as the levels of PAI-1 and total fibrin. Both active and total PAI-1 levels in BALF, and mRNA levels from tissue, became increasingly elevated with time, with the highest levels occurring at 48 h (Fig. 6), when total fibrin levels were also greatest (Fig. 8). These results are consistent with the dysregulated alveolar fibrin accumulation that has long been recognized to occur in ALI (4), manifesting as impaired fibrinolysis and increased procoagulant activity within the BALF of patients with ALI (8, 15, 22, 29). Fibrin formation and clearance in the lung is in part governed by the relative quantities and activities of fibrinolysis inhibitors, such as the serine protease PAI-1 (28), which can

Fig. 7. Representative images from lung specimens following fibrinogen immunohistochemical staining, marked with vector red. A: representative image obtained from a lung specimen of an acid-exposed mouse 4 h after injury, shown at ×200 magnification and at ×400 magnification (inset). B: representative image obtained from a lung specimen of an acid-exposed mouse 48 h after injury.

Fig. 8. A: mean (±SE) BALF D-dimer levels for acid-injured (solid circles) and saline control (open circles) mice at each time point. *Significant difference from saline controls and mice studied at 4-h time point (P < 0.05). B: progressive increase over time in total mean (±SE) lung tissue fibrin levels (ng/mg tissue) in 24-h saline controls and acid-injured mice at each sequential time point. *Significantly greater than mice at 4-h and in 24-h saline controls (P < 0.05). C: representative image of the immunoblotting technique for the β-chain of fibrin, with mouse fibrin standards and tissue extractions visualized at a level corresponding to a 57-kDa protein size.
prevent fibrinolysis via direct binding and inhibition of tissue
and urokinase plasminogen activator (58). Inhibition of uroki-
nase plasminogen activator activity by PAI-1 in the BALF of
ALI patients was first noted by Bertozzi and colleagues in 1990
(8), and it has subsequently been shown that mice genetically
deficient in PAI-1 fail to accumulate alveolar fibrin and are
more resistant to hyperoxia-induced ALI (5). Elevated plasma
and edema fluid levels of PAI-1 in human subjects with ALI
have also recently been linked to higher mortality (48, 59),
imparting value to PAI-1 as a prognostic marker in ALI.

Our findings of a temporal variation in the response of the
lung to DI and PEEP are in agreement with clinical findings
that lasting responses to recruitment maneuvers in ALI patients
are typically confined to the early course of their disease (9,
21). The lung mechanical derangement of ALI manifests
mainly as an increase in pulmonary elastance, particularly in
the more direct injury forms of ALI such as pneumonia (17).
At the bedside, this increase in elastance is typically observed
as an increase in peak and plateau airway pressures, but it can
also be observed as an expansion in the hysteresis of PV curves
obtained during graded inflation of the lung (47). Increased
elastance is thought to reflect both an increase in surface
tension within the alveolar lining fluid and a reduction in lung
volume from alveolar flooding and collapse, the latter process
being colloquially termed “baby lung” (16). Affected regions
of the lungs may often be so damaged that they remain
fluid-filled or collapsed throughout the entire course of infla-
tion (27), and CT imaging has demonstrated elastance to
 correspond to the amount of remaining aerated tissue in the
lung (18). This has lead to the notion that the increased lung
elastance seen in ALI is more a reflection of the degree of lung
derecruitment than of changes in intrinsic tissue stiffness (16).

We have made the same assumption in our laboratory’s pre-
vious work (3) and continue to do so in the present study by
using G and H as surrogate markers of open lung. This
assumption is supported by previous findings that derecruit-
ment causes G and H to increase in the same proportion (3),
such that their ratio (G/H), also termed “hysteresivity”(14),
remains relatively unchanged over time. However, it should
also be noted that such rises in H, and particularly G, could also
represent an element of increasing heterogeneity within the
lung, which has been shown to occur in models of ALI (33)
and may, in turn, contribute to variations in mechanical stress
and bioinjury (53). In turn, if the process of derecruitment
occurs during the measurement of Zrs, we have shown that
hysteresivity can potentially even decrease (6). Nevertheless,
the strong correlation between the rise in H and CT estimates
of VTG shown in Fig. 9B suggest that the majority of these
changes represents derecruitment of lung volume. This can be
further appreciated by both CT-based calculations of VTG
(Fig. 9A) and the air surface reconstructions from acid-injured
lungs (Fig. 9C), which demonstrate that the effects of DI and
PEEP become less effective at recruiting lung volume between
4 and 48 h following acid aspiration.

Our mouse model of acid aspiration was designed to simu-
late a widely recognized and common cause of ALI in humans,
namely aspiration of acidic gastric contents (60). Acid aspira-
tion involves a direct injury to the airways and parenchyma,
and thus represents a subset of direct or “pulmonary” ALI in
which recruitment has been historically shown to be less
effective (44), particularly in its later phases (21). One poten-
tial explanation for the differential effect of recruitment on
early and late injury in our model is a divergence in the
character of alveolar injury over time. For instance, the early

Fig. 9. A: bar graph demonstrating mean VTG measurements (±SE), interpolated from high-resolution CT scans of mice at 4 and 48 h following acid aspiration. *Statistically smaller mean VTG value at 48 h compared with 4 h. B: rise in H (over 7-min ventilation at PEEP 1 cmH2O) for individual mice at 4 and 48 h after injury, plotted against corresponding postventilation estimates for VTG. Open circles, 4-h acid ALI group; shaded triangles, 48-h acid ALI group. Linear regression against all points yielded an inverse correlation with an R of 0.90 (P < 0.001). C: representative three-dimensional air isosurface rendering from images from mice at 4 and 48 h after injury are shown to the right. Images were obtained following DI and ventilation at PEEP 1 cmH2O for 7 min before nitrogen gas fixation (see METHODS).
phase of acid injury in our model is characterized by an immediate disruption of epithelial barrier function, with an early increase in permeability and alveolar protein, but negligible accumulation of neutrophil exudates or fibrin. This likely leads to an injury pattern dominated by edema and altelectasis, a type of injury demonstrated in previous studies to be more responsive to recruitment and PEEP (57). Conversely, the later phase of injury in our model was characterized by an accumulation of fibrin-laden exudates (Fig. 7). This may be a histological pattern more in keeping with the late phase of ALI (21) or direct forms of ALI (44) that are typically less responsive to recruitment and PEEP.

We assert that the progressively increased transcription and elaboration of PAI-1 and increasing alveolar fibrin accumulation in our mice further support their validity as a clinically relevant animal model of ALI. Indeed, this model may have a number of advantages over the commonly used approach of delivering endotoxin to the air spaces. Although endotoxin reliably generates a robust accumulation of alveolar neutrophils (1, 55), the injury it causes often subsides within 24–48 h (42, 43). Furthermore, repeated endotoxin exposures are often required to generate significant derangement in lung mechanical function (31) and sometimes fail to generate any significant derangement at all (1). Even intravenous endotoxin, which generates a profound inflammatory response with increased permeability and derangement in lung function (12), subsides within 48 h (49). In contrast, acid aspiration appears to instigate a crescendo of injury in the form of increasing air space protein, permeability, and fibrin with a corresponding derangement in lung function that continues to evolve through to 48 h. Interestingly, the number of air space neutrophils remains stable between 14 and 48 h, possibly due to an attenuation of neutrophil apoptosis and clearance (41).

Nevertheless, there are aspects of our mouse model of ALI that were unexpected. In particular, rat models of acid-induced lung injury (35) have been shown to recover barrier function by 14 h and to exhibit a peak in neutrophil accumulation by 4 h. Both features of our mouse model extended much further out from the time of aspiration. The reasons for these differences are unclear. One possibility is that a delayed neutrophil response in our mice may have helped drive a correspondingly delayed derangement in epithelial/endothelial barrier function (13, 19, 37). Some studies have demonstrated a negligible contribution of neutrophil elastases to the disruption of barrier function during the earlier phase of acid-induced lung injury (56), suggesting that early injury is due to a direct caustic effect of acid on the epithelium. Other studies examining acid injury at a later time point (24 h) have demonstrated a more important role for neutrophils by demonstrating an attenuation in injury through neutrophil inhibition and depletion (13). The concept that neutrophils might drive a second phase of lung injury is also supported by the finding that neutrophil- elastase inhibitors only protect the lung in regions remote from the original site of aspiration injury (19). It is possible, therefore, that neutrophil dynamics following acid aspiration differ between mice and rats. However, little is known about this. Although mouse models of acid aspiration are becoming more widely utilized (30, 61), few investigators (36) have followed this model out as far as in the present study. Furthermore, none to our knowledge has investigated the effect of this injury on lung mechanical function, an important clinical marker of injury.

In conclusion, we have developed an acid-aspiration mouse model of ALI that exhibits many of the key physiological and biological features of direct ALI in patients. The injury in this model continues to worsen out to 48 h after aspiration, as reflected in progressive derangements in lung mechanical function with commensurate reductions in the effectiveness of DI recruitment maneuvers and PEEP. Furthermore, the mice exhibited increasing indexes of permeability and accumulation of alveolar fibrin, the latter known to be pathognomonic for human ALI and likely involved in the inhibition of surfactant function (51). Increasing levels of pulmonary PAI-1 and PAI-1 mRNA indicate that alveolar fibrin accumulation may have been further augmented through inhibition of fibrinolysis. Our study thus helps to elucidate the complex interplay between host defense mechanisms in the lung and their possible pathological consequences following a sufficiently egregious insult, and in particular supports the notion that alveolar protein and fibrin accumulation primarily determine the mechanical response of the lung to recruitment maneuvers and PEEP.

REFERENCES

This study was supported by National Institutes of Health Grants K08HL074107, R01 HL75593, and Centers of Biomedical Research Excellence (COBRE) P20RR15557.


