Extracellular Histones Play an Inflammatory Role in Acid Aspiration-induced Acute Respiratory Distress Syndrome

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ABSTRACT

Background: Systemic inflammation is a key feature in acid aspiration-induced acute respiratory distress syndrome (ARDS), but the factors that trigger inflammation are unclear. The authors hypothesize that extracellular histones, a newly identified inflammatory mediator, play important roles in the pathogenesis of ARDS.

Methods: The authors used a hydrochloric acid aspiration-induced ARDS model to investigate whether extracellular histones are pathogenic and whether targeting histones are protective. Exogenous histones and antihistone antibody were administered to mice. Heparin can bind to histones, so the authors studied whether heparin could protect from ARDS using cell and mouse models. Furthermore, the authors analyzed whether extracellular histones are clinically involved in ARDS patients caused by gastric aspiration.

Results: Extracellular histones in bronchoalveolar lavage fluid of acid-treated mice were significantly higher (1.832 ± 0.698) at 3 h after injury than in sham-treated group (0.63 ± 0.153; \( P = 0.0252 \), \( n = 5 \) per group). Elevated histones may originate from damaged lung cells and neutrophil infiltration. Exogenous histones aggravated lung injury, whereas antihistone antibody markedly attenuated the intensity of ARDS. Notably, heparin provided a similar protective effect against ARDS. Analysis of plasma from ARDS patients (\( n = 21 \)) showed elevated histones were significantly correlated with the degree of ARDS and were higher in nonsurvivors (2.723 ± 0.2933, \( n = 7 \)) than in survivors (1.725 ± 0.1787, \( P = 0.006, n = 14 \)).

Conclusion: Extracellular histones may play a contributory role toward ARDS by promoting tissue damage and systemic inflammation and may become a novel marker reflecting disease activity. Targeting histones by neutralizing antibody or heparin shows potent protective effects, suggesting a potentially therapeutic strategy. (Anesthesiology 2015; 122:127-39)

A CUTE lung injury is a serious clinical condition characterized by acute diffuse, inflammatory lung injury leading to enhanced alveolar-capillary permeability, edema, hypoxemia, or hemorrhage. Acute respiratory distress syndrome (ARDS) is the most severe form of acute lung injury.\(^1,2\) Notably, a recent report—the Berlin Definition—recommends use of three categories of ARDS, based on the degree of hypoxemia, to replace the definition of acute lung injury. Currently, most treatment for ARDS is just supportive care. Of these, lung-protective ventilation and fluid-conservative management have proven to be able to reduce mortality and morbidity, respectively.\(^5,5\) However, despite the advances in basic and clinical research, ARDS still represents a life-threatening problem among intensive care unit patients, with an in-hospital mortality of 40% or more.\(^1,2\)

The most common causes of ARDS are lung infection, aspiration of gastric contents, sepsis, multiple trauma, and other insults, all of which are thought to initiate a dysregulated inflammation and inappropriate accumulation and activation of leukocytes within the lungs that lead to alveolar barrier disruption, and subsequently a severe condition such as respiratory failure.\(^2,6\) It is noticeable that systemic inflammation is a common pathological feature shared by different etiologies-caused ARDS as demonstrated by a rapid influx of leukocytes and releases of proinflammatory cytokines.\(^2,7\) However, the factors or mechanisms that trigger systemic inflammation in ARDS are largely unclear. Identification of key mediators that may modulate ARDS-associated inflammation is highly desirable.

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What We Already Know about This Topic

• Acid aspiration is known to be a cause of acute lung injury.

What This Article Tells Us That Is New

• Extracellular histones were significantly elevated in the bronchoalveolar lavage from mice with acid-induced lung injury versus sham mice and in human patients who died from acute lung injury compared to survivors with acute lung injury. Extracellular histones may be causal, and targeting histones may be a reasonable therapeutic strategy.
More recently, extracellular histones are recognized as a pivotal mediator in systemic inflammatory diseases, both infectious and noninfectious, including sepsis, acute ischemia–reperfusion injuries of the kidney or liver, and trauma-associated lung injury.9-10 It reveals that extracellular histones have many functions including induction of endothelial damage, coagulation activation, platelet aggregation, and cytokine production,11-14 all of which are likely involved in the pathogenesis of inflammatory organ injuries such as ARDS.

We speculate that extracellular histones play a contributory role in the initiation and progression of ARDS and modulation of histones may have some therapeutic potential. To test this hypothesis, we prepared a mouse model of acid aspiration-induced ARDS, which is clinically relevant model of acute gastric aspiration injury.6,15 to investigate whether extracellular histones released after lung injury have pathogenic roles. Besides, targeting histones by specific antihistone antibody or activated protein C has been shown to be protective.8,16 We question whether neutralization of histones by other chemicals such as heparin may have similar beneficial effects, which could yield new strategies for management of ARDS. Heparin is commonly used as a potent anticoagulant, but it is also found to have antiinflammatory effects.17,18 However, the molecular mechanism of heparin-mediated antiinflammation remains to be defined. It reveals that histones have high affinity toward heparin19 and some histone-induced effects such as platelet aggregation could be inhibited in the presence of heparin.11 Therefore, we sought to examine whether heparin has the potential to attenuate acid aspiration-induced lung injury through binding to histones. Furthermore, we recruited 21 patients with ARDS caused by aspiration of gastric contents to analyze the clinical relevance of extracellular histones.

Induction of Lung Injury with Acid Aspiration in Mice
Before acid aspiration, mice fasted overnight but were allowed water ad libitum. For the induction of acid-induced lung injury, mice were anesthetized using sodium pentobarbital (50 mg/kg), and injured by intratracheal instillation of hydrochloric acid (HCl, 0.1N, pH 1.5) into the lung via a tracheal catheter. The sham mice underwent the same procedure but received sterile saline instead.

Assessment of ARDS in Mice
The extent of ARDS was assessed by blood gas analysis, pulmonary edema, and lung histology. An abdominal aorta catheter with sodium citrate was inserted to obtain arterial blood and arterial partial oxygen tension (PaO₂) was analyzed with a gas analyzer (Ciba Corning-170 blood gas analyzer, Ciba Corning, Etobicoke, ON, Canada) at different time points after acid aspiration.

The ratio of wet to dry lung weights was calculated to assess pulmonary edema. Lung tissues were rapidly excised and rinsed in phosphate-buffered saline (PBS) to remove contaminating blood. After removal of excessive phosphate-buffered saline by careful drying on tissue paper, lung tissues were weighed (wet weight). Then the tissues were dried in an oven at 60°C for 72 h, followed by a second weighing (dry weight).

For lung histology, part of lung tissues were fixed with 10% buffered formalin, embedded in paraffin, and 5 μm sections were obtained and stained with hematoxylin and eosin. The stained sections were evaluated and scored by pathologists who were blinded to the experimental protocol. The severity of lung injury was scored according to the following parameters: hemorrhage, alveolar edema, alveolar exudates, necrosis, and leukocyte infiltration.

Preparation of Mouse Samples
In another group of mice, the lungs were flushed with 1 ml phosphate-buffered saline to obtain bronchoalveolar lavage fluid (BALF). BALF was centrifuged at 1,000×g for 10 min at 4°C and supernatants were stored at −80°C until further analysis. The lung lobes were rapidly excised, flash frozen in liquid nitrogen, and stored at −80°C. Mouse blood was collected by retro-orbital bleeding in a tube containing sodium citrate as anticoagulant and centrifuged to separate plasma, and then kept at −80°C.

Measurement of Extracellular Histones in BALF and Plasma of Mice
As nucleosomes are complexed with histones and DNA, assaying the concentrations of nucleosomes allows for the relative quantification of histones.16,21 We first measured the concentrations of nucleosome in BALF and plasma of mice using a Cell Death Detection ELISA kit (Roche Applied Science, Mannheim, Germany). We further characterized extracellular histones, especially histone H4 by ELISA and Western blotting because histone H4 is believed to play a significant role in the pathogenesis of inflammatory lung injury.
central role in mediating cytotoxicity in contrast to other individual histones including H1, H2A, H2B, and H38,9.

Quantification of Lactate Dehydrogenase Activity and Neutrophil Activation

It has been suggested that extracellular histones can be released from severely injured tissues or inflammatory leukocytes, especially neutrophils. Lactate dehydrogenase (LDH) is a cytoplasmic enzyme and its activity reflects the degree of tissue damage. We measured LDH activity in BALF and plasma of mice using an Automated Multi-parameteric Analyzer (AU 5400, Olympus, Tokyo, Japan) according to an automated procedure. For determination of neutrophil activation, we assayed myeloperoxidase activity, an index of neutrophil, monocyte/macrophage infiltration,22,23 in BALF and plasma of mice using a commercial kit (BioVision, Milpitas, CA) according to the manufacturer’s recommended protocol. In addition, to strengthen the specificity, we stained paraffin-embedded lung sections with anti-Ly6G (Abcam, Cambridge, United Kingdom), which is commonly used as a surface marker for neutrophils24 to visualize neutrophil infiltration.

Treatment of Exogenous Histones, Antihistone Antibody, and Heparin

In a separate set of experiment, we sought to examine whether histones released after acid-caused lung injury play a major role in the pathogenesis of ARDS. To this end, a mixture of all individual histones (20 mg/kg) isolated from calf thymus or human recombinant histone H4 (5 mg/kg) was given by pulmonary instillation to mice shortly after HCl challenge. Meanwhile, another group of mice received antihistone H4 mAb (20 mg/kg) intravenously or heparin (250 IU/kg) by subcutaneous injection immediately after acid aspiration, with an aim of further confirming the pathogenic role of histones as well as exploring possible treatment strategies. The dosage of histones, antihistone H4 mAb, or heparin was adopted on the basis of our previous experiments.

Measurement of Cytokines

Frozen lung tissues of mice were homogenized in lysis buffer containing 50 mM Tris-HCl, 150 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate and cocktail protease inhibitors. Lung homogenates were centrifuged at 10,000g at 4°C for 15 min, and supernatants were stored at −80°C until assays were performed. We quantified interleukin (IL)-1β, IL-6, IL-10 and tumor necrosis factor (TNF)-α levels in BALF, plasma and lung homogenates using the ProcartaPlex Multiplex Immunoassay from eBioscience (Vienna, Austria), according to the manufacturer’s protocol.

Assay for Histone Cytotoxicity In Vitro

Human lung epithelial cells (BEAS-2B) were obtained from American Type Culture Collection (ATCC: CRL9609). They were cultured in Dulbecco’s modified eagle medium supplemented with 10% fetal bovine serum, and 1% penicillin-streptomycin. After the cells grew to 70–80% confluence, they were stimulated with calf thymus histones (50 μg/ml) or recombinant histone H4 (20 μg/ml) for 1 h at 37°C. In another set of experiment, calf thymus histones or recombinant histone H4 were preincubated with antihistone H4 antibody (20 μg/ml) or heparin (200 μg/ml) for 1 h at room temperature before administration to cells. Cells were harvested and stained with propidium iodide to analyze cell damage via flow cytometry. In addition, cell culture supernatants were analyzed for IL-1β, IL-6, IL-10, and TNF-α levels using the ProcartaPlex Multiplex Immunoassay.

Measurement of Extracellular Histones and Inflammatory Markers in Patients with ARDS

A total of 21 ARDS patients caused by aspiration of gastric contents who were admitted to the emergency ward were recruited from Shanghai Pulmonary Hospital, Tongji University School of Medicine, Shanghai, People’s Republic of China. These patients met the criteria for ARDS defined by the Berlin Definition.3 Inclusion criteria were as follows: (1) acute onset of lung injury, (2) arterial oxygen tension/fraction of inspired oxygen (PaO2/FiO2) less than or equal to 300 mmHg, (3) infiltrative shadow seen on chest radiography. All the patients were observed early in the course of ARDS. Patients who had sepsis or ARDS for more than 3 days were not included. All subjects or their relatives gave their informed consent for inclusion before they participated in the study. The study was approved by the Ethics Committee and Institutional Review Board of Shanghai Pulmonary Hospital, Shanghai, People’s Republic of China, which followed the recommendations of the Declaration of Helsinki for biomedical research involving human subjects. Fifteen age- and sex-matched healthy volunteers were included as controls. The blood samples were collected from patients at admission, plasma was separated, aliquoted and stored at −80°C for future analysis. Given that the incidence of gastric aspiration-induced ARDS is relatively low, and it was just a preliminary evaluation instead of a randomized, controlled study for human patients, so the sample-size calculation of patients was not conducted.

Statistical Analysis

For animal survival analysis, the Log-rank (Mantel-Cox) test was applied, with all data censored by 24 h. All values were expressed as mean ± SD. Data were analyzed using unpaired Student t test (for two groups), one-way analysis of variance followed by Tukey post-tests (for more than two groups). In addition, data that were obtained at multiple time points throughout the experiment were analyzed using a two-way analysis of variance for PaO2, lung dry/wet ratio, and nucleosomes/histones, LDH, and myeloperoxidase, respectively. Multiple comparisons were corrected by Bonferroni post-tests, in which group allocation (acid aspiration vs. sham) and time were treated as two factors. Correlations between variables were assessed using Pearson correlation.
analysis. The number of mice in each group was selected based on our preliminary experiments. The samples sizes (n) are indicated in the figures. Results were considered statistically significant when \( P < 0.05 \). All statistical analyses were calculated using GraphPad Prism v5 (GraphPad Software, Inc., San Diego, CA).

**Results**

**Extracellular Histones Are Dramatically Released in Mice with Acid Aspiration-induced Lung Injury**

It showed that administration of HCl to mice produced significant lung injury, as demonstrated by the decreased \( \text{PaO}_2 \) and a remarkable increase of lung wet/dry weight ratio, both of which were viewed as the hallmark of ARDS. \(^2,^4\) (fig. 1, A and B). Lung histological examination at 6 h after HCl challenge showed multifocal alveolar hemorrhage, disruption of alveolar wall, and massive infiltration of inflammatory cells in mice (fig. 1C). After lung injury, the levels of extracellular nucleosome that consist of histones and DNA were found to be increased in BALF of mice, with parallel results obtained from mice plasma (fig. 1, D and E). The release of nucleosome into BALF and plasma occurred in a time-dependent manner, which correlated with the severity of ARDS.

Further analysis of histones revealed that individual histone H2A, H2B, H3, and H4 were simultaneously detected in plasma of mice with ARDS (data not shown and fig. 1F). Considering that histone H4 is a primary factor exerting toxic effects, \(^8,^16\) we further measured the concentrations of histone H4 by ELISA in BALF and plasma of mice. Parallel BALF levels and plasma levels of histone H4 were observed to be increased markedly in mice with ARDS (fig. 1, G and H), which was in line with the changes of nucleosomes. Both assays for nucleosome and histone H4 were comparable (BALF and plasma nucleosome vs. BALF and plasma histone H4: Pearson correlation coefficient \( r = 0.7117, \quad P < 0.001; \quad r = 0.6368, \quad P = 0.0002 \)), indicating that nucleosome or histone H4 can be a shared marker for ARDS. Collectively, these data suggest that large quantities of nucleosomes are released into circulation during the course of severe lung injury, which can be presented in the form of individual histones including H4.

**Massive Injured Tissue Cells and Neutrophil Activation may Cause the Released Extracellular Histones**

Extracellular histones can be derived from dying tissue cells or from the degradation of inflammatory cells. \(^3,^25\) Neutrophil extracellular traps formed in response to systemic inflammation may be an important source of released histones found in the circulation. \(^23,^26,^27\) We first assayed LDH activity that reflects the degree of tissue damage, and as expected we observed a time-dependent increase of LDH activity in BALF and plasma of acid aspiration-treated mice (fig. 2, A and B), suggesting an occurrence of drastic cell death. Then, we measured neutrophil activation, which is considered to be a major mechanism for inflammation associated with lung injury.\(^27,^28\) by measuring myeloperoxidase activity in BALF and plasma of mice. After acid aspiration, parallel BALF and plasma myeloperoxidase activities were found to be increased markedly in a time-dependent manner (fig. 2, C and D). Moreover, immunohistochemical analysis of Ly6G staining confirmed a significant neutrophil infiltration at 6 h in the lung sections of acid-treated mice (fig. 2E). There was a strong correlation of BALF nucleosome levels with BALF LDH \( (r = 0.7406, \quad P < 0.0001) \), and with BALF myeloperoxidase activity \( (r = 0.7080, \quad P < 0.0001) \) (Supplemental Digital Content 1, fig. 1, http://links.lww.com/ALN/B88). Taken together, it suggested that the increased levels of extracellular histones were likely derived from dying lung tissue cells as well as neutrophil infiltration in response to HCl challenge in mice.

**Exogenous Histones Aggravate Tissue Damage and Systemic Inflammation in Mice with ARDS**

In further experiments, we aimed to ascertain whether extracellular histones released during the course of ARDS contributed to inflammation and lung injury in this model. We showed that pulmonary instillation of low doses of calf thymus histones (20 mg/kg) or recombinant histone H4 (5 mg/kg) alone resulted in mild lung damage in mice, as evidenced by an increase of BALF LDH levels at 6 h \( (431.2 \pm 47.88 \text{ vs } 366.3 \pm 27.02) \) in contrast to sham-treated mice \( (213.4 \pm 22.59; \quad P = 0.007, \quad P = 0.004) \) (fig. 3A). Notably, administration of exogenous histone mixture or recombinant histone H4 in conjunction with HCl significantly increased the death rate of mice, as compared with only HCl-treated group (fig. 3, B and C). Histone mixture or recombinant histone H4 remarkably aggravated acid-induced lung injury and systemic inflammation in mice, as evidenced by enhanced lung injury scores (fig. 3D) and the increased levels of inflammatory cytokines such as TNF-\( \alpha \), IL-1\( \beta \), IL-6 and IL-10 in BALF and lung homogenates (fig. 3, E–H and Supplemental Digital Content 1, fig. 2, http://links.lww.com/ALN/B88). Several of these cytokines are known to be associated with development and progression of ARDS in humans.\(^29,^30\) Therefore, our data confirmed that extracellular histones released from lung cell death or neutrophil infiltration are a major contributor to ARDS in mice after acid aspiration.

**Neutralization of Histones Is Protective in Mice with Lung Injury**

Targeting extracellular histones by specific neutralizing antibody (e.g., antihistone H4 antibody) or activated protein C was previously shown to be protective in several inflammatory conditions.\(^8,^9\) In this study, we sought to examine whether heparin, which is capable of binding to histones\(^19,^31\) (Supplemental Digital Content 1, fig. 3, http://links.lww.com/ALN/B88) has similarly protective potentials. Antihistone H4 antibody was administered separately as a positive control. It showed that either heparin or antihistone antibody significantly reduced lung injury in acid-treated mice,
demonstrated by improved lung histology (fig. 4, A and B). Parallel BALF and tissue levels of TNF-α, IL-1, IL-6, and IL-10 levels were found to be reduced remarkably with blockade of extracellular histones (fig. 4, C–F and Supplemental Digital Content 1, fig. 4, http://links.lww.com/ALN/B88). Collectively, this confirms that extracellular histones play a detrimental role in the progression of ARDS and targeting histones by neutralizing antibody or heparin is protective.

**In Vitro Studies**

We further elucidated whether heparin mediated-protection is dependent on binding to extracellular histones. To address this issue, we first stimulated human lung epithelial cells (BEAS-2B) with calf thymus histones or recombinant histone H4 and observed that administration of exogenous histones caused enhanced rate of cell death, confirming that extracellular histones possess direct cytotoxic effects. Preincubation of exogenous histones or recombinant histone H4 with heparin lowered cytotoxicity, as demonstrated by significantly improved cell viability rate and reduced inflammatory cytokines levels in the supernatant of culture medium (fig. 5). Antihistone antibody was also administered as a positive control. These results confirmed that heparin mediates cytoprotection via targeting histones.

**Extracellular Histones Indicate Disease Severity in Patients with Gastric Aspiration-induced ARDS**

Having shown that extracellular histones were released notably in mice with acid-induced ARDS and aggravated...
systemic inflammation, we checked whether this is similar to gastric aspiration-induced ARDS in patients. Totally, 21 ARDS patients (15 men and 6 women with a mean age of 58 ± 8.3 years) caused by gastric aspiration were enrolled in the study. These patients were evidenced to have had gastric aspiration events, and diagnosed by identification of an infiltrative shadow on a chest x-ray, and the relevant aspiration signs and symptoms including wheezing, shortness of breath, cyanosis, hypotension, and hypoxia. Baseline characteristics, demographic details, and biochemical parameters of each patient were routinely recorded and summarized in Supplemental Digital Content 1, table 1, http://links.lww.com/ALN/B88.

Fig. 2. Extracellular histones were likely derived from dying lung cells and infiltrated neutrophils. Quantification of (A) BALF and (B) plasma LDH activity, a marker reflecting tissue damage, was determined using an Automated Multiparametric Analyzer. Quantification of (C) BALF and (D) plasma myeloperoxidase activity, a marker of granules in neutrophils and monocytes, was determined by ELISA. Both LDH and myeloperoxidase activity were increased remarkably in mice after HCl aspiration. *P < 0.01 vs. the sham-treated mice. Immunohistochemical analysis of Ly6G staining at 6 h in the lung sections of (E) sham-treated mice and (F) HCl-treated mice. Large number of Ly6G-stained cells was observed in the lungs of HCl-treated mice, suggesting massive neutrophil infiltration. Arrowheads indicate positive cells. Scale bars: 50 μm. There was a strong correlation of BALF nucleosome levels with BALF, LDH, and BALF myeloperoxidase, suggesting that cell death and neutrophils, monocytes, or both contributed to the release of nucleosomes/histones in mice (Supplemental Digital Content fig. 1, http://links.lww.com/ALN/B88). BALF = bronchoalveolar lavage fluid; ELISA = enzyme-linked immunosorbent assay; HCl = hydrochloric acid; LDH = lactate dehydrogenase.

We measured levels of extracellular nucleosomes/histone H4, LDH, myeloperoxidase, and inflammatory cytokines in the plasma of these patients. It showed that mean extracellular nucleosome or histone H4 levels were significantly higher in ARDS patients (2.109 ± 0.179 or 14.78 ± 1.134)
as compared with healthy controls (0.526 ± 0.513 or 0.466 ± 0.076; both P < 0.0001). Moreover, their levels correlated well with the degree of ARDS (all P < 0.05; fig. 6, A and B). Notably, nucleosome or histone H4 levels in non-surviving patients (2.723 ± 0.2933 or 17.58 ± 1.555) were significantly higher than those in survivors (1.725 ± 0.1787 or 11.81 ± 1.261, P = 0.006 or P = 0.012; fig. 6, C and D). We also observed that LDH, myeloperoxidase, and inflammatory cytokines including TNF-α, IL-1β, IL-6, and IL-10 were all increased markedly in patients with ARDS (Supplemental Digital Content 1, fig. 4 and fig. 5, http://links.lww.com/ALN/B88). However, their levels did not correlate with the degree of ARDS, except for myeloperoxidase activity between moderate and severe ARDS (589.4 ± 150.4 vs. 1427 ± 321.2, P = 0.017; Supplemental Digital Content 1, fig. 5C, http://links.lww.com/ALN/B88). Moreover, there were significant differences in the levels of myeloperoxidase (551.8 ± 135.3 vs. 1249 ± 355.4, P = 0.0378), TNF-α (19.72 ± 4.21 vs. 56.13 ± 9.42, P = 0.0006), and IL-6 (27.25 ± 3.17 vs. 46.6 ± 10.94, P = 0.041).
between survivors and nonsurviving ARDS patients (Supplemental Digital Content 1, figs. 5 and 6, http://links.lww.com/ALN/B88). There was a significant correlation of plasma nucleosomes with plasma myeloperoxidase ($r = 0.4905$, $P = 0.025$), and IL-6 ($r = 0.6158$, $P = 0.002$) levels (table 1), suggesting an inflammatory role of extracellular nucleosomes/histones in ARDS. We concluded that extracellular histones could reflect disease severity of ARDS in clinical situations.

**Discussion**

So far, ARDS is still the leading cause for respiratory failure in critically ill patients with high morbidity and mortality. Aspiration of gastric contents is one of the major causes for ARDS. There is a broad range of conditions that predispose to gastric aspiration-induced ARDS (e.g., general anesthesia, alcohol and narcotic abuse, and neurologic disorders). Despite therapy, 30–50% of gastric aspiration-induced ARDS patients die as a result of respiratory failure. However, unlike sepsis-associated ARDS, the underlying mechanism for acid aspiration-induced ARDS is less well-studied.

Accumulating evidence reveals that uncontrolled and persistent inflammation is deeply implicated in the progress of ARDS, characterized by massive leukocyte activation, free radical production, and cytokine release. Moreover, damage-associated molecular pattern molecules such as extracellular histones, high mobility group box-1 protein, mitochondrial DNA, and formyl peptides have been suggested as a primary culprit of uncontrolled inflammation in addition to pathogen. Among these, extracellular histones were particularly identified as a novel damage-associated molecular pattern molecule involved in a series of inflammatory events. Histones are a group of nuclear proteins that form heterooctamers to wind up the double-stranded DNA to form nucleosomes. Histones play important roles in the regulation of DNA repair, gene transcription, and chromatin condensation.

**Fig. 4.** Antihistone antibody or heparin provides similar protection against ARDS. (A) Administration of antihistone H4 antibody or heparin significantly reduced lung damage of HCl-treated mice. The representative of H&E-stained sections indicated a significant improvement in the lung of antihistone antibody or heparin-treated mice at 6h. (a) sham-treated mice, (b) HCl-treated mice, (c) antihistone antibody plus HCl-treated mice, (d) heparin plus HCl-treated mice. Scale bars: 100 μm. (B) Lung histological scores were lower in antihistone antibody or heparin plus HCl groups than in HCl alone group ($P < 0.01$). BALF (C) TNF-α (D) IL-1β and (E) IL-6 were notably decreased in antihistone antibody- or heparin-treated groups as compared with HCl-treated group ($P < 0.05$). (F) There was no difference in IL-10 levels between antihistone antibody or heparin-treated groups and HCl-treated group. ARDS = acute respiratory distress syndrome; HCl = hydrochloric acid; H&E = hematoxylin and eosin; IL-1β = interleukin-1β; IL-6 = interleukin-6; IL-10 = interleukin-10; TNF-α = tumor necrosis factor-α.
act as bystanders or are active mediators of disease remains unclear. Recently Xu et al. for the first time reported that extracellular histones are key mediators of cell damage and organ dysfunction during the hyperinflammatory reactions such as sepsis. Extracellular histones were increased markedly in response to sepsis and induced endothelial cytotoxicity and triggered an inflammatory and thrombotic response that eventually led to multiple organ dysfunction syndrome and death. Subsequently Huang et al. studied the role of extracellular histones in sterile inflammatory liver injury and showed that endogenous histones derived from necrotic hepatocytes following hepatic ischemia–reperfusion injury serve as a crucial link between initial tissue damage and activation of inflammation. Moreover Allam et al. revealed that circulating histones from dying renal cells aggravated acute kidney injury in mice and neutralization of histones using a mAb significantly protected against injury. Fuchs et al. found that circulating histones could directly activate platelets and lead to thrombocytopenia in vivo, which may be another important mechanism of organ damage. All of these findings indicate a strikingly novel role of extracellular histones in systemic inflammation and organ dysfunction. However, the role of extracellular histones in the pathogenesis of ARDS caused by acid aspiration is still not clear.

Here, we first prepared a murine model of HCl aspiration-induced ARDS to study the role of extracellular histones, as aspiration of gastric acid is clinically relevant and is associated with high mortality rates of ARDS. HCl has been reported to initially damage pulmonary airway epithelia, which in turn triggers an inflammatory response followed by edema formation and disruption of the alveolar capillary membrane. We demonstrated that HCl aspiration caused severe lung injury in mice, together with the significantly increased levels of extracellular histones in BALF and

Fig. 5. Heparin can inhibit histone-mediated cell death in vitro. (A) PI staining of cell death via flow cytometry indicated that preincubation of exogenous histones or histone H4 with antihistone antibody or heparin could significantly decrease cell death in vitro. (B) Administration of histone mixture or recombinant histone H4 increased the levels of TNF-α, IL-1β, IL-6, and IL-10 in the supernatant of culture medium, whereas antihistone antibody or heparin preincubation remarkably decreased these cytokines (P < 0.01). IL-1β = interleukin-1β; IL-6 = interleukin-6; IL-10 = interleukin-10; PI = propidium iodide; TNF-α = tumor necrosis factor-α.
Extracellular Histones Promote ARDS

plasma of mice, which correlated with the severity of ARDS. We next determined the source of extracellular histones by assaying LDH activity and neutrophil infiltration. LDH is a classic marker representing the extent of tissue damage. The significantly increased levels of LDH in BALF and plasma suggested an occurrence of prominent damage of the lungs during the course of acid-induced ARDS. Myeloperoxidase is a heme protein stored in granules of neutrophils and monocytes, and thus is a classic marker for neutrophil or macrophage infiltration. We showed that parallel BALF and plasma myeloperoxidase activities were elevated markedly after lung injury, indicating a significant activation of neutrophils or macrophages. Besides, immunohistochemical staining of Ly6G, a commonly used marker for neutrophils, indicated a prominent infiltration of neutrophils, further supporting these findings. Collectively, we concluded that the elevated levels of extracellular histones might originate from dying lung cells or neutrophil infiltration in this model.

Large quantities of histones in the extracellular milieu have pathological importance. It is reported that histones have direct cytotoxicity by binding phospholipids, disrupting cell membranes, and causing calcium influx. In addition, histones can serve as a damage-associated molecular pattern molecule to promote inflammation by mediating downstream inflammatory responses leading to cytokine production. Consistent with these studies, we found that a low dose of exogenous histones or recombinant histone H4 aggravated HCl-induced ARDS in mice, whereas blockade of histones by either a neutralizing antibody or heparin provided significant protection against ARDS. It thus confirmed an important pathological and targetable role of extracellular histones in acid-induced ARDS. Based on our results, we propose a hypothetical role of extracellular histones in mediating inflammation and lung injury after acid challenge (fig. 7). In brief, dying lung tissue cells as well as neutrophil infiltration in response to HCl challenge release large quantities of histones, which are directly cytotoxic or act as an endogenous damage-associated molecular pattern molecule to promote innate immunity and systemic inflammation. Furthermore, histones may in turn attract more innate cells to inflammation site and amplify inflammation

Table 1. Correlation of Plasma Nucleosomes with Various Variables in ARDS Patients

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* P < 0.05 was considered to be statistically significant.

ARDS = acute respiratory distress syndrome; IL = interleukin; LDH = lactate dehydrogenase; TNF-α = tumor necrosis factor-α.

Fig. 6. Extracellular histones were observed to increase in patients with ARDS caused by gastric aspiration. Plasma (A) nucleosome (B) histone H4 levels were significantly higher in ARDS patients than in healthy controls (*P < 0.001). There were also statistical differences in the levels of nucleosome or histone H4 between mild, moderate and severe ARDS (*P < 0.05). Plasma (C) nucleosomes (D) histone H4 levels were significantly higher in nonsurviving patients than in survivors (*P < 0.05). ARDS = acute respiratory distress syndrome.
by promoting more cytokine production, all of which eventually add an important factor contributing to ARDS (fig. 7).

Notably, heparin provides a similar protective effect as a neutralizing histone H4 antibody in vivo and in vitro experiments. Heparin is a glycosaminoglycan well known for its anticoagulant properties. Heparin also possesses anti-inflammatory effects and has been successfully used for the treatment of inflammatory conditions such as ischemia-reperfusion injury. However, the mechanisms responsible for the antiinflammatory effects of heparin are not well understood. It has been reported that heparin binds to many cellular proteins including histones through electrostatic interactions of high affinity. This occurs because heparin is highly sulfated and has strong negative charges, whereas histones are positively charged. However, no one attributed the binding of heparin to histones to its antiinflammation property. In this study, we reported that heparin-treated mice with ARDS had a significantly improved survival rate and reduced lung inflammation compared to control mice, and heparin directly interfered with histone-induced cell death in human lung epithelial cells. We thus raise the possibility that the binding of heparin to histones is responsible for the antiinflammatory effects of heparin, which adds a novel explanation for the underlying mechanisms related to heparin-mediated antiinflammation. It also suggests that drugs targeting histones may be a therapeutic option in patients with ARDS. However, a concern that heparin treatment could increase bleeding risk in patients should not be ignored. To address such an issue, we intend to explore the protective effect of nonanticoagulant N-Acetyl heparin in the future, which excludes the anticoagulant property while retaining its antiinflammation ability.

ARDS remains a serious clinical concern around the world. In some cases, patients after gastric aspiration events are at high risk of pulmonary dysfunction and the development of severe ARDS. Over the years, no therapeutic agents have demonstrated a clear benefit during ARDS treatment. Here, we questioned whether there was a clinical relevance of extracellular histones in patients with ARDS caused by acid aspiration. We found that extracellular histones were low or even undetectable in healthy subjects but increased drastically in ARDS patients and the levels of histones correlated well with the degree of ARDS. Moreover, we observed the statistical difference in plasma histone levels among nonsurvivors or survivors. Thus, we conclude that extracellular histones may serve as an important marker for

Fig. 7. The hypothesized model of extracellular histones in mediating inflammation and lung injury after acid challenge. It is proposed that extracellular histones may originate from cell damage or infiltrated neutrophils as a result of acid aspiration, causing direct cytotoxicity and activation of innate immunity, which in turn attracts more inflammatory cells and amplify inflammation by promoting cytokine production that eventually contribute to the pathogenesis of ARDS. ARDS = acute respiratory distress syndrome; DAMPs = damage associated molecular patterns.
clinically monitoring the severity of lung injury in humans. Animal studies suggest that extracellular histones could become a potential therapeutic target. So it is conceivable that therapeutic strategies targeting histones will provide novel pharmacological approaches to treat ARDS in humans. Anti-histone therapies such as administration of neutralizing antibody, activated protein C or heparin may complement the available strategies for management of ARDS. But currently, no relevant data is available regarding whether ARDS patients receiving heparin or activated protein C intervention are protected. Future epidemiological studies are needed to address this possibility. Likewise, the development of specific neutralizing antihistone antibody is warranted.

In summary, our data clearly show that extracellular histones play an inflammatory role in acid aspiration-induced ARDS by stimulating systemic inflammation and contributing to lung damage. The levels of extracellular histones may act as a novel marker indicating disease activity in mice and humans with ARDS. Blockade of histones by specific neutralizing antibody or heparin may be a potentially therapeutic strategy in the treatment of ARDS.

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Competing Interests
The authors declare no competing interests.

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