Increased Concentrations of Extracellular Histones in Patients with Tuberculous Pleural Effusion

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Background:
Extracellular histones have recently been suggested as critical mediators in many inflammatory diseases. However, the role of extracellular histones in tuberculous pleural effusion (TPE) is unclear. The goal of this study was to explore the potential involvement of extracellular histones in patients with TPE.

Material/Methods:
Samples of pleural effusion and peripheral blood were obtained from 58 patients with tuberculosis. Extracellular histones were determined in both TPE and serum samples. Moreover, the biomarkers for cellular damage, inflammatory cell activation, and systemic inflammation including lactate dehydrogenase (LDH), myeloperoxidase (MPO), S100A8/A9, as well as multiple inflammatory cytokines were measured.

Results:
Extracellular histone levels were significantly elevated in TPE (4.762 mg/mL [3.336, 7.307]) and serum samples (1.502 mg/mL [1.084, 2.478]) from tuberculosis patients as compared with the serum (0.585 mg/mL [0.285, 0.949]) from healthy controls. Notably, extracellular histones in TPE were also much higher than in serum of patients (P=0.002). LDH, MPO, and S100A8/A9 levels were all increased in TPE, along with a remarkable elevation of various cytokines. A correlation analysis showed that extracellular histones were positively associated with LDH, MPO, and S100A8/A9, and a panel of inflammatory cytokines in TPE.

Conclusions:
These results suggest that high concentrations of extracellular histones are markedly present in TPE, which may play an inflammatory role towards the progression of tuberculosis.

MeSH Keywords:
Histones • Inflammation Mediators • Tuberculosis, Pleural

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Background

Tuberculosis is a serious public health problem around the world, which accounts for millions of active disease cases and deaths each year [1]. Although pulmonary form is the most common manifestation of TB, the disease may also result in tuberculous pleuritis, which usually presents as an acute illness with fever, cough, pleuritic chest pain, and tuberculous pleural effusion (TPE) [1,2]. TPE is actually caused by *Mycobacterium tuberculosis* infection of the pleura and a resultant inflammatory response may result in increased pleural vascular permeability, leading to an accumulation of fluid that is enriched in proteins, and an influx of inflammatory cells into the pleural space [2,3]. TPE will accelerate an active form of tuberculosis if untreated [4]. Currently, the understanding of the inflammatory processes in TPE is limited.

Extracellular histones, mainly derived from dying cells or inflammatory cell infiltration, have recently been discovered as critical mediators implicated in systemic inflammation, and tissue and organ injury [5–7]. It has been described that extracellular histones may possess a variety of toxic effects including direct vascular endothelial damage [8–10], erythrocyte fragility [11], platelet aggregation and coagulation activation [12,13], as well as cytokine elevation [14,15], all of which may result in enhanced inflammation. More importantly, therapeutic modulation of extracellular histones appears to be effective in the treatment of various inflammatory injuries [6,16,17]. In this report, we sought to explore whether extracellular histones participate in the inflammatory processes associated with TPE, with a hypothesis that extracellular histones may act as inflammatory mediators involved with disease progression. Insights from this study may help diagnose, monitor TPE or improve its therapeutic strategy.

Material and Methods

Study participants

Fifty-eight patients diagnosed newly with TPE were recruited for this study. In addition, 18 healthy donors were recruited as healthy controls. The Ethics Committee of Shanghai Pulmonary Hospital, Tongji University School of Medicine (Shanghai, P.R. China) approved this study, which followed the recommendations of the Declaration of Helsinki for biomedical research regarding human subjects. Written informed consent was obtained from patients or their next of kin.

TPE was diagnosed based on: 1) typical clinical symptoms (fever and chest pain) and B-mode ultrasound showing pleural effusion; 2) high levels of adenosine deaminase (ADA) in the pleural effusion (40–80 U/L); 3) positive tuberculin test result; 4) positive histopathological examination of a pleural biopsy specimen; and/or 5) clinical symptoms were rapidly relieved after anti-tuberculosis therapy [4]. Exclusion criteria were as follows: 1) autoimmune disease; 2) human immunodeficiency virus (HIV) infection; 3) cancer for TPE patients; 4) pregnancy; 5) ongoing infection other than pleural tuberculosis for TPE patients; or 6) any systemic diseases involving immunity [4].

Sample collection and processing

TPE from patients were collected within 24 hours of admission or on a symptomatic day before treatment. Pleural fluids were transferred to 50 mL tubes and centrifuged (3000×g for 10 min) within 1 hour of collection. Serum samples were isolated from the participants’ peripheral venous blood by centrifuging at 3000×g for 10 min. All supernatants were stored at −80°C until further analyses. In addition, to avoid experimental infection with tuberculosis from TPE samples, the samples were filtered by 0.22 mm filters to remove bacteria before the analysis in a class 2 biosafety cabinet within a BSL2 laboratory.

Extracellular histones assay

We assessed extracellular histones in TPE and serum samples by using an enzyme-linked immunosorbent assay (ELISA) kit (Roche Applied Science, Germany), which uses a capturing antibody against an epitope shared by all histones and a detection antibody against DNA [18,19]. Purified exogenous histones of calf thymus were used to produce standard curves [19].

Measurement of cell damage and inflammatory cell infiltration

Lactate dehydrogenase (LDH) is a cytoplasmic enzyme whose activity reflects the extent of cellular damage [20]. We assessed LDH activities in these samples with a commercially available kit (Roche, Germany), according to the manufacturer’s instructions. Myeloperoxidase (MPO) serves as an index for neutrophil and monocyte/macrophage infiltration, whereas the calcium-binding protein S100A8/A9 complex (S100A8/A9), which is abundantly stored in neutrophil cytoplasm, serves as another important marker for phagocyte [20,21]. Thereby, we quantified MPO and S100A8/A9 levels to analyze inflammatory cell infiltration/activation in these samples.

Assay for multiple cytokines

For detection of multiple cytokines, the ProcartaPlex™ Multiplex Immunoassay from Affymetrix eBioscience (San Diego, CA, USA) was used on a Luminox/Bioplex-200 System, which permits a simultaneous measurement of various cytokines in a single sample [9,19]. The following cytokines were measured: IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12p70, IL-13, IL-17A, IL-18, IL-21, IL-22, IL-23, IL-27, IP-10, IFN-γ, TNF-α, MCP-1, and VEGF-A.
Increased concentrations of extracellular histones in patients with tuberculous pleural effusion

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Abstract

Increased concentrations of extracellular histones in patients with tuberculous pleural effusion (TPE). ELISA analysis showed that the concentrations of extracellular histones were significantly higher in TPE as compared with the serum from the corresponding tuberculosis patients or the healthy controls. Data are expressed as median (interquartile range).

Statistical analysis

GraphPad Prism 6 (GraphPad Software, Inc., San Diego, CA) was used for statistical analysis. Unless otherwise stated, all values were expressed as medians and interquartile ranges. Data were analyzed by 2-tailed Student’s t-test or one-way analysis of variance (ANOVA). Correlations between variables were evaluated using Spearman’s rank correlation analysis. Results were considered statistically significant when P<0.05.

Results

Patients’ characteristics

Tuberculosis patients were 43.67±2.8 years old, including 39 males and 19 females. Healthy controls were 40.50±3.4 years old. The participants’ baseline characteristics are shown in Table 1. No patient had severe tuberculosis such as hematogenous disseminated pulmonary tuberculosis, or tuberculous meningitis. All patients were HIV-negative, and no patient had been diagnosed with immunodeficiencies. Furthermore, clinical symptoms of all patients were rapidly relieved after 1 month of anti-tuberculosis chemotherapy.

Table 1. The characteristics of the study subjects.

| Characteristics   | TB patients | Healthy controls |
|-------------------|-------------|------------------|
| Sample sizes      | 58          | 18               |
| Sex (Male/Female) | 39/19       | 10/8             |
| Age (years)       | 43.67±2.8   | 40.50±3.4        |
| Blood             |             |                  |
| ADA (U/L)         | 17.63±6.9   | 14.23±5.7        |
| Total protein (g/L)| 26.7±9.2   | 21.4±6.5         |
| TPE               |             |                  |
| ADA(U/L)          | 68.9±8.4    |                  |
| Total protein (g/L)| 42.3±7.6   |                  |

Extracellular histone levels are elevated in TPE

We first detected extracellular histone levels in TPE and serum samples. We found that extracellular histones in TPE samples (median value, 4.762 mg/mL [3.336, 7.307]) were significantly higher than in serum samples from tuberculosis patients (median value, 1.502 mg/mL [1.084, 2.478], P<0.001) or the serum from normal controls (median value, 0.585 mg/mL [0.285, 0.949], P<0.001) (Figure 1). In addition, the levels of extracellular histones were also remarkably higher in serum of tuberculosis patients than in the serum of normal controls (P=0.002).

High levels of LDH, MPO, and S100A8/A9 in TPE

Previous research has suggested that extracellular histones may originate from necrotic and apoptotic cells or from inflammatory cell infiltration/degradation [6,22]. Thus, we checked cellular origins of extracellular histones by measuring LDH, MPO, and S100A8/A9 activities in these samples. We found that LDH, MPO, and S100A8/A9 were all remarkably elevated in TPE as compared with the serum of tuberculosis patients or the serum of healthy controls (Figure 2). In contrast, MPO was also significantly elevated in the serum of tuberculosis patients as compared to healthy controls (P=0.008), whereas no statistical difference was detected in LDH and S100A8/A9 levels between the serum samples of tuberculosis patients and normal controls (both P>0.05). Notably, there was a positive correlation of extracellular histones with LDH (r=0.3267, P<0.0001), MPO (r=0.6525, P<0.0001), and S100A8/A9 (r=0.5108, P=0.0017), thereby suggesting that the increased extracellular histones were possibly released from damaged pleural mesothelium or the degradation of inflammatory cells during tuberculosis infection.

Increased concentrations of multiple cytokines in TPE

It has been demonstrated that high concentrations of extracellular histones could aggravate systemic inflammation in patients [9,23]. To assess the severity of systemic inflammation in TPE, we measured a panel of multiple cytokines. It showed that there were totally 12 cytokines markedly increased in TPE as compared with the corresponding serum samples or the controls (Figure 3). Moreover, there were 4 cytokines (IL-6, IL-10, IL-27, and IFN-γ) observed to be different in serum samples...
between tuberculosis patients and healthy controls. A correlation analysis indicated that the concentrations of extracellular histones were positively correlated with most of the detected cytokines, which are actually classical markers for systemic inflammation (Table 2). Taken together, these results indicated that high concentrations of extracellular histones were closely associated with the inflammatory processes in TPE, which may aggravate disease progression.

**Discussion**

The primary purpose of this study was to explore the possible involvement of extracellular histones in TPE. Until recently, extracellular histones, high mobility group box-1 protein (HMGB1), mitochondrial DNA, and formyl peptides are known to act as damage-associated molecular pattern (DAMP) molecules that can enhance inflammatory response in addition to pathogen [22]. Of these, extracellular histones were specifically discovered as a novel type of tissue-damaging products implicated in many inflammatory insults [11,22–24]. Histones are basic unit structure components of chromatin [6,22]. Besides having nuclear function, histones can be liberated into extracellular milieu after massive cell apoptosis or necrosis and turn out to be endogenous danger signals [6]. Mounting evidence unravels many biological effects of extracellular histones such as direct cytotoxicity by disrupting cell membranes and causing calcium influx, platelet aggregation, and coagulation activation, and cytokine surge, which is, more importantly, responsible for enhanced inflammatory state [5]. In addition, increased concentrations of extracellular histones have also been clinically associated with disease progression and outcome in patients with sepsis, cancer, stroke, organ injury, anemia, virus/bacterial infections, and autoimmune diseases [6,7,11,18,23–25]. It is known that tuberculosis-related pleural effusions may result from the infiltration of the pleural space by *M. tuberculosis* antigens or bacilli [2]. However, it is uncertain whether extracellular histones are involved in disease progression related to TPE.
Collectively these studies provide evidence supporting that cellular histones and high levels of inflammatory cytokines in indicated a positive correlation between high levels of extracellular histones and enhanced inflammation in TPE. A further correlation analysis served that a couple of canonical pro-inflammatory cytokines are known to possess many biological effects, particularly in a toxic role associated with disease progression, because they may be considered as a potential biomarker for TPE, and could be used to diagnose, monitor or improve the prognosis of TPE.

**Conclusions**

High concentrations of extracellular histones were predominantly present in TPE as compared with the serum from the same patients or healthy controls, and massive cellular death or inflammatory cell degradation may be the origins of extracellular histones. Extracellular histones may aggravate the inflammatory processes involved with TPE. Extracellular histones may be considered as a potential biomarker for TPE, and could be used to diagnose, monitor or improve the prognosis of TPE. More studies are needed to explore their functions.

**Conflict of interest**

None.
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