Elevated uric acid and adenosine triphosphate concentrations in bronchoalveolar lavage fluid of eosinophilic pneumonia

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Abbreviations:
AEP, acute eosinophilic pneumonia;
ATP, adenosine triphosphate;
BALF, bronchoalveolar lavage fluid;
CEP, chronic eosinophilic pneumonia;
DAMP, damage-associated molecular pattern molecule;
DC, dendritic cell;
EDN, eosinophil derived neurotoxin;
ELISA, enzyme-linked immunosorbent assay;
EP, eosinophilic pneumonia;
HP, hypersensitivity pneumonitis;
IL, interleukin;
ILC2, type 2 innate lymphoid cell;
LDH, lactate dehydrogenase;
Th, T helper;
UA, uric acid

Abstract

Background: Recent evidence has suggested that the innate immune response may play a role in the development of eosinophilic airway inflammation. We previously reported that uric acid (UA) and adenosine triphosphate (ATP), two important damage-associated molecular pattern molecules (DAMPs), activate eosinophil functions, suggesting that these molecules may be involved in the development of eosinophilic airway inflammation. The objective of this study was to measure the concentrations of DAMPs including UA and ATP in the bronchoalveolar lavage fluid (BALF) of patients with eosinophilic pneumonia (EP).

Methods: BAL was performed in patients with EP including acute and chronic eosinophilic pneumonia, and in patients with hypersensitivity pneumonia, and sarcoidosis. UA, ATP, and cytokine concentrations in the BALF were then measured.

Results: The UA concentration was increased in the BALF of EP patients. UA concentrations correlated with eosinophil numbers, and with eosinophil-derived neurotoxin and interleukin (IL)-5 concentrations. Furthermore, the ATP concentration was increased in the BALF of EP patients and ATP concentrations correlated with UA concentrations. Moreover, IL-33 was increased in EP patients and IL-33 concentrations correlated with UA and ATP concentrations.

Conclusions: The UA and ATP concentration was increased in the BALF of EP patients. UA concentrations correlated with eosinophil numbers, and with ATP and IL-33 concentrations. Our findings suggest that DAMPs such as UA and ATP play a role in the pathogenesis of EP.

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Introduction

Eosinophilic airway inflammation has been thought to be induced by adaptive immune responses. After antigen presentation by dendritic cells (DCs), T cells are polarized into allergen specific T helper (Th) 2 cells. Th2 cells then produce cytokines such as interleukin (IL)-5 that regulate the recruitment of eosinophils. In support of this mechanism, IL-5-producing T cells are increased in the airway of bronchial asthma. However, recent evidences suggested...
that innate immune responses are also involved in the development of eosinophilic airway inflammation. Type 2 innate lymphoid cells (ILC2) and epithelial-related cytokines such as IL-33, IL-25 and thymic stromal lymphopoietin play important roles in this process.16–18 In mouse models, instillation of IL-33 into the airway can directly induce eosinophilic inflammation even in recombination activating gene 2 knockout mice,19 suggesting that eosinophilic inflammation can be induced even without adaptive immunity.

Eosinophilic pneumonia (EP) is a disease that is characterized by infiltration of eosinophils into the airway.7,8 There are several types of eosinophilic pneumonia including acute eosinophilic pneumonia (AEP) and chronic eosinophilic pneumonia (CEP).4 Although the exact cause and mechanism of EP have not been fully clarified, there are some known causes of EP including certain drugs, chemical fumes, molds, parasites and cigarette smoke.7,8 Therefore, not only an adaptive immune response but also an innate immune response may play an important role in the pathogenesis of EP.

When cells are stressed or damaged, they release damage-associated molecular pattern molecules (DAMPs), which function as endogenous danger signals that alert the innate immune system to unscheduled cell death and microbial invasion.14–17 Recently, the role of DAMPs in the activation of eosinophils has been highlighted.15–17 Stenfeldt et al. reported that damaged epithelial cells can induce eosinophilic migration, degranulation, and cytokine production, probably through the release of DAMPs from damaged cells.15 Cormier et al. reported that signals from necrotic cells can induce eosinophil chemotaxis and degranulation in tumor drugs.15 Furthermore, we reported that uric acid (UA), an important DAMP, can activate eosinophil functions such as degranulation and cytokine production.16 We also reported that adenosine triphosphate (ATP), another important DAMP, also induces eosinophil activation.17 However, the role of these molecules in the pathogenesis of EP has not been fully elucidated.

In this study, we measured the concentration of DAMPs such as UA and ATP in the bronchoalveolar lavage fluid (BALF) of EP patients. We found that the concentration of UA and ATP was increased in the BALF of EP patients. UA concentrations correlated with the number of eosinophils and with eosinophil derived neurotoxin (EDN), IL-5, and eotaxin concentrations. There was also a significant correlation between UA concentrations and ATP and IL-33 concentrations. These findings suggest an important role of DAMPs such as UA and ATP in the pathogenesis of EP.

Methods

Patients

This study included 33 patients with EP (14 with AEP and 19 with CEP), 35 patients with sarcoidosis (who exhibited bilateral hilar lymphadenopathy but not interstitial lung disease), and 6 patients with hypersensitivity pneumonitis (HP) who were admitted to our department from 2003 to 2013 (Table 1, 2). The diagnosis of AEP, as described by Allen et al.,9 was made by the following criteria: acute onset of respiratory failure, diffuse pulmonary infiltrates on chest X-ray films, and an increased number of eosinophils in BALF (>25% of the total cells). The diagnosis of CEP was made according to the criteria by Carrington et al., which included typical clinical features of CEP, dense and multiple foci of consolidation in the peripheral lung fields on chest radiography, increased percentage of eosinophils in BALF, absence of other possible causes, and favorable response to corticosteroid therapy.10 The time interval from disease onset of AEP and CEP to BAL was 10.3 ± 1.5 days and 36.6 ± 2.9 days, respectively. This study was performed with the approval of the Institutional Review Board of Saitama Medical University Hospital and written informed consent was obtained from patients.

BALF

A bronchofiberscope (Olympus, Tokyo, Japan) was wedged into a subsegmental bronchus of either the right middle lobe or the left lingula, and lavage was performed by a combination of infusion of 50 ml of saline and subsequent gentle aspiration, repeated four times. Fluid recovered from the third and fourth lavages was used as BALF specimens. Cytocentrifuged slides were stained with May–Giemsa to determine cell differential counts. BALF supernatants were immediately stored at ~80 °C until analysis. Patients for whom the percentage of recovery of BALF was less than 50% were excluded from this study and therefore, the percentage of recovery of BALF was more than 50% in all patients. There was no difference in recovery rate between EP, sarcoidosis, and HP (data not shown).

Measurement of ATP, UA, albumin, lactate dehydrogenase (LDH), and cytokines/chemokines

ATP concentrations in BALF were measured using an ATP Determination Kit (BioAssay Systems, Hayward, CA, USA) and a luminometer as described previously.16 UA concentrations were measured by a uricase assay using an automatic clinical chemistry analyzer (LABSPECT 008, Hitachi, Tokyo, Japan). Albumin concentrations in BALF or serum, and LDH activity in BALF were measured using LABSPECT 008. IL-5, IL-33, and eotaxin concentrations in BALF were measured using enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems, Minneapolis, MN, USA) or Bio-Plex cytokine assay kits (Bio-Rad, Mississauga, Canada). EDN concentrations in BALF were measured using an ELISA (Medical and Biological Laboratory, Nagoya, Japan).

Statistical analysis

Values are expressed as means ± SEM. For statistical analyses, Student’s t-test or the Mann–Whitney U-test was used for paired comparisons, after checking for normality using the Shapiro–Wilk

<table>
<thead>
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<th>Table 1</th>
<th>Patient characteristics.</th>
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</tr>
<tr>
<td>Age</td>
<td>49.0 ± 1.7</td>
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<td>Sex (man/female)</td>
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<tr>
<td>BAL</td>
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<td>White blood cell ( × 10⁶/ml)</td>
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<td>BAL</td>
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test. One-way factorial analysis of variance with Tukey’s test or the Kruskal–Wallis test with the Steel–Dwass test were used for comparison of more than two variables. Correlations between data were evaluated by Pearson’s correlation coefficient. Values of $P < 0.05$ were considered statistically significant.

Results

The UA concentration is increased in the BALF of EP patients

To examine the role of DAMPs in the pathogenesis of EP, we first measured the concentration of UA, an important DAMP, in the BALF of EP, HP, and sarcoidosis patients. The UA concentration was significantly increased in the BALF of EP and HP patients compared with that of sarcoidosis patients (sarcoidosis 61.8 ± 6.6 µg/ml; HP 214.3 ± 18.4 µg/ml, $P < 0.01$ versus sarcoidosis; EP 463.4 ± 34.2 µg/ml, $P < 0.01$ versus sarcoidosis) (Fig. 1A). We next measured albumin concentrations to assess whether UA was produced in the airway or migrated from the serum into the bronchial lumen. Albumin was chosen as this reference standard because it is not synthesized in the lung and its presence in BALF reflects transudation from the intravascular compartment.18 The albumin concentration was significantly increased in the BALF of HP patients, but not of EP patients, compared with sarcoidosis patients ($P < 0.05$) (Fig. 1B). The BALF (UA/albumin)/serum (UA/albumin) ratio of EP patients was significantly higher than that of HP and sarcoidosis patients (sarcoidosis 3.4 ± 1.2, HP 2.9 ± 0.4, EP 10.8 ± 2.7; $P < 0.05$ for both) (Fig. 1C), suggesting that UA is likely produced locally in EP according to the formula by Reynolds et al.19

We next assessed the involvement of tissue damage in the pathogenesis of EP by measuring LDH activity.20 LDH activity in BALF was significantly increased in EP patients compared with that of HP and sarcoidosis patients (sarcoidosis 0.1 ± 0.0 IU/l, HP 0.4 ± 0.2 IU/l, EP 1.7 ± 0.4 IU/l; $P < 0.01$ versus sarcoidosis and $P < 0.05$ versus HP) (Fig. 1D).

UA concentrations correlate with eosinophil numbers

Next, we investigated factors that correlated with UA concentration in the BALF of EP patients. UA concentrations significantly correlated with the number of eosinophils ($r = 0.85$, $P < 0.01$) (Fig. 2A) and with the EDN concentrations ($r = 0.86$, $P < 0.01$) (Fig. 2B), suggesting that an increase in UA was associated with eosinophil activation. There was also a significant correlation between UA concentrations and IL-5 concentrations ($r = 0.87$, $P < 0.05$) (Fig. 2C), which is an important Th2 cytokine in eosinophil activation, and eotaxin concentrations ($r = 0.88$, $P < 0.05$) (Fig. 2D), which is an important chemokine in eosinophil migration. These findings suggest that UA may be involved in the development of airway eosinophilic inflammation in EP.

The ATP concentration is increased in the BALF of EP patients

We next measured the concentration of ATP, which is also an important DAMP and is produced from eosinophils by UA stimulation,17 in BALF. The concentration of ATP was increased in the BALF of EP patients compared with that of HP and sarcoidosis patients (6.8 ± 0.9 µM; HP 1.0 ± 0.8 µM; EP 40.0 ± 5.4 µM; $P < 0.01$ for both) (Fig. 3A). Furthermore, ATP concentrations correlated with the number of eosinophils ($r = 0.94$, $P < 0.01$) (Fig. 3B), and with the concentrations of UA ($r = 0.80$, $P < 0.05$) (Fig. 3C), and IL-5 ($r = 0.80$, $P < 0.01$) (Fig. 3D), suggesting that ATP may also play a role in the development of airway eosinophilic inflammation in EP.

The IL-33 concentration is increased in the BALF of EP patients

We next measured the concentration of IL-33 in BALF. IL-33 is another important DAMP and is produced from airway epithelial cells by ATP stimulation.21 The concentration of IL-33 was significantly increased in the BALF of EP patients compared with that of HP and sarcoidosis patients (sarcoidosis, 33.2 ± 1.6 pg/ml; HP, 23.4 ± 1.2 pg/ml; EP, 111.2 ± 12.4 pg/ml; $P < 0.01$ for both) (Fig. 3A). Furthermore, IL-33 concentrations correlated with the number of eosinophils ($r = 0.81$, $P < 0.01$) (Fig. 3B), and with the concentrations of UA ($r = 0.80$, $P < 0.05$) (Fig. 3C), and IL-5 ($r = 0.84$, $P < 0.01$) (Fig. 3D), suggesting that IL-33 may also play a role in the development of airway eosinophilic inflammation in EP.
Fig. 2. Correlations between UA concentrations and eosinophil count and cytokine concentrations. The relationship between UA concentrations and eosinophil counts (A), EDN (B), IL-5 (C), and eotaxin (D) concentrations in the BALF of EP patients are shown (n = 33).

Fig. 3. The concentration of ATP in the BALF. (A) The ATP concentration in patients with sarcoidosis (n = 35), HP (n = 6), and EP (n = 33). (B–D) Correlations between ATP concentrations and eosinophil counts (B), UA (C), and IL-5 (D) concentrations in the BALF of EP patients are shown (n = 33).
6.9 ± 16.4 pg/ml; EP, 468.9 ± 28.9 pg/ml; P < 0.01 for both) (Fig. 4A). Furthermore, IL-33 concentrations correlated with the number of eosinophils (r = 0.80, P < 0.05) (Fig. 4B) and with the concentrations of UA (r = 0.95, P < 0.01) (Fig. 4C), and ATP (r = 0.79, P < 0.05) (Fig. 4D).

**The concentration of DAMPs in the BALF of AEP and CEP**

In EP patients, the BALF UA concentration of AEP patients was higher than that of CEP patients (AEP, 524.3 ± 76.6 μg/ml; CEP, 402.1 ± 31.5 μg/ml; P < 0.01) (Fig. 5A). The BALF ATP or IL-33 concentration of AEP patients was higher than that of CEP patients (ATP: AEP, 68.1 ± 11.9 μM; CEP, 19.5 ± 1.8 μM; P < 0.01. IL-33: AEP 685.7 ± 64.7 pg/ml; CEP 310.5 ± 11.4 pg/ml; P < 0.01) (Fig. 5B, C). Furthermore, BALF LDH activity of AEP patients was higher than that of CEP patients (AEP, 3.0 ± 1.1 IU/l; CEP, 0.6 ± 0.3 IU/l; P < 0.05) (Fig. 5D). We next assessed the difference in local production of DAMPs between AEP and CEP using the formula of Reynolds et al. The BALF (UA/albumin)/serum (UA/albumin) ratio of AEP was slightly higher than that of CEP, however the difference was not significant (Fig. 5E), suggesting that although the UA concentration was higher in AEP BALF than in CEP BALF, the degree of local production of UA did not differ between AEP and CEP.

**Discussion**

In this study, we found that the UA concentration was increased in the BALF of EP patients. UA concentrations significantly correlated with eosinophil numbers and with EDN and IL-5 concentrations. We also found that the ATP concentration was significantly increased in the BALF of EP patients and that ATP concentrations significantly correlated with UA concentrations. Furthermore, IL-33 was significantly increased in EP patients and IL-33 concentrations significantly correlated with UA and ATP concentrations. These findings suggested that DAMPs such as UA, ATP, and IL-33 play important roles in the pathogenesis of EP.

LDH activity was increased in the BALF of EP patients (Fig. 1D), suggesting possibility the tissue injury may induce or augment eosinophilic inflammation in EP. DAMPs, induced by tissue injury, interact predominantly with host pattern recognition receptors and induce inflammation and immune responses. Among known DAMPs, the role of UA in the development of immune responses has been highlighted. UA is a major endogenous danger signal released from injured cells, and large concentrations of UA can be produced in vivo. Although cellular receptors for UA have not yet been identified, a major target of UA through which it activates the immune response is DCs. For example, Behrens et al. reported that stimulation of DCs with UA increased the production of IL-5 from DO11.10 CD4 T cells when incubated with DCs and ovalbumin, suggesting an important role of UA as a Th2 adjuvant. UA-mediated DC activation plays important roles in the immunological actions of the Th2-type adjuvant aluminum hydroxide. In this study, the UA concentration was significantly increased in the BALF of EP patients (Fig. 1A). Furthermore, UA concentrations significantly correlated with eosinophil numbers and with EDN, IL-5, and eotaxin concentrations (Fig. 2), suggesting that UA may act as a Th2 adjuvant in the development of eosinophilic airway inflammation in EP. We recently reported that UA directly activates eosinophil functions such as degranulation and cytokine production. Therefore, UA may play important roles in the pathogenesis of EP not only through DC-mediated activation of Th2 immune responses, but also through direct activation of eosinophils.

ATP has been implicated as an important DAMP in acute and chronic inflammation. Extracellular ATP may act as an autocrine regulator of epithelial cells. Although ATP is constantly...
released from epithelial cells, inflammatory stimuli such as DAMPs enhance ATP release from epithelial cells. Furthermore, extracellular ATP may also act as a chemoattractant for DCs and induce DC maturation, thereby inducing Th2 mediated-immune responses. \(^33\)–\(^36\) Idzko et al. reported that the BALF ATP level is increased in asthma after allergen challenge in humans and mice. \(^33\) They also reported that neutralization of intrapulmonary ATP levels or the application of unselective purinergic receptor antagonists can abrogate all of the cardinal features of experimental asthma in mice. \(^33\) In this study, we found that the ATP concentration was significantly increased in the BALF of EP patients (Fig. 3A). Furthermore, ATP concentrations significantly correlated with eosinophil counts and with UA and IL-5 concentrations (Fig. 3B–D). Therefore, ATP may also contribute to the pathogenesis of EP not only through upregulation of Th2 response through activated DCs or epithelial cells, but also through direct activation of eosinophil functions.

IL-33 is rapidly and passively released from damaged cells in response to stress conditions such as infection, injury, and inflammation. \(^37\) IL-33 plays an important role in the development of eosinophilic airway inflammation, mainly by activation and accumulation of ILC2. \(^4\)–\(^6\) Furthermore, IL-33 directly activates eosinophil functions such as eosinophil superoxide anion generation, degranulation, and cytokine production. \(^38\) In this study, the IL-33 concentration was significantly increased in the BALF of EP patients (Fig. 4A). Moreover IL-33 concentrations significantly correlated with eosinophil counts and with UA and ATP concentrations (Fig. 4B–D). Therefore, IL-33 may also play an important role in the pathogenesis of EP through activation of ILC2 and eosinophils.

The exact cause and mechanism of EP has not been fully clarified. \(^7\) In AEP, inhaled agents such as cigarettes and chemical agents may induce eosinophil inflammation. \(^7\)–\(^9\) For example, a relationship was observed between the recent onset of cigarette smoking (for the first time or following a period of smoking cessation) and the development of AEP. \(^30\)–\(^32\) As inhaled agents are considered to work in a non-specific (non-allergic) manner, this relationship suggests that an innate immune response rather than an adaptive immune response may play an important role in the pathogenesis of AEP. In this study, the BALF (UA/albumin)/serum (UA/albumin) ratio of EP was higher than that of sarcoidosis or HP (Fig. 1C); however, the ratio of AEP did not differ from that of CEP (Fig. 5E). These data suggested that the degree of local production of UA did not differ between AEP and CEP. There is a possibility that...
the migration of DAMPs from the serum into the bronchial lumen is higher in AEP than in CEP, since some reports have suggested increased permeability of blood vessels in AEP.21 It is reasonable that UA, ATP, and IL-33 are simultaneously induced by an unidentified inhaled agent(s) in EP. However, other mechanisms may also be involved in this process. For example, we previously reported that UA induced ATP production in eosinophils.22 Furthermore, Kouzaki et al. reported that ATP induced IL-33 production in bronchial epithelial cells.23 Therefore, we speculate that DAMPs can upregulate other DAMPs and induce subsequent Th2 responses and eosinophil inflammation, although additional studies are necessary to confirm this hypothesis.

A limitation of this study is that we could not identify the actual role of DAMPs in EP. Although we speculate that DAMPs released by an unidentified inhaled agent(s), probably from epithelial cells, activate DCs and eosinophils, there is also a second possibility. Thus, it is possible that eosinophils, activated by an unknown mechanism(s), release DAMPs thereby increasing the concentration of DAMPs in the BALF of EP. Of the known DAMPs, we previously reported that eosinophils can release ATP before cell death and that activated eosinophils themselves in an autocrine manner.16 However, there are no data regarding the release of UA or IL-33 from eosinophils before cell death or regarding autocrine eosinophil activation through UA or IL-33. Therefore, if these DAMPs are released from eosinophils, they are probably derived from dead eosinophils. Although we did not obtain evidence regarding an increase in dead eosinophils in the BALF of EP in this study, eosinophil degranulation (EDN concentration) was increased in this BALF and correlated with UA concentration (Fig. 2B). Overall, we speculate that induced DAMPs that are probably derived from epithelial cells, play roles in the development of eosinophil inflammation, and that activated eosinophils release DAMPs after cell death, which further increase their concentration in the BALF of EP. These hypotheses should be examined in the future.

In conclusion, the concentration of both UA and ATP was significantly increased in the BALF of EP patients. Furthermore, UA concentrations significantly correlated with EDN, IL-5, ATP, and IL-33 concentrations in the BALF of EP patients. Therefore, DAMPs including UA and ATP play important roles in the pathogenesis of EP.

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Conflict of interest

The authors have no conflict of interest to declare.

Authors’ contributions

TK carried out the experiments, analyzed the data, and created the figures. KN contributed to study design, analyzed the data, and wrote the manuscript. TN, KK, and YU carried out the experiments. TS, KL, and HN participated in the data analyses. MN contributed to study design and edited the manuscript. All authors read and approved the final manuscript.

References


