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Diversity of protein carbonylation in allergic airway inflammation

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Abstract

Oxidative stress is involved in asthma. This study assessed the carbonylation of sputum proteins in 23 uncontrolled adult asthmatic patients and 23 healthy controls. Carbonylated proteins (68 kDa and 53 kDa) were elevated in asthmatics when compared to controls and the 68-kDa carbonylated protein was significantly correlated with sputum eosinophilia. The kinetics of protein carbonylation in bronchoalveolar lavage fluid (BALF) were then examined in a mouse ovalbumin-induced allergic inflammation model. It was found that the carbonylation of various BALF proteins did not uniformly occur after challenge. The appearance of the 53-kDa carbonylated protein was limited within 24 h, while carbonylation of 68-kDa protein peaked at 48 h and was associated with BALF eosinophilia. Thus, it was demonstrated that the 68-kDa and 53-kDa proteins, corresponding to albumin and α1-antitrypsin, respectively, were specifically carbonylated in allergic inflammation in humans and in mice and that eosinophils may play a role in mediating carbonylation of albumin.

Keywords: *Protein carbonyls, asthma, airway inflammation, oxidative stress*

Introduction

Asthma is a chronic inflammatory disorder of the airways. A growing body of evidence suggests that oxidative stress is involved in allergic airway inflammation in asthma [1,2] and thus a variety of markers have been demonstrated to reflect the oxidative stress in asthma [3–6]. It is known that protein carbonylation reflects the oxidation of Lys, Arg or Pro residues in proteins and protein carbonyl content is the most commonly used marker for protein oxidation in body fluids [7–9]. However, the formation of protein carbonyls in asthmatic airways is not fully understood. Nadeem et al. [3] demonstrated an increase in plasma protein carbonyls in asthmatics. Foreman et al. [10] found increased levels of protein carbonyls among BALF proteins in atopic asthmatic adults 18 h after allergen challenge. In asthmatic children, the number of inflammatory cells in bronchoalveolar lavage fluid (BALF) was significantly correlated with the concentration of protein carbonyls [11]. On the other hand, some reports have demonstrated no increase in protein carbonyls in sputum from patients with mild asthma [12] or in BALF from asthmatic children [11] when compared to healthy subjects. Few studies have focused on specifying the individual proteins that are carbonylated in asthmatics [10].

Sputum eosinophilia is a hallmark of uncontrolled asthmatic status [13] and a significant correlation has

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been reported between the frequency of asthma exacerbation and the percentage of sputum eosinophils [14]. Excess reactive oxygen species (ROS) during allergic inflammation in the lungs is largely attributed to eosinophils [15,16], although the contribution of other sources of ROS, such as neutrophils, lymphocytes or structural lung cells, remains uncertain.

The specific aims of this study are to assess the status of sputum protein carbonylation and its relationship with the appearance of inflammatory cells in sputum from uncontrolled adult asthmatic patients and to address the question of whether protein carbonylation uniformly occurs during the course of airway inflammation in a mouse ovalbumin (OVA)-induced allergic model.

Methods

Subjects

Twenty-three patients with bronchial asthma attending the institute's outpatient clinic were included in the study. Diagnosis was established based on current guidelines for asthma, the Global Initiative for Asthma (GINA) guidelines [17]. All patients were receiving inhaled budesonide or fluticasone combined with salmeterol and salbutamol as the rescue bronchodilator based on the appropriate treatment step as per GINA guidelines. A total of 23 community-based asymptomatic volunteers were recruited as controls among the patients in our non-pulmonary clinics or among employees or trainees at our medical school. None of the volunteers had a history of asthma or other allergic disorders. All subjects were free of clinically apparent respiratory infections within the preceding 2 months and were evaluated by physical examination and spirometry. All subjects provided written informed consent and the study was approved by the Ethics Committee of Hokkaido University School of Medicine.

Sputum induction

Before sputum induction, subjects inhaled 200 μg of salbutamol from a metered dose inhaler. Sputum induction was performed by inhalation of 4.5% NaCl for 20 min from an ultrasonic nebulizer [18]. Subjects rinsed their mouths with distilled water. Subjects were encouraged to cough throughout the procedure and regularly interrupted their inhalation of hypertonic saline in order to expectorate sputum into previously weighed, 50-ml sterile ampules. As dithiothreitol (DTT), a mucolytic agent, is reported to block immunoassay [19], we preliminarily tested whether the treatment of sputum with DTT might affect the detection of protein carbonyls. At all three concentrations of DTT tested, 3.2 mM (0.05%), 6.5 mM (0.1%) and 12.8 mM (0.2%), the appearance of carbonylated proteins in sputum did not differ, as compared to the sample without DTT (data not shown). Thus, all of the sputum samples in this study were weighed (g) in a balance and diluted 3-fold with 0.1% DTT (Sigma Chemical Co., St. Louis, MO). Samples were then mixed gently with a vortex mixer and placed in a water bath at 37°C for 30 min in order to ensure complete homogenization. Samples were removed from the water bath periodically for additional vortex mixing. The homogenized sputum was centrifuged at $800 \times g$ for 15 min in order to separate the supernatants from the cell pellets. Supernatants were aspirated and frozen at −70°C for later analysis, while cell pellets were used for total cell counts and differentials, as described previously [20].

Mice

C57BL/6 mice were purchased from Clea Japan (Tokyo, Japan) and were used at 8–12 weeks of age. All mice were kept in plastic chambers with free access to food and water. Experimental protocols and procedures were approved by the Ethics Committee on Animal Research of the Hokkaido University School of Medicine.

Sensitization and challenge of mice

For induction of experimental allergic lung disease, sensitization and challenge was performed using the method of Li et al. [21], with some modification. Briefly, mice were immunized intraperitoneally (i.p.) with 20 μg of OVA (Sigma-Aldrich, St. Louis, MO) mixed with 4 mg of aluminum hydroxide (Imject Alum; Pierce, South Iselin, NJ) in 200 μl of PBS. Animals received booster i.p. injections of the alum-OVA mixture at 7 and 14 days. After the third i.p. injection at 21 days, mice were intratracheally challenged with 20 μg of OVA dissolved in 20 μl of PBS.

Bronchoalveolar lavage (BAL)

Animals were sacrificed at 0, 8, 24, 72 or 96 h after intratracheal OVA administration ($n = 5 \sim 8$ at each time point). The trachea was dissected from underlying soft tissues and a 0.58-mm-diameter tube was inserted through a small incision. BAL was performed with two 1-ml aliquots of saline. A total of 1.8–1.9 ml of BALF was consistently recovered by this technique. BALF was centrifuged at 1500 rpm for 5 min at 4°C and the supernatant was stored −80°C. For differential cell count, cytospin preparations were made and stained with Diff-Quik (Green Cross, Osaka, Japan). The number and specificity of cell types recruited into the airspaces were determined by BAL, as described previously [22].

Total protein and albumin assay

Total protein concentration in sputum and in BALF was quantified using a Micro BCA Protein Assay Reagent kit (Pierce Biotech, IL). Concentration of albumin was analysed by turbidimetric immunoassay (Autokit Micro Albumin, Wako, Osaka, Japan) as described previously for human sputum [20] and by using ELISA (Exocell Inc, Philadelphia, PA) for mouse BALF.

Assessment of protein carbonyls

Carbonylation of sputum or BALF proteins was assessed, as described previously [23]. Briefly, 4 μg of sputum supernatant protein or 16 μl of unconcentrated BALF was derivatized with dinitrophenylhydrazine (DNP) using the OxyBlot Protein Oxidation Detection Kit (Chemicon International, Temecula, CA) and was separated by electrophoresis on 10% SDS-polyacrylamide electrophoresis gels. Western blots were performed using anti-DNP antibody, followed by scanning with a GT-9500 scanner (Epson, Nagano, Japan); the intensity of the bands was calculated using NIH Image software (version 1.62). On each blot, the recorded total DNP intensity of all bands detected in each lane or bands detected for the same molecular weight was divided by that of a standard sample. The carbonyl content is given in terms of Arbitrary Units (AU).

Identification of 68-kDa carbonylated protein

The size of carbonylated proteins was analysed by Western blots before and after removing albumin from the samples. Albumin was removed using Vivapure Anti-HSA/IgG Kits (Sartorius AG, Goettingen, Germany). Briefly, samples were added to 5- or 10-times the amount of HSA Affinity Resin on the spin column and were incubated on a rotary shaker for 15 min. Depleted-albumin samples were collected after flowing through the spin column. The albumin depletion rate was $\sim$ 95% in human sputum and 83% in BALF from mice. Sample volume was adjusted by the rate of dilution and carbonylated protein was assessed as described above.

Estimation of 53-kDa carbonylated protein

The molecular mass of human and mouse α1-antitrypsin was determined by Western blotting on the same membrane used for OxyBlot. Specifically, after blotting for carbonyl proteins, the anti-DNP antibody was removed, followed by incubation with α1-antitrypsin antibody (1:3000, GeneTex, San Antonio, TX) and horseradish peroxidase-conjugated rabbit anti-chicken IgY antibody (1:20 000, Genway, San Diego, CA).

Statistical analyses

All data are shown as means $\pm$ standard error (SE). Differences between two groups were analysed by unpaired *t*-test. More than two means were compared by the Dannett method. All tests were performed using SPSS Version 12.0 (SPSS Inc., Chicago, IL). A value of $p < 0.05$ was considered to be statistically significant.

Results

Subject characteristics

Clinical characteristics and pulmonary function data for subjects enrolled in this study are summarized in Table I. None of the subjects were current smokers. There were no difference in age and pack years of smoking between asthmatics and controls. According to the clinical entities described by GINA, the 23 asthma patients enrolled consisted of seven step 3 patients and 16 step 4 patients. Sputum sampling of patients was not performed during asthma attacks. No severe adverse effects or complications were observed during sputum induction in asthmatic patients or in controls. Forced expiratory volume in 1 s,% predicted value (%FEV_1) and forced expiratory volume in 1 s forced vital capacity (FEV_1/FVC) were significantly lower in asthmatics than in controls (Table I). The percentage of eosinophils in sputum from asthmatic patients was significantly elevated when compared to that from controls (Table II).

Carbonylated proteins in sputum

In order to assess the relationship between oxidation of proteins and airway inflammation, we examined protein carbonyls in sputum from 23 patients with asthma and 23 controls. Immunoblot analysis revealed multiple carbonylated protein bands at molecular weights of 80 kDa, 68 kDa, 53 kDa and 29 kDa in most subjects when 4 μg of sputum supernatant protein was separated on 10% SDS-polyacrylamide electrophoresis gels. Total carbonylated proteins in sputum from asthmatics was significantly higher than in controls (66.6 ± 11.5 AU vs 28.9 ± 7.1, $p = 0.0076$) (Figure 1A). Carbonylated proteins at 80 kDa and 29 kDa did not differ between the patients and controls ($p = 0.2145$, $p = 0.8733$, NS,

Table I. Subject characteristics

	Controls ($n = 23$)	Asthmatics ($n = 23$)
Male/female	18/5	13/10
Age, y	43 ± 4	52 ± 3
Never/Ex	16/7	14/9
Step 1/2/3/4	–	0/0/7/16
FEV_1, % pred.	102.4 ± 2.7	78.7 ± 4.9*
FEV_1/FVC, %	87.4 ± 2.2	67.5 ± 2.3*

*$p < 0.05$ vs. Controls. Data are presented *n* or means $\pm$ SE.

Table II. Sputum characteristics

	Controls ($n=23$)	Asthmatics ($n=23$)
Total protein, mg/dl	77 ± 10	155 ± 28*
Neutrophils,%	30 ± 5	33 ± 5
Eosinophils,%	2 ± 1	23 ± 5*
Macrophages,%	38 ± 4	21 ± 4*
Lymphocytes,%	29 ± 4	24 ± 4

*$p<0.05$ vs. Controls. Data are presented means $\pm$ SE.

respectively, data not shown). However, carbonyl proteins with molecular weights of 68 kDa and 53 kDa were significantly higher in asthma patients than in controls (68-kDa; 21.9 ± 5.3 AU vs 5.4 ± 1.6, $p=0.0049$, 53-kDa; 22.8 ± 4.1 AU vs 10.9 ± 2.8, $p=0.0210$, respectively) (Figure 1B–D). The percentage of eosinophils was significantly correlated with the 68-kDa carbonylated protein ($n=46$, $r=0.552$, $p<0.001$: Figure 2A), but not with the 53-kDa carbonylated protein ($n=46$, $r=0.155$, $p=0.9041$: Figure 2B). However, there were no significant correlations between the percentage of neutrophils and the 68-kDa carbonylated protein or the 53-kDa carbonylated protein (data not shown). These data suggest that the eosinophilic airway inflammation preferentially carbonylates the 68-kDa protein in the airways of asthmatics.

Protein carbonyls in BALF of OVA challenged mice

We then assessed whether the carbonylation of 68-kDa and 53-kDa proteins observed in sputum from human asthmatics was also observed in BALF from a mouse OVA-induced allergic model. Western blot analysis of BALF proteins revealed the appearance of multiple sized-carbonylated proteins at various time points after single intratracheal OVA challenge in OVA-sensitized mice. The major carbonylated protein in BALF was 68 kDa and this was detected at all times, even before OVA challenge (Figure 3A), although it gradually increased at 24 h after OVA challenge and returned to baseline levels at 96 h, which coincided with the appearance of eosinophils in BALF (Figure 3B) (Table III). In contrast, carbonylation of the 53-kDa protein in BALF was prominent at 24 h and disappeared at 48 h after OVA challenge (Figure 3C), which coincided with the transient influx of neutrophils, preceding eosinophils, in BALF (Table III). Thus, the kinetic analysis revealed that the various BALF proteins were dynamically targeted for carbonylation at different time

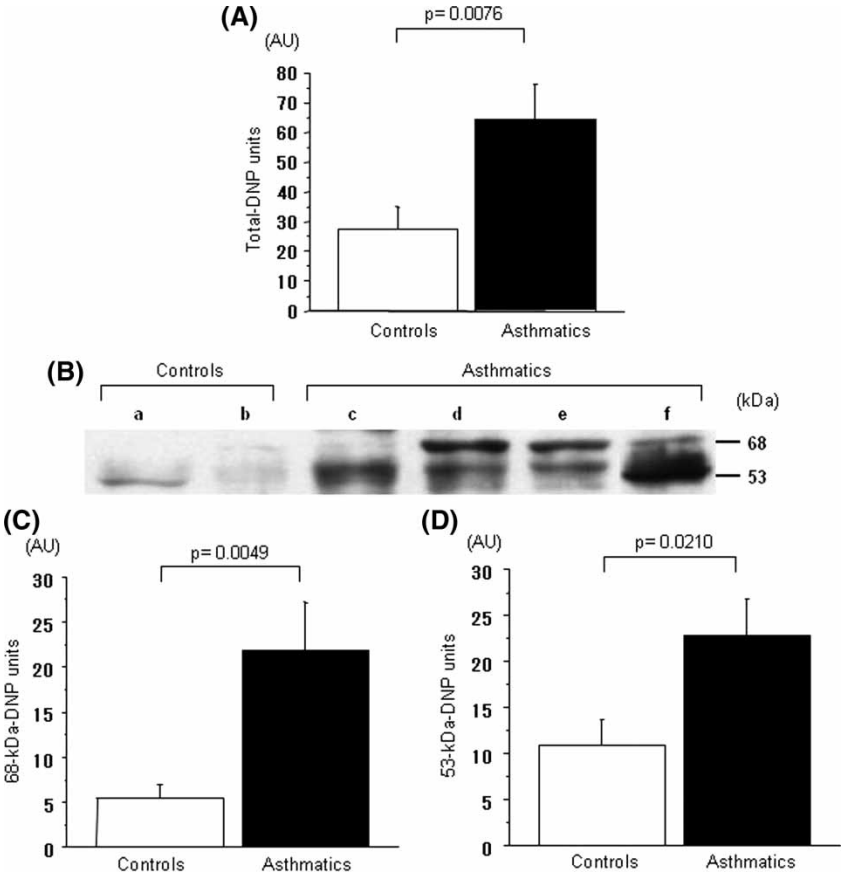

Figure 1. Protein carbonyls in sputum. (A) Comparison of total carbonylated proteins in sputum between asthmatics and controls. (B) Representative Western blot for protein carbonyls in sputum. The 68-kDa and 53-kDa carbonylated proteins were seen in sputum of most subjects (Lanes a and b, control subjects; Lanes c–f, patients with asthma). (C) Comparison of 68-kDa carbonylated protein in sputum between asthmatics and controls. (D) Comparison of the 53-kDa carbonylated protein in sputum between asthmatics and controls.

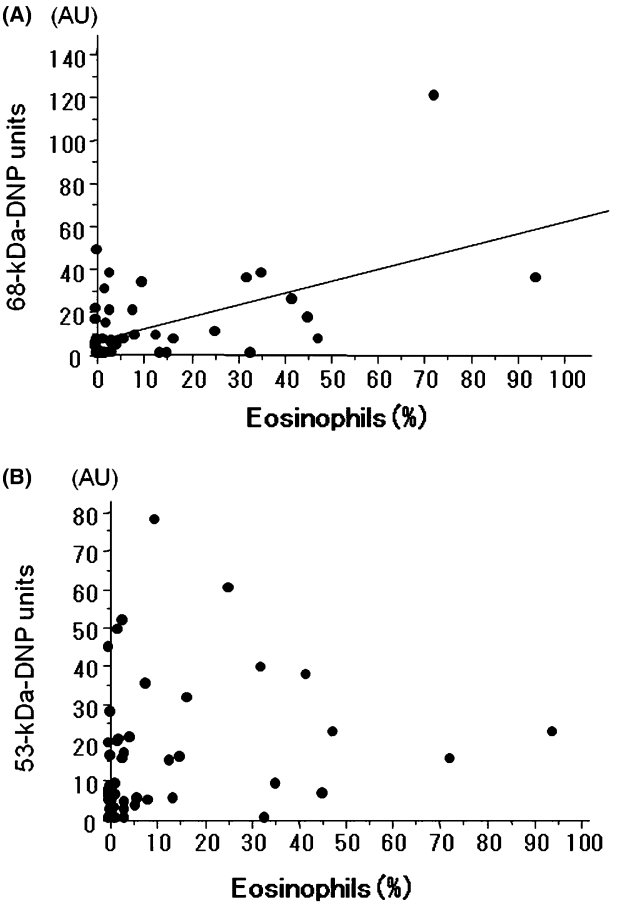

Figure 2. Relationships between the percentage of eosinophils and the levels of 68-kDa carbonylated protein (Aa) or 53-kDa carbonylated protein (B) in sputum.

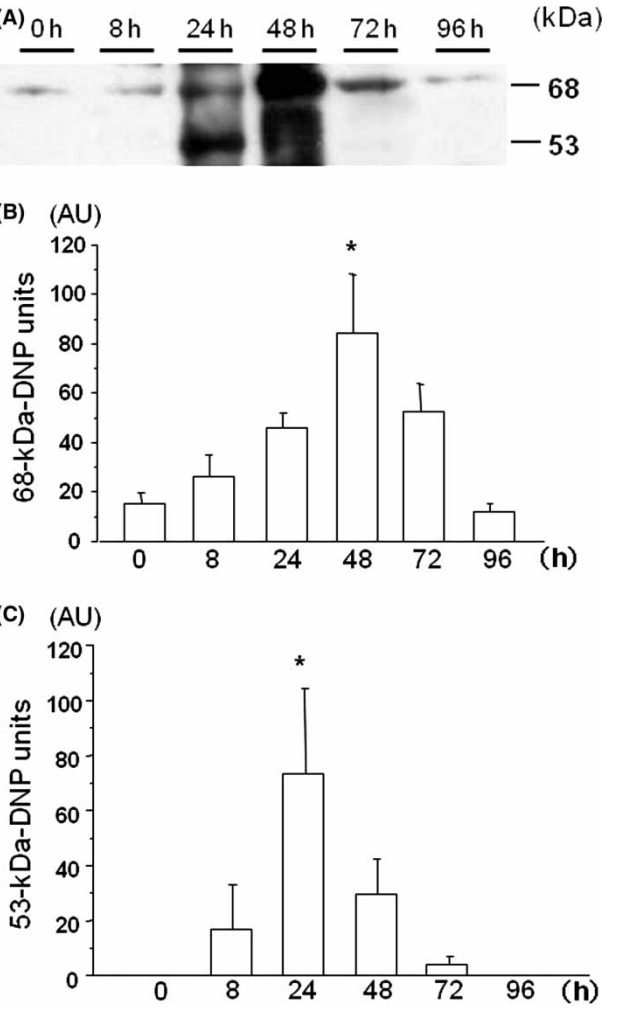

Figure 3. Time course of protein carbonyls in BALF after OVA challenge in mice. (A) Representative Western blot for protein carbonyls in mouse BALF. (B) 68-kDa carbonylated protein (* $p = 0.008$, 84.6 ± 23.5 AU vs 15.3 ± 4.2 at 0 h). (C) 53-kDa carbonylated protein (* $p = 0.010$, 73.4 ± 31.3 AU vs 0 ± 0 at 0 h).

points and may be associated with the inflammatory cell profile in the airway lumen in this model.

Estimation of 68-kDa and 53-kDa carbonylated proteins

The 68-kDa and 53-kDa carbonylated proteins were expected to correspond to albumin and α1-antitrypsin, respectively, in accordance with previous reports [10,24,25]. To confirm whether the 68-kDa protein in this study is albumin, band intensities were compared before and after albumin removal with OxyBlot. As shown in Figure 4A and B, the carbonylated protein at 68 kDa selectively disappeared after albumin removal in human sputum and in mouse BALF, thus suggesting that the carbonylated protein at 68 kDa was albumin. The membrane used for OxyBlot was then re-subjected to Western blot for α1-antitrypsin after stripping anti-DNP antibody and α1-antitrypsin was detected at 53 kDa in sputum samples (Figure 5A) and in mouse BALF samples (Figure 5B). An additional band at a molecular weight lower than 53 kDa in sputum was speculated to be a truncated form of α1-antitrypsin, as reported

Table III. BALF characteristics

Time after OVA i.t.	0 h	8 h	24 h	48 h	72 h	96 h
Total cells (10^4/ml)	0.8 ± 0.1	9.9 ± 5.4	43.1 ± 7.3*	21.9 ± 7.3	48.7 ± 13.6*	10.3 ± 3.0
Macrophages (10^4/ml[%])	0.8 ± 0.1 [96.3]	1.7 ± 0.2 [57.3]	4.0 ± 0.6 [17.1]	6.0 ± 2.2 [32.4]	7.7 ± 2.3* [17.4]	2.0 ± 0.3 [35.8]
Neutrophils (10^4/ml[%])	0.0 ± 0.0 [2.3]	7.9 ± 5.3 [40.0]	33.6 ± 4.4* [74.0]	10.39 ± 5.3 [38.8]	0.0 ± 0.0 [0.1]	0.0 ± 0.0 [0.4]
Eosinophils (10^4/ml[%])	0.0 ± 0.0 [0]	0.3 ± 0.2 [1.4]	5.5 ± 2.7 [8.9]	5.3 ± 2.2 [27.5]	40.2 ± 12.1* [81.2]	8.0 ± 2.8 [61.0]
Lymphocytes (10^4/ml[%])	0.0 ± 0.0 [1.4]	0.1 ± 0.1 [0.3]	0.0 ± 0.0 [0]	0.2 ± 0.0 [1.3]	0.8 ± 0.4* [1.3]	0.3 ± 0.1 [2.8]
Total protein (μg/ml)	237 ± 20	419 ± 121*	493 ± 123*	360 ± 52	310 ± 32	150 ± 20
Albumin (μg/ml)	53 ± 6	130 ± 40*	113 ± 27	84 ± 8	108 ± 27	27 ± 6

*$p < 0.05$ vs. 0 h. Data are presented means $\pm$ SE.

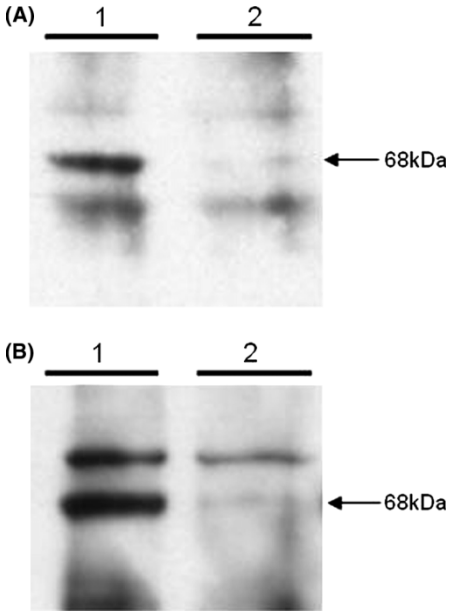

Figure 4. Representative Western blots for protein carbonyls before and after albumin removal. (A) Carbonylated proteins in sputum from a asthmatic patient before (lane 1) and after (lane 2) albumin removal, showing disappearance of the 68-kDa band. (B) Carbonylated proteins in mouse BALF at 48 h after OVA challenge before (lane 1) and after (lane 2) albumin removal, showing the selective deletion of the 68-kDa band.

previously for human BALF [20]. Thus, the carbonylated protein at 53 kDa is, at least partly, α1-antitrypsin.

Ratio of carbonylated albumin in OVA challenged mice

On ELISA, we found a significantly elevated concentration of albumin in BALF at 8 h after intratracheal OVA challenge, suggesting that plasma albumin flowed rapidly into the alveolar space

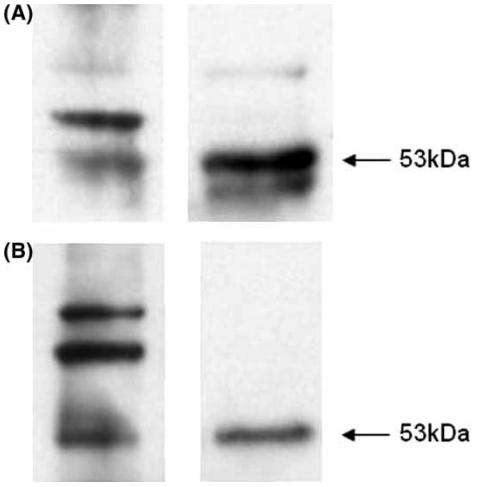

Figure 5. Representative Western blots for protein carbonyls and for α1 antitrypsin. After stripping the anti-DNP antibody, Western blotting was carried out on the same membrane using anti-α1 antitrypsin antibody. (A) sputum from asthmatic patient. (B) BALF from mouse at 48 h after OVA challenge (Left; anti-DNP antibody, Right; anti-α1 antitrypsin antibody).

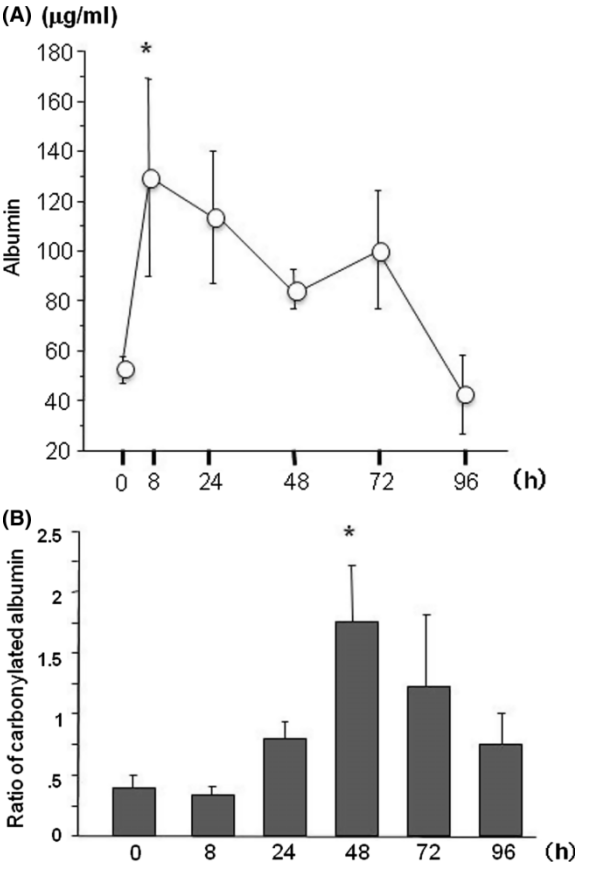

Figure 6. Time course of albumin concentration and carbonylated albumin in BALF after OVA challenge. (A) Levels of albumin in BALF (* $p = 0.031$, 129.3 ± 39.6 μg/ml vs 52.8 ± 6.3 at 0 h). (B) Ratio of carbonylated albumin (* $p = 0.039$, 1.8 ± 0.5 vs 0.4 ± 0.1 at 0 h).

(Figure 6A). However, little carbonylation was seen at that time (Figure 3B). The ratio of 68-kDa carbonylated protein to albumin was therefore determined. The ratio of carbonylated albumin was significantly increased at 48 h after OVA challenge, as compared to before challenge ($p = 0.039$) (Figure 6B). However, the increase on carbonylation of albumin was not parallel to the increase in albumin concentration in BALF.

Discussion

Epithelial lining fluid (ELF) acts as an interface between the airspace epithelium and the external environment and therefore affords protection against epithelial cell injury [26]. Because the constituents of ELF form the primary targets of inflammatory ROS generated within the airway lumen, oxidative modification of certain protein targets and their functional consequences have received considerable attention [10,23,27,28].

In this study, we demonstrated for the first time that various protein carbonyls are present in the sputum of asthmatic patients and that a 68-kDa

carbonylated protein, corresponding to albumin, is specifically correlated to the percentages of eosinophils in sputum. In the OVA-induced allergic mouse model, the carbonylation of BALF protein did not uniformly occur and each target protein had dynamic time frames of carbonylation following a single OVA challenge. The appearance of 53-kDa carbonylated protein, corresponding to α1-antitrypsin, was limited to within 24 h after OVA challenge, which coincided with the transient influx of neutrophils in BALF. On the other hand, carbonylation of the 68-kDa protein, albumin, peaked at 48 h after OVA challenge, which was associated with the increase of eosinophils in BALF.

It should be noted that the lack of carbonylation of albumin from plasma in the early phase of allergic reaction suggests that carbonylation of BALF proteins occurs *de novo* in the lungs. Protein clearance rate via the respiratory epithelial tract lining, the distal air spaces of the lung is 1–2%/h for albumin [29], but the clearance rate increases with inflammation [30]. Albumin is a quantitatively important antioxidant in the blood and extracellular fluids [31,32] and is known to be a carbonylation-susceptible protein in the plasma of mice, rats and rhesus monkeys [33]. In humans, albumin is also the major target protein for carbonylation in the plasma in uremia [34]. It has been reported that the diabetes mellitus-derived serum albumin also contains higher levels of carbonyls than normal albumin. Carbonylation of albumin is known to be a pro-oxidant risk factor in diabetic patients [35]. Exogenous administration of albumin decreases concentrations of protein carbonyls in patients with acute lung injury [36]. It is also considered that albumin carbonylation protects other proteins from oxidative damage [37,38]. The characterization of oxidative status of albumin would thus provide, not only useful information regarding the local and/or systemic redox state of the body, but also changes in the conformation and function of albumin, which may result in modification of its biological properties.

The increase in the 53-kDa carbonylated protein, corresponding to α1-antitrypsin, in the sputum of asthmatic patients and in the BALF of OVA-challenged mice is comparable to findings by Foreman et al. [10]. They reported that the majority of the protein carbonyl residues were found on a 53-kDa protein in BALF obtained 18 h after allergen challenge. Interestingly, in this study, the carbonylation of 53-kDa protein did not correlate with sputum eosinophililia or neutrophilia in humans and preceded the recruitment of eosinophils in the alveolar space in the OVA-mouse model, which is in sharp contrast to the carbonylation of albumin. Considering that sputum sampling was not performed during acute asthma attack and that the OVA-induced allergic mouse model did not mimic recurrent asthmatic status in humans, it is not surprising to see no correlation between the 53-kDa carbonylated protein, α1-antitrypsin and sputum neutrophilia in humans.

In general, the potential sources of ROS include monocytes, macrophages, eosinophils and neutrophils during inflammation. Protein carbonyls are reported to be closely associated with a variety of inflammatory states [39–41]. There was a significant correlation between relative BALF neutrophil count and protein carbonyls in children with chronic pulmonary diseases, such as pulmonary alveolar proteinosis, interstitial lung disease and chronic bronchitis [42]. However, inflammatory cells may not be the sole effectors of BALF protein carbonylation in the lungs. In chronic lung diseases, such as idiopathic pulmonary fibrosis and eosinophilic pneumonia, no significant correlations were observed between carbonylated protein concentrations and cell populations in BALF [43,44]. We also previously demonstrated that BALF protein carbonylation in smokers is age-dependent and that carbonylation is observed only in aged smokers, but not in young smokers, even though there is no difference in the inflammatory cell profiles in BALF between the two age groups [23]. The interaction between various ROS generated by inflammatory as well as structural cells and its vulnerability to each target protein may dynamically determine the formation of protein carbonyls in ELF.

In summary, this study demonstrates for the first time the diversity in protein carbonylation in allergic airway inflammation and the possible role of eosinophils as an important effector cell type to mediate carbonylation specific to albumin. These findings raise further questions on the mechanisms to address the diversity of protein carbonylation in airways. Further work is required to better understand how protein carbonylation shapes the allergic inflammatory response.

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