Free Radical Research, November–December 2008; 42(11–12): 921–929

informa
healthcare

Diversity of protein carbonylation in allergic airway inflammation

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Accepted by Professor N. Taniguchi

(Received 3 September 2008; revised 18 September 2008)

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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ISSN 1071-5762 print/ISSN 1029-2470 online
DOI: 10.1080/10715760802555585

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_3 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 ~ 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 ± 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 ± 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±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

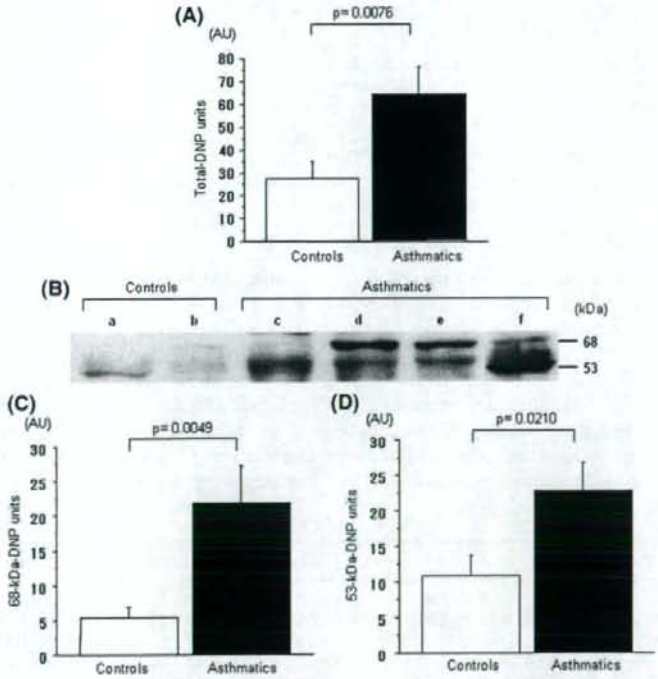

Figure 1. Protein carbonyls in sputum. (A) Comparison of total carbonylated proteins in sputum between asthmatics and controls. (B) Representative Western blot for protein carbonyls in sputum. The 68-kDa and 53-kDa carbonylated proteins were seen in sputum of most subjects (Lanes a and b, control subjects;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

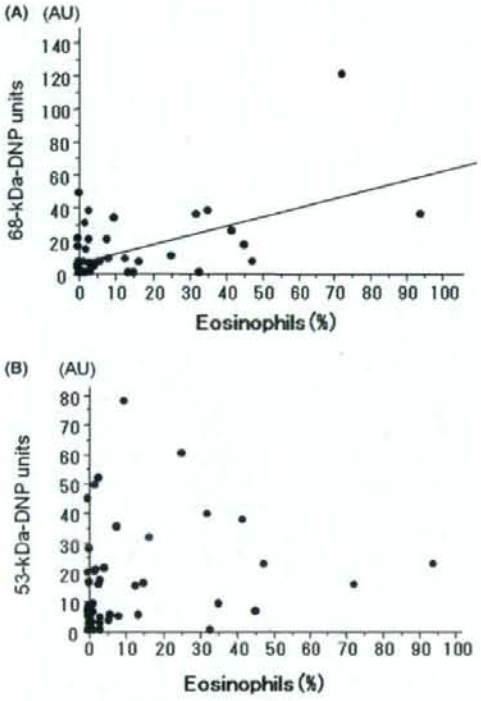

Figure 2. Relationships between the percentage of eosinophils and the levels of 68-kDa carbonylated protein (Aa) or 53-kDa carbonylated protein (B) in sputum.

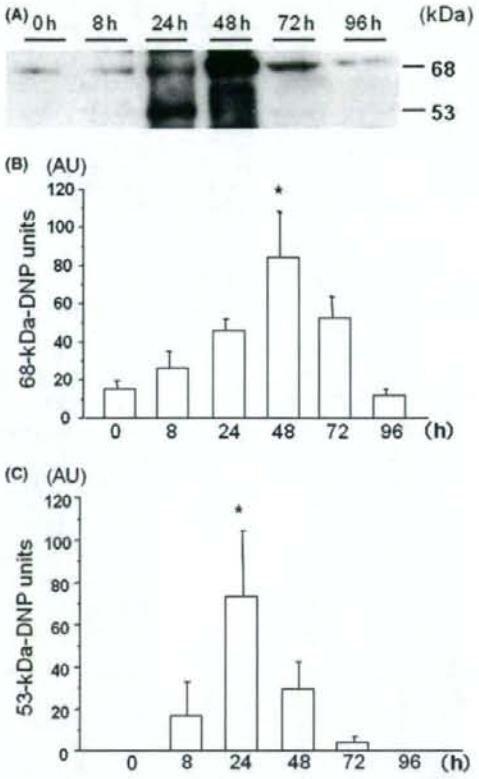

Figure 3. Time course of protein carbonyls in BALF after OVA challenge in mice. (A) Representative Western blot for protein carbonyls in mouse BALF. (B) 68-kDa carbonylated protein (* $p = 0.008$, 84.6±23.5 AU vs 15.3±4.2 at 0 h). (C) 53-kDa carbonylated protein (* $p = 0.010$, 73.4±31.3 AU vs 0±0 at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

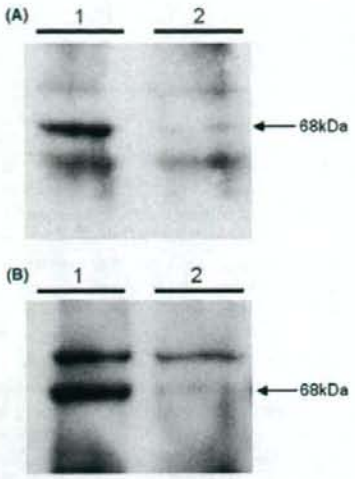

Figure 4. Representative Western blots for protein carbonyls before and after albumin removal. (A) Carbonylated proteins in sputum from a asthmatic patient before (lane 1) and after (lane 2) albumin removal, showing disappearance of the 68-kDa band. (B) Carbonylated proteins in mouse BALF at 48 h after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

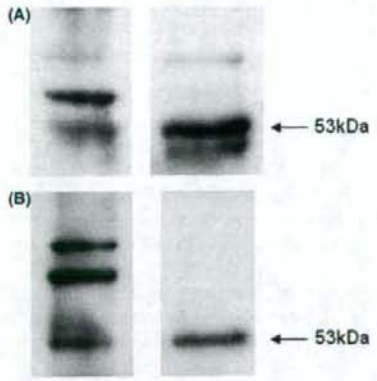

Figure 5. Representative Western blots for protein carbonyls and for α1 antitrypsin. After stripping the anti-DNP antibody, Western blotting was carried out on the same membrane using anti-α1 antitrypsin antibody. (A) sputum from asthmatic patient. (B) BALF from mouse at 48 h after OVA challenge (Left;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

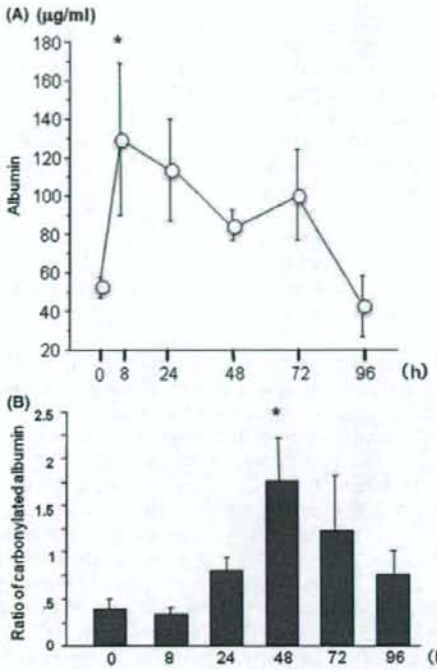

Figure 6. Time course of albumin concentration and carbonylated albumin in BALF after OVA challenge. (A) Levels of albumin in BALF (* $p = 0.031$, 129.3 ± 39.6 μg/ml vs 52.8 ± 6.3 at 0 h). (B) Ratio of carbonylated albumin (* $p = 0.039$, 1.8 ± 0.5 vs 0.4 ± 0.1 at 0 h).

(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.

Acknowledgements

This research was supported by scientific research grants from the Ministry of Education, Culture, Sports, Science and Technology of Japan (No. 19390221 to M.N. and No. 19590877 to T.B.).

***Declaration of interest*:** The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

References

[1] Talati M, Meyrick B, Peebles RS Jr, Davies SS, Dworski R, Mernaugh R, Mitchell D, Boothby M, Roberts LJ 2nd, Sheller JR. Oxidant stress modulates murine allergic airway responses. Free Radic Biol Med 2006;40:1210–1219.

[2] Wood LG, Gibson PG, Garg ML. Biomarkers of lipid peroxidation, airway inflammation and asthma. Eur Respir J 2003;21:177–186.
[3] Nadeem A, Chhabra SK, Masood A, Raj HG. Increased oxidative stress and altered levels of antioxidants in asthma. J Allergy Clin Immunol 2003;111:72–78.
[4] Kelly FJ, Mudway L, Blomberg A, Frew A, Sandstorm T. Altered lung antioxidant status in patients with mild asthma. Lancet 1999;354:482–483.
[5] Wood LG, Garg ML, Simpson JL, Mori TA, Croft KD, Wark PA, Gibson PG. Induced sputum 8-isoprostane concentrations in inflammatory airway diseases. Am J Respir Crit Care Med 2005;171:426–430.
[6] Rahman I, Morrison D, Donaldson K, Mac Nee W. Systemic oxidative stress in asthma, COPD and smokers. Am J Respir Crit Care Med 1996;159:1055–1060.
[7] Dalle-Donne I, Rossi R, Giustarini D, Milzani A, Colombo R. Protein carbonyl groups as biomarkers of oxidative stress. Clin Chim Acta 2003;329:23–38.
[8] Buss IH, Winterbourn CC. Protein carbonyl measurement by ELISA. Methods Mol Biol 2002;186:123–128.
[9] Chevion M, Berenshtein E, Stadtman E. Human studies related to protein oxidation: protein carbonyl content as a marker of damage. Free Radic Res 2000;33:99–108.
[10] Foreman R, Mercer P, Kroegel C, Warner J. Role of the eosinophil in protein oxidation in asthma: possible effects on proteinase/antiproteinase balance. Int Arch Allergy Immunol 1999;118:183–186.
[11] Schock BC, Young IS, Brown V, Fitch PS, Shields MD, Ennis M. Antioxidants and oxidative stress in BALF of atopic asthmatic children. Pediatr Res 2003;53:375–381.
[12] Aldridge RE, Chan T, van Dalen CJ, Senthilmohan R, Winn M, Venge P, Town GI, Kettle AJ. Eosinophil peroxidase produces hypobromous acid in the airways of stable asthmatics. Free Radic Biol Med 2002;33:847–856.
[13] Green RH, Brightling CE, McKenna S, Hargadon B, Parker D, Bradding P, Wardlaw AJ, Pavord ID. Asthma exacerbations and sputum eosinophil counts: a randomised controlled trial. Lancet 2002;360:1715–1721.
[14] Lemiere C, Ernst P, Olivenstein R, Yamauchi Y, Govindaraju K, Ludwig MS, Martin JG, Hamid Q. Airway inflammation assessed by invasive and noninvasive means in severe asthma: eosinophilic and noneosinophilic phenotypes. J Allergy Clin Immunol 2006;118:1033–1039.
[15] Owen S, Pearson D, Suarez-Mendez V, O'Driscoll R, Woodcock A. Evidence of free radical activity in asthma. N Engl J Med 1991;325:586–587.
[16] Sanders SP, Zweier JL, Harrison SJ, Trush MA, Rembish SJ, Liu MC. Spontaneous oxygen radical production at sites of antigen challenge in allergic subjects. Am J Respir Crit Care Med 1995;151:1725–1733.
[17] National Institutes of Health (NIH), National Heart Lung and Blood Institute (NHLBI). Global Initiative for Asthma (GINA): Global Strategy for Asthma Management and Prevention. Updated. 2005. Available online at: www.ginasthma.com (Accessed 17 October 2005).
[18] Pin I, Gibson PG, Kolendowicz R, Girgis-Gabardo A, Denburg JA, Hargreave FE, Dolovich J. Use of induced sputum cell counts to investigate airway inflammation in asthma. Thorax 1992;47:25–29.
[19] Erin EM, Jenkins GR, Kon OM, Zacharasiewicz AS, Nicholson GC, Neighbour H, Tennant RC, Tan AJ, Leaker BR, Bush A, Jose PJ, Barnes PJ, Hansel TT. Optimized dialysis and protease inhibition of sputum dithiothreitol supernatants. Am J Respir Crit Care Med 2008;177:132–141.
[20] Yoshioka A, Betsuyaku T, Nishimura M, Miyamoto K, Kondo T, Kawakami Y. Excessive neutrophil elastase in bronchoalveolar lavage fluid in subclinical emphysema. Am J Respir Crit Care Med 1995;152:2127–2132.
[21] Li XM, Schofield BH, Wang QF, Kim KH, Huang SK. Induction of pulmonary allergic responses by antigen-specific Th2 cells. J Immunol 1998;160:1378–1384.
[22] Betsuyaku T, Shipley JM, Liu Z, Senior RM. Neutrophil emigration in the lungs, peritoneum, and skin does not require gelatinase B. Am J Respir Cell Mol Biol 1999;20:1303–1309.
[23] Nagai K, Betsuyaku T, Kondo T, Nasuhara Y, Nishimura M. Long term smoking with age builds up excessive oxidative stress in bronchoalveolar lavage fluid. Thorax 2006;61:496–502.
[24] Van Vyve T, Chanez P, Bernard A, Bousquet J, Godard P, Lauwerijs R, Sibille Y. Protein content in bronchoalveolar lavage fluid of patients with asthma and control. J Allergy Clin Immunol 1995;95:60–68.
[25] Wattiez R, Hermans C, Bernard A, Lesur O, Falmagne P. Human bronchoalveolar lavage fluid: two-dimensional gel electrophoresis, amino acid microsequencing and identification of major proteins. Electrophoresis 1999;20:1634–1645.
[26] Wattiez R, Falmagne P. Proteomics of bronchoalveolar lavage fluid. J Chromatogr B Analyt Technol Biomed Life Sci 2005;815:169–178.
[27] Beatty K, Bieth J, Travis J. Kinetics of association of serine proteinases with native and oxidized alpha-1-proteinase inhibitor and alpha-1-antichymotrypsin. J Biol Chem 1980;255:3931–3934.
[28] Davies KJ. Protein damage and degradation by oxygen radicals. I. General aspects. J Biol Chem 1987;262:9895–9901.
[29] Folkesson HG, Matthay MA, Weström BR, Kim KJ, Karlsson BW, Hastings RH. Alveolar epithelial clearance of protein. J Appl Physiol 1996;80:1431–1445.
[30] Hastings RH, Folkesson HG, Matthay MA. Mechanisms of alveolar protein clearance in the intact lung. Am J Physiol Lung Cell Mol Physiol 2004;286:679–689.
[31] Wayner DD, Burton GW, Ingold KU, Locke S. Quantitative measurement of total, peroxyl radical-trapping antioxidant capability of human blood plasma by controlled peroxidation. FEBS Lett 1985;187:33–37.
[32] Halliwell B, Gutteridge JM. The antioxidants of human extracellular fluids. Arch Biochem Biophys 1990;280:1–8.
[33] Jana CK, Das N, Sohal RS. Specificity of age-related carbonylation of plasma proteins in the mouse and rat. Arch Biochem Biophys 2002;397:433–439.
[34] Himmelfarb J, McMonagle E. Albumin is the major plasma protein target of oxidant stress in uremia. Kidney Int 2001;60:358–363.
[35] Rasheed Z, Ali R. Reactive oxygen species damaged human serum albumin in patients with type 1 diabetes mellitus: biochemical and immunological studies. Life Sci 2006;79:2320–2328.
[36] Quinlan GJ, Mumby S, Martin GS, Bernard GR, Gutteridge JM, Evans TW. Albumin influences total plasma antioxidant capacity favorably in patients with acute lung injury. Crit Care Med 2004;32:755–759.
[37] Thomas CE. The influence of medium components on Cu(2+)-dependent oxidation of low-density lipoproteins and its sensitivity to superoxide dismutase. Biochim Biophys Acta 1992;1128:50–57.
[38] Wratten ML, Sereni L, Tetta C. Oxidation of albumin is enhanced in the presence of uremic toxins. Ren Fail 2001;23:563–571.
[39] Winterbourn CC, Buss IH, Chan TP, Plank LD, Clark MA, Windsor JA. Protein carbonyl measurements show evidence of early oxidative stress in critically ill patients. Crit Care Med 2000;28:143–149.
[40] Witko-Sarsat V, Friedlander M, Nguyen Khoa T, Capeillère-Blandin C, Nguyen AT, Canteloup S, Dayer JM, Jungers P, Drüeke T, Descamps-Latscha B. Advanced oxidation protein

products as novel mediators of inflammation and monocyte activation in chronic renal failure. J Immunol 1998;161:2524–2532.

[41] Li L, Shoji W, Takano H, Nishimura N, Aoki Y, Takahashi R, Goto S, Kaifu T, Takai T, Obinata M. Increased susceptibility of MER5 (peroxiredoxin III) knockout mice to LPS-induced oxidative stress. Biochem Biophys Res Commun 2007;355:715–721.

[42] Starosta V, Griese M. Protein oxidation by chronic pulmonary diseases in children. Pediatr Pulmonol 2006;41:67–73.

[43] Bargagli E, Penza F, Vagaggini C, Magi B, Perari MG, Rottoli P. Analysis of carbonylated proteins in bronchoalveolar lavage of patients with diffuse lung diseases. Lung 2007;185:139–144.

[44] Rottoli P, Magi B, Cianti R, Bargagli E, Vagaggini C, Nikiforakis N, Pallini V, Bini L. Carbonylated proteins in BAL of patients with sarcoidosis, pulmonary fibrosis associated with systemic sclerosis and idiopathic pulmonary fibrosis. Proteomics 2005;5:2612–2618.

This paper was first published online on iFirst on 28 November 2008.

Respiratory Medicine (2009) 103, 124–129

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Matrix metalloproteinases in blood from patients with LAM

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Received 26 December 2007; accepted 15 July 2008
Available online 29 August 2008

KEYWORDS
Lymphangioleiomyomatosis;
MMP-2;
MMP-9;
Serum;
Plasma

Summary
Pulmonary lymphangioleiomyomatosis (LAM) is characterized by the proliferation of abnormal smooth muscle cells (LAM cells) and destruction of alveolar structure. Immunohistochemical studies suggest that excess matrix metalloproteinases (MMPs) synthesized by LAM cells function in the proteolytic mechanisms of this disease. We postulated MMP levels in the blood are elevated in LAM patients. Serum samples were collected from 36 LAM patients and 25 controls, and gelatinolytic activities were semi-quantified by gelatin zymography. The reliability of serum data for MMP-9 was confirmed by the measurement of MMP-9 concentration in plasma by enzyme-linked immunosorbent assay as well as by gelatin zymography. Serum levels of MMP-9 (0.7 ± 0.1 AU), but not MMP-2, were significantly elevated in LAM patients compared with controls (0.1 ± 0 AU). Plasma and serum levels of MMP-9 significantly correlated. These results suggest the involvement of MMP-9 in LAM.

Introduction

Lymphangioleiomyomatosis (LAM) is a rare disease characterized by recurrent spontaneous pneumothorax, chylothorax, chylous ascites and angiomyolipomas that primarily affects women of childbearing age.[1] The primary pathological findings in LAM include the abnormal proliferation of smooth muscle-like cells (LAM cells) in the pulmonary interstitium and along the axial lymphatics in the thorax and abdomen.[2]

Abbreviations: AU, arbitrary unit(s); DLCO, diffusing capacity for carbon monoxide; ELISA, enzyme-linked immunosorbent assay; FEV_1, forced expiratory volume in 1 s; FEV_1/FVC, forced expiratory volume in 1 s/forced vital capacity; GnRH, gonadotropin-releasing hormone; HRCT, high-resolution computed tomography; LAM, lymphangioleiomyomatosis; LOH, loss of heterozygosity; MMPs, matrix metalloproteinases; NA, not available; TSC, tuberous sclerosis complex; VC, vital capacity.

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doi:10.1016/j.rmed.2008.07.017

Matrix metalloproteinases (MMPs) comprise a family of at least 25 extracellular matrix-degrading, zinc-dependent enzymes. Although MMPs play an important physiological role in tissue remodeling and repair, their overexpression is linked to various chronic pulmonary diseases.[3] The gelatinases, MMP-2 and MMP-9, can degrade native type IV collagen, denatured type I collagen (gelatin) and elastic fiber.[4,5] Immunohistochemical studies have found that MMP-2 and MMP-9 are predominantly expressed in LAM cells, implying that they are involved in the destructive cystic formation associated with pulmonary LAM.[6,7]

Until recently, LAM cells have been considered histologically benign not only in sporadic LAM, but also in tuberous sclerosis complex (TSC)-related LAM. However, compelling evidence such as the recurrence of LAM after lung transplantation and the presence of LAM cells with the same mutation in the lung, lymph nodes and kidney suggests that LAM cells can migrate and metastasize.[8–11] Crooks et al. also demonstrated that disseminated LAM cells carry a *TSC2* loss of heterozygosity (LOH) in the body fluids of patients with LAM.[12] Kumasaka et al. also recently reported that lymphangiogenesis is prominent in regions of LAM and that LAM cells are shed into the blood circulation via lymphatic circulation.[13,14]

Considering that LAM cells which over-produce MMPs are not restricted to the lungs, levels of circulating MMPs might also be augmented, presumably as a result of their release from affected organs and/or from circulating LAM cells. We therefore postulated that MMP-9 and MMP-2 levels are elevated in the blood circulation of patients with pulmonary LAM.

The present study initially validated the usefulness of measuring serum MMP levels by gelatin zymography, that is, the pre-analytical condition of serum, the potential effect of the menstrual cycle, and a correlation between serum and plasma samples. We then quantified serum levels of MMP-9 and MMP-2 by gelatin zymography in patients with pulmonary LAM.

Material and methods

Participants

The study population consisted of 36 female patients with LAM and age-matched 25 healthy females of childbearing age (controls). Among the 36 LAM patients, 24 were from Juntendo University and four from Kyushu University. We diagnosed LAM based on standard histopathological findings in lung biopsy tissues obtained from 24 patients using video-assisted thoracoscopy. We diagnosed LAM in the others based on a combination of multiple thin-walled cysts in the lungs on computed tomography and clinical diagnostic criteria,[15–17] such as recurrent pneumothorax and/or chylothorax, chylous ascites, abdominal lymphangioleiomyomas and/or angiomyolipomas. We graded the severity of cystic lung disease from HRCT as described.[18] Briefly, the extent of cystic involvement in each zone was graded as follows: mild, cysts involving less than one-third of the lungs; moderate, cysts involving one- to two-thirds of the lungs; and severe, cysts involving more than two-thirds of the lungs. Patients with infections or cancers were excluded from this study. Spirometry was performed according to the standard method. Vital capacity, forced expiratory volume in 1 s and diffusing capacity for carbon monoxide were measured in all clinically stable patients within 6 months of blood sampling (CHESTAC-55V; Chest Co., Tokyo, Japan). The normal controls were volunteers from among the medical staff and students recruited using various public advertisements at Hokkaido University School of Medicine. Written informed consent was obtained from each patient and volunteer participating in the study, and the Ethics Committees of Hokkaido, Juntendo and Kyushu Universities approved the study.

Blood sampling

Venous blood samples were collected into Vacutainer™ tubes containing clot activator and dipotassium EDTA for serum and plasma preparations, respectively. The tubes were centrifuged at 3000 rpm at 4 °C for 10 min and stored at −80 °C.

Gelatin zymography

We assessed the gelatinolytic activities of blood by gelatin zymography as described.[19] In brief, samples were resolved by electrophoresis on 8% (w/v) polyacrylamide gels containing 1 mg/dl gelatin in the presence of sodium dodecyl sulfate under non-reducing conditions. The gels were washed in 2.5% Triton X-100 for 30 min, rinsed briefly, and incubated at 37 °C for 18 h in a buffer containing 50 mM Tris–HCl, pH 8.0, 5 mM $CaCl_2$ and 1 mM $ZnCl_2$. The gels were stained with Coomassie blue R-250 and destained, then enzyme activity was detected as unstained bands against a blue background.

Semi-quantitative analysis of MMP-9 and MMP-2 by gelatin zymography

To semi-quantify gelatinolytic activities, zymograms were captured using a Scan Jet II cx/T (Hewlett–Packard, Palo Alto, CA) and band intensity was calculated using NIH Image software (version 1.54, ML). The relationship between known amounts of serially diluted MMP-9 and MMP-2 (CC073; Chemicon) and the intensity of gelatinolytic bands at 92 and 72 kDa was assessed within their respective linear ranges, which were repeated for each assay (Fig. 1A and B). Serum and plasma samples were diluted 40-fold. The activities of MMPs in each sample were calculated by dividing the optical intensity of bands at 92 or 72 kDa by the value of the standard MMPs on each zymogram and are described in arbitrary units (AU).

Enzyme-linked immunosorbent assays (ELISAs) of plasma MMP-9

We purchased ELISA kits for MMP-9 from Amersham Pharmacia Biotech (Little Chalfont, UK). Levels of MMP-9 in plasma were measured as the recognition of latent forms or of complexes with tissue inhibitor of metalloproteinase-1. The detection limit of the assay was 0.6 ng/ml. All samples

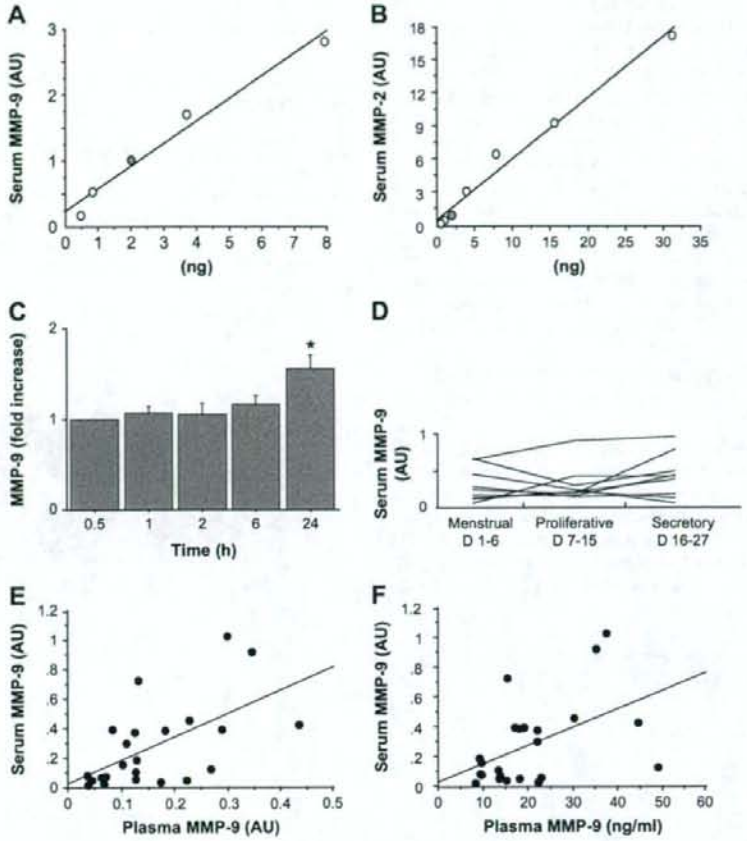

Figure 1 Evaluation of linear range between amounts of MMPs and integrated optical density (AU) on gelatin zymography and comparison of serum samples with blood sampling time, menstrual cycle, and plasma samples. (A) Amounts of MMP-9 integrated optical density (AU) ($r = 1.0$). (B) Amounts of MMP-2 significantly correlated with integrated optical density (AU) ($r = 1.0$). Gray circle, standard sample loaded on each gel. (C) Levels of MMP-9 were elevated at 24 h after blood sampling. Up to 6 h between blood sampling and centrifugation did not affect levels of MMP-9. Data are mean (SE) values for each group. *Difference from 0.5 h ($p = 0.0015$ for 24 h). (D) Menstrual phase also did not affect serum MMP-9 levels. (E) Serum MMP-9 was positively related to plasma MMP-9 level determined by gelatin zymography ($r = 0.6$, $p = 0.0017$). (F) Serum levels of MMP-9 and plasma level of MMP-9 determined by ELISA were positively related ($r = 0.5$, $p = 0.0187$).

were assayed in duplicate and values fell within the linear portion of the standard curve.

Statistical analysis

Results are expressed as means ± standard error of the mean (SEM). Data were statistically analyzed using the Bonferroni/Dunn test or Student's paired *t*-test for pre-analytical studies. Differences between two groups were compared using Student's unpaired *t*-test. Pearson's correlation coefficients or Spearman's correlation coefficients were calculated to assess relationships between two parameters (StatView J 5.0; SAS Institute Inc., Cary, NC). A value of $p < 0.05$ was considered statistically significant.

Results

Characteristics of the participants

Table 1 and Table E1 (Supplementary data) show the clinical characteristics of all patients with LAM who participated in the entire study. Airflow limitation was frequent in the patients.

Pre-analytical conditions for MMP-9 measurements in blood

To eliminate any artifactual effects on serum MMP-9 levels, we first examined the effects of time between blood

sampling and centrifugation on the serum levels of MMP-9 or MMP-2. We collected the tubes of blood which were centrifuged after 0.5, 1, 2, 6 or 24 h at room temperature from four controls. The serum MMP-9 levels were 1.6 ± 0.1 fold higher only when the blood remained at room temperature for over 24 h after sampling compared with 0.5 h ($p = 0.0015$; Fig. 1C). In contrast, the level of MMP-2 in serum did not change over time between sampling and centrifugation (data not shown). We then examined whether the menstrual cycle affects serum levels of MMP-9 or MMP-2. We collected three serum samples from each of nine normal controls at the menstrual, proliferative, and secretory phases of the menstrual cycle. Serum levels of MMP-9 and MMP-2 remained constant throughout the menstrual cycle (Fig. 1D and data not shown, respectively).

Correlation between serum and plasma MMP-9 levels

Meisser et al. have recently reported that plasma is preferable to serum for measuring MMPs.[20] We thus evaluated whether levels of serum and plasma MMP-9 correlate in using nine samples from the patients with LAM, and 14 from normal controls. Gelatin zymography and ELISA results showed that the serum and plasma levels of MMP-9 significantly correlated ($r = 0.6$, $p = 0.0029$; Fig. 1E and $r = 0.5$, $p = 0.0187$; Fig. 1F, respectively), suggesting that serum data of MMPs are also reliable as plasma ones.

Table 1 Clinical characteristics of LAM patients.

Number of participants (female/male)	36 (36/0)
Age (y)	34 ± 1
Smoker	8/36
Tuberous sclerosis (TS)	5/36
Angiomyolipoma	12/36
Abdominal lymphangioleiomyoma	4/36
Clinical features	
Dyspnea	9/36
Pneumothorax	15/36
Chylothorax	3/36
Pathological diagnosis of LAM	24/36
HRCT findings (multiple cyst)	36/36
HRCT grades	
Mild	12/36
Moderate	9/36
Severe	15/36
Pulmonary function	
VC (% predicted)	94 ± 3 (38–126)
FEV_1 (ml)	1899 ± 125 (510–3200)
FEV_1 (% predicted)	71 ± 5 (20–111)
FEV_1/FVC (%)	68 ± 3 (34–95)
DLCO (% predicted)	44 ± 5 (14–112)
Hormonal manipulation	
GnRH	16/36
Progesterone	2/36

Mean ± SEM (range), DLCO: diffusing capacity for carbon monoxide, FEV_1: forced expiratory volume in 1 s, FEV_1/FVC: forced expiratory volume in 1 s/forced vital capacity, GnRH: gonadotropin-releasing hormone, HRCT: high-resolution computed tomography scans, LAM: lymphangioleiomyomatosis, VC: vital capacity.

Serum levels of MMP-9 and MMP-2 in patients with LAM and controls

Fig. 2A shows a representative zymogram of serum from patients with LAM and from normal controls. Three distinct gelatinolytic bands with molecular masses of 92, 130 and 220 kDa migrated, although the 92-kDa band was the most prominent. These bands were considered to correspond to latent MMP-9, human neutrophil lipocalin/MMP-9 complex,[19] and the multiple form of MMP-9, respectively. Based on significant Pearson's correlation coefficients

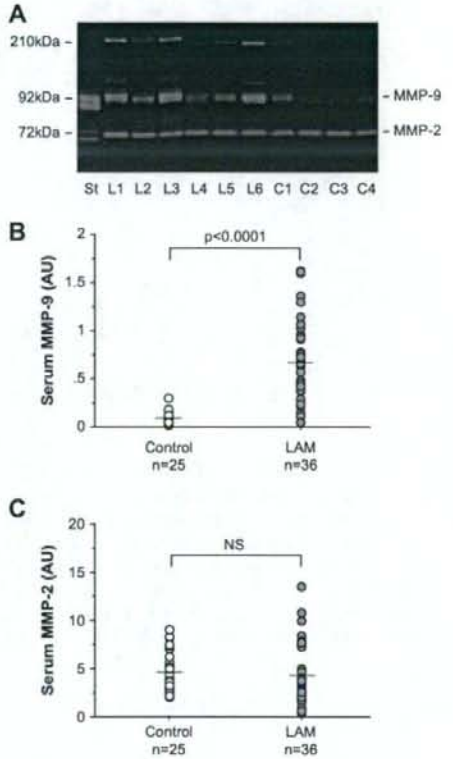

Figure 2 Serum levels of MMP-9 and MMP-2 in patients with LAM determined by gelatin zymography. (A) Representative zymographic profile shows relative amounts of MMP-9 (92 kDa) and MMP-2 (72 kDa) in serum from six LAM patients and four normal controls. L, LAM patient; C, normal control; St, standard. (B) Serum levels of MMP-9 were significantly elevated in LAM patients compared with those in normal controls (0.7 ± 0.1 vs. 0.1 ± 0 AU, $p < 0.0001$). (C) Levels of MMP-2 did not differ between LAM patients and normal controls (4.2 ± 0.5 AU vs. 4.7 ± 0.4, NS). Horizontal line indicates the mean value of each group.

between two observations ($r = 0.4$, $p = 0.0452$), the latter data set is presented.

Levels of MMP-9 were significantly elevated in serum from LAM patients compared with that from normal controls (0.7 ± 0.1 vs. 0.1 ± 0 AU, $p < 0.0001$; Fig. 2B). On the other hand, serum levels of MMP-2 did not differ between LAM patients and normal controls (4.2 ± 0.5 vs. 4.7 ± 0.4 AU, NS; Fig. 2C). Serum MMP-9 and MMP-2 values also did not differ between LAM patients who smoked and those who had never smoked (0.6 ± 0.1 vs. 0.7 ± 0.1 AU, NS and 3.2 ± 0.7 vs. 4.5 ± 0.6 AU, NS, respectively). The statistical significance persisted in the serum levels of MMP-9 between nonsmoking LAM patients ($n = 28$) and normal controls (0.7 ± 0.1 vs. 0.1 ± 0 AU, $p < 0.0001$). Serum MMP-9 or MMP-2 levels also did not differ between patients with and without angiomyolipoma (MMP-9: 0.8 ± 0.2 vs. 0.6 ± 0.1 AU, NS and MMP-2: 3.8 ± 0.9 vs. 4.4 ± 0.7 AU, NS), between patients who received hormonal therapy and those who did not or between those with or without TSC (data not shown).

Discussion

We discovered that serum levels of MMP-9, but not of MMP-2, were significantly higher in the patients with LAM than in healthy controls.

MMP-9 levels in circulating blood are elevated in various pathologic states such as asthma,[21] pulmonary emphysema[22] and lung cancer,[23] as well as in other malignancies[24] and ischemic heart disease.[25] Accordingly, our data imply that LAM is one of the diseases that MMP-9 is elevated in serum, and MMP-9 in serum is driven by circulating and/or pulmonary LAM cells. A recent case report by Moses et al. demonstrated that urinary MMPs comprise a potential biomarker with which to monitor the therapeutic efficacy of doxycycline in patients with LAM.[26] Assessment of a larger sample of patients should deepen understanding of the involvement of MMPs in the mechanism of the disease.

In contrast to levels of serum MMP-9, those of serum MMP-2 in patients with LAM and in normal controls did not significantly differ. One speculation is that the constitutive synthesis of MMP-2 by various mesenchymal cells such as fibroblasts and endothelial cells under normal conditions masks the increase of MMP-2 driven by LAM cells.

Various methods have been developed to quantify circulating MMP-9 and -2, such as ELISA, gelatin zymography, and Western blotting of either serum or plasma samples. Several investigators have recently raised concerns about MMP-9 analysis in the clinical environment, such as the pre-analytical condition of the blood,[20] and the background condition of patients, since circulating MMP-9 is reportedly elevated even in healthy smokers,[27] and during the menstrual phase in healthy women of childbearing age.[28] The present study therefore validated the feasibility of measuring MMPs in serum. We showed that time between blood sampling and centrifugation did not affect the level of MMP-9 or MMP-2 in serum, and that fluctuation associated with menstrual phase did not affect serum MMP-9 levels, suggesting that this parameter (at least in normal controls) is not affected by either the artifactual effects of blood sampling or the dynamics of sex hormones. Furthermore, we confirmed that the levels of MMP-9 in serum were parallel to those in plasma, regardless of whether MMP-9 was measured by gelatin zymography or ELISA. Overall, our data confirmed that MMP-9 can be reliably semi-quantified in clinical serum samples by gelatin zymography that, when prepared in a routine manner, would simplify multi-center clinical studies.

The mechanisms responsible for the elevated serum MMP-9 levels remain obscure, but a protein that upregulates MMPs, such as extracellular matrix metalloproteinase inducer (EMMPRIN), might be linked to the pathogenesis of LAM, as well as fibrosing pulmonary diseases.[29,30]

We concluded that the levels of serum MMP-9 were significantly elevated in patients with pulmonary LAM than in healthy controls. Although MMP-9 is not a specific disease marker of LAM, prospective studies could help to elucidate the significance of serum MMP-9.

Conflict of interest

None of the authors has a commercial association that might be perceived as a conflict of interest.

Acknowledgement

This study was partly funded by scientific research grants from the Ministry of Health, Labor, and Welfare of Japan to the Respiratory Failure Research Group.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.rmed.2008.07.017.

References

1. Corrin B, Liebow AA, Friedman PJ. Pulmonary lymphangiomyomatosis. A review. *Am J Pathol* 1975;**79**:348–82.
2. Ferrans VJ, Yu ZX, Nelson WK, Valencia JC, Tatsuguchi A, Avila NA, et al. Lymphangioleiomyomatosis (LAM). A review of clinical and morphological features. *J Nippon Med Sch* 2000; **67**:311–29.
3. Shapiro SD. Matrix metalloproteinase degradation of extracellular matrix: biological consequences. *Curr Opin Cell Biol* 1998;**10**:602–8.
4. Senior RM, Griffin GL, Fliszar CJ, Shapiro SD, Goldberg GI, Welgus HG. Human 92- and 72-kilodalton type IV collagenases are elastases. *J Biol Chem* 1991;**266**:7870–5.
5. Atkinson JJ, Senior RM. Matrix metalloproteinase-9 in lung remodeling. *Am J Respir Cell Mol Biol* 2003;**28**:12–24.
6. Hayashi T, Fleming MV, Stetler-Stevenson WG, Liotta LA, Moss J, Ferrans VJ, et al. Immunohistochemical study of matrix metalloproteinases (MMPs) and their tissue inhibitors (TIMPs) in pulmonary lymphangioleiomyomatosis (LAM). *Hum Pathol* 1997;**28**:1071–8.
7. Matsui K, Takeda K, Yu ZX, Travis WD, Ferrans VJ. Role for activation of matrix metalloproteinases in the pathogenesis of pulmonary lymphangioleiomyomatosis. *Arch Pathol Lab Med* 2000;**124**:267–75.

8. Yu J, Astrinidis A, Henske EP. Chromosome 16 loss of heterozygosity in tuberous sclerosis and sporadic lymphangiomyomatosis. *Am J Respir Crit Care Med* 2001;**164**: 1537–40.
9. Sato T, Seyama K, Fujii H, Maruyama H, Setoguchi Y, Iwakami S, et al. Mutation analysis of the TSC1 and TSC2 genes in Japanese patients with pulmonary lymphangioleiomyomatosis. *J Hum Genet* 2002;**47**:20–8.
10. Karbowniczek M, Astrinidis A, Balsara BR, Testa JR, Lium JH, Colby TV, et al. Recurrent lymphangiomyomatosis after transplantation: genetic analyses reveal a metastatic mechanism. *Am J Respir Crit Care Med* 2003;**167**:976–82.
11. Henske EP. Metastasis of benign tumor cells in tuberous sclerosis complex. *Genes Chromosomes Cancer* 2003;**38**: 376–81.
12. Crooks DM, Pacheco-Rodriguez G, DeCastro RM, McCoy Jr JP, Wang J, Kumaki F, et al. Molecular and genetic analysis of disseminated neoplastic cells in lymphangioleiomyomatosis. *Proc Natl Acad Sci U S A* 2004;**101**:17462–7.
13. Kumasaka T, Seyama K, Mitani K, Sato T, Souma S, Kondo T, et al. Lymphangiogenesis in lymphangioleiomyomatosis. Its implication in the progression of lymphangioleiomyomatosis. *Am J Surg Pathol* 2004;**28**:1007–16.
14. Kumasaka T, Seyama K, Mitani K, Souma S, Kashiwagi S, Hebisawa A, et al. Lymphangiogenesis-mediated shedding of LAM cell clusters as a mechanism for dissemination in lymphangioleiomyomatosis. *Am J Surg Pathol* 2005;**29**: 1356–66.
15. Sullivan EJ. Lymphangioleiomyomatosis: a review. *Chest* 1998; **114**:1689–703.
16. Johnson SR. Lymphangioleiomyomatosis. *Eur Respir J* 2006;**27**: 1056–65.
17. Ryu JH, Moss J, Beck GJ, Lee JC, Brown KK, Chapman JT, et al. NHLBI LAM Registry Group. The NHLBI lymphangioleiomyomatosis registry: characteristics of 230 patients at enrollment. *Am J Respir Crit Care Med* 2006;**173**:105–11.
18. Avila NA, Dwyer AJ, Rabel A, Moss J. Sporadic lymphangioleiomyomatosis and tuberous sclerosis complex with lymphangioleiomyomatosis: comparison of CT features. *Radiology* 2007;**242**:277–85.
19. Betsuyaku T, Nishimura M, Takeyabu K, Tanino M, Venge P, Xu S, et al. Neutrophil granule proteins in bronchoalveolar lavage fluid from subjects with subclinical emphysema. *Am J Respir Crit Care Med* 1999;**159**:1985–91.
20. Meisser A, Cohen M, Bischof P. Concentrations of circulating gelatinases (matrix metalloproteinase-2 and -9) are dependent on the conditions of blood collection. *Clin Chem* 2005;**51**:274–6.
21. Ko FWS, Diba C, Roth M, McKay K, Johnson PRA, Salome C, et al. A comparison of airway and serum matrix metalloproteinase-9 activity among normal subjects, asthmatic patients, and patients with asthmatic mucus hypersecretion. *Chest* 2005;**127**:1919–27.
22. Mao JT, Tashkin DP, Belloni PN, Baileyhealy I, Baratelli F, Roth MD. All-trans retinoic acid modulates the balance of matrix metalloproteinase-9 and tissue inhibitor of metalloproteinase-1 in patients with emphysema. *Chest* 2003;**124**:1724–32.
23. Jumper C, Cobos E, Lox C. Determination of the serum matrix metalloproteinase-9 (MMP-9) and tissue inhibitor of matrix metalloproteinase-1 (TIMP-1) in patients with either advanced small-cell lung cancer or non-small-cell lung cancer prior to treatment. *Respir Med* 2004;**98**:173–7.
24. Nikkola J, Vihinen P, Vuoristo MS, Kellokumpu-Lehtinen P, Kahari VM, Pyrhonen S. High serum levels of matrix metalloproteinase-9 and matrix metalloproteinase-1 are associated with rapid progression in patients with metastatic melanoma. *Clin Cancer Res* 2005;**11**:5158–66.
25. Fukuda D, Shimada K, Tanaka A, Kusuyama T, Yamashita H, Ehara S, et al. Comparison of levels of serum matrix metalloproteinase-9 in patients with acute myocardial infarction versus unstable angina pectoris versus stable angina pectoris. *Am J Cardiol* 2006;**97**:175–80.
26. Moses MA, Harper J, Folkman J. Doxycycline treatment for lymphangioleiomyomatosis with urinary monitoring for MMPs. *N Engl J Med* 2006;**354**:2621–2.
27. Raitio A, Tuomas H, Kokkonen N, Salo T, Sorsa T, Hanemaaijer R, et al. Levels of matrix metalloproteinase-2, -9 and -8 in the skin, serum and saliva of smokers and non-smokers. *Arch Dermatol Res* 2005;**297**:242–8.
28. Abdallah MA, Nakajima ST, Lin PC, Gercel-Taylor C. Plasma matrix metalloproteinase activity during the menstrual cycle of healthy ovulatory patients who underwent tubal ligation. *Fertil Steril* 2005;**84**:1766–7.
29. Betsuyaku T, Kadomatsu K, Griffin GL, Muramatsu T, Senior RM. Increased basigin in bleomycin-induced lung injury. *Am J Respir Cell Mol Biol* 2003;**28**:600–6.
30. Odajima N, Betsuyaku T, Nasuhara Y, Itoh T, Fukuda Y, Senior RM, et al. Extracellular matrix metalloproteinase inducer in interstitial pneumonias. *Hum Pathol* 2006;**37**:1058–65.

Biogerontology (2009) 10:173–180
DOI 10.1007/s10522-008-9164-4

RESEARCH ARTICLE

Aging affects lipopolysaccharide-induced upregulation of heme oxygenase-1 in the lungs and alveolar macrophages

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Received: 18 March 2008 / Accepted: 24 July 2008 / Published online: 9 August 2008

Abstract Lung injuries are generally more serious and cause high mortality in aged humans and animals. Heme Oxygenase-1 (HO-1) is known to be readily inducible in alveolar macrophages (AMs) and airway epithelial cells to confer cytoprotection against oxidative stress. We thus investigated whether aging impairs the stress-induced upregulation of HO-1. In this study, we first quantified basal levels of HO-1 expression in lungs from male ICR mice of various ages. Second, young (9–11 weeks) and old (65–66 weeks) mice were subjected to intratracheal administration of lipopolysaccharide (LPS) and expression of HO-1 in the lungs was quantified at 2, 24 and 72 h. HO-1 expression in bronchiolar epithelial cells harvested by laser capture microdissection (LCM) was also specifically quantified in the two age groups. Third, we examined HO-1 expression in AMs lavaged from 22-week-old and 86–96-week-old male ICR mice in response to LPS for 24 h in vitro. We found that basal expression of HO-1 in the lungs did not differ with age. LPS-induced HO-1 upregulation was significantly impaired in the lungs of 65–66-week-old mice than in 9–11-week-old mice at 2 and 24 h, although there were no differences in the magnitude of HO-1 upregulation in bronchiolar epithelium at 2 h. LPS-induced upregulation of HO-1 was observed in AMs from 22-week-old mice (1.8-fold), but not in AMs from 86–96-week-old mice in vitro. In summary, we demonstrated age-related defects in HO-1 induction in the whole lungs and in AMs in response to LPS.

Keywords Acute lung injury · Aging · HO-1 · LPS

Abbreviations

AMs	Alveolar macrophages
ARDS	Acute respiratory distress syndrome
BAL	Bronchoalveolar lavage
β2MG	β2-Microglobulin
BGUS	β-Glucuronidase
HO-1	Heme oxygenase-1
HPRT	Hypoxanthine ribosyltransferase
LPS	Lipopolysaccharide
LCM	Laser capture microdissection
Nrf2	Nuclear factor erythroid 2-related factor
PCR	Polymerase chain reaction
SE	Standard error

Introduction

Heme oxygenase-1 (HO-1) is the rate-limiting enzyme that catalyzes heme, and is known to be

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readily inducible to confer cytoprotection against oxidative stress (Morse and Choi 2002). The HO-1 gene is strongly induced in response to changes in cellular redox status (Ryter and Choi 2002) and its expression is primarily regulated at the transcriptional level (Camhi et al. 1995; Morse and Choi 2002). HO-1 has been shown to have anti-inflammatory, anti-apoptotic and anti-proliferative effects (Morse and Choi 2002) and the products of heme metabolism, bilirubin, ferritin and carbon monoxide (CO) also functions as antioxidants (Stocker et al. 1987; Choi and Alam 1996; Maines 1997; Minetti et al. 1998; Oberle et al. 1999). Increased HO-1 expression has been demonstrated in lung tissue and bronchoalveolar lavage (BAL) fluid from patients with acute respiratory distress syndrome (ARDS) (Mumby et al. 2004). In addition, HO-1 mediates protective effects in animal models of hyperoxic lung injury (Otterbein et al. 1999a, b, c), ischemic lung injury (Fujita et al. 2001), ventilator-induced lung injury (Dolinay et al. 2004) and endotoxin-induced lung injury (Otterbein et al. 1995, 1997). Moreover, chemical inhibition of HO-1 activity increases the susceptibility of rats to lung injury from endotoxin (Otterbein et al. 1995). Overexpression of HO-1 attenuates lipopolysaccharide (LPS)-induced neutrophilic inflammation in the lungs, whereas inhibition of HO-1 activity mitigates the effect of overexpression (Inoue et al. 2001). Taken together, these findings suggest that HO-1 is upregulated in various injury setting to exert protective effects on the lungs, and that the impaired upregulation of HO-1 may worsen damaging influences on the lungs.

In humans and animals, acute lung injury is generally more serious and is associated with higher mortality at advanced ages (Antonini et al. 2001; Ely et al. 2002; Gomez et al. 2007). We have previously reported prolonged and enhanced neutrophilic inflammation in the lungs of aged mice, as compared with young mice, after LPS intratracheal administration, in which age-related decreases in lung VEGF expression and prolonged overexpression of neutrophilic chemokines were observed (Ito et al. 2005, 2007). However, the mechanism by which aging is a risk factor of higher mortality in lung injury still remains to be elucidated.

A possible link between physiological aging and HO-1 expression has recently been reported. Age-related changes in HO-1 expression have been investigated in brain and liver in rodents (Schipper 2000; Lavrovsky et al. 2000; Ewing and Maines 2006; Patriarca et al. 2007), although these results were contradictory depending on differences in the species used and tissues evaluated. To date, however, the effects of aging in HO-1 expression have not been investigated in the lungs. Therefore, in the first part of this study, we examined HO-1 expression in murine lungs at various ages. We then investigated the age-related differences in the magnitude of HO-1 upregulation in response to LPS in the lungs. HO-1 is known to be expressed in alveolar macrophages (AMs), airway epithelial cells, fibroblasts, and endothelial cells (Donnelly and Barnes 2001; Lim et al. 2000; Li et al. 2000). As cell-specific effects often exist, it is important to determine whether LPS-induced changes in HO-1 expression are influenced by age in specific cell types within the lungs. Therefore, we investigated the expression of HO-1 in laser-capture microdissection (LCM)-harvested bronchiolar epithelium, as well as in whole lung homogenates in the 9–11-week-old and the 65–66-week-old mice after intratracheal instillation of LPS. We then examined whether AMs harvested from aged mice respond differently to LPS in vitro.

Materials and methods

Animals

Male ICR mice of various ages were obtained from Japan Clea (Tokyo, Japan). All mice were kept in plastic chambers with free access to food and water. None of the mice were found to have gross pathological lesions. Experimental protocols and procedures were approved by the Ethical Committee on Animal Research of the Hokkaido University School of Medicine.

LPS-induced acute lung injury model in mice

Fifty microliters of saline containing LPS (200 μg/animal) (Sigma Chemical Co, St. Louis, MO) was intratracheally administered to mice aged 9–11 weeks ($n = 27$) and 65–66 weeks ($n = 22$) under anesthesia with a mixture of ketamin and xylazine, as described previously (Betsuyaku et al. 1999; Ito et al. 2005,

2007). Age-matched, untreated normal mice were served as controls.

Sampling of lung tissues

At 2, 24 or 72 h after LPS injection ($n = 5$–8 at each time point), animals were sacrificed. Lungs were removed, inflated with diluted Tissue-Tek OCT (Sakura Finetek U.S.A., Torrance, CA) (50% vol/vol in ribonuclease (RNase)-free PBS containing 10% sucrose), immediately frozen on dry ice as described previously (Betsuyaku et al. 2001) and stored at −80°C. For preparation of tissue homogenates, individual lung tissues were dissolved in buffer containing the protease inhibitors phenylmethylsulfonyl fluoride (100 mM), antipain (1.2 mg/ml), aprotinin (2 mg/ml), pepstatin A (0.5 mg/ml) and leupeptin (1 mg/ml). Samples were centrifuged at 15,000 rpm for 10 min at 4°C and the supernatant was stored −80°C.

Collection of mouse bronchiolar epithelial cells by LCM

Using the frozen lung tissues described above, LCM was performed on 7 μm frozen sections in order to retrieve cells within 100 μm of the bronchoalveolar junction using the PixCell II System (Arcturus Engineering, Mountain View, CA) with the following parameters, as described previously: laser diameter, 30 μm; pulse duration, 5 ms; and amplitude, 50 mW (Betsuyaku et al. 2001). After the samples were captured on transfer films (CapSure Macro LCM Caps, LCM0211; Arcturus Engineering), nonspecific attached components were removed with adhesive tape (CapSure Cleanup Pad, LCM0206; Arcturus Engineering). Approximately 5,000 laser bursts were used to collect cells for RNA isolation from each mouse.

Collections of AMs

In order to harvest AMs, bronchoalveolar lavage (BAL) was performed as described elsewhere (Betsuyaku et al. 1999) in the young and old mice (aged 22-weeks and 86–96 weeks, respectively). BAL fluid was centrifuged and the supernatant was removed as described above, and the pellet was resuspended in RPMI culture medium (Invitrogen, Grand Island, NY). BAL cells were incubated at 37°C in a humidified atmosphere with 5% CO_2 for 30 min in 24-well CELLSTAR tissue culture plates (5×10^4 cells/well), supernatant was removed and cells were washed. There were no contaminating neutrophils in any of the culture cell preparations.

In vitro exposure to LPS in AMs

AMs purified as described above were incubated with or without 1 μg/ml LPS at 37°C in a humidified atmosphere with 5% CO_2 for 24 h in 24-well CELLSTAR tissue culture plates. After exposure to LPS for the period indicated, cells were washed and subjected to lysis buffer for RNA extraction (Qiagen, Hilden, Germany).

Quantitative reverse transcription-polymerase chain reaction (PCR) for HO-1

Total RNA was extracted from LCM-retrieved bronchiolar epithelial cells, whole lung homogenates, or AMs using an RNeasy Mini kit (Qiagen). Quantity and quality of RNA were determined using a NanoDrop spectrophotometer (NanoDrop Inc., Wilmington, DE). RNA was reverse transcribed using the TaqMan Reverse Transcription Reagents kit (Applied Biosystems, Foster City, CA). The resulting first-strand cDNAs were used as templates for quantitative real-time RT-PCR using the ABI Prism 7700 Sequence Detector (Applied Biosystems) and gene-specific TaqMan Gene Expression Assays probes (Applied Biosystems), as described previously (Ito et al. 2005). Probes for mouse HO-1 (Assay ID: Mm 00516007_m1) were derived from the boundary of exons 4 and 5 of the mouse HO-1 gene (accession number mCG14997) (Applied Biosystems). Relative amounts of target mRNA in the samples was assessed by interpolation of threshold cycles from a standard curve. Signal levels for each gene were normalized against beta2-microglobulin (β2MG) mRNA (Assay ID: Mm 00437764_m1). In some experiments, β-glucuronidase (BGUS) (Assay ID: Mm 00446953_m1) and hypoxanthine ribosyltransferase (HPRT) mRNA (Assay ID: Mm 00446968_m1) were also used for normalization, as described previously (Ito et al. 2007). In addition, in order to confirm the specific amplification by those probes and primers used for the quantitative real-time RT-PCR, the PCR products

after 40 cycles for HO-1 and β2MG were separated by 2% agarose gel electrophoresis.

Western blot analysis

Protein (10 μg) was denatured and reduced in the presence of 2-mercaptoethanol, and was then electrophoresed on 12% sodium-dodecylsulphate-polyacrylamide gels using prestained molecular weight markers (Bio-Rad, Hercules, CA). Proteins were then transferred to Immun-Blot PVDF membranes (Bio-Rad, Hercules, CA), using a semi-dry transfer cell (AE-6677, ATTO, Tokyo, Japan). Nonspecific binding sites were blocked with Tris-buffered saline-containing 0.1% Tween 20 (TBS-T) and 5% nonfat dried milk for 1 h at room temperature. Membranes were incubated with rabbit polyclonal antibody against HO-1 (Stressgen, Victoria, B·C., Canada) at 1:1,000 dilution overnight at 4°C. After three washes with 0.1%TBS-T, membranes were incubated in horseradish peroxidase-conjugated anti-rabbit immunoglobulin at 1:12,500 dilution (DAKO) for 1 h at room temperature. Antibody binding was visualized using the Immobilon Western blotting detection system (Millipore Corporation, Billerica, MA). Blots were scanned with a GT-9500 scanner (Epson, Nagano, Japan); the intensity of the bands was calculated using NIH Image software (version 1.62).

Statistical analysis

Data are presented as means ± standard error of the mean (SEM). More than two groups were compared using the Kruskal–Wallis test followed by the Mann–Whitney *U* test. Differences were analyzed using unpaired *t*-test for in vivo study between the two age groups. Paired *t*-test was used for the in vitro study to compare samples before and after LPS treatment. Statistical significance was set at $P < 0.05$.

Results

HO-1 mRNA levels in the lungs of various ages

First, in order to assess whether physiological aging affect the levels of HO-1 mRNA expression in the lungs, we quantified the HO-1 expression in steady-state the lungs at 9, 45, 66 and 75 weeks of age. It has not been elucidated which genes are the most appropriate internal control for lung cells during the aging process; thus we performed the analyses using different house keeping genes to confirm our findings. There were no significant differences in the expression of HO-1 mRNA with age, regardless of housekeeping genes used for normalization, including β2-MG, BGUS and HPRT (Fig. 1a–c). Therefore, β2-MG was selected for subsequent experiments.

Expression of HO-1 at mRNA and protein levels in LPS-induced lung injury model

We then examined age-related differences in the regulation of HO-1 in response to LPS in the lungs. As was previously reported (Camhi et al. 1995), expression of HO-1 was upregulated in the lungs in response to LPS. It should be noted that expression of HO-1 in lungs from 65–66-week-old mice was significantly lower than in 9–11-week-old mice at 2 h (1.3 ± 0.3 standard error (SE) vs. 2.5 ± 0.3, $P = 0.0247$) and at 24 h (3.3 ± 0.8SE vs. 10.2 ± 0.9, $P = 0.0003$), however, there were no differences in HO-1 expression at 72 h between the two age groups (Fig. 2a). The PCR products for HO-1 or β2-MG were shown as a single band at predicted size, respectively. However, it should be noted that the amplification reaches to plateau, there is no obvious difference in the band intensities between the samples applied for the gel analysis (Fig. 2b).

Western blot analysis confirmed a band at ~32 kDa corresponding to HO-1 in lung homogenates. As shown in Fig. 2c, d, HO-1 bands were weaker in the lungs from 65–66-week-old mice as compared to those from 9–11-week-old mice at 24 h after LPS instillation (0.5 ± 0.3SE vs. 1.0 ± 0.1, $P = 0.0350$) (Fig. 2c, d).

Expression of HO-1 mRNA in bronchiolar epithelial cells

We next focused on bronchiolar epithelial cells as a potential source of HO-1 in the lungs. We first quantified HO-1 mRNA expression in the bronchiolar epithelial cells harvested by LCM after intratracheal LPS instillation in the 9–11-week-old mice. Expression of HO-1 in bronchiolar epithelial cells was significantly upregulated at 2 h when compared to untreated mice (2.6 ± 0.5SE vs. 1.0 ± 0.1, $P = 0.0147$), while

1996; Villar et al. 1994). Several studies have demonstrated age-related deficiencies in HO-1 response against stress in brain and liver in animal models. Patriarca et al. reported that young rats (aged 2.5 month) respond to acute ethanol intoxication by displaying increased expression of liver HO-1 mRNA, while 18-month-old rats do not show any response (Patriarca et al. 2007). The ability of 20-month-old rat brain tissue to respond to hypoxic/hyperthermia was compromised at the HO-1 protein and mRNA levels when compared to 2-month-old rats (Ewing and Maines 2006). We demonstrated the impaired upregulation of HO-1 expression in aged mice at 2 and 24 h after LPS administration, whereas no difference was observed at 72 h. Accordingly, aging may have an impaired capacity for transcriptional upregulation of HO-1 in early phase in response to stress in the lungs.

In this study, we further investigated whether bronchiolar epithelial cells in the lungs are responsible for the age-related changes in the expression of HO-1 in response to LPS. In bronchiolar epithelial cells, HO-1 was rapidly upregulated after LPS administration, and returned to near baseline levels at 24 h. When we compared bronchiolar HO-1 expression at 2 h between the young and the old mice, no differences were observed with age. Because the bronchiolar HO-1 upregulation is not retained for 24 h, bronchiolar epithelial cells are not likely to be a particular cellular source of increased HO-1 expression at 24 h in the whole lung homogenates, suggesting that other cell source of HO-1 such as AMs, fibroblasts or endothelial cells might be responsible for the age-related difference in HO-1 expression in response to LPS in the lungs. Although we found the impaired function of AMs harvested from aged mice to upregulate HO-1 in response to LPS in vitro, we cannot imply that AMs are a responsible cell type for the age-related changes in the expression of HO-1 in the lungs in vivo.

Rushworth et al. recently demonstrated that LPS-induced HO-1 expression in human monocytic cells requires nuclear factor erythroid 2-related factor (Nrf2), a key antioxidant transcription factor (Rushworth et al. 2005). Our laboratory recently found the reduced expression of Nrf2 in AMs from aged mice in association with impaired-upregulation in several downstream antioxidant genes, including HO-1, in response to cigarette smoke extract (Suzuki et al. in press). However, the regulatory mechanism of age-dependent differences in the upregulation of HO-1 remains to be elucidated in response to LPS.

In summary, our data indicate that HO-1 expression in the lung is not altered by aging, thus suggesting that its mRNA levels are not considered as a possible biomarker of aging. However, we demonstrated age-related defects in HO-1 induction in the lungs and in AMs in response to LPS, which indicates an impaired capacity for the transcriptional response to stress in aged lungs.

Acknowledgements This research was supported by a scientific research grant from the Ministry of Education, Culture, Sports, Science and Technology of Japan (No. 19590877 to T.B.). The authors would like to thank Ms. Yoko Suzuki for her invaluable technical assistance.

References

Antonini JM, Roberts JR, Clarke RW, Yang HM, Barger MW, Mark JY et al (2001) Effect of age on respiratory defense mechanisms: pulmonary bacterial clearance in Fischer 344 rats after intratracheal instillation of Listeria monocytogenes. Chest 120:240–249. doi:10.1378/chest.120.1.240

Betsuyaku T, Shipley JM, Liu Z, Senior RM (1999) Neutrophil emigration in the lungs, peritoneum, and skin does not require gelatinase B. Am J Respir Cell Mol Biol 20:1303–1309

Betsuyaku T, Griffin GL, Watson MA, Senior RM (2001) Laser capture microdissection and real-time reverse transcriptase/polymerase chain reaction of bronchiolar epithelium after bleomycin. Am J Respir Cell Mol Biol 25:278–284

Camhi SL, Alam J, Otterbein L, Sylvester SL, Choi AM (1995) Induction of heme oxygenase-1 gene expression by lipopolysaccharide is mediated by AP-1 activation. Am J Respir Cell Mol Biol 13:387–398

Choi AM, Alam J (1996) Heme oxygenase-1: function, regulation, and implication of a novel stress-inducible protein in oxidant-induced lung injury. Am J Respir Cell Mol Biol 15:9–19

Dolinay T, Szilasi M, Liu M, Choi AM (2004) Inhaled carbon monoxide confers antiinflammatory effects against ventilator-induced lung injury. Am J Respir Crit Care Med 170:613–620. doi:10.1164/rccm.200401-023OC

Donnelly LE, Barnes PJ (2001) Expression of heme oxygenase in human airway epithelial cells. Am J Respir Cell Mol Biol 24:295–303

Ely EW, Wheeler AP, Thompson BT, Ancukiewicz M, Steinberg KP, Bernard GR (2002) Recovery rate and prognosis in older persons who develop acute lung injury and the acute respiratory distress syndrome. Ann Intern Med 136:25–36

Ewing JF, Maines MD (2006) Regulation and expression of heme oxygenase enzymes in aged-rat brain: age related