Original article

Potential roles of pentosidine in age-related and disease-related impairment of pulmonary functions in patients with asthma

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Background: Pentosidine is well established as an intermolecular cross-linking type of advanced glycation end products, and it accumulates with aging in various connective tissues.

Objective: To determine whether pentosidine contributes to age-related and disease-related impairment of pulmonary functions in patients with asthma.

Methods: We measured pentosidine levels in induced sputum from young to elderly patients with asthma and assessed the slope of the nitrogen (N_2) alveolar plateau (delta N_2), closing volume (CV), and closing capacity (CC) from a nitrogen washout curve in a single breath.

Results: Pentosidine levels in induced sputum were significantly higher in patients with asthma than in normal controls (patients with asthma: median, 20.1, interquartile range, 16.7-26.5 ng/mL; normal controls: median, 3.0, interquartile range, 0.7-7.5 ng/mL; $P < .001$). The levels were closely correlated with age in both normal controls and patients with asthma. However, the slope of age-related increase in pentosidine levels was markedly steeper in patients with asthma than in normal controls. CV/vital capacity, CC/total lung capacity, and delta N_2 increased with aging in both normal controls and patients with asthma. Moreover, in each range of age (21-40, 41-60, 61-80 years), CV/vital capacity, CC/total lung capacity, and delta N_2 were significantly higher in patients with asthma than in normal controls. In addition, pentosidine levels in patients with asthma were closely correlated with each of these variables.

Conclusion: Our results demonstrated the association between sputum levels of pentosidine and age-related small airways function in both normal controls and patients with asthma. Moreover, the age-related increase in pentosidine levels was more pronounced in patients with asthma. These findings will herald new era in the pathophysiology of elderly asthma. (J Allergy Clin Immunol 2010;■■■:■■■-■■■.)

Key words: *Pentosidine, elderly asthma, lung elasticity, small airways, aging*

Asthma is a chronic airway inflammatory disease characterized by variable airflow obstruction and airway hyperresponsiveness attributed to an underlying inflammatory process and airway structural changes.[1] The structural alterations of airway walls in asthma include epithelial desquamation, goblet cell hyperplasia, thickening of the reticular basement membrane, smooth muscle hypertrophy/hyperplasia, and the growth and proliferation of new blood vessels.[2] These structural changes are referred to as *airway remodeling* and potentially lead to irreversible airflow obstruction. Indeed, a subgroup of patients with asthma has seemingly irreversible airflow obstruction despite aggressive treatment.[3] In this regard, previous studies determined that reduced lung elasticity is primarily responsible for the development of irreversible airflow obstruction in chronic persistent asthma.[4,5] Thus, it is easily conceivable that the relative contribution of reduced lung elasticity to irreversible airflow obstruction is of high importance. Reduced lung elasticity was also reported in stable, asymptomatic patients with asthma as well as in patients with severe exacerbations of the disease.[6] Moreover, it is also reported that even patients with asthma with no actual airway obstruction have reduced lung elasticity.[7] Therefore, it is likely that loss of lung elasticity may occur after the onset of asthma and lead to irreversible airflow obstruction over many years. In addition, reduced lung elasticity allows small airway closure or near closure, thereby precipitating airway obstruction in asthma.[8] These findings suggest that intrinsic airway obstruction, loss of lung elasticity, or both might contribute to limiting expiratory airflow in asthma.

Asthma is commonly considered a disease of the young; however, epidemiologic evidence indicates that asthma is not rare in elderly subjects.[9] Despite the improvements in the management of asthma, elderly patients with asthma have the high rates of mortality and hospitalization among all adult patients with this disease.[10] Until recently, the potential role of reduced lung elasticity with a subsequent decline in expiratory driving pressure on airflow obstruction has been largely neglected in elderly patients with asthma. The aging lung may undergo various degrees of change in airway structures and functions. These age-related changes are mainly derived from loss of lung elasticity, suggesting that mechanical lung properties are impaired with aging.[11] Thus, age-related loss of lung elasticity is a limiting factor for the decrease in expiratory airway caliber in elderly patients with asthma. Despite our inability to explain completely the loss of lung elasticity in elderly patients with asthma, its high incidence and contributory

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Abbreviations used
CC: Closing capacity
CV: Closing volume
Delta N_2: Slope of the nitrogen alveolar plateau
$FEF_{25–75}$: Forced expiratory flow between 25% and 75% of forced viral capacity
FVC: Forced viral capacity
IQR: Interquartile range
NO: Nitric oxide
RV: Residual volume
TLC: Total lung capacity
VC: Vital capacity

role on airflow obstruction in these patients suggest a greater role than previously appreciated.

It is known that the extracellular matrix undergoes progressive changes during senescence. These changes particularly affect collagen-rich tissues like arteries and lungs and are thought to result from the formation of age-related intermolecular cross-linking of the extracellular matrix.[12] Therefore, elucidation of the nature of extracellular cross-linking in aging is of both clinical and theoretical interest. Pentosidine is well established as an intermolecular cross-linking type of advanced glycation end products, and it accumulates with aging in various connective tissues.[13] In animal and human studies, pentosidine has been found to be responsible for collagen cross-linking in lung tissues and increased with aging,[14,15] suggesting that high levels of pentosidine might explain the age-associated loss of lung elasticity. Thus, it is plausible that pentosidine is of significance for pulmonary gerontologic research. Therefore, we hypothesized that pentosidine contributes to the age-related loss of lung elasticity by intermolecular cross-linking of the extracellular matrix in asthma. To test our hypothesis, we measured pentosidine levels in induced sputum from young to elderly patients with asthma and examined the potential mechanisms of reduced lung elasticity in elderly patients with asthma.

METHODS

Subjects

Nonsmoking subjects with clinically stable asthma, age 21 to 80 years, receiving inhaled corticosteroids (800 μg/d beclomethasone equivalent) for more than 12 months were recruited to participate in this study. Exclusion criteria included current smokers or exsmokers with more than a 5 pack-year history, body mass index >25 $kg \cdot m^{-2}$, clinically significant medical disorders except for asthma, concurrent use of certain respiratory medications (long-acting β_2-agonists, leukotriene modifiers, and systemic corticosteroids), and recent asthma exacerbation or respiratory tract infection (within 4 weeks). Inhaled short-acting β_2-agonists were withdrawn for at least 24 hours before the pulmonary function test. All normal controls were healthy, lifelong nonsmoking volunteers who had no history of lung disease. We randomly selected 10 to 20 subjects from each 20-year range (21-40, 41-60, 61-80 years) among both patients with asthma and normal controls. Methacholine inhalation challenge testing was performed for all study subjects. Exhaled nitric oxide (NO) levels were also measured for all subjects with a chemiluminescence analyzer with a resolution of 1 parts per billion, in accordance with American Thoracic Society standards.[16] All subjects gave their written informed consent for participation in this study, which was approved by the Ethics Committee of Osaka City University, Japan.

Sputum induction and processing

Sputum induction was performed as we previously described.[17] In this study, sputum induction was safely performed even in elderly patients with asthma. The volume of sputum samples was measured, and the sample was diluted with phosphate-buffered solution containing dithiothreitol (final concentration, 1 mmol/L; WAKO Pure Chemical Industries Ltd, Osaka, Japan) and gently vortexed at room temperature for 20 minutes. After centrifugation at 400*g* for 10 minutes, the cell pellet was resuspended. The slides were prepared by using a cytospin (Cytospin 3; Shandon, Tokyo, Japan) and stained with May-Grunwald-Giemsa stain for differential cell counts. Pentosidine levels in induced sputum were measured by using commercial ELISA kits (Fushimi Pharmaceutical Co, Marugame, Japan). We preliminarily examined reproducibility and found no detrimental effects of dithiothreitol on measurement of pentosidine levels in induced sputum. In this assay system, the limit of detection for pentosidine was 0.5 ng/mL.

Pulmonary function test

All subjects underwent pulmonary function tests just before sputum induction by using a CHESTAC-8800 unit (Chest, Tokyo, Japan) according to the recommendations of the American Thoracic Society/European Respiratory Society.[18,19] Forced vital capacity (FVC), FEV_1, and forced expiratory flow between 25% and 75% of FVC ($FEF_{25–75}$) were examined. The best of 3 consecutive attempts at spirometric measurements was recorded. Total lung capacity (TLC) and residual volume (RV) were determined by the helium gas dilution method. Small airways function was assessed by the single breath nitrogen test using a dry rolling seal spirometer filled with 100% oxygen and equipped with a nitrogen meter connected to the mouthpiece, allowing continuous sampling.[20] During the test, each patient performed a slow, full inspiratory and expiratory vital capacity (VC) maneuver at a flow rate of 0.5 L/s. The constant flow was ensured by a mechanical flow regulator. The expiratory nitrogen concentration ($\%N_2$) was plotted against lung volume (between TLC and RV), producing the expiratory nitrogen washout curve. The slope of the nitrogen alveolar plateau (delta N_2, phase III) was calculated by drawing the best fit line through phase III of the expiratory volume-concentration curve and was expressed as $\%N_2$/L. One expert operator, who was unaware of the subjects' characteristics, manually drew all lines. This procedure has been validated in our laboratory, showing good within-observer and between-observer reproducibility in the slope of phase III and in closing volume (CV). The first departure from this straight line was considered as indicative of airway closure, allowing the calculation of CV (phase IV) and closing capacity (CC = RV + CV). The measurements were accepted only if the VC during the single-breath nitrogen test was within a 10% range of VC measured by spirometric testing. CV and CC were expressed as the ratio of VC (CV/VC) and TLC (CC/TLC), respectively.

Statistical analysis

All data are presented as median and interquartile range (IQR). When multiple comparisons of nonparametric data were performed between groups, significant intergroup variability was first established by using the Kruskal-Wallis test. A Mann-Whitney *U* test was then used for intergroup comparisons. Differences between the slopes of the age-related increase in pentosidine levels were analyzed by using analysis of covariance. The significance of correlation was evaluated by determining Spearman rank correlation coefficients. A *P* value <.05 was considered significant.

RESULTS

Comparisons of pentosidine levels between normal controls and patients with asthma

The clinical characteristics of the 51 patients with asthma and 30 normal controls are shown in Table I. The 2 groups were well matched for age and body mass index. However, the patients with asthma in this study had greater airflow obstruction and exhibited airway hyperresponsiveness to methacholine. Pentosidine levels in induced sputum were significantly higher in patients with asthma than in normal controls (patients with asthma: median, 20.1 [IQR, 16.7-26.5] ng/mL; normal controls: 3.0 [0.7-7.5] ng/mL; $P < .001$;

TABLE I. Clinical characteristics of study subjects

	Normal controls	Patients with asthma
Subject no. (male/female)	30 (12/18)	51 (22/29)
Age (y)	46 (34-62)	51 (36-66)
BMI	21.9 (20.9-23.7)	23.0 (21.6-23.8)
FEV_1 (% predicted)	106 (98-114)	75 (69-81)*
FEV_1/FVC (%)	86 (80-89)	71 (65-75)*
$FEF_{25–75}$ (% predicted)	86 (82-91)	72 (55-80)*
PC_{20} methacholine (mg/mL)	>10	5.8 (5.0-6.7)*
Sputum eosinophils (%)	0.1 (0-0.3)	1.1 (0.4-1.5)*
Exhaled NO (ppb)	15.3 (13.9-16.8)	21.0 (18.2-24.7)*

BMI, Body mass index.
All values are median (IQR) unless otherwise noted.
*$P < .01$ compared with normal controls.

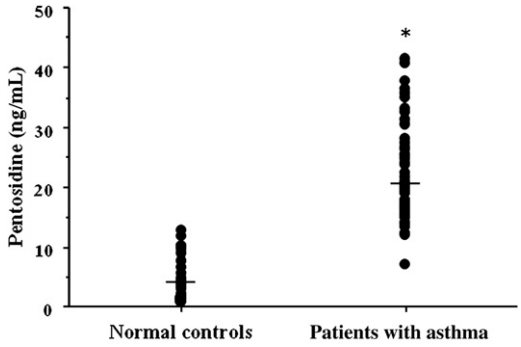

FIG 1. Comparison of pentosidine levels in induced sputum from normal controls and patients with asthma. Each bar represents the median value. *$P < .01$ compared with normal controls.

Fig 1). Similarly, levels of exhaled NO and sputum eosinophils were also significantly higher in patients with asthma than in normal controls. However, in patients with asthma, pentosidine levels in induced sputum were not significantly correlated with levels of exhaled NO, sputum eosinophils, and airway hyperresponsiveness to methacholine (Fig 2). Moreover, pentosidine levels were not significantly correlated with values of FEV_1/FVC (Fig 3). In contrast, we found a significant correlation between pentosidine level and $FEF_{25–75}$.

Role of pentosidine on age-related changes in pulmonary functions

In normal controls, pentosidine levels in induced sputum increased with aging (21-40 years: 0 [0-0.7] ng/mL; 41-60 years: 3.3 [2.0-4.6] ng/mL; 61-80 years: 10.0 [8.2-10.6] ng/mL). Moreover, levels in normal controls were closely correlated with age ($r = 0.90$; $P < .001$; Fig 4). Similarly, pentosidine levels in patients with asthma increased with aging (21-40 years: 16.0 [13.8-18.3] ng/mL; 41-60 years: 19.7 [16.9-25.0] ng/mL; 61-80 years: 28.0 [23.1-35.2] ng/mL) and were significantly higher than those in normal controls in each range of age ($P < .001$). Moreover, levels in patients with asthma were also significantly correlated with age ($r = 0.82$; $P < .001$). Pentosidine levels in elderly control subjects were relatively lower than those in young patients with asthma. In addition, the slope of age-related increase in pentosidine level was markedly steeper in patients with asthma than in normal controls (slope in patients with asthma, 0.4, vs in normal controls, 0.2; $P = .004$).

Role of pentosidine in disease-related changes in pulmonary functions

In normal controls, FEV_1/FVC and $FEF_{25–75}$ did not significantly change with aging (Table II). In patients with asthma, there was also no significant change in FEV_1/FVC between young and elderly patients with asthma. However, $FEF_{25–75}$ was significantly lower in elderly (61-80 years) than in young (21-40 years) subjects ($P = .02$). On the other hand, CV/VC, CC/TLC, and delta N_2 increased with aging in both normal controls and patients with asthma. Moreover, in each range of age, CV/VC, CC/TLC, and delta N_2 were significantly higher in patients with asthma than in normal controls. Pentosidine levels in patients with asthma were closely correlated with each of these variables (CV/VC: $r = 0.73$, $P < .001$; CC/TLC: $r = 0.78$, $P < .001$; delta N_2: $r = 0.79$, $P < .001$; Fig 5).

DISCUSSION

In this study, we selected clinically stable patients with asthma from each 20-year range (20-40, 41-60, 61-80 years) who had already been receiving inhaled corticosteroid therapy for more than 12 months. Therefore, exhaled NO concentrations and percentages of eosinophils in induced sputum were considerably low, but airway inflammation could not be completely inhibited despite long-term of inhaled corticosteroid therapy. Our novel finding in this study is that pentosidine levels in induced sputum were significantly higher in patients with asthma than in normal controls. However, pentosidine level in patients with asthma was not significantly correlated with level of exhaled NO or sputum eosinophils, indicating that pentosidine levels in asthmatic airways were not directly associated with the magnitude of airway inflammation.

Moreover, levels were not significantly correlated with FEV_1/FVC or airway hyperresponsiveness to methacholine. These findings appear consistent with the concept that pentosidine levels are not reflective of other specific perturbations related to asthmatic airway disease *per se*. In contrast, levels were significantly correlated with $FEF_{25–75}$, an index of small airways function. This fact is responsible for the possible role of pentosidine on functional impairment of small airways. Interestingly, we found that pentosidine levels in induced sputum increased with aging in subjects with and without asthma. Nevertheless, pentosidine levels in young patients with asthma were higher than those in elderly control subjects. In addition, its levels in each range of age were significantly higher in patients with asthma than in normal controls, and the slope of age-related increase in airway pentosidine levels was steeper in patients with asthma. These findings suggest that pentosidine in the airways is a novel biomarker of airway senescence in human subjects and that asthma itself might lead to accelerate the rate of progression of airway senescence.

Nitrogen washout can distinguish between ventilation inhomogeneity originating in the peripheral airways versus the central airways.[21] Analysis of nitrogen washout curve generated in the single-breath or multiple-breath tests can actually provide information regarding distal lung abnormalities. In the single-breath test in particular, increased CV or CC and increase in the phase III slope of the washout curve (delta N_2) reflect air trapping because of small

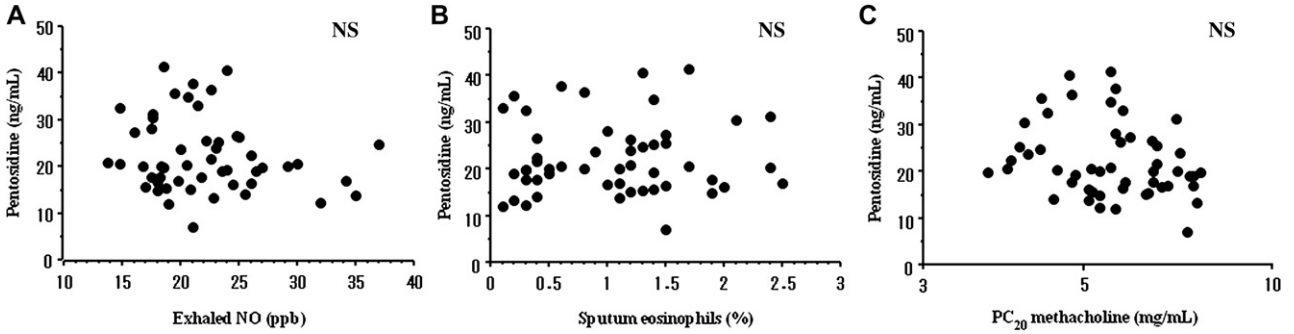

FIG 2. Correlations between pentosidine levels in induced sputum and exhaled NO **(A)**, sputum eosinophils **(B)**, PC_{20} methacholine **(C)** in patients with asthma. *NS*, Not significant.

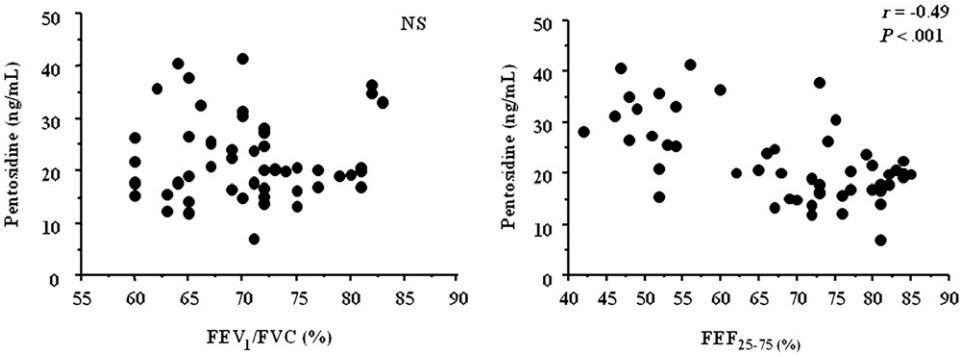

FIG 3. Correlations between pentosidine levels in induced sputum and FEV_1/FVC *(left)* and $FEF_{25–75}$ *(right)* in patients with asthma. *NS*, Not significant.

airway closure or near-closure. Previous studies have suggested that increased airway closure in asthma is an important marker of disease severity.[22] Although airway closure is difficult to measure directly, it has been estimated indirectly using a range of physiologic techniques such as nitrogen washout.[23] In this study, we found that increase in the delta N_2 could be observed with aging, and that values of delta N_2 in each range of age were significantly higher in patients with asthma than in normal controls. Moreover, pentosidine levels in induced sputum were closely correlated with value of delta N_2 in patients with asthma. These findings suggest that high levels of pentosidine might be associated with small airway closure or near closure, eventually leading to expiratory fixed airway obstruction in patients with asthma.

Although physiologic consequences of reduced lung elasticity result in premature airway closure and fixed expiratory airway obstruction, the mechanisms of loss of lung elasticity remain elusive. The outward manifestations of tissue aging in the elderly primarily involve the 2 major structural proteins, collagen and elastin. The changes in these proteins are associated with intermolecular cross-linking. Intermolecular cross-linking involves 2 different mechanisms, a precise enzyme process during development and maturation, and a subsequent nonenzymatic adventitious reaction with glucose during aging.[24] The latter glycation reactions are the major cause of tissue dysfunction in the elderly as a result of intermolecular cross-linking, which impairs functions of the heart, vascular system, and lungs, mainly by altering their elasticity.[25] In fact, previous studies reported that the marked loss of lung elasticity in patients with asthma accounted for more than half of the reduction in total maximum airflow.[26,27] In this study, we found that

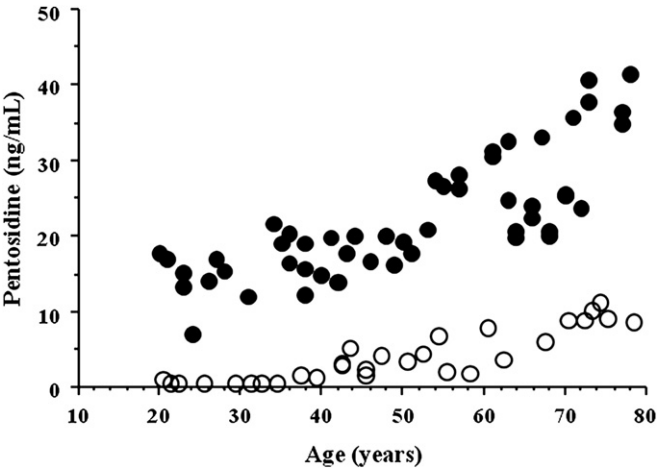

FIG 4. Correlations between pentosidine levels in induced sputum and age in normal controls *(open circles)* and patients with asthma *(closed circles)*.

pentosidine levels may be expressed, at least partially, in the rate of aging of airway extracellular matrix, and that the progressive increase in age-related impairment of pulmonary functions may be associated with loss of lung elasticity in elderly patients with asthma.

Most of the elderly patients with asthma in this study had a long duration of asthma. Furthermore, it is plausible that prolonged duration of asthma is associated with irreversible expiratory airflow limitation caused by loss of lung elasticity. Moreover, it is conceivable that chronic use of corticosteroids, particularly in the

TABLE II. Age-related changes in pulmonary functions

	Normal controls			Patients with asthma		
Range (y)	**21-40**	**41-60**	**61-80**	**21-40**	**41-60**	**61-80**
Subject no.	10	12	8	17	14	20
FEV_1/FVC (%)	85 (80-91)	86 (84-89)	81 (79-88)	67 (64-74)**	72 (68-74)**	70 (67-78)**
FEF_{2575} (% predicted)	86 (84-89)	84 (81-93)	85 (82-90)	76 (72-80)**	73 (55-82)**	63 (53-74)**
CV/VC (%)	9 (6-14)	11 (9-12)	18 (16-19)	14 (11-18)*	25 (23-29)**	36 (33-38)**
CC/TLC (%)	26 (24-28)	28 (26-34)	36 (30-39)	35 (29-36)**	37 (35-42)**	46 (43-53)**
Delta N_2 (%N_2/L)	0.9 (0.7-1.0)	1.1 (1.0-1.2)	1.5 (1.3-1.5)	1.1 (0.9-1.3)*	1.9 (1.7-2.2)**	2.5 (2.4-2.8)**

All values are median (IQR) unless otherwise noted.
*$P < .05$, **$P < .01$ compared with normal controls.

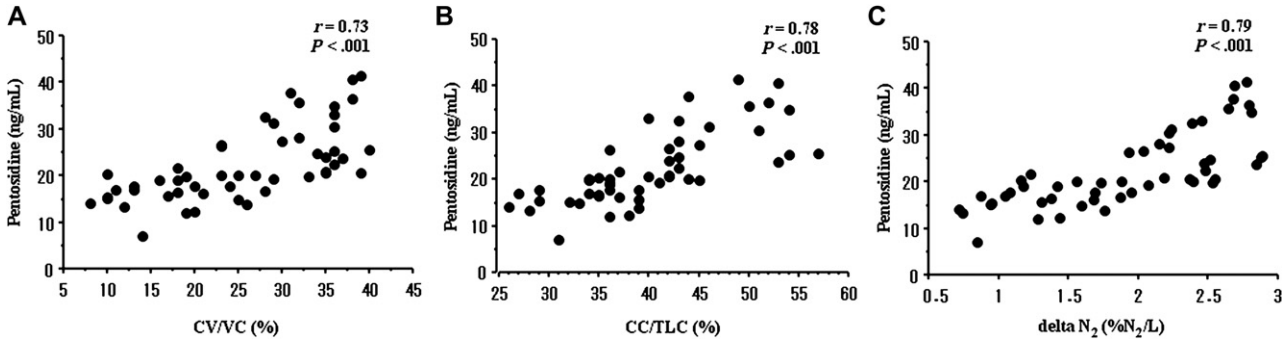

FIG 5. Correlations between pentosidine levels in induced sputum and CV/VC **(A)**, CC/TLC **(B)**, and delta N_2 **(C)** in patients with asthma.

aging subjects, may have affected lung elasticity and, therefore, our results in this study. However, previous studies reported that patients with asthma have exhibited loss of lung elasticity with age despite optimal inhaled corticosteroid therapy.[4,5] The discovery of an age-related increase in pentosidine in asthmatic airways is the first molecular evidence for the involvement of intermolecular cross-linking in airway extracellular matrix.

However, irreversible expiratory airflow limitation can also be readily explained by pathologic large and small airway remodeling that includes deposition of collagen in the subepithelial matrix. Interestingly, pentosidine may rapidly accumulate in active remodeled subepithelial matrix. In the future, we will be required to examine the differences in airway wall thickness and total amount of pentosidine accumulation in airway walls between young and elderly subjects with asthma and to clarify directly the morphology-function relationship in asthmatic airways.

Collagen and elastin are the most abundant proteins in the lung extracellular matrix. The increased pentosidine in lung collagen and elastin probably interferes with the functional properties of extracellular matrix components, not only changing their mechanical properties but also altering the normal cell-matrix interactions in the lung. The significant correlation between age and pentosidine levels in the lung suggests the implication of the intermolecular cross-linking of pulmonary connective tissue in the age-related impairment of pulmonary mechanical properties. It needs to be emphasized that our study was simultaneously performed over a range from young to elderly patients with asthma in 1 study. Our results support the hypothesis that pentosidine levels increase in the aging lung and that this increase is accelerated in the asthmatic lung in relation to altered small airways function. As in other tissues, this may be a cause of the progressive decrease in lung elasticity and, possibly, of small airways stiffness, which have been observed in the aged lung.

What are the clinical implications of our study? First, our findings underline the relevance of assessing lung elasticity even in patients with mild degrees of asthma. Second, our results support a role for a surrogate marker as an index of lung elasticity that appears to be complementary to spirometry in monitoring patients with asthma. However, the current study has several limitations. First, we have simply demonstrated the existence of an association between pentosidine levels and altered small airways function with age and asthma without investigating the nature of this association. Further studies will be required to represent directly causal relationships between them. Second, we should also consider the limitations in using pentosidine levels as a surrogate marker of small airway disorder in asthma because, as an advanced glycation end product, pentosidine can accumulate under different conditions, including in age-related degenerative and other diseases. In summary, our results will call for more studies to assess the relationship between pentosidine levels and the mechanical properties of airway parenchyma. This work will provide new insights into the mechanisms of airway senescence by demonstrating the presence of pentosidine in asthmatic airways and its increase with aging. This may be of great use in understanding the age-related impairment of pulmonary functions in patients with asthma.

Clinical implications: Our results determined that pentosidine increases in asthmatic airways with aging, and that this may be a cause of the reduced lung elasticity in elderly patients with asthma.

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