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ORIGINAL ARTICLE
Pulmonary Circulation

Maternal Outcome in Pregnancy Complicated With Pulmonary Arterial Hypertension

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Background: Pulmonary arterial hypertension (PAH), including Eisenmenger syndrome, has a risk of mortality in pregnancy of 10–40%. The aim of this study was to investigate whether pulmonary artery blood pressure (PABP) is a prognostic factor for pregnancy outcome in patients with PAH.

Methods and Results: The subjects were 42 patients with PAH during pregnancy. Severe and mild cases were defined by PABP before and during the first 14 weeks of pregnancy, with severe cases having mean PABP >40 mmHg by catheterization or systolic PABP >50 mmHg on echocardiography. Eighteen women chose termination of pregnancy before 14 weeks, leaving 24 women (10 mild, 14 severe) for analysis. The women with severe PAH delivered earlier (35.4 vs. 31.5 weeks, P<0.05) and had higher rates of small-for-gestational-age infants (0/10 vs. 7/14, P<0.01). Among the women with severe PAH, the New York Heart Association class dropped by 1 in 9 cases, by 2 in 3 cases, and remained the same in 2 cases as pregnancy progressed, whereas among the women with mild PAH, the class dropped by 1 in 1 case and 9 women remained in the same class. Among the severe cases, 1 woman died and there was 1 fetal death; PABP markedly increased in later pregnancy from 54 to 74 mmHg (catheter measurement) and from 78 to 93 mmHg (echocardiography) (P<0.05).

Conclusions: The level of PABP before or in the early stage of pregnancy is an important predictor of pregnancy outcome. (*Circ J* 2012; **76:** 2249–2254)

Key Words: Eisenmenger syndrome; Pregnancy; Pulmonary arterial hypertension

Pulmonary arterial hypertension (PAH) is a complex disorder in which pulmonary arterial obstruction leads to elevated pulmonary arterial resistance and right ventricular failure.[1–4] Elevation of the pulmonary arterial pressure correlates with progressive damage to the pulmonary artery.[3,4] Before the development of surgical treatment for ventricular septal defect, atrial septal defect and patent ductus arteriosus, most patients died around the age 40, with right-sided cardiac failure being the main cause of death.[5–7] Treatment with drugs such as epoprostenol, sildenafil, and bosentan causes vasodilatation of the pulmonary vasculature, which reduces pulmonary resistance and allows survival until about 60 years of age, [8–12] and lung transplantation can also increase survival.[13,14]

Pregnancy is strongly associated with life-threatening problems in patients with PAH. The risk of cardiac failure during and after pregnancy increases and sudden cardiac arrest may occur during cesarean section or soon after birth.[15–18] The rate of maternal death in pregnancies complicated by PAH is variouslyreported to be 20–60%.[18–21] Predictors of cardiac failure during pregnancy are elevated pulmonary arterial blood pressure (PABP),[22,23] elevated level of brain natriuretic peptide,[24,25] and increased size of the right ventricle.[26,27] There may also be a genetic predisposition.[28,29] Elliot et al. reported that pregnancy in women with PAH seems to be relatively safe up to a PABP of approximately 40 mmHg.[30] However, Bédard et al found that even patients with mild PAH can develop cardiac failure or die postpartum (within 3 months after delivery) in up to 30% of cases.[31]

Most reports of PAH in pregnancy have only examined PABP pre-pregnancy and do not mention changes in New York Heart

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Table 1. Baseline Demographic and Obstetric Characteristics of Patients With PAH

	Mild PAH (n=14)	Severe PAH (n=28)	P value
Maternal age (years)	29.5±3.5	30.1±4.0	NS
Nulli/multiparous	8/6	15/13	NS
Miscarriage/delivered	4/10	14/14	NS
Week of delivery*	36.4±4.0	31.4±2.8	<0.005
Birth weight (g)*	2543±350	1464±290	<0.005
SGA*	0	8	<0.05
Delivery mode*			<0.05
Vaginal	6	2	
Cesarean section	4	12	
Regional/general anesthesia	0/4	0/12	NS
BMI	21.2±1.5	22.1±1.8	NS
DM	1	3	NS
Hypertension	2	3	NS
Smoking	1	2	NS

*Only for delivery cases: mild group (n=10), severe group (n=14).
P<0.05 indicates a significant difference. Maternal age, week of delivery, birth weight, and BMI are shown as mean± SD and were analyzed by Student's t-test. Other data were analyzed by chi-square test and Fisher exact test.
PAH, pulmonary arterial hypertension; NS, not significant; SGA, small for gestational age; BMI, body mass index; DM, diabetes mellitus.

Table 2. Underlying Diseases in Patients With Mild and Severe PAH

Category	Mild PAH (n=14)		Severe PAH (n=28)	
	Miscarriage (n=4)	Delivered (n=10)	Miscarriage (n=14)	Delivered (n=14)
IPAH	2	–	2	3
Congenital heart disease	2	8	1	6
ASD (pre/post-op)	1 (0/1)	3 (1/2)	1 (0/1)	1 (0/1)
VSD (pre/post-op)	0	3 (1/2)	0	3 (2/1)
PDA (pre/post-op)	1 (0/1)	1 (1/0)	0	2 (0/2)
ECD (pre/post-op)	0	1 (0/1)	0	0
Eisenmenger syndrome	–	–	10*	4*
ASD			3	0
VSD	–	–	5	3
PDA			2	1
Collagen disease	–	2	–	–
Other	–	–	1	1

Data were analyzed by chi-square test and Fisher's exact test. *P<0.05.
PAH, pulmonary arterial hypertension; IPAH, idiopathic PAH; ASD, atrial septal defect; pre/post-op, pre/post operation; VSD, ventricular septal defect; PDA, patent ductus arteriosus; ECD, endocardial cushion defect.

Association (NYHA) classification or PABP during pregnancy or postpartum. Furthermore, there are no reports of the effects of PABP and maternal cardiac performance in pregnant Japanese women, and fetal growth has not been well studied. Therefore, we investigated the relationship of PABP before and during pregnancy to subsequent maternal cardiac function and neonatal outcome.

Methods

To study mortality and morbidity in maternal outcomes following PAH, we examined the charts of 42 pregnant women with PAH from January 1982 to December 2007. Cardiac function was evaluated using right-sided pulmonary catheterization and echocardiography, although in some cases of mild PAH only echocardiography was used. In the middle of the pregnancy, echocardiography was mainly used for the evaluation of PAH. The patients were divided into mild cases (systolic PABP ≥30 and <50 mmHg on echocardiography[3,3] or mean PABP ≥25 and <40 mmHg by catheterization[3,3]) and severe cases (systolic PABP ≥50 mmHg on echocardiography or mean PABP ≥40 mmHg by catheterization). Cardiac function was evaluated during pregnancy and after delivery. Some women chose early termination of pregnancy to avoid risk. Vaginal delivery was attempted for women with spontaneous labor, whereas cesarean section was selected for those with a need for early delivery because of an immature cervix. The NYHA classification was used to evaluate cardiac status.[3,4]

Data Collection

Data were collected for family history (sudden death, PAH), maternal age, height, body weight, parity, presence of hypertension, diabetes mellitus, change in PABP during and after pregnancy, right and left ventricular function, delivery mode (cesarean section or vaginal delivery), time of delivery (gestational week), and birth weight.

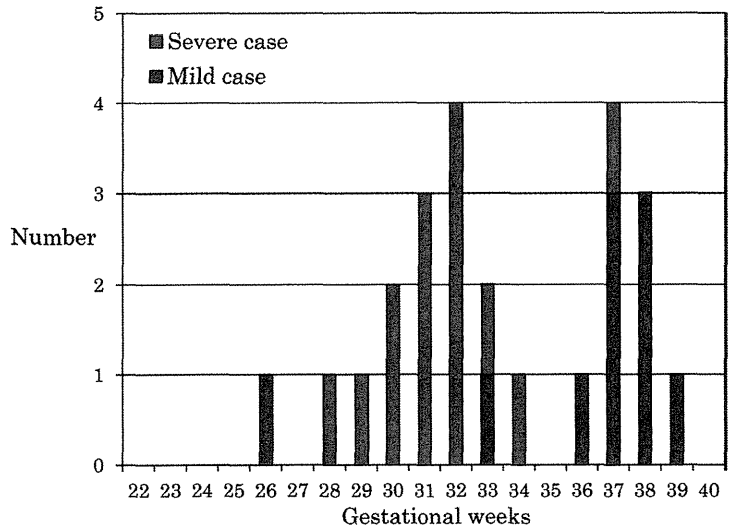

Figure 1. Week of delivery in pregnancies complicated with pulmonary arterial hypertension (PAH). Patients with mild PAH mostly delivered at term, whereas those with severe PAH had earlier deliveries.

Statistical Analysis

For continuous variables, a Student t-test was performed for analysis of normally distributed data, otherwise a Wilcoxon test was used. Chi-square test and Fisher's exact test were performed to compare categorical variables between the mild and severe cases. All statistical analyses were performed using JMP 7 (SAS Institute, Cary, NC, USA). $P<0.05$ was considered statistically significant.

Results

The baseline clinical and obstetrical characteristics of the 42 subjects are shown in Table 1. Overall, 42 cases of pregnancy complicated with PAH were analyzed, including 14 mild cases and 28 severe cases. Of the 42 patients, 18 (mild 4, severe 14) selected termination of pregnancy, and 24 (mild 10, severe 14) selected to continue after counseling. The number of patients in each PAH category is shown in Table 2.

Idiopathic PAH

There were 3 cases of severe idiopathic PAH. The maternal ages were 30, 38, and 20 years. All were referred because of exacerbated exertional fatigue, dyspnea, and pretibial edema at 25–30 weeks gestational age. On admission, the patients' respective PaO_2 level was 75, 66, and 86 mmHg; PABP was 72/30, 61/31, and 82/42 mmHg; and NYHA class was IV, IV, and III. Delivery by cesarean section was performed at 32, 28, and 32 weeks' gestation under general anesthesia with continuous Swan-Ganz catheter and systemic BP (via a radial arterial line) monitoring. Percutaneous cardiopulmonary support (PCPS) was ready in each case for use in an emergency. In the first case (in 1985), the mother died intraoperatively. Emergency cesarean section had been planned because of an abnormal fetal heart rate pattern, but the mother died of hypotension soon after intubation, despite attempts at resuscitation including PCPS. In the other two cases, which occurred in 2000 and 2003, the women survived to leave hospital. We attribute these outcomes to improved management using continuous infusion of epoprostenol. In the 2003 case, postpartum right-sided pulmonary catheterization showed PABP of 68/32. Dobutamine hydrochloride was started at $1\,\mu g \cdot kg^{-1} \cdot min^{-1}$ for severely low cardiac function, after which subjective symptoms such as shortness of breath during walking disappeared. Epoprostenol infusion therapy was then started at $0.5\,ng \cdot kg^{-1} \cdot min^{-1}$ and gradually increased in increments of $0.5\,ng \cdot kg^{-1} \cdot min^{-1}$ twice weekly until reaching a dose of $7\,ng \cdot kg^{-1} \cdot min^{-1}$. During the course the patient felt lower jaw pain as a side effect, but this gradually disappeared. Pretibial pitting edema and PAH evaluated by echocardiography and right-heart catheterization both improved and the patient was discharged from hospital on the 12th postpartum day.

Pregnancy Outcomes for Mild and Severe Cases of PAH

Gestational length at delivery showed a bimodal distribution (Figure 1). Patients with mild PAH mostly delivered at term, whereas those with severe PAH delivered earlier. The indications for delivery in patients with severe PAH were acute dyspnea (3 cases), fatigue and cough (3 cases), elevation of PABP (6 cases), and 2 women went into labor spontaneously. The gestational age at delivery and birth weights were significantly higher in the patients with mild PAH compared with those having severe PAH: 35.4 vs. 31.5 weeks, $P<0.05$; 2,543±350 vs. 1,464±290 g, $P<0.05$; respectively. More cases of fetal restricted growth were observed among the patients with severe PAH than among the mild PAH group: 0/10 vs. 8/15, $P<0.05$. Amniotic volume was adequate in all cases examined in both groups during pregnancy.

Echocardiographic and Cardiac Catheter Data

Among the patients with severe PAH, the average PABP increased as pregnancy progressed, based on the mean PABP pre-pregnancy and in the later stage of pregnancy measured by cardiac catheter (53.5±12.3 vs. 72.8±13.3 mmHg, $P<0.05$) and echocardiography (68.2±11.1 vs. 95.8±18.5 mmHg, $P<0.05$) (Figure 2). In the women with mild cases, PABP increased as pregnancy progressed, but did not reach statistical significance (Table 3).

NYHA Class

In 7 of the 10 women with mild PAH, NYHA class I was maintained throughout pregnancy (Figure 3). In these patients, elevation of PABP was not significant during pregnancy. The remaining 3 women were already NYHA class II in the pre-pregnancy period and 2 remained in NYHA class II until the postpartum period and 1 changed to NYHA class III. Of the 14

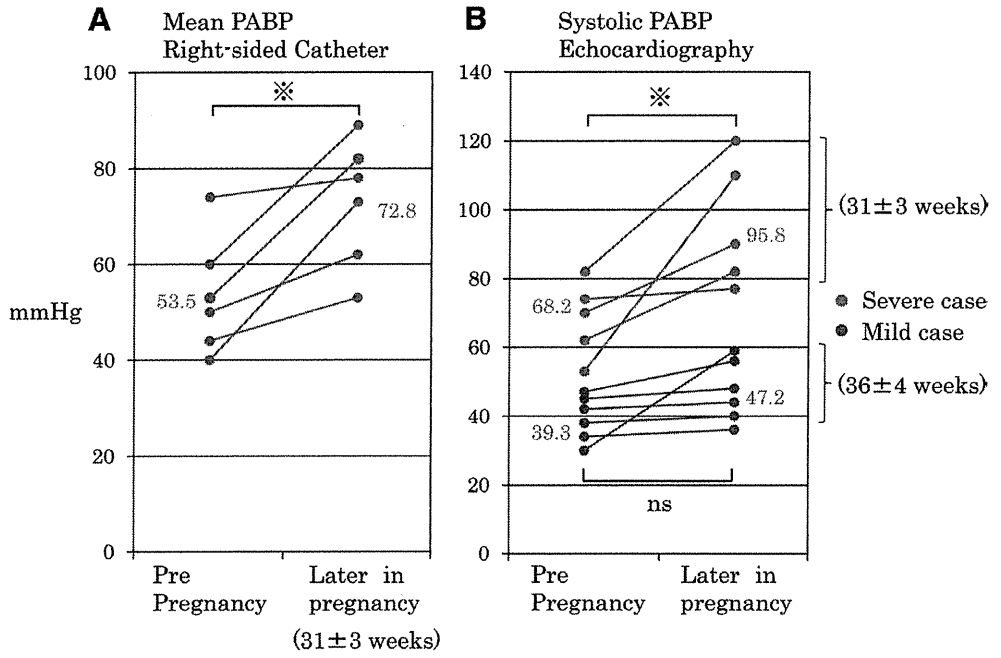

Figure 2. Changes in PABP during pregnancy. (A) Change in the mean PABP measured by a pulmonary cardiac catheter. (B) Change in the systolic PABP measured by echocardiography. Patients with severe PAH showed a significant increase in PABP later in pregnancy. PABP, pulmonary arterial blood pressure; PAH, pulmonary arterial hypertension.

Table 3. Echocardiographic Findings in Patients With Mild and Severe PAH Measured Early in Pregnancy (Except Systolic PABP)

	Mild PAH (n=14)	Severe PAH (n=28)	P value
Systolic PABP			
Pre-pregnancy	39.3±6.6	68.2±11.1	<0.05
Late-stage pregnancy	47.2±9.2	95.8±18.5	<0.05
Tricuspid valve regurgitation			
None-mild	9	8	<0.05
Moderate-severe	5	20	
LVDs	31.1±4.7	30.1±4.6	NS
Pulmonary artery valve regurgitation	2	3	NS
%FS	36.5±5.6	37.5±4.6	NS
RA cavity enlarged	2	17	<0.05
RV cavity enlarged	2	18	<0.05

LVDd, LVDs, %FS, and systolic PABP were analyzed by Student's t-test and are shown as the mean±SD. Other data were analyzed by chi-square test and Fisher's exact test. P<0.05 indicates a significant difference.
PAH, pulmonary arterial hypertension; PABP, pulmonary arterial hypertension; LVDs, left ventricular end-systolic dimension; NS, not significant; %FS, fractional shortening; RA, right atrium; RV, right ventricle; LVDd, left ventricular end-diastolic dimension.

severe cases, 1 woman was in NYHA class I, 12 women were in class II, and 1 was in class III early in pregnancy. The NYHA class worsened in all but 2 patients as pregnancy progressed. In these women the elevation of PABP was significant during pregnancy. At delivery, 1 patient died soon after intubation in the operation room, 11 were in class III, and 2 were in class IV with severe heart failure.

Discussion

We believe this is the first study in which the change in PABP was monitored during pregnancies complicated by PAH. PABP increased in the later stage of pregnancy in comparison with pre-pregnancy in patients with severe PAH, but not in those with mild PAH. PABP increased in all cases of severe PAH, from a mean of 53.5 mmHg pre-pregnancy to 72.8 mmHg in the later stage of pregnancy. Because pulmonary vascular resistance is elevated in PAH patients, pregnancy continuation may lead to right-heart failure. Circulating blood volume gradually increases by approximately 50% up to around 30 weeks of gestation, and then reaches a plateau.[35] In severe cases, this early rise leads to decompensation and the need for delivery.

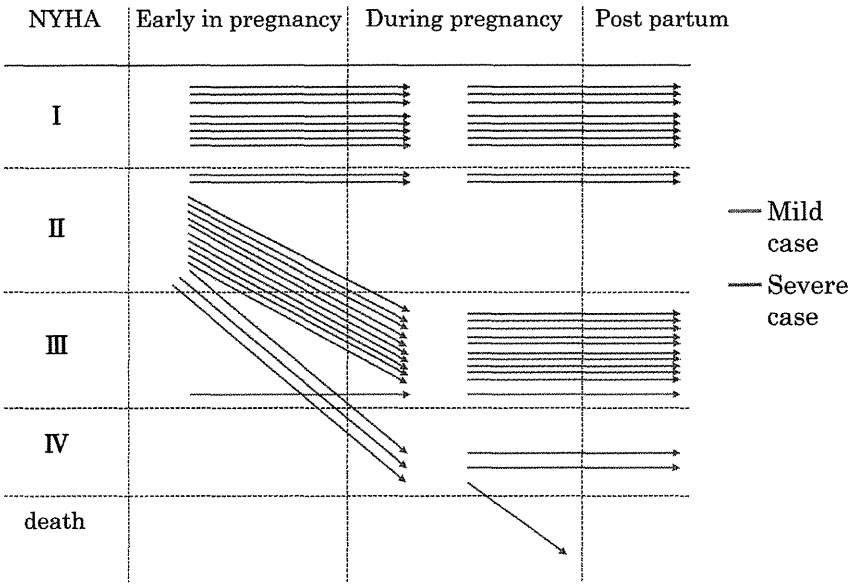

Figure 3. Changes in the New York Heart Association classification over the course of pregnancy.

The signs of decompensation are dyspnea, exertional fatigue, and pretibial edema. Perhaps surprisingly, signs and symptoms of right-heart failure, arrhythmia or angina (resulting from right ventricular ischemia) did not occur in the study subjects, although they might have done so if the pregnancies had been allowed to continue.

Although 70% of maternal deaths reported in the literature occur postpartum, there were no such deaths in our series and no deterioration of NYHA class postpartum. We attribute these improved results to 3 factors. The first is early termination of pregnancy around 30 weeks gestation in severe cases. Improvement of treatment in the NICU facilitated this decision, because all the preterm infants survived without neurological disorders, despite weighing only 1,000–1,500 g with prematurity of most organs. The second factor is the introduction of new drugs for the treatment of pulmonary hypertension, including beraprost, sildenafil, and epoprostenol; and the third is the improvement in anesthetic management. When PABP became higher than systemic BP during cesarean section, especially after removal of the placenta, the anesthetists were ready to reduce the blood volume by 100 ml in a few minutes from a Swan-Ganz catheter and use neosynesin (0.2 mg IV) to raise BP. The women with severe PAH had a higher rate of small-for-gestational-age babies compared with the women with mild cases, which was probably related to reduced cardiac output. However, some babies born to mothers with severe PAH grew adequately.

Patients with mild PAH mostly delivered at or near term, and tolerated the increased heart rate and circulating blood volume of pregnancy well. They were asymptomatic and showed no significant elevation of PABP. These findings indicate that PAH patients with mildly elevated PABP can be advised that pregnancy is appropriate. However, in 8 of 10 mild cases of PAH, the condition was associated with congenital heart disease. Thus, further studies are required to determine the safety of pregnancy for patients with mild idiopathic PAH, including analysis of the need for continuous treatment with epoprostenol (prostacyclin) or oral sildenafil. This study also indicates the significance of evaluating PAH before or in the early stage of pregnancy.

The NYHA class is used as the general standard for rating exercise tolerance in women with heart disease. One patient with severe PAH went from class I to class III during pregnancy and 15 patients with mild or severe PAH in class II pre-pregnancy went to class III during pregnancy (and 1 died), so special care has to be taken of patients who are already class II pre-pregnancy. In contrast, NYHA class I in a woman with mild PAH predicts continuation of pregnancy until term. The disease severity of the present patients may have been higher than that of general patients with PAH because the National Cerebral and Cardiovascular Center is a referral center for cardiovascular diseases. Many patients with severe PAH are referred for genetic analysis because of a family history of pulmonary hypertension. Because PAH is relatively rare, we were only able to include 42 patients in this study. The small number of subjects prevented correction of the results for the effects of potential confounding factors such as hypertension and previous obstetric history, performance of multifactorial analysis, and analysis of the effects of different etiologies of PAH (Table 2). However, measurements of the ventricles and atria, and the degree of tricuspid valve regurgitation, were better defined in the present study compared with other multicenter studies. In future work, we plan to investigate a larger cohort of patients to clarify the risk factors in female patients with PAH for cardiac dysfunction during pregnancy. The outcomes for these patients are improving because of the introduction of intravenous treatment with epoprostenol and/or oral sildenafil[36] during pregnancy. In some cases of severe PAH, use of this treatment results in PABP not increasing during pregnancy and appropriate birth weights for gestational age.

Study Limitations

The definition of PAH is a mean PABP ≥25 mmHg and diagnosis requires confirmation by right-sided catheterization. In

most cases in our study, right-heart catheterization was performed before pregnancy, but not during pregnancy provided the patient was not symptomatic, because this examination is invasive for both mother and fetus. For this reason, we are unable to show changes in PAH evaluated by right-sided catheterization, only the changes determined by echocardiography. Therefore, PABP may have been overestimated, because the mean pulmonary artery pressure has been shown to be significantly overestimated by echocardiography compared with catheterization.[37]

Conclusions

Among the cases of severe PAH in this study, PABP increased during pregnancy and there was 1 maternal death during cesarean delivery. The NYHA class in most cases of severe PAH was III or worse in later pregnancy. Early delivery was required and the rate of small-for-gestational age babies was significantly higher. Pregnancy may be safe for PAH patients with mildly elevated PABP. However, in 8 of 10 cases of mild PAH, the women had associated congenital heart disease, indicating that further studies are needed to determine the appropriateness of pregnancy in patients with idiopathic PAH, even if the condition is mild.

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Disclosure

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Clinical, Physiological and Anti-Inflammatory Effect of Montelukast in Patients with Cough Variant Asthma

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Key Words
Cough variant asthma · Leukotriene receptor antagonist · Montelukast · Induced sputum · Cysteinyl leukotrienes · Eosinophils · Cough receptor sensitivity

Abstract
Background: Cough variant asthma (CVA) is a phenotype of asthma presenting solely with coughing, characterized by airway hyperresponsiveness, eosinophilic inflammation and a cough response to bronchodilators. Leukotriene receptor antagonists (LTRAs) are antiasthma medications with anti-inflammatory and bronchodilatory properties. Although LTRAs exert antitussive effects in CVA, the mechanisms involved are unknown. ***Objectives:*** This study aimed to clarify the antitussive mechanisms of LTRAs in CVA patients. ***Methods:*** We prospectively observed the effect of montelukast (10 mg) daily for 4 weeks in 23 consecutive nonsmoking adults with anti-inflammatory treatment-naive CVA. We evaluated, before and after treatment, the cough visual analogue scale (VAS), pulmonary function (spirometry and impulse oscillation), methacholine airway responsiveness, cough receptor sensitivity, expressed by the concentration of capsaicin inducing 2 or more (C2) and 5 or more (C5) coughs, sputum eosinophil counts and levels of inflammatory mediators, including cysteinyl leukotrienes, leukotriene B_4, prostaglandin (PG) D_2, PGE_2, $PGF_{2\alpha}$ and thromboxane B_2. We compared the baseline characteristics of the patients based on the symptomatic response to montelukast, defined as a decrease in the cough VAS of >25% (n = 15) or ≤25% (n = 8). ***Results:*** Montelukast significantly decreased the cough VAS (p = 0.0008), sputum eosinophil count (p = 0.013) and cough sensitivity (C2: p = 0.007; C5: p = 0.039), whereas pulmonary function, airway responsiveness and sputum mediator levels remained unchanged. Multivariate analysis showed that a better response to montelukast was associated solely with younger age (p = 0.032). ***Conclusion:*** The antitussive effect of montelukast in CVA may be attributed to the attenuation of eosinophilic inflammation rather than its bronchodilatory properties.

Introduction

Cough variant asthma (CVA) is a variant form of asthma that presents solely with cough [1] and is one of the most common causes of chronic cough worldwide [2]. Pa-

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tients with CVA have mild airway hyperresponsiveness (AHR), as demonstrated by methacholine challenge, and bronchodilators such as inhaled β_2-agonists and/or oral sustained-release theophyllines are effective against the cough [1]. Modest but variable airflow limitation indicates that bronchial constriction is involved in the mechanism of cough in CVA [3].

Findings in induced sputum, bronchoalveolar lavage and bronchial biopsies have shown that eosinophilic inflammation is involved in CVA as well as in classic asthma with wheezing [4, 5]. Several inflammatory mediators are also implicated in CVA [6, 7]. Prostanoids such as prostaglandin (PG) E_2, $PGF_{2\alpha}$ and thromboxane (TX) A_2 modulate airway caliber [8, 9] and enhance the cough response to capsaicin [10, 11], which is produced by various cells including eosinophils [12]. Metabolites of lipoxygenase products have recently been identified as ligands of the transient receptor potential vanilloid 1 receptor, also known as the capsaicin receptor [13, 14].

Cysteinyl leukotriene (cys-LT) receptor antagonists (LTRAs) are antiasthma medications that reduce clinical symptoms and improve pulmonary function as well as airway inflammation in patients with classic asthma [15, 16]. An uncontrolled observational study has shown that the LTRA montelukast reduces airway levels of cys-LTs in association with improvements in the quality of life of patients with classic asthma already under treatment with inhaled corticosteroids (ICS) [17]. Although LTRAs exert antitussive effects in CVA [18–20], details of the mechanisms underlying these effects, including anti-inflammatory effects, remain unknown.

The present study examined the effect of montelukast on cough symptoms, pulmonary function, capsaicin cough receptor sensitivity, AHR and sputum inflammatory indices including numbers of eosinophils and levels of inflammatory mediators [cys-LTs, leukotriene B_4 (LTB_4), PGD_2, PGE_2, $PGF_{2\alpha}$ and TXB_2] in patients with CVA, in order to investigate its antitussive mechanisms.

Patients and Methods

Study Design

This was a prospective observational study. To elucidate the antitussive mechanism of montelukast, we measured, before and after treatment, cough symptoms, pulmonary function, capsaicin cough receptor sensitivity, AHR and sputum inflammatory indices including eosinophil counts and levels of mediators such as cys-LTs, LTB_4, PGD_2, PGE_2, $PGF_{2\alpha}$ and TXB_2 in patients with CVA. The study was approved by the Institutional Review Board of Kyoto University Hospital, and written informed consent was obtained from all participants.

Patients

Twenty-three consecutive patients with CVA (9 males and 14 females; mean age (SD) 46 (16) years) who were referred to the asthma and chronic cough clinic at Kyoto University Hospital were recruited between March 2002 and June 2005. The duration of cough was 3.5 (3.3) years. Nineteen of the patients had never smoked. Four had smoked less than 5 pack-years but had stopped smoking for more than 3 years before entry into the study.

CVA was diagnosed on the basis of chronic cough persisting for >8 weeks, the absence of wheezing or dyspnea, AHR to methacholine and a symptomatic response of coughing to inhaled β_2-agonists (used as needed or 4 times per day) [1, 4, 21]. Wheezing or rhonchi were not audible on chest auscultation, even with forced expiration. The patients had normal chest radiographs and no history of asthma or other respiratory diseases. No other causes of cough were present, such as gastroesophageal reflux disease or sinobronchial syndrome. None of the patients had ever been administered oral corticosteroids or ICS, antileukotriene drugs, sustained-release theophyllines, TX synthase inhibitors or receptor antagonists, cyclooxygenase inhibitors or angiotensin-converting enzyme inhibitors, or had had an upper respiratory infection within the previous 8 weeks.

Study Protocol

Prebronchodilator pulmonary function was initially determined by impulse oscillometry (IOS) [22] and spirometry, methacholine challenge and sputum induction, in that order. Seven days later, the patients indicated their cough severity during the previous 7 days on a linear visual analogue scale (VAS) on which 0 mm represents no cough and 100 mm the worst cough [23]. Capsaicin cough challenge was performed on the same day, and montelukast (Kipres, Kyorin Pharmaceutical Co. Ltd., Tokyo, Japan; 10 mg/day) was administered in an uncontrolled, open-label fashion for 4 weeks. This is considered long enough to examine the effect of montelukast on cough symptoms and cough receptor hypersensitivity in CVA [18, 19]. After 4 weeks, spirometry, IOS, methacholine challenge and sputum induction were repeated, followed by capsaicin cough challenge 1 week later while the patients were still receiving montelukast.

Sputum Induction and Processing

Sputum was induced and processed as described previously [24, 25]. Briefly, after premedication with inhaled sulbutamol, the subjects inhaled hypertonic (3%) saline solution for 15 min, delivered using an ultrasonic nebulizer. The sputum was processed for dispersed cell total and differential counts. Supernatants were stored at –80°C for subsequent mediator measurements. Sputum induction was carried out within 1 h after the methacholine challenge test. Methacholine does not alter the cellular and biochemical constituents of sputum [26].

Measurement of Sputum Levels of Inflammatory Mediators

Concentrations of cys-LTs, LTB_4, PGD_2-methoxime, PGE_2, $PGF_{2\alpha}$ and TXB_2 in the supernatant of induced sputum were measured with sandwich enzyme immunoassay kits (cys-LTs and PGE_2: Amersham Biosciences Corp., N.J., USA; LTB_4, PGD_2-methoxime, $PGF_{2\alpha}$ and TXB_2: Cayman Chemical, Ann Arbor, Mich., USA) according to the manufacturers' instructions. Duplicate measurements were averaged for analysis. Because PGD_2 and TXA_2 are both relatively unstable, we measured PGD_2-methox-

ime and TXB_2, which are stable derivatives of PGD_2 and TXA_2, respectively [7]. The detection limit of each mediator was 10, 13, 3.1, 40, 8 and 11 pg/ml for cys-LTs, LTB_4, PGD_2-methoxime, PGE_2, $PGF_{2\alpha}$ and TXB_2, respectively.

Pulmonary Function

We conducted IOS using an MS-IOS (Erich Jaeger, Hoechberg, Germany) as described elsewhere [25, 27–29] and according to standard recommendations [30]. We measured R5 and R20 as indices of total and proximal airway resistance, respectively, and considered the difference between them (R5 – R20) a surrogate marker of small airway resistance. Reactance at 5 Hz (X5) was also measured as an index of peripheral airway abnormalities [22, 25, 29, 30]. IOS is a simple and noninvasive method of assessing pulmonary function without forced maneuvers, and the indices respond to therapeutic intervention or correlate with pathophysiological indices more sensitively than those of spirometry [25, 27–29, 31].

The spirometric indices forced expiratory volume in 1 s (FEV_1) and mid-forced expiratory flow ($FEF_{25-75\%}$), as well as the reversibility of FEV_1 with a β-agonist, were measured according to recent recommendations [32].

Capsaicin Cough Challenge

Capsaicin cough receptor sensitivity was measured as described previously with slight modifications [33]. Briefly, 10 doubling concentrations of capsaicin (0.61–312.5 μM) were inhaled during tidal breathing until 2 or more coughs and 5 or more coughs were induced (cough thresholds C2 and C5, respectively). The patients inhaled a saline control followed by progressively increasing concentrations of capsaicin. Each 15-second capsaicin inhalation was interspersed with saline inhalation for 45 s as a blind.

Methacholine AHR

AHR was examined by continuous methacholine inhalation with simultaneous measurement of respiratory resistance (Rrs; cm H_2O/l/s) (Astograph; Chest, Tokyo, Japan) [34]. Briefly, twofold increasing concentrations of methacholine solution in 10 dose steps (49–25,000 μg/ml) were prepared. They were inhaled during tidal breathing from nebulizers with an output of 0.15 ml/min. After recording the baseline Rrs during inhalation of physiologic saline for 1 min, methacholine was inhaled sequentially, starting from the lowest concentration, at 1-min intervals. The cumulative dose of inhaled methacholine at the inflection point at which Rrs begins to increase (Dmin) was used as the index of airway sensitivity. This variable was measured in terms of a unit defined as 1-min inhalation of 1 mg/ml methacholine. Inhalation of methacholine was continued until Rrs reached twice the baseline value. The slope of the methacholine-Rrs dose-response curve (SRrs) was used as the measure of airway reactivity. The total cumulative dose of methacholine after inhalation of the highest dose was 50 units.

This method was developed and established by Takishima et al. [34] and further validated by our group [35].

Response to Montelukast

Patients were assigned to groups according to their response to montelukast. A better symptomatic response to montelukast was arbitrarily defined as a >25% decrease in VAS (ΔVAS >25%).

Table 1. Changes in 23 patients treated with montelukast for 4 weeks

	Montelukast treatment		p value
	before	after	
Cough VAS, mm	40.3 (22.1)	19.3 (14.8)	0.0008
FEV_1, % predicted	103 (13)	106 (16)	0.13
$FEF_{25-75\%}$, % predicted	96 (30)	101 (32)	0.65
R5, kPa/l/s	0.31 (0.10)	0.33 (0.11)	0.89
R20, kPa/l/s	0.27 (0.07)	0.28 (0.07)	0.96
R5 – R20, kPa/l/s	0.04 (0.04)	0.05 (0.05)	0.42
X5, kPa/l/s	–0.12 (0.06)	–0.11 (0.06)	0.60
log C2, μM	0.49 (0.72)	0.77 (0.75)	0.007
log C5, μM	0.79 (0.75)	0.98 (0.74)	0.039
log Dmin, units	0.32 (1.08)	0.70 (0.58)	0.51
SRrs, cm H_2O/l/s/min	2.08 (1.64)	1.93 (1.48)	0.65
Sputum indices			
Eosinophils, $\times 10^5$/g	1.23 (3.36)	0.55 (1.25)	0.013
cys-LTs, ng/ml	18.4 (15.8)	12.8 (14.7)	0.50
LTB_4, ng/ml	5.24 (6.37)	4.86 (6.94)	0.96
PGD_2, ng/ml	0.14 (0.29)	0.05 (0.05)	0.21
PGE_2, ng/ml	1.25 (1.11)	1.11 (0.91)	0.69
$PGF_{2\alpha}$, ng/ml	0.83 (0.62)	0.74 (0.49)	0.80
TXB_2, ng/ml	1.94 (1.79)	1.58 (1.41)	0.59

Data are means (SD).

Baseline characteristics, including physiological and inflammatory indices, of these patients were compared with those of the remaining patients (ΔVAS ≤25%).

Statistical Analysis

Values are expressed as means (SD). Changes in symptoms after treatment with montelukast were analyzed using the Wilcoxon signed-rank test. Subgroups classified according to the response to montelukast were compared by univariate analysis using the Mann-Whitney U test or Fisher's exact probability test. Independent factors associated with the response to treatment were tested using logistic regression analysis. p values of <0.05 were considered statistically significant.

Results

Effect of Montelukast

Table 1 and figure 1 show changes in symptoms, physiological indices and inflammatory parameters of sputum before and after treatment with montelukast. The cough VAS improved from 40.3 (22.1) to 19.3 (14.8) mm after 4 weeks (p = 0.008), with a mean (SD) improvement rate of 52.2% (33.0). With respect to cough receptor sensitivity, C2 increased in 13 of the 23 patients (57%) after

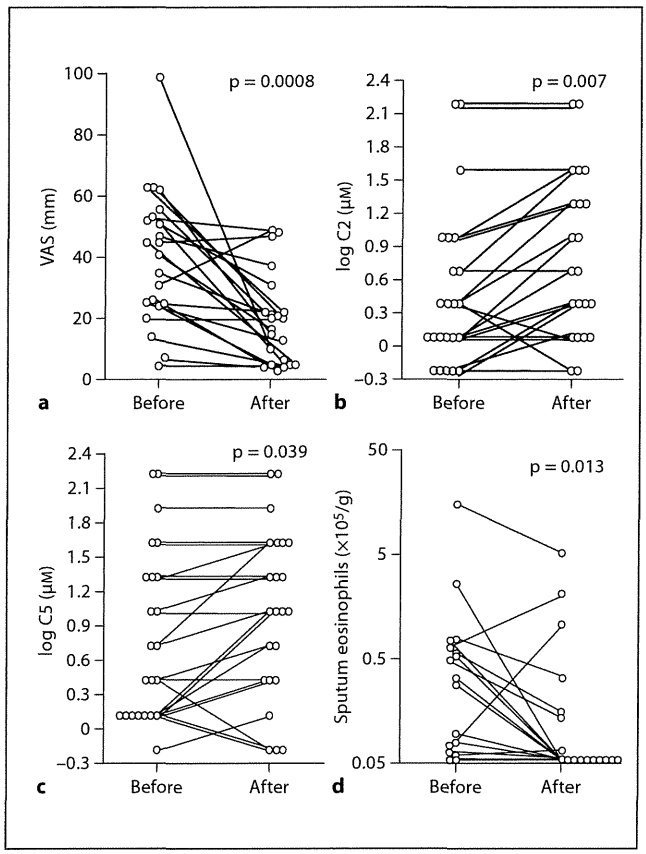

Fig. 1. Effects of montelukast for 4 weeks on cough VAS (**a**), log C2 (**b**), log C5 (**c**) and sputum eosinophil counts (**d**) in patients with CVA.

Table 2. Baseline characteristics of patient subgroups according to montelukast response

	Response to montelukast		p value
	ΔVAS >25%	ΔVAS ≤25%	
Male/female, n	5/10	4/4	0.66
Age, years	42 (13)	56 (13)	0.026
Disease duration, years	3.6 (4.0)	3.2 (2.0)	0.56
Nonsmokers/ex-smokers, n	13/2	6/2	0.59
Atopy/nonatopy[a], n	9/6	4/4	0.68
FEV_1, % predicted	100 (12)	110 (12)	0.053
$FEF_{25-75\%}$, % predicted	91 (29)	104 (31)	0.20
FEV_1 β_2 reversibility, %	4.4 (6.1)	2.1 (3.6)	0.24
R5, kPa/l/s	0.31 (0.11)	0.31 (0.08)	0.61
R20, kPa/l/s	0.26 (0.08)	0.27 (0.07)	0.75
R5 – R20, kPa/l/s	0.04 (0.04)	0.04 (0.03)	0.97
X5, kPa/l/s	−0.14 (0.07)	−0.09 (0.03)	0.14
log C2, μM	0.3 (0.5)	0.8 (0.9)	0.19
log C5, μM	0.8 (0.7)	1.0 (0.9)	0.77
log Dmin, units	0.047 (0.93)	0.77 (1.2)	0.030
SRrs, cm H_2O/l/s/min	2.26 (1.94)	1.71 (0.58)	0.54
Sputum indices	(n = 9)	(n = 8)	
Eosinophils, $\times 10^5$/g	0.54 (0.73)	2.00 (4.9)	0.81
cys-LTs, ng/ml	17.6 (16.8)	19.6 (15.5)	0.85
LTB_4, ng/ml	3.55 (3.76)	7.14 (8.23)	0.56
PGD_2, ng/ml	0.21 (0.40)	0.06 (0.05)	0.44
PGE_2, ng/ml	1.16 (1.12)	1.37 (1.16)	0.70
$PGF_{2\alpha}$, ng/ml	0.85 (0.70)	0.80 (0.55)	>0.99
TXB_2, ng/ml	2.18 (2.10)	1.60 (1.32)	0.77

Data are means (SD).
[a] Atopy is defined as the presence of at least one positive specific IgE response against eight common allergens [61].

treatment but did not change in 8 and decreased in 2, while log C2 improved from 0.49 (0.72) to 0.77 (0.75) μM (p = 0.007). Also, C5 increased in 11 patients (48%) but was unchanged in 9 and decreased in 3; log C5 improved from 0.79 (0.75) to 0.98 (0.74) μM (p = 0.039). Adequate pairs of sputum samples were collected from 17 of the 23 patients before and after treatment. The number of sputum eosinophils decreased with treatment from 1.23 (3.36) to 0.55 (1.25) $\times 10^5$/g (p = 0.013). However, spirometric and IOS indices, airway sensitivity and reactivity, and sputum levels of cys-LTs, LTB_4, PGD_2, PGE_2, $PGF_{2\alpha}$ and TXB_2 did not change significantly (table 1).

Baseline Clinical Characteristics according to Response to Montelukast

The symptomatic response to montelukast was better (ΔVAS >25%) in 15 of the 23 patients (65%). The results of the univariate analysis showed that the better respondents were significantly younger (p = 0.026) and more sensitive to methacholine (p = 0.030) than those with ΔVAS ≤25% (table 2). Other indices, including IOS and spirometry, log C5, airway reactivity, sputum eosinophil count and sputum mediator levels including that of cys-LTs, did not differ between the subgroups. Montelukast responses, assessed as the percentage change in physiological indices (FEV_1, $FEF_{25-75\%}$, R5, R20, R5 – R20, X5, log C2, log C5, log Dmin or SRrs) and the number of sputum eosinophils, also did not differ between subgroups (data not shown). Multiple linear regression analysis including age and log Dmin identified younger age as the sole predictor of a better responsiveness to montelukast (table 3).

An analysis of 17 patients who had adequate pairs of sputum samples before and after treatment revealed no

Table 3. Multiple regression model for better response to montelukast

	Odds ratio	p value
Age	0.89 (0.78–0.99)	0.032
log Dmin	0.26 (0.063–1.08)	0.064

Values in parentheses represent 95% confidence intervals.

significant correlations among percentage changes in VAS, sputum eosinophils, log C2 and log C5 (data not shown). In addition, percentage changes in the sputum eosinophil count were unrelated to those in sputum mediator levels (data not shown). In all of the 23 subjects, percentage changes in VAS were also unrelated to those in C2 or C5 (data not shown).

Discussion

We investigated the clinical, physiological and anti-inflammatory effects of montelukast in patients with CVA. Effects on the cough VAS and cough receptor sensitivity were confirmed as in other studies [18, 19]. We also discovered a significant decrease in sputum eosinophil count, although IOS and spirometry indices remained unchanged.

Ample evidence supports the effectiveness of LTRAs against classic asthma [15, 16, 36], and their value against CVA has also been reported [18–20, 37]. A placebo-controlled crossover study found that 2 weeks of treatment with zafirlukast significantly improved cough scores and capsaicin cough receptor sensitivity in 8 patients with CVA, while FEV_1 values remained unchanged [18]. Four weeks of treatment with montelukast improved subjective and objective measures of cough frequency and scores on the Asthma Quality of Life questionnaire without a concomitant change in FEV_1 in 8 patients with CVA, as compared with 6 who received a placebo [19]. However, the antitussive mechanisms of the LTRAs were not examined in these small studies, except for one analysis of bronchial tissue from a subset of patients (n = 13), which showed that responders to montelukast had increased numbers of CD63-positive cells (activated mast cells) compared with nonresponders [37].

Airflow limitation is usually minimal in CVA, as it was in our patients, while being involved in the pathogenesis of cough in CVA that is attenuated by bronchodilators [1, 3, 4, 21]. Airway caliber changes that may be missed by spirometry can be sensitively detected by IOS [22, 25, 27–29, 31], but we found no significant changes in indices of IOS, nor in indices of spirometry, after treatment with montelukast, which was consistent with previous findings [18, 19]. The antitussive effect of montelukast in CVA may thus be attributable to its anti-inflammatory properties that attenuate eosinophilic inflammation rather than to bronchodilation.

Tussive inflammatory mediators, which may activate afferent sensory nerve endings, have been implicated in the pathogenesis of cough [7, 38, 39]. Inhalation studies in healthy individuals [9, 10] and inhibition studies of the specific antagonists of inflammatory mediators in asthmatic patients [11] have shown that prostanoids such as PGE_2, $PGF_{2\alpha}$ and TXA_2 are associated with the cough reflex. A recent in vitro study revealed that the metabolites of lipoxygenase products such as 12- and 15-(*S*)-hydroperoxyeicosatetraenoic acids, 5- and 15-(*S*)-hydroxyeicosatetraenoic acids and LTB_4 might be ligands for transient receptor potential vanilloid 1 receptors [13]. The expression of such receptors is increased in the airways of patients with chronic cough, including those with CVA [40], indicating their involvement in cough hypersensitivity [14]. However, evidence supporting direct tussive effects of cys-LTs on 'cough receptors' is scarce.

Montelukast decreases cys-LT levels in the exhaled breath of patients with classic asthma [17, 41]. To our knowledge, this is the first study to investigate the detailed anti-inflammatory effect of an LTRA in CVA, and we revealed that montelukast significantly decreased sputum eosinophils as well as the cough VAS and capsaicin sensitivity but did not affect sputum levels of cys-LTs, LTB_4, PGD_2, PGE_2, $PGF_{2\alpha}$ and TXB_2. Since eosinophils are a source of various prostanoids [12] and cys-LTs [42], we postulated that montelukast exerts antitussive effects by attenuating eosinophilic inflammation with a resultant fall in some prostanoid and cys-LT levels in CVA. However, our results disproved this notion. Percentage changes in sputum eosinophils were unrelated to those of sputum levels of mediators, including cys-LTs.

With respect to cys-LTs, the discrepancy of the results between the previous breath condensate studies conducted in classic asthma patients presenting with wheezing [17, 41] and our sputum study of CVA patients may have derived from possible pathophysiological differences between CVA and classic asthma. In CVA and the related condition eosinophilic bronchitis, activation of

mast cells has been indicated by the increased sputum levels of the mast cell-derived tussive mediators histamine and PGD_2 [7], while this is not a feature of classic asthma [43]. A recent bronchial biopsy study has also implicated the activation of mast cells in the pathophysiology of CVA [37]. Such evidence may suggest that mast cells rather than eosinophils are a relatively more important source of cys-LTs in patients with CVA as compared with those with classic asthma. As for other mediators measured in this study, major cellular sources are cells other than eosinophils (e.g. mast cells for PGD_2, alveolar macrophages for PGE_2 and neutrophils for LTB_4) [44] which are poorly responsive to LTRAs, unlike eosinophils. This may explain why our mechanistic hypothesis was not verified. Since ICS, which are potent inhibitors of eosinophilic inflammation, do not alter cough sensitivity in CVA [45], whereas LTRAs do, as we and others [18] have shown, LTRAs might exert a different antitussive mechanism from ICS.

In a study by Birring et al. [7], sputum mediator levels were measured in 18 healthy subjects and in three subgroups of chronic cough patients (CVA or eosinophilic bronchitis, n = 20; various causes of nonasthmatic cough, n = 20; idiopathic cough, n = 22). Levels of PGD_2 and PGE_2 were significantly higher in all cough subgroups compared with controls, and histamine levels were also increased in the CVA and eosinophilic bronchitis and idiopathic cough subgroups, whereas cys-LT levels were increased only in the CVA and eosinophilic bronchitis subgroup. These results indicate that cys-LTs are specifically involved in cough associated with eosinophilic airway disorders, whereas PGD_2, PGE_2 and histamine are universally involved in the common mechanism of cough.

One possible mechanism for the association of cys-LTs with cough in CVA is via the tussive mediator substance P. The expression of substance P in the airway epithelium is increased in CVA patients compared with classic asthma patients and healthy subjects, both of whom express similar amounts of substance P [46]. We have also reported that plasma levels of substance P are elevated in patients with CVA and cough-predominant asthma as compared with healthy subjects [47]. cys-LTs induce the release of substance P through the stimulation of airway afferent nerve fibers [48], and LTRAs inhibit such release [48, 49]. Evidence that LTRAs attenuate cough receptor sensitivity in CVA [18] but not classic asthma [50] might be consistent with this hypothesis, but the details remain to be clarified.

The effect of LTRAs in classic asthma is variable. This has been attributed to the effects of genetics [51], smoking [52] and also age. Symptoms and lung function respond better to zafirlukast in patients aged <65 years than in older patients [53]. The effect of zafirlukast is better among patients aged <50 years [54, 55]. Our results are consistent with these findings, but the mechanisms involved are unknown. Sputum levels of mediators mainly derived from mast cells, such as PGD_2, were not increased in our patients with better responses to montelukast, which contradicts the suggested hypothesis [37].

Some limitations of our study should be noted. We arbitrarily defined a better response to montelukast as a >25% decrease of VAS based on our clinical experience, not on evidence. The European Respiratory Society guidelines on the assessment of cough recommend using the cough VAS for assessment of chronic cough severity [23]. Actually, there is evidence that the cough VAS score is highly reproducible [56] and responsive to intervention [57] when used as an outcome measure in clinical studies of chronic cough. However, the minimal change in VAS needed to judge the efficacy of intervention remains to be determined. The British Thoracic Society guidelines of cough have defined the minimal significant improvement of cough as a change in VAS of 15 mm [58]. However, this is not even based on evidence. We readily admit that future validation studies are essential for this issue. The lack of a control group is another limitation of our study. This might have affected the precise evaluation of treatment effects. However, a placebo effect of montelukast upon sputum eosinophils and coughing that had persisted for an average of 3.5 years seems unlikely. We believe that the observed efficacy of montelukast on the cough VAS, cough sensitivity and sputum eosinophilia but not on pulmonary function measures is relevant in the absence of controls. Moreover, this short-term study could not investigate the effect of treatment on the long-term consequences of disease such as the development of irreversible airflow obstruction [1], airway remodeling [5, 59] and progression to classic asthma, which might be preventable by ICS [60].

In conclusion, we found that the cough VAS, sputum eosinophil counts and cough sensitivity significantly improved in CVA patients treated with montelukast for 4 weeks, while pulmonary function did not change. The antitussive effect of montelukast in CVA might be attributable to its anti-inflammatory ability rather than bronchodilation. However, the detailed mechanisms remain to be clarified by future controlled studies.

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DNA damage as a molecular link in the pathogenesis of COPD in smokers

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ABSTRACT: **In this study, we investigated whether DNA double-strand breaks (DSBs) contribute to the pathogenesis of chronic obstructive pulmonary disease (COPD).**

We immunofluorescence-stained lung tissue samples obtained from COPD patients, asymptomatic smokers and nonsmokers for markers of DSBs.

The numbers of DSB foci (phosphorylated histone 2AX (γH2AX), phosphorylated ATM (ataxia telangiectasia mutated) substrate and phosphorylated p53-binding protein-1 foci) per cell in alveolar type I and II cells and endothelial cells were higher in the COPD patients than in the asymptomatic smokers and nonsmokers. The lung tissue in which type II cells contained higher numbers of γH2AX foci per cell had higher percentages of type II cells that expressed $p16^{INK4a}$ (p16), phosphorylated nuclear factor (NF)-κB and interleukin (IL)-6, and of alveolar wall cells that expressed active caspase-3. The type II cells that contained higher numbers of γH2AX foci per cell had higher rates of expression of p16, phosphorylated NF-κB, and IL-6. Half of the alveolar wall cells that expressed active-caspase-3 contained γH2AX foci. Type II cells that stained positive for 8-hydroxy-2-deoxyguanosine contained a higher number of γH2AX foci per cell than the type II cells that stained negative.

In conclusion, DSBs, at least in part caused by oxidative stress, appear to contribute to the pathogenesis of COPD by inducing apoptosis, cell senescence and pro-inflammatory responses.

KEYWORDS: **Apoptosis, cell senescence, chronic obstructive pulmonary disease, DNA damage, interleukin-6, nuclear factor-κB**

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Chronic obstructive pulmonary disease (COPD) is characterised by a persistent abnormal inflammatory response to noxious environmental stimuli, most commonly to cigarette smoke. Recent evidence indicates that the pathogenesis of COPD involves oxidative stress, apoptosis and cell senescence, as well as inflammation [1]. These multiple pathobiological processes in COPD are thought to be associated with the generation of interactive feedback loops that contribute to alveolar destruction, airway remodelling and ineffective tissue repair [1]. Although cigarette smoking directly causes inflammation, as well as oxidative stress, apoptosis and cell senescence, only a minority of smokers develop clinically significant COPD, and the inflammation and decline in pulmonary function continue even after smoking cessation [1]. This suggests the presence of specific mechanisms that are responsible for the susceptibility to damage by smoking, persistent inflammation and the chronic, progressive nature of the structural derangement in the lungs of COPD patients. We conducted the present study to test our hypothesis that DNA damage that alters the behaviour of pulmonary parenchymal cells underlies COPD-specific mechanisms.

Cigarette smoke is the most important environmental pollutant that causes DNA damage [2–4]. DNA double-strand breaks (DSBs) are among the most lethal forms of DNA damage caused by smoking [5], and if left unrepaired they can lead to apoptosis, cell senescence, pro-inflammatory responses and oncogenesis [6–8]. When a DSB is induced, the histone 2A (H2A)X becomes rapidly phosphorylated at serine 139 by ATM (ataxia telangiectasia mutated)/ATR (ataxia telangiectasia and Rad3-related) and DNA-dependent protein kinase. This modified form, called γH2AX, is easily identified by staining with antibodies and serves as a reliable, sensitive indicator of DSBs [7–10]. Since each DSB corresponds to one γH2AX focus, immunofluorescence-based assays that allow visualisation of discrete nuclear γH2AX foci are ⩾100 times more sensitive than

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other methods of detecting DSBs, such as pulsed-field gel electrophoresis and the alkaline comet assay [7–10]. γH2AX then recruits DNA repair proteins, including p53-binding protein (53BP)1, to the sites of DSBs and initiates DNA damage signal transduction that determines whether the DNA will be repaired; if repair is impossible, apoptosis, senescence or a pro-inflammatory response will be activated [6, 11, 12]. Thus, the persistence of γH2AX foci is regarded as indicating that some of the DNA damage remains unrepaired.

In the present study, we performed immunofluorescence staining for γH2AX foci, a marker of the presence of DSBs, to determine the intensity of DSB damage in the lungs of COPD patients. The results showed the presence of significantly more γH2AX foci in the alveolar type I and II cells and endothelial cells of the lungs of COPD patients than in the lungs of non-COPD smokers and nonsmokers, and that the higher numbers of γH2AX foci were associated with apoptosis, cell senescence, pro-inflammatory phenotypic changes and DNA oxidation. These results suggest that DNA damage that is, at least in part, caused by oxidative stress contributes to the molecular pathogenesis of COPD by inducing apoptosis, cell senescence and pro-inflammatory responses.

METHODS

Human lung tissue samples

The protocol of the study conformed to the Declaration of Helsinki and approval was obtained from the Tokyo Women's Medical University Institutional Review Board, Tokyo, Japan (grant number 1783). Lung tissue blocks were obtained from COPD patients who were smokers (COPD smokers; n=14) during lung volume reduction surgery (n=13) or pulmonary resection for localised lung cancer (n=1), smokers who did not have COPD (non-COPD smokers; n=10), and nonsmokers (n=10) during pulmonary resection for localised lung cancer. Each tissue block was fixed in 10% formalin, embedded in paraffin and cut into sections 3 μm thick. All of the COPD and non-COPD smokers had stopped smoking ⩾3 months before the surgery. The patients' clinical information is shown in table 1.

Immunofluorescence staining

Deparaffinised tissue sections were double or triple immunofluorescence stained using primary antibodies against γH2AX, 53BP1, phosphorylated ATM/ATR substrate, $p16^{INK4a}$ (p16), cleaved (active) caspase-3, phosphorylated nuclear factor (NF)-κB, interleukin (IL)-6, 8-hydroxy-2-deoxyguanosine (8-OHdG), surfactant protein (SP)-C, aquaporin (AQP)-5 and CD31 (see online supplementary material for details).

We examined ⩾40 randomly selected microscopic fields on each slide with an Olympus BX60 epifluorescence microscope (Olympus Optical Co. Ltd, Tokyo, Japan) equipped with a 100× objective lens, and visually counted the numbers of γH2AX foci, phosphorylated 53BP1 foci and phosphorylated ATM substrate foci per cell in SP-C-positive cells, the number of γH2AX foci per cell in AQP5-positive cells, and the number of γH2AX foci per cell in CD31-positive cells. We also counted the number of γH2AX foci per cell in SP-C-positive cells according to whether they expressed p16, phosphorylated NF-κB, IL-6 or 8-OHdG, and the number of γH2AX foci per cell in alveolar wall cells according to whether they expressed active caspase-3.

Cell culture and irradiation

Normal human lung microvascular endothelial cells were irradiated with a 10-Gy X-ray dose (see online supplementary material for details).

Guinea pig exposure to cigarette smoke

Hartley-strain guinea pigs were exposed to air or the smoke of 10 cigarettes 5 days a week for 10 weeks (see online supplementary material for details).

Statistical analysis

Statistical analyses were performed using Excel X (Microsoft Corp., Redmond, WA, USA) with the Statcel 3 add-in (OMS, Tokyo, Japan). Clinical, cell culture and animal experiment data are presented as mean±SEM, and human histological data are presented as medians. Differences in clinical data were analysed by ANOVA and if the results were significant, the Tukey–Kramer test was used as a multiple comparison *post hoc* test. Differences in human histological data were analysed by the Kruskall–Wallis test and if the results were significant, the Steel–Dwass multiple comparison test was used. Differences in cell culture data and animal experiment data were analysed by unpaired t-tests. Correlations were analysed by the Spearman rank correlation test. A p-value of <0.05 was considered statistically significant.

RESULTS

Increased level of DSBs in the lungs of COPD patients

A single γH2AX focus reflects hundreds to thousands of γH2AX protein molecules concentrated around one DSB [9]. As shown in figure 1a, immunofluorescence staining for γH2AX showed that the nuclei of many of the alveolar wall cells in the lungs of the COPD patients contained multiple γH2AX foci. No signal was visible when the anti-γH2AX antibody was omitted or the antibody was pre-incubated with

TABLE 1 Characteristics of the subjects

	COPD patients	Smokers	Nonsmokers
Subjects	14	10	10
Males/females	14/0	10/0	3/7
Age yrs	63.2±2.4	61.6±4.2	66.3±3.3
Smoking pack-yrs	74.4±13.2	47.0±7.2	0±0
FEV1 L	0.90±0.11**	2.37±0.20	2.05±0.11
FEV1/FVC %	33.4±3.3**	75.7±2.2	75.3±1.8
FEV1 % pred	33.7±3.8**	94.6±4.0	97.2±5.2
COPD severity			
GOLD stage II	2		
GOLD stage III	4		
GOLD stage IV	8		

Data are presented as n or mean±SEM. All chronic obstructive pulmonary disease (COPD) patients and smokers were ex-smokers. FEV1: forced expiratory volume in 1 s; FVC: forced vital capacity; % pred: % predicted; GOLD: Global Initiative for Chronic Obstructive Lung Disease. **: $p<0.01$ compared with asymptomatic smokers and nonsmokers.

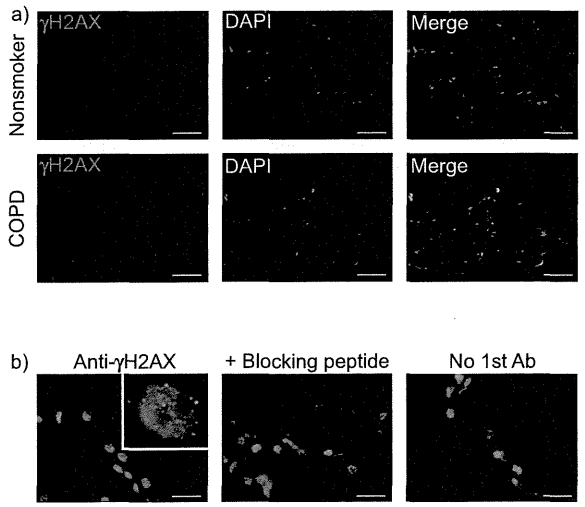

FIGURE 1. a) Representative images of anti-phosphorylated histone 2AX (γH2AX) immunofluorescence-stained sections of lung tissue obtained from asymptomatic nonsmokers and chronic obstructive pulmonary disease (COPD) patients. Nuclear DNA was counterstained with 4,6-diamidino-2-phenylindole (DAPI). Scale bars=50 μm. b) Specificity of γH2AX immunofluorescence staining. Discrete γH2AX foci in the nuclei of alveolar wall cells in the lungs of COPD patients were no longer visible when the antibody was pre-incubated with a blocking peptide before application of the secondary antibody or the anti-γH2AX antibody was omitted. Ab: antibody. Scale bars=20 μm.

a blocking peptide, thereby verifying the specificity of the γH2AX signal (fig. 1b). As shown in figure 2b, the γH2AX foci were co-localised with other DSB markers, including phosphorylated 53BP1 and phosphorylated ATM/ATR substrates [13, 14], thereby corroborating the validity of using γH2AX as a biomarker to measure DSB levels.

Quantitative analyses showed greater numbers of γH2AX foci per cell in AQP5-positive type I cells, SP-C-positive type II cells and CD31-positive endothelial cells in the COPD patients than in the non-COPD smokers and the nonsmokers (figs 2a and 3a–c). Similarly, the numbers of phosphorylated 53BP1 foci and phosphorylated ATM/ATR substrate foci per cell in type II cells were significantly higher in the COPD smokers than in the non-COPD smokers and nonsmokers (figs 2a, and 3a and e). However, the type I and II cells and endothelial cells of the non-COPD smokers and nonsmokers were found to contain similar numbers of γH2AX foci, phosphorylated 53BP1 foci and phosphorylated ATM/ATR substrate foci per cell (fig. 3). The numbers of γH2AX foci per cell in type II cells were closely correlated with the numbers of phosphorylated 53BP1 foci per cell (r=0.83) and phosphorylated ATM/ATR substrate foci per cell (r=0.78) in type II cells (fig. S1). These results suggest the presence of more severe DNA damage in the alveolar type I and II cells and endothelial cells of the COPD patients than in those of the non-COPD smokers and nonsmokers.

Tissue-level analyses of the relationship between DSBs and apoptosis, cell senescence, inflammation and oxidative stress

Consistent with the results of previous studies [15–21], we found that the lungs of the COPD patients contained higher percentages of alveolar wall cells that expressed active caspase-3 (a marker of apoptosis) (fig. 4a), type II cells that expressed p16 (a marker of senescence) (fig. 4b), type II cells that expressed phosphorylated NF-κB (fig. 4c) and IL-6 (fig. 4d) (markers of pro-inflammatory phenotypic changes), and type II cells that expressed 8-OHdG (a marker of oxidative stress) (fig. 4e) than the lungs of the non-COPD smokers and nonsmokers. Since DSB damage is a strong inducer of apoptosis, cell senescence and pro-inflammatory cytokine production [6, 11, 12], we investigated whether the presence of γH2AX, a marker of DSBs, was associated with apoptosis, cell senescence and pro-inflammatory phenotypic changes in type II cells. When all of the subjects were included in a tissue-level correlation analysis, the lung tissue with type II cells containing higher numbers of γH2AX foci contained higher percentages of alveolar wall cells that expressed active caspase-3 (fig. 5a), and higher percentages of type II cells that expressed p16 (fig. 5b), type II cells that expressed phosphorylated NF-κB (fig. 5c) and type II cells that expressed IL-6 (fig. 5d). These results indicate that more severe DNA damage in lung tissue was associated with greater numbers of type II cells undergoing apoptosis, senescence, pro-inflammatory phenotypic changes and DNA oxidation.

Cellular level analyses of the relationship between DSBs and apoptosis, cell senescence, inflammation and oxidative stress

Next, we conducted a cellular level analysis to investigate whether the DNA damage was directly related to apoptosis, cell senescence and pro-inflammatory phenotypic changes. Analysis of the type II cells of the COPD patients showed that the cells that contained higher numbers of γH2AX foci had higher rates of expression of p16 (fig. 6a), phosphorylated NF-κB (fig. 6b) and IL-6 (fig. 6c). Almost all of the type II cells that contained ⩾21 γH2AX foci expressed p16 and phosphorylated NF-κB. Moreover, about half of the alveolar wall cells that expressed active caspase-3 contained γH2AX foci (focal staining pattern), which indicated that the DNA damage had triggered apoptosis, whereas the remainder exhibited a diffuse γH2AX staining pattern, which indicated that DNA fragmentation was occurring during apoptosis (fig. 6d) [22]. These findings suggest that DNA damage is directly linked to the induction of apoptosis, cell senescence and pro-inflammatory phenotypic changes in the lungs of COPD patients.

Since oxidative stress is among the major causes of DSBs [23], we attempted to determine whether there was a correlation between DNA oxidation and the presence of γH2AX foci. A tissue-level analysis of the lungs of all of the subjects as a whole showed that the lung tissue with type II cells containing higher numbers of γH2AX foci per cell had higher percentages of type II cells that expressed 8-OHdG (fig. 5e). Moreover, a cellular level analysis of the lungs of the COPD patients alone showed that the type II cells that expressed 8-OHdG contained significantly more γH2AX foci than the type II cells that did not express 8-OHdG (fig. 6e). These results suggest that oxidative stress is at least partly responsible for DNA DSBs in the parenchymal cells of the lungs of COPD patients.

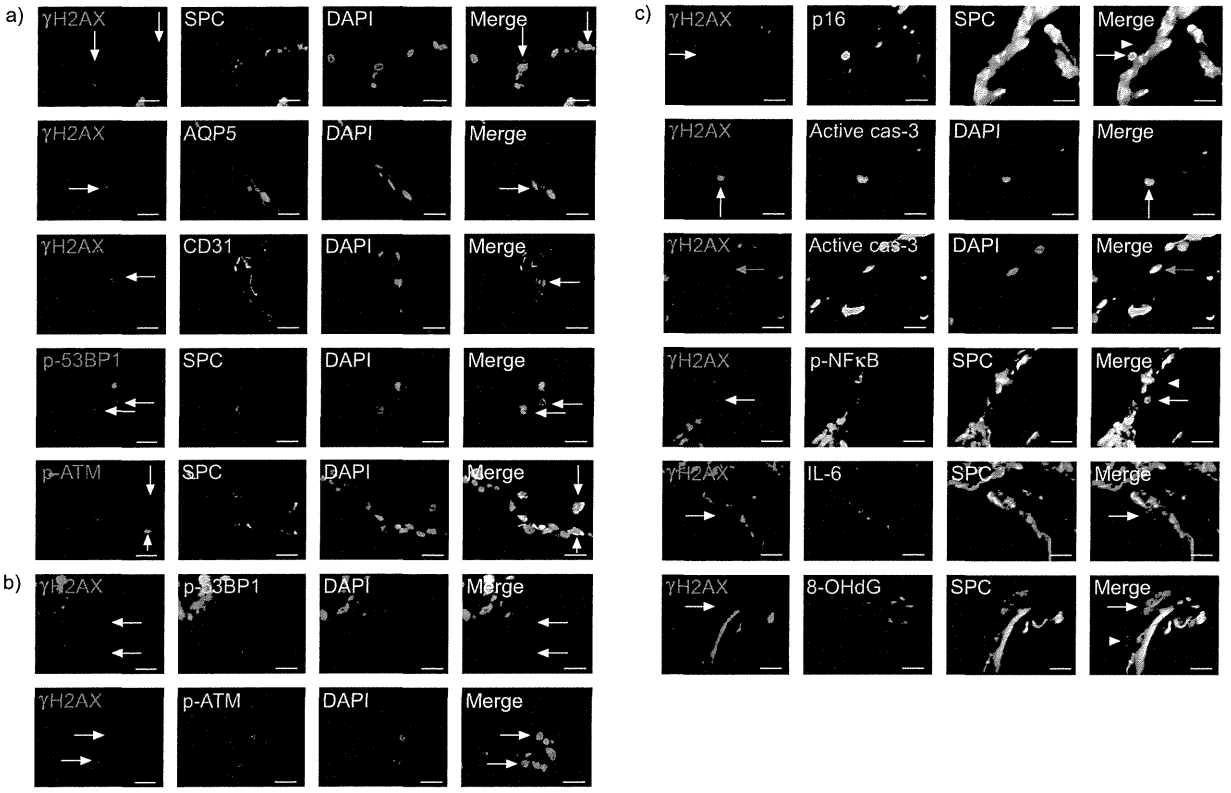

FIGURE 2. Representative images of double and triple immunofluorescence-stained sections of lung tissue obtained from chronic obstructive pulmonary disease patients. a) Double immunofluorescence staining for the double-strand break markers phosphorylated histone 2AX (γH2AX), phosphorylated p53-binding protein 1 (p-53BP1) or phosphorylated ataxia telangiectasia mutated/ataxia telangiectasia and Rad3-related substrate (p-ATM) and for one of the cell type-specific markers surfactant protein (SP)-C, aquaporin (AQP)5 or CD31. Nuclear DNA was counterstained with 4,6-diamidino-2-phenylindole (DAPI). White arrows indicate double-immunopositive cells. b) Double immunofluorescence staining for γH2AX and p-53BP1, and for γH2AX and p-ATM. Nuclear DNA was counter-stained with DAPI. White arrows indicate co-localisation. c) Triple immunofluorescence staining for γH2AX, SP-C and either $p16^{INK4a}$ (p16), phosphorylated nuclear factor-κB (p-NF-κB), interleukin (IL)-6 or 8-hydroxy-2-deoxyguanosine (8-OHdG), and double immunofluorescence staining for γH2AX and active caspase (cas)-3, followed by DNA counterstaining with DAPI. White arrows indicate triple-immunopositive cells. White arrowheads indicate SP-C-positive cells that did not stain for γH2AX or p16, for γH2AX or p-NF-κB, or for γH2AX or 8-OHdG. The nuclei of active cas-3-positive cells stained diffusely (yellow arrows) or focally (red arrows) for γH2AX. Scale bars=20 μm.

In vitro *effects of DSBs on apoptosis, cell senescence and inflammation*

To confirm that DNA DSBs directly cause apoptosis, cell senescence and a pro-inflammatory response, we irradiated normal human lung microvascular endothelial cells with a single 10-Gy X-ray dose. This dose increased the numbers of γH2AX foci per cell three-fold and the numbers of phosphorylated 53BP1 foci per cells six-fold, thereby validating the induction of DSBs by X-irradiation (fig. S2). X-irradiation also increased the percentages of lung microvascular endothelial cells that stained positively for active caspase-3, p16 and phosphorylated NF-κB, and the amount of IL-6 secreted by lung microvascular endothelial cells (fig. S2). These results indicate that DNA DSBs directly caused apoptosis, cell senescence and a pro-inflammatory response in lung microvascular endothelial cells. We also treated the alveolar type II cell-like epithelial cell line A549 cells with bleomycin, which is known to induce both single-strand breaks and DSBs [24]. A549 cells express p21 but not p16 [25]. We found that bleomycin treatment also increased the numbers of γH2AX and phosphorylated 53BP1 foci per cell, the percentages of A549 cells that stained positively for active caspase-3, p21 and phosphorylated NF-κB, and the amount of IL-6 secreted by A549 cells (fig. S3).

Increased DSBs in a guinea pig model of cigarette smoke-induced emphysema

We also determined whether exposing guinea pigs to cigarette smoke causes DNA DSBs in the alveolar wall cells of their emphysematous lungs. Histological examination of samples of the lung tissue of the guinea pigs exposed to cigarette smoke for 10 weeks showed enlargement of the alveolar air spaces and the Lm value (mean airspace size) was 45% higher than in the nonsmoking control group ($p<0.01$) (fig. S4). The alveolar wall cells of the lungs of the cigarette smoke-exposed group contained twice as many γH2AX foci per cell as the alveolar wall cells of the lungs of the control group (mean±SEM 2.05±0.26 *versus* 1.06±0.17; $p<0.01$) (fig. S4). These results indicate that DSBs occurred in the alveolar wall cells of emphysematous lungs of guinea pigs exposed to cigarette smoke.

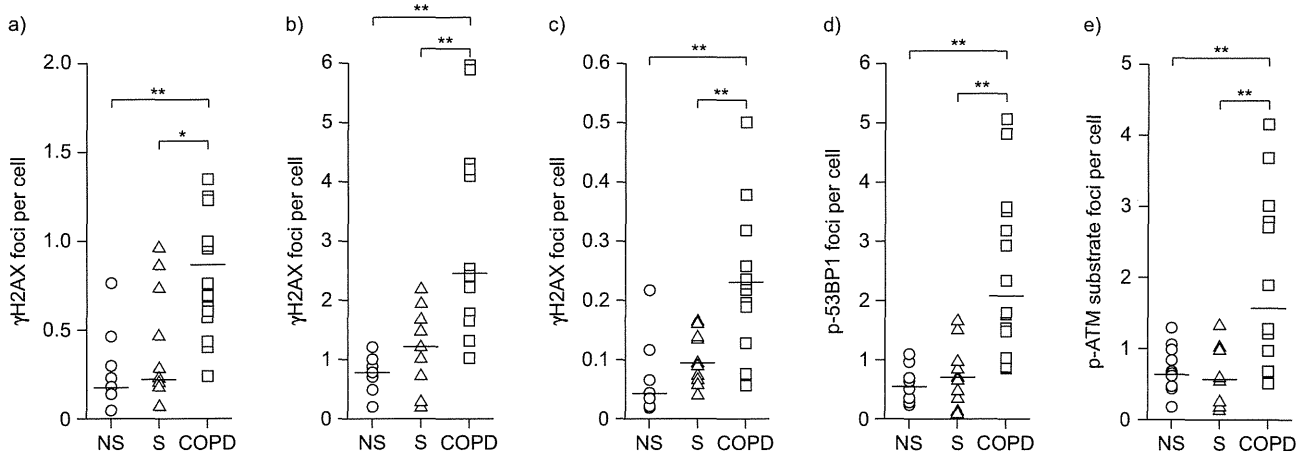

FIGURE 3. Quantitative analyses of DNA double-strand breaks in alveolar wall cells of the lungs of chronic obstructive pulmonary disease (COPD) patients, and asymptomatic smokers (S) and nonsmokers (NS). a) Phosphorylated histone 2AX (γH2AX) foci per cell in aquaporin 5-positive type I cells, b) surfactant protein (SP)-C-positive type II cells and c) CD31-positive endothelial cells, and the numbers of d) phosphorylated p53-binding protein 1 (p-53BP1) foci and e) phosphorylated ataxia telangiectasia mutated (p-ATM) substrate foci per cell in SP-C-positive type II cells. —: median. *: $p<0.05$. **: $p<0.01$.

DISCUSSION

Immunofluorescence-based assays of γH2AX provide a sensitive, efficient and reproducible method of measuring the number of DSBs [7–10]. Since persistence of γH2AX foci after the initial induction of DNA DSBs indicates that some of the damage remains unrepaired, the persistence of foci is widely used as a biomarker of DNA damage in various tissues [7–10]. In the present study, we found significantly increased numbers of γH2AX foci in the alveolar wall cells, including type I and II cells, and endothelial cells of COPD smokers than in non-COPD smokers and nonsmokers. The presence of γH2AX foci in type II cells was associated with apoptosis, cell senescence, pro-inflammatory phenotypic changes and oxidative stress. The results of this study also showed that the γH2AX foci were co-localised with DNA damage repair proteins, *i.e.* phosphorylated ATM/ATR substrates and phosphorylated 53BP1, and that the numbers of γH2AX foci per cell were closely correlated with the numbers of phosphorylated 53BP1 foci and phosphorylated ATM/ATR substrate foci per cell, thereby demonstrating the validity of γH2AX number as a biomarker of DNA damage. Taken together, the results of the present study suggest that DNA damage, in particular DSBs caused at least in part by oxidative stress, contributes to the molecular pathogenesis of COPD by inducing apoptosis, cell senescence and pro-inflammatory responses.

The results of this study provide evidence that DSBs are a prominent feature of the alveolar wall cells, including type I and II cells and endothelial cells, of COPD patients. This evidence obtained in the human lung tissue study was supported by the results of our animal study in guinea pigs that showed significantly higher numbers of γH2AX foci in the

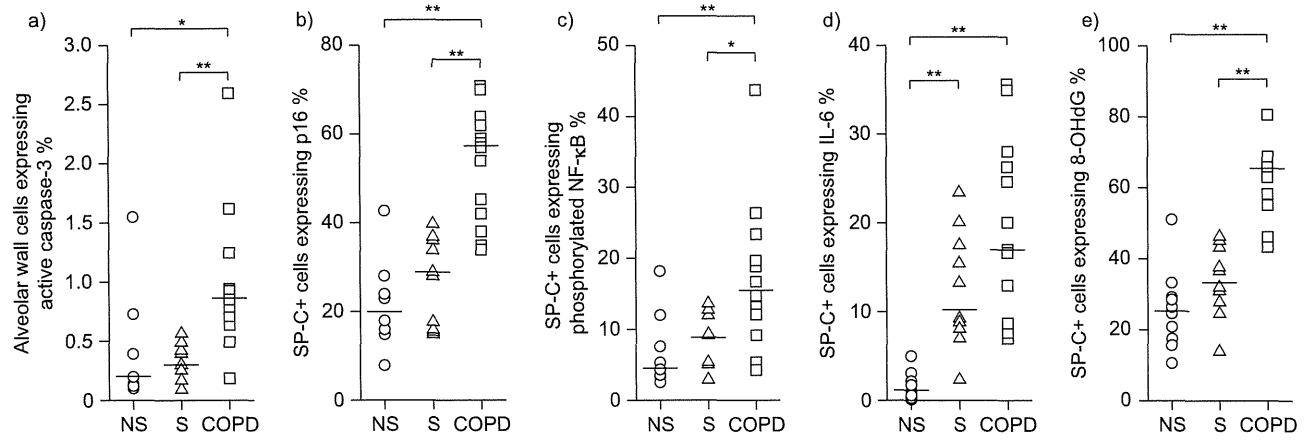

FIGURE 4. Quantitative analyses of apoptosis, cell senescence, pro-inflammatory phenotypic changes and oxidative stress in the lungs of chronic obstructive pulmonary disease (COPD) patients, and asymptomatic smokers (S) and nonsmokers (NS). The panels show the percentages of a) alveolar wall cells that expressed active caspase-3, and the percentages of type II cells that expressed b) $p16^{INK4a}$ (p16), c) phosphorylated nuclear factor (NF)-κB, d) interleukin (IL)-6 and e) 8-hydroxy-2-deoxyguanosine (8-OHdG). —: median values. SP: surfactant protein. *: $p<0.05$. **: $p<0.01$.