DNA DAMAGE AS A MOLECULAR LINK IN THE PATHOGENESIS OF COPD IN SMOKERS

Kazutetsu Aoshiba¹,², Fang Zhou¹, Takao Tsuji², Atsushi Nagai².

¹Pulmonary Division, Graduate School of Medical Science, Tokyo Women’s Medical University
²First Department of Medicine, Tokyo Women’s Medical University
8-1 Kawada-cho, Shinjuku-ku, Tokyo 162-8666, Japan

Corresponding author: Kazutetsu Aoshiba, M.D.
First Department of Medicine, Tokyo Women’s Medical University
8-1 Kawada-cho, Shinjuku-ku, Tokyo 162-8666, Japan
Tel. 81-3-3353-8111; Fax. 81-3-5379-5457; e-mail. kaoshiba@chi.twmu.ac.jp
ABSTRACT

In this study we investigated whether DNA double-strand breaks (DSBs) contribute to the pathogenesis of COPD.

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

The numbers of DSB foci (γH2AX, phosphorylated ATM substrate, and phosphorylated 53BP1 foci) per cell in alveolar type I cells, type II cells, and endothelial cells were higher in the COPD patients than in the asymptomatic smokers and nonsmokers. The lung tissue whose type II cells contained higher numbers of γH2AX foci per cell had higher percentages of type II cells that expressed p16, phosphorylated NFκB, and 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 NFκB, 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-OHdG 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 proinflammatory responses. (198 words)
Key Words: apoptosis, cell senescence, chronic obstructive pulmonary disease, DNA damage, interleukin-6, nuclear factor-κB

INTRODUCTION

Chronic obstructive pulmonary disease (COPD) is characterized by 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 pathobiologic processes in COPD are thought to be associated with the generation of interactive feedback loops that contribute to alveolar destruction, airway remodeling, 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 behavior 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, proinflammatory responses, and oncogenesis [6-8]. When a DSB is induced, the histone H2A variant H2AX becomes rapidly phosphorylated at serine 139 by ataxia telangiectasia mutated kinase/ataxia telangiectasia and Rad3-related kinase (ATM/ATR) and DNA-dependent protein kinase (DNA-PK). 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 visualization of discrete nuclear γH2AX foci is at least 100 times sensitive than 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 1 (53BP1), to the sites of DSBs and initiates DNA damage signal transduction that determines whether the DNA will be repaired, or if repair is impossible, apoptosis, senescence, or a proinflammatory 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 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 cells, type 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, proinflammatory 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 proinflammatory 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 (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 localized lung cancer (\( n = 1 \)), and smokers who did not have COPD (non-COPD smokers, \( n = 10 \)), and nonsmokers (\( n = 10 \)) during pulmonary resection for localized lung cancer. Each tissue block was fixed in 10% formalin, embedded in paraffin, and cut into sections 3 \( \mu \)m thick. All of the COPD smokers and non-COPD smokers had stopped smoking at least three months before the surgery. The patients’ clinical information regarding these patients is shown in Table 1.

Immunofluorescence Staining

Deparaffinized tissue sections were double- or triple-immunofluorescence-stained by using primary antibodies against phospho-histone H2AX (\( \gamma H2AX \)), phospho-p53-binding protein 1 (53BP1), phospho-ataxia telangiectasia mutated kinase (ATM)/ataxia telangiectasia and Rad3-related kinase (ATR) substrate, p16\(^{INK4a} \) (p16),
cleaved-caspase-3 (active caspase-3), phospho-nuclear factor (NF)-κB, IL-6, 
8-hydroxy-2-deoxyguanosine (8-OHdG), surfactant protein-C (SPC), aquaporin-5 
(AQP5), and CD31. (See online supplementary material for details.)

We examined at least 40 randomly selected microscopic fields on each slide 
with an Olympus BX60 epifluorescence microscope equipped with a 100x objective, and 
visually counted the numbers of γ 2AX foci, phosphorylated 53BP1 foci, and 
phosphorylated ATM substrate foci per cell in SPC-positive cells, the number of γ 2AX foci per cell in AQP5-positive cells, and the number of γ 2AX foci per cell in 
CD31-positive cells. We also counted the number of γ 2AX foci per cell in SPC-positive 
cells according to whether they expressed p16, phosphorylated NFκB, IL-6, or 8-OHdG, 
and the number of γ 2AX 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 on 5 days a 
week for 10 weeks. (See online supplementary material for details.)

Statistical Analysis

Statistical analyses were performed by using the Excel X software program with the 
add-in software Statcel 3 (OMS, Tokyo, Japan). Clinical data, cell culture data, and
animal experiment data are expressed as the mean ± SEM and human histologic data are expressed as the median. Differences in clinical data were analyzed by an analysis of variance (ANOVA), and if the results were significant, the Tukey-Kramer test was used as a multiple comparison post hoc test. Differences in human histologic data were analyzed by the Kruskall-Wallis test, and if the Kruskall-Wallis test showed significance, the Steel-Dwass multiple comparison test was used. Differences in cell culture data and animal experiment data were analyzed by the Student’s t-test. Correlations were analyzed 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 proteins 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 immunosignals were visible when the anti-γH2AX antibody was omitted or the antibody was preincubated with a blocking peptide, thereby verifying the specificity of the γH2AX immunosignals (Figure 1B). As shown in Figure 2B, the γH2AX foci were colocalized 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 $\gamma$H2AX foci per cell in AQP5-positive type I cells, in SPC-positive type II cells, and in CD31-positive endothelial cells in the COPD patients than in the non-COPD smokers and the nonsmokers (Figures 2A, 3A, 3B, and 3C). 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 (Figures 2A, 3D, and 3E). However, the type I cells, type II cells, and endothelial cells of the non-COPD smokers and the nonsmokers were found to contain similar numbers of $\gamma$H2AX foci, phosphorylated 53BP1 foci, and phosphorylated ATM/ATR substrate foci per cell (Figure 3). The numbers of $\gamma$H2AX 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 (Figure E1 in the online supplementary material). These results suggest the presence of more severe DNA damage in the alveolar type I cells, type 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) (Figure 4A), type II cells that expressed p16 (a marker of senescence) (Figure 4B), type II cells that expressed phosphorylated NFκB
(Figure 4C) and IL-6 (Figure 4D) (markers of proinflammatory phenotypic changes), and type II cells that expressed 8-OHdG (a marker of oxidative stress) (Figure 4E) than the lungs of the non-COPD smokers and nonsmokers. Since DSB damage is a strong inducer of apoptosis, cell senescence, and proinflammatory cytokine production [6, 11, 12], we investigated whether the presence of γH2AX, a marker of DSBs, was associated with apoptosis, cell senescence, and proinflammatory phenotypic changes in type II cells. When all of the subjects were included in a tissue-level correlation analysis, the lung tissue whose type II cells contained higher numbers of γH2AX foci contained higher percentages of alveolar wall cells that expressed active caspase-3 (Figure 5A) and higher percentages of type II cells that expressed p16 (Figure 5B), type II cells that expressed phosphorylated NFκB (Figure 5C), and type II cells that expressed IL-6 (Figure 5D). These results indicate that severer DNA damage in lung tissue was associated with greater numbers of type II cells undergoing apoptosis, senescence, proinflammatory 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 proinflammatory 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 (Figure 6A), phosphorylated NFκB (Figure 6B), and IL-6 (Figure 6C). Almost all of the type II cells that contained 21 or more γH2AX foci expressed p16 and phosphorylated
NFκB. 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 the execution of apoptosis (Figure 6D) [22]. These findings suggest that DNA damage is directly linked to the induction of apoptosis, cell senescence, and proinflammatory 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 whose type II cells contained higher numbers of γH2AX foci per cell had higher percentages of type II cells that expressed 8-OHdG (Figure 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 (Figure 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.

In Vitro Effects of DSBs on Apoptosis, Cell Senescence, Inflammation

To confirm that DNA DSBs directly cause apoptosis, cell senescence, and a proinflammatory 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 (Figure E2 in the online supplementary material). X-irradiation also increased the percentages of lung microvascular endothelial cells that stained positive for active-caspase-3, p16, and phosphorylated NFκB, and the amount of IL-6 secreted by lung microvascular endothelial cells (Figure E2). These results indicate that DNA DSBs directly caused apoptosis, cell senescence, and a proinflammatory response in lung microvascular endothelial cells. We also treated alveolar type II-like epithelial cell line A549 cells with bleomycin, which is known to induce both single- and double-strand breaks [24]. A549 cells express p21 but not p16 [25]. We found that bleomycin treatment also increased the numbers of γH2AX foci per cell, the numbers of phosphorylated 53BP1 foci per cells, the percentages of A549 cells that stained positive for active-caspase-3, p21, and phosphorylated NFκB, and the amount of IL-6 secreted by A549 cells (Figure E3 in the online supplementary material).

**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) (Figure E4 in the online supplementary material). The alveolar wall cells of the lungs of cigarette-smoke-exposed group contained twice as many γH2AX foci per cell as the alveolar wall cells of the lungs of the control group (2.05 ± 0.26 vs. 1.06 ± 0.17,
P<0.01) (Figure E4). These results indicate that DSBs occurred in the alveolar wall cells of emphysematous lungs of guinea pigs exposed to cigarette smoke.

**DISCUSSION**

Immunofluorescence-based assays of γH2AX (phosphorylated H2AX) 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 γH2AX foci in the alveolar wall cells, including type I cells, type 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, proinflammatory phenotypic changes, and oxidative stress. The results of this study also showed that the γH2AX foci were co-localized with DNA damage repair proteins, i.e., phosphorylated ATM/ATR substrates and phosphorylated 53BP1, as well as 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 proinflammatory responses.
The results of this study provide evidence that DSBs are a prominent feature of the alveolar wall cells, including type I cells, type 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 alveolar wall cells of the lungs of guinea pigs with cigarette-smoke-induced emphysema than in the lungs of sham-exposed animals (Figure E4 in the online supplementary material). Our findings are consistent with those of Siafakas and colleagues, who detected DNA lesions in the form of microsatellite instability (MSI) and loss of heterozygosity (LOH) in the sputum cells and bronchoalveolar fluid (BALF) cells obtained from COPD patients [26-28]. Cigarette smoke contains many genotoxins, including benzo(a)pyrene, nitrosamines, aldehydes, and oxidants, that induce various forms of DNA adducts [5]. The DNA damage accumulates at multiple genetic loci, and sometimes induces DSBs, the most cytotoxic damage, and the DSBs trigger the formation of γH2AX foci at the break site [29]. The results of the present study revealed significantly higher numbers of γH2AX foci per cell in the alveolar wall cells of COPD smokers but not of non-COPD smokers than in non-COPD nonsmokers. This finding corroborates the findings of Siafakas and colleagues who detected MSI and LOH in sputum cells and BALF cells of the COPD smokers but not in any of the non-COPD smokers [26-28]. Their findings together with our own strongly suggest that the DNA damage in the lungs of COPD smokers is amplified and/or remains unrepaired, and this results in gradual accumulation of DNA damage in their lungs.
The current theory of the pathogenesis of COPD suggests that alveolar destruction is caused by interactions between several pathobiologic processes, including inflammation, apoptosis, cell senescence, and oxidative stress [1]. By carefully analyzing correlations at both the tissue and cellular level, we found that DNA damage is correlated with apoptosis, cell senescence, and proinflammatory phenotypic changes in the lungs of COPD patients, thereby underscoring DNA damage as a molecular link between the pathobiologic processes thought to be involved in the alveolar destruction in COPD.

Since this study is an observational study of human lung tissue specimens, it was impossible to establish a causal connection between DNA damage and the development of apoptosis, cell senescence, and proinflammatory phenotypic changes in this study. Some of the DNA damage observed in this study may have been the result of apoptosis, cell senescence, and inflammation rather than their cause. However, it is well established that DNA damage, in particular DSBs, is a strong inducer of apoptosis, cell senescence, and proinflammatory responses in various types of cells and tissues [6-12]. In fact, many studies have shown that activation of an ATM/\gamma H2AX–mediated signal transduction pathway in response to unrepaired DSBs causes apoptosis and cell senescence, thereby eliminating the DNA-damaged cells from the tissue and preventing their oncogenic transformation [6, 9]. Furthermore, Rodier and colleagues have recently discovered a novel ATM/\gamma H2AX-mediated proinflammatory signal transduction pathway in response to DSBs, and that activates NFκB and C/EBPβ transcriptional activity and stimulates secretion of proinflammatory cytokines, including IL-6, which in turn acts in an autocrine feedback loop to reinforce the senescence growth arrest [11, 12].
In the present study we showed that DSBs caused by X irradiation of cultured lung microvascular endothelial cells induced proinflammatory responses, such as NF-κB phosphorylation and IL-6 production, as well as caspase-3 activation and p16 expression, suggesting that DSBs are direct causes of proinflammatory responses, apoptosis, and cell senescence (Figure E2 in the online supplementary material). We also showed that treatment of A549 cells with bleomycin, which induced γH2AX focus formation, caused NF-κB phosphorylation and IL-6 production, as well as caspase-3 activation and p21 expression (Figure E3 in the online supplementary material). We also recently found that persistent DNA damage in Clara cells induced by repeated injection of mice with naphthalene and bromodeoxyuridine caused airway epithelial senescence accompanied by a p38-mitogen-activated protein kinase (MAPK)-dependent airway inflammatory response [30]. These lines of evidence, although not wholly conclusive, support the hypothesis that DNA damage plays a causative role in the apoptosis, cell senescence, and proinflammatory responses observed in the lungs of COPD patients. However, future studies on animal models of COPD will be needed to show whether this view is correct.

Oxidative stress is among the major causes of DSBs [23]. In the present study we demonstrated an association between the presence of γH2AX foci and the presence of 8-OHdG, suggesting that oxidative stress is responsible for the DSBs in the lungs of COPD patients. It has been proposed that oxidative stress contributes to apoptosis, cell senescence, and inflammation in COPD [1, 31], and recent evidence suggests that oxidative stress induces inflammation via multiple mechanisms that involve oxidative activation of c-Jun activated kinase, p38-MAPK, NFκB, and activator protein-1, and
oxidative inhibition of histone deacetylase activity [31]. Our findings in this study show that oxidative DNA damage may also contribute to the mechanism of persistent inflammation in COPD.

The results of the present study showed that γH2AX focus formation was also associated with IL-6 production by type II cells. This finding corroborates with recent evidence that “danger signals” from injured cells elicit an immune response [32] and that IL-6 is produced by DNA-damaged cells and is a major secreted factor in their proinflammatory phenotype [11, 12, 33]. It has been shown that IL-6 is involved in pulmonary and systemic inflammation in COPD [34] and that higher serum IL-6 levels are associated with lower FEV1 [35]. Overexpression of IL-6 in mice was found to result in the development of emphysema and airway fibrosis [36]. In view of these findings, we suggest that IL-6 may be a key mediator that links DNA damage to the inflammatory responses in COPD.

There were several limitations to this study. First, the COPD patients had a 1.6 times greater smoking-pack-years history than the non-COPD smokers, although the difference between the two groups was not statistically significant (Table 1) (P = 0.12, Student’s t-test). This possible mismatch regarding smoking intensity, however, is unlikely to have accounted for the higher numbers of γH2AX foci per cell in the COPD smokers, because no significant correlations were found between the smoking-pack-years and the numbers of γH2AX foci per cell in type I cells (r = 0.16, P = 0.58), type II cells (r = 0.001, P = 0.99), or endothelial cells (r = 0.18, P = 0.22). Second, it remains uncertain whether DNA damage is an early pathobiologic event in COPD, because most of the
COPD patients included in the present study had advanced COPD that received lung volume reduction surgery. A further study evaluating the number of γH2AX foci in all COPD stages, in subjects with different degrees of smoking exposure, and in COPD patients treated with different medications would enable us to better elucidate the DNA damage in COPD. Third, this study may have overestimated the level of DNA damage in the lungs of the non-COPD smokers and nonsmokers, because a comorbid cancer might have promoted the formation of γH2AX foci in the surrounding lung tissue through the bystander effect [37]. Fourth, it remains unknown whether DNA damage in non-parenchymal cells, such as airway epithelial cells and pulmonary arterial endothelial cells, is also more severe in COPD patients.

Based on the results of the present study, we hypothesized that the apoptosis, cell senescence, and inflammation, which are thought to represent the pathobiological processes of COPD [1], are at least partly attributable to DNA damage (Figure 7). The traditional theory of the pathogenesis of COPD suggests that activation of inflammation by inhaled cigarette smoke and other pollutants plays a central role in airway wall thickening, alveolar destruction, airspace enlargement, and vascular remodeling [38]. Our hypothesis that DNA damage underlies the molecular mechanism of COPD seems to suggest answers to several important questions that the traditional theory does not address. The first question is, why does COPD take decades to develop? The answer based on our DNA damage hypothesis would be that the DNA damage caused by long-term smoking needs to accumulate over several decades before COPD develops, by analogy to the development of lung cancer. The second question is, why does
inflammation persist after ceasing to smoke? The answer is that it probably persists because smoking-induced DNA damage persists long after smoking cessation, as is reported previously [3, 4]. Third, why do corticosteroids have little impact on the inflammation in COPD? The answer may be that corticosteroids do not restore the DNA damage. Fourth, why is it that some smokers develop COPD while others do not, and, fifth, why are COPD smokers more prone to develop lung cancer than non-COPD smokers? The answers to the last two questions would be that the greater susceptibility to DNA damage due to smoking may be genetically determined just as greater susceptibility to smoking-induced lung cancer, so that smokers who are more susceptible to DNA damage may be predisposed to both COPD and lung cancer. However, the results of the current study do not sustain all these answers. Most likely longitudinal studies will be needed that include a larger number of COPD patients with different stages of disease severity.

Two independent groups of investigators have recently proposed a somatic mutation hypothesis of COPD, which differs from our hypothesis in requiring a "somatic gene mutation" to explain enhanced inflammatory responses in COPD. In 2003, Anderson and Bozinovski proposed that acquired somatic mutations in p53, Ras, EGFR, and PTEN induced by cigarette smoke carcinogens may contribute to the pathogenesis of COPD by causing aberrant inflammatory responses [39]. In 2009, Tzortzaki and Siafakas proposed that acquired somatic mutations, such as MSI and LOH, may lead to altered lung epithelial barrier cells, which are in turn misinterpreted by the host immune system as “nonselF”, leading to an aberrant immune response and the clonal expansion of
cytotoxic CD8+ cells, ultimately resulting in apoptosis and/or necrosis [40]. Although both our DNA damage hypothesis and the somatic mutation hypothesis require further experimental supports, we think that both of these hypotheses suggest a new previously overlooked role of genetic damage in the pathogenesis of COPD.

In conclusion, the results of the present study strongly suggest that DNA damage underlies the molecular pathogenesis of COPD. The DNA damage hypothesis may help to better understand the pathogenetic mechanism of COPD and to better target new drugs, such as drugs to prevent DNA damage and to modulate responses to DNA damage that lead to the pathobiologic processes of COPD.
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REFERENCES


FIGURE LEGENDS

Figure 1. Representative images of anti-γH2AX immunofluorescence stained sections of lung tissue obtained from COPD patients and asymptomatic nonsmokers (A). Nuclear DNA was counterstained with 4,6-diamidino-2-phenylindole (DAPI). (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 anti-γH2AX antibody was omitted or the antibody was preincubated with a blocking peptide before application of the secondary antibody.
Figure 1

A
Non-smoker

COPD

B
Anti-γH2AX + Blocking peptide No 1st ab
Figure 2. Representative images of double- and triple-immunofluorescence stained sections of lung tissue obtained from COPD patients. (A) Double immunofluorescence staining for one of the DSB markers γH2AX (red), phosphorylated 53BP1 (red), or phosphorylated ATM/ATR substrates (red) and for one of the cell type-specific markers SPC, AQP5, or CD31 (green). Nuclear DNA was counterstained with DAPI (blue). White arrows indicate double-immunopositive cells. (B) Double immunofluorescence staining for γH2AX (red) and phosphorylated 53BP1 (green) and for γH2AX (red) and phosphorylated ATM/ATR substrates (green). Nuclear DNA was counter-stained with DAPI (blue). White arrows indicate co-localization of double immunosignals. (C) Triple immunofluorescence staining for γH2AX (red), SPC (blue), and either p16 (green), phosphorylated NFκB (green), IL-6 (green), or 8-OHdG (green), and double immunofluorescence staining for γH2AX (red) and active caspase-3 (green) followed by DNA counterstaining with DAPI (blue). White arrows indicate triple-immunopositive cells. White arrowheads indicate SPC-positive cells that did not stain for γH2AX or p16, for γH2AX or phosphorylated NFκB, or for γH2AX or 8-OHdG. The nuclei of active caspase-3-positive cells stained diffusely (yellow arrows) or focally (red arrows) for γH2AX.
Figure 2

A

γH2AX  SPC  DAPI  Merge

γH2AX  AQP5  DAPI  Merge

γH2AX  CD31  DAPI  Merge

p-53BP1  SPC  DAPI  Merge

p-ATM  SPC  DAPI  Merge

B

γH2AX  p-53BP1  DAPI  Merge

γH2AX  p-ATM  DAPI  Merge

20 μm
**Figure 3.** Quantitative analyses of DNA DSBs in alveolar wall cells of the lungs of COPD patients (*COPD*), asymptomatic smokers (*S*), and asymptomatic nonsmokers (*NS*). Panels show the numbers of γH2AX foci per cell in AQP5-positive type I cells (*A*), SPC-positive type II cells (*B*), and CD31-positive endothelial cells (*C*), and the numbers of phosphorylated 53BP1 foci (*D*) and phosphorylated ATM substrate foci (*E*) per cell in SPC-positive type II cells. *Bars* = median values.
Figure 3

A

B

C

D

E

number of γH2AX foci per cell

number of γH2AX foci per cell

number of γH2AX foci per cell

number of phospho-53BP1 foci per cell

number of phospho-ATM substrate foci per cell

NS  S  COPD

NS  S  COPD

NS  S  COPD

NS  S  COPD

NS  S  COPD

P<0.01

P<0.05

P<0.01

P<0.01

P<0.01

P<0.01

P<0.01

P<0.01

P<0.01
Figure 4. Quantitative analyses of apoptosis, cell senescence, proinflammatory phenotypic changes, and oxidative stress in the lungs of COPD patients (COPD), asymptomatic smokers (S), and asymptomatic nonsmokers (NS). The panels show the percentages of alveolar wall cells that expressed active caspase-3 (A) and the percentages of type II cells that expressed p16 (B), phosphorylated NFκB (C), IL-6 (D), and 8-OHdG (E). Bars = median values.
**Figure 5.** Tissue-level analyses of the relationships between the numbers of γH2AX foci per cell and the percentages of cells that stained positive for markers of apoptosis, cell senescence, proinflammatory phenotypic changes, and oxidative stress in the entire group of subjects. The panels show positive correlations between the numbers of γH2AX foci per cell in type II cells and percentages of alveolar wall cells that expressed active caspase-3 (A), and between the number of γH2AX foci per cell in type II cells and percentages of type II cells that expressed p16 (B), phosphorylated NFκB (C), IL-6 (D), and 8-OHdG (E). Circles = asymptomatic smokers; triangles = asymptomatic smokers; squares = COPD patients.
Figure 6. Cellular level analyses of the relationships between the numbers of γH2AX foci per cell and the percentages of cells that stained positive for markers of apoptosis, cell senescence, proinflammatory phenotypic changes, and oxidative stress in COPD patients. Panels A, B, and C show the percentages of type II cells that expressed p16 (A), phosphorylated NFκB (B), and IL-6 (C) according to the numbers of γH2AX foci per cell. Panel D shows the proportion of active-caspase-3-positive alveolar wall cells that exhibited no γH2AX staining, focal γH2AX staining, and diffuse γH2AX staining (see text for explanation). Panel E shows the number of γH2AX foci per cell in type II cells that expressed or did not express 8-OHdG. Each circle indicates the mean value of variables obtained in an individual COPD patient. Bars = median values.
Figure 7. A DNA damage hypothesis for the pathogenetic mechanism of COPD in smokers. Cigarette smoke causes DNA damage at least in part through oxidative stress. If left unrepaired, DNA damage induces apoptosis, cell senescence, and a proinflammatory response of the alveolar wall cells, all of which contribute to the development of COPD. Unrepaired DNA damage also promotes carcinogenesis.
Table 1. Characteristics of the subjects

<table>
<thead>
<tr>
<th>Feature</th>
<th>COPD patients $(n = 14)$</th>
<th>Smokers $(n = 10)$</th>
<th>Nonsmokers $(n = 10)$</th>
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<td>14/0</td>
<td>10/0</td>
<td>3/7</td>
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<tr>
<td>Age, years</td>
<td>$63.2 \pm 2.4$</td>
<td>$61.6 \pm 4.2$</td>
<td>$66.3 \pm 3.3$</td>
</tr>
<tr>
<td>Smoking, pack years</td>
<td>$74.4 \pm 13.2$</td>
<td>$47.0 \pm 7.2$</td>
<td>$0 \pm 0$</td>
</tr>
<tr>
<td>FEV1, liters</td>
<td>$0.90 \pm 0.11^{**}$</td>
<td>$2.37 \pm 0.20$</td>
<td>$2.05 \pm 0.11$</td>
</tr>
<tr>
<td>FEV1/FVC, %</td>
<td>$33.4 \pm 3.3^{**}$</td>
<td>$75.7 \pm 2.2$</td>
<td>$75.3 \pm 1.8$</td>
</tr>
<tr>
<td>FEV1, % predicted</td>
<td>$33.7 \pm 3.8^{**}$</td>
<td>$94.6 \pm 4.0$</td>
<td>$97.2 \pm 5.2$</td>
</tr>
</tbody>
</table>

GOLD stage of COPD severity: II, 2; III, 4; IV, 8

All COPD patients and smokers were ex-smokers. **P<0.01 compared to asymptomatic smokers and nonsmokers.