Cigarette Smoke-Induced Emphysema in A/J mice is Associated with Pulmonary Oxidative Stress, Apoptosis of Lung Cells, and Global Alterations in Gene Expression

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Running Title: Pulmonary Gene Expression Profiling of CS-exposed A/J Mice

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Nonstandard Abbreviations Used: Bronchoalveolar lavage (BAL); Chronic Obstructive Pulmonary Disease (COPD); Cigarette smoke (CS); Months (mos); terminal deoxynucleotidyl transferase-mediated dUTP end-labeling (TUNEL); 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxo-dG)
Abstract

Cigarette smoking is the major risk factor for developing chronic obstructive pulmonary disease, the fourth leading cause of deaths in the United States. Despite recent advances, the molecular mechanisms involved in the initiation and progression of this disease remain elusive. We used Affymetrix Gene Chip arrays to determine the temporal alterations in global gene expression during the progression of pulmonary emphysema in A/J mice. Chronic cigarette smoke (CS) exposure caused pulmonary emphysema in A/J mice, which was associated with pronounced bronchoalveolar inflammation, enhanced oxidative stress, and increased apoptosis of alveolar septal cells. Microarray analysis revealed the up-regulation of 1190, 715, 260, and 246 genes and the down-regulation of 1840, 730, 442, and 236 genes in the lungs of mice exposed to CS for 5 h, 8 days, 1.5 and 6 months, respectively. Most of the genes belong to the functional categories of Phase I genes, Nrf2-regulated antioxidant and Phase II genes, Phase III detoxification genes and others including immune/inflammatory response genes. Induction of the genes encoding multiple Phase I enzymes was markedly higher in the emphysematous lungs while reduced expression of various cytoprotective genes constituting ubiquitin-proteasome complex, cell survival pathways, solute carriers and transporters, transcription factors and Nrf2-regulated antioxidant and Phase II-responsive genes was noted. Our data indicate that the progression of CS-induced emphysema is associated with a steady decline in the expression of various genes involved in multiple pathways in the lungs of A/J mice. Many of the genes discovered in this study could rationally play an important role in the susceptibility to CS-induced emphysema.
Keywords: Microarray, cigarette smoke exposure, genomics, emphysema, lung cancer.
Introduction

Chronic obstructive pulmonary disease (COPD) is the fourth leading cause of death in the United States and is projected to be the number three cause of deaths globally by 2020 (1, 13). COPD is characterized by limitation of airflow that is not fully reversible, which usually progresses together with an abnormal inflammatory response to noxious particles or gases. Pulmonary emphysema is a major manifestation of COPD. The characteristic features of pulmonary emphysema in most patients with COPD are alveolar destruction and abnormal repair (23). A large number of COPD patients suffer from lung cancer (5). Cigarette smoking is the undisputed risk factor for the development of COPD and lung cancer. Despite the well-documented role of cigarette smoking in the etiopathogenesis of the disease, it is unclear what steps are common or different in the pathogenesis of COPD and lung cancer. It remains unclear why only 10–20% of smokers actually develop COPD (34).

Current hypotheses for the development of emphysema include an imbalance between protease and antiprotease activity, and oxidants/antioxidants that are interlinked to the inflammation in the pathogenesis of COPD (3, 7, 31, 44, 45, 58). Oxidant stress is believed to play an important role in the pathogenesis of cigarette smoke (CS)-induced emphysema by potentiating proteolytic damage, inducing endothelial and epithelial cell death, and inhibiting lung repair mechanisms (39, 58). The combined effect of enhanced proteolytic damage, increased cell death, and decreased lung repair leads to emphysematous lung (50).

Studies in animals have revealed the mechanisms by which cigarette smoking leads to the irreversible destruction of terminal airspaces of the lung. Transgenic and null-mutant mouse studies have identified a number of genes and pathways that when altered, result in the morphologic changes of emphysema (14, 32). These studies are limited to demonstrating the
protective or destructive effects attributed to the abrogation or gross over expression of a single
gene. Many studies have aimed at defining the pathogenesis of emphysema using gene
expression profiles of human emphysematous lung tissue (18, 20, 36, 38, 46, 47). However, to
our knowledge, no studies exist utilize temporal global gene expression profile of
emphysematous lung tissues of chronic CS-exposed mice, through the progression of
emphysema. The aim of this study was to determine the differential expression of genes during
the progression of pulmonary emphysema in A/J mice strain, which develop emphysema after
chronic CS exposure.
Materials and Methods

Antibodies and Reagents

Antibodies and reagents used are listed in the materials and methods section of the online supplement of this article.

Animals

Male A/J mice (8 weeks of age) were purchased from Jackson Laboratories (Bar Harbor, ME) and were housed in the animal facilities at Johns Hopkins University. Mice were fed AIN-76A diet and water ad libitum and housed under controlled conditions (23 ± 2°C; 12/12 h light/dark periods). Experiments on animals were conducted in compliance with protocols approved by the Animal Care and Use Committee of the Johns Hopkins Medical Institutions.

Exposure to Cigarette Smoke

A/J mice were divided into five groups: Group I mice (n = 80) were kept in filtered air environment, and Group II, Group III, Group IV, and Group V mice were exposed to 1 day, 8 days, 1.5 months (mos), and 6 mos CS (n = 35 mice/group), respectively, as previously described (39). The cigarette smoke exposure protocol is described in detail in the materials and methods section of the online supplement.

Bronchoalveolar Lavage, Phenotyping and Localization of Macrophages in Lungs

For bronchoalveolar lavage and phenotyping, the mice (n = 7 per group) exposed to acute (1 day) or chronic (6 months) CS were anesthetized and differential count was performed as described in the materials and methods section of the online supplement.

The macrophages in the lung tissues (n=5 mice/group) were stained using Griffonia (Bandeiraea) simplicifolia lectin I isolectin B4 (Vector Laboratories, Burlingame, CA) and
quantified by immunohistochemistry using the procedure described in the materials and methods section of the online supplement.

**Lung Morphometric Measurements**

CS-induced alveolar destruction in the lung was measured using computer-assisted topometric measurements (39). For lung morphometric measurements, the mice exposed to 1.5 months and 6 months of CS or filtered room air were anesthetized with halothane, and the lungs were immediately inflated with 0.5% low-melting agarose at a constant pressure of 25 cm of H$_2$O as previously described (39). The agarose-inflated lungs were then fixed in 10% buffered formalin and embedded in paraffin. Lung sections (5 µm) were stained with hematoxylin and eosin. The mean linear intercept (MLI) and the increase in alveolar diameter (AD) were determined by computer-assisted morphometry with Image Pro Plus software (Media Cybernetics, Silver Spring, MD) (39). The lung sections in each group were coded, and representative images (15 per lung section) were acquired by an investigator blinded to the identity of the slides, using a Nikon E800 microscope using 10X lens.

**Immunohistochemical Detection of 8-oxo-dG**

The occurrence of oxidative stress in the lung sections of the CS-exposed (6 months) or age-matched air-exposed A/J mice was assessed by measuring the level of oxidative stress marker, 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxo-dG) using mouse-anti-8-oxo-dG antibody (QED Bioscience, San Diego, CA) (39). The lung sections were then stained with InnoGenex™ Iso-IHC DAB kit. Normal mouse-IgG1 antibody was used as a negative control. The images of the lung sections were acquired with Nikon E800 microscope using 20X lens. The 8-oxo-dG-positive cells were counted manually.
TUNEL Assay

Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) kit (Oncogene Research Products, San Diego, CA) was used to detect the apoptotic cells in the agarose-inflated lung sections (n = 5 per group) of the 6 months CS-exposed and age-matched air-exposed A/J mice as described in our previous publication (39). The number of apoptotic cells was normalized by the total number of DAPI-positive cells.

Identification of Alveolar Apoptotic Cell Populations in the Lungs

The apoptotic type II epithelial cells and endothelial cells in the lungs were identified by incubating the TUNEL-labeled lung sections with anti-mouse SpC antibody and anti-mouse CD34 antibody. These procedures are described in the materials and methods section of the online supplement.

Immunohistochemical Localization of Active Caspase-3 in the Lungs

Active caspase-3 in the lungs sections from the 6 months CS-exposed mice or age-matched air-exposed mice was localized by immunohistochemical staining using anti-active caspase-3 antibody by following our protocol described earlier (39). Images of the lung sections (10 fields/per lung section) were captured using Nikon Eclipse E800 microscope (Nikon) with a 20X lens, and the number of active caspase-3 positive cells was counted manually.

Oligonucleotide Microarray

Total RNA was extracted from mice lungs using TRIZOL reagent (Invitrogen, Carlsbad, CA). The extracted RNA was purified using the RNeasy mini kit (Qiagen, Valencia, CA). The quality of the RNA was assessed using the RNA 6000 nano assay kits (Agilent Technologies, Palo Alto, CA). The isolated RNA was applied to Mouse Genome 430 2.0 GeneChip arrays (Affymetrix,
Santa Clara, CA) (n = 3 per group) as described previously (39). Scanned output files were analyzed using Affymetrix GeneChip Operating Software (GCOS) Version 1.3 and were independently normalized to an average intensity of 500. Analysis and classification of up-regulated and down-regulated genes in the lungs of A/J mice exposed to air or CS for various time points are explained in the materials and methods section of the online supplement.

**Quantitative Real-Time RT-PCR**

We used quantitative real-time PCR (RT-PCR) to determine the transcriptional induction of antioxidant genes such as heme oxygenase 1 (HO-1), glutathione cysteine ligase catalytic (GCLc) subunit, and glutathione reductase (GSR) in the lungs of air- or CS-exposed mice. The reverse transcription reaction was performed by using the Superscript First-Strand Synthesis System (Invitrogen, Carlsbad, CA) as described previously (40). These analyses were performed using assay-on-demand primers and probe sets in the ABI 7000 Taqman system (Applied Biosystems, Foster city, CA). GAPDH was used for normalization, and all PCRs were assayed in triplicate.

**Statistical Analysis**

Statistical analysis was performed by multiple analysis of variance (ANOVA) using SigmaStat version 2.03 and differences between groups were determined by Student's *t*-test using InStat program.
Results

Histological and Lung Morphometric Studies

H&E staining of the lung sections from the air-exposed A/J mice showed normal alveolar structure (Fig. 1A). However, histologic evaluation revealed enlargement of the air spaces accompanied by the destruction of the normal alveolar architecture in A/J mice after 6 months CS exposure (Fig. 1A). Lung morphometric measurements using Image Pro Plus software revealed a significant increase in the AD and MLI in 6 months CS-exposed mice (AD = 58.2 ± 2.4 µm; MLI = 73.4 ± 3.9 µm) compared to the AD and MLI in the age-matched air-exposed mice (AD = 47.3 ± 1.6 µm; MLI = 55.8 ± 2.3 µm). On the contrary, the lungs from 1.5 months CS-exposed mice showed minimal alveolar destruction. The AD (49.2 ± 1.6 µm) and MLI (60.2 ± 3.2 µm) in 1.5 months CS-exposed mice were significantly lower than the AD and MLI in the 6 months CS-exposed mice (Fig. 1B and C). Histological examination of the lung sections did not reveal any tumors in the lungs of air or sub acute/chronic CS-exposed A/J mice.

Inflammatory Cells in BAL Fluid and Lungs

We analyzed the inflammatory cell population in the BAL fluid using Diff-quick reagent and tissue macrophages using lectin staining. The total number of inflammatory cells (predominantly macrophages) in the BAL fluid from 6 months CS-exposed mice was significantly higher than the acute CS-exposed mice (see supplemental Fig. 1A and B and the result section of the online supplement). Immunohistochemical staining revealed an increased infiltration of macrophages in the (see supplemental Fig. 1C and D and the result section of the online supplement) emphysematous lungs (herein emphysematous lungs refer to the lungs from the mice exposed to 6 months CS) of A/J mice.
Increased Markers of Oxidative Stress in the Emphysematous Lungs

The occurrence of oxidative stress in the lung tissues from 6 months CS-exposed mice was determined using anti–8-oxo-7,8-dihydro-2′-deoxyguanosine (anti–8-oxo-dG) antibody. Immunohistochemical staining revealed a significantly (P ≤ 0.05) increased number of 8-oxo-dG positive cells in the lung sections from the mice exposed to 6 months CS (28.3 anti-8 Oxo-dG positive cells/10 fields) (Fig. 2A and B). Lung sections from air-exposed mice showed few 8-oxo-dG-positive cells (4.2 anti-8 Oxo-dG positive cells/10 fields). Immunohistochemical staining with normal mouse IgG antibody did not show any IgG-reactive cells in the lungs of air- or CS-exposed mice. These results indicated that chronic CS-induced alveolar destruction was associated with enhanced oxidative stress in the lungs of the A/J mice.

Increased Apoptosis of Lung Cells Due to Chronic CS Exposure

Oxidants are key mediators of apoptosis, and apoptosis is thought to contribute to the development of emphysema in animal models and in humans (39, 50). Therefore, we evaluated chronic CS-exposed (6 months) mice for DNA strand breaks in situ by fluorescent TUNEL staining (Fig. 3 A and B) and found an increased number of TUNEL-positive cells in the lungs of chronic CS-exposed mice (87.6 ± 6.3 TUNEL-positive cells/1000 DAPI positive cells) compared with the lungs of age-matched air-exposed mice (6.2 ± 0.48 TUNEL-positive cells/1000 DAPI positive cells). Double staining of the TUNEL-labeled lung sections with anti-SpC (for type II epithelial cells) and anti-CD34 (for endothelial cells) antibodies revealed that apoptosis of these cells occurred in the emphysematous lungs of A/J mice (Fig. 3C).

The occurrence of apoptosis in the emphysematous lungs was further demonstrated by staining the lung sections using anti-active caspase-3 antibody (Fig. 3D and E). Immunohistochemical analysis showed a significantly (P ≤ 0.05) higher number of caspase-3–
positive cells in the alveolar septa of 6 months CS-exposed mice (38.7 ± 3.4 active caspase-3–
positive cells/10 field) than in the age-matched air-exposed mice (4.5 ± 0.5 active caspase-3–
positive cells/10 fields).

**Microarray Analysis During the Course of Development of CS-induced Emphysema**

It is clearly evident from the graph (Fig. 4) that there is a steady temporal decline in the
numbers of genes induced or repressed at each progressive time-point (/length of exposure). In
addition, the numbers of genes up-regulated (see supplemental Table 1) and down-regulated (see
supplemental Table 2) for each individual time-point seem to be in the same order of magnitude.

Figure 5A shows the up-regulation of 1190, 715, 260, and 246 genes in the lungs of A/J
mice exposed to CS for 5 h, 8 days, 1.5 months, and 6 months, respectively. Twenty-one genes
were commonly up-regulated in the lungs during all time points of CS exposure (see
supplemental Table 3). The commonly up-regulated genes include three antioxidant genes
(thioredoxin reductase 1, transcobalamin 2, and selenium binding protein 1), various Phase I
genes [cytochrome p450 (CYP) 1A1, CYP1B1, carbonyl reductase 3, aldehyde dehydrogenase
family 3, subfamily A1, and alcohol dehydrogenase 7], transcription factor forkhead box O3a,
ATP-binding cassette (subfamily A), and semaphorin 7A. Figure 5B shows the down-regulation
of 1840, 730, 442, and 236 genes in the lungs of A/J mice exposed to CS for 5 h, 8 days, 1.5
months, and 6 months, respectively.

**Pathway Changes in Response to Cigarette Smoke**

We have classified the up-regulated (see supplemental Table 1) and down-regulated
genes (see supplemental Table 2) genes into different categories based on their functions. The
total number of up-regulated or down-regulated genes in each functional category are
represented in supplemental Table 4. Cluster analysis of the expression of Phase I genes,
antioxidant and Phase II detoxification genes, Phase III genes, growth factors, genes constituting the ubiquitin-proteasome complex, genes involved in cell survival, apoptosis, and extracellular matrix maintenance are represented in Figure 6. Genes constituting Phase I detoxification pathways remained up-regulated during the course of the study (see supplemental Table 4). However, by day 8 of CS exposure, the expression of more than 39% of the genes constituting multiple cellular pathways were significantly reduced. After 1.5 months and 6 months of CS-exposure, the expression of genes constituting different biological pathways were markedly reduced (see supplemental Table 4).

Comparison with previously published studies of genome wide transcriptional analysis of human COPD (18, 36) and rat (48) showed little overlap for differentially expressed genes (see supplemental Table 5). Down-regulated genes in the lungs of A/J mice exposed to CS whose deletion or overexpression has been shown to contribute to the pathogenesis of spontaneous emphysema in various mice models are listed in the supplemental Table 6.

Validation of Antioxidant Gene Expression by Real Time RT-PCR

We used real time RT-PCR analysis to confirm the relative expression of three classical antioxidant and phase II detoxification genes such as HO-1, GCLc, and GSR. Real time RT-PCR results revealed a significantly ($P \leq 0.05$) reduced expression of these three antioxidant and phase II detoxification genes in the lungs of the mice exposed to CS for 8 days, 1.5 months, and 6 months compared to the lungs of the mice exposed to CS for 1 day (see supplemental Fig. 2) and were in agreement with the microarray results (see supplemental Table 1).
Discussion

Due to the poor understanding of pathogenesis of COPD, effective therapies are not available, and there is an urgent need to develop new therapeutic targets for treatment of COPD. The aim of this study was to identify molecular pathways, which could be responsible for the damaging consequences of cigarette smoke exposure in the lungs of A/J mice (susceptible to lung cancer) for various time periods using microarray analysis.

The current hypothesis of emphysema disease pathogenesis suggests that inflammation is a contributing factor for the genesis of pulmonary emphysema, which is associated with a chronic inflammatory response predominantly in small airways and lung parenchyma and is characterized by increased number of macrophages, neutrophils, and T lymphocytes. Many inflammatory proteases, peptides, chemokines, cytokines, lipid mediators, reactive oxygen, and nitrogen species, and growth factors are involved in orchestrating the complex inflammatory process that results in alveolar destruction (44, 58). Analysis of differential cell counts in the BAL fluid revealed a significant increase in the number of total inflammatory cells, and macrophages were predominantly higher in the lungs of 6 months CS-exposed mice compared to age-matched air-exposed mice. These BAL alterations in response to chronic CS exposure were associated with increased number of macrophages in the lung tissues.

CS-induced inflammatory response was associated with the expression of more than 56 and 23 inflammatory genes in the lungs of acute and 6 months CS-exposed mice, respectively. Multiple cytokines/chemokines and their receptors [such as monocyte chemo attractant protein 1 (MCP-1 or CCL2), chemokine (C-C motif) ligand 5 (CCL5), chemokine (C-C motif) ligand 6 (CCL6), chemokine (C-C motif) receptor 2 (CCR2)], growth factors [platelet derived growth factor D (PDGF-D) and vascular endothelial growth factor (VEGF)], and endothelin were
induced by exposure to CS. All these genes have been shown to be involved in the trafficking/accumulation of macrophages. PDGF-D attracts macrophages (51) and so are the chemokines like MCP-1 (12), CCL5 (52), CCL6 (29) and endothelin (2, 4). MCP-1 and CCR2 were involved in the recruitment of macrophages and mast cells into the airway epithelium in COPD (12). Microarray analysis revealed the expression of endothelin in the lungs of day 1 and 1.5 months CS-exposed mice and expression of CCL6 in the lungs of 6 months CS-exposed mice (see supplemental Table 1). Intraperitoneal injection of thioglycollate increased peritoneal CCL6, and neutralization of CCL6 significantly inhibited the macrophage infiltration in a murine model of acute peritonitis (29). Endothelin play a critical role in the recruitment of monocyte/macrophages (4, 15) and neutrophils (4) in animal models. Both matrix metalloproteinase-12 (MMP-12) and elastin fragments generated by MMP-12 have shown to contribute to the accumulation of macrophages and the pathogenesis of CS-induced emphysema in mice (21, 25). However, CS did not up-regulate or down-regulate the expression of MMP-12 in the lungs of A/J mice.

Even though acute CS exposure induced the expression of more than 50 genes concerned with inflammatory processes, surprisingly only 50% of these genes were up-regulated in the emphysematous lung tissues of A/J mice. Reduction in the differential expression of these genes could be due to the loss of lung tissues or due to the immunosuppressive effects caused by chronic CS. The following genes were up-regulated more than 5 fold in response to acute CS: interleukin 6, interleukin 1 receptor (type II), double C2 (beta), chemokine (C-X-C motif) ligand 5 (CXCL5), suppressor of cytokine signaling 3 (SOCS3), calcitonin/calcitonin-related polypeptide (alpha), cathelicidin antimicrobial peptide, pentaxin related gene, prostaglandin-endoperoxide synthase 2, and nuclear factor, interleukin 3, regulated gene. CXCL5 was the
predominantly expressed inflammatory gene both in acute and 6 months CS-exposed lungs. Other inflammatory genes expressed in the emphysematous lung tissues include pre-B-cell colony-enhancing factor 1 (PBEF), inhibitor of kappa B kinase gamma, CXCL 5, the RANTES receptor (chemokine [C-C motif] receptor 1), ankyrin repeat and SOCS box-containing protein 5, SOCS3, CD163 antigen, and CD14 antigen. PBEF is a novel candidate gene induced in mechanical stress-induced acute lung injury (57). The inhibitor of kappa B kinase gamma is the regulatory subunit of Ikappa B kinase (IKK) complex, which serves as the master regulator for the activation of NF-kappa B by various stimuli (43). SOCS3 is an important negative regulator of IL-6. Mice in which the SOCS3 gene was deleted in all hematopoietic cells developed neutrophilia and a variety of inflammatory pathologies (59). CD163 is a member of the macrophage scavenger receptors and is expressed on most subpopulations of mature tissue macrophages. CD14 is a well characterized pattern recognition receptor, which binds to LPS and other bacterial derived components and plays a significant role in the development of Th1 response (60). CXCL5 is an epithelial cell-derived neutrophil activating peptide (ENA-78) and has been shown to activate neutrophils and possesses angiogenic properties (28, 49). Cigarette smoke exposure significantly inhibited the expression of various genes involved in humoral immune responses, different B cell, T cell and dendritic cell receptors/cell surface antigens, and various inflammatory cytokines and chemokines. Cigarette smoke exposure also down-regulated more than 20 interferon-regulated genes, toll-like receptor (Tlr)1, Tlr3, and Tlr4, which play a key role in the innate immune system (see GEO Accession #: GSE8790). Mice deficient in Tlr4 develop spontaneous emphysema (63).

Emphysema associated with the adult lung is thought to result from the progressive proteolytic destruction of extracellular matrix without adequate repair, occurring through an
imbalance in proteinase-antiproteinase activity (23, 24). Studies using knock out and transgenic animal models have revealed contribution of neutrophil elastase, macrophage metalloproteinases, and cysteine proteinases in the development of CS-induced emphysema (23, 45, 58). Acute CS exposure induced the expression of 30 genes involved in extra cellular matrix homeostasis. The genes that were up-regulated both in the acute and chronic CS-exposed lung tissues included a disintegrin and metalloprotease domain 8, ADAM metallopeptidase with thrombospondin type 1 motif, 15 (Adamts15), elastin, and serine proteinase inhibitor, clade A, lysyl oxidase, and naked cuticle 1 homolog. Cathepsin K and fibrinogen gamma polypeptide were uniquely expressed in the emphysematous lung tissues. Cathepsin K is the most potent mammalian elastase yet described (9, 17). Cathepsin K also possesses a unique collagenolytic activity and has been shown to cleave both type I and type II collagen (9, 17). A recent study (6) using cathepsin K knockout mice demonstrated the pivotal role of cathepsin K in collagen metabolism and lung matrix homeostasis. Chronic CS exposure also resulted in the down regulation of 51 genes involved in the maintenance of the lung extracellular matrix. The genes down-regulated in the emphysematous lungs include various types of collagen genes; elastin microfibril interface 1; transforming growth factor, beta 2; latent transforming growth factor beta binding protein 4; matrix metalloproteinases 11 (Mmp11) and Mmp15; serine (or cysteine) proteinase inhibitor, clade H, member 1; a disintegrin-like and metalloproteinase with thrombospondin type 1 motif 1 (Adamts1); and lysyl oxidase like 1 gene (see GEO accession #: GSE8790). Mice lacking the protein lysyl oxidase–like 1 do not deposit normal elastic fibers and develop airspace enlargement in the lungs (30).

Oxidative stress plays an important role in the pathogenesis of pulmonary emphysema. Like protease/antiprotease and inflammation, oxidant/antioxidant imbalance is central to the
process of tissue destruction and apoptosis in CS-induced emphysema (39, 58). There is overwhelming evidence of presence of markers of oxidative stress in smoker's lung, which is caused by chronic inflammation and oxidants in the smoke (31). Oxidative stress enhances inflammation, inactivates critical antiproteinase inhibitors such as AAT, and enhances apoptosis of alveolar cells (58). The transcription factor Nrf2 plays a critical role in protecting the lungs against CS-induced inflammation and oxidative stress, and alveolar cell apoptosis by up-regulating multiple antioxidant, Phase II and Phase III detoxification genes. The importance of antioxidants in emphysema susceptibility was supported by our recent work using the mice deficient in transcription factor Nrf2 (39). We observed an increased expression of 8-oxo-dG, the marker of oxidative stress, in the emphysematous lung tissues of chronic CS-exposed mice. The increased oxidative stress in the emphysematous lung tissues of susceptible A/J mice was associated with reduced differential expression of various Nrf2-regulated pulmonary antioxidant genes, Phase II and Phase III detoxification genes as well as various Nrf2-binding partners. In response to acute CS exposure, 27 antioxidant and Phase II detoxification genes were up-regulated in the lung tissues of A/J mice, and the majority of these genes were regulated by Nrf2 (see supplemental Table 1). Of these 27 genes, 10 genes were up-regulated only in the acute CS-exposed lungs, and there was a significantly reduced expression of 6 Nrf2-regulated genes (heme oxygenase 1, glutathione S-transferase, alpha 2 [GSTα2], metallothionein 1, metallothionein 2, thioredoxin reductase 1, and glutathione reductase 1) in the emphysematous lung tissues compared to the acute CS-exposed lungs. This reduced expression was consistent with the low level induction of v-maf musculoaponeurotic fibrosarcoma oncogene family, protein F (MafF), the positive regulator of Nrf2, as well as reduced differential expression of Nrf2-phosphorylating casein kinases and other positive regulators of Nrf2 such as MafG, MafK, CCAAT/enhancer
binding protein (C/EBP beta), C/EBP delta and activating transcription factor IV. Among the Phase II detoxification genes, NAD(P)H dehydrogenase, [quinone] 1 (Nqo1) was the only gene with significantly higher expression in the emphysematous lung tissues compared to the acute CS-exposed lungs. The genes up-regulated only in the emphysematous lung tissues were ferritin light chain 2, ceruloplasmin 1, uridine 5’-diphospho (UDP)-glucuronosyltransferase 1 family, member 2, and 4 glutathione S-transferases (GSTp2, GSTμ1, GSTα3, and microsomal GSTα2).

Similar to antioxidant and Phase II genes, acute CS induced the expression of more than 13 Phase I detoxification genes in the lungs. The oxidative metabolism process in the Phase I system is mainly mediated by the Cytochrome (CYP) P-450 family or flavin function oxidases. Among the Phase I genes, CYP1A1, CYP1B1, and aldehyde dehydrogenase family 3 were highly expressed in the lungs of acute and 6 months CS-exposed mice. In contrast to classical Nrf2-regulated antioxidant and Phase II detoxification genes, the expression of Phase I genes such as CYP1A1, CYP1B1, carboxyl esterase 1, and alcohol dehydrogenase 7 were significantly increased in the emphysematous lungs. Numerous carcinogens such as polycyclic aromatic hydrocarbons and nitrosamines are metabolized by cytochrome P-450 enzymes. Among all up-regulated genes, CYP1A1 is the predominant gene with more than 100-fold expression in the emphysematous lungs. However, microarray analysis revealed inhibition of aryl hydrocarbon receptor, reduced expression of aryl hydrocarbon receptor nuclear translocator, and higher expression (30-fold) of aryl-hydrocarbon receptor repressor in the emphysematous lung tissues. These results suggest the possible role of other transcription factor(s) in the transcriptional induction of CYP1A1 and 1B1 genes and warrant further investigation.

In response to acute CS exposure, 40 genes constituting solute carrier and channel proteins and Phase III multidrug transporters were up-regulated in the lungs of A/J mice.
compared to the up-regulation of only 7 such genes in the emphysematous lungs. Acute CS exposure also down-regulated 9 antioxidant and Phase II detoxification genes, 27 Phase I genes, and 98 solute carrier, and Phase III genes. Several ABC transporter proteins are believed to play an important role in preventing the accumulation of potentially harmful xenobiotics in the lung (42). Even though potassium channel openers (37) and inhibitors of calcium-activated chloride channels (41) are being developed for the treatment of COPD, very little is known about the role of solute carrier and channel proteins in the pathogenesis of CS-induced emphysema.

Pathogenesis of lung diseases such as COPD and lung cancer is tightly linked to environmental chemicals, most notably tobacco smoke. Many of the compounds associated with these diseases require Phase I enzymatic activation to exert their deleterious effects on pulmonary cells. These activated hydrophobic xenobiotics are converted into hydrophilic forms via conjugation reactions catalyzed by Phase II enzymes. The metabolites generated by Phase I and Phase II reactions are excreted from the body with the aid of Phase III detoxification systems. The results of the present study revealed that the pathogenesis of pulmonary emphysema in A/J mice is clearly associated with increased expression of major Phase I genes and reduced expression of various Phase III and Nrf2-regulated antioxidant and Phase II detoxification genes in the emphysematous lung tissues. Our findings are similar to the findings in the recent study in humans (38), which revealed the up-regulation of CYP1A1 and CYP1B1 genes in both comparisons i) COPD vs heavy smokers and ii) heavy smokers vs non-smokers. The expression of a number of genes involved in oxidant stress responses were increased in heavy smokers compared with non-smokers. This expression of oxidant response genes further increased in individuals with chronic productive cough, but no significant airflow limitation (i.e. COPD Stage
0) with even further increase in Stage 1 COPD was noted. In more severe COPD, there was a fall in mean expression levels noted in Stage 0.

Apoptosis plays an important role in the pathophysiology of lung diseases (22, 58). Gene expression profiling revealed the expression of various genes constituting both the death receptor-induced extrinsic apoptotic pathway and the mitochondrial-regulated intrinsic apoptotic pathway in the lungs after CS exposure. CS induced the expression of 13 and 7 genes in the lungs of acute and chronic CS-exposed mice, respectively. Death associated protein kinase 1 and 2,3,7,8-Tetrachlorodibenzo-p-dioxin -inducible (TCDD)-inducible poly(ADP-ribose) polymerase were expressed in the lungs of the mice exposed to CS for 1 day, 1.5 months, and 6 months. Death associated protein kinase 1 is a calcium/calmodulin-dependent serine/threonine kinase and functions as a positive mediator of apoptosis induced by a variety of stimuli, such as Fas, TNF-α, transforming growth factor beta (TGF)-β, ceramide, and cellular myelocytomatosis viral oncogene (c-myc). The apoptotic genes uniquely up-regulated in the emphysematous lungs were Bcl-2/adenovirus E1B 19 kDa-interacting protein 1, NIP3 (BNIP3), p53 apoptosis effector related to PMP-22 (PERP)-TP53 apoptosis effector, cytochrome c oxidase, subunit VIIIb, cell death-inducing DNA fragmentation factor, alpha subunit-like effector A (CIDE-A), and cell death-inducing DFFA-like effector c. BNIP3, a pro-apoptotic protein in the Bcl-2 family, is a central regulator of mitochondrial membrane permeability (61). PERP is an apoptosis-associated target of the p53 tumor suppressor gene and is involved in DNA damage-induced apoptosis (26). CIDE-A is a 40 kDa caspase-3-activated nuclease that is associated with the regulation of the apoptosis/DNA fragmentation pathway (27). Forkhead box O3 is implicated in the regulation of a variety of cellular processes, including cell cycle, p53 dependent apoptosis, DNA repair, and stress resistance (16). Chronic exposure to CS resulted in increased apoptosis of alveolar septal
cells in the lungs of A/J mice. Staining of the TUNEL-labeled lung sections with anti-SPC and anti-CD34 antibodies revealed the apoptosis of type II epithelial and endothelial cells, respectively, in the emphysematous lungs. Alveolar cell apoptosis has been recognized as a critically important mechanism of alveolar septal destruction in emphysema. The presence of enhanced apoptosis in the lungs of chronic CS-exposed mice might be related to enhanced oxidative stress, inflammation, or excessive lung proteolysis (58). Apoptosis in the emphysematous lung tissues was consistent with reduced differential expression of various genes constituting the cell survival pathways. Microarray analysis revealed the up-regulation of 43 and 5 cell survival genes in the lungs from acute and 6 months CS-exposed mice, respectively. Most of the genes up-regulated in the acute CS-exposed lungs constitute the cell survival pathways mediated by phosphoinositide 3 kinase (PI3K)/Akt (protein kinase B). Endothelial cell survival by Akt is driven by the modulation of a series of intrinsic cellular pathways that include protein tyrosine kinase and the G protein-coupled receptor, PI3K, the receptors for vascular endothelial growth factor (VEGF) such as FMS-like tyrosine kinase 1 (Flt 1) and FMS-like tyrosine kinase 4 (Flt 4), Ras/mitogen activated protein kinases, endothelial nitric oxide synthase 3, and Bcl-2. Several growth factors, such as epidermal growth factor and basic fibroblast growth factor, which were induced in the acute CS-exposed lungs, are known to provide endothelial cell survival by inhibiting endothelial cell apoptosis. These growth factors inhibit endothelial cell apoptosis by upregulating the expression of the anti-apoptotic proteins Bcl-2 and endothelial nitric oxide synthase by activating PI3/Akt pathways (10). Furthermore, the interaction of cells with the extracellular matrix via integrin beta-1 also provides a potent survival signal (35). Association of the alpha1beta1 integrin with the adaptor protein Shc can regulate cell survival and cell cycle progression via the Ras/MAPK/extracellular signal-regulated kinase pathway (10).
The cell survival genes up-regulated in the emphysematous lung tissues were fibroblast growth factor binding protein 1, fibroblast growth factor 13, angiopoietin-like 4, mitogen-activated protein kinase kinase kinase 6, and forkhead box O3a.

Strain A/J mice have a relatively high spontaneous adenoma/adenocarcinoma incidence and following exposure to a carcinogen, readily develop lung tumors (33). Even though CS induced the expression of more than 54 cancer associated genes in the acute CS-exposed lungs and 5 oncogenes in the emphysematous lungs of A/J mice (see supplemental Table 1), histological examination did not reveal any tumors in the lungs of CS/age-matched air-exposed control mice. Recent studies (53, 56) indicate that ETS is a lung carcinogen in A/J mice. However, following ETS exposure, a recovery period of air is necessary to fully reveal the tumorigenic action of cigarette smoke in A/J mice (56).

The net result of alveolar cell death on alveolar structure depends on the lung's ability to undergo cell proliferation, which is pivotal for the maintenance of normal tissue homeostasis. The data on the balance of apoptosis versus cell proliferation have been discrepant (58). We observed the expression of 52 genes involved in cell adhesion and cell cycle/proliferation in acute CS-exposed lungs compared to the expression of 7 genes in the emphysematous lungs. The cell cycle regulator cyclin-dependent kinase inhibitor 1A (p21) gene is predominantly expressed in both acute (~46-fold) and 6 months (~9-fold) CS-exposed lungs of A/J mice. Progression through the cell cycle is regulated by cyclins and cyclin-dependent kinases. The cyclin kinase inhibitor p21 can induce G1 arrest and senescence in a variety of cell types and has been shown to augment TGFβ, cigarette smoke and LPS induced inflammation and alveolar destruction in mice (54, 55). Microarray analysis also showed reduced differential expression of various growth factors, ubiquitin-proteasome complex, heat shock proteins (see the discussion section of the online
supplement), transcription factors and multiple genes involved in signal transduction, cytoskeleton reorganization, and lipid metabolism in the emphysematous lungs compared to the lungs of the acute CS-exposed mice (see supplemental Table 1).

We compared the differentially expressed genes in the emphysematous lungs of AJ mice with the gene expression profile of lung tissues from human COPD (18, 36) and pulmonary gene expression profile of chronic CS-exposed rats (48). In a report, using serial analysis of gene expression (SAGE) and microarray analysis, Ning et al. (36) have identified several genes and many of them have not been previously associated with COPD. The 11 commonly expressed genes reported by Ning et al. (36) and the present study were as follows: cyclin-dependent kinase inhibitor 1A (P21), fibrinogen, gamma polypeptide, heme oxygenase (decycling) 1, lectin, galactose binding, soluble 3, phosphogluconate dehydrogenase, protein tyrosine phosphatase 4a1, receptor (calcitonin) activity modifying protein 2, receptor (calcitonin) activity modifying protein 3, secretoglobin, family 3A, member 1, selenium binding protein 1, and solute carrier family 40 (iron-regulated transporter), member 1. Early growth response 1 gene (Egr-1) gene has been shown to be expressed in lung tissues from patients with late stage emphysema (62). Egr-1 plays an important role in the regulation of matrix metalloproteinase 9 (MMP-9) (36) and cigarette smoke induced autophagy (11). Acute CS exposure significantly induced the expression of Egr-1 in the lungs of A/J mice. However, Egr-1 was not induced in the lungs from the mice exposed to subacute and chronic CS exposure. There was no commonality in the genes (up-regulated) reported in this study and reported by Golpon et al. (18). Comparison of microarray data identified 11 genes commonly expressed in the emphysematous lungs of A/J mice and in the lungs of chronic CS-exposed rats (48). The commonly expressed genes were aldehyde dehydrogenase family 3 (subfamily A1), CYP1B1, GCLc, GCLm, glutamate-ammonia ligase,
hexokinase 2, interleukin 1 receptor (type II), metallothionein 1, NAD(P)H dehydrogenase, quinone 1, and thioredoxin reductase I components of the cigarette smoke. These results clearly suggest the need for caution when extrapolating data on emphysema development in one species of rodents to another and also for its implications in humans.

Mice strains differ in their susceptibility to CS-induced emphysema. The role of strain difference in the response to cigarette smoke was investigated in various mice models (8, 19). Mice of the strains C57BL/6J, SJ/L, and A/J were moderately susceptible to CS-mediated emphysema and AKR/J mice were super susceptible to pulmonary emphysema. However, NZWLac/J and ICR did not develop emphysema after chronic CS exposure. The factor(s) which determines the resistance to CS-emphysema was largely not known. In our previous study (39), using ICR mice deficient in the transcription factor Nrf2, we showed the importance of intrinsic antioxidant defenses in protecting the lungs against CS-induced emphysema. Disruption of the Nrf2 gene in the emphysema resistant ICR mice strain led to earlier-onset and more extensive CS-induced emphysema after chronic CS exposure. Microarray analysis revealed the expression of as many as 120 genes and most of them are antioxidant and Phase II detoxification genes in the lungs of ICR mice after acute CS exposure. In contrast, acute CS induced the expression of more than 1190 genes in the lungs of emphysema susceptible A/J mice strain. These results clearly suggest the marked difference in the gene expression pattern in the lungs of emphysema resistant and emphysema susceptible mice strains. Similar to the up-regulated genes, microarray analysis revealed a steady decline in the number of down-regulated genes during the progression of CS-induced pulmonary emphysema. We have classified the down-regulated genes into different functional categories. Some of the down-regulated genes whose deletion or overexpression has been shown to cause spontaneous/age dependant emphysema are listed in the supplemental Table.
6. Future studies are needed to delineate the molecular pathways that are altered in different emphysema resistant and susceptible mice strains using microarray and proteomic technologies.

Animal models play an important role in the understanding of the pathogenesis COPD. However, both transgenic and gene-targeted models suffer limitations and their applicability to COPD in human may depend on several factors, including the disease model and similarities in mouse structure and function between species. Mouse and human clearly share many basic physiological processes, but the details of how gas exchange is achieved will determine how closely findings in mice can be applied to human (2). Hence, careful translation of the findings from studies in mouse to human is required.

Conclusions

In conclusion, CS exposure significantly influenced the expression of numerous genes in the lungs of A/J mice. Importantly, the progression of pulmonary emphysema is associated with a steady decline in the differential of expression of various genes constituting multiple pathways, speculatively participating in the pathogenesis of COPD. Acute CS exposure induced the expression of more than thousand genes in the lungs. A decline in the differential expression of 40% genes in the lungs after subacute CS exposure and more than 80% genes in the lungs after subchronic and chronic CS exposure was noted. The decline in the expression of genes during subacute to chronic CS exposure is consistent with the reduction in the differential expression of more than 75 – 90% transcription factors in lungs. Further investigation is needed to delineate the role of factors contributing to the reduced differential expression of genes due to exposure to CS.

The expression of genes constituting cell survival pathways, ubiquitin-proteasome complex, and various heat shock proteins was significantly affected as early as during subacute
CS exposure. However, the expression of the majority of growth factors, solute carrier and Phase III genes, inflammatory genes; genes involved in cell adhesion/cell cycle/cell proliferation; and genes constituting signal transduction pathways were affected during subchronic and chronic CS exposure. In contrast, CS did not significantly affect the expression of multiple genes constituting Phase I detoxification pathway, and Cyp1A1 was predominantly expressed in the emphysematous lungs of A/J mice. The strength of expression of multiple antioxidant and Phase II detoxification genes in the emphysematous lungs was reduced after chronic CS exposure, which is consistent with the enhanced expression of marker of oxidative stress, increased infiltration of inflammatory cells and apoptosis of alveolar septal cells. Exposure of the mice to CS also resulted in the down regulation of multiple key genes such as lysyl oxidase-like 1, macrophage-colony stimulating factor, platelet derived growth factor alpha, latent transforming growth factor beta binding protein 4, integrin β6, toll-like receptor 4, interleukin 1 beta, elastin, and fibrillin 1 in the lungs. Deletion or overexpression of these down-regulated genes has been shown to contribute to the pathogenesis of pulmonary emphysema in various mice models. Our data indicate that the pathogenesis of emphysema in susceptible mice strain is unlikely entirely caused by inflammation or deletion of a single gene. Instead, reduction in the differential expression of various genes constituting cell survival pathways, ubiquitin-proteasome complex, heat shock proteins and multiple transcription factors during subacute CS exposure along with alterations in the expression of various genes involved in cell adhesion/cell cycle/cell proliferation, cytoskeletal reorganization, extracellular matrix production, solute carrier and Phase III genes, and Phase II detoxification and antioxidant genes during subchronic and chronic CS exposure are likely to be involved in the pathogenesis of pulmonary emphysema. Results of the present study not only support the possible involvement of some of the previously reported
genes and pathways in the pathogenesis of the disease, but also suggest the alterations of some novel potential pathways during the progression of CS-mediated pulmonary emphysema.

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Footnotes

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References


Figure Legends

Figure 1 Increased air space enlargement in A/J mice exposed to chronic CS. A) H&E stained lung sections from A/J mice exposed to room air or CS at the indicated time points. Lung sections from the 6 months CS-exposed mice show increased alveolar destruction and air space enlargement compared with the lung sections from age-matched air-exposed A/J mice. Sections from the age-matched air-exposed mice show normal alveolar structure (n = 5 mice per group); Original magnification, X20. The images (15 fields/slide) of the H&E stained lung sections from the air- and CS-exposed mice were acquired with a Nikon E800 microscope and alveolar diameter (B) and mean linear intercepts (C) were determined by computer-assisted morphometry with the Image Pro Plus software. Six months CS-exposed A/J mice show a significantly increased alveolar diameter (B) and mean linear intercept (C) compared to 1.5 months CS-exposed mice. Data are mean ± SEM. p ≤ 0.05.

Figure 2 Increased oxidative stress in the lungs of chronic CS-exposed A/J mice. A) The occurrence of oxidative stress in the lungs of 6 months CS-exposed mice was determined by immunohistochemistry with anti-8 Oxo-dG antibody. Lung sections from the CS-exposed A/J mice show increased staining for 8-oxo-dG (indicated by arrows) compared with lung sections from the age-matched air-exposed control mice. Normal mouse IgG1 was used as a negative control (magnification, X20). (B) Quantification of 8-oxo-dG–positive cells in lungs after 6 months of CS exposure. Increased number (28.3 anti-8 Oxo-dG positive cells/10 fields) of 8 Oxo-dG positive cells were detected in the lungs of CS-exposed mice. Few (4.2 anti-8 Oxo-dG positive cells/10 fields) 8 Oxo-dG positive cells were detected in the lung tissues of air-exposed mice (n = 5 mice per group). Values (positive cells/10 fields) are represented as mean ± SEM, *p ≤ 0.05 vs air exposed A/J mice.
Figure 3 Chronic cigarette smoke exposure causes alveolar cell apoptosis in A/J mice. (A) Lung sections (n = 5) from air or CS-exposed (6 months) mice was subjected to TUNEL (middle column) and DAPI (left column) staining. Merged images are shown in the right column. Overlapping DAPI in blue and FITC in green create a magenta, apoptotic-positive signal. (B) CS-exposed mice (6 months) showed abundant TUNEL-positive alveolar septal cells (arrows) compared to air-exposed mice (n = 5 mice per group). In each image, the number of DAPI-positive (blue signal) and apoptotic cells (magenta) were counted manually. Apoptotic cells were normalized by the total number of DAPI-positive cells. Values are represented as mean ± SEM. p ≤ 0.05. (C) Identification of apoptotic type II epithelial cells (upper panel) and endothelial cells (lower panel) in the lungs of CS-exposed mice (6 months). Type II epithelial cells and endothelial cells were detected using anti-SpC and anti-CD34 antibodies, respectively. Nuclei were detected with DAPI (blue). The merged images with colocalization of cell-specific markers (cytoplasmic red signal) with apoptosis (green FITC + blue DAPI) signal, resulting in lavender-like signal (yellow arrows) are shown. D) Active caspase-3 expression in lung sections from chronic CS-exposed (6 months) mice. CS-exposed A/J mice show increased numbers of caspase-3–positive cells (indicated by arrows) in the lungs (n = 5 per group) (magnification, X20). (E) Number of caspase-3–positive cells in the lungs of age-matched air or CS-exposed mice. Caspase-3–positive cells were significantly (P ≤ 0.05) higher in the lungs of 6 months CS-exposed mice than the air exposed mice. Values are represented as mean ± SEM.

Figure 4 Graph depicting the numbers of differentially expressed genes (up-regulated or down-regulated) at each exposure time-point. Solid black bars above the horizontal line represent the number of up-regulated genes at each time point. Similarly, hollow bars below the horizontal line represent the number of down-regulated genes at the same time points.
Figure 5 Venn diagrams depicting the extent of overlap between the gene expression profiles from the lungs of mice exposed to CS for various time periods. Groups were compared and genes that satisfied the criteria of fold change ≥ 1.5 and \( p \leq 0.05 \) were designated as significantly changed genes. The number of genes summarized in the Venn plot are as follows: circles shaded red, green, blue, and grey represent the total numbers of genes up-regulated (A) or down-regulated (B) with CS exposure at 5 h, 8 days, 1.5 months, and 6 months, respectively.

Figure 6 Pulmonary gene expression profiles of A/J mice exposed to CS. Cluster analysis shows the expression of Phase I genes, antioxidant and Phase II detoxification genes, Phase III genes, growth factors, genes constituting the ubiquitin-proteasome complex, genes involved in cell survival, apoptosis, and extracellular matrix maintenance. The genes are visualized by Treeview. Green indicates up-regulated genes and black represents no significant change in expression. Gene symbols are provided.
Fig. 4

![Bar chart showing the number of upregulated (black) and downregulated (white) genes. The total number of genes is 1840, with 1190 upregulated and 715 downregulated. In the second stage, 260 genes are upregulated and 442 are downregulated. In the final stage, 246 genes are upregulated and 236 are downregulated.](image-url)