Molecular Processes that Drive Cigarette Smoke–Induced Epithelial Cell Fate of the Lung

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Abstract

Cigarette smoke contains numerous chemical compounds, including abundant reactive oxygen/nitrogen species and aldehydes, and many other carcinogens. Long-term cigarette smoking significantly increases the risk of various lung diseases, including chronic obstructive pulmonary disease and lung cancer, and contributes to premature death. Many *in vitro* and *in vivo* studies have elucidated mechanisms involved in cigarette smoke–induced inflammation,

DNA damage, and autophagy, and the subsequent cell fates, including cell death, cellular senescence, and transformation. In this Translational Review, we summarize the known pathways underlying these processes in airway epithelial cells to help reveal future challenges and describe possible directions of research that could lead to better management and treatment of these diseases.

Keywords: cigarette smoke; inflammation; autophagy; DNA damage; cellular senescence

A number of *in vitro* studies have been conducted to determine the effects of cigarette smoke (CS) on various endpoints, including gene expression (1, 2), inflammation (3, 4), DNA damage (5, 6), and cell fates in lung epithelial cells (7–11). Fewer studies have evaluated the CS effects on epithelial cells harvested by bronchial brushings from humans (e.g., transcriptome analyses) (12–14). Certainly *in vivo* studies provide very valuable data that may help us understand the complex effects of CS in whole-organ settings that involve, for example, cell–cell interactions. However, human airway epithelial cells (HAECs) cultured at the air–liquid interface can recapitulate many of the airway transcriptome alterations in airway epithelial cells (AECs) in smokers (1). Therefore, *in vitro* models, especially when they replicate target signals at similar magnitudes to those observed by CS *in vivo*,

are crucial for the following reasons. First, *in vitro* systems are more feasible and convenient for biochemical studies, such as expression analyses starting from transcriptional and translational regulation, protein stability, and protein–protein interaction, because a single cell type can be studied more readily than in *in vivo* systems. They provide the possibility to modulate the target protein expression by various molecular tools to determine the biological relevance of altered expression. Second, *in vitro* studies can be done under well controlled conditions with easy adjustments to the dose and time of CS exposure, without the many confounding factors that need to be considered in human studies, including the intermittent nature of exposures to CS and other air pollutants that, together with age, sex, comorbidities, state and type of infection, and use of medications, can strongly alter the

biological response of epithelial cells to CS. Third, *in vitro* systems are more economical, practical, and reproducible for proof-of-concept drug discovery studies that can then be tested in the more complex setting of *in vivo* systems (15).

However, results from *in vitro* studies should always be viewed with caution when trying to extrapolate to settings *in vivo*. Selection of cell lines should be carefully considered, because the methods of immortalization may influence certain endpoints of interest. For example, cell lines immortalized by the E6E7 oncoprotein, derived from human papilloma virus, are not suitable for a model of normal DNA damage response (DDR), as E6E7 inhibits p53 and retinoblastoma protein (Rb) (16). Because *in vitro* systems usually lack complex interactions with other cell types, the findings *in vitro* may not reflect responses *in vivo*. Disease process, such as

(Received in original form August 5, 2013; accepted in final form October 7, 2013)

This work was supported by American Lung Association biomedical research grant RG-231988-N, AG037768 (T.N.), HL068111 and ES015482 (Y.T.), and the Tobacco Master Settlement through a cooperative research agreement with the University of New Mexico.

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Am J Respir Cell Mol Biol Vol 50, Iss 3, pp 471–482, Mar 2014

Published 2014 by the American Thoracic Society

Originally Published in Press as DOI: 10.1165/rcmb.2013-0348TR on October 10, 2013

Internet address: www.atsjournals.org

those that occur in chronic obstructive pulmonary disease (COPD), can be affected by the complex cytokine milieu established by inflammatory cells, in addition to the CS exposure. Furthermore, a modifying factor of the CS effects on airway inflammation is extracellular components of airway secretions. For example, CS exposure enhances the deposition of hyaluronan (HA) fragments in the lungs (17). Through the HA receptors, layilin and Toll-like receptors (TLRs) 2 and 4, HA modulates inflammatory responses via layilin-mediated down-regulation of E-cadherin (18) or TLR2/4-mediated activation of NF- κ B (19).

Here, we review CS-induced effects on inflammation, DNA damage, and autophagy, and the subsequent fates of the cells, including cell death and cellular senescence. Because the many cell types of the lung can respond differently to CS, this *Review* focuses primarily on epithelial cells of the lung *in vitro* and *in vivo*. Due to space limitations, we discuss various cell fates that potentially lead to transformation, but transformation is not addressed in depth here (for recent reviews see Refs. 20, 21).

Contribution of Epithelial Cells to CS-Induced Inflammatory Response

Epithelial cells, with the assistance of various pattern-recognition receptors, recognize the inhaled CS components and mount a defense response. The TLRs are one major family of pattern-recognition receptors involved with the innate immune response. Because TLR2 is activated, TLR2 deficiency provides some protection against the CS-induced inflammation in mice (22). In addition, both TLR4 and the IL-1 receptor are involved in the acute response to CS in mice (23, 24). As the first responders to CS, AECs fulfill important roles in several ways. First, both ciliated and secretory cells serve as a physicochemical barrier, and assist in mucociliary clearance by mechanically trapping and transporting particulate matter out of the respiratory tract (25–27). Second, in response to CS, AECs are capable of producing a number of inflammatory mediators that include various growth factors, chemokines, cytokines, and lipid mediators to stimulate the innate and adaptive immune system (28, 29).

Proinflammatory mediators produced by AECs include IL-1 β , IL-6, TNF- α , granulocyte/monocyte colony-stimulating factor, a soluble form of intracellular adhesion molecule-1 and C-X-C-motif ligand (CXCL) 8 (30–32). In addition, epithelial cells are the primary source of various chemokines, such as CXCL1, CXCL2, CXCL5, CXCL9, CXCL10, C-C-motif ligand (CCL) 11, CCL24, and CCL26, CCL17, CCL22, and C-X3-C-motif ligand 1 (33, 34). These mediators facilitate recruitment and activation of leukocytes to help clear the inhaled foreign matter (30, 35–40). Various laboratories have reported conflicting results for responses of alveolar epithelial type (AT) II cells treated with CS extract (CSE). Some studies report that secretion of IL-1 β , IL-6, granulocyte/monocyte colony-stimulating factor, IL-8, CXCL1, CCL2, CCL3, and CCL5 (41 and 42) is suppressed, whereas others report induced expression of IL-6 and IL-8 (3). Primary HAECs exposed to CS *ex vivo* show a proinflammatory response characterized by increased expression of IL-6, IL-8, and matrix metalloproteinase-1 (37, 43). However, when analyzed in a combination exposure together with LPS, or microorganisms, CS suppresses the inflammatory responses of epithelial cells (4, 39). IL-1-related cytokines, including IL-1 α , IL-1 β , IL-18, and IL-33, are key players in the regulation of inflammation. Both IL-1 β and IL-18 are secreted as pro forms, and the activated forms are released by the proteolytic activity of caspase-1 as the component of a multiprotein complex referred to as inflammasome. B cell lymphoma (Bcl)-2 and Bcl-X_L, primarily known as cell death regulators, are increased in AECs in response to CS or LPS (44–46), and modulate inflammatory responses by interacting with inflammasome and inhibiting processing of pro-IL-1 β and pro-IL-18 (47). Structure-function studies have shown that a 50-aa flexible loop between the first and second α helices of Bcl-2 and Bcl-X_L inhibit oligomerization of nucleotide-binding oligomerization domain-like receptor (NLR) pyrin domain containing 1, a prominent member of NLRs and a central component of inflammasome (48).

Epithelial cells also produce various ligands, including amphiregulin, heparin-binding epidermal growth factor-like growth factor (EGF), and tissue growth factor- α , in response to CS that cause aberrant activation

of EGF receptor (EGFR) and lead to epithelial cell hyperplasia and mucous cell metaplasia (36, 49–51). Several cytokines, including IL-1 α , IL-1 β , IL-8, IL-13, and TNF- α , and various TLR ligands modulate EGFR pathways to regulate airway mucous production (36, 52, 53). The expression and activation of various peptidases, such as matrix metalloproteinases and a disintegrin and metalloprotease domain, also known to modulate EGFR activity and tissue remodeling (54), are also affected by CS (55).

Another proinflammatory cytokine, IL-17, has recently caught more attention in chronic airway diseases (56). AECs express IL-17 during CS-induced and allergic airway inflammation (57, 58) that is attenuated by IL-17-neutralizing antibodies (58). IL-17 was discovered as a proinflammatory cytokine synthesized mainly by activated T cells and, in certain conditions, by activated neutrophils and eosinophils. IL-17 recruits and activates neutrophils by induction of CXCL-8 from epithelial cells, and also induces mucin 5, subtypes A and C expression together with IL-1 β , TNF- α , CXCL1, granulocyte colony-stimulating factor, and intracellular adhesion molecule-1 (59–63). Furthermore, the IL-17A signaling appears to be crucial for the formation of CS-induced emphysema, as the genetic deletion of IL-17A or the receptor prevents CS-induced emphysema *in vivo* (64, 65).

Among the six structurally related IL-17 cytokines (66), several studies demonstrate that IL-17C and -F are expressed in AECs both *in vitro* as well as in *in vivo* settings (67, 68). Furthermore, increased expression of immunoreactive IL-17C and -F are reported in AECs of subjects with COPD (69, 70). Epithelial-specific IL-17F expression is associated with the severity of asthma (71), and is induced by treatment of AECs with IL-33. IL-33 is an inflammatory mediator secreted by AECs that induces IL-17F in epithelial cells in an autocrine manner via suppression of tumorigenicity 2/extracellular signal-regulated kinase1/2/mitogen- and stress-activated protein kinase 1 signaling pathway (68). Expression of many inflammatory factors is driven by the activation or up-regulation of various transcription factors. CS activates several transcription factors, including NF- κ B (72, 73), activator protein 1 (74), cAMP response element-binding protein (CBP) (75), CCAAT/enhancer-binding protein-b

(76), and peroxisome proliferator-activated receptor γ (77). As a master regulator of inflammation, methods to inhibit NF- κ B activation have been of prime interest for developing anti-inflammatory approaches for chronic diseases, such as COPD and cancer, and, recently, functional polymorphisms in NF- κ B and I κ B α have been associated with increased risk of COPD or cancer (78).

CS-Induced DNA Damage

As components of CS, the RONS and other carcinogens, such as polycyclic aromatic hydrocarbons and N-nitrosamines (79, 80) disturb the oxidant:antioxidant balance and induce various types of DNA damage, including nucleotide oxidation, apurinic/apyrimidinic sites, bulky base adducts, DNA cross-links, and double- and single-strand breaks (81). Exposure of normal human lung fibroblasts (NHLFs), primary HAECs, and BEAS-2B cells to CSE induces DNA breaks/fragmentation, as evidenced by the terminal dUTP-biotin nick-end labeling or Comet assays (5). CSE-induced DNA breaks are reversible in cultured NHLFs and BEAS-2B cells if the dose and length of CSE exposure are limited (5, 6).

CS induces DNA strand breaks (82) and also nucleic acid oxidation in the lungs of mice (83). Alveolar wall cells obtained from smokers with severe emphysema exhibit more prominent oxidation of both DNA and RNA compared with those from donors without COPD (84). Caramori and colleagues (85) also confirmed a significant increase in DNA oxidation in the lungs of smokers with and without COPD compared with those of nonsmokers. However, DNA oxidation between smokers with and without COPD was not different. In contrast, the number of apurinic/apyrimidinic sites that are common DNA lesions generated during the course of base excision repair of oxidized, deaminated, or alkylated bases, is increased in smokers with COPD compared with nonsmokers and smokers without COPD, implying a significant role of the DNA damage and repair in COPD pathogenesis (85). The link of DNA damage to the development of lung cancer is established by numerous studies. For example, some carcinogens in CS, such as polycyclic aromatic hydrocarbons or acrolein, induce the formation of DNA adducts. The DNA damage subsequently

causes loss-of-function mutations of p53, a critical tumor suppressor, or gain-of-function mutations of K-ras, a proto-oncogene encoding a member of the small GTPase subfamily, which contributes to lung carcinogenesis (86–88).

In response to CS, lung cells exhibit an orchestrated signaling process called the DDR to sense DNA damage and initiate DNA repair to maintain genomic integrity (89). The DDR signaling is mediated by the phosphoinositide 3-kinase–related protein kinases (PIKKs), including ataxia telangiectasia mutated (ATM), DNA dependent protein kinase (DNA-PK), and ATM- and Rad3-related. Both ATM and DNA-PK are primarily activated by DNA double-strand breaks (DSBs), whereas ATM- and Rad3-related is activated by single-strand breaks (SSBs). Activation of the PIKKs inhibits cyclin-dependent kinase (CDK) activity by activating p53 and the protein kinases, such as checkpoint kinase (CHK)1 and CHK2. As part of the response to arrest the cell cycle, p53 transcriptionally induces p21, a broad-spectrum CDK inhibitor (90). Suppression of CDK activity delays cell cycle progression and allows the cells to repair DNA damage (89). The PIKKs also phosphorylate serine 139 of the histone H2A variant (γ H2AX) on chromatin flanking DSB sites, which is crucial for subsequent DDR signaling and DNA repair (89).

CS activates the ATM-p53-p21 in cultured NHLFs (91) and lung tissues of mice (92) or the ATM-CHK2 pathway in cultured primary AECs and A549 cells (93). CSE-exposed primary AECs and A549 cells also exhibit γ H2AX mediated by ATM, but not DNA-PK (94). Notably, CSE causes cell cycle arrest in S phase without cell death in cultured HAECs (95) and NHLFs (91). Consistent with these data, the DDR signaling is also more prominent in S phase than in G1 or G2/M phase cells (93).

Given the rapid kinetics in response to genotoxin, γ H2AX has been widely used as a biomarker to monitor DNA damage, especially DSBs, and repair in translational research (96). In human studies, the number of DSBs evidenced by γ H2AX in alveolar epithelial cells is increased in smokers with COPD compared with those without COPD (97). Another human study also demonstrated persistent DNA damage (γ H2AX) in the lungs of patients with severe COPD, despite smoking cessation over at least 6 months (98). These studies

suggest a potential role of persistent DNA damage in COPD pathogenesis.

CS-Induced Cell Death

The pathways that drive cell death have been reviewed extensively (99–101); therefore, we do not repeat the basic pathways in this *Review*. Two modalities of programmed cell death in the lung are apoptosis and necrosis. Apoptosis is a process by which tissues remove unwanted or damaged cells. This type of cell death is an important part of healthy tissue homeostasis and mammalian development, but, when dysregulated, can result in necrosis and disease (102). HAEC death occurs through both apoptosis and necrosis in patients with COPD (103). The lung consists of different cell types, including ATI and ATII cells, alveolar macrophages, and the cells of the AECs, which include ciliated cells, mucus-secreting cells, and basal cells (104). These cell types have unique phenotypes, and respond differently to cell death-inducing stimuli, such as CS; consequently, the disease components that ultimately manifest as COPD, emphysema, and chronic bronchitis are driven by different molecular pathways.

Emphysema, largely caused by cigarette smoking, is a condition characterized by alveolar wall destruction (105). Consistent with this idea, apoptosis and proliferation of alveolar wall cells are significantly higher in patients with emphysema than in asymptomatic smokers and nonsmokers (106), and alveolar cells show an increased turnover compared with healthy lungs (106). These findings are supported by reports from *in vitro* studies showing that CS induces apoptosis and/or necrosis in various cell lines, such as A549, an alveolar epithelial cell line (7), NHLFs (107), and alveolar macrophages (108). Although cells of the alveolar walls undergo cell death in response to CS, other cell types of the lung can respond differently. Because this *Review* focuses on AECs, the remainder of this section focuses on studies related to AECs.

Interestingly, *in vitro* and *in vivo* studies with AECs exposed to CS show vastly different results. Some studies report that CS has deleterious effects on AECs, whereas others show that these cells are seemingly resilient to CS. These discrepancies could be due to differences

in CSE composition or state of AEC differentiation, but also reveal how poorly the molecular mechanisms affecting CS-induced death of AECs are understood, despite the known adverse effects that smoking has on the lungs.

One of the major factors contributing to the difference in effects on AECs of CS is the confluency of the cells. Nonconfluent cultures of human AECs are susceptible, whereas confluent cultures are resistant to cell death (8). These differences were attributed to the thioredoxin-apoptosis signal-regulating kinase-1-c-Jun N-terminal kinase pathway, which may be important in mediating CSE-induced cell death in nonconfluent cultures (8). CSE increases epithelial tight junction permeability through activation of Rho kinase and tyrosine kinase (2, 18, 109, 110). However, whether the formation of tight junctions and the related molecular mechanisms in confluent cultures protects from CS-induced cell death is still unknown. In the context of cell survival, tight junction may not be necessary, as some transformed or epithelial-mesenchymal transition cells that lose cell contact are often resistant to CSE-induced cell death.

BEAS-2B cells and HAECs succumb to DNA damage by CS. However, CS-induced DNA damage is not necessarily lethal. These cells show increased SSBs, as evidenced by terminal dUTP-biotin nick-end labeling positivity, but do not undergo necrosis or apoptosis (5). SSBs are repaired and not detectable at 72 hours after a transient CSE exposure (for 6 h). Chemical inhibition of poly (ADP-ribose) polymerase blocks the DNA repair and results in persistent SSBs for 72 hours. However, the persistent SSBs do not induce cell death in cultured AECs (5).

In contrast, other studies suggest that AECs undergo apoptosis in response to CS. CS suppresses expression of a proapoptotic Bcl-2 family member, Bcl-2 interacting killer (Bik) (111). Bik levels are reduced in bronchial epithelial cells obtained by bronchial brushings from patients with chronic bronchitis compared with those without. In addition, exposure of mice to CS reduces Bik expression and increases the numbers of AECs per millimeter basal lamina, resulting in AEC hyperplasia. This hyperplasia is resolved when Bik is introduced using adenoviral vectors. Finally, Bik-induced cell death is mediated by blocking extracellular signal-regulated

kinase1/2 translocation to the nucleus. These results suggest that CS causes an increase in AEC numbers due to an inflammatory response and suppression of a proapoptotic killer.

Furthermore, *in vitro* studies in the immortalized human bronchial epithelial cell line showed that CS activates neutral sphingomyelinase 2, producing ceramide, which induces apoptosis in AECs. This type of apoptosis can be blocked by an antioxidant, glutathione, through blocking neutral sphingomyelinase 2 (112). In addition, microarray analyses of AECs obtained through airway brushings from smokers and nonsmokers show changes in apoptotic genes (113). Pirin, one of the genes that is up-regulated by smoking, encodes for a transcription cofactor that, when overexpressed, causes death of nonconfluent AECs *in vitro*.

In other studies, CS concentrations of 30% or below caused apoptosis, whereas concentrations greater than 30% caused necrosis in BEAS-2B cells. Heme oxygenase-1, an enzyme that catalyzes the rate-limiting step in the oxidative degradation of heme, also functions to protect cells against apoptosis; it was protective against CSE-induced AEC death by localizing to the mitochondria and preserving ATP production. In addition, *in vivo* studies support these findings, showing that heme oxygenase-1 mRNA is detected at higher levels in lungs of mice chronically exposed to CS (114).

Although these studies present conflicting results as to whether AECs undergo cell death by CS, they point to important mechanisms that are activated. Future studies should clarify how these findings can be reconciled, and expand our understanding of mechanisms involved in *in vivo* settings.

CS-Induced Autophagy

Macroautophagy, hereafter referred to as autophagy, is an evolutionarily conserved, catabolic process that serves to sequester cytoplasmic proteins and/or organelles in double-membrane vesicles, also called autophagosomes, and degrades them by fusing with lysosomes (115). The degraded products, including ATP and essential building blocks, are then recycled for the maintenance of cellular functions during nutrient deprivation or metabolic stress (116, 117).

Over the last decade, more than 30 Autophagy-related (ATG) genes have been discovered in yeast from genetic screenings, and many of these genes have mammalian homologs (118–121). The Bcl-2 interacting protein, beclin 1 (*Atg6* in yeast) and the microtubule-associated protein-1 light chain 3B (LC3B; *Atg8* in yeast) represent major regulators of autophagy in mammalian cells (122, 123). In mammals, the conversion of LC3B-I (the cytoplasmic free form) to LC3B-II by conjugation to phosphatidylethanolamine and the LC3-II binding to the autophagosome membrane is regarded as a good marker of the autophagosome formation (115). Once this pathway is initiated, the isolation membrane, or phagophore, forms in the cytosol to enclose and sequester cytoplasmic components for autophagic degradation. The mechanism for the formation of the autophagosome is not yet well defined, although a number of other ATG proteins have been characterized in mammalian cells (e.g., *Atg5*, *Atg12*, *Atg7*) that have distinct roles in the induction and progression of the autophagic pathway (124), particularly in the formation of the autophagosomes (125). Autophagosomes subsequently fuse with lysosomes to form a single-membrane vesicle, the autolysosome. The lysosomes are then rederived from autolysosomes by a process called autophagic lysosome reformation (118, 126).

The main role of autophagy is to maintain general homeostatic removal, degradation, and recycling of damaged proteins and organelles necessary during many specific physiological and pathological processes, such as development, immunity, energy homeostasis, cell death and carcinogenesis. Autophagy has been implicated in both health and disease (127), and it is generally believed that it plays a protective role in overcoming exogenous stress, but prolonged and excessive autophagy can lead to cell death (127, 128). Recent studies have shown that increased autophagy occurs in the lungs of patients with COPD, in the lungs of mice exposed to CS, and in HAECs exposed to CSE (9, 10). Interestingly, increased expression of ATG proteins is also observed in the genetic variant of COPD, α 1-antitrypsin deficiency, the etiology of which is independent of smoke or particle inhalation (9), suggesting that, in addition to direct cellular responses

to CS, other pathways may activate autophagy in the lungs of patients with COPD. Although suppression of autophagic proteins (LC3B or beclin-1) inhibits apoptosis in response to CS *in vitro* (9), the functional role for CS-induced autophagy in various cell types of the lungs of patients with COPD is not fully understood and requires further research.

Increasing evidence suggests cross-talk between autophagy and apoptosis, as several apoptosis-related factors are critically involved in autophagy (129, 130).

Concurrent up-regulation of both autophagy and apoptosis has been observed in lung epithelial cells subjected to CSE treatment (10). CS initiates the extrinsic apoptosis pathway involving assembly of the Fas-dependent death-inducing signal complex formation and activation of caspase-8, induces the expression and conversion of the autophagic protein, LC3B, increases autophagosome formation, and ultimately increases caspase-3 activation in epithelial cells (10). In clinical samples from patients with COPD, whereas autophagic markers are elevated in early stages of the disease, caspases are activated at the later stages, suggesting that autophagy may precede apoptosis as a general response to chronic CS stress in the lung (9). These observations suggest that autophagy can promote either cell survival or death, depending on the specific stimuli, environmental conditions, and cell type (136).

The transcription factor, early growth response-1 (Egr-1), a promoter of CS-induced autophagy and apoptosis (9), is up-regulated in lung tissues of mice exposed to chronic CS (9), of patients with COPD, and in NHLFs after CSE exposure (25). CSE suppresses HDAC activity, resulting in enhanced binding of Egr-1 and E2F factors to the LC3B promoter, and increased LC3B expression. Knockdown or deletion of Egr-1 in mice inhibits CS-induced LC3B and Atg4B expression (9). Collectively, these studies demonstrate the critical role of Egr-1 in COPD pathogenesis by mediating CS-induced autophagy and apoptosis.

Caveolin-1 (Cav-1), a candidate tumor suppressor, is a 21-kD protein phosphorylated on tyrosine 14 by avian sarcoma (Schmidt-Ruppin A-2) viral oncogene homolog tyrosine kinase (131, 132) has been implicated in modulating signal transduction by inhibiting a number of enzymes (132–134). Chen and colleagues (135) have described a role for Cav-1 in the

regulation of CS-induced autophagy and apoptosis. Cav-1 acts as a plasma membrane anchor for LC3B and Fas, and, through this dynamic interaction, plays a regulatory role in the extrinsic apoptotic pathway. CSE promotes dissociation of LC3B and Fas from Cav-1, allowing the progression of the extrinsic apoptotic pathway. Suppression of LC3B levels inhibits apoptosis by increasing Cav-1-dependent Fas sequestration. The knockdown of Cav-1 sensitizes epithelial cells to CS-induced apoptosis, and Cav-1^{-/-} mice present higher levels of autophagy and apoptosis in the lungs that is augmented in response to chronic CS exposure *in vivo* (135). These studies show that Cav-1 controls autophagy, cell death, and emphysema development, and that LC3B protein not only plays a pivotal role in autophagy, but also in the extrinsic apoptosis pathway activated during CS-induced lung cell death.

Recently, the participation of TLR4 as a protective regulator of CS-induced autophagy and emphysema development has been described (136). Lung tissues from patients with COPD and immortalized HAECs exposed to CSE display increased TLR4 expression, together with increased autophagic and apoptotic markers. Suppression of TLR4 causes increased expression of LC3B that is further increased in response to CSE. A1H cells from TLR4^{-/-} mice exhibit an increase in LC3B, accompanied by induction of apoptosis, caspase-3 activation, and enlargement of airspace that is further increased after CS exposure for 2 months. These data demonstrate that TLR4 has a protective role controlling autophagy, apoptosis, and emphysema development in mice exposed to CS. In the lungs of patients with COPD, the higher expression of TLR4 is probably a mechanism that down-regulates the activated autophagic and apoptotic pathways.

p62 brings the ubiquitinated substrates to the autophagosome, and is degraded together with the cargo in the autolysosome. Fujii and colleagues (137) reported that CSE induces autophagy transiently in HAECs with accumulation of p62 and ubiquitinated proteins at longer times of CSE exposure. In addition, autophagy induction by a mammalian target of rapamycin inhibitor suppresses p62 accumulation and ubiquitinated proteins in CSE-exposed HAECs, and the opposite happens when autophagic degradation is inhibited with

bafilomycin A1, or by suppression of LC3B or Atg5. Furthermore, primary AECs isolated from patients with COPD exhibit an increase in LC3 conversion as compared with AECs from patients without COPD, but, at the same time, the lung homogenates from patients with COPD exhibit accumulation of ubiquitinated proteins and p62. These findings suggest that even when autophagy and autophagic degradation are induced by CS in the lungs of patients with COPD, the autophagic degradation is not sufficient to eliminate the amount of ubiquitinated proteins and p62.

CS-Induced Cellular Senescence

Cellular senescence is a viable cell state with complete and irreversible loss of replicative capacity (138). In tissue culture, NHLFs or primary HAECs stop proliferation after roughly 50 or 10 population doublings, respectively (139–141). The endpoint of this proliferation limit is referred to as replicative senescence (142), and is due to the attrition of telomeres located at the end of DNA strands (143–145). Cellular senescence can also be induced by various stresses, including hydrogen peroxide, hyperoxia, ultraviolet radiation, γ -irradiation (146), CS (91), and oncogenic stimulation (147, 148). The other phenotypes of cellular senescence include: (1) a distinct, flat, and enlarged cell morphology (149); (2) resistance to apoptosis (150, 151); (3) altered production of inflammatory and growth mediators (152); and (4) an increase in senescence-associated β -galactosidase (SA β -gal) activity (149).

Cells are prone to undergo cellular senescence when DNA damage overwhelms the repair pathway (153), and cellular senescence may be induced through either or both the p53 and p16-Rb pathways (154, 155). As stated in the section “CS-Induced DNA Damage”, CS causes the DDR that activates the ATM-p53-p21 pathways. The p16-Rb pathway is also sufficient to induce cell cycle arrest (156, 157). DNA damage can induce transcriptional activation of p16 via an alternative splicing mechanism of the INK4a/alternate reading frame locus (155), and p16 binds to CDK4 and CDK6 and suppresses the CDK activity by interfering with their association with D-type cyclins (158). Consequently, activated Rb (the

hypophosphorylated form) induces chromatin modifications to suppress growth-promoting transcription factors, such as E2F (154). In contrast, alternate reading frame activates the p53 pathway through the binding to the mouse double minute 2 protein (an E3 ubiquitin ligase), which subsequently inhibits mouse double minute 2-mediated degradation of p53 (155).

CSE induces cellular senescence in cultured primary ATII cells and A549 cells, as evidenced by a dose- and time-dependent increase in the SA β -gal activity and p21 accumulation (11). Exposure of cultured NHLFs to CSE for 14 days is sufficient to induce cellular senescence accompanied by p53-p21 and p16-Rb up-regulation (91).

Hallmarks of cellular senescence have been detected in cellular samples from patients with COPD (92, 159, 160). Several human studies demonstrated that smokers with COPD exhibit a higher prevalence of cellular senescence in NHLFs (159, 161). Senescence markers used were SA β -gal activity and reduced proliferative capacity, shown by both p16 and p21 positivity using immunohistochemistry, of ATII cells and endothelial cells (160). Indeed, cellular

senescence can intertwine with chronic inflammation (162), redox imbalance (163), and loss of reparative capacity (164), all of which are important factors in the COPD pathogenesis (165). Senescence of ATII cells is increased fivefold in mice exposed to CS for 2 weeks, as detected by SA β -gal activity and p21 expression (11), suggesting that CS-induced alveolar cell senescence precedes the development of emphysema.

The increased DNA damage signals are associated with alveolar cell inflammation, apoptosis, and cellular senescence, the latter of which represents a state in which cells are metabolically active, but permanently unable to divide (97), further supporting a potential role of DNA damage in the pathogenesis of COPD.

Shortening of telomere length has been recognized as an important risk factor for CS-induced emphysema, because telomerase-deficient mice were more resistant to developing emphysema (166). In contrast, human studies demonstrated complicated results. Although telomere length of ATII cells in smokers was significantly shorter than in nonsmokers (160), there was no significant difference of

the telomere length between smokers with and without COPD (160). Muller and colleagues (159) also reported that there is no significant change in telomere length of NHLFs obtained from smokers with COPD compared with those from smokers without COPD. These data suggest that the mechanisms of cellular senescence in the lungs of patients with COPD are more complex than simple telomere attrition. Therefore, the precise mechanism of senescence in COPD still remains to be elucidated. Although the causative role of cellular senescence remains unclear, emerging evidence suggests that accumulation of senescent cells clearly causes organ dysfunction (likely from a decreased regenerative capacity) and altered secretory phenotype, which promotes chronic inflammation and carcinogenesis (152).

Technical Limitations of *In Vitro* Modeling

To investigate CS-induced effects on AECs, numerous *in vitro* and *in vivo* studies have

Figure 1. Molecular processes that drive cigarette smoke (CS)-induced epithelial cell fate of the lung. CS exposure induces inflammation, DNA damage, and autophagy that cause lung epithelial cells to undergo cell death, cellular senescence, and/or transformation.

been conducted. Unfortunately, these studies are often marred by variable and even contradictory results being reported by various laboratories. One major factor for these contrasting data may be the different methods employed to generate CS or CSE for *in vitro* and *in vivo* exposure (51, 83, 167–174). However, recent technological advancement has shed some light on the difficult standardization of CS exposure *in vitro*. The smoking robots are able to generate side- and mainstream CS from standardized cigarettes with a reproducible dose adjustment (175, 176). This type of tool will likely make comparisons between different laboratories feasible.

Conclusions and Future Directions

In this Review, we summarize effects of CS on inflammation, DNA damage, and autophagy and the subsequent cell fates (Figure 1). These complex biological responses may be primarily modulated by a redox imbalance driven by CS-induced reactive oxygen species. Inhibiting reactive oxygen species using n-acetyl cysteine or other mediators attenuates CS-induced inflammation, DNA damage, and

autophagy, and may rescue an irreversible cell fate, such as cellular senescence or cell death (9, 91, 112). The cell fate of CS-exposed cells likely depends on the cell type, state of cell cycle, and the concentration and duration of CSE exposure (7, 8, 91, 107, 108). However, there are several unresolved issues regarding experimental approaches both *in vitro* and *in vivo* aside from the technical limitations stated previously here. First, adaptive and maladaptive responses to CS should be differentiated. Although acute CS exposure modulates expression of more than 3,000 genes in the mouse lung (177), the majority of these alterations may represent an adaptive response. Because most smoking-related lung diseases manifest after decades of smoking, it is likely that certain maladaptive responses to CS are responsible for the development of chronic lung diseases. Identification of the molecular pathways that represent these maladaptive responses and play a role in pathological processes, ultimately causing the clinical phenotype, remains challenging. In addition, because only a subset of smokers develops chronic lung diseases, it is assumed that genetic susceptibility factors play a role (178–180). The functional importance of these genes and

how they may be related to the maladaptive responses remains to be elucidated. Second, it is important to identify the molecular mechanisms of COPD that persist despite smoking cessation. Although smoking cessation is an important intervention for patients with COPD, and improves respiratory symptoms and slows lung function decline (181, 182), smoking cessation does not restore lost lung function or halt emphysema progression (183). Given the complex effect of CS on AEC biology (e.g., affected by the type, dose, and duration of CS), it may be fruitful to study AECs of ex-smokers with COPD to identify the irreparable effects of CS on molecular pathways. Understanding processes that determine the long-term effect of CS could help manage these deadly diseases in susceptible individuals before their onset, and may even provide better treatment modalities for those with COPD and lung cancer. ■

Author disclosures are available with the text of this article at www.atsjournals.org.

Acknowledgments: The authors thank Mr. Thomas Gagliano, Resource Manager, Lovelace Respiratory Research Institute (Albuquerque, NM) for assistance with preparing the figure.

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