

# Roles of Apoptosis in Airway Epithelia

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The airway epithelium functions primarily as a barrier to foreign particles and as a modulator of inflammation. Apoptosis is induced in airway epithelial cells (AECs) by viral and bacterial infections, destruction of the cytoskeleton, or by exposure to toxins such as high oxygen and polycyclic hydrocarbons. Various growth factors and cytokines including TGF- $\beta$ , IFN- $\gamma$ , or the activators of the death receptors, TNF- $\alpha$  and FasL, also induce apoptosis in AECs. However, cell death is observed in maximally 15% of AECs after 24 h of treatment. Preincubation with IFN- $\gamma$  or a zinc deficiency increases the percentage of apoptotic AECs in response to TNF- $\alpha$  or FasL, suggesting that AECs have mechanisms to protect them from cell death. Apoptosis of AECs is a major mechanism in reducing cell numbers after hyperplastic changes in airway epithelia that may arise due to major injuries in response to LPS or allergen exposures. Resolution of hyperplastic changes or changes during prolonged exposure to an allergen is primarily regulated by the Bcl-2 family of proteins. Fas and FasL are both expressed in AECs, and their main function may be to control inflammation by inducing Fas-induced death in inflammatory cells without inducing apoptosis in neighboring cells. Furthermore, AECs engulf dying eosinophils to clear them by phagocytosis. Therefore, in the airway epithelium apoptosis serves three main roles: (1) to eliminate damaged cells; (2) to restore homeostasis following hyperplastic changes; and (3) to control inflammation, and thereby support the barrier and anti-inflammatory functions.

**Keywords:** cytokines; infection; inflammation; phagocytosis; mucus cell hyperplasia

The airway epithelium (AE) is developmentally programmed to protect itself from environmental toxins and injury by forming a barrier, trapping foreign particles that enter the airway, and allowing their removal by ciliary action. This primary role explains why secretory and ciliated cells are the major cell types within the airway epithelium.

In recent years, it has become increasingly clear that the AE also is key modulator of inflammatory responses and plays a crucial role for normal immune function. For this purpose, the AE not only produces a broad range of cytokines and chemokines but also generates lipids and peptide mediators (enzymes and enzyme inhibitors) and reactive oxygen species (ROS) that enhance the infiltration of the lung airspaces with leukocytes (1). Mediators secreted by airway epithelial cells (AECs) and infiltrating leukocytes in turn affect the differentiation of the AE and the cell death processes of epithelial cells.

This review focuses on what is known about the apoptotic signaling mechanisms in AECs in the context of the various roles airway epithelial cell death contributes to the normal func-

tioning of the epithelium. Because of the different natures of the type I and type II alveolar cells, apoptosis related to alveolar epithelial cells will not be discussed in the present review.

## THE BASIC PATHWAYS OF APOPTOSIS

To make this review on apoptotic pathways in airway epithelial cell death easily understandable for readers not familiar with this subject, a brief summary of the major pathways known to elicit programmed cell death in many cell types is provided. The studies summarized were primarily conducted in cells of the hematopoietic compartment and have laid the foundation for studies in neuronal and epithelial cells from various organs (2). Two pathways broadly categorize the sequence of events that culminate in the activation of aspartate-specific cysteine proteases, caspases (cysteinyl, aspartate-specific proteases): the extrinsic and the intrinsic pathways.

The extrinsic pathway can be initiated by one of several cell surface death receptors when bound by the appropriate ligand (3). TNF receptor 1 (TNFR-1) and Fas receptors contain death domains (DDs) and recruit the DD-containing adaptor molecules, TNFR-1-associated death domain (TRADD), and Fas-associated death domain (FADD), respectively. Homotypic interaction between the DDs of Fas and FADD induces the recruitment and self-activation of pro-caspase-8 (4). In TNF signaling, TRADD recruits FADD after formation and release of a TNFR-1 complex to initiate pro-caspase-8 activation (5, 6). The receptors for TNF-related apoptosis-inducing ligand (TRAIL), TRAIL-R1 (also known as death receptor-5, DR-5), also recruit and activate pro-caspase-8 (7) in a FADD-dependent manner (8).

The intrinsic pathway is characterized by the permeabilization of the outer mitochondrial membrane and the release of several pro-apoptotic factors into the cytosol. These factors include cytochrome c (9, 10), Smac/Diablo (11, 12), apoptosis-inducing factor (AIF) (13), endonuclease G (14), and HtrA2/Omi (15). The release of these mediators is regulated by the Bcl-2 family (16). Once released, cytochrome c binds to an adaptor protein, Apaf-1, which self-oligomerizes and recruits pro-caspase-9 to form the apoptosome complex (17). This promotes the autoprocessing of caspase-9, which in turn recruits and cleaves pro-caspase-3, which degrades proteolytically targeted substrates and activates DNases.

Proteins in the Bcl-2 family are major regulators of the intrinsic pathway. Family members include both pro- and anti-apoptotic proteins, and they share homology in four conserved regions, termed Bcl-2 homology (BH) domains 1–4 (18). The family can be divided into three main subclasses:

1. The anti-apoptotic proteins that include Bcl-2 and Bcl-x<sub>L</sub> are all multi-domain, sharing homology throughout all four BH domains. Bcl-2 and Bcl-x<sub>L</sub> enhance cell survival by inhibiting apoptosis induced under a wide variety of circumstances. Bcl-2 and Bcl-x<sub>L</sub> extend cell survival by preventing cell death in different cell types and in response to different stimuli, suggesting that Bcl-2 and Bcl-x<sub>L</sub> act at a central control point in the pathway to apoptotic cell death (18, 19, 2).
2. The multidomain pro-apoptotic proteins such as Bax and Bak possess sequence homology in at least three BH

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domains. Bax and Bak have overlapping roles in the regulation of apoptosis during mammalian development and tissue homeostasis (20). Activation of these “multidomain” pro-apoptotic members is an essential gateway required for cell death in response to diverse stimuli (21).

3. The third subset of this family includes Bid, Bad, Noxa, Bik, Bmf, Blk, Hrk, Bnip-3, Puma, and Bim, all of which lack BH-1, -2, and -4 (22). BH-3-only proteins are pro-apoptotic and share sequence homology within the amphipathic  $\alpha$ -helical BH-3 region (23–25). Bid is cleaved by caspase-8 to generate active truncated Bid (tBid), which leads to the Bax-dependent release of pro-apoptotic factors from mitochondria. Thus, this event integrates the extrinsic and intrinsic pathways (26). The BH3 region is required for the pro-apoptotic members to cause binding to multidomain Bcl-2 family members and induce death (27). BH-3 molecules display some selectivity for multiple domain Bcl-2 members (24, 28). Inactivation of the anti-apoptotic proteins Bcl-2 and Bcl-x<sub>L</sub> by BH-3 domain-only members results in the activation of the “multidomain” pro-apoptotic members Bax and Bak (29). The pro-apoptotic activity of BH-3-only molecules is kept in check by either p53-dependent transcriptional control (25), post-translational modification, or by binding to the dynein light chain in myosin V filamentous actin, thereby being sequestered from binding to Bcl-2 (30, 31). Association of BH-3 proteins with actin filaments enables them to sense death signals and initiate anoikis (30, 32). When cells receive a stimulus to undergo apoptosis, the BH-3-only members translocate to inactivate Bcl-2 or to enable Bax and Bak and initiate a cascade of events leading to activation of caspases and DNases.

Members of all three classes of the Bcl-2 family can localize to the endoplasmic reticulum (ER) membrane and influence ER homeostasis (33). Calcium release from the ER can either directly activate effectors of caspases or sensitize mitochondria to a variety of extrinsic and intrinsic death stimuli.

## APOPTOSIS AS A MECHANISM OF REMOVING DAMAGED AECs

The epithelial barrier represents a critical line of defense against the environment, so AECs are likely designed to be refractory to a number of potentially apoptotic stimuli, including cell death receptor activators such as TNF- $\alpha$  and FasL. The relative resistance of AECs to apoptosis is likely helpful in maintaining the integrity of the epithelial barrier during an inflammatory response when immune cells that express or secrete these death receptor ligands are trafficking through the lung. These characteristics may be the basis for the inherent properties of the airway epithelium, which is a stable mucosal surface with relatively low rates of cell proliferation under normal conditions (34).

### Cell Death by Viral Infection

Apoptosis in response to viral infection likely occurs as part of the host response in an attempt to limit virus replication and to prevent persistent infection and spread of the virus. Thus, in the context of a virus infection, damage to the epithelial barrier may be the lesser evil. As a consequence, many viruses have evolved various mechanisms to inhibit or evade apoptosis. In addition, death by apoptosis enhances the efficiency of capture of viral antigens by antigen-presenting cells and presentation to T cells. Infection of AECs with the adenoviral vector Ad-CMV-lacZ (35), or with influenza A virus (36), induces apoptotic cell death. Respiratory syncytial virus (RSV)-induced apoptosis of airway

epithelial cells has been reported; however, the Fas pathway appears to not be important (37). Other studies have implicated RSV in inducing anti-apoptotic pathways. Treatment of RSV-infected cells with an inhibitor of phosphatidylinositol 3-kinase (PI3-k) resulted in more rapid apoptosis, implying that under normal conditions signaling through the PI3-k pathway mediates an inhibition of RSV-induced apoptosis (38). This is consistent with another report showing that apoptosis is inhibited or delayed in RSV-infected cells because a minimal cytopathic effect was observed in response to RSV infection in primary human AECs that had differentiated into a pseudostratified mucociliary epithelium *in vitro* (39). Analysis of apoptotic genes expressed in RSV-infected cells showed that cells may be susceptible to killing through the TRAIL pathway and Mcl-1 might account for the delayed induction of apoptosis in RSV-infected cells (40).

### Cell Death by Bacterial Infection

Under normal conditions, primary AECs in culture form tight junctions that render them resistant to apoptosis by an inoculum of *Pseudomonas aeruginosa* PAO1 (only 5–10% of cells showed mitochondrial permeability changes) at doses that are sufficient to activate proinflammatory cytokine expression. Resistance is lost, and up to 50% of the population undergo apoptosis after 6 h of exposure once the tight junctions are disrupted with EGTA or in cell lines such as 9HTEo- cells that do not form tight junctions (41). Specific adhesions, complete LPS, and functional type II secretion system by the *P. aeruginosa* are necessary to evoke apoptosis even in the susceptible cells that were unable to form tight junctions.

Cystic fibrosis transmembrane conductance regulator (CFTR) mutation is attributed to the accumulation of CFTR in the ER and activation of the NF- $\kappa$ B, which is anti-apoptotic (42). In addition, caspase activity may be inhibited by impaired pH regulation in CFTR cells (43). However, AECs with CFTR dysfunction ( $\Delta$ F508 – W1282X or  $\Delta$ F508) show no differences in apoptosis compared with normal cells after treatment with *P. aeruginosa* (41). Similarly, CFTR +/+ and –/– mice show few apoptotic cells in the airway epithelium after *P. aeruginosa* infection. It is possible that *P. aeruginosa* toxins induce apoptosis by disturbing actin polymerization and cell–cell contacts, and may not be related to CFTR (44).

Vernooy and coworkers (45) report that instillation of LPS induces apoptosis in bronchial epithelial cells within 2 h and peaking at 24 h and that TNF- $\alpha$  is not involved in this cell death process. However, this report does not exclude the possibility that TUNEL positivity in the AE could stem from dying inflammatory cells. *P. aeruginosa* stimulates transcription of IL-8, the major attractant for polymorphonuclear leukocytes (PMN) by stimulating translocation of NF- $\kappa$ B. NF- $\kappa$ B also inhibits cell death by inducing TRAF-1 and TRAF-2 and cIAP1 and cIAP2 to suppress caspase-8 activation (46, 47), and also regulates expression of genes that inhibit cell death in part by inducing the anti-apoptotic Bcl-2 (48). Therefore, other pro-apoptotic pathways may be activated to override the anti-apoptotic function of NF- $\kappa$ B to induce cell death in primary AECs (41).

Bacterial exoproducts (especially exotoxin, ETA) from *P. aeruginosa* induce death in 16HBE14o– cells 24 and 48 h after treatment with 1,000 ng/ml as measured by the dimethylthiazole 2,5 diphenyl tetrazolium bromide (MTT) assay. This cell death is not affected by zVAD-fmk (a broad spectrum inhibitor for caspases), condensed nuclei are not observed, and mitochondria of dying cells were highly condensed (49). The absence of mitochondrial swelling suggests that mitochondrial permeability transition played a minor role in this cell death because large transmembrane pores are likely to open in response to mitochondrial dysfunction. The authors conclude that ETA induces cell

death in AECs by a mechanism different than that classically described as apoptosis. Because ETA did not induce apoptosis in primary cultures of human nasal polyp epithelial cells, and the role of tight junctions was not tested, the significance of ETA to cause cell death under physiologic conditions is not clear.

### Cell Death by Destruction of Cytoskeletal Support

Disruption of the elongation or the aggregation of actin filament with cytochalasin D (0.5  $\mu\text{g/ml}$ ) or with jasplakinolide (3  $\mu\text{M}$ ), respectively, induces apoptosis as measured by TUNEL positivity in 30–50% 1HAE $^{-}$  and in 10–15% primary normal human bronchial epithelial (NHBE) cells within 5 h (50). This death was abrogated by z-VAD-fmk but not by blocking the Fas receptor. Pro-caspase-8 was cleaved by cytochalasin D but not by jasplakinolide, suggesting that disruption of actin filament integrity by the two agents causes cell death by different mechanisms. Further support for the significance of actin filaments in affecting cell death stems from inhibition studies of Rho kinase, which regulates the formation of stress fibers and focal adhesions (51). Inactivating Rho kinase with Y-27632 or HA1077 or by overexpressing a mutant Rho kinase causes membrane ruffling and apoptosis (as measured by TUNEL staining in 15–25% of primary AECs). While the signaling of cell death induced by destruction of actin filaments is not known, there is increasing evidence that in other cell types disruption of microtubule turnover by taxol or vincristine (which phosphorylate Raf-1 and Bcl-2) leads to apoptosis and detachment (52, 53). Activation of caspase-3 cleaves the actin filament, severing the protein gelsolin and leading to morphologic changes of the dying cells (54). However, it is not known whether the cleavage of actin cytoskeletal network itself causes apoptosis or if this is just one of the manifestations of the cell's shape. Furthermore, BH-3-only proteins are associated with actin filaments, and disruption of their interaction may enable them to sense death signals and initiate detachment of AECs (30, 32).

### Cell Death by Oxygen Exposure

AECs are constantly exposed to high oxygen pressure and have developed an antioxidant capacity that is sustained with antioxidant enzymes to detoxify reactive oxygen species (ROS). However, when this protective system is overwhelmed, apoptosis is induced with a minimal amount of necrosis (measured by the release of lactic dehydrogenase, LDH), as shown for primary small airway epithelial cells (SAEs) exposed to 95%  $\text{O}_2$  for 2 d (55). However, A549 cells primarily undergo necrosis, which is why it is necessary to test the effect of each stimulus in primary cells to validate their importance under physiologic conditions.

When adult male C57Bl/6J mice are exposed to 95%  $\text{O}_2$  for 72 h, AECs (most prominently the AECs of the distal bronchiolar epithelium) and alveolar cells show increased p53 protein levels and loss of DNA integrity as detected by TUNEL (56). DNA damage induced by ionizing radiation, bleomycin, or drugs that inhibit DNA topoisomerase activity prolong p53 protein half-life, and this accumulation of p53 induces a  $G_1$  arrest (57, 58). However, when DNA damage is extensive, p53 can induce cell death by inducing Bax (59) or by directly damaging mitochondrial integrity (60).

### Death of AECs by IFN- $\gamma$ , TNF- $\alpha$ , and Fas

Exposure of NHBEs to IFN- $\gamma$  causes Bax translocation to the ER, calcium release (data not shown), and caspase activation (61). Although IFN- $\gamma$  has induced Fas in keratinocytes (62), we did not observe induction of Fas in NHBEs exposed to IFN- $\gamma$ . Co-treatment with Fas-antibody does not enhance IFN- $\gamma$ -induced cell death in NHBEs. IFN- $\gamma$  has been shown to induce

apoptosis in a variety of cell types, including colon adenocarcinoma cells (63, 64), primary human keratinocytes (62), HeLa cells (65), breast tumor cells (66), and fibroblasts (67). IFN- $\gamma$ -enhanced signaling proteins that are responsible for cell death remain largely unknown (68), and various pathways appear to be involved in IFN- $\gamma$ -induced apoptosis.

TNFR-1 and -2 are expressed in AECs, and their levels are increased in response to TNF- $\alpha$  (69); however, preincubation of these cells with TNF- $\alpha$ , IL-1 $\beta$ , or IFN- $\gamma$  did not effect expression of Fas or the mRNA for the adaptor proteins FADD, TRADD, or caspase-8 (70). AECs also express Fas receptor and FasL (69, 71, 72). Therefore, several studies have tested whether Fas or TNF- $\alpha$  induce cell death in AECs. Only 10–15% of primary AECs or A549 cells undergo cell death in response to anti-Fas ligation of the receptor (73, 74, 72) or to TNF- $\alpha$  (69). While all SAEs expressed Fas receptor to a similar extent as did NHBEs, 40% of distal SAEs showed cell death, and only 10% of proximal NHBEs showed TUNEL positivity when exposed to soluble FasL (500 ng/ml) (70).

While NHBEs are refractory to Fas or TNF- $\alpha$ , they can be sensitized by other cytokines to readily undergo cell death to these mediators. Activated T cells and eosinophils from healthy and atypical individuals induce apoptosis in AECs, and cell death was blocked when activated T cells and eosinophils were preincubated with antibodies to IFN- $\gamma$  and TNF- $\alpha$  (69). However, eosinophils induced apoptosis in AECs only when cells were pretreated with IFN- $\gamma$ . Interestingly, apoptosis induced in AECs by T cell supernatants was prevented by caspase inhibitors but was not affected by Fas or FasL, suggesting that the FasL-Fas pathway plays a less important role in the death of AECs. A549 (72), NT-1, and the cystic fibrosis cell lines CFT-1 and CFT-2 (75) are also not susceptible to anti-Fas mAb, although they express the Fas receptor. However, up to 74% of these cell lines, primary NHBEs, and SAEs show cell death when pretreated with 10 ng/ml IFN- $\gamma$  for 24 h (69). A minimum of 6–8 h of pretreatment was sufficient to increase cell death in A549 cells by anti-Fas (72). Collectively, these studies suggest that IFN- $\gamma$  sensitizes AECs to TNF- $\alpha$ - or FasL-induced cell death. However, the mechanisms of this sensitization process are not known.

### TGF- $\beta$ and Death of AECs

In NHBEs, TGF- $\beta$  induces apoptosis by increasing the p38 MAP kinase phosphorylation, but also by increasing the phosphorylation of ERK 1/2 and JNK. This cell death was inhibited by budesonide or with inhibitors of the MAP kinases P38, ERK 1/2, and JNK (76). Another report showed that TGF- $\beta$ 1 protected NHBEs from Fas-induced cell death that was in the range of 10–15%, and this protection was blocked by inhibition of the Smad pathway (73). These contradictory findings may suggest that the definition of inducing apoptosis in a cell population is somewhat vague, or that future studies need to clarify whether TGF- $\beta$  has a protective role when it is present together with other inducers of apoptosis.

### Corticosteroids and Apoptosis of AECs

Corticosteroids elicit apoptosis in eosinophils and in T lymphocytes in a rapid and massive manner (77–79). Several studies from the same group report that corticosteroids elicit apoptosis in AECs where the magnitude of apoptosis in response to dexamethasone treatment for 24 h reached a maximum  $\sim$  5–10% as measured by TUNEL positivity (80–82). The authors acknowledge that this proportion is small compared with apoptosis elicited by the same agents in hematopoietic cells, but we propose that epithelial damage over prolonged treatment with corticosteroids may lead to a significant degree of airway mucosal damage. These studies used concentrations of corticosteroids at the high



end of what might be achieved in a clinical setting (80). While apoptosis of AECs in the AE of an individual with asthma can be detected after collection by endobronchial biopsy, other studies have not observed epithelial cell apoptosis by individuals with asthma (83). Therefore, the physiologic significance still remains to be elucidated. The  $\beta$ -adrenergic agonist, albuterol, and both PKA activators, forskolin and dibutyryl cAMP, significantly inhibited the 5% apoptosis induced by corticosteroids. And an inhibitor of PKA, H-89, also blocked the protective effect of albuterol, suggesting that the inhibition was through the PKA pathway. Because treatment with these  $\beta$ -agonists for longer than 4 h after treatment with corticosteroids was not protective, the authors recognize that the timing of the protective effect of albuterol (an agent that is relatively short in half-life for biological effect when inhaled) may be a concern. While the mitochondrial pathway and cytochrome c release is suggested, Bcl-x<sub>L</sub> does not affect this cell death, and absence of blocking experiments make a conclusive understanding of the mechanism of action impossible at present. Fas receptor-activated cell death of AECs was not affected by  $\beta$ -adrenergic agonists, indicating that the pathways for the corticosteroid receptor and Fas receptor-induced cell death are different. However, the Fas-induced cell death (5–15% of cells showed TUNEL positivity at 24 h) is also relatively minor.

Corticosteroid treatment of BALB/c mice over prolonged periods of up to 4 wk caused increased presence of AECs in the lavage fluid. Furthermore, the concentration of dexamethasone at 1 mg/kg, sufficient to reduce the allergen-induced inflammation, did not reverse airway epithelial shedding. Analysis of the epithelial cells by histology showed increased TUNEL positivity and also increased detection of cleaved p85 by immunohistochemistry. Dorscheid and colleagues (84) concluded that corticosteroid treatment over prolonged periods may induce apoptotic cell death in airway epithelium *in vivo*, and while sufficient to reduce the allergen inflammatory response, did not alter the number of AECs that are shed during an asthmatic phenotype.

In contrast to these studies, several reports show that glucocorticoids inhibit cell death induced by cytokines, such as IFN- $\gamma$  or TGF- $\beta$ . Dexamethasone at a concentration of 1 mM induced the hIAP mRNA significantly and inhibited cell death in A549 cells by IFN- $\gamma$  from 38% to 7% and by IFN- $\gamma$  plus anti-Fas from 81% to 10% (72). IFN- $\gamma$  further increased dexamethasone-induced hIAP. Cell death by IFN- $\gamma$  and Fas was successfully inhibited by zVAD-fmk, suggesting that caspases are involved in this cell death process. IFN- $\gamma$  induced IL-1 $\beta$ -converting enzyme (ICE) or caspase-1; however, the cleavage product of caspase-1 was not detected. Dexamethasone did not affect IFN- $\gamma$ -induced caspase-1 expression. Because zVAD-fmk inhibited IFN- $\gamma$  and anti-Fas-induced apoptosis the authors conclude that caspase 3 and possibly caspase 7 were involved in this cell death process. Whether dexamethasone-induced hIAP suppresses apoptosis by IFN- $\gamma$  and Fas is not clear. Also, the TGF- $\beta$ -induced MAP kinase activation and apoptosis in AECs are effectively inhibited by budesonide, suggesting that inhaled glucocorticoids have a protective role against epithelial injury (76).

#### Zinc as Anti-Apoptotic Factor

When primary AECs are deprived of zinc, a nominal increase in caspase activation and apoptosis is observed in 10% of cells; however, the barrier function of differentiated cultures is not disrupted (85). Zinc depletion combined with treatment with TNF- $\alpha$ , IFN- $\gamma$ , and FasL abruptly decreases trans epithelial resistance and increases apoptosis to 60–70% as measured by caspase-3 activity. This apoptosis was suppressed by adding zinc or magnesium sulfates. Interestingly, zinc increased phosphory-

lation of Akt within 2 h of treatment, and suppression of Akt phosphorylation with LY249002 suppressed the protective function of zinc. This study suggests that subacute zinc deficiency may place the AE at a disadvantage during periods of inflammation by reducing the refractory state of the AE. While zinc activates PI3 kinase signaling it is not clear whether other signaling pathways may be activated by zinc to protect AECs from cell death. Identification of the pathways that make a normal AE resistant to many inducers of apoptosis is a crucial field of study to fully understand deficiencies that may provide sustained damage and inflammation in chronic diseases.

#### APOPTOSIS TO RESTORE HOMEOSTASIS AFTER HYPERPLASTIC CHANGES IN THE AIRWAY EPITHELIUM

Exposure of the lungs to harmful substances such as bacteria or cigarette smoke induces acute lung inflammation characterized by infiltration with neutrophils and macrophages (86–88), followed by proliferation of nonciliated columnar epithelial cells (89–93). Inflammatory cells secrete a number of proteins (including cytokines, chemokines, and proteases) that promote the differentiation of proliferating and pre-existing epithelial cells into mucous cells by inducing mucin biosynthesis (94–96). Therefore, after injury, the airway epithelium presents goblet cell metaplasia and hyperplasia (97).

Goblet cell metaplasia (GCM) can occur as a result of pre-existing Clara or serous cells that begin to express MUC5AC (98) in the absence epithelial cell proliferation. Another study (92) reported that 10% of mucous cells proliferate in conjunction with a compensatory decrease in Clara and ciliated cell numbers. Our studies show an increase in total AECs together with increased GCM (93, 99, 100). The difference between the three studies appears to be the extent of injury and inflammatory response due to length of allergen exposure or dose of LPS administered. The resolution of GCM under conditions where epithelial cells have not proliferated is associated with downregulation of MUC5AC expression, which leads to the reversion of mucous cells to Clara or serous cells. When injury is extensive and epithelial cell proliferation has occurred, in the absence of further insult to the epithelium, inflammation is cleared, and mucous cell numbers are reduced by programmed cell death (74, 101, 102).

#### Resolution of Hyperplastic Changes after a Single Injury to the Airway Epithelium

After a single intratracheal instillation of LPS, the number of AECs per millimeter of basal lamina exceeds the number found in normal epithelium by 30–40% (93, 99). Resolution of these hyperplastic changes involves reduction of AEC numbers by 30% together with downregulation of MUC5AC expression and differentiation of mucus-producing cells into Clara, serous, or other types of cells.

Classic apoptotic epithelial cells are not obvious during the recovery of the airway epithelium; whether some of the metaplastic cells slough off during this process is still under investigation. It is possible that selected cells are removed by extrusion and may be sloughed off before the classic apoptotic morphology appears. Cell death involving detachment from the basement membrane is also called anoikis (103). In an attempt to understand the mechanisms involved in reducing the number of metaplastic mucous cells, we analyzed the expression of regulators of apoptosis and found that metaplastic mucous cells transiently express Bcl-2, an inhibitor of apoptosis (104, 102). Bcl-2 is expressed in 30% of LPS-induced metaplastic goblet cells in airway epithelia (102) or in  $\sim$  50% of ozone-induced metaplastic goblet

cells in epithelia lining the maxillo-turbinate (104). Metaplastic goblet cells are heterogeneous in their ability to express Bcl-2, which suggests that mechanisms may be in place to selectively eliminate AECs. However, these selection mechanisms are currently unknown.

Bcl-2 is expressed in proliferating and nonproliferating mucous cells, as was shown by identifying proliferating AECs using BrdU (93). The presence of Bcl-2 in BrdU-negative cells shows that Bcl-2 is expressed in pre-existing, nonproliferating mucous cells and that it does not have cell cycle-regulatory functions as was shown in other cell systems (105–107).

The following observations show that Bcl-2 expression sustains GCM. (1) After a single intratracheal instillation of LPS to F344/N or brown Norway rats, the percentage of Bcl-2-positive mucous cells decreases to background levels at least 2 d before the decrease of GCM (99, 102). These data suggest that anti-apoptotic Bcl-2 must be downregulated before mucous cell numbers can be reduced. (2) Downregulation of Bcl-2 mRNA levels, as shown by *in situ* hybridization, reduces epithelial mucosubstances in organ cultures and rats *in vivo*. The reduction of mucous cell numbers when Bcl-2 levels are decreased directly links Bcl-2 to the sustenance of metaplastic mucous cells. Conversely, sustained expression of Bcl-2 in transgenic mice caused increased LPS-induced GCM over a prolonged period compared with wild-type mice (99).

Treatment of rats with antisense oligodeoxynucleotide (ASODN) inhibits Bcl-2 expression and reduces GCM (99), indicating that pro-apoptotic pressures are present and initiate cell death once Bcl-2 levels are reduced. We have identified BH-3 domain-only proteins that are important in airway epithelial cell death, but their importance in the resolution process remains to be elucidated. This family of proteins is also known to sense death signals associated with detachment-induced cell death (30, 32) (Figure 1).

Disruption of this recovery process may persistently elevate mucous cell numbers and contribute to mucous hypersecretion and airway obstruction found in chronic lung diseases such as CF, chronic bronchitis, or asthma (108, 109). Various studies show the importance of goblet cell hyperplasias as the major source of mucin in the tracheobronchial tree (110, 111). Therefore, the possible significance of Bcl-2 expression in chronic diseases associated with chronic mucin hypersecretion was studied. Horses with reactive airway obstruction (RAO) have increased mucous secretions (112), and persistent mucus overproduction is an important component of airway obstruction (113). These RAO horses have a significantly higher percentage of Bcl-2-positive mucous cells compared with control horses (personal observation).

Furthermore, a significantly higher percentage of mucous cells immunostain with Bcl-2 antibodies in humans with CF compared

with control subjects without disease and to subjects with other lung diseases (99), suggesting that GCM may be sustained and not transient if the expression of Bcl-2 is dysregulated. Another group of researchers used microarray approaches to identify Bcl-2 as one of the genes expressed after a smoke-induced injury to human AECs (114). Collectively, these studies demonstrate that Bcl-2 expression in mucous cells is observed in a variety of species, including human, horse, rat, and mouse, and that such regulation is conserved among different species. Studies of how the epithelium removes excess epithelial cells after inflammatory responses may help identify molecular deficiencies that lead to excess mucous cell numbers under pathologic conditions.

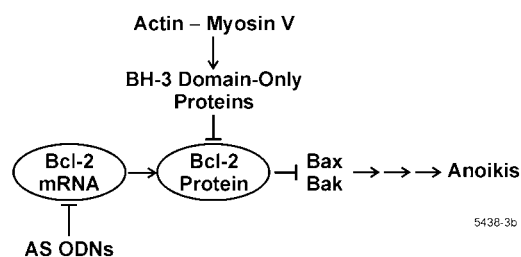
While O'Reilly and coworkers (56) describe the cell death that occurs directly in response to hyperoxia, it is also known that relief of oxidant stress is often associated with significant epithelial cell proliferation and differentiation to replace sick and dying cells that were injured during the exposure period (115). Because hyperoxia alters cell proliferation and survival, the proliferating cells may be enhanced to result in increased cell numbers per millimeter of basal lamina compared with normal epithelia. Therefore, reduction of these cell numbers is important during the recovery process. These observations support the notion that in chronic inflammation proliferation and apoptosis are balanced in airway epithelia. Activation of cell proliferation may also activate the cellular apoptotic program that may counter-regulate survival signals. These signals may be provided externally by nearby cells providing cytokines such as TNF- $\alpha$  and IFN- $\gamma$  that cause cell death or by growth factors such as EGF that inhibit apoptosis by inducing anti-apoptotic proteins.

### Resolution of Allergen-Induced Hyperplastic Changes

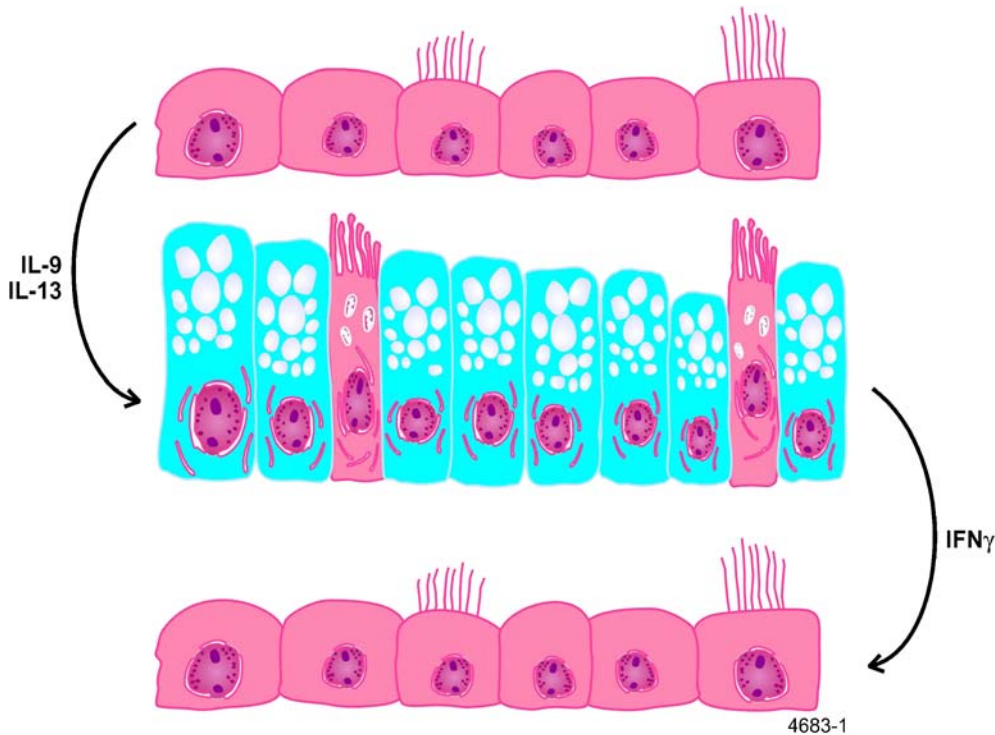
In rodents, chronic exposure to an allergen initially induces inflammation, which decreases over time (116, 117), whereas in humans with allergic asthma, inflammation persists and is chronic during their lifetime (118). This transition from antigen sensitization to immunologic tolerance is accompanied by a shift in the lymphocyte content in the lung tissue and bronchial lavage fluid (BALF) (119, 116). Antigen-specific regulatory T cells are believed to produce IL-10 transiently and to thereby inhibit the asthma phenotype during the development of tolerance (120, 121). Prolonged exposure of mice to an allergen causes the cytokine IL-13 that is secreted by T helper cell type 2 (Th2) to decrease and IFN- $\gamma$ , which is secreted by Th1 cells, to increase in the bronchoalveolar lavage fluid (61). IFN- $\gamma$  mediates the reduction of IL-9- and IL-13-induced GCM by inducing programmed cell death (74) (Figure 2).

While Bcl-2 is not detected, the percentage of Bax-immunopositive mucous cells increases from ~3–25%, while the number of metaplastic mucous cells decreases (61). The fact that Bcl-2 expression is associated with the appearance of LPS-induced GCM and that Bcl-2 is not detected in allergen-induced GCM in mice suggests different resolution mechanisms in these two experimental systems. Overall, ~25–35% of mucous cells express Bax after repeated exposure to allergen for 15 d. Trifilieff and colleagues (122) found that by 3 d after allergen exposure, ~30% of epithelial cell nuclei are BrdU-positive, a marker for cells that undergo DNA synthesis during the cell cycle. Taken together, the observed Bax positivity in ~25–35% of mucus cells during the resolution of allergen-induced GCM suggests that the Bax-positive mucous cells may represent cells that must be eliminated to reconstitute the original cell number of the repaired epithelium.

One could speculate that humans with allergic asthma are deficient in developing such immune deviation to specific compounds or in IFN- $\gamma$  signaling to reduce GCM. In some instances, serum levels of IFN- $\gamma$  can be increased in severe asthma



**Figure 1.** Regulation of the anti-apoptotic activity of Bcl-2 by expression levels and by BH-3 domain-only proteins that sense the death signals from the actin-myosin filaments.



**Figure 2.** Allergic inflammation induces epithelial cell hyperplasias by IL-9 and IL-13, and the resolution occurs when IFN- $\gamma$  is present at increased concentrations compared with the Th2 cytokines.

cases (123), and IFN- $\gamma$  levels in BALF are sometimes found even in mild asthma (124, 125). Although these data seem paradoxical, a reasonable hypothesis would explain a failure of IFN- $\gamma$  in these patients with asthma to drive the resolution of airway hyperreactivity and GCM. For instance, IFN- $\gamma$  levels may not be high enough to reverse the Bax inhibitory effects of IL-13. Supporting this hypothesis, the instillation of 100 ng IFN- $\gamma$  causes reduction of GCM, while 50 ng does not (74). It is also possible that in a subpopulation of individuals with asthma, a deficiency in the IFN- $\gamma$ -signaling pathway may render IFN- $\gamma$  incapable of inducing cell death in epithelial cells. This hypothesis is supported by recent reports that polymorphisms in genes encoding for IFN- $\gamma$  and IFN regulatory factor-1 (IRF-1) confer genetic susceptibility to allergic asthma in Japanese children (126) and that Stat 1 is constitutively activated in epithelial cells of individuals with asthma (127). These alterations may contribute to deficient signaling of IFN- $\gamma$  and sustained increased levels of GCM even in the presence of IFN- $\gamma$ . Supporting this hypothesis, mice deficient in Stat 1 (an obligatory protein for IFN- $\gamma$  signaling) do not resolve GCM after repeated and prolonged exposure to an allergen (74). These animals may represent appropriate models to study chronic inflammatory airway disease that resembles human asthma and to test immunomodulatory therapies to reverse established airway remodeling.

### AECS CONTROL INFLAMMATION BY APOPTOTIC MECHANISMS

#### Fas and FasL on AECs Reduce Inflammation

Several studies have confirmed by flow cytometry and immunohistochemistry that Fas and FasL are expressed in AECs (69, 71). Fas and FasL expression are similar in both the SAEs and NHBES (70). Coexpression of Fas and FasL in the same cells is uncommon for mammalian cells and has only been described for corneal epithelium (128) and crypt cells in ulcerative colitis (129). The functional role of this coexpression of epithelial cells

is still not clear. A possible role of the FasL in causing adjacent cell death should be manifested by rapid cell turnover. However, AE has estimated turnover rates of < 1% in normal tissue (130). AECs must be refractory to FasL-induced death signals. Fas signaling may have functions unrelated to the cell death process (131), but whether these functions are important in AECs remains to be determined.

Another role for FasL expression in AECs may be to control the numbers of inflammatory cells by inducing cell death. Support for the anti-inflammatory role of FasL is found from observation in mice and from reports showing that the AE is capable of controlling the number of inflammatory cells and to actively reduce the inflammatory response once the insult is removed (132). In mice, expression of FasL, as shown by *in situ* hybridization, was found to be primarily restricted to bronchial Clara cells and nonciliated columnar AECs (71). Some squamous cells of the upper airways also showed FasL expression. In *gld* mutant mice, where FasL is nonfunctional, a diffuse accumulation of mononuclear inflammatory cells is observed in the lung that is different from inflammation typified by bronchus-associated lymphoid tissues (BALT). The finding is in agreement with other reports showing that in *lpr* or *gld* mice that have nonfunctional Fas and FasL, respectively, lymphocytes accumulate in epithelial tissues such as the small intestine, female genital tract, the lung, and T cells express markers of chronic activation including CD69 (133). These findings support the hypothesis that FasL has an immune regulatory role in all epithelial tissues. FasL mRNA expression was reduced in mice exposed to allergen where airway inflammation is increased, suggesting that FasL on AECs control the infiltration by T cells of the lung (71).

#### Fas and FasL in Diseased Airway Epithelia

FasL expression is markedly more intense in ciliated and submucosal gland of CF airways compared with that of normal subjects and in the CF cell line (CF-T2) compared with the non-CF cell line (NT) (134). However, expression of Fas is similar in both



airway tissues and cell lines from subjects with CF and control subjects. The findings are consistent with others reporting Fas and FasL expression in bronchial epithelial cells (135–137). Increased FasL expression in patients with CF could be due to various reasons: (1) chronic inflammation (although IFN- $\gamma$  and TNF- $\alpha$  did not increase Fas and FasL expression in their studies, it is possible that other cytokines may be responsible); (2) extracellular matrix consisting of subepithelial fibrosis and degradation of collagenic and elastic fibers in CF may induce expression (138). Whether induction of FasL is directly signaled by CFTR deficiency or by upregulating cytokine expression is not known.

In serum of patients with CF, soluble FasL was significantly lower than in serum obtained from control subjects (138), although the significance of this finding is unclear. Expression of Fas and FasL in the same epithelium is also found in autoimmune diseases, where their interaction leads to epithelial destruction. FasL may also induce apoptosis in inflammatory immune cells (71, 135, 136). FasL may confer immune suppression in malignancy by inducing apoptosis of Fas bearing immune cells such as lymphocytes and dendritic cells (139). FasL can also be proinflammatory by activating PMNs (140). Because PMNs are predominant phagocytes in CF (141), death of PMNs may increase DNA that alters viscosity of CF airway secretions (142).

Hamann and coworkers (135) report that Fas expression appears to be greater in epithelial regions where some epithelial damage was present. Overexpression of Fas may be proinflammatory in chronic inflammation such as asthma and chronic bronchitis and, as a result, may play a role in the overall epithelial integrity. This observation may explain why increased epithelial cell turnover has been documented in chronic bronchitis (130). On the other hand, the expression of FasL may function to prevent infiltration of Fas-bearing inflammatory cells such as eosinophils in the epithelial layer. A role for Fas and FasL in immune privilege has been suggested for other tissues including testes (143), brain (144), and placenta (145). Decreased FasL expression or the inheritance of a weak form of FasL may predispose to increased inflammation and a weakening of this immune barrier, leading to conditions of chronic inflammation such as asthma.

### AECs as Phagocytes

Walsh and coworkers (146) and Sexton and colleagues (147) showed by electron microscopy and confocal microscopy that resting and cytokine-stimulated human SAEs and NHBs recognize and ingest apoptotic eosinophils. Furthermore, the same group showed that A549 also phagocytose apoptotic eosinophils by recognizing integrin, lectin, and phosphatidylserine membrane receptors, and that this process can be upregulated by dexamethasone (148). Treatment with cytochalasin D at 10  $\mu$ M concentration completely abolishes the uptake of apoptotic eosinophils by SAEs, NHBs, or macrophages. This apoptosis was confirmed by the lack of adhesive interactions between viable eosinophils and neutrophils with airway epithelial cells. Interestingly, the uptake of eosinophils was inhibited by preincubation of apoptotic eosinophils with the amino sugars. Ligation of CD44 receptors on SAEs or NHBs or treatment of these cells with IL-1 (this study did not differentiate whether this was IL-1 $\alpha$  or IL-1 $\beta$ ) increased the phagocytosis ability. However, apoptotic neutrophils were not subjected to phagocytosis by AECs even in the presence of cytokines such as IL-1. Interestingly, cell lines of mammary epithelium (ZR-75-1) and colon epithelium (HT-60) also revealed no ingestion of apoptotic neutrophils but of apoptotic eosinophils. These studies suggest that AECs are active participants in the removal of apoptotic eosinophils and may be important in the resolution of eosinophilic inflammation in the asthmatic lung.

### SUMMARY AND FUTURE DIRECTIONS

Many studies have shown that T lymphocytes, neutrophils, and eosinophils undergo cell death in massive numbers within 3–6 h after cytokine deprivation or stimulation with death agonists. However, AECs undergo cell death in significant numbers only 24 h after exposure to cell death agonists, and in most instances the percentage of dying cells is in the range of 5–15%. We find that 60% of AECs die 4 d after exposure to IFN- $\gamma$  (74). These findings suggest that cell death in AECs is either not mediated by a direct pathway and requires protein synthesis or checkpoints to stop direct signaling to activate effector caspases are in place. Elimination of these checkpoints such as by pretreatment with IFN- $\gamma$  may increase susceptibility and the appearance of massive cell death to Fas or TNF- $\alpha$ . It is also feasible that the airway epithelium has developed various mechanisms as protection from undergoing cell death in response to various injurious substances: formation of tight junctions (which may give stability by coordinating the structural actin filaments) or the readiness to activate I $\kappa$ B, which causes the proinflammatory cytokine expression and also expression of pro-survival proteins including the caspase inhibitors c-IAP1 (149) and c-IAP-2 (150).

Many environmental toxins, including PAHs and their derivatives such as PAH epoxides, cause release of calcium from the ER. This ER calcium can mimic signaling for cell proliferation. However, it is possible that once AECs are sensitized to undergo cell death by certain conditions, or when Bax is in the right cytosolic compartment, the same signal may induce cell death. It is conceivable that the refractory nature of AECs to apoptosis may stem from their ability to successfully sequester proapoptotic mediators so they need to have other signals to activate them for activating downstream caspases.

In physiologic settings, the slow induction of cell death in AECs is critical because rapid cell death would not allow for closure of the basement membrane by neighboring cells. One could postulate that neighboring cells have mechanisms to sense the death of AECs to cover the open space created by the dying cell(s). Furthermore, it is still not clear whether dying AECs are removed by phagocytosis by neighboring AECs or macrophages or by sloughing off into the airway lumen and undergoing detachment-induced cell death, also called anoikis.

It is crucial to understand the mechanisms that make AECs refractory to cell death. Fas and FasL are expressed, but AECs do not undergo cell death by this pathway. In fact, our studies suggest that FasL induces proliferation of AECs in mice that have been exposed to allergen (personal observation).

SAEs appear to be more sensitive to Fas ligation than NHBs (70), suggesting that one needs to be very careful when reporting what cells are being used in the cell death process and indicate that proximal and distal airway epithelial cells may activate different pathways to their cell death process. Furthermore, while Hagimoto and coworkers (151) report that activation of Fas with recombinant human FasL induces IL-8 secretion in NHBs, Nakamura and colleagues (70) did not find increased IL-8 by activating Fas with Fas antibody. These studies suggest that various means of activation of Fas could result in different outcomes.

It is possible that GCM could be reduced by targeting cell death processes in cases where intrinsic changes in airway epithelia sustain GCM independent of inflammation. However, in chronic diseases, GCM may be a result of continuous inflammation and repeated injury and repair to the airway epithelium. Therefore, it is important to determine how altering the half-life of goblet cells without removal of the continuous inflammation may affect the dynamics of the airway epithelia. It may be crucial to use Bcl-2 and/or the pro-apoptotic players to reduce GCM as part of a general therapeutic effort to reduce

inflammation. Chronic inflammation in animal models needs to be established to adequately address these complex issues of GCM in chronic inflammation.

In the context of apoptosis to restore homeostasis in hyperplastic epithelium, it remains unclear how the epithelium determines the number of cells that must be eliminated to restore the original condition. Is it possible that epithelial cells sense intercellular pressure and signal cell death to eliminate cells and create enough space? Furthermore, it is unknown whether the discarded cells represent abnormal or damaged epithelial cells and whether there are signals that determine which cells should be discarded from the airway epithelium. Finally, how the epithelium restores the normal proportions of epithelial cell types is unclear. Why do a certain number of the remaining cells differentiate into serous, Clara, ciliated, and mucous cells to reconstitute the proportions found in normal epithelia?

Future studies will also focus on the ability of the AE to control inflammation. Deficiency in this ability may be crucial for sustained inflammation in airways and could provide novel targets for developing new therapies.

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