

# Apoptosis-Regulatory Factors as Potential Drug Targets in the Epithelium of Normal and Inflamed Airways

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**Abstract:** Airway epithelium (AE) lines the conducting airways of the respiratory system and functions to maintain airway integrity by providing both a physical barrier to inhaled noxious agents and a mechanism for their clearance *via* the mucociliary escalator. Normal AE cells are relatively refractory to a number of apoptotic stimuli and survival mechanisms are in place to maintain the integrity of the epithelial barrier that is exposed to agents such as reactive oxygen species (ROS) and death receptor ligands secreted by immune cells during inflammation. When damage to AE does occur, there is increased AE apoptosis, such as in the airway damage that occurs in the chronically inflamed airways in diseases like asthma where rates of AE apoptosis can be increased many-fold. The usual treatment for persistent asthma in humans involves a combination of bronchodilator and inhaled corticosteroid; there is however a need to develop strategies to better control other aspects of the disease, including minimizing the ongoing damage to AE and consequent airway remodeling. Targeting of the major apoptosis-regulatory factors in AE may be one such strategy. Here we review what is known about apoptosis and its regulatory factors in normal AE and abnormalities in these factors in the inflamed airways of mice and humans.

**Keywords:** Airway epithelium, Apoptosis, Necrosis, Airway damage, Inflammation, Asthma, Caspases, Therapy.

## 1. INTRODUCTION

Apoptosis is a tightly-regulated mechanism of cell death that is critical for normal tissue homeostasis and often dysregulated in disease [1]. Contributing to disease pathogenesis are both insufficient apoptosis (as in many tumours and autoimmune disease) as well as excessive or inappropriate apoptosis (as in neurodegenerative disease and some immunodeficiencies). As a consequence of this, there is considerable interest in the concept of targeting apoptosis signalling pathways as a means to improve therapy or prevent disease. This review concerns the occurrence and targeting of apoptosis in the normal and inflamed airway epithelial lining. Death of other airway cells and tissues (e.g. alveolar epithelial cells, smooth muscle cells, fibroblasts and pulmonary endothelial cells) as well as epithelial cell death in other airway diseases (such as cystic fibrosis and chronic obstructive pulmonary disease (COPD)) are beyond the scope of this review and are mentioned only briefly. The review commences with brief overviews of airway epithelium and disease (Section 2) and apoptosis and necrosis (Section 3). Section 4 discusses apoptosis of normal airway epithelial cells (AEC) while Section 5 focuses on death of AEC in asthma, a major airway inflammatory disease. Section 6 considers the possibilities and limitations of strategies to target death of AEC in diseased airways.

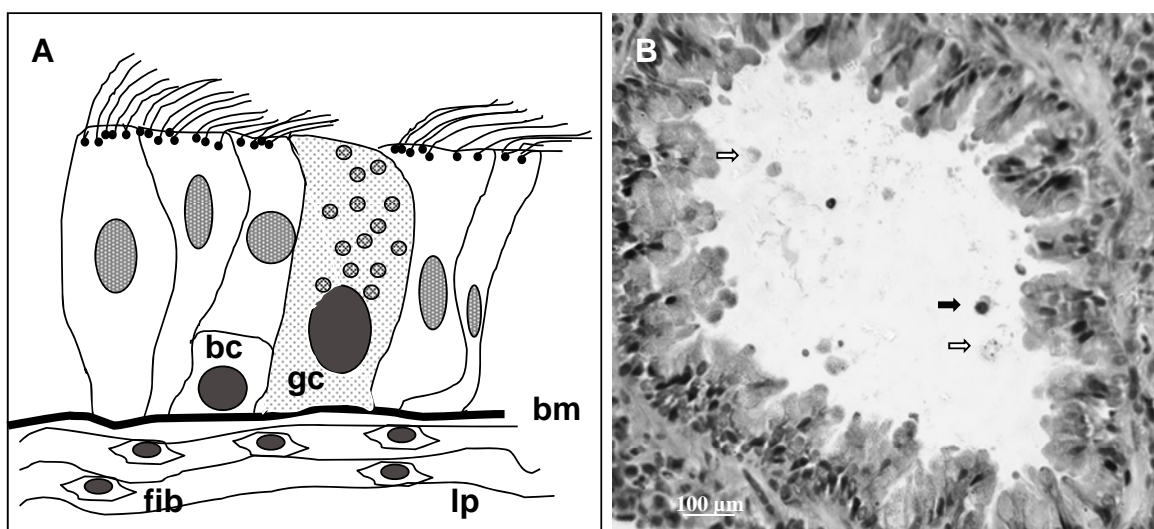
## 2. AIRWAY EPITHELIUM AND AIRWAY DISEASE

The airways that conduct oxygen to the lung alveoli include the nasal cavity, trachea, bronchi and bronchioles. All but the most terminal bronchioles are lined by a columnar, pseudostratified AE (Fig. 1A) comprising three major cell types:- ciliated columnar cells (the predominant cell type),

muco-secretory cells and basal cells. The latter are believed to serve as precursors of the other two cell types [2]. AE is critical for normal integrity and function of the respiratory system [3]. It forms a physical barrier to inhaled oxidants, allergens and other noxious substances, which might otherwise damage underlying tissues such as smooth muscle and airway vasculature, or trigger immune and inflammatory responses. It also removes foreign particles and infectious agents from the airway lumen, *via* the mucociliary escalator. Mucopolysaccharides secreted into the lumen from non-ciliated epithelial cells (e.g. goblet cells) trap inhaled foreign matter [4]. The latter is propelled up the airways by the beating of cilia on the apical surface of the columnar cells [5]. A third function of AE is to secrete a number of regulatory cytokines and other mediators which are important in immune regulation and bronchodilation [5, 6].

The normal architecture and function of AE is disturbed in chronic airway inflammatory diseases such as bronchial asthma. Asthma is characterized by recurrent episodes of wheezing, breathlessness and chest tightness because of airway narrowing due to inflammation. It affects a steadily increasing proportion of the population, especially in the developing world, and has been labelled a "disease of a modern lifestyle" [7]. There are several acute and chronic animal models of airway inflammation, including murine models in which Balb/c mice that have been sensitized to ovalbumin (OVA) and then challenged with aerosolized OVA develop a pronounced airway eosinophilia associated with airway hyper-responsiveness, mucous hyperplasia, damage to AE and collagen deposition [8, 9]. The pathology of allergic asthma involves immediate IgE-dependent responses which trigger mediator release causing bronchoconstriction as well as chronic infiltration of airways by inflammatory cells (especially eosinophils, but sometimes neutrophils). Cytokines, proteases and oxyradicals released from infiltrating eosinophils, neutrophils and immune cells, as well as from

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**Fig. (1). AE and its damage in murine airway inflammation:**

**A:** Cartoon showing the three major cell types in normal AE. Ciliated epithelial cells predominate and usually extend across the entire epithelium; their cilia, which may number several tens to hundreds per cell, beat in a coordinated manner and are anchored in the apical cytoplasm by basal bodies (depicted as black spheres). Goblet cells (gc) contain numerous secretory granules and secrete mucopolysaccharides which trap foreign particles at the luminal surface. Basal cells (bc), which are precursors of the other two cell types, lie at the base of the epithelium. Beneath the basement membrane (bm), fibroblasts (fib) secrete a matrix of extracellular proteins in the lamina propria (lp).

**B:** Stained lung section (Martius Scarlet Blue) from OVA-treated mice showing heavily swollen, irregular bronchiolar AE with shedding of apoptotic (black arrow) and necrotic (white arrow) AEC into the lumen.

activated, resident tissue macrophages and mast cells, cause proteolytic and oxidative damage to AE [3, 10]. A damaged and partially denuded AE may not only fail in maintaining sterile airways and in protecting underlying structures, but it can also actively participate in the pathogenesis of airway disease by further promoting airway inflammation [6, 11, 12]. In chronic asthma, repeated episodes of damage and repair lead to airway remodelling with smooth muscle hyperplasia and extensive collagen deposition that further limit airflow [11].

Not only is there increased exposure to cytotoxic agents during airway inflammation but, for reasons still not properly understood, the AEC themselves acquire a heightened susceptibility to die compared with normal AEC exposed to the same agents (See Section 5). There is therefore a need to better understand the mechanisms underlying AEC death and the mechanisms that render them sensitive to death-inducing signalling pathways.

### 3. APOPTOSIS AND NECROSIS, MECHANISMS OF CELL DEATH

#### 3.1. Apoptosis and Caspases

Apoptosis is a distinct mechanism of cell death enabling the removal of superfluous, mutant or moderately damaged cells without release of cellular contents that would otherwise damage neighbouring cells or provoke an immune or inflammatory response. It is normally tightly-regulated but can become deregulated in disease, the altered regulation often contributing to the pathogenesis [13]. Apoptosis is an active, energy-dependent process characterized by cell

shrinkage, condensation and fragmentation of the cytoplasm and nucleus, inter-nucleosomal DNA fragmentation, changes in the phospholipid distribution in the plasma membrane and eventual formation of apoptotic bodies which are cleared *in vivo* by phagocytosis or shed into luminal cavities [1]. There are diverse input signalling pathways originating from events such as ligation of death receptors in the plasma membrane (e.g. Fas), cytoskeletal disruption, DNA damage and oxyradical-induced lipid oxidation [14]. A number of current therapeutic strategies are aimed at modulating these pathways in disease to either suppress or restore apoptosis where required.

Although caspase-independent forms of apoptosis exist [15], most examples of apoptosis involve both initiator and effector caspases [16]. The major initiator caspases -8 and -9 transduce signals from specific input pathways (e.g. Fas ligation results in caspase-8 activation while caspase-9 activation is triggered by release of cytochrome *c* from mitochondria). Cytochrome *c* release triggers the formation of a structure called the apoptosome that provides the scaffold and cofactors for procaspase activation [17]. Pro-caspase-9 is activated by formation of a complex with Apaf-1 (apoptosis protease-activating factor) and with cytochrome *c*. Activated caspase-9 then cleaves and activates pro-caspase-3. Cleavage of critical substrates (at caspase-specific tetra-peptide sites) by the executioner caspases leads to the downstream events of apoptosis. Model synthetic fluorogenic substrates based on these motifs (e.g. zDEVD-AFC for caspase-3) enable specific types of caspase activity to be measured in cell lysates while cell-permeable substrates enable measurement of caspase activity in intact cells as well as pharmacological manipulation of apoptosis [18, 19].

Caspases are subject to terminal regulation by the family of cellular proteins called the Inhibitors of Apoptosis proteins (IAPs) [20]. Mammalian IAPs include cIAP<sub>1</sub>, cIAP<sub>2</sub> and the X-linked XIAP. Most IAPs are able to suppress caspases by one or more of a variety of mechanisms, although different members of the family may have distinct mechanisms [21]. These include i) binding to and inactivation of the active catalytic site of effector caspases, ii) binding to the processed amino terminus of monomeric caspase-9 thereby preventing the dimerization required for effector caspase activation, iii) sequestering of mitochondrial Smac/Diablo and thereby preventing them from acting as antagonists for other IAPs and iv) targeting procaspases or caspases for ubiquitylation and degradation by proteasomes.

### 3.2. Necrosis and MMP

Necrosis is cell death due to severe cell damage resulting in a cessation of cellular synthetic functions, and involving disruptions of the internal and external membranes of the cell [22]. It has often been considered to be accidental cell death but more recent studies suggest a number of biochemical mechanisms in common with apoptosis, especially involving alterations in mitochondrial membrane permeability (MMP) [23, 24]. Necrosis is thought to result from the opening of a pore in the inner mitochondrial membrane known as the mitochondrial permeability transition pore, as a consequence of accumulation of calcium ions inside the mitochondria or high levels of ROS [25]. Sustained opening of the pore results in ATP depletion and loss of plasma membrane integrity. In the case of apoptosis, it is proposed that the pore opens transiently, allowing maintenance of ATP production (required for apoptosis) but release of certain factors (cytochrome c, Smac, Diablo) that promote activation of the caspases [25]. Other factors released may promote caspase-independent apoptosis [26].

The existence of divergent pathways leading from MMP to both apoptosis and necrosis has a number of important implications. Agents which suppress MMP, or steps upstream of it, should block both mechanisms of cell death. This can be seen in the capacity of mitochondrial-associated anti-apoptotic proteins (e.g. Bcl-2) and pro-apoptotic proteins (e.g. Bax), which regulate some of these permeability-associated events, to also regulate both apoptosis and necrosis [24]. In addition, agents which act downstream of MMP are likely to regulate only one of the two cell death processes. Thus, caspase inhibitors effectively block caspase-dependent apoptosis but do not prevent cell death; instead, the cells die by necrosis [27].

## 4. WHAT DO WE KNOW ABOUT APOPTOSIS OF NORMAL AEC?

This review largely focuses on airway epithelial cell lines or primary ciliated AE cells. There is limited information on apoptosis of mucin-secreting cells and almost nothing on basal cells. Although apoptosis of alveolar AE cells has been studied in more detail, especially in the context of acute lung injury, it is beyond the scope of this mini-review and only discussed in relation to the issue of why upper AE cells appear to be more resistant to apoptosis than lower AE.

### 4.1. Beneficial Roles of Apoptosis in AE

While apoptosis may be part of the damage induced by noxious agents and airway inflammation, it also has a number of important functions in AE (Table 1). The latter have been the subject of several recent reviews. Tesfaigzi [28] has proposed that apoptosis serves three main roles in the airways. First, it provides a mechanism to safely remove damaged AE cells. Second, it regulates AEC numbers. For an excellent review on the balance between apoptosis and proliferation in AE homeostasis in both physiologic and pathological conditions, see Ref [29]. In normal bronchial epithelium, the cell turnover is low with about 1% of cells in cycle [30]. Allergens and irritants can enhance AE proliferation and induce hyperplastic changes such as mucous cell hyperplasia (seen in asthma) and mucous cell metaplasia (seen in damaged airways); during recovery, the epithelium reduces the number of epithelial cells to the original state [31]. The third role proposed by Tesfaigzi [28] for apoptosis in the airways is to control inflammation, by inducing death of inflammatory cells. Although this role does not involve apoptosis of the AEC, nevertheless, FasL and other death-inducing ligands on their surfaces are involved. Since death of inflammatory cells hastens resolution of the inflammation, this is very important from the context of new therapeutic strategies for airway inflammatory disease and has been nicely reviewed elsewhere [32-34]. It has been argued, from studies of influenza A infection of human nasal and bronchiolar epithelial cells, that another role for AE apoptosis is in limiting viral cytokine production and release and thereby minimizing airway inflammation [35]. Removal of infected AE cells by apoptosis might also limit the spread of the virus [36].

**Table 1. Significances of Airway Epithelial Apoptosis**

Role	Refs
Mechanism for normal epithelial turnover	[29]
Marker of epithelial damage	[65]
Mechanism for removal of damaged AEC	[28]
Anti-inflammatory	[28]
Mechanism to limit viral infection of epithelium	[35, 36]
Contributes to epithelial damage and airway inflammation	[42, 64]

### 4.2. Apoptogens for AE

In addition to physiological apoptosis in AE, a wide variety of noxious agents or stresses induce apoptosis of AE cells, *in vitro* and *in vivo*. As well as causing apoptosis, these may also induce necrosis (especially at higher concentrations). Tissue cell death can be considered a continuum between apoptosis, at one end of the spectrum, and necrosis, at the other end. Apoptogens for AE include *inhaled substances* such as cigarette smoke [37], *cytokines* such as TGF- $\beta$ 1, which is thought to be a major mediator of damage to AE in lung injury [38], activators of the death receptors such as TNF- $\alpha$  and FasL [39, 40], *viral and bacterial pathogens* [36, 41] and *hyperoxia* [42]. Apoptotic death of epithelial cells is also seen as a result of prolonged exercise (as in the damage and repair associated with endurance training in mice [43])

and in lung transplant rejection [44]. Mice given the neutrophil-derived product cathelicidin LL-37 by the intra-tracheal route had extensive damage to AE in both upper and lower airways, accompanied by marked increases in TUNEL-positive apoptotic cells within the epithelium as well as shedding of epithelial cells into the lumen [45].

### 4.3. Spatial Aspects of AE Damage and Cell Death

It is convenient to consider these insults to AE as primarily acting at one of three sites: i) *The threat from above*: This class of apoptogens primarily acts at the apical membrane and the noxious factors are derived as a result of inhalation. They include ozone, diesel exhaust pollutants, cigarette smoke, heavy metals, allergens and infectious agents. Oxidative damage is a mechanism common to some of these agents [46]. ii) *The threat from below*: The major threat at the basal membrane is from inflammatory cells infiltrating the airways, especially during episodes of chronic airway inflammation as well as in heavy smokers. As inflammatory cells can also cross the basement membrane and AE, damage may not always be restricted to the basal membranes of the AEC [47]. Damage induced by inflammatory cells can be *via* direct cellular interaction with the AE cell or more likely *via* factors they release such as proteases, ROS and cytokines [3, 47, 48]. iii) *The threat from within*: In addition to external insults, ciliated AEC are threatened by ROS released from mitochondria as a by-product of energy production for the beating of the cilia [49]. These mitochondria are typically located in the apical cytoplasm, below the cilia and associated basal bodies, and therefore apical membranes and the apical cytoskeleton are most at risk. To counteract ROS, ciliated AEC contain a number of anti-oxidants, including enzymes (e.g. Cu/Zn superoxide dismutase, Mn superoxide dismutase, glutathione peroxidase), low molecular weight thiols (glutathione) and anti-oxidant metal ions selenium (Se) and zinc (Zn). In the normal airways, some of these factors appear to be concentrated in the apical cytoplasm [50, 51].

### 4.4. Regulation of Apoptosis in Normal AE Cells

There is yet to be a detailed, systematic investigation of the pathways of apoptosis and their regulation in AE. However, we have been afforded glimpses of the process. As with apoptosis in other cells and tissues, caspases are very important effectors. This has been demonstrated in a number of AE models, *in vitro* and *in vivo*, and by various assays such as cleavage of fluorogenic peptide substrates, cleavage of the caspase-3 substrate cytokeratin 18 (CK18) and immunohistochemical evidence for presence of active caspase-3 [40, 52-54]. A novel *in vivo* demonstration of the importance of caspases in AE apoptosis in mice was the induction of apoptosis in ciliated AEC after a single intra-tracheal administration of active caspase-3; apoptosis was dependent upon uptake of active caspase-3 into the AE, as the caspase had to be given in association with a protein transfection agent (known as chariot); neither chariot nor caspase-3 alone was effective, but in combination resulted in release of significant numbers of apoptotic AEC into the lumen (22% of the cells in bronchoalveolar lung wash-out (BAL) were apoptotic within 2h of administration); apoptosis was effectively blocked by co-administration of a peptide caspase inhibitor [55].

Although bronchial epithelium can be induced to apoptose by a diverse range of apoptogens, there is evidence that these cells in their normal setting may have a relatively high resistance to cell death. This would help to maintain the integrity of the epithelial barrier. Factors implicated in resistance of these cells to apoptosis include Se and Zn. Thus, influenza-induced apoptosis was greater in human AEC that were Se-deficient [56]. Elegant studies by Bao and Knoell [39, 40] have shown an important role for Zn in AE survival. They reported that *in vitro* Zn deprivation sensitized both transformed and primary human airway and alveolar epithelial cells to apoptosis induced by TNF- $\alpha$ , IFN- $\gamma$ , and Fas receptor ligation. Increased apoptosis in AE monolayers was associated with proteolysis of cellular proteins involved in tight junction formation and both a rapid decrease in transepithelial resistance and an increased paracellular leak (indicating enhanced permeability), both of which were reversed by addition of Zn sulphate. These studies confirmed our own findings showing synergy between Zn chelation and pro-oxidants in apoptosis of primary human and animal AE cells, *in vitro* [53, 57]. These findings are consistent with the hypothesis that Zn protects upper respiratory epithelial cells and may have implications for human asthma where there is often both hypozincemia and airway epithelial damage [reviewed in 51].

Resistance may also depend on maintenance of cell-cell adhesion and tight junctions within the epithelial lining. Thus, AE monolayers were highly resistant to induction of apoptosis by *Pseudomonas aeruginosa* whereas AE cell lines that did not form tight junctions were susceptible [41]. While it is possible that the decreased resistance was due to other factors inherent to these cell lines rather than to their lack of capacity to form monolayers, it is well known that disruption of cell-cell adhesion renders some cells highly susceptible to apoptosis (a phenomenon known as anoikis [58]). It is highly likely that anti-apoptotic cellular proteins, such as the Bcl-2 and IAP families, also play important roles in resistance of AEC to apoptosis. This is an area which has yet to receive systematic investigation. Interestingly, tracheal and bronchiolar epithelium exhibited some of the strongest immunostaining for IAPs of the many human tissues tested [59]. While current studies do not indicate whether AE has high levels of Bcl-2, decreased expression of Bcl-2 in AE of severe asthmatics has been reported (see Section 5).

It will be interesting also to compare the susceptibility of upper airway AEC to apoptosis with that of distal bronchiolar epithelium and alveolar epithelium. There is a considerable literature on alveolar lung injury caused by agents such as hyperoxia [60, 61] and bleomycin [62]. For example, exposure of mice to high concentrations of oxygen led to diffuse alveolar epithelial damage and a breakdown of the alveolar-capillary barrier; epithelial cell death occurred by both apoptosis and necrosis [61]. While apoptosis in upper AE is also stimulated by oxidative stress (see section 4.2), the overall damage to upper AE appears to be much less. Other factors, in addition to or of greater importance than, differences in intrinsic susceptibility of upper and lower AEC to apoptogens may also be involved. First, there are differences in tissue architecture between upper and lower airways, such as the close association between endothelium and epithelium in the alveoli required for gas exchange. In hyperoxic mice,

alveolar epithelial damage was preceded by damage to the endothelium, and might be mediated, in part, by agents released from the latter [61]. Second, since alveolar epithelial damage results in a substantial leak of fluid into the alveoli, interfering with gas exchange and leading to respiratory failure [61], the consequences are more evident, dramatic and life-threatening than AE damage higher up in the airways.

As discussed earlier, one of the threats to AEC is from ROS released from their apical mitochondria and therefore the integrity of AE is highly dependent on their anti-oxidant defences. Critical to this defence is the action of the mitochondrial anti-oxidant enzyme Mn superoxide dismutase. Inhibition or si-RNA-mediated down-regulation of this enzyme in a bronchial epithelial cell line resulted in opening of mitochondrial pores, release of cytochrome c, caspase activation and increased cell death [54].

#### 4.5. Polarized AE as a Paradigm for Studying Spatio-Temporal Mechanisms of Apoptosis

The main thrust of this review is directed at applying what we know about apoptosis, in general, to increase our understanding of the role of AE apoptosis in airway damage and inflammation. At the same time, because of the polarized nature of AE, these studies may also reveal new insights into how apoptosis, itself, is regulated; this information is less easily derived from apoptosis experiments on less polarized cells such as cancer cell lines and lymphocytes. During our initial immunocytochemical studies of caspases in normal human, ovine and murine AE, we observed the pronounced apical distribution of procaspase-3 [reviewed in 50]. Although small amounts of procaspase-3 were seen in the nuclei of these cells, the bulk of this caspase precursor was concentrated in the region just below the cilia. We have rationalized this pattern of subcellular distribution on the basis that much of the threat to ciliated AE in normal circumstances comes either from the lumen or from ROS released by apical mitochondria. It makes good sense to have the caspase-3 precursor in this same region, ready to be activated in the event that the normal defences of the cell are breached and the cell is damaged beyond repair. Otherwise, as described earlier, necrosis would be more likely to occur, with increased risk of further damage to the epithelium by cytotoxins released from necrotic cells and by chemotoxins leading to inflammation. Rapid removal of the damaged cell by apoptosis would minimize the risk of release of proteases and other factors that might promote tissue disaggregation or otherwise trigger an immune or inflammatory response; in the special case of an infected cell, apoptosis might help to minimize infection of neighbouring cells. If procaspase-3 and other procaspases are apically localized, then apoptosis regulatory factors might also be nearby. It was therefore interesting to find that both Cu/Zn superoxide dismutase and Zn, an anti-oxidant and caspase inhibitor, were also localized to the same region of the AEC [53]. More recently, our unpublished studies have also shown strong expression of the caspase-regulatory IAPs in the apical cytoplasm of human nasal epithelial cells. This unique spatial localization of pro- and anti-apoptotic factors implies the existence of mechanisms to sequester these factors. The apical cytoskeleton, with its very dense network of cytokeratin filaments and microtubules [63] may provide such a scaffold.

## 5. ALTERED REGULATION OF AE APOPTOSIS AND AIRWAY INFLAMMATION?

### 5.1. Apoptosis and AE Damage

Increased apoptosis of AE cells is a consistent feature of damaged and inflamed airways. It is generally believed that enhanced AE apoptosis contributes to airway damage, such as in acute lung injury [42] or asthma [64]. The reasons for this are that excessive apoptosis of AE cells will lead to a partially denuded epithelium with exposure of underlying structures, decreased mucociliary function and impaired production of regulatory factors. Increased AE apoptosis may also contribute to airway inflammation and therefore airway damage. In addition, apoptosis of basal precursor cells, especially in situations where AE cell turnover is enhanced, would be expected to interfere with normal epithelial turnover and repair. This is a complex issue, however, since the increased apoptosis of AE cells in damaged or inflamed airways may also simply be a consequence of damage and reflect the removal of damaged cells as part of repair processes.

A measure of AE apoptosis provides a potential surrogate measure of AE damage. This assumes that most of the cell death is *via* apoptosis rather than necrosis or at least that the rate of apoptosis is proportional to the amount of damage. While necrosis can be recognized morphologically, especially at the ultrastructural level, it is much less easy to quantify. Apoptosis can be measured either by enumerating apoptotic cells or by measuring some factor associated with apoptosis. *In vivo*, apoptotic AEC may be enumerated in lung biopsies, BAL and induced sputum while soluble factors associated with apoptosis (such as the anti-apoptotic soluble Fas and pro-apoptotic soluble Fas ligand) can be measured by ELISA in relevant biological fluids such as BAL, induced sputum, nasal washings and plasma/serum [65].

It would be very convenient to have an *in vivo* means to assess airway damage in humans based on measurement of an apoptosis- or necrosis-related soluble factor in plasma, induced sputum or exhaled breath condensate. Unfortunately, Fas and Fas ligand are not specific for AE, being present in other tissues including pulmonary endothelium [61]. More promising is cleaved cytokeratin 18 (CK18). Cleavage of CK18 by caspases during apoptosis results in release of a soluble fragment that can be assayed by ELISA [66]. The important things here are that CK18 is only present in epithelial cells, its cleavage, as far as we are aware, only occurs during apoptosis and there are commercial antibodies that recognize a neo-epitope that is only present on the cleaved protein. This technique has been used successfully to quantify epithelial damage in patients with epithelial tumours following drug therapy [67] and needs to be tested on relevant airway-associated fluids to determine its potential diagnostic usefulness in airway inflammatory disease.

### 5.2. AE Apoptosis and Asthma

While a variable part of the damage seen in asthmatic AE has been attributed to artefacts associated with the collection of the biopsies or airway fluids [68], there is well-documented evidence that in human and animal models of

asthma, the AE becomes fragile and easily damaged, with shedding of dead and dying cells into the lumen (Fig. 1B) [11, 69, 70]. There is however variability in the extent of the damage and some cases of mild asthma show no evidence of AE injury [70]. Similarly, there is variability in the extent of AE apoptosis in asthma. While Druihle and colleagues [71] found no increase in apoptotic cells by TUNEL assay in human asthmatic bronchial biopsies, a number of studies have now shown elevated levels of apoptosis in the AE of asthmatics, using a variety of techniques to identify apoptotic cells. Cohen *et al.* [72] found a greater level of apoptotic activity (by TUNEL) in AE of subjects with severe asthma as compared with normal subjects and linked this to a down-regulation of Bcl-2 expression in the epithelium. It should be pointed out, however, that TUNEL does not distinguish between apoptosis and necrosis, and therefore the reported frequencies of apoptotic cells are likely to be over-estimated in these studies. Apoptosis (assessed by staining for active caspase-3) was enhanced in the bronchial epithelium of steroid-untreated asthmatics, as compared with control subjects [73]. Another study reported a mean percentage of apoptotic cells in asthmatic human bronchial biopsies (n=13 subjects) of 10.5 compared with 0.4 in normals (n=9), using detection of cleaved poly ADP-ribose polymerase as an early apoptosis marker; in addition, the AE cells cultured from asthmatics had a greater than 2-fold increase in susceptibility to hydrogen peroxide-induced apoptosis compared with AEC from normals, suggesting an intrinsic heightened susceptibility of the cells to apoptosis induced by the oxygen-rich environment of the cultured cells [74]. Interestingly, bronchial epithelial cells from asthmatics did not have increased susceptibility to apoptosis by actinomycin D and they had decreased apoptosis in response to rhinovirus infection; these findings have led the authors to speculate that the increased susceptibility might be specific to apoptosis induced by oxidants and that decreased apoptosis of virally-infected AEC might promote viral infection and be a factor in virus-induced asthma exacerbations [74, 75].

In a mouse model of allergic airway inflammation, we observed a 20-fold increase in apoptotic cells in AE of OVA-challenged mice, compared to control mice. Apoptosis was assessed by staining for activated caspase-3 and for the cleavage product of CK18. There was also a striking synergy between nutritional Zn deficiency and OVA challenge in inducing AE apoptosis. Increased apoptosis was associated with various features of airway inflammation, including airway hyper-responsiveness, eosinophilia and mucus cell hyperplasia [52].

The increased apoptosis in asthma is partly due to increased exposure of the AE cells to a number of cytotoxic factors, mainly derived from immune and inflammatory cells. These include IFN- $\gamma$  from activated T cells, elastase from neutrophils, major basic protein, eosinophil peroxidase and eosinophil cationic protein from eosinophils and ROS released from neutrophils and eosinophils [6, 48, 76-78]. These mediators may induce both apoptosis and necrosis, depending on the severity of the damage inflicted [78]. Increased apoptosis in asthma is also partly due to increased sensitivity of the AE cells to cytotoxic factors, such as ROS [74]. This increased vulnerability is likely due to both environmental and genetic factors. Our studies have shown both

depletion of AE Zn in murine airway inflammation as well as potent synergy between nutritional Zn restriction and allergen treatment in induction of AE apoptosis [52]. Evidence for hypozincaemia in human asthma [reviewed in 51] reinforces links between dietary anti-oxidants and susceptibility to airway inflammation and damage. Other studies have linked inactivation of Mn superoxide dismutase to AE apoptosis in the asthmatic airway and shown correlations with asthma severity [54].

It will be important to determine whether any of the susceptibility genes in asthma relate to increased susceptibility to apoptosis. One gene that is interesting in this context is the Death-associated protein-3 (DAP3) gene. In a case-control study involving 1341 human adults subjects, Hirota and colleagues [76] have reported an interesting association between bronchial asthma, serum IgE concentrations and single-nucleotide polymorphisms in the DAP3 gene. DAP3 expression in normal bronchial epithelial cells is inducible by IFN- $\gamma$  and there is some evidence that the encoded protein mediates IFN- $\gamma$ -induced death of AEC. Interestingly, however, there was no association with childhood asthma [76].

Other airway diseases in which AE apoptosis may be relevant to pathogenic mechanisms are cystic fibrosis [79-81] and COPD [82]. In a rat model of cigarette smoke-induced COPD, apoptotic AE cells were increased several fold [83]. The damage and cell death in COPD, however, is primarily targeted to the lower airways and alveolar epithelium.

## 6. POSSIBILITIES AND LIMITATIONS OF STRATEGIES TO TARGET APOPTOSIS OF AE IN AIRWAY DISEASE

The usual treatment for persistent asthma in humans involves a combination of bronchodilator and inhaled corticosteroid [84]; there is however a need for strategies to better control other aspects of the disease, including minimizing the ongoing damage to AE and airway remodelling. Development of such strategies will come from a better understanding of the mechanisms by which AE cells become fragile and die during episodes of airway inflammation.

### Caspases

Since effector caspases are terminal regulators of apoptosis, in the sense that, once activated, apoptotic cell death is very likely to follow, one might expect these caspases to be the ideal target for preventing unscheduled apoptosis. The studies by Brydon *et al.* [35] and Bao and Knoell [39, 40] throw further light on this issue.

In bronchial epithelial cells infected with influenza virus A, cell death was inhibited by both a specific caspase-8 peptide inhibitor and a pan-caspase inhibitor and, at the same time, these inhibitors significantly increased the levels of secreted pro-inflammatory cytokines [35]. In virally-infected AE, therefore, apoptosis of infected cells is likely to be beneficial both by preventing viral spread and by minimizing cytokine release. This is a scenario in which suppressing AE apoptosis is not good. The other message from this study is that cell death induced by the virus is triggered by caspase

activation rather than a consequence of general damage to the cells leading to caspase-activation. If the latter was occurring then suppressing caspases would not prevent the damage caused by the virus and the caspase-blocked cells would likely die from necrosis. Since the assay for cell death used by Brydon and colleagues measured cytotoxicity, as well as TUNEL, necrosis would also have been detected.

The studies of Bao and Knoell [39, 40] clearly show the problems with targeting caspases directly in AE cells that are dying in response to some agents. In primary AE monolayer cultures, caspase peptide inhibitor reduced cytokine/death receptor-induced apoptosis but did not prevent the cells dying; rather, they now died by necrosis. Enhanced epithelial permeability also remained. Therefore, all caspase inhibitors achieved was switching cell death from the preferred mechanism (apoptosis) to the less preferred mechanism (necrosis).

The message from both of these studies together, as well as that involving apoptosis of AEC *in vitro* by cathelicidin LL-37 [45], is that caspase inhibitors are very effective agents in targeting apoptosis, at least *in vitro*, but caution needs to be exercised concerning their potential therapeutic use *in vivo*. First, one needs to question the relevance of the *in vitro* findings to *in vivo* models. Second, assuming the *in vitro* findings are relevant, caspase inhibitors could well do more harm than good, depending on what factors are driving the AE cell death. There appear to be only two published studies in which caspase inhibitors have been used *in vivo* in the context of airway disease. The pan-caspase peptide inhibitor ZVAD-fmk was effective in blocking alveolar and airway epithelial cell apoptosis (by 81% and 63%, respectively) as well as lung fibrosis (by 85%) in a rat model of bleomycin-induced lung injury [62]. However, it was not clear whether the rescued AE cells eventually died by necrosis. Iwata and colleagues [85] administered the same caspase inhibitor to mice by iv injection before allergen challenge, with the intention of testing whether suppression of caspase activation in inflammatory cells would delay their removal during resolution of the inflammatory episode and thereby exacerbate the inflammation. In fact, they found the opposite, in that caspase inhibitors attenuated the eosinophil accumulation, mucus production, and Th2 cytokine release. Since T cells removed from caspase-inhibitor treated mice were much less readily activated by CD3 ligation *in vitro*, they concluded that the caspase inhibitors were primarily acting by suppressing T cell activation. It cannot be excluded however that some of the actions of this inhibitor were directed at suppressing apoptosis in AE. AE apoptosis was not studied.

## Zinc

Our own studies [86], as well as another [87] have shown that the caspase inhibitor Zn also attenuates eosinophil accumulation in the mouse airway inflammation model, even when Zn was given after induction of airway inflammation. Unfortunately, neither of these studies determined whether Zn supplements directly affect apoptosis in the AE of the allergen-treated mice. Zn is much more than a caspase inhibitor; it is an anti-oxidant and a membrane stabilizer [50]. Bao and Knoell [40] have shown that Zn blocks both mechanisms of cell death, as well as maintaining epithelial integrity

and they have suggested that Zn supplements may therefore be beneficial in strategies to prevent acute lung injury. Zn appeared to act *via* activation of the phosphatidylinositol 3-kinase/Akt signalling pathway, indicating a role for this pathway in protection against AE damage [40].

## Bcl-2

The Bcl-2 family of cell survival proteins plays a key role in a number of diseases and targeting of Bcl-2 by drugs like ABT-737 (which is a BH3 mimetic) is providing a new therapeutic tool in the treatment of cancer [88]. There are potential roles for targeting these proteins in airway disease. The most studied airway disease to date has been metaplasia of mucous-secreting cells in AE; increased expression of Bcl-2 occurred in this condition when metaplasia was induced by exposure to ozone, endotoxin, cigarette smoke or allergens [89, 90]. Increased expression of Bcl-2 was also associated with mucous hyperplasia in humans with cystic fibrosis and in recurrent airway obstruction in an animal model; down-regulating Bcl-2 expression reduced goblet cell hyperplasia [31, 81]. Other anti-apoptotic Bcl-2 family members, as well as the pro-apoptotic Bax-like members, may also play important roles in AE repair and remodelling [28], although little information is currently available on this. Decreased expression of Bcl-2 and increased apoptosis was found in AE in severe asthma (n = 31) [72]. Elucidation of the role of these proteins in protection of AE and in development of airway inflammation may reveal new ways to target AEC death.

## MMP

Opening of the mitochondrial membrane pores leads to cell death by both apoptosis and necrosis [24]. By targeting this step, it is possible not only to block apoptosis of AEC but at the same time prevent them from going on to die by necrosis. The caveat here is that for this approach to be therapeutically effective it is important that it is not used in situations where the cell is likely to be extensively damaged. If irreversible damage to a cell (e.g. by oxidation) cannot be prevented then that cell needs to be removed from the epithelium and any attempts to block MMP would be counter-productive. Although much more work needs to be done in this area, it is likely that targeting MMP is not the way to go where damage to the epithelium is caused by such things as pro-oxidants, infection and cigarette smoke but may be ideal where excessive AE cell death is induced by such things as cytokines or death receptor ligands, acting primarily through signalling pathways and altered gene expression.

## Fas Receptor and its Ligand

Fas (CD95) and Fas ligand (FasL) are members of the tumor necrosis factor receptor family expressed on columnar, secretory and basal cells of human airways [70, 91]. Based on immunohistochemical and *in situ* hybridization techniques, it was concluded that FasL was mainly expressed on Clara cells in the small airways [92]. This was somewhat contradicted by other evidence suggesting that Fas/FasL are involved in AE cell death, especially in the upper airways and that soluble FasL was present throughout the airways

[93]. The latter study also showed that proinflammatory cytokines sensitize AE to Fas-induced death. Tesfaigzi [28] has argued for a major role for FasL on AEC in triggering death of inflammatory cells and therefore in helping to control airway inflammation in asthmatic lungs. Mutant mice with defects in FasL had submucosal and peribronchial infiltration of inflammatory cells in both the upper and lower airways, while markedly decreased FasL mRNA and protein was seen in the AE of mice in the allergic airway inflammation model [92]. There is potential therefore to use targeted expression of FasL on AEC or administration of soluble FasL to induce death of inflammatory cells and therefore better control airway inflammation. Much more needs to be learned about the role of these proteins in controlling death of AEC.

## p21

p21(Cip1/WAF1) is a cyclin-dependent kinase inhibitor that controls both cell proliferation and cell death. In response to damage-induced G1 arrest, there is up-regulation of p53 and this, in turn, activates transcription of p21 [94]. Thus, AEC isolated from p21-null mice had increased cell proliferation and apoptosis compared to AEC from wild-type mice [95]. Heightened expression of p21 in the bronchial epithelium of asthmatics, especially in the nuclei of the proliferative, basal cell compartment [64], suggests that p21 may be a factor in the abnormal AE cell turnover in asthma and therefore another candidate therapeutic target in asthma. p21 expression was unaffected by corticosteroid treatment [64].

## Stress Response Proteins

Stress response proteins (such as the heat shock protein Hsp-70) protect cells against a variety of noxious agents and stresses. A role for stress proteins in protection of AE against proteases released from neutrophils was demonstrated recently [96]. These investigators found that over-expression of Hsp-70 in bronchial epithelial cells reduced cell injury induced by neutrophil elastase. When Hsp-70 protein was delivered to these cells by liposomal delivery, both necrosis and apoptosis of the cells by elastase were decreased compared with untreated control AEC [96].

## Pirin and Peroxiredoxin V

Strategies to target AEC death in response to cigarette smoke may emerge from current research into how cigarette smoke induces AE damage and cell death and what factors regulate it. Oxidative stress is a major contributor to AE damage and the need to prevent oxidative damage is central to strategies to make the epithelium more resilient. One factor that has emerged recently is peroxiredoxin V (PRXV), a potent antioxidant protein that is highly expressed in AE [97]. Exposure of isolated tracheal segments or monolayer cultures of AEC to cigarette smoke extract resulted in transcriptional down-regulation of PRXV protein, glutathione and protein thiol oxidation and increased epithelial permeability. Since siRNA-mediated down-regulation of PRXV resulted in increased cell death and protein oxidation in response to hydrogen peroxide [97], it is likely that peroxire-

doxin is an important factor in AE resilience to oxidative damage.

Other recent research has uncovered a role for the iron-binding transcription factor pirin in cigarette smoke-induced apoptosis of AEC. Up-regulation of pirin was shown to occur in human bronchial epithelium exposed to cigarette smoke, *in vitro* and *in vivo*, and this was associated with a marked increase in AE apoptosis [37].

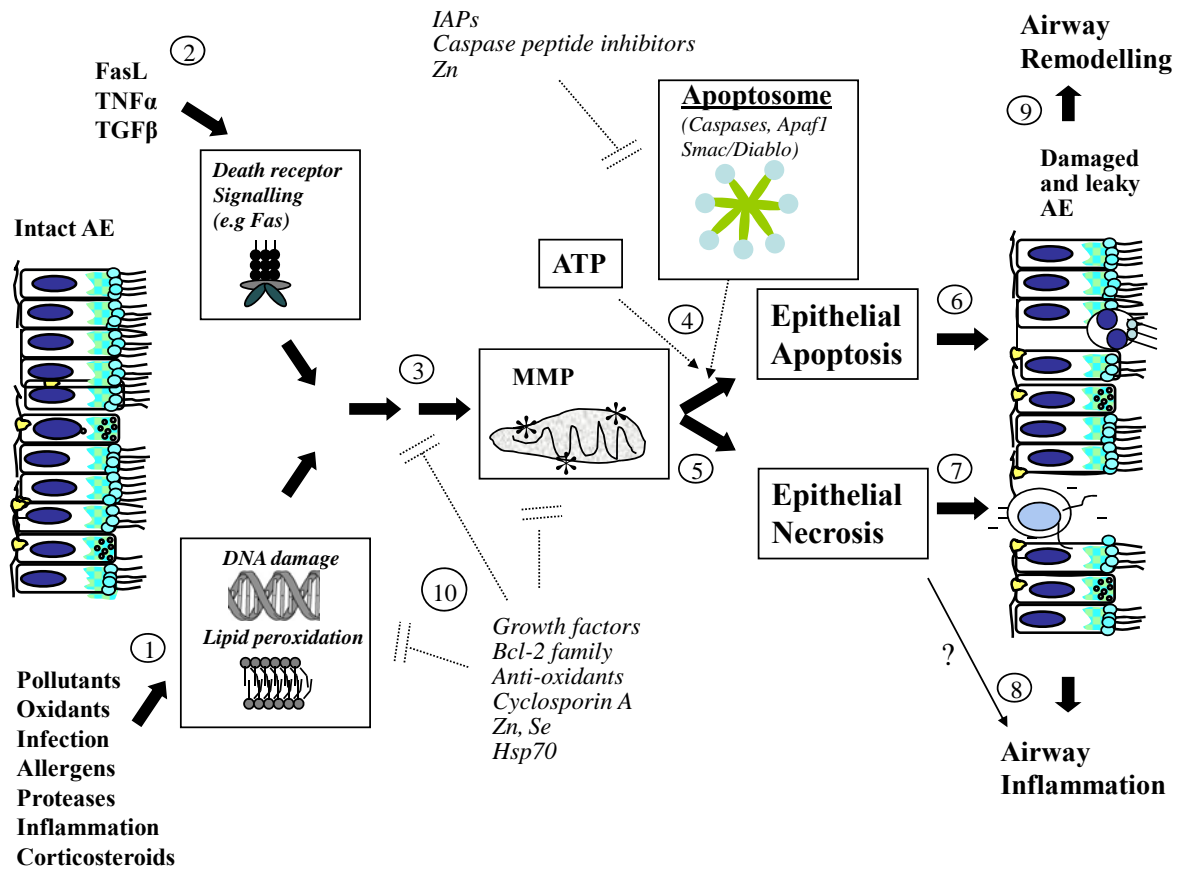
## Corticosteroids

Considering the pro- and anti-apoptotic factors discussed in this section, it will be important to determine not only whether future drugs might be used to target them but also whether they are influenced by the current therapeutic drugs used in treatment of airway inflammatory disease. Corticosteroids are interesting from this perspective. Reports of increased apoptosis in the AE of human asthmatics have involved subjects both on, and without, corticosteroid treatment, suggesting that the increased cell death is not simply a consequence of drug treatment. There is some *in vitro* evidence, however, that therapeutically-relevant concentrations of corticosteroids induce disruption of mitochondrial polarity, activation of caspases and apoptosis in primary AEC cultures and AEC lines [98-100]. These authors have argued that part of the damage and cell death in AE in asthma might therefore result from the treatment rather than the disease. On the other hand, Wen and colleagues [101] found that dexamethasone, acting *via* up-regulation of cIAP2, was a potent inhibitor of apoptosis induced by IFN- $\gamma$  and Fas ligation in A549 lung epithelial cells.

## 7. CONCLUSIONS AND FUTURE PRIORITIES

In an excellent review of the history of asthma research, Persson [102] has discussed how early recognition of the processes of airway epithelial shedding and airway eosinophilia in the 19<sup>th</sup> century has shaped the direction of asthma research over the following century. Apoptosis was not recognized as a distinct mechanism of cell death until the mid 20<sup>th</sup> century, when the concept of cells dying in a programmed manner emerged from studies of early organ and tissue development and, in the 1960-70s, pathologists first recognized cells dying in a morphologically distinct manner [1]. There quickly followed an appreciation of the importance of apoptosis for disease processes and therapeutics, including diseases involving the epithelial tissues of the body. Apoptosis in AE has only recently been studied, perhaps largely because of the relative inaccessibility of the tissues. This still remains a major obstacle to research in this area, although studies of induced sputum as well as nasal and bronchial brushings and biopsies are providing important new information.

Fig. (2) presents a summary model for the roles of apoptosis and necrosis in AE damage and inflammation (Sections 4 and 5) and the sites at which therapeutic strategies may be directed (Section 6). The validity of this model needs to be tested by carefully designed experiments. As pointed out by Persson [102], there remain considerable gaps in our understanding of the role the epithelium plays in pathogenesis of asthma. Clearly, much needs to be learned about oxidative



**Fig. (2). Schema of events leading to AEC death and sites that might be targeted:** AE is exposed to a variety of noxious insults, both in normal and inflamed airways. These insults may either directly induce damage, such as oxidative injury or DNA damage (step 1), or they may act more subtly by triggering cell death signalling pathways (e.g. *via* TNF- $\alpha$  or Fas receptor/ligand ligation, step 2). Regardless of the cause, the pathways converge onto a central pathway involving a crucial change in the permeability of the mitochondrial membrane (known as MMP, Step 3). Factors released from the mitochondria, the duration of the pore openings and the cellular levels of ATP determine whether the cell dies by apoptosis or necrosis. If pore opening is transient, ATP levels are maintained and the appropriate apoptosis-inducing cofactors are released from the mitochondria, apoptosomes form, caspases are activated and apoptosis ensues (step 4). Apoptosis is the preferred mechanism of death, because the dying cell is rapidly removed by phagocytosis or shedding without lysis. If pore opening is sustained, ATP levels are low and there are substantial calcium fluxes, then necrosis occurs, with cell lysis (Step 5). Both mechanisms of cell death lead to epithelial denudation which may provoke further damage and barrier disruption (Step 6, 7) and lead to airway inflammation (Step 8). Necrosis may also contribute directly to airway inflammation *via* release of factors which damage neighbouring cells and attract inflammatory cells. Repeated episodes of AE damage and repair in chronic asthma can lead to airway remodelling (Step 9). Simply blocking caspase activation after MMP has been initiated is not ideal since the cells may still die by necrosis. For therapeutic strategies to be most effective in maintaining AE integrity, they need to be directed at preventing MMP and/or upstream events (cell damage, death receptor signalling). A number of protective agents discussed in Section 6 potentially fall into this category (Step 10).

stress, DNA damage and response to injury in AE exposed to the different noxious agents. What pathways are unique to each, what pathways are shared and how the pathways converge with each other and with the death receptor signalling pathways all need investigation. The latter are very interesting in that they induce cell death in otherwise healthy cells. Inappropriate triggering of these pathways could have a major impact on AE integrity and permeability without evidence of much cellular damage. Much of what we know about cell death in AE is likely based on death of the columnar ciliated AEC, as these are the major cell type. The secretory and basal AEC also play critical roles in AE integrity and future experiments should identify the mechanisms by which their death is regulated in both physiological and pathological states. There is a need to measure necrosis both in AE cultures and in biopsy sections of AE to complement the apoptosis data. There is also a need to monitor AE cell

death *in vivo* in human subjects by minimally invasive techniques. This may provide a further measure of how well airway inflammation is under control in treated patients. Measurement of levels of soluble FasL and soluble cleaved CK18 in induced sputum or exhaled breath condensates may prove useful. In view of the very interesting data from the groups of Bao & Knoell [39, 40] and Davies & Holgate [74] on the relative resistance of normal AE cells to death and heightened susceptibility of AE cells in asthma to death by oxidants and other agents, we need to develop techniques to measure AE susceptibility. Whether nasal epithelial brushings can be used to predict what happens regarding AE cell death lower in the airways is a topic for further investigation. The work of Hirota and colleagues [76] on the DAP3 gene and asthma susceptibility should lead to systematic studies of the roles of cell death susceptibility genes in airway inflammatory disease. With regard to regulation of AE cell death

signalling pathways and epithelial repair, the biggest gap in our current understanding may be at the level of growth factor biology. Growth and differentiation factors are critical to regulation of cell death and tissue homeostasis and identification and characterization of these factors for AE will provide important insights not only into AE cell death but also mechanisms of AE repair and airway remodelling. There is a lot still to be learned about the cellular biology of AEC. The recent report from the Puchelle group [103] on the role of the Trefoil Factor Family peptides is exciting in two ways:- first, in relation to ciliogenesis in AEC; and, second, since these peptides are secreted by mucous cells, from the viewpoint of how ciliated and mucosecretory AE cells communicate and influence each other's behaviour. The death of ciliated and secretory cells in AE is also very likely to be influenced by their physical and biochemical interactions with each other.

Clearly, there is much work ahead to achieve the goal of targeting specific cell death pathways to help maintain the integrity of AE in inflamed airways. Such studies will not only uncover new therapeutic agents in asthma and other airway inflammatory diseases but will also reveal the extent to which the current therapeutic drugs (e.g. corticosteroids and salbutamol) influence AE vulnerability to damage, death receptor signalling and effector pathways of cell death.

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#### ABBREVIATIONS

AE	=	Airway epithelium
AEC	=	Airway epithelial cells
Apaf-1	=	Apoptosis protease-activating factor
BAL	=	Bronchoalveolar lung wash-out
CK18	=	Cytokeratin 18
COPD	=	Chronic obstructive pulmonary disease
DAP3	=	Death-associated protein-3
FasL	=	Fas ligand
IAPs	=	Inhibitors of Apoptosis proteins
MMP	=	Mitochondrial membrane permeability
OVA	=	Ovalbumin
ROS	=	Reactive oxygen species
Se	=	Selenium
Zn	=	Zinc

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