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Apoptosis in the normal and inflamed airway epithelium: role of zinc in epithelial protection and procaspase-3 regulation

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Abstract

The epithelium lining the airways is a physical barrier as well as a regulator of physiological and pathological events in the respiratory system. Damage to the epithelium by oxidants released from inflammatory cells is a critical factor in the pathogenesis of airway inflammatory diseases such as bronchial asthma. In these diseases, excessive apoptosis may be a likely mechanism responsible for damage to, and sloughing, of airway epithelial cells. Factors that increase the airway epithelium's resilience to apoptosis are likely to lessen the severity of this disease. One such factor is the dietary metal zinc. A special role for labile intracellular pools of zinc as anti-apoptotic agents in the regulation of the caspases, has emerged over the past two decades. This review focuses on caspase-inhibitory functions of zinc in airway epithelial cells, apparent abnormalities of zinc homeostasis in asthmatics and studies from the authors' laboratory which showed that zinc was strategically localized in the apical cytoplasm of airway epithelium to control caspase-3 activated apoptosis. These findings are discussed in the context of recent data from a murine model of allergic asthma, showing that loss of airway epithelial zinc was accompanied by changes in levels of both procaspase-3 and active caspase-3 and that nutritional zinc deprivation further increased airway epithelial apoptosis. We hypothesize that zinc has a protective role for the airway epithelium against oxyradicals and other noxious agents, with important implications for asthma and other inflammatory diseases where the epithelial barrier is vulnerable and compromised.

Keywords: Airway epithelium; Asthma; Apoptosis; Caspase; Zinc; Antioxidant

1. Introduction

Sequential activation of the family of procaspase zymogens is a critical event in the apoptosis pathway which is tightly regulated at several levels. A major focus of current research is the elucidation of the mechanisms and temporal relationships underlying the activation and regulation of these procaspases. Our studies have particularly focused on AEC which we believe are excellent examples for studying the spatiotemporal relationships between oxidative stress, procaspase activation and downstream events in apoptosis. This review begins with an overview of the airway epithelium and its vulnerability to oxidant-induced damage and cell death, especially in asthma, a major disease of airway inflammation. This is followed by a brief description of the cellular biology of Zn, leading to a consideration of the cytoprotective functions of this metal ion in epithelial tissues, including those of the airways as well as evidence, largely from human studies, suggesting that asthmatics have low circulating Zn levels. The commentary then focuses on four aspects relating to PC3 regulation that have emerged from our studies: firstly, the unique spatial distributions of both PC3 and labile Zn (one of its regulators), in the apical cytoplasm of AEC; secondly, the synergistic interactions between AEC Zn depletion and oxidants *in vitro* in stimulating PC3 activation; thirdly, the changes in levels and distribution of PC3 and labile Zn in AEC in a murine model of allergic airway inflammation; and finally, the interactions between nutritional Zn deprivation and allergic airway inflammation on airway epithelial damage and apoptosis. These findings have implications for the mechanisms by which the apoptotic machinery and its regulators are spatially polarized in some

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types of cell and the potential for these pathways to be disturbed in chronic disease, further exacerbating the pathogenic mechanisms. The commentary concludes with an attempt to identify some of the critical pressing questions that most need addressing in the next few years.

2. Airway epithelium, oxidative stress and apoptosis

The airway epithelium is a pseudostratified, columnar epithelium that lines our nasal passages and the conducting airways from the trachea to the larger bronchioles. As well as being a protective barrier that separates the airways from the external environment, the epithelium produces many pro- and anti-inflammatory substances [1–3]. Damage to the airway epithelium by inhaled noxious agents (e.g. environmental pollutants and allergens), not only compromises its protective barrier function but also results in decreased production of smooth muscle relaxant factor and increased release of pro-inflammatory cytokines such as IL-6 and TNF-α [1,2]. The airway epithelium is especially vulnerable to damage by ROS, arising from endogenous sources (such as the mitochondria), as well as from exogenous sources (such as inflammatory cells, air pollution and cigarette smoke) [4]. Interestingly, only a proportion of smokers may be susceptible to the airway damage that converts into physiological decline in airway function [5]. ROS-induced damage to the epithelium likely involves apoptosis as well as necrosis. Apoptosis is the body's preferred mechanism of cell death in epithelial tissues, since apoptotic cells undergo morphological and biochemical changes which facilitate their rapid clearance by phagocytosis or shedding into lumenal cavities [6]. These cells are cleared by the mucociliary transport system and ultimately are either swallowed or coughed up. AEC are susceptible to apoptosis by various stimuli, including corticosteroids, pro-inflammatory cytokines and ROS [7–9]. In these studies, dying airway cells exhibited various features of apoptosis, which included cellular shrinkage, DNA condensation, nuclear fragmentation and activation of PC3 to AC3. However, while apoptosis of damaged AEC is likely to be beneficial in the normal respiratory tract, excessive and inappropriate apoptosis of AEC may contribute to the pathogenesis of chronic inflammatory respiratory diseases.

3. Asthma and AEC apoptosis

Asthma is a chronic inflammatory disorder of the airways affecting an increasing proportion of our society, including up to 25% of children in Westernised countries (Australian statistics) [10]. This disease is characterized by chronic infiltration of the airways by eosinophils, mucus hyperplasia, variable airways obstruction, AHR and the shedding and desquamation of the airway epithelium. The latter is caused by proteases and ROS released from eosinophils [3]. As a consequence of repeated damage and repair, the epithelium becomes altered and over-produces mucus, growth factors and pro-inflammatory cytokines such as TNFα, which act to further aggravate the inflammation and increase AHR [2]. Bronchial biopsies taken from asthmatic patients commonly demonstrate loss of the mucosal epithelium, and the swelling of ciliated columnar cells giving the epithelium a "fragile appearance" [11]. In these cases, apoptosis may contribute to the denuding of the respiratory epithelium and increased exposure of the basement membrane to toxic mediators of inflammatory cells, therefore exacerbating inflammation while retarding airway repair. Disruption of junctional complexes and epithelial cell shedding are common features noted in both asthma [2,11] and apoptosis [12]. In addition, apoptosis, as assessed by several markers (e.g. AC3 and cleaved poly(ADP-ribose) polymerase) was shown to be increased up to 20-fold in the bronchial epithelium and submucosa of human asthmatics when compared with non-asthmatic subjects [7,9,13]. This finding was also recently confirmed and extended in cultures of bronchial human epithelial cells, where cells from subjects with asthma were 2–3-fold more susceptible to the induction of apoptosis by H_2O_2 than cells from normal control subjects [9]. There is now clearly a need to identify factors which contribute to this heightened susceptibility to apoptosis in asthmatic airway epithelium. One such factor may be the levels of Hsp27, a heat shock protein which protects human bronchial epithelial 16-HBE cells against oxidative stress and, when down-regulated, results in an increased susceptibility to apoptosis induced by H_2O_2 [14]. Another factor contributing to the increased frequency of AEC apoptosis in asthmatics may be low AEC intracellular labile Zn levels.

4. Labile Zn and Zinquin

Ionic Zn^{2+} is highly charged and cannot cross biological membranes by passive diffusion; intracellular homeostasis of this trace element is achieved by the activity of specific proteins involved in uptake, efflux and intracellular compartmentalisation. One group of transporters thought to be primarily involved in Zn uptake are the ZIP proteins; another group comprising seven members in mammals, the ZnT family, mediates intracellular Zn trafficking [15,16]. Zn is required for housekeeping functions in all cells, such as tightly-bound, poorly-exchangeable pools involved in metalloenzyme catalysis and as structural elements that stabilize DNA binding domains of Zn finger transcription factors [17]. These constitute up to 90% of cellular Zn. In addition, dynamic, free or loosely-bound (labile) pools of Zn are also involved in the regulation of many organ-specific Zn-dependent processes, which include signal transduction, apoptosis, secretion, fertilization and neurotransmission [18,19]. The labile Zn pools are also rapidly depleted in Zn deficiency, whereas the tightly

bound pools of Zn often remain unaltered. We have previously visualized the labile Zn pools using the highly Zn-specific fluorophore Zinquin (ethyl-[2-methyl-8-*p*-toluenesulphonamido-6-quinolyloxy]acetate). Zinquin is a non-toxic, membrane-permeable fluorophore which is sensitive to nanomolar free Zn and preferentially reactive with the most labile Zn pools. Zinquin forms two complexes with labile Zn as determined by X-ray crystallography. 1:1 (log K_{a1} 6.4) and 2:1 (log K_{a2} 7.1) [20]. Under UV light (optimal excitation wavelength of 364 nm), these complexes emit a vivid blue fluorescence (peak emission wavelength of 485 nm), giving a stable signal in intact cells, as well as in frozen tissues. Specificity for Zn is confirmed by showing quenching of Zinquin fluorescence with the membrane permeable Zn chelator TPEN which binds Zn more tightly (log K_a 15.6) [18].

5. Labile Zn as an anti-apoptotic factor

Zn exhibits a close relationship with the regulation of apoptosis since imbalances in Zn levels can either suppress or increase apoptosis. It has been shown that very high concentrations of Zn may, in some cells, trigger cell death either by apoptosis or necrosis, while physiological concentrations of Zn suppress apoptosis *in vitro* and *in vivo* (reviewed in [19]). Perhaps the best studies that show Zn as an important regulator of apoptosis are those that report an increase in apoptosis in cells or animals made Zn-deficient. For instance, the frequency of apoptotic cells is markedly increased in many tissues and organs in Zn-deficient animals including the epithelia of intestine, retinal pigment, skin and fetal neural tube (reviewed in [21]). In addition, increased apoptosis occurs in cells depleted of Zn by culture in Zn-depleted medium or where intracellular Zn has been chelated by treatment with TPEN [18,19,21]. We have previously demonstrated an inverse correlation between the level of intracellular labile Zn in lymphocytes and their susceptibility to undergo apoptotic DNA fragmentation [18]. In these studies, Zn-deficiency induced apoptosis, *in vitro* and *in vivo*, had all of the major biochemical and morphological features of apoptosis (including DNA fragmentation, caspase activation, substrate cleavage, nuclear fragmentation, chromatin condensation and apoptotic body formation). There are likely to be two aspects to the anti-apoptotic mechanisms of Zn.

5.1. Inhibitor of oxidative stress

Apoptosis is closely linked with oxidative stress and may well have evolved primarily to rid the body of oxidatively damaged cells [22]. Many agents which induce apoptosis are either oxidants or stimulators of cellular oxidative metabolism. These include ROS, such as superoxide anion, hydroxyl radical and peroxynitrite, all of which are highly unstable compounds capable of oxidizing lipids, proteins and nucleic acids [19,22]. One anti-apoptotic mechanism of Zn is its capacity to minimize oxidative damage to cellular organelles, thereby suppressing major signalling pathways leading to caspase activation and apoptosis [19]. Zn can protect organelles against oxidants by several mechanisms, including stabilization of sulphydryls from oxidant-induced disulphide bond formation and stabilization of membrane phospholipids [23]. In Zn-deficient animals and cells, there is a substantial increase in oxidative stress, manifested by lipid peroxidation, protein sulphydryl oxidation to disulphides and protein tyrosine nitration [23]. Not only can Zn deficiency induce oxidative damage *per se* but it can greatly enhance lung oxidative damage caused by high concentrations of oxygen [24]. These observations were further confirmed in the skin and intestinal villi of rodents, where a strong link was found between Zn deficiency and increased oxidative stress, tissue damage (manifested by enhanced microvascular permeability and appearance of inflammatory lesions), and apoptosis [25,26]. Antioxidants given in the drinking water were able to suppress these changes.

5.2. Inhibitor of PC3 activation and AC3 activity

Zn can also directly suppress some of the apoptotic effectors, principally the endonucleases and (pro)caspase enzymes. Several groups have reported AC3-dependent cleavage of substrates to be relatively sensitive to inhibition by low micromolar or nanomolar concentrations of Zn, typical of those found in mammalian cells (reviewed in [18]). For example, Maret *et al.* showed that removal of stoichiometric amounts of Zn from caspase-3 by thionein resulted in activation of caspase catalytic activity [27]. Redox-dependent regulation of proteins vis binding of Zn to cysteine sulphydryl groups is an area of current interest [28]. On the other hand, we have proposed that Zn suppresses a step immediately prior to, or during, the activation of PC3 [19]. There is now a need to determine precise intracellular concentrations of Zn in the microenvironment of this proenzyme. The two mechanisms of action of Zn described above (i.e. inhibitor of oxidative stress and inhibitor of PC3 activation or AC3 catalytic activity may, in fact, be closely related since both proteolytic processing of precursor caspases and caspase enzymatic activity are influenced by the redox state of the cell and, part of the cytoprotective role of Zn may be to protect (mask) essential sulphydryls of the caspases, themselves [19]).

6. Co-localization of PC3 and labile Zn in airway epithelial cells

6.1. PC3

One relatively neglected area of apoptosis research has been the spatial aspects of procaspase activation: that is,

where these zymogens are localized at a subcellular level, not only in relationship to themselves and other pro- and anti-apoptotic regulators, but also in relationship to regions of the cell which are most vulnerable to damage. Our finding that PC3 is almost exclusively concentrated beneath the apical plasma membrane of human and animal AEC [29] extends a previous finding by Krajewska *et al.* using human autopsy tissue where they showed preferential localization of PC3 on the lumenal side of the tracheobronchial epithelium [30]. This regional distribution of PC3 may have important implications for spatial and temporal aspects of caspase activation and apoptosis in these cells, since the initial damage leading to apoptosis may more likely be initiated at the apical surface which is not only exposed to pollutants, allergens and oxidants in inspired air, but also is close to abundant apical mitochondria which release oxyradicals as a by-product of energy production for ciliary beating. Hence, the localization of PC3 to the apical cytoplasm may provide an early response mechanism to initial cell damage, allowing the cell to rapidly die by apoptosis before succumbing to damage sufficient to interfere with ion homeostasis and plasma membrane integrity.

6.2. *Labile Zn*

Our studies have shown animal and human AEC to be rich in labile Zn [29,31] compared with the low levels of labile Zn in many other cells (such as lymphocytes and fibroblasts) [32]. Of the human and sheep AEC we analysed at high magnification, more than 95% had intense apical Zinquin fluorescence immediately beneath the ciliary apparatus and, in some AEC, within the basal bodies and cilia [29]. Consistent with this pattern, Zinquin fluorescence was most intense beneath the apical surface of the trachea (F) and bronchi in cryostat sections [31].

The close proximity of labile Zn to PC3 in the apical cytoplasm of AEC raises the question of whether Zn is strategically located to maintain PC3 in an inactive state until the enzyme is required. The mechanisms that constrain both Zn and PC3 at these sites and how these change during a normal apoptotic response now need to be elucidated.

7. Effects of *in vitro* changes in AEC Zn levels on lipid peroxidation and PC3 activation

Previous studies in our laboratory showed that chelation of intracellular Zn by TPEN, rendered AEC highly susceptible to oxidative damage, especially in the apical membranes, as determined by immunocytochemical determination of hydroxynonenal, a marker of lipid peroxidation; this was associated with increased AC3-dependent apoptosis [29,31]. There was a lag phase of about 60 min between decline in available Zn (induced by TPEN) and rise in cytosolic AC3 levels [31], suggesting that a reduction below a threshold concentration in the apical labile Zn pool may trigger an event, or series of events, immediately prior to the processing of PC3. This event may be related to increased oxidative damage or it may be due to a relocation of PC3 in the cells. The effects of TPEN on induction of AC3 were decreased (50–90%) when cells were pre-incubated with various antioxidants [29], indicating that other antioxidants can compensate for Zn chelation. Of the antioxidants used, the most effective was *N*-acetylcysteine, which is readily absorbed by cells and converted to glutathione. Partial beneficial effects were observed with vitamin C, which scavenges ROS such as superoxide anion and hydroxy radicals as well as with vitamin E, which protects membrane lipid from oxidation (discussed in [29]). A variety of cellular antioxidants in the apical cytoplasm of AEC may cooperate to minimize oxidative stress.

Interestingly, Zn chelation by TPEN not only increased the rate of apoptosis in AEC but also synergistically enhanced the induction of AC3 activity by peroxynitrite and H_2O_2 [31]. Thus, a severe reduction of intracellular labile Zn can directly induce apoptosis while smaller decreases render AEC more vulnerable to apoptosis by ROS. In view of evidence (described below) that asthmatics may have an underlying Zn deficiency or at least disturbances in Zn homeostasis, low AEC Zn levels may contribute to the susceptibility of AEC from asthmatic patients to oxidant-induced damage and cell death.

8. Altered zinc homeostasis and human asthma

Zn deficiency is a global problem affecting all age groups, but especially the very young and the very old [33]. It is prevalent not only in third world countries but also in developed countries including USA and Australia. In addition, a number of studies have revealed that many asthmatics have hypozincaemia and/or low hair Zn levels, suggestive of an underlying Zn deficiency or, at least, altered Zn metabolism (reviewed in [34]). For example, DiToro *et al.* [35] reported a significant decrease in hair Zn status in 43 asthmatic children compared with healthy children and Kadrabova *et al.* [36] showed a significant drop in serum Zn levels in 22 asthmatics. The NHANES II survey of 9074 adults in the general population in USA found a negative relationship between wheezing and the serum Zn:Cu ratio (the ratio was measured in this study since low serum Zn is often associated with high Cu) [37]. Furthermore, Soutar *et al.* [38], in a case control study in Scotland, investigated the relationship between allergic diseases and dietary antioxidants and noted an increase in the presence of atopy, bronchial reactivity and the risk of allergic type symptoms in adults with the lowest intake of dietary Zn. These studies do not indicate whether Zn deficiency is *pre-existing* in asthmatics (e.g. caused by

low dietary Zn intake or abnormal Zn transport mechanisms), or whether it is a *secondary consequence* of the disease (e.g. as is evident in diabetes mellitus where there is excessive loss of Zn in the urine [39]).

Several factors may contribute to a low Zn status in asthmatics. Firstly, a redistribution of plasma Zn to the liver can occur during excessive stress. This has been attributed to a cytokine-directed movement of Zn from plasma to hepatocytes [40]. Secondly, the immune system is very dependent on the availability of Zn for maintaining its homeostasis. As a result, inflammatory diseases can cause an increase in the demand for Zn, which is needed for the production of thymic hormone thymulin, natural killer cells and granulocytes [41]. Thirdly, excessive loss of Zn-rich AEC and inflammatory cells, due to turnover and shedding into the airway lumen, will eventually deplete body stores of Zn, especially if these losses of Zn are not compensated by increased dietary intake. Finally, there may be abnormalities in expression of Zn transporters in the AEC of asthmatics.

9. *In vivo* studies in a mouse model of "asthma"

To further investigate these issues, we used a well-established murine model of allergic airway inflammation, characterized by blood and airway eosinophilia, mucous hyperplasia and AHR, to address the following two questions: (1) are there changes in Zn levels and PC3 activation in the airway epithelium of the allergic mice? and (2) does mild nutritional Zn deprivation increase AEC apoptosis and worsen the disease? Groups of 5-week-old Balb/c mice ($N = 8$) were given either a Zn normal diet (50 ppm Zn, ZN mice) or a Zn-limiting diet (14 ppm Zn, ZL mice) to induce a mild state of Zn deficiency. The mice received no other source of Zn since their drinking water was free of Zn, they were maintained on raised stainless steel grid floors in their cages to minimize coprophagy and their cages were washed daily with Zn-free water. Under these stringent conditions, mice were readily rendered Zn-deficient by a lowering of the Zn content of the diet [42]. Growth inhibition is a sensitive marker of Zn deficiency [17] and the growth curves reported [42] clearly showed that Zn was the only limiting factor to weight gain in these mice. The rationale behind inducing a mild, rather than a more severe, Zn deficiency in these mice is that this more adequately reflects the suboptimal levels found in asthmatics [34]. Mice on the ZN or ZL diets were then sensitised to OVA, by two intraperitoneal injections at days 0 and 12, followed by repeated challenge with aerosolised OVA (three 30 min cycles per day, every second day), from days 22 to 30 to induce a state of allergic asthma; control mice received SAL instead of OVA. Zn deficiency was demonstrated by reduction in growth rates within 4 days of administration of the ZL diet. This period also coincided with the periods of OVA sensitisation. On day 31, mice were tested for AHR to methacholine, before sacrifice for tissue collection.

9.1. Labile Zn, PC3 and apoptosis in the airway epithelium

Sensitization and nebulization with OVA, resulted in a 2–3-fold decrease in apical Zn in the bronchial epithelium of both the ZN and ZL mice ($P \leq 0.001$, Fig. 1A) [42]. These decreases caused by induction of airway inflammation were, in fact, much greater than the small reduction in Zinquin fluorescence due to the ZL diet alone (column 2 in Fig. 1A). The reduction in available Zn in the allergic mice was homogenous and occurred in both the apical and basal compartments of the epithelium which suggests that there was an overall loss of Zn from the epithelium rather than from one cell type. However, it must be formally determined whether this loss is predominantly in the ciliated cells, in the mucus cells or in both, since in the OVA-treated mice approximately 70% of the airway epithelium consists of mucus-secreting cells. It is technically difficult to address this issue since Zinquin fluorescence can only be done on frozen tissues and not in conjunction with morphological stains. One possible solution would be by a double labelling technique using Zinquin fluorescence in conjunction with antibodies to specific epithelial cell markers such as muc5b. The mechanism behind the marked decrease in available Zn in the airway epithelium of OVA-treated mice is not known. It may represent a net reduction in epithelial Zn concentration and/or an incorporation of a greater proportion of Zn into metallothionein, which attenuates Zinquin fluorescence [43]. If Zn has indeed been lost from the airway epithelium, then several factors may play a part. Firstly, losses of cellular Zn may result from shedding of Zn-rich AEC and eosinophils into the airway lumen. Secondly, Zn may be lost from the AEC by secretion into the epithelial lining fluid or by binding to the negatively charged mucopolysaccharides in the mucin layer. Thirdly, redistribution of Zn from certain tissues via the plasma to the liver is a common feature of inflammation [17,40], although this is unlikely, given our finding of a trend towards decreased liver Zn in the OVA-treated mice, compared to SAL-treated mice [42].

Decreases in AEC Zn in the ZN OVA mice were accompanied by a 20% increase in apical PC3 levels (from 207.8 ± 5.2 gray scale units in the non-allergic mice to 240.5 ± 2.2 gray scale units in the allergic mice, $P \leq 0.001$, Fig. 1B) [42]. The distribution of this procaspase also changed from a predominantly apical pattern in the control mice to a more homogeneous pattern. Changes in PC3 levels were also associated with its activation to AC3, as detected by antibodies specific for the active form of this caspase and for the cleaved form of CK18, an AC3 substrate. Overall, there was a 4-fold increased frequency of apoptosis in the airway epithelium of the ZN OVA-treated mice compared with the SAL control mice (Fig. 1C)

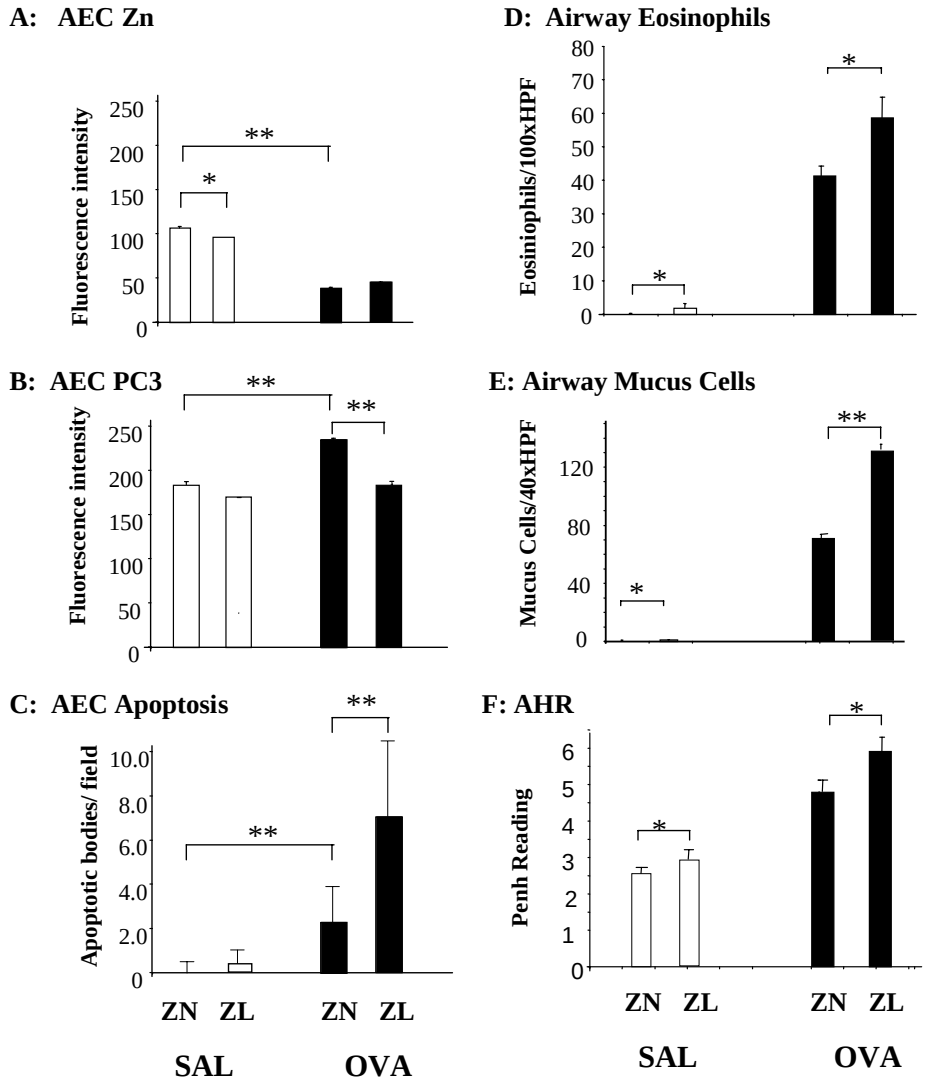

Fig. 1. Changes in airway epithelial Zn and apoptosis in a combined murine model of nutritional Zn deprivation and allergic airway inflammation. Four groups of mice (N = 8) were set up according to (1) Zn content of diet (ZN: 50 ppm Zn or ZL: 14 ppm Zn) and (2) treatment with OVA (to induce airway inflammation, filled columns) or SAL (no airway inflammation, open columns), as described in the text. Mice were tested for AHR on day 31 and sacrificed for collection of tissues. Zn and PC3 were measured by Zinquin fluorescence and PC3 immunofluorescence in airway epithelium of cryosections of lung, respectively. Apoptosis was measured by enumeration of apoptotic bodies in anti-cleaved CK18-labelled cryosections of airway epithelium. Tissue eosinophils and mucus cells were enumerated in high power fields of paraffin-embedded lung stained with Carbol's chromotrophe hematoxylin and alcian blue/periodic acid-Schiff, respectively. AHR was measured by Buxco technique (methacholine 50 mg/mL) [42]. (A) Loss of AEC Zn in OVA-treated ZN or ZL mice; (B) increase in AEC PC3 protein in OVA-treated ZN mice, but not in ZL OVA mice; (C) moderate and substantial increases in AEC apoptosis in ZN OVA and ZL OVA mice, respectively; (D) increase in airway eosinophilia in ZN OVA mice and further increase in ZL OVA mice; (E) increase in airway mucus cells in ZN OVA mice and further increase in ZL OVA mice; (F) increase in airway reactivity in ZN OVA mice and further increase in ZL OVA mice. Significances for certain comparisons between the groups of mice are shown. These were determined by student *t*-test: $^{*}P < 0.05$; $^{**}P < 0.005$.

[42]. In the ZL allergic mice, substantial amounts of AC3 and cleaved CK18 were seen in a localised pattern particularly along the basement membrane which may indicate that this is the first site of damage in the airway epithelium [42]. AC3 was also increased in the lamina propria, within regions where there was an accumulation of eosinophils. Whether this reflects direct apoptosis of eosinophils or damage to surrounding tissue fibroblasts by factors released from eosinophils is not known. There was also a high frequency of apoptotic bodies at the lumenal surface of the epithelium and in the lumen. Apoptotic bodies were enumerated under high magnification (2500×) in 20 fields from duplicate slides for the different groups of mice [42]. The mean number (±SD) of apoptotic bodies per high-powered field were 0.1 ± 0.4 for ZN non-allergic mice and 0.3 ± 0.7 for ZL non-allergic mice (not significant). However, there were significant increases in allergic mice ($P \leq 0.005$) where ZN allergic mice had 2.2 ± 1.7 apoptotic bodies and the ZL allergic mice had 7.0 ± 3.3. The value for ZL allergic mice was also significantly higher

than that of the ZN allergic mice ($P \leq 0.005$) suggesting that dietary restriction of Zn further increases airway epithelial apoptosis over that due to the allergen treatment alone. It is not yet possible to conclude that the ''loss'' of Zinquin-stainable Zn in the airway epithelium of the allergic mice is directly related either to the changes in PC3 levels and distribution, or to the increased activation of this effector enzyme. Since it was not possible to determine the levels of necrotic cell death in the epithelium of the Zn-deprived allergic mice, it is not clear whether Zn deprivation also facilitates AEC death by necrosis.

9.2. Nutritional Zn deprivation and inflammation?

Increased epithelial apoptosis in OVA mice having mild Zn deficiency, over that in OVA mice on a normal Zn diet, was associated with, and may partly be responsible for, significantly increased airway inflammation and AHR [42]. For example, the ZL OVA mice had a 1.6-fold increase in airway eosinophils (Fig. 1D) and a 1.9-fold increase in airway mucus-containing cells (Fig. 1E), compared with ZN OVA-treated mice [42]. AHR was increased correspondingly (Fig. 1F, $P \leq 0.05$) [42]. Zn deficiency alone also increased AHR, modestly. For example, airway reactivity (as measured in penh units, where penh is a dimensionless parameter that reflects changes in waveform of the pressure signal from the plethysmography chamber combined with a timing comparison of early and late expiration) in the ZL SAL-treated mice was greater than that of the ZN SAL-treated mice by 49.7% compared with an increase of 106.2% due to allergy alone [42]. This shows a direct effect of change in dietary Zn levels on airway function, which was approximately half of that observed due to allergen treatment, suggesting that even mild Zn deficiency has a significant influence on airway function.

9.3. The paradox

The studies with the combined Zn deficiency and airway inflammation suggested that there may not be a simple relationship between loss of Zinquin-stainable Zn in AEC of OVA-treated mice and increased susceptibility to apoptosis. Importantly, ZN OVA mice had a substantial decrease in Zinquin-stainable apical Zn and this was not further lowered in the ZL OVA mice. However, the latter had substantially more apoptosis than the ZN OVA mice. These results may indicate that there are two pools of labile Zn, one depleted in airway inflammation and the other depleted only by nutritional Zn deprivation. Only the latter would be responsible for regulation of PC3. Alternatively, labile Zn may not be functionally lost in the AEC of the OVA-treated mice. Rather the Zn may be still present but invisible to Zinquin, perhaps because of binding to a stronger ligand such as metallothionein. At present, it is not yet possible to distinguish between these and other possibilities.

10. Model

A schema depicting the interactions between Zn and PC3 in AEC and in asthma is shown in Fig. 2. In this model, we propose the following scenario: (1) Zn is taken up across the basolateral plasma membrane of AEC from subepithelial capillaries via an unidentified specific transporter(s). (2) This Zn is incorporated into vesicles with the aid of one of the vesicular ZnT transporters (see inset). (3) These vesicles deliver Zn to the apical cytoplasm. The sequestering of Zn provides a mechanism to keep Zn away from cytoplasmic enzymes, which it otherwise might inhibit. Such vesicles have been observed in the perinuclear region and apical cytoplasm of Zinquin-stained AEC [29,31]. (4) Apical cellular Zn protects the mucociliary apparatus (cilia, basal bodies) and other organelles from damage by oxidants, that would otherwise trigger PC3 activation to AC3 and downstream events in apoptosis. Zn may also directly stabilize PC3 proteins. The process, however, is bi-directional since Zn not only blocks actions of ROS, but ROS can release Zn ions from redox-sensitive Zn proteins such as metallothionein [44]. (5) In asthma, ROS released from eosinophils cause oxidative damage to airway epithelium. (6) This damage triggers PC3 activation to AC3 and subsequent events in apoptosis. (7) Excessive apoptosis in airway epithelium leads to epithelial shedding and contributes to the ongoing inflammation. (8) Nutritional depletion of Zn, or loss of Zn by other mechanisms, act synergistically with ROS to cause oxidative damage. Zn depletion also directly facilitates PC3 activation because this enzyme is inhibited by binding of Zn to an essential sulphydryl group [19].

11. Where to now?

The studies discussed here have raised a number of questions which need to be addressed. Firstly, how are Zn and PC3 constrained in the apical cytoplasm of AEC and do they physically interact with each other? At present, very little is known about the mechanisms that target PC3 to its subcellular sites. The enzyme has no membrane-spanning domain and any association with cellular membranes is likely to be peripheral. Our previous studies have shown that Zn binds to, and sequesters, protein kinase C in the cytoskeleton of lymphocytes [45] and Zn could also play a similar role in procaspase localization. This could explain the apparent redistribution of PC3 away from an apical pool in the Zn-poor airway epithelium of the asthmatic mice [42]. The finding of large amounts of Zn within the microtubular structures of basal bodies and cilia in AEC raises the question of whether Zn plays an important role in ciliary beating; this can be directly tested by measuring ciliary beat frequency of AEC before and after treatment with TPEN and/or Zn supplements *in vitro*. While Zinquin has proven very useful for revealing the

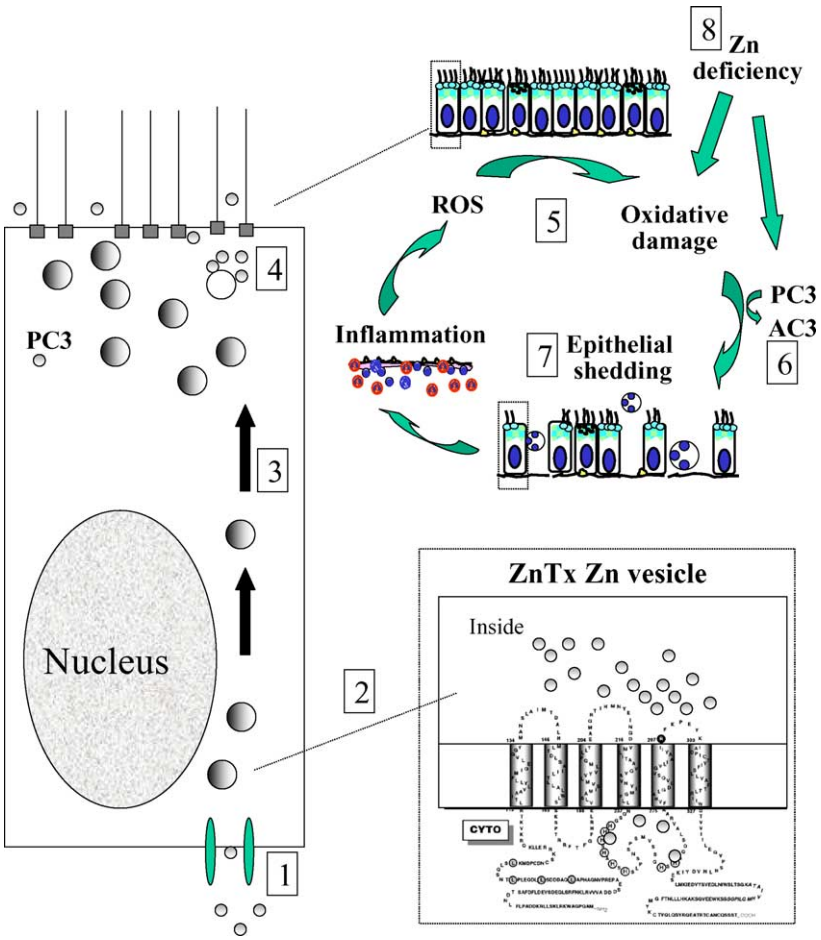

Fig. 2. Schema incorporating a model of Zn regulation of PC3 in AEC. On the left hand side, a typical AEC is shown. Step 1: Zn is taken up across the basolateral plasma membrane from sub-epithelial capillaries via an unidentified specific membrane Zn transporter(s). Step 2 (lower right figure): Free Zn is potentially toxic to sulphydryl-containing enzymes in the cytoplasm and is therefore sequestered inside membrane-bound vesicles with the aid of one of the ZnT family of vesicular Zn transporters. The ZnT proteins have six transmembrane domains, with N and C termini located on the cytoplasmic side of the membrane. They also have a conserved histidine-rich domain between trans-membrane segments IV and V which is thought to bind Zn [16]. Step 3: These vesicles traffic to the apical cytoplasm where they release their Zn for incorporation into basal bodies, cilia and PC3. Step 4: By virtue of its antioxidant properties, apical cellular Zn protects the mucociliary apparatus (cilia, basal bodies) from damage by ROS, that would otherwise trigger PC3 activation. Upper right figure shows damage to the airway epithelium in asthma. Step 5: ROS released from inflammatory cells in asthma cause oxidative damage to organelles in the airway epithelium. Step 6: Oxidative damage triggers PC3 activation to AC3 and downstream events in apoptosis. Step 7: Excessive apoptosis in airway epithelium leads to epithelial shedding and contributes to the ongoing inflammation. Step 8: Zn deficiency depletes AEC Zn and has two major effects on PC3 activation; it acts synergistically with ROS to cause oxidative damage to the airway epithelium thereby indirectly triggering PC3 activation and it also directly facilitates PC3 activation by removal of inhibitory Zn.

apical distribution of Zn in airway epithelium and the localization of some of this Zn to either cytoplasmic vesicles or ciliary basal bodies, further characterization of this Zn requires methods which allow visualization of Zn at the ultrastructural level. One such method is that of autometallography [46] which can also be used in combination with immunogold labelling of antigens, to study the relationship of Zn to PC3 and Zn transporters. Clues to the understanding of Zn physiology and the role of Zn transporters in determining Zn homeostasis in AEC should come from defining the relative times at which Zn transporters are expressed and Zn levels change. This might best be done in primary cultures of AEC which undergo ciliogenesis and allow the analysis of temporal changes in Zn, Zn transporter and PC3 levels with mucociliary differentiation. Particularly important will be studies which correlate functional responses with real concentrations of labile Zn within subcellular compartments. Previous studies from our laboratory indicated that approximately 10% of total cellular Zn in lymphocytes was in the labile pool, and that levels of this labile Zn correlated inversely with susceptibility of the cells to undergo toxin-induced apoptosis [19,32]. Lymphocytes are relatively poor in labile Zn compared with AEC, based on Zinquin fluorescence intensity. Similar determinations of the size of the labile vs. fixed pools of Zn need to be performed in AEC, so that we can begin to address the issue of whether cells with larger pools of labile Zn have increased threshold sensitivities to induction of apoptosis, and to what the extent these pools need to be reduced to confer the high sensitivity to induc-

tion of apoptosis seen in AEC of asthmatic humans [9] and mice [42]. Such studies need also to compare effects on apoptosis of rapid Zn deprivation induced by TPEN with Zn deprivation induced more slowly, as would occur in clinical Zn deficiency. Relevant to this issue, Rudolf *et al.* [47] have shown that in long-term cultures of Hep-2 cells there was an inverse relationship between concentration of extracellular Zn (varied from physiological to pharmacological levels) and induction of apoptosis, confirming the effects of TPEN.

The animal studies described in this review now need to be extended and designed to test the alternative hypotheses: (1) *Zn deficiency predisposes to asthma* and (2) *Zn deficiency arises as a consequence of asthma*. It is likely that if daily losses of Zn in these chronic respiratory diseases exceed dietary intake of this metal, systemic Zn deficiency will eventuate. If the second hypothesis is correct, the prediction would be that if the mice continue to be challenged with allergen over a longer period (setting up a chronic airway inflammation more like that found in human asthma), then a systemic Zn deficiency will eventuate; that is, the longer the mice have their airway inflammation, the more Zn will be lost through the airways and the lower will be their blood and tissue Zn levels. Related questions are (1) how quickly is AEC Zn lost in the asthmatic mice? (2) do the asthmatic mice eventually succumb to systemic Zn deficiency? (3) are there excessive losses of Zn in either the airways or the urine of asthmatic mice? (4) does the loss of Zn occur in the ciliated columnar cells or in secretory AEC (e.g. goblet cells) or in both? (5) what are the long-term effects of mild Zn deficiency alone, or in combination with allergen, on airway epithelial parameters and AHR? and, (6) will Zn supplementation restore airway epithelial Zn levels and have beneficial effects in the murine model?

It could be argued that the changes in plasma or serum Zn in human asthma are transient and reflect redistribution, rather than a real loss. Whether or not this is the case, our studies using a mouse model of asthma suggest that there is a real deficit of Zn in the airway epithelium in this disease [42]. These findings now need to be confirmed in human asthmatics and in other forms of chronic inflammatory respiratory disease. It is essential that reliable techniques are developed to assess airway epithelial Zn levels in patients. It will then be important to determine whether, in human asthma, intracellular Zn levels in AEC fall below threshold levels required to suppress oxidative stress and apoptosis.

In conclusion, we propose that some of the pathogenic changes causing asthma, such as AHR, are also facilitated as a consequence of enhanced oxidative damage and AEC death in subjects with Zn deficiency. If this is true, then increased epithelial apoptosis resulting from Zn deficiency may either be one of the multiple factors rendering individuals more susceptible to asthma or it may exacerbate the symptoms of this disease.

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