BioMetals **14:** 315–330, 2001.

Review

The role of zinc in caspase activation and apoptotic cell death

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Received 5 January 2001; accepted 15 April 2001

Key words: apoptosis, caspase, ROS, sulfhydryl, zinc

Abstract

In addition to its diverse role in many physiological systems, zinc (Zn) has now been shown to be an important regulator of apoptosis. The purpose of this review is to integrate previously published knowledge on Zn and apoptosis with current attempts to elucidate the mechanisms of action of this biometal. This paper begins with an introduction to apoptosis and then briefly reviews the evidence relating Zn to apoptosis. The major focus of this review is the mechanistic actions of Zn and its candidate intracellular targets. In particular, we examine the cytoprotective functions of Zn which suppress major pathways leading to apoptosis, as well as the more direct influence of Zn on the apoptotic regulators, especially the caspase family of enzymes. These two mechanisms are closely related since a decline in intracellular Zn below a critical threshold level may not only trigger pathways leading to caspase activation but may also facilitate the process by which the caspases are activated. Studies by our laboratory in airway epithelial cells show that Zn is co-localized with the precursor form of caspase-3, mitochondria and microtubules, suggesting this Zn is critically placed to control apoptosis. Further understanding the different pools of Zn and how they interact with apoptotic pathways should have importance in human disease.

Abbreviations: AEC – Airway epithelial cells; Cu/Zn SOD – Copper zinc superoxide dismutase; z-DEVD-AFC – boc-Asp-Glu-Val-Asp 7-amino-4-trifluoromethyl coumarin; H_2O_2 – Hydrogen peroxide; HNE – Hydroxynonenal; L-NAME – NG-nitro-L-arginine methyl ester; PARP – Poly (ADP-ribose) polymerase; ROS – Reactive oxygen species; TPEN – N,N,N′,N′-tetrakis-2-pyridylmethyl-ethylenediamine; Zn – Zinc.

Introduction

In the last two and a half decades, substantial evidence *in vitro* and *in vivo* has accumulated linking Zn deficiency with a markedly increased susceptibility of cells and tissues to die by apoptosis. This form of cellular demise is an active, tightly-regulated process that involves a series of cytoskeletal, membrane, nuclear and cytoplasmic changes that culminate in condensation and fragmentation of the cell into apoptotic bodies, which are eventually cleared by phagocytosis. Increase in apoptosis in Zn deficiency is directly related to a decrease in intracellular Zn within the cells fated to die. On the other hand, supplementing cells with exogenous Zn *in vitro*, and possibly also *in vivo*, decreases the susceptibility of cells and tissues to spontaneous or toxin-induced apoptosis, even when the cells apparently have a normal Zn status. The pools of Zn which influence cell susceptibility to apoptosis are the more exchangeable (labile) Zn, which are readily depleted in Zn deficiency and augmented following Zn supplementation. The subcellular distribution of these labile pools is amenable to studies using Zn-specific fluorophores or the Neo-Timm's silver sulphide stain for electron microscopy (Frederickson 1989).

This review will discuss aspects of Zn physiology and its possible beneficial actions in regulating

apoptosis. There are a number of issues concerning the role of Zn in protection against apoptosis which need to be considered in the context of normal cellular physiology. Amongst these issues are (1) the distribution and homeostasis of the subcellular pools of labile Zn that mediate cellular protection, (2) the mechanisms for delivery of this Zn to critical target(s) of the induction and/or effector pathways of apoptosis, (3) the precise mechanism by which Zn regulates the processing and/or catalytic activity of the caspases, (4) the relationship of Zn to other regulators of apoptosis e.g. Bcl-2 and (5) the role of enhanced apoptosis in increased vulnerability to disease (e.g. diabetes mellitus, Alzheimer's dementia and asthma), where there is often an underlying subclinical or overt state of Zn deficiency. For detailed reviews on the earlier papers relating Zn and apoptosis, the reader is referred to the following reports (Zalewski & Forbes 1993; Sunderman 1995; Fraker & Telford 1997; Chai *et al.* 1999; Truong-Tran *et al.* 2000b).

This review highlights more recent studies and aims to integrate this knowledge with current concepts of the cellular biology of this biometal. It also takes a critical look at some of the interpretations that have emerged and attempts to identify some of the gaps in our understanding of Zn and apoptosis. We begin with a brief overview of apoptosis and then explore the mechanisms by which it is influenced by Zn. Also discussed are the homeostatic mechanisms which control the intracellular levels and subcellular distribution of Zn.

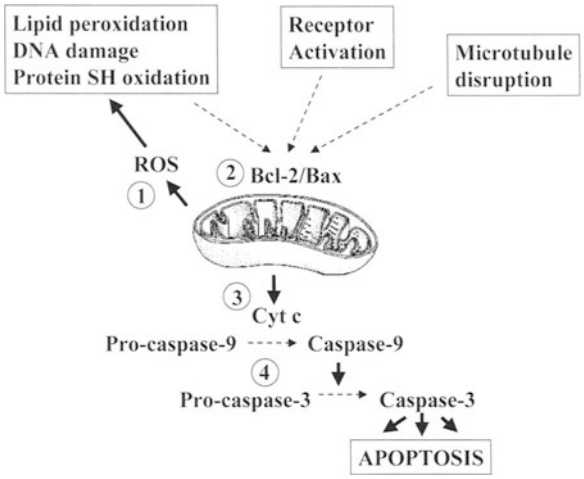

Fig. 1. The mitochondrion as a central controller of apoptosis. Figure shows the central role of the mitochondria in the regulation of apoptosis. (1) Mitochondria release ROS during aerobic respiration, potentially causing oxidation of lipids, DNA and protein sulfhydryls. Up to 5% of the oxygen consumed by mitochondria is thought to be converted to ROS, however under normal conditions these are rapidly removed by anti-oxidants (Halliwell 1991). (2) Mitochondria also influence the process of apoptosis by coordinating the various input signaling pathways and channeling them onto a central pathway which is governed by mitochondrial-associated anti-apoptotic (e.g., Bcl-2) and pro-apoptotic (e.g., Bax) families of regulators (Strasser *et al.* 2000). Whether the cell proceeds into apoptosis is determined by the ratio of Bcl-2/Bax proteins; this has also been referred to as the Bcl-2/Bax rheostat and serves as a major check-point for commitment of the cell to death (Korsmeyer *et al.* 1993). (3) Once a cell has passed through this checkpoint cytochrome c is released through mitochondrial pores, triggering (4) the activation of the caspase cascade leading to morphological changes of apoptosis.

Apoptosis (gene-directed cell death)

Although the finding that some cells in the body die as a part of normal development, instead of injury, emerged from studies several decades ago, it is only relatively recently that we have come to the realization that the mechanism of this programmed cell death shares many features with that of cells dying as a consequence of mild damage (e.g., by oxyradicals or microtubule poisons), starvation of growth factors and activation of certain membrane receptors (for review see Kerr *et al.* 1987). This process, referred to as apoptosis or gene-directed cell death, is the major mechanism of cell death in the body, enabling the removal of superfluous, mutant or moderately damaged cells. Distinct from necrosis, due to severe physical, chemical or osmotic cell damage, apoptosis deletes cells without release of their contents that would otherwise damage neighboring cells and provoke an inflammatory response.

Apoptosis is an active, energy-dependent process divided into two phases, biochemical and morphological. The biochemical phase involves diverse input signaling pathways originating from the plasma membrane (e.g., Fas receptor ligation, lipid peroxidation), the nucleus (e.g., newly-transcribed gene products such as *reaper*, DNA damage or mutations) or the cytoskeleton (e.g., disruption of microtubules) (Sun *et al.* 1999). As depicted in Figure 1, the mitochondrion is a major player in the induction, regulation and execution of apoptosis. Mitochondria coordinate apoptosis by channeling the input pathways onto a central pathway which is governed by mitochondrial-associated anti-apoptotic (e.g., Bcl-2) and pro-apoptotic (e.g., Bax) families of regulators and by providing a scaffolding for the proteolytic events that trigger processing and activation of various members of the caspase enzyme

family (Strasser *et al.* 2000). Caspases are proteases which recognize and cleave their substrate proteins at tetra-peptide sites with characteristic sequences, e.g., the DXXD motif for caspase-3, where X is any amino acid (Thornberry & Lazebnik 1998). They comprise two families: (1) the initiator caspases (such as caspases-8 and -9) which act upstream and transduce signals from specific input pathways (e.g., Fas ligation results in caspase-8 activation while release of cytochrome c from mitochondria triggers caspase-9 activation) and (2) the executioner caspases (such as caspases-3 and -6) which are activated by initiator caspases and, in turn, cleave critical substrates leading to downstream events of apoptosis. For example, caspase-3 cleaves ICAD (inhibitor of calcium-activated DNAase) liberating the endonuclease and enabling it to cleave DNA at inter-nucleosomal sites (Janicke *et al.* 1998). Caspase-6, previously known as Mch2α, cleaves the nuclear lamin scaffold proteins as a prerequisite for fragmentation of the nucleus (Takahashi *et al.* 1996). Action of the caspases leads to the morphological changes such as cell shrinkage, condensation and fragmentation of the cytoplasm and nucleus and formation of membrane-enclosed apoptotic bodies, which contain the contents of the cell and are cleared *in vivo* by phagocytosis (Song & Steller 1999; Strasser *et al.* 2000).

Apoptosis is tightly regulated and its dysregulation is central to the pathogenesis of a number of diseases, being excessive in neurodegenerative disorders, AIDS and diabetes mellitus and inadequate in autoimmune disease and malignancies (Thompson 1995; Wyllie 1997). A number of therapeutic strategies are aimed at correcting these imbalances and, as a consequence, the factors regulating the induction and execution phases of apoptosis are being intensively studied. One such factor is zinc.

Zinc and apoptosis

This section considers the evidence relating labile pools of Zn to regulation of caspase-dependent apoptosis. Although high concentrations of Zn may, in some cells, trigger cell death either by apoptosis or necrosis (Sensi *et al.* 1999; Hamatake *et al.* 2000; Untergasser *et al.* 2000), the bulk of evidence indicates that Zn is a physiological suppressor of apoptosis.

Zn deficiency increases apoptosis

The most convincing evidence for a physiological role for Zn in suppression of apoptosis comes from Zn deprivation animal studies. Although systematic studies of apoptosis in Zn deficient animals are still lacking, it is clear that the frequency of apoptotic cells is markedly increased in certain tissues and organs, including the intestinal and retinal pigment epithelium, skin, thymic lymphocytes, testis and pancreatic acinar cells of adult animals (reviewed in Duvall & Wyllie 1986; Zalewski & Forbes 1993) as well as the neuroepithelium of foetal rats (Rogers *et al.* 1995). The latter, which occurred within 4 days of maternal Zn deficiency, was particularly evident in the neural crest cells and interfered with neural tube closure leading to severe congenital abnormalities of the nervous system. In nearly all examples of Zn deficiency-induced apoptosis *in vivo*, increased cell death appears to be a direct consequence of a lowering of intracellular levels of Zn in the affected tissues. One exception is the involution of the thymus in Zn deficient mice which is at least partly due to increased circulating glucocorticoids produced in response to the stress associated with Zn deficiency (Fraker & Telford 1997).

Numerous *in vitro* studies have now shown that depletion of intracellular Zn by culture of cells in Zn-depleted medium or by treatment of cells with N,N,N′,N′-tetrakis-2-pyridylmethyl-ethylenediamine (TPEN) results in apoptosis (Martin *et al.* 1991; Zalewski *et al.* 1991,1993; Treves *et al.* 1994). Zn deficiency-induced apoptosis, *in vitro* and *in vivo*, has all of the major morphological features of apoptosis, including DNA and nuclear fragmentation, chromatin condensation and apoptotic body formation (Truong-Tran *et al.* 2000b). In addition, Zn deficiency-induced apoptosis is dependent on caspase-3 activation, since cytosolic caspase-3 activity is increased in Zn deficient cells while the specific caspase-3 inhibitor z-DEVD-fmk can partially suppress apoptosis (Chai *et al.* 2000). There is also now at least one *in vivo* example of this where Zn deficiency-induced apoptosis in rat embryos was associated with increased caspase-3 activity (Jankowski-Hennig *et al.* 2000).

Zn depletion renders cells more susceptible to apoptosis by toxins

Zn depletion not only increases the rate of apoptosis but there is a potent synergy, in the induction of apoptosis, between Zn depletion and other apoptotic inducers such as colchicine, tumour necrosis factor

and HIV-1 Tat protein (Zalewski *et al.* 1993; Seve *et al.* 1999; Meerarani *et al.* 2000). Furthermore, studies by our laboratory have shown that Zn deficiency increases the susceptibility of respiratory epithelial cells to H_2O_2-induced apoptosis (Truong-Tran *et al.* 2000a). Thus, a severe reduction of intracellular labile Zn can directly induce apoptosis while smaller decreases may simply render cells more vulnerable to apoptosis by other toxins. The latter has important clinical implications for the relationships between mild or sub-clinical states of Zn deficiency and disease severity and incidence (see later).

Exogenous Zn suppresses apoptosis

There are several examples of increased resistance of Zn-supplemented animals to toxic apoptotic inducers. These include the protective effects of Zn salts against whole body irradiation in mice (Floersheim *et al.* 1992), sporidesmin-induced immunosuppression in sheep (Waring *et al.* 1990), neuronal apoptosis following transient forebrain ischemia in the hippocampus of primates (Matsushita *et al.* 1996) and apoptosis of the anterior and stromal keratinocytes in the eye following superficial keratectomy in rabbits (Kuo *et al.* 1997). Recently, Kown and colleagues (2000) demonstrated that Zn suppresses caspase-3 activity and apoptosis *in vivo* using rats transplanted in the abdomen with allogeneic hearts. They found that i.p. injections of 1–5 mg/kg of $ZnCl_2$ suppressed caspase-3 activity by up to 3.7-fold and apoptosis by up to 2-fold. The latter was assessed by (99m)Tc-Annexin V labelling in the allografts. Survival rates of the allografts were also increased from 6.4 days to 11.5 days ($P \leq 0.005$). These findings are consistent with the cytoprotective and anti-apoptotic effects of exogenous Zn *in vitro* in diverse cellular models. In particular, it should be noted that Zn blocks apoptosis induced by all apoptosis-inducing treatments tested, indicating that it suppresses a common event (reviewed in Zalewski & Forbes 1993; Sunderman 1995).

However, interpretation of the Zn supplementation studies remains unclear. While the *in vivo* studies suggest that intracellular Zn levels can be manipulated sufficiently to influence vulnerability of cells and tissues to apoptosis-inducing agents, there has been no attempt as yet to correlate changes in intracellular Zn content with resistance to toxins *in vivo*. Another concern is that most studies of Zn suppression of apoptosis *in vitro* have used supraphysiological concentrations of Zn salts in the medium (e.g., 5 mM in the study of Barbieri *et al.* 1992). One of the reasons for this is the relatively poor uptake of ionic Zn across the plasma cell membrane. However, mM concentrations of Zn will cross-link proteins non-specifically and no useful information can be gained from such experiments. Unfortunately, this includes most published studies. Our laboratory has attempted to address this by showing suppression of apoptosis using low concentrations of Zn sulphate (typical of those found in extracellular fluids) and given in combination with a Zn ionophore such as pyrithione (Zalewski *et al.* 1991). However, this approach also has the limitation in that intracellular levels of Zn in these Zn-loaded cells may very likely be much higher than would occur *in vivo*. This is not a trivial issue since metabolically available Zn is distributed non-uniformly throughout the cell, existing in nM-pM concentrations in the cytosol and up to mM concentrations within vesicles (Frederickson 1989). It is not known whether the pools of Zn responsible for suppression of apoptosis are compartmentalized and, if so, what the local available concentrations of Zn are. Finally, it is not known whether Zn supplementation affects the same pools and molecular targets within the apoptosis pathway as does Zn depletion. The various supplementation and depletion studies reported in the literature may be measuring different aspects of the influence of Zn on apoptosis, especially if there are multiple targets of Zn such as the nucleases, caspases and the Bcl-2 family (see below).

Zn and necrosis

While Zn depletion triggers caspase activation leading to apoptosis, other cell death can also occur. In some cells, e.g., T cell leukaemic Molt-3 cells, Zn deprivation resulted in necrosis (Martin *et al.* 1991). The reason for this is not clear but may depend, in part, on the functional state of the caspases. For example, in a recent study of TPEN-induced Zn deficiency in human renal cell carcinoma cell lines, mutant cell lines lacking caspases-3, -7, -8 and -10 died by necrosis rather than apoptosis (Kolenko *et al.* 1999). Therefore, we should not view Zn as a specific regulator of apoptosis but rather as a cytoprotectant that, when lacking, renders the cell vulnerable to death both by apoptosis and necrosis. A similar argument has been advanced for cytoprotection by Bcl-2 against both necrosis and apoptosis (Okuno *et al.* 1998).

It has been proposed that commitment to cell death can be regulated at a point upstream from caspases, probably at the level of the mitochondria since phar-

macological agents which cause increased permeability of mitochondrial membranes cause both necrosis and apoptosis, depending on the activity of the caspases (reviewed in Kroemer & Reed 2000). Caspase inhibitors often fail to prevent cell death, rather shifting the mechanism of death from apoptosis to necrosis. For agents to be cytoprotective, they must act at the pre-mitochondrial or mitochondrial stages of apoptosis, rather than at the level of the caspases (Jacotot *et al.* 1999). This issue needs addressing in relation to the mechanism of action of Zn. Even if increase in intracellular Zn does specifically suppress apoptosis-related biochemical events, the cells may still die in the longer term. The evidence that Zn fails to block cell death in many systems has recently been reviewed (Fraker & Telford 1997). There are two separate issues to consider here. First, by suppressing apoptosis, Zn may simply divert irreversibly-damaged cells into necrosis. Secondly, the apoptotic program may be regulated by multiple factors and complete cytoprotection may require all of these in addition to Zn. A critical issue that has scarcely received attention is how the different anti-apoptotic factors cooperate with each other. Rather than simply testing the effect of high, pharmacological or even toxic, concentrations of Zn in isolation, experiments are now required to test the effects of smaller, more physiological Zn fluxes in combination with different levels of other cellular survival factors.

Mechanisms of the anti-apoptotic actions of zinc

There are very likely to be two aspects to the anti-apoptotic mechanisms of action of Zn. Firstly, it limits the extent of damage induced by oxyradicals and other toxins and thereby suppresses some of the signaling pathways leading to caspase activation and apoptosis. Secondly, it directly affects some of the apoptotic regulators, principally the caspase enzymes. These two actions may, in fact, be 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 sulfhydryls of the caspases.

The intention of this section is to bring together the evidence for the role of Zn as both a cytoprotectant and an anti-apoptotic agent, principally acting at the level of the mitochondrion. The final parts of this section concern the possible inter-relationships between Zn and other anti-apoptotic factors in cells.

Zn is a cytoprotectant against oxidative stress

Zn is well known as a cytoprotectant, protecting and stabilizing cellular molecules (e.g., proteins and DNA), macromolecular complexes (e.g., microtubules) and subcellular organelles (e.g., membranes) (Vallee & Falchuk 1993). Central to this role is its capacity to minimize oxidative damage. It has been proposed that Zn became especially important at the time of evolution of cellular respiration when the hazards of oxidative stress became manifest (Da Silva & Williams 1991).

Apoptosis is closely linked with oxidative stress and may well have evolved primarily to rid the body of oxidatively-damaged cells (Frade & Michaelidis 1997). Many agents which induce apoptosis are either oxidants or stimulators of cellular oxidative metabolism. These include reactive oxygen species (ROS), such as superoxide anion, hydroxyl radical and peroxynitrite which are highly unstable compounds with unpaired electrons, capable of oxidizing lipids, proteins and nucleic acids (Halliwell 1991). Likewise, some anti-apoptotic factors (e.g., Bcl-2) also protect cellular organelles from oxidative damage and typical anti-oxidants (e.g., vitamin E and vitamin C and N-acetylcysteine) are often found to be anti-apoptotic (Dimmeler *et al.* 1997; Hennig *et al.* 1999).

Many previous studies have linked Zn deficiency in animals with enhanced rates of oxidative damage such as in testes and erythrocytes; supplementation with other anti-oxidants (vitamin C, vitamin E or β-carotene) reversed these effects and protected against the development of skin lesions and some other manifestations of Zn deficiency (Bettger and O'Dell 1981; Taylor *et al.* 1990; Oteiza *et al.* 1995; Kraus *et al.* 1997). Similarly, Zn supplementation also protects against oxidative damage. Thus, Zn acexamate, an anti-ulcer agent, inhibited lipid peroxidation and lesions in rat gastric mucosa *in vitro* and *in vivo* (Tsutsui *et al.* 1999).

Two recent studies have further contributed to our understanding of the link between Zn deficiency, oxidative stress and apoptosis. Firstly, Oteiza and colleagues (2000) investigated the effects of Zn deficiency on oxidative stress in 3T3 cells using a novel fluorescent probe (carboxy-2′7′-dichlorodihydrofluorescein diacetate) to assess for oxidative damage. When cells were exposed to Zn manipulated media the fluorescence intensity was increased up to 15-fold in the Zn deficient cells over that of untreated cells. Hence, they concluded that

oxidative stress is induced by Zn deficiency in 3T3 cells. Secondly, Cui and colleagues (1999, 2000) have provided a strong link between (1) increased oxidative stress, due to increased levels and/or activity of inducible nitric oxide synthase and consequent nitric oxide production, (2) increased tissue damage, manifested by enhanced microvascular permeability and appearance of inflammatory lesions, and (3) increased apoptosis (as assessed by terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling), in the skin and intestinal villi of Zn deficient mice and rats. The nitric oxide synthase inhibitor, NG-nitro-L-arginine methyl ester (L-NAME), given in the drinking water suppressed all of these changes. In airway epithelial cells (AEC) and in the malignant human lung epithelial cell lines A549 and NCI-H292, we have shown that TPEN acts synergistically with low concentrations of ROS (peroxynitrite or H_2O_2) in the induction of caspase-3 activity (Truong-Tran *et al.* 2000a; unpublished observations). Furthermore, the effects of TPEN were decreased by 50%–60% when cells were pre-incubated with various anti-oxidants (vitamin C, vitamin E or N-acetylcysteine, unpublished observations). In preliminary studies, we have found, using immunocytochemistry with antibody to hydroxynonenal (a lipid peroxidation marker), increased lipid peroxidation in the membranes of TPEN-treated primary AEC (see later).

Mechanisms by which Zn blocks oxidative damage

We believe it is unlikely that Zn protects against oxidative damage via its role in Cu/Zn superoxide dismutase (Cu/Zn SOD), an enzyme which removes the superoxide anion radical. The role of Zn in Cu/Zn SOD may simply be structural since severe depletion of intracellular Zn in keratinocytes by treatment with TPEN *in vitro* resulted in apoptosis but did not affect the Cu/Zn SOD activity of cells (Parat *et al.* 1997); in addition, lung Cu/Zn SOD was not responsible for the cytoprotection of Zn in a mouse model of hyperoxia; in fact, activity of this enzyme was paradoxically increased in Zn deficiency (Oteiza *et al.* 1996).

It is more likely that the cytoprotective Zn is to be found in the more labile and exchangeable cellular pools of Zn. This Zn may both directly and indirectly protect cells from oxidative damage. Directly, Zn is a well-known stabilizer of lipids and proteins. Thus, Zn protects cellular membranes and macromolecules against oxidative damage (Bettger and O'Dell 1981; Hennig *et al.* 1992, 1993; Kraus *et al.* 1997; Taylor *et al.* 1997; Powell 2000). Zn protects sulfhydryl groups in proteins from oxidation by forming strong, yet readily reversible, thiolate complexes (Williams 1987). Zn, thereby, affords protection to thiol-dependent enzymes such as δ-aminolevulinic acid dehydratase and dihydroorotase. It will also protect other cellular proteins with essential thiols such as tubulin, where sulfhydryls are required for polymerization into microtubules (Roychowdhury *et al.* 2000). The finding of large amounts of Zn within the microtubular structures of basal bodies and cilia in airway epithelial cells (Truong-Tran *et al.* 2000a) raises the question of whether Zn is there to protect their sulfhydryls against ROS released from their abundant mitochondria. Zn is a stabilizer of microtubules (Hesketh 1982) and microtubular disruption occurs in Zn deficiency (Nickolson & Veldstra 1972), oxidative stress (Banan *et al.* 2000) and in the early stages of apoptosis (Martin & Cotter 1990).

Indirectly, Zn may act via effects on glutathione, the main intracellular anti-oxidant. Recent studies have implicated glutathione in protection against inappropriate apoptosis. In view of its general cellular role as a redox regulator, glutathione could influence a number of events in the apoptotic pathway. One of these is the release of cytochrome c from mitochondria, which serves to initiate caspase-9-dependent processing and activation of caspase-3 (Grutter 2000). Appearance of cytochrome c in the cytosol is a cellular response to the depletion of glutathione, induced by inhibition of glutathione biosynthesis (Ghibelli *et al.* 1999). In this context, the reports by Parat and colleagues (1997) and Nakatani and colleagues (2000), showing that TPEN depletes intracellular glutathione in cultured keratinocytes and hepatocytes, respectively, are interesting. In keratinocytes, TPEN reduced glutathione content from 65 to 8 μmoles/g protein. In the study by Nakatani *et al.*, the glutathione depletion, caspase-3 activation and apoptosis that occurred in TPEN-treated hepatocytes were not only suppressed by Zn supplementation but also by addition of N-acetylcysteine, a precursor of glutathione. This suggests that the increase in caspase-dependent apoptosis in response to Zn depletion, was due to the decline in glutathione levels. However, this hypothesis needs substantiation from time course studies that show glutathione depletion *follows* the decrease in cellular Zn, but *precedes* the rise in caspase-3 levels. Furthermore, the kinetic relationships between depletion of Zn and glutathione, and onset of oxidative damage, mitochon-

drial cytochrome c release and caspase-3 activation need to be determined.

Effects on the processing and catalytic activity of caspase-3

In addition to blocking oxidative stress, Zn may also influence apoptosis by directly interacting with other apoptotic regulators. Historically, Zn was first shown to be a potent inhibitor of the endonuclease responsible for apoptotic DNA fragmentation and it was assumed that this was the critical target affected by Zn deficiency (Duvall & Wyllie 1986). At the time this seemed logical, since our whole understanding of the biochemistry of apoptosis centered on this enzyme and the nuclear changes. Furthermore, data presented showed that treatment of isolated nuclei with Zn salts could suppress Ca^{2+}-induced apoptotic changes within nuclei (Cohen & Duke 1984). For a variety of reasons, we now know that the endonucleases are only a secondary target. Apoptosis can occur without DNA fragmentation and even in cytoplasts lacking a nucleus; yet Zn is still suppressive in these models (reviewed in Truong-Tran *et al.* 2000b). Moreover, the concentrations of Zn required to inhibit the endonuclease are relatively high.

The possibility that Zn may suppress a step prior to activation of the endonucleases was first shown by Lazebnik and colleagues (1993), who used a cell-free model of apoptosis in which cytosols prepared from cells primed to undergo apoptosis were added to intact nuclei to produce nuclear condensation and DNA fragmentation. Importantly, the apoptosis-associated morphological changes of the nuclei were suppressed by concentrations of Zn lower than those required to suppress the fragmentation of DNA by the endonucleases. In addition, experiments in which cytosols and nuclei were separately pre-treated with Zn, indicated that the primary target of Zn was cytoplasmic rather than nuclear. Subsequent studies identified the cytosolic target of Zn as an aspartate-specific protease CPP-32 (renamed caspase-3), although they did not establish whether Zn blocked caspase-3 enzymatic activity or the steps leading to its activation (Faleiro *et al.* 1997).

This remains a controversial issue. We, and others, have found caspase-3 to be relatively insensitive to exogenous Zn (Takahashi *et al.* 1996; Truong-Tran *et al.* 2000b), although the activation of pro-caspase-3 in cell-free models was very sensitive to Zn. Using a cell-free system (described in Liu *et al.* 1996) in which addition of cytochrome c to cytosol of healthy cells triggers proteolytic conversion of pro-caspase-3 to the active enzyme, Mesner and colleagues (1999) found that Zn (>500 μM total concentration) inhibited pro-caspase-3 activation. We observed that addition of 800 nM *free* Zn blocked activation of caspase-3 by 50% in this model; there was no effect of Zn when added 90 min after cytochrome c but prior to addition of fluorogenic caspase-3 substrate, confirming that Zn blocks the process of caspase-3 activation rather than the already activated enzyme (Truong-Tran *et al.* 2000b). A similar finding using Western blotting to track caspase-3 processing in HL60 cells has also been reported (Aiuchi *et al.* 1998).

Caspase-3 requires an essential sulfhydryl for enzymatic activity (Nicholson *et al.* 1995). Its failure to be directly inhibited by Zn in the above mentioned studies may be a consequence of the use of metal chelators and thiol-reducing agents (e.g., dithiothreitol) in the cell extraction buffers, since these would otherwise bind Zn and lower the available Zn concentration. Using a cell-free system consisting of purified bovine poly (ADP) ribose polymerase (PARP) as a substrate and an apoptotic extract or recombinant caspase-3, Perry and colleagues (1997) reported that Zn inhibits PARP-proteolysis by caspase-3 in the low μM range. Furthermore, they found that cleavage of the caspase-3 tetrapeptide substrate was even more sensitive to Zn, being inhibited in the nM range. It is relevant that the extraction buffer used in this study lacked metal chelators and thiol reducing agents. Stennicke and Salvesen (1997) derived an inhibitory value of 0.15 μM for caspase-3 and concluded that Zn is a good caspase inhibitor, albeit very dependent on the thiol content and therefore presumably the redox potential of the cell. Another important paper supporting the role of Zn as a physiological caspase inhibitor was that of Maret *et al.* (1999) who found a 50% inhibition of caspase-3 activity by 1.7 nM Zn, under conditions where they used highly purified metal free buffers and no thiol reducing agents and a 1:1 stoichiometric relationship in the inhibition of caspase-3 by exogenous Zn. The latter implies that Zn may interact with only one sulfhydryl group in caspase-3, presumably the sulfhydryl required for catalytic activity.

Effects on caspases-6 and -9

Since the proteolytic processing and activation of caspase-3 is dependent on other caspases, including caspase-6 and caspase-9 (see earlier), these enzymes,

and or their mechanisms of activation, may be the critical targets of Zn. It is of interest, that both of these caspases are reported to be very sensitive to inhibition by Zn. At least two recent studies have shown that although Zn inhibits all of the different caspases that have been tested, at lower, more physiological concentrations, it is a selective inhibitor of caspase-6/Mch2-α; complete inhibition was observed at 10 μM Zn (Takahashi *et al.* 1996; Stennicke & Salvesen 1997). Thus, Zn was found to block cleavage of lamin A by pro-apoptotic cell extracts and by recombinant caspase-6 but did not block cleavage of PARP by caspase 3. A 50% inhibition occurred at about 400 μM total added Zn, however the cell extracts contained metal chelators and the actual free Zn concentration was not determined. These effects on caspase-6 are interesting in light of studies by Cohen and colleagues (1992) where, using electron microscopy, they showed that Zn supplementation of dexamethasone-treated thymocytes *in vitro* blocked the transition from peripheral nuclear chromatin condensation to nuclear collapse. Since caspase-6 is responsible for nuclear lamin cleavage leading to nuclear fragmentation (see earlier), Zn-mediated suppression of this caspase may be responsible for the morphological effects.

Zn may also suppress caspase-3 activation by initially blocking caspase-9. At least one study has reported Zn-dependent suppression of cleavage of the specific tetrapeptide fluorogenic substrate LEHD-AFC by recombinant human caspase-9 in the 300–1000 μM range. Zn was also found to inhibit the generation of active caspase-9 as detected by immunoblotting and enzymatic activity in intact cells (Mesner *et al.* 1999; Wolf & Eastman 1999). Interestingly, both caspase-6 and caspase-9 belong to the group III subset of caspases which preferentially cleave at (I/V/L)E*X*D tetrapeptide sequences (Earnshaw *et al.* 1999). Other members of this group include caspase-8 and granzyme B. There is no information yet on effects of Zn on caspase-8 but Zn does not block recombinant murine granzyme B, although neither do peptide caspase inhibitors (Pham *et al.* 1998).

TPEN might act by stripping Zn from either or both of caspases 6 and 9, enhancing their activity. However, we were unable to show any significant activation of caspase-6 prior to activation of caspase-3. Caspase-6 was activated in TPEN-treated cells, but only at time-points later than that of caspase-3 activation (Truong-Tran *et al.* 2000a). It is possible that only small amounts of caspase-6 are required for processing of caspase-3 and that these levels were too low to be detected by our fluorogenic substrate assay.

New insights into the compartmentalization of caspase processing and activation in cells coupled with colocalization studies of labile Zn and caspases (especially caspase-6) may provide further clues. How many of the other 14 caspases are as sensitive to Zn as caspase-6 and caspase-9 is not known. There is also a particular need for determining whether Zn deprivation *in vitro* and *in vivo* directly activates other suicide enzymes, as it does caspase-3.

Does Zn protect caspase sulfhydryls from oxidation?

In cells in which the caspases are dysfunctional, due to knock-out of the genes or after blockade by specific caspase peptide inhibitors, damage often results in necrosis rather than apoptosis (Kolenko *et al.* 1999). Necrosis is injurious to the body since the plasma membrane of the dying cell is disrupted, allowing release of phospholipases, proteases and other agents that can damage neighbouring cells and trigger an inflammatory response. Caspases, therefore, play a critical role in the commitment to apoptosis rather than necrosis. In cells damaged by ROS, there is an increased risk of cell death by necrosis since the caspase enzymes, themselves, contain an essential sulfhydryl (Nicholson *et al.* 1995) which is susceptible to oxidation (Sen *et al.* 1999) and S-nitrosation (Rossig *et al.* 1999). How then can caspases operate effectively in cells that are undergoing apoptosis triggered by an onslaught of ROS? We speculate that Zn may interact in a reversible manner with the essential sulfhydryl of Cys163 of the human pro-caspase-3 molecule (Figure 2). This would have two effects: the sulfhydryl would be protected from oxidation to a disulphide and the caspase activity would be temporarily suppressed.

In cells committed to apoptose, the masking Zn may be removed by changes in the redox state of the cell and/or by exchange of Zn with thionein. Maret *et al.* (1999) reported that Zn can be transferred from metallothionein to the apoforms of Zn metalloenzymes forming thionein. In addition, thionein was able to reactivate some Zn-inhibited enzymes, suggesting that it is an effective, intracellular chelator and may regulate a number of Zn-dependent cellular processes. Of particular relevance here, Maret and colleagues (1999) found that thionein was a potent activator of Zn-inhibited caspase-3; 100 nM thionein was as effective as 1 mM EDTA in reactivating this caspase (W. Maret, personal communication). It will be impor-

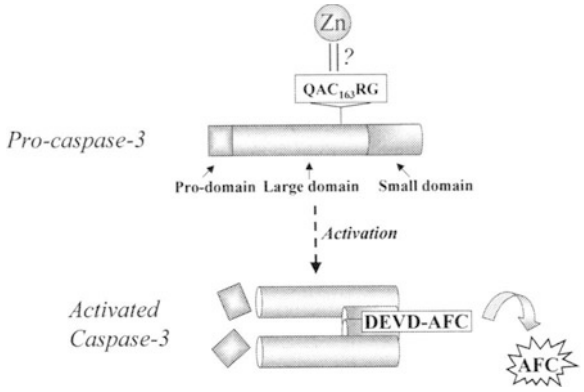

Fig. 2. Model for inactivation of caspases by Zn. The diagram shows the pro-caspase-3 polypeptide containing three domains (N-terminal prodomain, central large domain and C-terminal short domain) and the highly conserved pentapeptide $QAC_{163}RG$ within the long domain. Activation of caspase-3 involves the removal of the pro-domain and separation of large and short domains which reassemble to form the active enzyme. The activated enzyme is now able to cleave substrates such as the fluorogenic tetrapeptide DEVD-AFC releasing the coumarin derivative AFC. Cys_{163} is essential for catalytic activity (Nicholson *et al.* 1995; Earnshaw *et al.* 1999) and may be inactivated by oxidation to a disulfide. There are several other cysteines within 20 amino acids of this Cys_{163} which could potentially form a disulfide bond with this cysteine; however its not clear whether these do form in the tertiary structure of the molecule. It is proposed that Zn binds to this sulfhydryl in Cys_{163}, protecting it from oxidation and reversibly inhibiting enzymatic activity.

tant now to determine whether thionein is capable of triggering caspase activation and downstream events of apoptosis in cell-free models and to what extent thionein and the redox state of cells influences the activity of the caspases *in vivo*.

Other potential targets of Zn

Pharmacological concentrations of Zn and other divalent cations have been shown to block the mitochondrial pore through which cytochrome c is released (Zamzami *et al.* 1996). However, it is not clear whether this suppression occurs at a physiological level of Zn within the vicinity of the mitochondria. This mechanism can not explain the suppressive effects of Zn in cell-free models which lack mitochondria and contain exogenous cytochrome c. Furthermore, Wolf & Eastman (1999) recently confirmed that Zn prevents the activation of downstream caspases but does not prevent release of mitochondrial cytochrome c, as assessed by Western blotting studies to measure release of this cofactor.

Alternatively, Zn may influence the levels of auxillary factors in the activation of caspase-3. Candidates include members of the Bcl-2 family of proteins which suppress caspase-3 activation. Fukamachi and colleagues (1998) showed that Zn supplementation of cells *in vitro* increased the Bcl-2/Bax ratio in a monocyte cell line U937, thereby increasing the resistance of the cells to apoptosis. However, a criticism of this study is that a very high concentration (1 mM) of Zn salt was used to supplement the cells and therefore the increase in the Bcl-2/Bax ratio may be partially due to a response to the stress of excessive levels of Zn rather than a physiological effect of Zn on the regulation of apoptosis. Further experiments using a more physiological concentration of Zn are required to confirm these findings. However, there may be an interesting synergistic relationship between Zn and Bcl-2 (see below).

Zn may also influence events at the level of gene expression since Zn deprivation-induced apoptosis was suppressed by cycloheximide in neurons (Ahn *et al.* 1998). One possible target of Zn in gene-dependent apoptosis may be the Zn finger proteins. Previously it was thought that the Zn in these Zn finger domains is so tightly bound that it is not influenced by Zn deficiency. We now know that this Zn is a kinetically-labile pool especially in an oxidative microenvironment (Berendji *et al.* 1997).

Relationship of Zn to other anti-apoptotic regulators

A key issue is how the different physiological anti-apoptotic factors in cells cooperate to suppress apoptosis. Decreases in the concentrations of one factor may be compensated for by increases in another factor, if there is a level of redundancy.

Vitamin E (alpha-tocopherol) and Zn may at least partially substitute for each other. Vitamin E, which occurs in membranes and lipoproteins, blocks the chain reaction of lipid peroxidation by scavenging intermediate peroxyl radicals, particularly in membranes that are rich in polyunsaturated lipids (Liebler 1993). Like Zn deficiency, depletion of vitamin E disrupts membranes (Hennig *et al.* 1993) and causes neurodegeneration (Vatassery *et al.* 1992). As discussed earlier, vitamin E can reduce oxidative damage and apoptosis in Zn-depleted cells *in vivo* and *in vitro*. Hennig and colleagues (1993) have interpreted the decrease in plasma concentrations of vitamin E in dietary Zn deficiency to indicate greater utilization of this vitamin as a substitute for Zn. Vitamin E, however, cannot mimic Zn in the protection of protein sulfhydryls from oxidation and this may explain

why vitamin E could only partially block apoptosis in Zn-depleted cells (unpublished observations).

The anti-apoptotic Bcl-2 family of proteins share a number of properties with Zn. Both are antagonists of a central mechanism in apoptotic cell death and therefore suppress apoptosis in response to a variety of inducers acting via diverse pathways. Like Zn, Bcl-2 is localized around mitochondria and protects against apoptosis and necrosis by multiple mechanisms including its protective effects against oxyradicals and interference with caspase processing (Korsmeyer *et al.* 1993; Reed *et al.* 1998; Thornberry & Lazebnik 1998; Esposti *et al.* 1999). Bcl-2 knock-out mice, like Zn-deficient rodents, exhibit massive apoptotic involution of thymus and spleen associated with depletion of $CD4^+$ T cells, are growth stunted and exhibit abnormal skin pigmentation (suggested, in Bcl-2 deficient mice, to be due to a defect in redox-regulated melanin synthesis (Veis *et al.* 1993)).

Zinc homeostasis and apoptosis

The relationship between Zn homeostasis and apoptosis needs further investigation and, in particular, the following questions need to be addressed. Firstly, why does Zn deficiency primarily increase apoptosis in those tissues undergoing rapid cell turnover (e.g., bone, thymus, epidermis, esophagus, testis, intestinal crypts and developing tissues of fetus)? We believe that cycling cells are highly prone to undergo apoptosis following Zn depletion. One mechanism may involve the cell cycle regulator $p21^{waf1/cip1}$ which is cleaved immediately following TPEN treatment of cells (Chai *et al.* 2000). This loss immediately followed activation of caspase-3 in Zn-depleted cells and proceeded in two steps: initial cleavage of p21 at D_{112} was followed by complete degradation of the protein. Since the turnover of p21 is regulated by the proteasome complex of proteases (Blagosklonny *et al.* 1996), the initial cleavage to p15 may expose sites that facilitate its ubiquitination and subsequent targeting to the proteasome. Loss of p21 leads to a dramatic induction of cdk2 activity which may result in premature entry of the cells into S-phase and apoptotic cell death, as occurs in some other forms of apoptosis (King & Cidlowski 1998). This may, in part, explain why *in vivo* Zn deficiency preferentially increases apoptotic cells in tissues where there is rapid cell turnover.

Secondly, do those cells in the body which are rich in exchangeable Zn (e.g., mast cells, pancreatic islet cells, ejaculated spermatozoa, certain hippocampal neurons and AEC) have increased resistance to apoptosis (Frederickson 1989; Zalewski *et al.* 1994a; Zalewski *et al.* 1996; Truong-Tran *et al.* 2000a)? By virtue of their specialized functions, these cells may be more exposed to ROS or other noxious agents and therefore need extra Zn for protection. The answer remains unknown but our laboratory is currently exploring this question using cell types with different concentrations of labile Zn. Only when the cellular biology of labile Zn is better understood will the full implications of Zn-related apoptosis become apparent. Thirdly, which of the subcellular pools of Zn participate in the suppression of apoptosis? Zn has diverse functions in cells which extend from structural and/or catalytic roles within metalloenzymes and Zn finger proteins to transient interactions with cellular signaling pathways (Vallee & Falchuk 1993). These functions can be sub-divided into those dependent on a largely fixed pool of cellular Zn which is poorly exchangeable (e.g., stoichiometric amounts of Zn that are tightly bound within the tertiary protein structure of metalloenzymes) and the more dynamic, labile Zn pools which are either loosely bound or tightly bound but kinetically labile (Frederickson 1989; Vallee & Falchuk 1993; Zalewski *et al.* 1993). These labile pools are sufficiently accessible and as a result are able to be visualized by specific Zn fluorophores (Zalewski *et al.* 1993) and by electron microscopy with the Neo-Timm's stain (Frederickson 1989). Since apoptosis is readily influenced by Zn deprivation or supplementation, it is likely that the more labile pools of Zn are involved in this regulation. Studies with mouse thymocytes and human chronic lymphocytic leukaemia cells revealed a strong inverse correlation between the level of intracellular labile Zn (increased or decreased by treatment with Zn ionophore or chelator, respectively) and the extent of apoptotic DNA fragmentation. Relatively small changes in labile Zn were able to cause large changes in susceptibility of cells to apoptosis and a threshold concentration in intracellular Zn may exist, below which apoptosis is induced (Zalewski *et al.* 1993).

Further clues to the identity of the key targets of Zn may come from a better understanding of the local interactions between Zn and apoptotic regulators in discrete subcellular pools. In addition to interactions between Zn and caspases, there may be distinct apoptosis-regulating functions of other cellular pools of Zn including intranuclear pools (Longin *et al.* 1997), microtubular and other cytoskeletal pools (Hes-

keth 1982; Zalewski *et al.* 1990), mitochondrial Zn (Untergasser *et al.* 2000), intravesicular Zn (Zalewski *et al.* 1993; Palmiter *et al.* 1996) and Zn within the cell membranes (Bettger & O'Dell 1981).

It is not clear whether intravesicular pools of Zn mediate protection against oxyradicals and interact with caspases and other molecular targets within the apoptosis pathway or whether this Zn is simply in the process of being transported to such sites. Vesicular traffic of molecules originating from the post-Golgi and endosomal sorting compartments is thought to be directed to their final destinations in the apical or basolateral domains and retained in place by microtubules and the intermediate filaments (Salas 1999). In this context, investigations performed by our laboratory using AEC may be relevant to studies concerning the importance of labile intracellular pools of Zn and its role in apoptosis.

Co-localization of mitochondria, lipid peroxides, Zn and procaspase-3 in the apical region of AEC

In our laboratory, we have utilized AEC as models to study Zn-regulated, oxidant-triggered, apoptosis (Truong-Tran *et al.* 2000a). AEC have a well-differentiated structure that is especially advantageous for study of the subcellular localization of the regulatory molecules and factors that govern apoptosis. They are polarized, with an apical plasma membrane that contains numerous beating cilia anchored to basal bodies (Figure 3A); the apical membrane faces the tracheobronchial airway lumen, *in vivo*, and the cilia function by propelling foreign particles, trapped in the extracellular mucin, back up the airways. To provide energy for cilial beating, AEC contain abundant mitochondria in the apical cytoplasm immediately below the basal bodies (Mills *et al.* 1999). Of clinical importance, AEC become fragile and easily damaged in asthma (Laitinen & Laitinen 1994), a disease with an underlying hypozincaemia (Di Toro *et al.* 1987; el-Kholy *et al.* 1990; Kadrabova *et al.* 1996). Although it is still controversial as to whether this hypozincaemia is a reflection of true Zn deficiency or rather a secondary response to the airway inflammation, an understanding of the mechanisms by which Zn protects these cells from apoptosis may have implications for the pathogenesis of a disease which affects 10% of adults and 25% of children in certain regions of the world (Adams *et al.* 1997).

The sub-cellular distribution of Zn was studied in AEC, using Zinquin, which detects the more labile pools of cellular Zn (Zalewski *et al.* 1993; 1994a, b; 1996). Labile Zn was found to be highly concentrated in the apical cytoplasm, both around the mitochondria and within the microtubular-derived basal bodies and cilia of sheep, pig and human AEC (Figure 3B) (Truong-Tran *et al.* 2000a). A similar pattern of Zn distribution was observed when cryostat sections of sheep or pig trachea and bronchi were labelled with Zinquin (Truong-Tran *et al.* 2000a). The cytoplasmic Zn was especially concentrated in perinuclear and apically-distributed vesicles as well as in basal bodies and cilia. Zn may be acquired from capillaries at the basal membranes of these cells, then packaged into vesicles and transported to the apical cytoplasm where at least some of it is incorporated into the microtubules of basal bodies and cilia (Truong-Tran *et al.* 2000a). Other Zn may be disposed around the mitochondria.

This same apical region was also rich in pro-caspase-3 as shown by immunocytochemistry (Figure 3C,D), using antibody to the precursor form of human caspase-3 (Krajewska *et al.* 1997). This suggests that pro-caspase-3 is strategically placed to trigger apoptosis should mitochondrial- or lumenal-derived ROS or other oxidants overwhelm the local defences. By immunocytochemistry with antibody to hydroxynonenal, there was some lipid peroxidation in the cell membranes, even in freshly isolated AEC from sheep trachea (Figure 3E). This was particularly evident in the apical membranes. Addition of exogenous H_2O_2 greatly increased lipid peroxidation throughout the cell.

In support of the hypothesis that the apical Zn is protecting the cells against ROS and caspase-3 activation, we found that depletion of Zn by treatment of AEC with TPEN resulted in increased lipid peroxidation (Figure 3F) and rapid activation of caspase-3 and morphological changes of apoptosis (Truong-Tran *et al.* 2000a). Interestingly, onset of caspase-3 activation followed a lag period of about 60 min after decline in intracellular Zn. It is essential now to understand the factors that govern the subcellular distribution of this family of enzymes. The precursor forms of caspases appear to exist in high molecular weight, multi-protein complexes (Cain *et al.* 1999), and may be anchored to components of the mitochondrial membranes and microtubules. Two recent studies (Zhivotovsky *et al.* 1999; Krebs *et al.* 2000) have also reported the localization of this caspase in mitochondria and/or associated membranes. Dual-labelling electron microscopy of AEC and other types of cells using Neo-Timm's stain for labile Zn and gold-

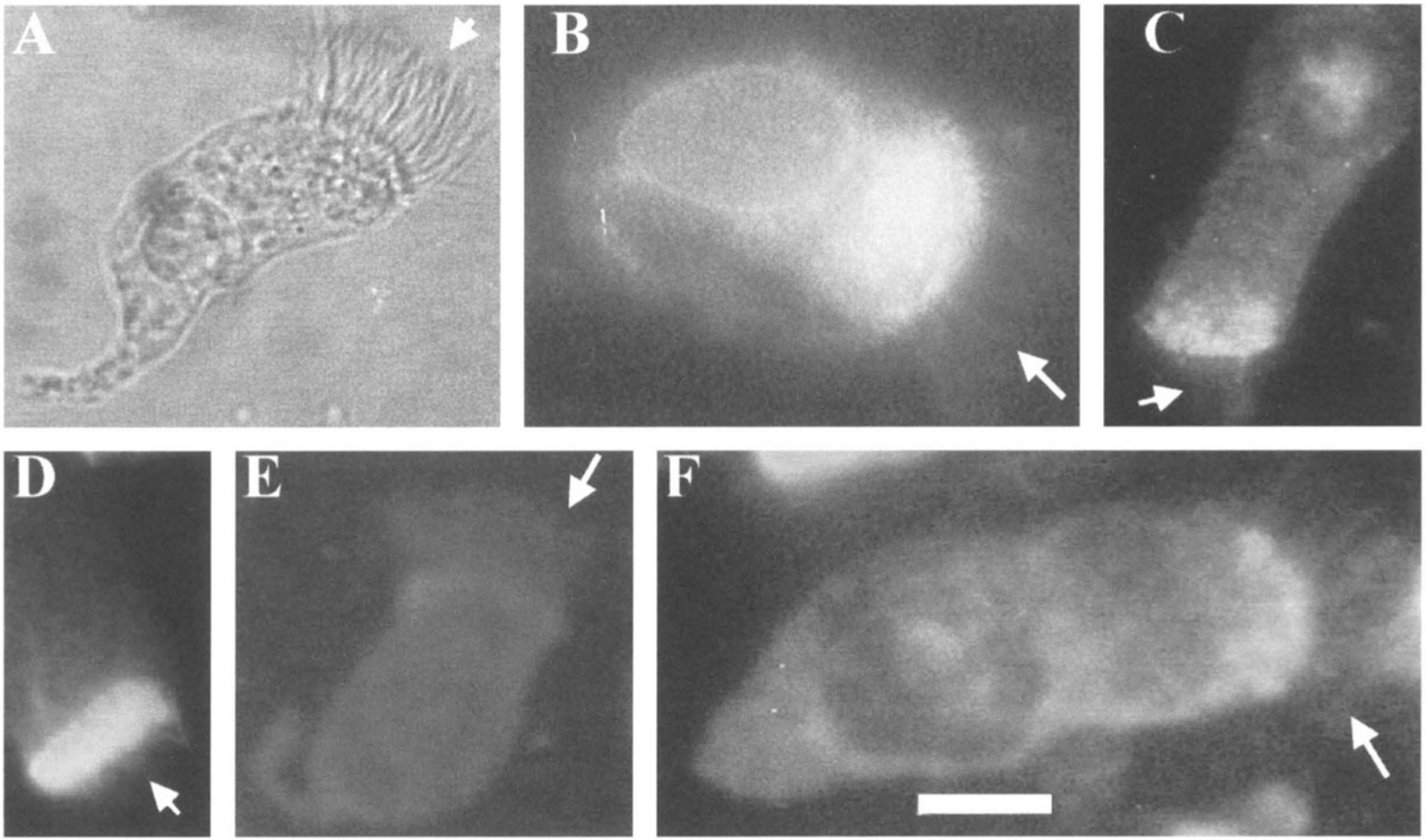

Fig. 3. Subcellular distribution of Zn, pro-caspase-3 and lipid peroxide in airway epithelial cells. Figure shows the co-localization of Zn, pro-caspase-3 and lipid peroxide HNE in the apical cytoplasm of AEC. A: Morphology of sheep AEC showing the columnar shape with distinct cilia at the apical membrane and the basally-located nucleus. B: Labile Zn concentrated in the apical cytoplasm and within the microtubular basal bodies of the cilia in a typical sheep AEC. Note also the perinuclear distribution of Zn. Zn was detected using 25 μM Zinquin and UV fluorescence microscopy. C: Pro-caspase-3 distribution in TPEN-treated sheep AEC showing predominate apical localization with some staining also in the nucleus. Cells were treated with 25 μM TPEN for 90 min and pro-caspase-3 was detected using anti-rabbit directed towards human pro-caspase-3 and visualized with goat anti-rabbit Ig FITC conjugated secondary antibody. D: Pro-caspase-3 distribution in untreated human AEC showing intense fluorescence at the apical region below the cilia. Same method of detection was used as in C. E: Low levels of lipid peroxide HNE in the apical membranes of untreated sheep AEC. HNE was detected using rabbit-anti human HNE and visualized with goat anti-rabbit Ig FITC conjugated secondary antibody. F: Increased levels of HNE especially in the apical region of TPEN-treated sheep AEC. Same method of detection was used as in E. Scale bar indicates 10 μm in panels A, C, D and E and 6 μm in panels B and F.

conjugated antibodies to detect caspases may provide important new information on the regulation of these enzymes by Zn.

Changes in Zn homeostasis during apoptosis

When considering the functions of anti-apoptotic regulators it is pertinent to ask what happens to their levels or distribution when cells do undergo apoptosis. In the case of Bcl-2, it appears to be down-regulated, probably by caspase-dependent cleavage (Fujita *et al.* 1998). Similarly, there may be a decline in intracellular Zn since several apoptosis-inducing agents cause a decrease in total intracellular Zn prior to induction of apoptosis (Treves *et al.* 1994). Somewhat paradoxically, the levels of intracellular labile Zn may rise dramatically during apoptosis as shown by an intense reaction with Zinquin (Zalewski *et al.* 1994b). We have proposed that the new pools of Zinquin-reactive Zn arise as a result of a change in the redox state of the cell which releases Zn bound to protein sulfhydryls when cells are in the later stages of apoptosis (Zalewski *et al.* 1994b). Since Zn is a structural building block in cells (Vallee & Falchuk 1993), the dismantling of cellular microtubules, chromatin and other components during apoptosis is likely to liberate large amounts of Zn which may be recycled or excreted from the body.

Conclusions

Life has evolved around metals like Zn and so apparently has the regulation of cellular death processes. We now need to understand the relationship between the physiological and pathological changes in altered Zn homeostasis and their control of apoptosis. There is relatively little knowledge on the differences in content and distribution of pools of Zn in different cells, tissues and organs at different stages of development, in different metabolic states and in local or systemic disease. It is essential that investigations are now focused on the cellular homeostasis of Zn in the normal and diseased states and how this directly or indirectly impacts on the pathways governing apoptosis.

This review has attempted to integrate the available information concerning Zn physiology and its involvement in regulating apoptosis. The advent of new technologies, such as the visualization of labile pools of Zn by Zn fluorophores, will enable new insights into the mechanisms by which Zn exerts these anti-apoptotic effects.

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