Zinc and Health: Current Status and Future Directions

Cellular Zinc Fluxes and the Regulation of Apoptosis/Gene-Directed Cell Death

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ABSTRACT The maintenance of discrete subcellular pools of zinc (Zn) is critical for the functional and structural integrity of cells. Among the important biological processes influenced by Zn is apoptosis, a process that is important in cellular homeostasis (an important cellular homeostatic process). It has also been identified as a major mechanism contributing to cell death in response to toxins and in disease, offering hope that novel therapies that target apoptotic pathways may be developed. Because Zn levels in the body can be increased in a relatively nontoxic manner, it may be possible to prevent or ameliorate degenerative disorders that are associated with high rates of apoptotic cell death. This review begins with brief introductions that address, first, the cellular biology of Zn, especially the critical labile Zn pools, and, second, the phenomenon of apoptosis. We then review the evidence relating Zn to apoptosis and address three major hypotheses: (1) that a specific pool or pools of intracellular labile Zn regulates apoptosis; (2) that systemic changes in Zn levels in the body, due to dietary factors, altered physiological states or disease, can influence cell susceptibility to apoptosis, and (3) that this altered susceptibility to apoptosis contributes to pathophysiological changes in the body. Other key issues are the identity of the molecular targets of Zn in the apoptotic cascade, the types of cells and tissues most susceptible to Zn-regulated apoptosis, the role of Zn as a coordinate regulator of mitosis and apoptosis and the apparent release of tightly bound intracellular pools of Zn during the later stages of apoptosis. This review concludes with a section highlighting areas of priority for future studies.

Key Words: • zinc • apoptosis • caspase • disease • Zinquin

The maintenance of discrete subcellular pools of zinc (Zn) is critical for the functional and structural integrity of cells and contributes to a number of important biological processes, including gene expression, DNA synthesis, enzymatic catalysis, hormonal storage and release, neurotransmission, memory and the visual process (Vallee and Falchuk 1993). Another fundamental cellular process that may be regulated by Zn is the cell suicide process of apoptosis (also known as gene-directed cell death or, less correctly, as programmed cell death) (reviewed in Wyllie 1997) (for detailed reviews on the earlier view of apoptosis and the role of Zn in apoptosis, see Zalewski et al. 1996 and Zalewski and Forbes 1993). This review focuses on more recent studies and attempts to relate Zn to the current concepts of the regulation of apoptosis. 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.

Any consideration of the cellular biology of Zn and its role in the regulation of apoptosis and other growth processes must recognize, by necessity, the diverse functions of this metal, which extend from highly stable structural roles within folded protein domains to transient interactions within cellular signaling pathways. These diverse functions can be classified broadly into those dependent on a largely fixed pool of cellular Zn (e.g., stoichiometric amounts of Zn that are tightly bound within the tertiary protein structure of metalloenzymes and poorly exchangeable) and the more dynamic, labile Zn pools that are subject to ionic fluxes and readily influenced by Zn deprivation or supplementation (Frederickson 1989, Vallee and Falchuk 1993, Zalewski et al. 1993). It is this labile pool that we focus on in the context of the regulation of apoptosis. To visualize and quantify this pool, we used a sulfonamidoquinoline-based UV-excitable Zn fluorophore, Zinquin. Studies with Zinquin, and the related fluorophore TS-Q, have revealed relatively slow (time scale of hours) Zn fluxes associated with a number of physiological and pathological processes, including gene expression, secretion, mitosis, apoptosis, spermatogenesis, fertilization, early embryonic development and inflammation (Frederickson 1989, Zalewski et al. 1993, 1994a, 1994b and 1996).
Apoptosis (gene-directed cell death)

Apoptosis is a regulated biological mechanism required for the removal and deletion of superfluous, mutant or moderately damaged cells. In most healthy adult mammalian tissues, it occurs at a low rate, thereby complementing mitosis in a steady state of kinetics to determine tissue and organ size and shape (Wyllie 1997). Apoptosis is prominent during embryonic and fetal development and in the immune response, among other processes, as well as being a target for growth factors, hormones and cytokines; in addition, it is a major mechanism of cell death in the body in response to toxic agents (e.g., x-irradiation), and its dysregulation (insufficient or excessive) is central to pathogenic mechanisms in many diseases (e.g., neurodegenerative disorders, acquired immune deficiency syndrome, autoimmune disease and malignancy) (Cohen and Duke 1993, Kerr et al. 1987, Wyllie 1997). As such, apoptosis has become a novel therapeutic target in medicine, and the factors regulating its induction and execution are being intensively studied.

Apoptosis occurs in two phases, consisting of (1) the biochemical signaling pathways that commit a cell to apoptosis and (2) the executional phase characterized by stereotypical morphological changes leading to cell death. Apoptosis results from the interaction between the initiating stimuli, which can be either physiological or injurious to the cell, and the factors determining the susceptibility of the cell to apoptosis. The input signaling pathways are diverse and may originate from plasma membrane receptors, newly transcribed gene products or disturbance of the microtubular cytoskeleton (Wyllie 1997). These converge onto a central pathway masterminded by the caspases and regulated by the proapoptotic Bax-like and antiapoptotic Bcl-2–like family of mitochondrial membrane proteins (Thornberry and Lazebnik 1998, Wyllie 1997). Members of the caspase family share two key features: they require an aspartic acid at the cleavage site, and they are all synthesized as proenzymes, which are activated by cleavage by other caspases, resulting in a cascade of proteolytic events (Thornberry and Lazebnik 1998). The 14 members of the caspase family are subdivided into the executioner caspases, which cleave particular substrates to commit the cell to irreversible cell death, and the initiator caspases, which act upstream to relay apoptosis-inducing signals by proteolytically activating proenzyme forms of the executioner caspases. The best studied of the executioner caspases is caspase-3, which cleaves proteins containing the consensus motif DXXD (where X is any amino acid). Substrates include the cell cycle regulator p21Waf1/Cip1 (Levkau et al. 1998) and the Ca/Mg-dependent endonuclease, caspase-activated deoxyribonuclease, leading to activation of a Ca/Mg-dependent endonuclease that cuts DNA into nucleosomal fragments, recognized as a “DNA ladder” on gels (Janicke et al. 1998). The other major executioner caspase is caspase-6, which cleaves the nuclear lamin scaffold proteins, resulting in collapse of the nucleus as well as participating in the proteolytic activation of caspase-3 (Srinivasula et al. 1996, Takahashi et al. 1996).

Cells entering death via apoptosis undergo a distinct set of structural changes that are consistent throughout all cell types (Kerr et al. 1987). These include the separation of the dying cell from its neighbors, loss of microvilli, blebbing of the membrane, condensation of cytoplasm, increased cell density and the compaction and segregation of the nuclear chromatin to form dense masses underlying the nuclear membrane. Condensation of the chromatin commences around the periphery of the nucleus and later involves most of the nucleus. This is followed by nuclear fragmentation and the budding of the cell to produce membrane-bound apoptotic bodies that are shed into luminal cavities or phagocytosed. The formation of apoptotic bodies keeps the dying cell out of direct contact of the remaining healthy tissue. This contrasts to the situation in necrosis, where cell death and lysis follow physical, chemical or osmotic damage, liberating potentially toxic cytoplasmic enzymes onto the dying cell’s neighbors as well as chemotactic factors that may initiate a local inflammatory reaction (Kerr et al. 1987).

Relationship of intracellular labile zinc to apoptosis

This section considers the evidence relating Zn deprivation and supplementation, intracellular labile Zn and susceptibility of cells to undergo apoptosis both in vitro and in vivo.

Zinc deprivation. Localized or systemic tissue Zn deficiency can be induced by malnutrition or the administration of metal chelators. It can also arise in a number of pathophysiological states, including malabsorption, increased Zn requirement (e.g., in pregnancy, lactation and severe burns), increased Zn loss (e.g., zincuria, sweating), heavy metal poisoning in which Zn is displaced by the metal, aging and disease (e.g., diabetes mellitus and Down’s syndrome) (Solomons 1988, Valle and Falchuk 1993). Although systematic studies of apoptosis in Zn-deficient animals are still lacking, it is clear that the frequency of apoptotic cells is increased markedly in certain tissues and organs, including the intestinal and retinal pigment epithelium, skin, thymic lymphocytes, testis and pancreatic acinar cells (reviewed in Duvall and Wyllie 1986, Sunderman 1995, Zalewski and Forbes 1993). Apoptosis is also markedly increased in conjunction with congenital abnormalities in fetal rats borne by Zn-deficient dams. This was emphasized in the neouroepithelium, where excessive apoptosis interfered with neural tube closure (Record et al. 1985). Rogers et al. (1995) concluded that apoptotic embryonal cell death, particularly in the neural crest cells, could arise within 4 d of maternal Zn deficiency. To what extent does increased apoptosis also occur in Zn-deficient humans? The answer to this question is not yet known, largely because of limited access to tissues for analysis. One approach would be to study the susceptibility of neutrophils to apoptosis in vivo because it is known that neutrophil Zn levels decline in even mildly Zn-deficient humans (Pat and Prasad 1988, Prasad et al. 1993). Interestingly, peripheral leukocytes of patients with Down’s syndrome, a disease associated with Zn deficiency, had a greatly increased frequency of cells with DNA nicks, which is thought to be an early stage of apoptosis; these cells were substantially decreased after Zn supplementation for 6 mo (Antonacci et al. 1997). Unclear, however, is whether the appearance of these cells was simply the result of reduced DNA repair rather than the promotion of apoptosis.

Increased apoptosis in vivo may be a direct consequence of a decrease in intracellular Zn concentration ([Zn$^{2+}$]) or indirectly via some other change. This issue has received some attention in the context of the involution of the thymus in Zn-deficient animals, where it was concluded that at least in part, the increased apoptosis of thymocytes was due to excessive levels of circulating glucocorticoids triggered by a Zn deficiency–associated stress response (Fraker and Telford 1997). However, the evidence from in vitro studies indicates that apoptosis can result directly from a decline in intracellular Zn within the same cells. Numerous in vitro studies have shown a direct stimulatory effect on apoptosis in cells whose levels of Zn have been depleted. Thus, apoptosis was induced in various types of cells when cultured in a Zn-free medium.
Intracellular labile Zn was decreased by treatment with a chelator or increased by treatment with an ionophore, there was good correlation between the content of labile Zn and the inhibition of DNA fragmentation (Zalewski et al. 1993), suggesting that a reduction below a threshold concentration in [Zn^{2+}], induces apoptosis. The steepness of the threshold curve indicates that relatively small changes in labile Zn can cause large changes in the susceptibility of cells to DNA fragmentation.

However, there are still some major reservations with this model. First, there remain concerns about the interpretation of the in vitro Zn supplementation studies, especially where excessively high Zn concentrations have been used. Unfortunately, this includes most published studies. Apoptosis is an active, energy-dependent process, and it will be blocked by excessive cell damage. Fraker and Telford (1997) have shown that even 100 μmol/L Zn is toxic for mouse thymocytes, although surprisingly the death was by apoptosis rather than by necrosis. We have preferred to limit extracellular Zn to 25 μmol/L and to instead use the ionophore pyrithione to transport Zn into the cells (Zalewski et al. 1993). However, even here, at higher concentrations of pyrithione resulting in excessive Zn overload, there is blebbing of the cells and detachment from the substratum. The second reservation is that even though an increase in [Zn^{2+}], specifically suppresses 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 been reviewed recently (Fraker and Telford 1997). An often-cited study is that of Barbeiri et al. (1992), who reported that Zn blocked dexamethasone-induced DNA fragmentation in thymocytes but did not prevent cell death. However, they used such an extremely high concentration of Zn (11.3 mmol/L) of Zn that it is scarcely surprising that the cells died.

There are two separate issues here. First, by suppressing apoptosis, Zn may simply divert cells into necrosis. This is not necessarily a trivial concern because necrosis of the cells will lead to a greater inflammatory response than if the cells were to undergo apoptosis and be rapidly cleared. Second, the complete apoptotic program may be regulated by multiple factors (see later). Full cytoprotection may require all of these in addition to Zn.

**Why zinc?** In considering the mechanisms by which Zn regulates apoptosis, it is pertinent to ask why Zn and not other metals? It has been argued that Zn has assumed, during evolution, a special role in mediating events associated with necrosis. It protects macromolecules (e.g., proteins and DNA) from oxidation and proteolysis, and stabilizes macromolecular complexes (e.g., microtubules) and subcellular organelles (e.g., membranes) (Vallee and Falchuk 1993). Its affinity for sulfydryl groups coupled with lack of redox activity enables it to reversibly suppress cysteine-dependent enzymes (including perhaps one or more of the caspases) without irreversible damage or inactivation.

Notwithstanding this, related metals in the periodic table may also play a role because in vivo depletion of Cu in rats led to a marked increase in apoptosis of the acinar cells (Kishimoto et al. 1994). On the other hand, Fraker and Telford (1997) found that supplementation of Cu (and Ni) at varying concentrations up to 500 μmol/L did not mimic Zn in the suppression of glucocorticoid inhibition of thymocyte apoptosis. Other metals may antagonize the role of Zn. Although its precise mechanism of action remains unclear, Ca is known to activate some proapoptotic enzymes (e.g., endonucleases, proteases and phospholipases) (McConkey & Orrenius, 1997),...
whereas both Cd and Au induce apoptosis (and necrosis) (Fraker and Telford 1997).

**Changes in intracellular zinc early and late in apoptosis.**

Assuming that Zn is a physiological regulator of apoptosis, it is pertinent to ask what happens to [Zn\(^{2+}\)], in cells that are induced to undergo apoptosis? Initially, there may be a decline in [Zn\(^{2+}\)]. Several apoptosis-inducing agents cause a decrease in [Zn\(^{2+}\)], (e.g., intracellular Zn was decreased before the induction of apoptosis in lymphoid cells with dexamethasone or ATP; Treves et al. 1994). However, during the process of apoptosis, [Zn\(^{2+}\)], rises as shown by an intense reaction with Zinquin (Zalewski et al. 1994b). This did not occur in necrosis. 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 that releases Zn bound to protein via Zn—S thiolate bonds (Zalewski et al. 1994b). Because apoptosis is a process by which the dying cell dismantles itself and because Zn is a structural building block in many cellular components (e.g., membrane, cytoskeleton and chromatin) (Vallee and Falchuk 1993), a release of Zn during apoptosis is perhaps not surprising. After phagocytosis, Zn may be recycled or excreted from the body. Whether the release is simply an effect of apoptosis or further accelerates the process (e.g., by destabilization of the microtubules and other structures, thereby facilitating action of caspases and endonucleases) is not known. Alternatively, the increase in fluorescence may result from an influx of extracellular Zn due to the loss of membrane integrity or decreased membrane efflux (as discussed in Cuajungco and Lees 1997). Mechanisms aside, Zn fluorophores may prove to be useful reagents in screening for apoptotic cells, particularly in tissues where the frequency of these cells is very low.

**Implications for conditions of altered zinc homeostasis**

One of the least understood but very important areas in Zn-related apoptosis is the relationship of apoptosis to physiological and pathological changes in altered Zn homeostasis. Why, for instance, does Zn deficiency primarily increase apoptosis in tissues undergoing rapid cell turnover (e.g., bone, thymus, epidermis, esophagus, testis, intestinal crypts and developing tissues of fetus)? Is it because the cells in these tissues are primed for apoptosis, or are cycling cells preferentially sensitive to Zn depletion? The relationship between the high basal cellular levels of labile Zn in pancreatic islet cells and their known resistance to apoptosis requires clarification (Zalewski et al. 1994a and references within). To what extent do manifestations of Zn deficiency (e.g., growth retardation, immunodeficiency, skin lesions, delayed sexual maturation and gastrointestinal problems) reflect an increase in apoptosis in the relevant tissues? Mori et al. (1996) proposed a role for increased apoptosis in the formation of vesicular skin lesions in patients with acquired zinc deficiency but not in the formation of hyperkeratotic skin lesions. To what extent increased apoptosis contributes to delayed wound healing in Zn deficiency is not known, but it may reflect changes in the rates of apoptosis in epithelial cells and fibroblasts. Certainly the atrophy of tissues in Zn-deficient animals is likely to be due not only to inhibition of cell proliferation but also to excessive apoptotic cell death, although there have been no systematic studies of this to date. If Zn is required to both promote mitosis (reviewed in Chesters 1989) and suppress apoptosis, it may serve to coordinate these two opposing growth-regulatory processes. Enhancement of mitosis and suppression of apoptosis occur in regenerating tissues, whereas tissue involution and atrophy result from the simultaneous suppression of mitosis and enhancement of apoptosis. Cellular Zn uptake is enhanced by growth factors, hormones and other agents that promote mitosis and suppress apoptosis (e.g., by epidermal growth factor in thymic epithelial cells; Coto et al. 1992). Because cells require both growth factors and Zn to pass the G1/S restriction point (a cell cycle decision point ~2–3 h before the onset of DNA synthesis), after which neither is required (Chester 1989), events close to this restriction point may be critical to the mechanism via which the two opposing growth regulatory processes are coordinated.

Does Zn play a functional role in development-associated programmed cell death? The only relevant study is that of Budzik et al. (1982), who showed that Zn chelation by EDTA mimicked millerian-inhibiting substance in causing apoptotic regression of the millerian duct in fetal organ cultures and that Zn was the only metal capable of blocking regression caused by both of these agents. The role of Zn in development requires further study.

If Zn is a physiological regulator of apoptosis, then there may be implications for degenerative changes in aging where decreased uptake and utilization of Zn by cells could contribute to the increased susceptibility of senescent cells to undergo apoptosis. In aging, Zn is decreased in the plasma, leukocytes, bone, epidermis, testis and kidney, associated with reduced activities of Zn-dependent enzymes and hormones as well as depression of Zn-dependent functions, including immunodeficiency, wound healing, smell and taste acuity (see Prasad et al. 1993 and references within). Is increased apoptosis in a Zn-deficient setting a factor in the heightened susceptibility of T-cells to antigens in aged animals to apoptosis (Aggarwal and Gupta 1998) or in the excessive apoptosis accompanying neurodegeneration in Alzheimer’s disease (Anderson et al. 1996)? In diseases complicated by secondary Zn deficiency, is there excessive apoptosis? For example, in diabetes mellitus, does the generalized cell death accompanying depletion of intracellular Zn (Pai and Prasad 1988) also affect the β-cells and render them more susceptible to apoptosis induced by autoimmune cytotoxic T-cells or by chemical diabetogens? This may be relevant to the mechanism by which Zn chelators cause permanent diabetes in experimental animals (Goldberg et al. 1990).

Finally, is labile Zn increased in some tumors, and if so, is this a factor in their relative resistance to apoptosis and accelerated growth? There have been relatively few reported studies of Zn in tumors and very little study of the relationship between level of Zn and tumor growth. In the sporadic cases reported so far, less Zn was observed in some tumors, but more was observed in others. For example, levels of Zn were reported to be increased 75% in rat colon tumors relative to the adjacent normal tissue (Song et al. 1993). However, cancer of the prostate contained less Zn than normal prostatic tissue (Byar 1974). Zn supplementation and Zn deficiency can have both inhibitory and augmenting effects on tumor formation, depending on the tumor and host species (Kasprzak and Waalkes 1986). Some tumor cells and transformed cell lines have acquired a greatly decreased dependency on extracellular Zn for growth compared with the normal primary cell cultures (e.g., kidney cells) (Chesters 1989).

**Biochemical mechanisms of zinc in apoptosis**

Identification of the biochemical mechanisms of action of Zn should throw further light on the possible physiological role of Zn in the control of apoptosis. The first part of this section describes the action of Zn on putative molecular targets, whereas the second part considers which of the subcellular pools of labile Zn may be most important.

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**SUPPLEMENT**

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Molecular targets of zinc. Initial interest focused on the Zn-mediated suppression of Ca/Mg-dependent endonuclease, which causes apoptotic DNA fragmentation by cutting linker regions between nucleosomes generating a ladder pattern (Cohen and Duke 1984). In studies from an unrelated laboratory, Cohen et al. (1992) observed that Zn blocked the transition from the morphological stage in which the chromatin is condensed around the periphery to that stage in which most of the nucleus is involved, and they also attributed this to specific inhibition of the Ca/Mg-dependent endonuclease. It has been proposed that the exchange of Zn for Ca within the nucleus may initiate the DNA fragmentation (Duvall and Wyllie 1986). However, there remain no studies of whether physiological concentrations of Zn influence the endonuclease and whether it is directly activated in Zn deficiency.

The possibility that Zn may suppress a step before activation of the endonuclease was first shown by Lazebnik et al. (1993). Using a cell-free model in which cytosol from cells primed to undergo apoptosis induces nuclear condensation and DNA fragmentation, they showed that morphological changes in the nuclei were suppressed by concentrations of Zn lower than those required to suppress the fragmentation of DNA and that the target was cytoplasmic rather than nuclear. Because the active cytosolic factor was subsequently identified as the protease CPP-32 (renamed caspase-3), it was suggested that Zn either may block the mechanism by which the inactive procaspase-3 is processed and thereby activated or may block the capacity of active caspase-3 to cleave its cellular substrates. Figure 1 shows that in colon cancers induced to undergo apoptosis by the addition of the histone deacetylase inhibitor butyrate, supplementation with Zn (using the Zn ionophore pyrithione plus 25 μmol/L ZnSO₄) suppressed a step before the activation of caspase-3.

At least two recent studies have shown that although Zn inhibits all of the different caspases that have been tested (e.g., caspase-3 at millimolar concentrations of Zn; Perry et al. 1997), at lower, more physiological concentrations, it is a selective inhibitor of caspase-6 (also known as Mch-2); complete inhibition was observed at 10 μmol/L Zn (Stennicke and Salvesen 1997, Takahashi et al. 1996). There is as yet no physiochemical explanation for the selective effects of Zn on this enzyme. Caspase-6 is, to date, the most sensitive apoptosis-related molecular target of Zn, although it remains to be determined whether this enzyme is influenced by normal concentrations of Zn present within its subcellular compartment.

Because caspase-6 is responsible for the cleavage of lamins and therefore is directly involved in the nuclear membrane dissolution (Srinivasula et al. 1996, Takahashi et al. 1996), Zn should retard these events, although this has not yet been directly tested. In addition, caspase-6 is known to cleave and activate the proenzyme form of caspase-3 (Srinivasula et al. 1996). Therefore, Zn should also retard caspase-3 activation.

We tested the latter using a cell-free system in which the addition of cytochrome c to the cytosol of healthy cells triggers the proteolytic conversion of procaspase-3 to the active enzyme. The addition of 800 nmol/L free Zn blocked the activation of caspase-3 by 50% in this model; there was no effect of Zn when added 90 min after cytochrome c but before the addition of fluorogenic caspase-3 substrate, confirming that Zn blocks the process of caspase-3 activation rather than the already activated enzyme (Fig. 1, inset). This was also shown by Auchi et al. (1998) with the use of Western blotting to track the caspase-processing. However, the role of caspase-6 in this model is not known.

Another highly conserved family of cellular proteins that regulate a common pathway of apoptosis are the antiapoptotic Bcl-2-like and proapoptotic Bax-like mitochondrial membrane proteins. The ratio of Bcl-2-like to Bax-like proteins acts as a cellular rheostat to determine in part survival or death of cells after an apoptotic stimulus (Korsmeyer et al. 1993). In a recent report, Fukamachi et al. (1998) showed that Zn supplementation of cells in vitro increased the Bcl-2/Bax ratio thereby increasing the resistance of the cells to apoptosis.

Other potential targets for Zn are the microtubular cytoskeleton, which is disrupted in both Zn deficiency (Nickolson and Veldstra, 1972) and apoptosis (Martin and Cotter 1990), oxidative stress (a central mechanism in the induction of apoptosis that may be influenced by the antioxidant properties of Zn) (Vallee and Falchuk 1993) and, specifically, glucocorticoid-induced apoptosis, suppression of glucocorticoid binding to its cytoplasmic receptors (Fraker and Telford 1997).

Still unclear is whether each of these candidate molecular targets of Zn reflects physiological, pharmacological or toxicological actions. A criticism with many of the studies to date is that only the effects of Zn supplementation (not depletion) were tested and that very high (supraphysiological concentrations of Zn) were present in the extracellular medium. The activity of caspase-6 and mechanisms involved in the activation of caspase-3 may be exceptions because they were suppressed by much lower concentrations of Zn. The effects of Zn chelators at the molecular level have been largely neglected. In fact, it remains uncertain whether Zn deprivation and Zn supplementation are affecting the same step in the cascade.

What are critical intracellular pools? Which of the subcellular pools of Zn participate in the suppression of apoptosis? Potential targets may exist in the nucleus (Ca/Mg endonuclease, gene promoters), cytosol (caspase-6, metallothionein, oxyradicals, Ca ions), cytoskeleton (microtubules) and mitochondria (Bcl-2/Bax). Multiple subcellular pools are probable because in cell-free models of apoptosis, Zn inhibits apoptosis regardless of whether it is added to isolated nuclei (Cohen and Duke 1984) or to cytosol (Lazebnik et al. 1993). When intracellular pools of Zn were increased by Zn ionophore under...
levels in the body, due to dietary factors, altered physiological states or disease, can sufficiently alter labile intracellular Zn to change cell susceptibility to apoptosis, and (3) that this altered susceptibility to apoptosis contributes to changes in the body. These hypotheses at best remain tenuous at this stage, and it is premature to conclude that Zn is a physiological regulator of apoptosis.

It may be instructive at this stage to compare Zn with the best known physiological suppressor of apoptosis Bcl-2 (and related members of the family). There are some interesting parallels between the two factors. 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. Both inhibitors appear to act on apoptosis at multiple overlapping sites. Thus, like Zn, Bcl-2 protects cells from oxidative stress (Korsmeyer et al. 1993) and directly suppresses caspase processing (Thornberry and Lazebnik 1998). Like severely Zn-deficient animals (Solomons 1988, Vallee and Falchuk 1993), bcl-2 knockout mice have stunted growth; immunodeficiency associated with greatly increased apoptosis within the thymus, sparing only the epithelial cells and macrophages; and hair hypopigmentation, thought to be due to an oxidation-related pathology (Korsmeyer et al. 1993). Finally, feedback mechanisms may ensue to release cells from suppression by both Zn and Bcl-2 once the cells enter the apoptotic pathway. In the case of Bcl-2–like proteins, they normally act to suppress the processing of caspases and restrict entry into apoptosis, but during apoptosis they become substrates for caspases (Fujita et al. 1998). This down-regulation may accelerate downstream events. Similarly, although Zn may normally suppress caspase activation in healthy cells, the dramatic changes in intracellular Zn homeostasis during apoptosis may relieve suppression and facilitate downstream events such as endonuclease activation. Whether Zn and Bcl-2 have overlapping, additive or synergistic functions in the control of apoptosis awaits determination.

The next problem will be in distinguishing the physiological, pharmacological and even toxicological effects of Zn. Characterization of the intracellular pool or pools of Zn that mediate suppression and a better understanding of how this pool or these pools interact with the apoptotic signaling and effector pathways are urgently needed. By analogy, the realization that Bcl-2 was largely a mitochondrial membrane protein led to the discovery of its role in cytochrome c release from mitochondria and subsequent activation of caspases. 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, identified to date, are as sensitive to Zn as caspase-6 is not known. Such studies, in association with site-directed mutagenesis of histidines and nonessential cysteines in caspase-6, should provide a better understanding of how Zn inhibits this enzyme. There also is a particular need to determine whether Zn deprivation in vitro and in vivo directly activates these suicide enzymes. It is not at all clear whether Zn supplementation and Zn deprivation affect the same targets in the apoptotic cascade, especially if there are multiple targets of Zn. Some targets may already be Zn saturated and therefore will be more influenced by Zn deficiency than by Zn supplementation. Well-defined cell-free models of caspase processing and apoptosis now exist (Thornberry and Lazebnik 1998) that can be used to study the role of Zn deprivation and supplementation under well-controlled conditions.

An area ripe for study is the influence of Zn supplementation in vivo on enhanced rates of apoptosis in primary and secondary tissues, and organ systems in pathophysiological states; and (2) that systemic changes in Zn conditions that suppress apoptosis, there was little increase in nuclear Zn, except for occasional intense labeling of nucleoli, but there was a strong increase in cytoplasmic fluorescence that appeared to be localized within membrane-enclosed vesicles (Fig. 2). This may indicate that the target or targets are primarily cytoplasmic (e.g., caspases). To what extent Zn interacts with the cytoplasmic pools of caspases and their precursors is not known, nor is it known whether the proenzyme and activated forms of the caspases are within or associated with Zn-rich vesicles and influenced by Zn supplementation or Zn deprivation.

Future priorities

Further understanding of the role of Zn in the regulation of apoptosis should benefit a number of areas: (1) in terms of understanding the control of apoptosis, with particular relevance to the coordinated growth and mechanism of action of growth factors; (2) in terms of whether changes in the rate of apoptosis due to changes in [Zn$^{2+}$], affect cells, tissues, organs and organ systems in pathophysiological states; and (3) regarding whether Zn can be administered therapeutically to prevent or ameliorate degenerative disorders associated with a high rate of apoptotic cell death.

A substantial body of evidence now implicates Zn in the regulation of apoptosis in vitro. This evidence includes studies of the effects of Zn deprivation or supplementation on either the spontaneous rate of apoptosis or the susceptibility of cells to the induction of apoptosis by other agents, as well as studies of the effects of Zn on various components of the apoptotic signaling pathway. We now need to obtain further evidence from a variety of models that address the following three hypotheses: (1) that a specific pool or pools of intracellular labile Zn regulate apoptosis; (2) that systemic changes in Zn
secondary Zn deficiency states. If Zn-related apoptosis is increased in Alzheimer's disease, late-onset diabetes mellitus and other diseases, there is a need for ongoing monitoring of intracellular Zn levels as we age. The priority should be to maintain healthy Zn levels during aging rather than to try and correct Zn deficiency after it has occurred. This is particularly important in the case of neuronal loss, where only limited regeneration is possible. In other situations, therapeutic manipulation of levels of intracellular labile Zn in vivo by supplementation where apoptosis is excessive (e.g., neurodegenerative disorders) or chelation where apoptosis is insufficient (e.g., autoimmunity and malignancy) may provide new therapeutic strategies. The issue will be how best to supplement the relevant intracellular pools in different affected tissues.

Another area that is very much underdeveloped is the cellular biology of Zn, in particular, the fluxes of labile Zn that either participate in or result from biological and pathological processes. There is relatively little knowledge of 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. Nor do we understand how these fluxes of Zn interact with the pathways of apoptosis. Much has yet to be learned about how cells handle and use Zn, concentrate it in organelles and insert it in Zn metalloproteins. How, for instance, do labile Zn-rich pancreatic islet cells differ in the Zn uptake, and how is Zn incorporated from relatively labile Zn-poor cells such as lymphocytes? After exocytosis, where and how is the secretory Zn reacquired? It is only when the cellular biology of labile Zn is better understood will the full implications of Zn-related apoptosis become apparent.

Fraker and Telford (1997) concluded their recent reappraisal of Zn and apoptosis with the comment, “Finally, the more important question may be whether so-called inhibitors of apoptosis actually provide cells with long term protection or only a very temporal blockade that is subsequently overridden.” Perhaps the issue that needs clarification is the extent to which Zn cooperates with Bcl-2 and other survival factors. We must be prepared to study the effect of small physiological Zn fluxes on the background of up-regulation of other apoptotic suppressors rather than to simply test the effect of high, potentially toxic concentrations of Zn in isolation.

LITERATURE CITED


