MINIREVIEW

A Reappraisal of the Role of Zinc in Life and Death Decisions of Cells (44132)

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Abstract. **There is a great deal of interest in chemicals and biochemicals that can modulate apoptosis. As will be discussed, zinc, an essential trace element, can induce as well as block apoptosis. High concentrations of extracellular zinc (500–1000 μ*M*) have frequently been used to block apoptosis or programmed cell death in a variety of systems. Early investigators provided evidence that this concentration of zinc could block DNA fragmentation that is often associated with apoptosis. Since zinc plays a role in many aspects of cell function, there are probably many sites in a death pathway that zinc could potentially modulate. In the case of glucocorticoid-mediated apoptotic death, new evidence presented herein indicates that high zinc can also block the binding of steroids to the glucocorticoid receptor thereby inhibiting the death signal itself. In this case, zinc probably binds to the vicinal cysteines in the receptor ligand binding site thereby blocking binding of glucocorticoid. Indeed, glucocorticoid-induced apoptosis in thymocytes has become one of the most frequently studied systems and is a focal point of this review. Studies herein will show that unlike zinc other trace-like metals such as nickel, copper, cadmium, and gold do not afford thymocytes protection against the DNA fragmentation induced by glucocorticoid-mediated cell death. Interestingly, in attempting to determine if lower or more physiological concentrations of zinc could provide protection against apoptosis, it was found that 80–200 μ*M* zinc could actually induce death in 40% of $CD4^+ CD8^+ \alpha\beta TCR^{lo} CD3^{lo}$ thymocytes. From these experiments one might have been optimistic that zinc could, indeed, be a modulator of cell death. However, this thought has been overshadowed by growing evidence that zinc does not provide long-term protection to so-called surviving cells.** [P.S.E.B.M. 1997, Vol 215]

Interest in apoptosis, or cell suicide, has grown enormously in the past few years, fostered by recognition that this form of programmed cell death plays a key role in a number of facets of immune function (1). For example, it is now evident that deleterious anti-self clones that could lead to autoimmunity are eliminated apoptotically as they arise (1). Likewise, the high proportion of nonsense clones produced by developing T and B cells that make nonproductive gene rearrangements of the T-cell receptor or immunoglobulin molecule appear to be eliminated by a suicide-like mechanism (1). The cytolytic responses of the immune system against target cells such as tumor cells or virally infected cells rely heavily on the induction of apoptosis in these cells (1). Phagocytic cells are also a key part of the cell death process since it is their job to recognize and clear dying cells. The large losses of T- and B-cell precursors subsequent to exposure to glucocorticoids or γ irradiation are clearly due to the induction of high levels of apoptosis in these cells (2). Indeed, glucocorticoid-induced apoptosis of mouse thymocytes has become a classical system for the study of apoptosis in the last decade (3).

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This work was supported by a grant from the National Institutes of Health (DK 52289).

0037-9727/97/2153-0229$10.50/0

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The list of diseases or conditions in which apoptosis plays a role has also grown substantially. Undesirable activation of apoptotic pathways occurs in AIDS, appearing to hasten the loss of helper T cells (4). In the case of autoimmune disease, much of the ongoing destruction of tissue is facilitated by apoptosis. Likewise, destruction of tissue in neurodegenerative diseases and ischemic strokes is accelerated by apoptosis (5). The efficacy with which cancers can be treated is clearly related to their susceptibility or resistance to apoptotic death. Indeed, some of the more potent chemotherapeutic agents act by inducing apoptosis in tumor cells (1). Growing evidence indicates that malnutrition can also induce apoptosis in certain cells and tissues, especially on precursor lymphocytes (6). Thus, learning to modulate apoptosis with enhancers or inhibitors has considerable clinical as well as scientific importance. For this reason, the potential of natural substances such as zinc to modulation of apoptosis is of interest and will be the topic of discussion herein.

Physical and Biochemical Changes in Immune Cells Undergoing Apoptosis

Cells of the immune system undergoing apoptosis have a characteristic set of changes almost regardless of the type of signal that initiated death. Indeed, the signals that can initiate programmed cell death or apoptosis are very diverse. In necrotic or accidental death, cells enlarge and lyse. In the case of apoptotic cell death, both the cell and its nucleus condense in size (1, 3). These morphological changes are sufficiently dramatic that they can be observed in an ordinary phase-contrast microscope. Higher optical resolution shows a dramatic change in the chromatin structure within the nucleus of the apoptotic cell. There are also marked changes in the cellular membrane that include ruffling or blebbing of the membrane, which at late stages develop into so-called apoptotic bodies (3). It is these morphological changes that pathologists consider the most reliable indicator of apoptosis. Yet, little information is available on the actual biochemical nature of these dramatic membrane changes. We know that there is expression of large amounts of phosphatidyl serine residues on the surface of the dying cell which are normally more internal to the cell (3). At some point, a variety of proteases are activated, including enzymes like ICE or interleukin-1–converting enzyme, serine proteases, calpain, etc. (7). In most but not all cases, a putative endogenous endonuclease is activated which digests the DNA at the nucleosomal linker regions resulting in the generation of "ladders of DNA," which are nucleotide fragments that are 200-bp multimers (3). It is this process that many found to be inhibitable by high zinc (8). The ability of inhibitors of translation and transcription to block apoptosis indicated that induction of an array of death genes was also integral to this type of cell death (3).

Advancement in Methods for Measuring Apoptosis

Throughout the 1980s most of the studies of apoptosis were limited to cell lines or tissues such as the thymus, which contains a fairly homogenous population of immature cells called thymocytes. This was primarily because the methodology for quantitating apoptosis was limited. For example, it was possible to count fields of cells, noting the proportion of cells with apoptotic morphology *via* use of phase-contrast microscopy or electron microscopy. This was cumbersome and labor intensive. More recently, cell biologists have used standard DNA gel electrophoresis to verify the presence of the DNA ladder in designated populations of cells presumed to have undergone apoptosis. An example of such a gel is given in Figure 1, where thymocytes were treated with and without corticosterone. As expected, the DNA of the untreated thymocytes remained at the top of the gel (Lane A), whereas multiple small DNA fragments are noted in the corticosterone-treated cells (Lane C). As mentioned, high zinc was able to block corticosterone-induced cell death (Lane B). This method is also time-consuming, requiring 3 or 4 days' effort, and is at best semiquantitative. Moreover, the source of the DNA fragments within the population under study cannot be determined by this method (Fig. 1). However, our lab has demonstrated that, by extending cell-cycle analysis using the flow cytometer, one can rapidly and quantitatively evaluate apoptosis using intact cells. The fixing-staining and analysis of the cells can be done in less than 2 hr (9–11). Furthermore, by incorporating fluorescently labeled phenotypic antibodies directed at unique membrane markers, it was also possible to compare and contrast the degree of apoptosis in

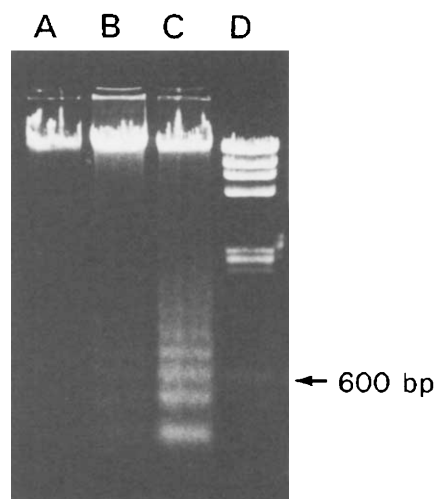

Figure 1. Thymocytes were incubated with or without 1 μ*M* corticosterone (CS) in the presence or absence of 700 μ*M* zinc sulfate for 8 hr. Cells were lysed and the DNA prepared and electrophoresed on 1.8% agarose gels and stained with ethidium bromide for detection of DNA fragmentation. Lane A, untreated thymocytes; Lane B, thymocytes treated with 1 μ*M* CS but also incubated with 700 μ*M* zinc; Lane C, thymocytes treated with CS alone; Lane D, molecular weight markers prepared by digesting λ DNA with *Hin*dIII.

different types of cells within a heterogenous population prepared from the blood, bone marrow, spleen, etc. (2). A sample of this technology is shown in Figure 2. The highly fragmented DNA of the apoptotic cell reduces their fluorescence so that they appear as a distinct peak separate from cells in G0/G1. The apoptotic peak increased in size with dose of glucocorticoid as shown here (9, 10). Furthermore, formation of this peak was blocked by standard inhibitors of cell death such as cycloheximide, actinomycin D, and high concentrations of zinc sulfate (9, 10). Care was taken to sort cells from the presumed apoptotic region and verify by end labeling that the DNA of cells in this region contained 180-bp multimers (10). In addition, the morphological analysis of the cells sorted from the hypodiploid or low fluorescence peak indicated that it contained predominantly apoptotic cells (>95%) (10). It should be pointed out that a variety of DNA dyes, such as propidium iodide, ethidium bromide, daunomycin, 7AAD, DAPI, etc., can be used with equally good results (11). Thus, one can select a DNA dye that does not interfere with the fluorochromes used for phenotypic analysis. This methodology greatly increased the systems, tissues, etc., that could be analyzed for apoptosis and made possible many of the studies reported herein.

Suppression of Apoptosis by High Concentrations of Extracellular Zinc

There is now a regiment of experiments used to verify that an apoptotic form of cell death has occurred. This includes employing a standard list of inhibitors known to block apoptosis as an indirect means of demonstrating that a death pathway was operating. The use of inhibitors of translation and transcription such as cycloheximide and actinomycin D that presumably block induction of death genes are used with the greatest frequency (1, 3). Aurintricarboxylic acid has been used on occasions since it supposedly inhibits endonuclease activity that causes the DNA (3, 9). Also, high on the list of effective inhibitors is the addition of extracellular zinc at concentrations of 500–1000 μM (1, 8, 9, 12). This is high concentration of zinc that is 10- to 100-fold greater than the concentration of zinc found in serum or tissues, but one which effectively blocks a wide

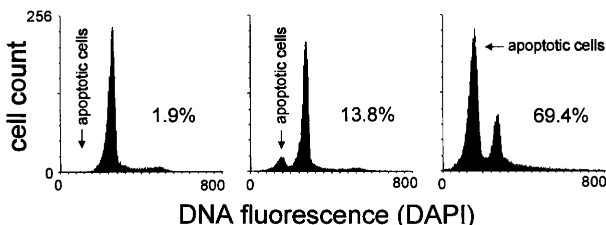

Figure 2. Fluorocytometric analysis of freshly prepared mouse thymocytes (left panel) and the thymocytes 8 hr later, after treatment with no steroid (middle panel) or 1 μM corticosterone (CS) (right panel). Cell-cycle analysis was performed on populations of cells stained with DAPI to determine the DNA content of cells. The regions of low DNA fluorescence that contain apoptotic cells are indicated with arrows.

array of cell death inducers for a variety of types of cells, as will be discussed below (12).

One of the earliest and most careful studies documenting the ability of high zinc to block apoptosis was done by Cohen and Duke (8). Using glucocorticoid-treated thymocytes, these investigators demonstrated that induction of apoptosis could be blocked by cycloheximide and actinomycin D, suggesting that both mRNA and protein synthesis were essential to cell death. They subsequently showed that the endonuclease central to DNA fragmentation was endogenous and could be induced by incubating thymic nuclei in the appropriate amounts of Mg^{++} or Ca^{++}. DNA fragmentation could, in turn, be blocked by incubating the nuclei in 500–5000 μM $ZnSO_4$. Likewise, induction of apoptosis in intact thymocytes could also be inhibited by incubating them in the high zinc (8). This was the beginning of the use of zinc as an inhibitor of apoptosis. This also appears to be the beginning of the presumption that high zinc specifically prevented activation of endonuclease. We offer evidence later that, while this may be one site of action for zinc, there are other possible sites in the cell where zinc could effectively block apoptosis.

Other Apoptotic Cues Antagonized by High Concentrations of Zinc

Subsequently high concentrations of zinc (>500 μM) have been shown to be able to block an array of apoptotic cues (12). As discussed, zinc is now a well-established inhibitor of glucocorticoid– and γ irradiation–induced death, not only in thymocytes, but in B-lineage marrow precursor cells as well (1, 2, 9). Zinc inhibited tumor necrosis factor–induced death in fibrosarcomas and also inhibited sporadesmin- and gliotoxin-induced death in T lymphoblasts and macrophages, respectively (13, 14). Likewise, etoposide-induced cell death in HL-60 promyelocytic leukemia cells and hyperthermia-induced death among human lymphoma cell lines were preventable if high concentrations of zinc were used (15, 16). Even the withdrawal of interleukin-2 from mature T lymphocytes and T-cell hybridomas, and the killing of target cells by cytolytic T cells were inhibitable by zinc (17, 18). Zinc has also been used to successfully block apoptosis in nonimmune cells such as fibroblasts, HeLa cells, etc. (19). Overall, this is a highly diverse range of inducers of cell death which were all presumably inhibited by the addition of 500–1000 μM zinc to the culture media. However, there were limitations to most of these studies. Few, if any, of these studies attempted to assess the intracellular concentration of zinc that was effective in blocking apoptosis. In addition, most of the cited studies relied solely on measuring DNA fragmentation to verify that apoptosis had occurred. Finally, the long-term health or survival of the so-called protected cells was apparently not examined. As will be seen, this may have lead to some erroneous conclusions.

Ability of Related Trace Metals To Inhibit Apoptosis: Specificity of Zinc

The ability of the flow cytometer to quantitative apoptosis in intact populations of cells also made it possible to do a number of important comparative studies. For example, we were interested in whether or not the ability of zinc to inhibit apoptosis was unique among trace like metals. To this end, we compared the ability of the sulfate salts of zinc, nickel, copper, cadmium, and gold to inhibit glucocorticoid-induced cell death in thymocytes using a range of concentrations. In this case, thymocytes from young mice were incubated for 8 hr with and without 0.1 μM dexamethasone in medium supplemented with various concentrations of the above metals as shown in Figure 3. The percentage of apoptotic cells in each treatment group was determined by FACS. From Figure 3, it can be readily seen that neither nickel nor copper provided any protection against dexamethasone-induced cell death. Conversely, at 500 μM zinc reduced dexamethasone-induced apoptosis from 70% to 23%, where background was 11%. The effects of cadmium and gold are not shown, since they were toxic at all concentrations tested and caused extensive necrotic death. Though an exhaustive list of metals was not tested, the other trace metals tested here were unable to provide protection against the DNA fragmentation associated with apoptosis.

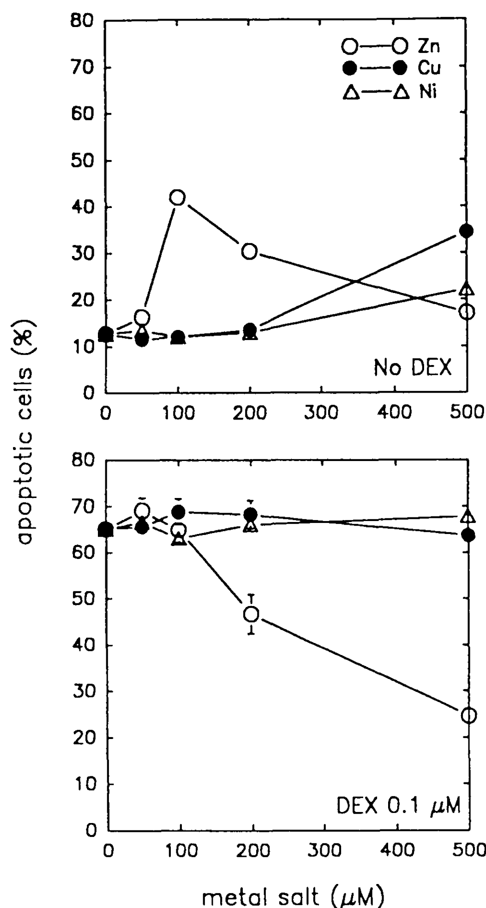

Figure 3. Dose-response curves for effects of zinc sulfate, copper sulfate, and nickel sulfate on dexamethasone-induced apoptosis in mouse thymocytes. Cells were incubated without (top panel) or with (bottom panel) dexamethasone (0.1 μM) for 8 hr. Metal salts were added simultaneously with steroid. The percentage of cells in the population that were apoptotic was determined by flow cytometry of the cell cycle using propidium iodide to stain the cells as per Figure 2.

Ability of Zinc To Block a Death Signal

As mentioned, glucocorticoid-induced cell death in thymocytes has become a common system for study of programmed cell death. In addition to the simplicity of the system, it offers the advantage of a fairly well defined signaling pathway compared with other apoptotic cues. The glucocorticoid receptor (GR) which is found in the cytoplasm of most mammalian cells is a member of the steroid superfamily of transcription factors. In its docked form in the cytoplasm, the receptor is surrounded by heat shock proteins, especially Hsp90, which appears to block the DNA binding region of the receptor (20). Upon binding of a glucocorticoid to the ligand binding region of the receptor, the Hsp90 is released and the receptor becomes mobile. A transportosome complex facilitates the movement of the receptor-ligand complex to the nucleus. The zinc fingers of the receptor facilitate the binding of the complex to specific glucocorticoid response elements in the DNA (20). In the case of thymocytes and precursor B-cells this results in the induction of so-called death genes and apoptosis (2, 3).

Metals were known to play a role in this pathway. For example, molybdate is associated with the docked complex being thought to help stabilize it. In addition, the literature indicated that metals such as cadmium could inhibit steroid binding *via* cross-linking of the vicinal thiols found in the ligand binding region (21). Indeed, site-directed mutagenesis of cysteine residue numbers 656 and 660 in the ligand-binding region confirmed the importance of these residues to steroid binding (22). Because of the well-known interplay between zinc and cysteine residues to form zinc fingers, it seemed probable that the high concentration of zinc used to block apoptosis might block steroid binding to the receptor. Using a glucocorticoid receptor preparation from the liver, we examined the effects of high concentrations of zinc on the glucocorticoid pathway. In cytosolic preparations, 50–100 μM zinc completely inhibited steroid binding to the glucocorticoid receptor (23). Although zinc inhibited receptor-ligand binding when added prior to or simultaneously with steroid, zinc was not able to dissociate already formed receptor ligand complexes. Thus, zinc is not a strong inhibitor. Furthermore, the inhibitory effect of zinc was rapidly reversed by the removal of zinc with Chelex 100, a metal chelating resin. In previous studies, the reducing agent DTT could overcome the inhibitory effects of transition metals and oxyanions that cross-linked the vicinal thiols of the glucocorticoid receptor. We found that DTT could also override the inhibitory effects of zinc. In sum, zinc was able to inhibit the formation of receptor ligand complexes in liver cytosolic preparations as well as immunoabsorbed re-

ceptor complexes (23). Collectively, the experiments suggest that zinc probably blocked ligand binding *via* complexing with cysteine 656 and 660 in the steroid binding domain as did cadmium (21). Thus, in the case of the glucocorticoid pathway, zinc has an additional potential site of action which is to block the sending of the death signal itself. Subsequent studies with intact thymocytes indicated that zinc blocked the binding of $[^3H]$dexamethasone to the cytoplasmic glucocorticoid receptors. This affirms that zinc can probably act at a number of sites in the cell when blocking apoptosis.

Ability of Moderate Concentrations of Zinc To Induce Apoptosis

Because the concentrations of zinc used to inhibit apoptosis were 10- to 50-fold greater than the concentrations of zinc found in plasma and tissues, the potentially artifactual nature of using high zinc as a protectant became of greater concern to us. Thus, it seemed important to carry out dose-kinetic assays to ascertain whether or not zinc could inhibit apoptosis at lower or more physiological concentrations. To this end, mouse thymocytes from young mice were treated with moderate amounts of glucocorticoids (1 μM corticosteroid) and a range of concentrations of zinc sulfate were tested for inhibition capacity. A companion set of wells contained no steroid but analogous concentrations of zinc. Control wells contained no supplements. The degree of apoptosis was determined 8 hr later using FACS analysis. As indicated by DNA analysis, zinc effectively prevented cell death at the 500–1000 μM range, where 700 μM was the optimal effective dose (24). Atomic absorption analysis indicated that cells incubated in the high zinc contained 6–7 μg Zn/10^9 cells compared with 1–1.5 μg Zn/10^9 cells incubated in normal media. Below 300 μM, zinc provided limited protection against glucocorticoid-induced death (24). Time course studies indicated that to provide maximum protection zinc had to be added at the same time as the steroid. A delay in the addition of zinc by 3–4 hr after steroid resulted in complete loss of protection. This indicates that zinc acts early to inhibit apoptosis, as our previous studies suggested, and/or that several hours are required before an effective concentration of zinc has entered the cell.

The surprise came when we began to examine the wells containing less than 300 μM zinc. We observed that 80–200 μM zinc was, in fact, a reasonable inducer of apoptosis in $CD4^+CD8^+$ murine thymocytes (24). Although apoptosis was observed in all populations within the thymus, the highest level of death was observed in the immature $CD4^+CD8^+\alpha\beta TCR^{lo}CD3^{lo}$ cells (24). A facsimile of these results is shown in Figure 4. Moreover, we subsequently observed that substantial amounts of programmed death were also induced among developing cells of the B-lineage in the marrow of mice by these intermediate concentrations of zinc (unpublished). Subsequently, a group in Italy made observations analogous to our own, also finding that lower

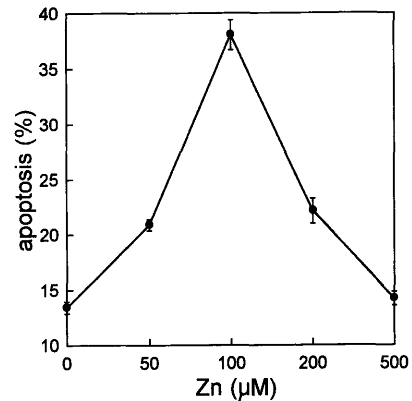

Figure 4. Dose-response curve for induction of apoptosis in mouse thymocytes by extracellular zinc sulfate. Cells were incubated for 8 hr in RPMI-1640 containing 2% heat-inactivated fetal bovine serum plus various amounts of zinc. The degree of apoptosis was determined by flow cytometry using propidium iodide to stain the DNA. Values are the standard deviation of triplicate samples being representative of three separate experiments.

concentrations of zinc induced DNA fragmentation in thymocytes from young mice (25).

Effect of Zinc Chelators on Apoptosis

In contrast to the above studies, which involved addition of extracellular zinc to cell cultures, a number of investigators showed an apoptotic state was also induced if the intracellular level of zinc was reduced using chelators. N,N,N′,N′-tetrakis-2-pyridyl methyl-ethyline diamine (TPEN) added to culture systems induced apoptosis in human peripheral blood lymphocytes and rat thymocytes and splenocytes (12, 26, 27). These experiments add additional support to the supposition that variations in intra- and extracellular zinc can modulate apoptosis. However, one must keep in mind that these chelators are not specific for zinc and may have chelated a variety of metals. Not unlike the experiments that employed high zinc as a protectant, these studies are also somewhat artificial in nature, since the rapid removal of zinc from multiple sites within the cell *via* chelation may or may not mimic any real life situations. Nevertheless, these studies also demonstrate that zinc plays a complex, dose- and time-dependent role in apoptosis.

Role for Zinc in Modulating Neuronal Function

A very recent paper demonstrates that changes in zinc concentration can also modulate life and death in neuronal tissue (28). During development, excess neurons are initially produced which are ultimately eliminated *via* apoptosis. Interestingly, fairly high levels of zinc are present in presynaptic vesicles. The actual role of zinc is not known, but it is thought to block current generation. However, excessive extracellular zinc can be neurotoxic, appearing to induce apoptosis. It was also recently noted that zinc was released during transient brain ischemia and accumulated in degenerating neurons. Interestingly, chelation of the released zinc greatly slowed neuronal degeneration, perhaps

by blocking apoptosis (28). Thus, zinc appears to play an important role in the normal function of neurons as well as in their pathogenesis. This is additional evidence in a divergent system that small changes in the location and concentration of zinc in a cell or its environment can modulate apoptosis.

Zinc Does Not Inhibit Cell Death in All Systems

There are several interesting cases where zinc failed to block apoptosis. Cyclophosphamide, an alkylating agent that can cross-link DNA at cytotoxic concentrations, readily induced apoptosis in mature human lymphocytes (29). The typical morphological changes associated with apoptosis, including DNA fragmentation, were noted. However, $ZnSO_4$ failed to suppress cyclophosphamide-induced apoptosis in this system. The failure of zinc to provide protection in this system would seem to be more related to its inability to prevent the structural changes in chromatin and DNA created by the cyclophosphamide than to its inability to block endonuclease activity (29).

DNA fragmentation and nuclear condensation also occur in a programmed manner during the life cycle of Tetrahymena, a primitive unicellular organism. However, addition of zinc sulfate failed to prevent DNA fragmentation. Interestingly, this fragmentation was not Ca^{++} dependent, as is the case for thymocytes, since addition of EGTA also failed to block DNA fragmentation (30). These authors conclude that the endonuclease of Tetrahymena has different requirements for activity than the mammalian version. Given the very different construction of the nucleus of this organism in comparison with mammals, it in fact might be further evidence that the blocking of DNA fragmentation by zinc may also be mediated through changes in the chromatin structure or DNA folding.

Zinc also failed to provide protection against apoptosis in several of the more typically studied models. Barbieri *et al.* were among the first to provide clear evidence that the protective effects of zinc were limited (31). They showed that in the case of spontaneous or dexamethasone induced death zinc did not really prevent apoptosis among rat thymocytes. It did, indeed, block DNA fragmentation, but it did not prevent the membrane and morphological changes characteristic of apoptosis (31). This was a warning of the problems of relying solely on assessments of DNA status when evaluating apoptosis. This was followed by a very similar observation made by a group in England under the leadership of Cohen *et al.* that also used rat thymocytes as their model system (32). In another case, HeLa cells treated with an etoposide derivative underwent apoptosis even in the presence of 0.25–1 m*M* $ZnSO_4$ though DNA fragmentation was blocked (33). However, the warnings set forth by these studies largely went unnoticed.

Failure of High Concentrations of Zinc To Provide Thymocytes with Long-Term Protection against Apoptosis

Because the extracellular concentration of zinc required to protect cells was so high, our lab also became concerned about possible side effects of zinc on so-called surviving cells. Most experiments, including our own, which used zinc as a protectant, examined cell viability and death over a period of only a few hours. Therefore, one wondered if zinc was able to provide the so-called surviving cells with long-term protection. While studying glucocorticoid-induced apoptosis among murine precursor B cells and thymocytes, we had noted poor viability and recovery of so-called zinc-protected cultures on several occasions when we extended the time period under study. To examine this question further, we incubated thymocytes from 8- to 12-week-old A/J males in standard RPMI-1640 media supplemented with 10% fetal bovine serum (2). Dexamethasone at 1 μ*M* was used to induce apoptosis with both zinc sulfate (700 μ*M*) and 2 μ*M* RU38486 used to block cell death. RU38486 is a well-known antagonist of the glucocorticoid receptor (2, 3). At 6 hr, all cells were harvested and washed twice to remove the additives and analyzed for degree of apoptosis. They were counted and replated. The cultures were reexamined at 16 hr. Thus, the cultures were examined at 6 and 16 hr for total cell numbers and cell viability *via* trypan blue exclusion with the degree of apoptosis determined by FACS and morphological analysis using phase microscopy. Thus, two different analyses were used for apoptosis. The results are shown in Table I.

Table I. Failure of High Zinc and the Glucocorticoid Receptor Antagonist RU38486 To Provide Lasting Protection to Thymocytes Exposed to Dexamethasone

Culture additives	6-hr incubation		16-hr incubation	
	% normal cells	% apoptotic cells	% normal cells	% apoptotic cells
No additions	91	10	76	24
700 μ*M* $ZnSO_4$	89	11	12	88
2 μ*M* RU38486	88	12	64	36
1 μ*M* Dex	39	61	12	88
Dex + RU38486	84	16	59	41
Dex + Zn	84	16	5.0	95

Note. Data are a result of five separate experiments where the proportion of apoptotic cells was verified both by morphology and FACS analysis. There was less than 10% variance in results obtained with the two methods. The FACS data are shown here. Background levels of necrosis were negligible being less than 5% in any of the treatment groups and are not shown here.

Cultures containing dexamethasone exhibited 61% apoptosis at 6 hr, with untreated cells exhibiting background levels of apoptosis (10%). By 16 hr, 88% of all cells were apoptotic in cultures that had contained dexamethasone. Interestingly, at 6 hr zinc gave the appearance of having reduced dexamethasone-mediated apoptosis to 16%. Both light microscopy or morphology and FACS gave similar results. However, at 16 hr 95% of all thymocytes in the so-called zinc-protected culture were also apoptotic. Thus, zinc was unable to provide extended protection of cells, with more cells being dead than in the dexamethasone-treated cultures. Moreover, cultures containing only zinc sulfate, which exhibited background levels of apoptosis at 6 hr (11%), had escalated to 88% apoptosis by 16 hr. This, of course, reflects changes in the dose-kinetic curve for zinc. It indicates that a concentration of zinc that was protective at 6 hr had gradually become an inducer by 16 hr. Note that thymocyte cultures without any additions exhibited 24% apoptosis at 16 hr. The latter is to be expected since over 90% of thymocytes are destined to die due to faulty rearrangement of the T-cell receptor genes and induction of apoptosis by endogenous glucocorticoids, etc.

The glucocorticoid receptor antagonist RU38486, which has been used for years to block glucocorticoid-mediated apoptosis among cells of the immune system, presented its own interesting dilemma (2, 3). At 6 hr, it provided considerable protection against steroid-mediated apoptosis, keeping levels of apoptosis to 16% of the population; however, 41% of thymocytes by 16 hr were apoptotic. Though better than zinc, this nevertheless represented a level of death that was nearly double the level of apoptosis for untreated controls. RU38486 alone, which initiated no apoptosis at 6 hr, had seemingly created its own potential for delayed initiation of apoptosis, because 36% of the cells at 16 hr were apoptotic.

Thus, neither inhibitor provided adequate long-term protection to cells from glucocorticoid-mediated death. Furthermore, zinc gradually became an inducer of apoptosis over time. This is not unlike cycloheximide, which is also frequently used to protect cells from apoptosis but can, under the right circumstances, also be a potent inducer of apoptosis (34). All of this seems to suggest that so-called inhibitors of apoptosis need to be used with greater scrutiny, especially in regards to the concentrations used. Moreover, it begins to appear that the so-called inhibition may be quite temporal or, at the very least, more limited than previous studies would suggest. Few studies have examined the actual state of surviving cells over a long period. Thus, how many of the so-called surviving cells actually live for an extended period of time is not known. How soon surviving cells can resume cycling, differentiating, proliferating, etc., hasn't really been rigorously examined either. Furthermore, little effort has been devoted to possible bystander effects. It may be hazardous for normal cells to have dying cells in their midst, especially *in vitro,* where their removal by phagocytosis is limited.

Conclusion

Inhibitors that actually override death signals would be of great value in the clinic as well as in the laboratory. As shown, high concentrations of zinc have frequently been used as an inhibitor of apoptosis. Whether or not such concentrations of zinc effectively block cell death is now open to question. Clearly, high extracellular zinc blocks DNA fragmentation in a variety of systems, which is in keeping with the assumptions of the earlier literature. However, reliance on DNA fragmentation as the sole indicator of apoptosis resulted in the failure of a number of investigators to note that cell death was not really blocked by high zinc in the case of glucocorticoid-initiated death (31–33). Only DNA fragmentation was blocked by zinc. Thus, it is not known how many other systems thought to be protected by high zinc actually underwent apoptosis. However, studies now suggest that zinc can also interfere with the death signal itself by blocking the binding of glucocorticoids to the GR. This makes the inability of zinc to provide long-term protection against apoptosis even more puzzling. Indeed, examination of so-called survivor cells for several hours indicated that, in fact, zinc provided little or no long-term protection to thymocytes previously exposed to glucocorticoids. Most thymocytes were dead in less than 24 hr regardless of whether they had been exposed to glucocorticoid alone, glucocorticoid with high zinc, or just high zinc. Given somewhat similar observations for other inhibitors, like cycloheximide and RU38486, concern is raised regarding the true nature of chemical inhibitors of apoptosis. Future studies of so-called inhibitors of apoptosis should take care to examine the effects of the inhibitor on cell survivors over a wide range of doses and time periods in order to ascertain if they ever become inducers of apoptosis. Moreover, doses of inhibitor presumed to protect cells should be carefully scrutinized to verify by both morphological and biochemical means that cell death was, indeed, blocked. 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 blockage that is subsequently overridden.

The authors thank Mark Lyons for the data in Table I and Teresa Vollmer for the preparation of the manuscript.

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