The Influence of Zinc on Apoptosis*†

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ABSTRACT

This review summarizes the evidence that apoptosis is modulated by intracellular excess or deficiency of Zn\(^{2+}\), considers mechanisms whereby Zn\(^{2+}\) may influence apoptosis, and delineates gaps in current knowledge and opportunities for research. The experimental evidence supports four major conclusions:

[1] Zinc deficiency, resulting from dietary deprivation of mice, or exposure of cultured cells to membrane-permeable Zn\(^{2+}\)-chelators, can induce apoptosis;

[2] Zinc supplementation, either by pretreating mice with ZnSO\(_4\), or adding Zn\(^{2+}\) to the media of cell cultures, can prevent apoptotic death. Zn\(^{2+}\) protects against the apoptosis induced by diverse physical, chemical, or immunologic stimuli in cultured cells of lymphoid, hepatic, or neoplastic origin;

[3] Zn\(^{2+}\) does not affect the triggering events or earliest signs of apoptosis, but acts later in the apoptotic pathway, preventing endonucleosomal fragmentation and subsequent cytolysis; and

[4] An intracellular pool of chelatable Zn\(^{2+}\) plays a critical role in apoptosis, possibly by modulating the activation or activity of endonuclease(s).

These conclusions should alert pharmacologists and physicians to the potential therapeutic applications of zinc compounds and zinc chelators in clinical disorders and diseases that involve apoptosis, and to the relevance of zinc nutrition in such conditions.

Introduction

ETYMOLOGY, DEFINITION, AND PRONUNCIATION

In Egyptian mythology, the diurnal cycle was ascribed to Apophis, the demon of darkness, who was dispatched each morning by Ra, the sun god. The concept of cyclical dismissal or demise was carried into classical Greek, where "αποπτωσις" denoted the annual shedding of foliage from trees or petals from flowers. In 1972, Kerr et al\(^1\) applied the word "apoptosis" to a physiological process whereby individual cells die without swelling, necrosis, or inflammation. Kerr et al\(^1\) advised that apoptosis be pronounced with stress on the penultimate syllable and that the second 'p' be silent (i.e., as in "ptosis," which refers to droop-
ZINC AND APOPTOSIS

Illustrative Examples and Morphological Signs

Apoptosis is considered responsible for selective deletion of cells during embryogenesis, homeostasis of cell populations in continuously renewing tissues (i.e., serving as a counterpoise to mitosis), and tissue involution in response to chemical or physical stimuli (e.g., thymic atrophy induced by glucocorticoids, irradiation, or oxidative stress). The morphological signs of apoptosis, based on examinations of histological sections and electron photomicrographs, include reduction of cell volume, condensation and margination of nuclear chromatin, convolution and blebbing of the cell surface, and compaction of cytoplasmic organelles into membrane-bound globular fragments ("apoptotic bodies") that are shed from epithelial surfaces or phagocytosed by macrophages and parenchymal cells.\(^3,4,5\)

Biochemical and Biophysical Indicators

As molecular mechanisms of apoptosis began to be elucidated, the morphological criteria were supplemented, and to some extent replaced, by biochemical and biophysical indicators, such as DNA fragmentation.\(^6,7,8\) Initially, a paramount indicator of apoptosis was endonucleosomal cleavage of chromatin DNA into 180 to 200 basepair (bp) multimers, visualized by agarose gel electrophoresis as an "oligonucleosomal ladder,"\(^9,10\) but oligonucleosomal laddering is now viewed as a late and inconstant sign of apoptosis.\(^11,12\) Earlier stages of apoptosis are evidenced by (1) high molecular DNA fragments (300 and 50 kbp) that are visualized by field-inversion gel electrophoresis,\(^13,14\) (2) low frequency DNA strand-breaks, demonstrable by the fluorometric DNA unwinding assay,\(^15\) by the random oligonucleotide primed synthesis (ROPS) assay,\(^16\) or by combined application of in situ tailing and nick translation assays,\(^17\) and (3) flow cytometry, which distinguishes normal and apoptotic cells on the basis of size, fluorescence, and forward light scattering.\(^18\)

Mechanistic Insights

According to current concepts, apoptosis is triggered by diverse, interacting signals, including (1) programmed or aberrant expression of oncogenes (e.g., bcl-2, c-myc) and tumor suppressor genes (e.g., p 53),\(^19,20,21,22,23\) (2) transient elevation of intranuclear free Ca\(^{2+}\) concentration, which affects chromatin organization, influences the cell cycle, and activates nuclear Ca\(^{2+}/Mg\(^{2+}\)-dependent endonuclease activity,\(^24,25,26,27,28\) and (3) intracellular acidification, which activates nuclear deoxyribonuclease II (DNase II) activity.\(^12,29\) The formation of apoptotic bodies (i.e., cell fragments that are insoluble in detergents and chaotropic agents) involves protein crosslinkage by Ca\(^{2+}\)-dependent transglutaminase activity.\(^30\) The intracellular processes that contribute to apoptosis are incompletely understood, but protease activation and proteolysis play important roles.\(^14,28,31,32,33,34\)

Review Articles

For background information about apoptosis, readers are referred to general reviews,\(^35,36,37,38,39,40\) supplemented by articles that discuss apoptosis in relation to genetics,\(^41,42\) endocrinology,\(^43\) immunology,\(^44,45\) toxicology,\(^46\) aging,\(^47\) and cancer.\(^48,49,50,51\) For a skeptical commen-
tary on apoptosis, readers should consult Farber's critique, which stresses the distinctive responses of different cell types, and the difficulty of distinguishing between antemortem processes that lead to cell death per se and structural-biochemical changes that follow cell death (i.e., postmortem changes).

TOPICS FOR DISCUSSION

This review summarizes the evidence that apoptosis is modulated by intracellular excess or deficiency of Zn\(^{2+}\), considers mechanisms whereby Zn\(^{2+}\) may influence apoptosis, and delineates gaps in current knowledge and opportunities for research. Since this article is specifically focused on the relationship of Zn\(^{2+}\) to apoptosis, readers should consult the original publications for information about other important facets of apoptosis in the various experimental systems. The primary goal of this essay is to alert pharmacologists and physicians to the potential therapeutic applications of zinc compounds and zinc chelators in clinical disorders and diseases that involve apoptosis, and to the relevance of zinc nutrition in such conditions.

Relation of Zinc to Apoptosis

ZINC DEFICIENCY

The earliest report that explicitly linked zinc to apoptosis was published in 1977 by Elmes, who observed massively increased numbers of apoptotic bodies in the crypt region of the mucosa of small intestine of zinc-deficient rats, compared to pair-fed controls that received a zinc-sufficient diet, suggesting that zinc deficiency induced apoptosis. Fraker et al described the rapid onset of thymic atrophy and defective T helper cell function in young adult A/J mice that were fed a zinc-deficient diet, consistent with an acute involutional response of thymocytes to zinc deprivation. Similar findings were reported by Fernandez et al, who studies three strains of mice (A/Jax, C57BL/Ks, and CBA/H) that were fed a zinc-deficient diet at age 6 to 8 weeks, in comparison to pair-fed controls on a zinc-sufficient diet. Many of the zinc-deprived mice developed thymic atrophy and signs of acrodermatitis enteropathica (i.e., diarrhea, skin lesions on the paws and tail, and stunted growth). Martin et al maintained three cell lines of human lymphoid (Molt-3 and Raji) or myeloid (HL-60) origin in vitro under Zn-sufficient or Zn-deficient conditions. Proliferative capacity and viability of the three cell types were all diminished by Zn-deficiency; death occurred mainly via apoptosis with internucleosomal DNA degradation in the HL-60 and Raji cells and via necrosis in the Molt-3 cells. These studies suggested that zinc deficiency can, under certain circumstances, induce apoptosis in vivo and in vitro.

INITIAL REPORT OF Zn\(^{2+}\)-PROTECTION AGAINST APOPTOSIS

In 1983, Duke et al reported that Zn\(^{2+}\) inhibited apoptosis of murine P815 mastocytoma cells. When the target tumor cells were exposed to effector cells (i.e., allogeneic cytotoxic T lymphocytes), the DNA of target cells became fragmented within 10 to 90 min, showing oligonucleosomal ladders with DNA bands in integral multiples of 200 bp subunits. Within 4 hr after exposure to effector cells, the target cells were lysed, as evidenced by 51Cr-release assays. Fragmentation of DNA and cytolysis were inhibited by adding ZnSO\(_4\) to the culture medium, with partial suppression at Zn\(^{2+}\) concentrations from 0.1 to 0.4 mM and complete prevention at 0.8 mM. Exposure to Zn\(^{2+}\), per se, was not cytotoxic, since the via-
bility of the target or effector cells in control experiments was unaffected by addition of ZnSO₄ (0.8 mM) to the medium.

APOPTOSIS OF THYMOCYTES

Cohen & Duke⁷ and Sellins & Cohen⁵⁷ showed that addition of Zn²⁺ (0.5 to 5 mM) to the medium inhibited DNA fragmentation and cytolysis of murine thymocytes, induced by exposure to dexamethasone or γ-irradiation. Beletsky et al⁵⁸ reported that addition of Zn²⁺ (1 or 2 mM) to the medium completely prevented the chromatin degradation that occurred during in vitro incubation of thymocytes from normal or γ-irradiated mice. Cohen et al¹⁸ reported that Zn²⁺ (1 mM) did not abrogate the early signs of apoptosis in rat thymocytes exposed to dexamethasone, as detected by flow cytometry, but Zn²⁺ prevented the subsequent development of oligonucleosomal ladders and the characteristic morphologic signs of apoptosis. Similarly, Raffray et al⁵⁹ found that pretreatment of rat thymocytes with Zn²⁺ (1 mM) did not influence the early disturbances of nuclear morphology induced by methylprednisone or bis(tributyltin) oxide, but prevented the subsequent chromatin condensation, DNA fragmentation, and cell death. Zhivotovsky et al⁶⁰ showed that addition of Zn²⁺ (0.1 mM) to the medium inhibited the formation of high molecular weight DNA fragments (300 and 50 kbp) in human thymocytes exposed to methylprednisone or thapsigargin, based on DNA fractionations by pulsed-field gel electrophoresis. These in vitro studies indicated that Zn²⁺ supplementation does not block the initiation of apoptosis, but prevents the subsequent DNA fragmentation, which requires endonuclease activity. Dose-dependent protection by Zn²⁺ against apoptosis of murine thymocytes was demonstrated in vivo by Thomas and Caffrey,⁶¹ who showed that pretreatment of NAW/W1 mice with Zn²⁺ (as zinc acetate, 1 to 10 mg Zn/kg, sc), three days prior to injection of S. typhimurium lipopolysaccharide, reduced the internucleosomal cleavage of thymic DNA.

APOPTOSIS OF OTHER LYMPHOID CELLS

Odaka et al⁶² reported that addition of Zn²⁺ (0.2 mM) to the medium partially inhibited the cell death that occurred when antigen-specific murine T cell hybridomas were exposed to immobilized anti-CD3 antibody. Waring et al⁶³ exposed murine peritoneal macrophages and concanavalin A-stimulated spleen cells to sporidesmin or gliotoxin, with or without addition of Zn²⁺ to the culture medium. Apoptotic bodies and other signs of apoptosis, including DNA laddering and eventual cytolysis, were partially suppressed at a low concentration of Zn²⁺ (0.3 mM) and completely prevented at a higher concentration (1 mM). Treves et al⁶⁴ investigated intracellular Zn²⁺ as a regulator of apoptosis in cultured human lymphocytes, with or without phytohemagglutinin (PHA) stimulation. Lysates of lymphocytes from peripheral blood were centrifuged and zinc concentrations in the supernatants were determined by ICP-MS analysis. Stimulation of PHA greatly increased the intracellular Zn²⁺ concentration and prevented spontaneous apoptotic changes (nuclear condensation and DNA fragmentation).

APOPTOSIS OF TUMOR CELLS

Flieger et al⁶⁵ reported that addition of Zn²⁺ (0.1 to 1 mM, as ZnSO₄ or ZnCl₂) to the medium partially or completely prevented DNA fragmentation, subsequent cytolysis, and death of murine fibrosarcoma cells exposed to tumor necrosis fac-
tor (TNF), whereas Cu^{2+} (0.4 mM as CuSO_4) gave no protection. Cytolysis of fibrosarcoma cells was abrogated even when Zn^{2+} (0.4 mM) was added 2 hr after TNF, suggesting that the protective effect of Zn^{2+} occurred at a late stage, after TNF had become bound to its receptor. Shimizu et al^{15} found that adding Zn^{2+} (1 mM) to the medium at 0, 1, or 2 hr after exposure of human promyelocytic leukemia cells (HL-60) to etoposide did not prevent the DNA strand breaks that were detected by a sensitive fluorimetric DNA unwinding assay, but completely prevented the subsequent formation of (1) oligonucleosomal ladders, (2) morphologic changes characteristic of apoptosis, and (3) stimulation of poly-ADP-ribose synthesis for DNA repair, although Zn^{2+} did not directly inhibit poly(ADP-ribose) polymerase activity. Thus, Zn^{2+} prevented apoptosis by inhibiting a metabolic process that occurred after the initial DNA strand breaks. Ojcieus et al^{66} showed that addition of Zn^{2+} (0.25 mM) to the medium, prior to exposure of murine mastocytoma cells (P815) to beauvericin or valinomycin, partially prevented the late signs of apoptosis, such as DNA fragmentation and cytolysis. Martin and Cotter^{67} reported that, following brief exposure of human promyelocytic leukemia cells (HL-60) to UV-irradiation, Zn^{2+} enrichment of the culture medium (0.05 to 0.8 mM) reduced, in a concentration-dependent fashion, the proportion of cells that developed morphologic signs of apoptosis.

**Effects of Zn^{2+}-Ionophores and Chelators**

McCabe et al^{68} described nuclear condensation and oligonucleosomal laddering in rat and human thymocytes exposed to a membrane-impermeable Zn^{2+}-chelator (diethylenetriamine pentacetic acid, DTPA). The TPEN-induced apoptosis was prevented when the metal-binding sites of TPEN were saturated by adding equimolar amounts of Zn^{2+} or Cu^{2+} to the medium. Similarly, Treves et al^{64} showed that exposure to TPEN lowered the Zn^{2+} content of human lymphocytes and caused the DNA to fragment into oligonucleosomes. These reports show that depletion of intracellular Zn^{2+} by chelation can trigger apoptosis in cells of lymphoid origin, resembling the apoptotic response of such cells to zinc-deficient culture medium. Zalewski et al^{69} studied the apoptosis that occurs spontaneously in a minor proportion of human chronic lymphatic leukemia cells (CLL) during culture for 18 hr, and in practically all CLL cells following exposure to colchicine. Spontaneous and colchicine-induced apoptosis was prevented by adding Zn^{2+}, alone (0.5 or 1 mM), or by adding Zn^{2+} at low concentration (5 to 25 μM) in combination with a Zn^{2+}-ionophore (pyrithione, 0.25 to 1 μM). When CLL cells or normal lymphocytes were exposed to an intracellular Zn^{2+}-chelator (1,10-phenanthroline), DNA fragmentation and cell death occurred unless Zn^{2+}, or another cation chelated by 1,10-phenanthroline, was simultaneously added to the medium. Based on these various studies, a chelatable pool of intracellular Zn^{2+} influences apoptosis, since influx of Zn^{2+} prevented apoptosis, while chelation of Zn^{2+} induced apoptosis.

**Ca^{2+}/Mg^{2+}-Endonuclease Activity**

Cohen and Duke^{7} reported that Ca^{2+}/Mg^{2+}-dependent endonuclease activity in isolated nuclei from murine thymocytes was inhibited when Zn^{2+} (0.5 mM) was added to the medium. Nieto and Lopez-Rivas^{70} showed that Zn^{2+} (0.1 to...
0.3 mM) suppressed DNA fragmentation by endogenous Ca$^{2+}$/Mg$^{2+}$-endonuclease activity in isolated nuclei of dexamethasone-treated murine leukemia cells (CTLL-2). Beletsky et al.\(^5\) noted that Ca$^{2+}$/Mg$^{2+}$-endonuclease activity of thymocyte nuclei from normal and γ-irradiated mice was completely inhibited in the presence of Zn$^{2+}$ (0.1 mM), whereas acid endonuclease activity was only partially inhibited by Zn$^{2+}$ under similar experimental conditions.

Giannakis et al.\(^8\) observed that the Ca$^{2+}$/Mg$^{2+}$-endonuclease activity of human chronic lymphocytic leukemia cells (CLL) increased six-fold during colchicine-induced apoptosis, coincident with the onset of DNA fragmentation. The Ca$^{2+}$/Mg$^{2+}$-endonuclease activity and DNA fragmentation were both blocked by adding low concentrations of Zn$^{2+}$ (0.05 to 0.25 mM) to the medium, together with a Zn-ionophore (pyrithione) to promote the cellular uptake of Zn$^{2+}$.

The Ca$^{2+}$/Mg$^{2+}$-endonuclease activity was inhibited even more strongly when Zn$^{2+}$ was added directly to cell-free extracts. Gaido and Cidlowski\(^71\) isolated at 18-kDa Ca$^{2+}$-dependent endonuclease (NUC18) from rat thymocyte nuclei and showed that NUC18 activity was inhibited by Zn$^{2+}$ (2 mM).

Lohmann and Beyersmann\(^72\) reported that DNA in nuclei isolated from bovine hepatocytes or thymocytes became fragmented after incubation for 1 hr with Ca$^{2+}$ (10 to 100 μM). The DNA damage was partially prevented when Zn$^{2+}$ (1 to 10 μM), Cd$^{2+}$ (1 to 10 μM), or Hg$^{2+}$ (0.01 to 1 nM) were added to the incubation medium. Zhivotovsky et al.\(^14\) observed high molecular weight DNA fragments (50 kbp) and oligonucleosomal ladders in rat hepatocyte nuclei that were incubated in medium that contained Ca$^{2+}$ and Mg$^{2+}$; the DNA damage was partially prevented when Zn$^{2+}$ (100 μM) was added to the medium. These studies are consistent with the hypothesis that Zn$^{2+}$ prevents apoptosis by blocking the activation or inhibiting the activity of Ca$^{2+}$/Mg$^{2+}$-endonuclease.

**ACID ENDONUCLEASE ACTIVITY**

Many studies have suggested that endonucleosomal cleavage during apoptosis is a Ca$^{2+}$-dependent process,\(^27,29,73\) but Kluck et al.\(^74\) cited several instances where apoptosis is not associated with elevated intracellular levels of ionized calcium. Barry and Eastman\(^24,28\) proposed that intracellular Ca$^{2+}$ is not always the signal for endonuclease activation in apoptosis, but that in some situations an acidic endonuclease (e.g., deoxyribonuclease II, DNase II) may be activated by intracellular acidification. In various models of apoptosis, Barry and Eastman\(^24,29\) observed that intracellular acidification coincided with the onset of DNA fragmentation. Although Barry and Eastman\(^20\) found that Zn$^{2+}$ did not directly inhibit DNase II activity, Morana et al.\(^75\) showed that Zn$^{2+}$ (0.1 to 1 mM) prevented intracellular acidification in etoposide-exposed human myeloid leukemia cells (ML-1) and thereby indirectly blocked the formation of oligonucleosomal DNA ladders. Protection against DNA cleavage was observed even when Zn$^{2+}$ addition was delayed 1 h after etoposide treatment. Morana et al.\(^75\) advised caution in implicating any specific endonuclease(s) in apoptosis, since Zn$^{2+}$ apparently influences the activity of both DNase II and Ca$^{2+}$/Mg$^{2+}$-endonuclease in intact cells.

**Discussion**

The experimental evidence that has been discussed supports four major conclusions:

1. Zinc deficiency, resulting from dietary deprivation of mice, or exposure of cultured cells to membrane-permeable Zn$^{2+}$-chelators, can induce apoptosis.
[2] Zinc supplementation, either by pre­
treating mice with ZnSO$_4$, or adding
Zn$^{2+}$ to the media of cell cultures,
can prevent apoptotic death. Zn$^{2+}$
protects against the apoptosis induced
by diverse physical, chemical, or
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[3] Zn$^{2+}$ does not affect the triggering
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Zn$^{2+}$ play a critical role in apoptosis,
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These conclusions should alert phar­
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disorders and diseases that involve apo­
ptosis, and to the relevance of zinc nutri­
tion in such conditions.

Research is needed to establish the
molecular mechanisms whereby zinc
deprivation and supplementation influ­
ence apoptosis. For instance, future
investigations should (1) purify Zn$^{2+}$-
sensitive endonucleases and determine
their zinc binding sites, dissociation con­
stants, and inhibition constants; (2) probe
the possible involvement of Zn-metallo­
proteinases in apoptosis; (3) elucidate the
cellular fluxes of zinc during apoptosis by
means of quantitative analysis, radio­
tracer assays with $^{65}$Zn$^{2+}$, and cytometric
applications of Zn$^{2+}$-chromophores and
-fluorophores; (4) study the roles of met­
allothionein and glutathione in apoptosis
as regulators of the intracellular pool of
chelatable Zn$^{2+}$, and (v) explore the
interactions of Zn$^{2+}$ with other metal­
ions (e.g., Cu$^{2+}$, Cd$^{2+}$, Co$^{2+}$, Ni$^{2+}$) in
experimental models of apoptosis. The
gaps in current knowledge about zinc in
apoptosis provide manifold opportunities
for basic research that seems likely to
yield clinical benefits.

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