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Zinc Induces Mixed Types of Cell Death, Necrosis, and Apoptosis, in Molt-4 Cells[1]

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To investigate the mode of zinc-induced cell death, the associated morphological changes, and biological events were examined in zinc-treated Molt-4 cells. Fluorescence microscope observations with double staining of zinc-treated cells with Hoechst 33342 and propidium iodide (PI) indicated that the metal induced both necrosis and apoptosis. To confirm this, cells were stained with both PI and FITC-labeled annexin V, which binds phosphatidylserine, and then analyzed by flow cytometry. The results also confirmed that zinc induces mixed types of cell death, necrosis and apoptosis, and that the former induction occurs earlier and at a greater frequency. Hallmarks of apoptosis such as abnormal chromosome condensation and release of cytochrome *c*, as well as the appearance of annexin-positive cells, appeared along with the expression of mitochondrial membrane protein 7A6. However, zinc did not induce increases in caspase-3 like protease and caspase-8 activities, and caused slightly hypodiploid cells. Furthermore, the induction of cell death and annexin-positive cells was not blocked by the caspase inhibitors Ac-YVAD-CHO and Ac-DEVD-CHO. These results indicate that zinc induces both necrosis and apoptosis, without caspase-3 activation.

Key words: annexin, caspase, caspase inhibitor, phosphatidylserine externalization, zinc.

Two distinct types of cell death, apoptosis and necrosis, are characterized by different morphological and biological features. Apoptotic cell death occurs during normal development, tissue homeostasis, and regulation of the immune system (*1*). Cells die in a highly coordinated manner with characteristic structural changes including a reduction in volume, apoptotic body formation, chromatin condensation, nuclear DNA fragmentation, and externalization of phosphatidylserine (*2*). Recent studies have revealed that sequential activation of caspases plays a critical role in the induction of apoptosis (*3*, *4*). One key enzyme, caspase-3, is activated through distinct pathways involving caspase-8 and the release of cytochrome *c* from mitochondria (*6*). Bcl-2 and Bcl-X_L are located primarily in the outer mitochondrial membrane and prevent cytochrome *c* release from the mitochondria (*5*, *7*, *8*), so that activation of caspases such as caspase-3 and caspase-9 does not occur (*9*). Necrosis is characterized by mitochondrial swelling, membrane lysis, and apparently intact nuclei (*10*).

Zinc, a trace element required for growth and maintenance of the immune system, functions as an essential cofactor for many enzymes involved in macro molecular synthesis or protein metabolism (*11*). Zinc inhibits apoptosis in human thymocytes and various tumor cells exposed to dexamethasone or antitumor drugs (*12–14*), and influences later stage events such as DNA fragmentation or chromosomal condensation (*12*, *13*, *15*). This inhibition of drug-induced apoptosis by zinc may be associated with inhibition of caspase-3 like protease (*16*), an increase in the Bcl-2/Bax ratio (*17*), or a protease (*18*), while zinc itself induces apoptosis in mouse thymocytes at relatively low concentrations (*19*, *20*).

We recently showed that zinc causes necrosis rather than apoptosis in prostate cells (*21*). In the present study, on the other hand, zinc induced phosphatidylserine externalization, a hallmark of apoptosis (*22*, *23*), in Molt-4 leukemic cells. We characterized the zinc-induced cell death in these cells, and found that both necrosis and apoptosis occur, with phosphatidylserine externalization independent of activation of caspase-3 like proteases. The latter result is in contrast with reports that the externalization involves activation of caspases (*24–28*) and is consistent with the suggestion of an independent process (*29*, *30*).

MATERIALS AND METHODS

Cell Culture—The human T cell acute lymphoblastic leukemic cell line, Molt-4 cells, was cultured in RPM1-1640 medium containing 10% calf serum under a humidified atmosphere of 5% CO_2 in air.

Peptide Inhibitors—Ac-YVAD-CHO, Ac-DEVD-CHO, Ac-

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Abbreviations: etoposide, 4′-demethyl-epipodophyllotoxin-9-(4,6-*O*-ethylidine-β-D-glycopyranoside); ECL, enhanced chemiluminescence; PI, propidium iodide.

DEVD-MCA, and Ac-IETD-MCA were purchased from the Peptide Institute, Osaka.

Assessment of Cell Viability—Cell proliferation was evaluated by measuring the fluorescence intensity in the presence of alamar blue (Wako Pure Chemical Industries) (*21*). Cells were seeded into 96-well multidishes (Costar Corporation) at a density of 1.5×10^4 cells per well in culture medium, and then incubated overnight before being treated with zinc. After the indicated time, 20 μl of alamar blue was added to each well and the plates were incubated for 4 h. The fluorescence intensity was measured using a Cytofluor 2350 with excitation at 530 nm and emission at 590 nm.

Nuclear Staining Assay—Cell viability was assayed morphologically. After 24 h treatment with zinc, Molt-4 cells were collected by centrifugation at 500 ×*g* for 5 min. The collected cells were stained with Hoechst 33342 (10 μM) and PI (10 μM) for 10 min in PBS, and then analyzed under a non-confocal fluorescence microscope (Nicon Microphoto-FXA) with excitation at UV (360 nm). Viable and apoptotic cells had blue, round nuclei and blue or pink fragmented nuclei, respectively (*32*).

Analysis of Hypodiploid Cell Induction—Flow cytometric analysis was performed to identify hypodiploid/apoptotic cells after PI staining in hypotonic buffer, as described (*34*). Briefly, cell pellets were suspended in 1 ml of a hypotonic fluorochrome solution (0.1% w/v sodium citrate, 0.1% v/v Triton X-100, and 50 μg/ml PI), and then the cells were analyzed using a FACScan (Becton and Dickinson) with Lysys-2.

Cytochrome c Release—Molt-4 cells were collected by cen-

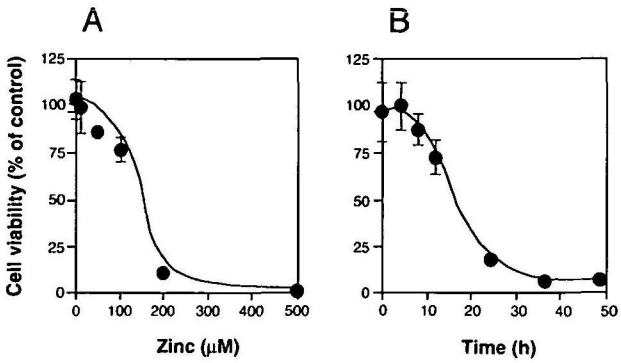

Fig. 1. **Dose- and time-dependence of growth inhibition in zinc-treated Molt-4 cells.** Cells were treated with increasing concentrations of zinc for 48 h (A) or were incubated with 300 μM zinc for the indicated times. Cell growth was examined by means of the alamar blue assay. Data are mean ± SD values for five incubations.

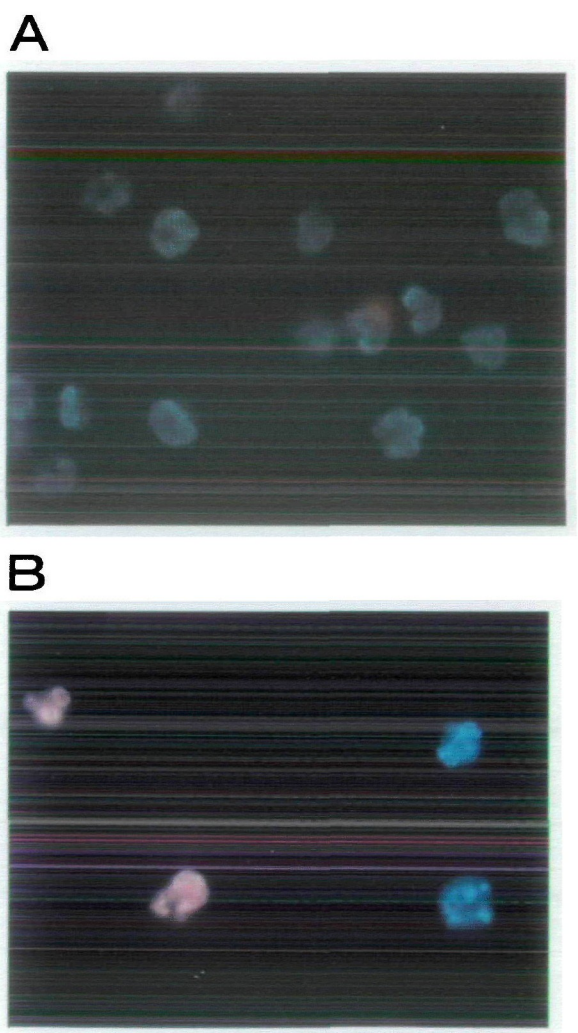

Fig. 2. **Morphological analysis of cells treated with zinc.** Cells were incubated in the absence (A) or presence of 100 μM (B) or 300 μM (C) zinc for 24 h, stained with Hoechst 33342 (blue) and PI (pink), and then analyzed under a non-confocal fluorescence microscope.

trifugation, rinsed twice with ice-cold PBS, and then resuspended in 100 μl of extraction buffer [50 mM Hepes-KOH, pH 7.4, 220 mM mannitol, 68 mM sucrose, 50 mM KCl, 5 mM EDTA, 2 mM $MgCl_2$, 1 mM dithiothreitol, and protease inhibitors (Complete Cocktail, Boehringer Mannheim)]. After chilling on ice for 30 min, the cells were disrupted with 40 strokes of a glass homogenizer. The homogenate was centrifuged at 12,000 ×*g* for 1 h and the supernatant was recovered. Cytosolic fractions were separated on 12% SDS–polyacrylamide electrophoresis gels, blotted onto nitrocellulose membranes and then exposed to a monoclonal antibody to cytochrome *c*, at a dilution of 1:2,000, followed by a horseradish peroxidase–conjugated goat anti-mouse secondary antibody (Sigma) at a dilution of 1:50,000. Immunoblots were examined by means of an enhanced chemiluminescence (ECL) assay (Amersham Life Science) according to the manufacturer's instructions.

Assaying of Enzyme Activity—Zinc-treated cells were collected by centrifugation, washed twice with ice-cold PBS, resuspended in extraction buffer as above, sonicated and then centrifuged at 10,000 ×*g*. The supernatant (30 μg protein) was incubated with 50 μM Ac-DEVD-MCA or Ac-IETD-MCA at 37°C for 30 min, the release of amino-4-methylcoumarin being monitored using a spectrofluorometer at an excitation wavelength of 380 nm and an emission wavelength of 460 nm.

Procaspase-3 Activation—Molt-4 cells (2×10^6 cells) were pelleted by centrifugation, rinsed twice with ice-cold PBS, resuspended in 20 μl of SDS-sample buffer and then boiled for 5 min. The cell extracts were subjected to SDS-PAGE with 12% polyacrylamide, blotted onto nitrocellulose membranes and then probed using an anti–caspase-3 rabbit polyclonal antibody (Pharmingen) at a dilution of 1:2,000. Immunoblots were detected using a horseradish peroxidase–conjugated goat anti–rabbit IgG, and an ECL detection system.

Analysis of Apoptosis by Annexin V Staining—Apoptosis was determined using an Apoptosis Detection Kit (R&D Systems). Briefly, after drug treatment, Molt-4 cells were collected, washed twice in cold PBS, and then resuspended in binding buffer (Hepes-buffered saline solution containing 2.5 mM calcium chloride) at a density of 1×10^5 cells/ml. Fluorescein-labeled annexin V and PI were added to the cells, and then samples were incubated for 15 min before being analysed with a FACScan. Annexin V-FITC generated signals were detected with a FITC signal detector (FL1, 525 nm). PI signals were monitored using a detector reserved for phycoerythrin emission (FL2, 575 nm).

Apo-2.7 Assay—Cells were incubated with 100 μg/ml digitonin in PBS for 20 min on ice and then permeabilized. After Apo 2.7 PE (Phycoerythrin) (Immunotech) had been added, samples were incubated for 15 min at room temperature in the dark and then analyzed by flow cytometry.

RESULTS

Effects of Zinc on Cell Growth—When Molt-4 cells were exposed to various concentrations of zinc for 48 h (Fig. 1A) or to 300 μM zinc for the indicated times (Fig. 1B), their viability decreased, in a dose- and time-dependent manner. With exposure to 100 and 200 μM zinc for 48 h, the viability was 75 and 10%, respectively, while it was reduced to 80 and 20% after exposure to 300 μM zinc for 10 and 24 h, respectively. The susceptibility of Molt-4 cells to zinc proved to be slightly lower than that of prostate carcinoma cells, as reported earlier (*21*).

Estimation of Apoptosis and Necrosis—Apoptosis is characterized by the appearance of DNA fragmentation, abnormal chromosome condensation and hypodiploid cells (*2*). Necrosis is defined by mitochondria swelling, membrane lysis and apparently intact nuclei (*10, 31*). To determine which type of cell death is induced by zinc, non-treated and

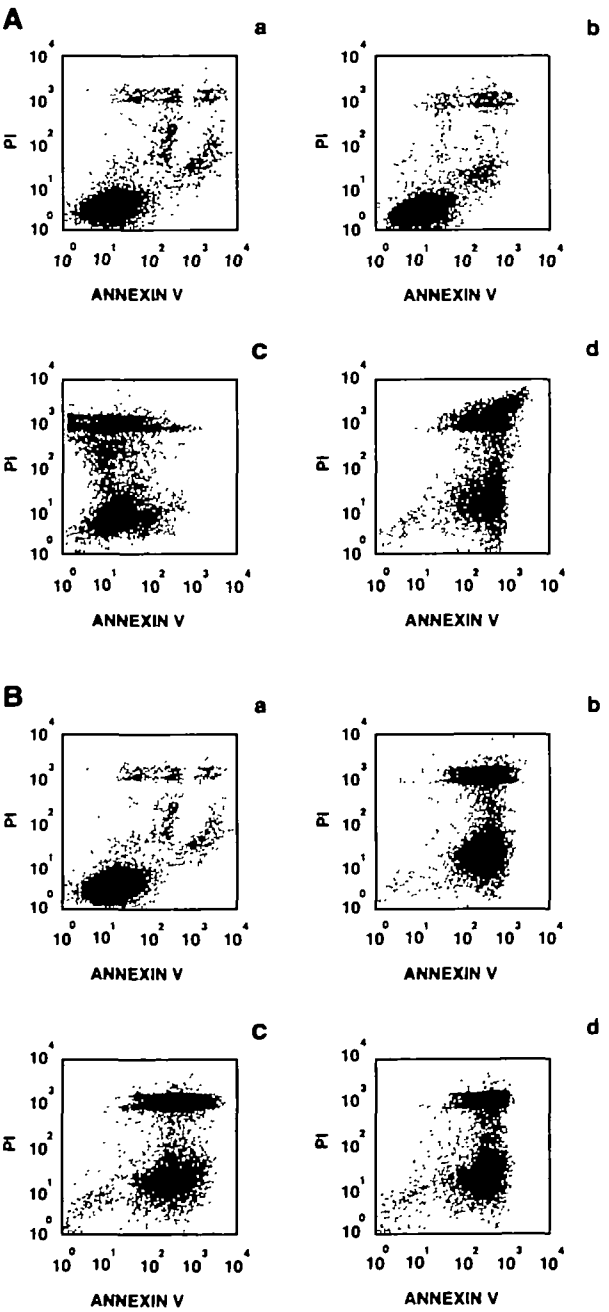

Fig. 3. **(A) Time course of induction of annexin V–stained cells.** Cells were incubated with no metal (a), or 300 μM zinc for 2 h (b), 9 h (c), or 24 h (d). **(B) Effects of caspase inhibitors on the induction of annexin V–stained cells.** Cells were preincubated with 100 μM Ac-YVAD-CHO (c) or 100 μM Ac-DEVD-CHO (d) for 10 min, and then incubated with 300 μM zinc for 24 h (b, c, d) or without the metal (a).

treated cells were stained with Hoechst 33342 and PI, and then analyzed by fluorescence microscopy. Hoechst 33342 can readily pass through all cell membranes, while PI can penetrate into necrotic or late apoptotic cells, but not viable or early apoptotic cells. Both drugs bind to DNA, and fluoresce blue and pink, respectively. Therefore, necrotic cells have pink and round nuclei, and apoptotic ones have pink or blue abnormally condensed chromatin (*32*). The results in Fig. 2 show that non-treated cells were all viable, while zinc-treated cells comprised a mixture of necrotic and apoptotic cells, among which necrotic cells with condensed chromatin were often observed.

To confirm that zinc induces both necrosis and apoptosis, cells were stained with both PI and FITC-labeled annexin V, which binds phosphatidylserine (*33*), and then analyzed by flow cytometry. In Fig. 3, the lower left population of cells, in each plot represents viable cells, which excluded PI and did not bind annexin V. The upper left population comprises necrotic cells, which did not exclude PI and were not stained with FITC-labeled annexin V. The lower and upper right populations correspond to apoptotic and late apoptotic cells, respectively. Non-treated control cells were annexin-negative, and annexin-positive cells were not increased at 2 and 9 h after exposure to 300 μM zinc (Fig. 3A, a–c). In contrast, a significant increase in necrotic cells was observed at 9 h (Fig. 3A, c). At 24 h, annexin-negative cells had decreased and subsequently the two types of positive cells had increased (Fig. 3A, d). The annexin-positive and PI-negative cells were apoptotic, while those positive for both are likely to be late apoptotic cells derived from the annexin-negative necrotic cells. The results indicate that zinc rapidly induced necrosis and then apoptosis at a later time. Next, we examined the effects of caspase inhibitors

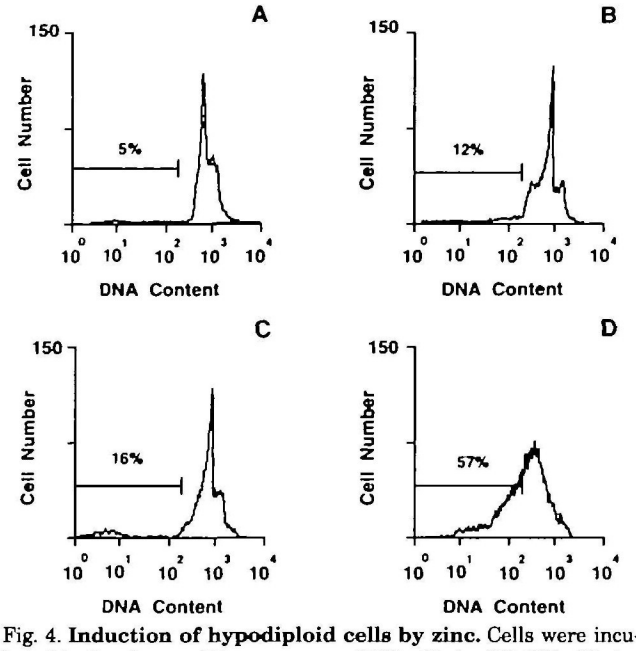

Fig. 4. **Induction of hypodiploid cells by zinc.** Cells were incubated in the absence (A) or presence of 100 μM zinc (B), 300 μM zinc (C), or 3 μM etoposide (D) for 24 h. DNA fluorescence histograms of PI-stained Molt-4 cells are shown.

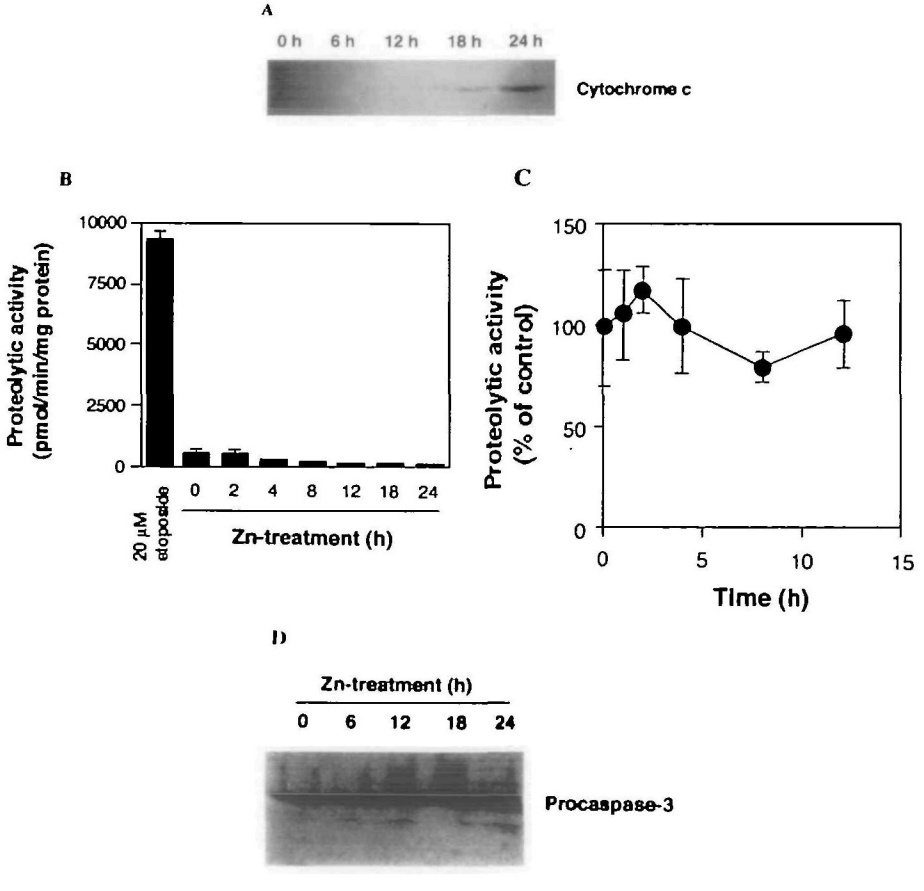

Fig. 5. **Effects of zinc on cytochrome *c* release and caspase induction.** Cells were treated with 300 μM zinc for the indicated times, and then cytosolic extracts or cell lysates were prepared. (A) The amounts of cytochrome *c* were determined by immunoblot analysis. Caspase-3 (B) and caspase-8 (C) activities were determined with Ac-DEVD-MCA and Ac-IETD-MCA as the substrate, respectively. As a reference, cells were treated with 20 μM etoposide for 6 h and then the increase in caspase-3 activity was examined (B). Proteolysis of precaspase-3 was detected using anti–caspase-3 antibodies (D).

(Fig. 3B). Ac-YVAD-CHO, an inhibitor of caspase-1 (IL-1β converting enzyme), and Ac-DEVD-CHO, an inhibitor of caspase-3, did not prevent the increase in annexin-positive cells (Fig. 3B, c and d). Ac-YVAD-CHO and Ac-DEVD-CHO could inhibit the caspases activity in Molt-4 cells because the agents inhibited apoptosis and the proteolytic cleavage of topoisomerase II in neocarzinostatin (an X-ray mimetic agent)-treated cells (data not shown).

The induction of hypodiploid cells (*34*) was also examined. Figure 4A shows the typical cell population with a 2C DNA content. A slight reduction in the number of cells with this normal DNA content was detected with treatment with 100 or 300 μM zinc for 24 h (Fig. 4, B and C), but increases in the numbers of hypodiploid cells and a concomitant reduction of cells with a normal DNA content were observed after treatment of cells with 3 μM etoposide, an inhibitor of topoisomerase II (Fig. 4D).

Activation of Caspases—The release of cytochrome *c* from the mitochondria into the cytosol and activation of caspase-3 were examined after exposure of cells to zinc. The release of cytochrome *c* was measured by immunoblot analysis of cytosol extracts and activation of caspase-3 was examined using Ac-DEVD-MCA as a substrate. Cytochrome *c* was not detected in cytosol from untreated Molt-4 cells (Fig. 5A). A slight increase was evident at 12 h after zinc treatment, and significant amounts of cytochrome *c* were detected at 24 h. There was no zinc-induced increase in caspase-3 activity, in contrast to in the case of etoposide treatment (Fig. 5B). As reported (*16*), zinc at 1 μg/ml inhibited caspase-3 activity in cell extracts, and the metal also inhibited purified caspase-3 at the nearly same concentration (data not shown). When caspase-8 activity was examined using Ac-IETD-MCA as a substrate in zinc-treated cells, no increase was evident (Fig. 5C). Caspases are synthesized as inactive proenzymes which are cleaved to give the active form. As zinc directly inhibits the activity of caspase-3, activation of caspase-3 was examined by means of proteolytic cleavage of procaspase-3. As shown in Fig. 5D, the 32 kDa precursor was not cleaved into the 17 kDa active form in the presence of zinc (*35*), although etoposide-induced conversion was detected (data not shown).

We also examined zinc-induced changes using Apo 2.7 antibodies (2.7 A6A3 clone), which react with a 38 kDa mitochondrial membrane protein (7A6 antigen) expressed as an early event in apoptosis (*36*, *37*). After exposure of Molt-4 cells to 200 μM zinc for 24 h, the number of Apo 2.7–positive cells was increased (Fig. 6C). A similar increase was also observed with 1 μM etoposide (Fig. 6D).

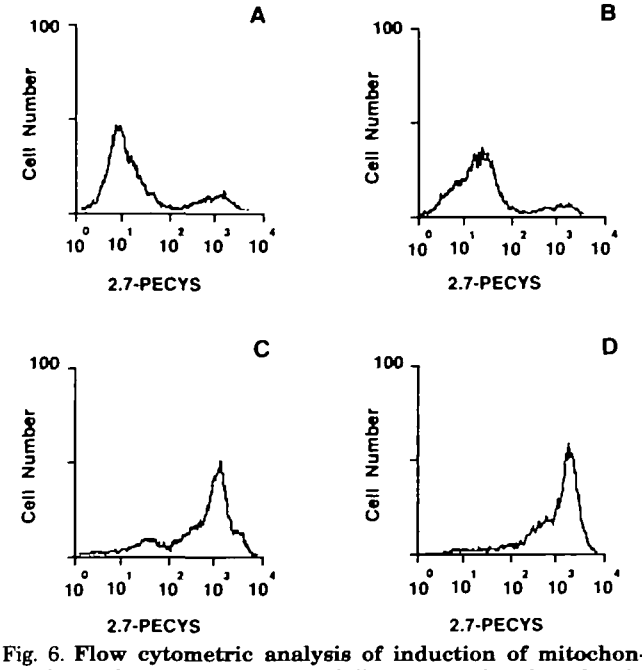

Fig. 6. **Flow cytometric analysis of induction of mitochondrial membrane protein 7A6.** Cells were incubated in the absence (A) or presence of 50 μM zinc (B), 200 μM zinc (C), or 1 μM etoposide (D) for 24 h.

DISCUSSION

Zinc at high concentrations has been shown to inhibit characteristic events in the late stages of apoptosis such as DNA fragmentation or induction of hypodiploid cells (*17*, *38*), while relatively low concentrations of the metal (80–200 μM) induced apoptosis in mouse thymocytes (*19*, *20*). We have reported that relatively high doses of zinc (200–500 μM) induce necrosis in human prostate carcinoma cells (*21*). In the present study, zinc at 100–300 μM induced necrosis and apoptosis, the cell death being independent of caspase activation. Furthermore, the induction of apoptosis was not inhibited by caspase inhibitors Ac-YVAD-CHO and Ac-DEVD-CHO. The concentration of zinc used here was only about 10-fold higher than that found in serum or tissue (*39*). Furthermore, the metal binds to serum proteins in the medium, and the concentrations required for cortical neuronal death could be reduced using a medium lacking serum (*40*). Perry *et al.* showed that 100 μM zinc caused the complete inhibition of etoposide-induced Poly(ADP-ribose) polymerase proteolysis, an apoptotic event in Molt-4 cells (*16*). We did not observe any zinc inhibition of cell growth at concentrations lower than 100 μM. These conflicting observations could be due to the fact that zinc-induced cell death mainly comprises necrosis, with some apoptosis occurring independent of caspase activation.

Apoptosis is characterized by morphological and physiological changes such as cell shrinkage, abnormal chromosome condensation, apoptotic body formation, and DNA fragmentation (*2*). However, not all cell strains exhibit the same series of events. For example, low-molecular weight fragmented DNA corresponding to nucleosomal ladders was not detected in topoisomerase II inhibitor–induced apoptotic Molt-4 cells (*41–43*). In the present study, the release from cytochrome *c*, and induction of annexin-positive, 7A6 antibody-reactive cells and abnormal chromosome condensation were observed, while activation of caspase-3 and caspase-8 were not detected, and induction of hypodiploid cells was low (Figs. 2, 4, and 6). There are at least two pathways for the activation of caspase-3. Upon anti-Fas treatment, autoproteolytic activation of caspase-8 occurs, which in turn activates other caspases such as caspase-3 and caspase-6 (*4*). We did not detect any zinc-induced increase in caspase-8 activity. Another mechanism might involve the release of cytochrome *c* from the mitochondria, an event which induces apoptosis by activating caspase-9 and caspase-3 (*35*, *44*). Since we detected zinc-induced release of cytochrome *c*, the two processes are not essentially linked. Although caspase activation and abnormal

chromosome condensation are characteristic features of apoptosis, both could be induced through separate pathways (*45*, *46*).

Annexin V, a protein with high affinity for phosphatidylserine, can bind to exposed phospholipids in apoptotic cells. Phosphatidylserine externalization is a feature of apoptosis induced by various drugs (*23*), and its recognition by macrophages promoted phagocytosis (*22*, *47*). Such externalization has been shown to be an early apoptotic event prevented by inhibitors of caspase or Bcl-2 (*24–28*). However, we found that zinc-induced annexin-positive cells appeared at a late stage rather than an early stage of apoptosis. Moreover, the induction was not prevented by caspase inhibitors, in contrast to the case of etoposide-induction. Like zinc-induced phosphatidylserine externalization, the externalization in anti-CD2 and staurosporine-treated cells was not inhibited by caspase-3 inhibitors. Thus a distinct mechanism of induction of annexin-positive cells is presumably involved, depending on cell death-induced agents. Recently, thymocytes undergoing necrosis were found to be associated with externalization of phosphatidylserine (*48*). The enzymes responsible for this process occurring at early and late stages of apoptosis have yet to be identified, although lipid scramblase (*49*) and aminophospholipid translocase (*50*) probably play roles in it. An unusual observation in this study was that many necrotic cells exhibited abnormal chromatin condensation (Fig. 2), since necrosis is in general not associated with induction of condensation, except with glutamate-induced necrosis in mouse cortical neurons (*51*).

We here obtained evidence that zinc causes mixed types of cell death, necrosis, and apoptosis, the latter occurring in annexin-positive and 7A6-reactive cells without the activation of caspase-3 and caspase-8, and the induction of hypodiploid cells. Zinc-induced phosphatidylserine externalization is independent of caspase activation, in contrast with reports of anti-cancer drugs or cytokine-induced externalization (*24–28*). The latter evidence suggests that phosphatidylserine externalization occurs in distinct pathways, caspase dependently and independently.

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