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Zinc Ions Prevent Processing of Caspase-3 during Apoptosis Induced by Geranylgeraniol in HL-60 Cells

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Geranylgeraniol (GGO) at 50 μM induces apoptosis in HL-60 cells. We examined the effects of Zn^{2+} ions on this process. Treatment of HL-60 cells with Zn^{2+} ions inhibited subsequent GGO-induced fragmentation of DNA. In a cell-free system that consisted of a specific substrate for caspase-3 and a lysate of HL-60 cells that had been treated with 50 μM GGO, Zn^{2+} ions at concentrations above 0.1 mM inhibited the activity of caspase-3. The effect of Zn^{2+} ions on the processing of caspase-3 during GGO-induced apoptosis was investigated by Western blotting, which revealed that an inactive 32-kDa precursor of caspase-3 was cleaved, in response to GGO, to yield an activated 17-kDa enzyme. Treatment of HL-60 cells with Zn^{2+} ions inhibited the cleavage of the precursor by a protease that was induced by treatment with GGO, and inhibition of this processing was well correlated with the inhibition by Zn^{2+} ions of caspase-3 activity in the cell-free system. In cell-extracted cytosols, Zn^{2+} ions inhibited the cleavage of the 32-kDa precursor by caspase-9 (Aapf-3) that was activated by addition of cytochrome *c* and dATP. These results indicate that inhibition of GGO-induced apoptosis in HL-60 cells by Zn^{2+} ions might be due to inhibition by Zn^{2+} ions of the processing of a precursor to caspase-3.

Key words: apoptosis, caspase-3, geranylgeraniol, HL-60 cells, zinc ions.

Geranylgeraniol (GGO) is a potent inducer of apoptosis in various lines of tumor cells (*1*), and the apoptosis in response to GGO has been characterized in detail (*2-4*). Identification of inhibitors of GGO-induced apoptosis should help us to understand the molecular mechanisms involved in this type of cell death. During apoptosis, several caspases are activated before the activation of endonucleases. We reported that an activity that resembled that of caspase-3 (CPP32) increased during GGO-induced apoptosis, while no activation of caspase-1 (ICE) was detectable (*4*). Zn^{2+} ions are known as a potent inhibitor of caspase-3 (*5, 6*) and pretreatment of cells with $ZnCl_2$ prevents apoptosis in response to various agents (*5, 7, 8*). Caspase-3 is expressed in cells as an inactive 32-kDa precursor, and proteolytic processing is required to generate the 17 and 12 kDa subunits that form the enzyme that is active during apoptosis (*9-11*). Some inhibitors of caspase-3, such as CrmA, TPCK, and acetyl-DEVD-aldehyde (*11, 12*), inhibit the proteolytic processing of caspase-3 (*11*).

In the present study, we examined the effects of Zn^{2+} ions on DNA fragmentation and the activity of caspase-3 (CPP32) during GGO-induced apoptosis. We found that Zn^{2+} ions suppressed the GGO-induced activation of caspase-3, and moreover, that Zn^{2+} ions inhibited the activity of an enzyme(s) that cleaved the precursor to activate caspase-3. Thus, inhibition by Zn^{2+} ions of GGO-induced fragmentation of DNA appears to be due to inhibition by Zn^{2+} ions of the maturation of caspase-3 (CPP32) rather than to inhibition of the activity of this enzyme.

MATERIALS AND METHODS

Chemicals—EDTA and HEPES were purchased from Dojindo (Kumamoto). RPMI 1640 culture medium and fetal bovine serum were purchased from Life Technologies (Rockville, MD, USA). GGO, TPCK, and DAPI were obtained from Sigma (St. Louis, MO, USA). All other chemicals were of reagent grade. A stock solution of 10 mM GGO was prepared in ethanol.

Cell Culture—Human promyelocytic HL-60 leukemia cells were maintained in RPMI 1640 medium that had been supplemented with 10% heat-inactivated fetal bovine serum, 100 units/ml of penicillin, and 0.1 mg/ml streptomycin. Cells were incubated in an atmosphere of 5% CO_2 in air at 37°C.

Quantitation of DNA Fragmentation—Cells were harvested by centrifugation at 200×*g* for 8 min, then lysed with a hypotonic solution that contained 10 mM Tris-HCl (pH 7.4), 3 mM EDTA, and 0.2% Triton X-100. The lysate was centrifuged for 30 min at 15,000×*g* to separate fragmented DNA from intact chromatin. The extent of DNA

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Abbreviations: DAPI, 4′,6-diamidino-2-phenylindole dihydrochloride; EDTA, ethylenediaminetetraacetic acid; GGO, geranylgeraniol; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; TPCK, *N*-α-tosyl-L-phenylalanine chloromethyl ketone; VP16, 4′-demethylepipodophyllotoxin ethylidene-β-D-glucoside.

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fragmentation was determined by a fluorometric method after reaction with DAPI (*1, 7*). The extent of DNA fragmentation was defined as the ratio of the amount of fragmented DNA to the total amount of DNA.

Measurement of Caspase-3 Activity—Cells were lysed in ice-cold hypotonic buffer that contained 20 mM Tris-HCl (pH 7.2), 1 mM EDTA, and protease inhibitors (*4*). Lysis was completed by two cycles of freezing at −80°C and thawing at 4°C, to prevent nonspecific cleavage of proteins. Homogenates were clarified by centrifugation for 10 min at 15,000×*g*. Supernatants were collected and diluted with 50 mM Tris-HCl (pH 7.5) that contained 0.1% CHAPS and 10 mM dithiothreitol for assays of enzymatic activity. Assays were performed in duplicate with MOCAc-DEVDA-PK(Dnp)-NH_2 (Peptide Institute, Osaka) as the substrate (*4*).

Cell-Free Model of Apoptosis—Cells were lysed in ice-cold buffer that contained 50 mM HEPES (pH 7.4), 50 mM KCl, 1 mM EGTA, 2 mM $MgCl_2$, 1 mM dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride. Lysis was completed by two cycles of freezing at −80°C and thawing at 4°C. Homogenates were clarified by centrifugation for 10 min at 15,000×*g*, and supernatants were collected and used as cytosol. Cytosols were diluted with an equal volume of the buffer, containing 10 mM HEPES (pH 7.4), 50 mM NaCl, 1 mM EGTA, 2 mM $MgCl_2$, 1 mM dithiothreitol, and 2 mM ATP, with or without 1 mM dATP and 0.3 mg/ml horse heart cytochrome *c* (*13, 14*). After 90 min at 30°C, samples were analyzed Western blotting analysis.

Western Blotting Analysis—Samples from control or GGO-treated cells were boiled in Laemmli's buffer and loaded onto a 15% polyacrylamide gel to monitor proteolysis of the precursor of caspase-3. After transfer of proteins to a polyvinylidene difluoride membrane (Immobilon P; Millipore Japan, Tokyo) in a wet electrical blotting apparatus, the membrane was blocked with 5% skimmed milk for 1 h at room temperature. The blocked membrane was incubated with a mouse monoclonal antibody against a peptide in the p20 domain of caspase-3, (Transduction Laboratories, Lexington, KY, USA) that had been diluted 1:1,000. After incubation for 1 h at room temperature, the membrane was washed extensively, then incubated with horse-radish peroxidase-conjugated second antibodies. After the membrane had been washed, immunoreactive proteins were visualized with an ECL detection system from Amersham (Buckinghamshire, UK).

RESULTS

Effects of Zn^{2+} Ions on GGO-Induced Apoptosis—Treatment of HL-60 cells with 50 μM GGO for 3 h induces internucleosomal fragmentation of DNA (*1*). Figure 1A shows the inhibitory effects of Zn^{2+} ions on the fragmentation of DNA induced by exposure of HL-60 cells to 50 μM GGO for 3 h. The HL-60 cells were treated with $ZnCl_2$ at various concentrations for 1 h prior to exposure to GGO. $ZnCl_2$ above 0.25 mM significantly prevented the fragmentation of DNA induced by GGO. Treatment with 0.9 mM $ZnCl_2$ for 60 min prior to exposure of cells to GGO also prevented the development of morphologic features of apoptosis, such as the condensation of chromatin and the fragmentation of nuclei, that are induced by GGO (data not shown). Simultaneous addition of 0.9 mM $ZnCl_2$ and 50 μM GGO did not inhibit GGO-induced endonucleolytic fragmentation of DNA. Much higher concentrations of $ZnCl_2$ were required to inhibit GGO-induced apoptosis when $ZnCl_2$ was added to cultures at the same time as GGO.

Caspase-3-like activity increases in HL-60 cells during GGO-induced apoptosis (*4*). Figure 1B shows the caspase-3 activity of extracts of HL-60 cells that had been treated with $ZnCl_2$ and then with GGO as described in the legend to Fig. 1A. $ZnCl_2$ inhibited the increase in caspase-3 activity that was induced by GGO, and the effect of $ZnCl_2$ was dependent on concentration (Fig. 1B).

Effects of Zn^{2+} Ions on GGO-Induced Activation of Caspase-3—Using the extracts of HL-60 cells that had

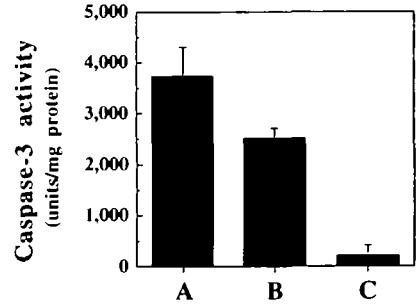
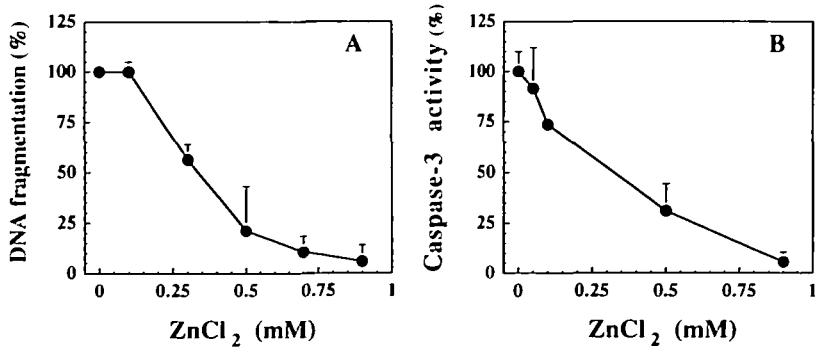


Fig. 2. **Effects of $ZnCl_2$ on the activity of caspase-3 in extracts of HL-60 cells that had been treated with GGO.** Caspase-3 activity in cell extracts was measured as described in the legend to Fig. 1. Results are means±SD from duplicate assays. (A) Caspase-3 activity in an extract of cells that had been treated with 50 μM GGO, (B) in the same extract to which 0.1 mM $ZnCl_2$ had been added, and (C) in the same extract to which 0.9 mM $ZnCl_2$ had been added.

Fig. 1. **Effects of the concentration of $ZnCl_2$ on the fragmentation of DNA and the activity of caspase-3 induced in HL-60 cells upon subsequent treatment with GGO.** HL-60 cells were incubated with $ZnCl_2$ at various concentrations for 1 h and then with 50 μM GGO for 3 h. (A) The fragmentation of DNA was determined by fluorometry after staining with DAPI. Results are means±SD of results from duplicate assays. (B) Caspase-3 activity in extracts of HL-60 cells that had been treated as described in (A) was measured with a specific fluorescent substrate. Results are means±SD from duplicate assays.



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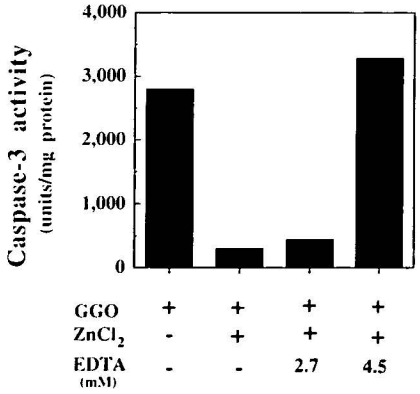

Fig. 3. **Effects of chelation of Zn^{2+} ions by EDTA.** Caspase-3 activity in an extract of cells that had been treated with 50 μM GGO was inhibited upon addition of 0.9 mM Zn^{2+} ions. Further addition of EDTA at 4.5 mM restored caspase-3 activity, but EDTA at 2.7 mM was ineffective.

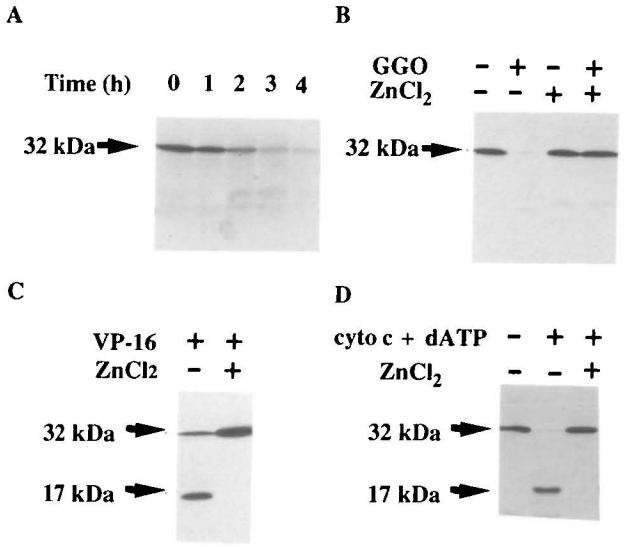

Fig. 4. **Effects of Zn^{2+} ions on processing of the precursor to caspase-3.** (A) HL-60 cells were incubated with 50 μM GGO. At the indicated times, the cells were harvested and lysed, and supernatants were analyzed for caspase-3 by Western blotting. (B) HL-60 cells were treated for 1 h with 0 to 0.9 mM $ZnCl_2$, then incubated with 50 μM GGO or (C) 40 μM VP-16. (D) Cytosol was extracted from HL-60 cells as described in "MATERIALS AND METHODS." Cytosol extracted was treated for 1 h with or without 0.3 mg/ml cytochrome *c* (cyto *c*), 1 mM dATP, and 2 mM $ZnCl_2$, then analyzed for caspase-3 by Western blotting.

been treated for 3 h with 50 μM GGO, we examined the effects of various divalent ions on caspase-3 activity. Zn^{2+} ions inhibited the caspase-3 activity in cell extracts and the effect was concentration-dependent (Fig. 2). This result indicated that Zn^{2+} ions directly inhibited the caspase-3-like activity, resembling previously reported results (*5, 6*). However, the concentration of $ZnCl_2$ required to inhibit caspase-3 was higher than that reported by Perry *et al.* (*5*). Ca^{2+}, Mn^{2+}, Ni^{2+}, and Mg^{2+} ions at concentrations as high as 1 mM had no inhibitory effect on caspase-3 like activity (data not shown).

The inhibitory effect of 0.9 mM $ZnCl_2$ on the caspase-3 activity in extracts of HL-60 cells that had been treated with 50 μM GGO was unaffected by addition of 2.5 mM EDTA to the extracts, but it was abolished by addition of 4.5 mM EDTA (Fig. 3). This result suggests that the interaction between Zn^{2+} ions and caspase-3 might be reversible but is rather stable.

Effects of $ZnCl_2$ on Processing of the Precursor to Caspase-3—Caspase-3 in HL-60 cells is present as an inactive 32-kDa precursor, and the enzyme is activated upon cleavage by an enzyme(s) in the caspase family, which yields subunits of 17 and 12 kDa (*9-11*). Figure 4A shows that the amount of the 32-kDa precursor was reduced by GGO, as determined with antibodies against caspase-3. However, pretreatment of HL-60 cells with $ZnCl_2$ prevented the GGO-induced proteolytic cleavage of the 32-kDa precursor (Fig. 4B). The antibodies against caspase-3 that we used in the present study could not detect the 17-kDa subunit in apoptosis induced by GGO (Fig. 4, A and B). The Western-blotting analysis, shown in Fig. 4, indicated that the GGO-induced processing of the precursor was inhibited by Zn^{2+} ions. Thus, prevention by Zn^{2+} ions of GGO-induced apoptosis can be attributed to inhibition of the maturation of caspase-3. For comparison, the effects of $ZnCl_2$ on the maturation of caspase-3 that is induced by VP-16 are shown in Fig. 4C. Zn^{2+} ions also prevented the cleavage of 32-kDa precursor to caspase-3 during apoptosis induced by VP-16.

Effects of $ZnCl_2$ on Processing of Caspase-3 Induced by Cytochrome c—Addition of cytochrome *c* and dATP to cytosolic extracts from some cells induced endogenous caspase-3 activation in the extracts (*13, 14*) and also that of HL-60 cells as shown in Fig. 4C. We tested whether $ZnCl_2$ would also inhibit cytochrome *c*- and dATP-dependent processing of caspase-3 in the cell-free model. $ZnCl_2$ inhibited processing of caspase-3 induced by cytochrome *c* (Fig. 4D). In this system, activated caspase-9 (Aapf-3) was shown to be cleaved and activate caspase-3 in the presence of cytochrome *c* and dATP, and this result indicates that Zn^{2+} ions can inhibit the protease activity and/or the activation of caspase-9.

DISCUSSION

Zn^{2+} ions inhibit the fragmentation of DNA and apoptosis that is induced by various inducers in various cell lines (*5, 7, 8*). These effects of Zn^{2+} ions have generally been attributed to the inhibition of an endonuclease(s) during apoptosis. Zn^{2+} ions were reported recently to be a potent inhibitor of caspase-3, and one target of inhibition by Zn^{2+} ions in apoptosis has been suggested to be caspase-3 (*5, 6*). In this study, we confirmed that Zn^{2+} ions inhibited caspase-3 (-like) activity in HL-60 cells that had also been treated with 50 μM GGO, and we found that Zn^{2+} ions prevented the conversion of the precursor of caspase-3 to an active form. The 32-kDa precursor of caspase-3 is cleaved proteolytically to generate active caspase-3 with subunits of 17 and 12 kDa by two different proteases in a two-step process. CrmA, which is a cowpox viral protein, and TPCK prevent the processing of the 32-kDa precursor (*11*). Our results suggest that Zn^{2+} ions inhibit the protease(s) that catalyzes the conversion of the precursor of caspase-3 to an active apoptosis-inducing protease, *via* a mechanism similar to that of inhibition by TPCK and

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CrmA. CrmA inhibits several members of the caspase family (*12, 15-17*), and Zn^{2+} ions might also inhibit some members of the caspase family that operate upstream of caspase-3 (*6*). Recently, Li *et al.* showed that the precursor of caspase-3 was cleaved and activated by caspase-9 (Aapf-3) activated by cytochrome *c* (*14*). The results shown in Fig. 4D suggest the possibility that Zn^{2+} ions may inhibit the process of activation of caspase-9 induced by cytochrome *c*.

The concentrations of $ZnCl_2$ required to inhibit caspase-3 in our study were above 0.1 mM, higher than those reported previously for inhibition in Molt4 cells incubated in the presence of 2% fetal calf serum (*5*). In the present study, HL-60 cells were incubated with Zn^{2+} ions in the presence of 10% fetal bovine serum. The difference in effective concentrations of $ZnCl_2$ might be due to the difference in cell lines used and/or to the concentration of serum, which contains numerous metal-binding proteins.

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