Activation of Caspase-3 in HL-60 Cells Treated with Pyrithione and Zinc

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Received November 19, 2004; accepted January 5, 2005

The transition metal zinc (Zn) is an endogenous regulator of apoptosis. The ability of Zn to modulate apoptosis is believed to be mediated by the regulation of caspase activity. Previously, we reported that an acute influx of labile Zn induced apoptosis *via* activation of caspase in human leukemia HL-60 cells treated with a Zn ionophore (Py, pyrithione) and Zn at 1 and 25 μM , respectively. In the present study, we investigated the involvement of caspase-3 in Py (1 μM)/Zn (25 μM)-induced apoptosis in HL-60 cells. Pro-caspase-3 is an inactive form of caspase-3. The processing of pro-caspase-3, a sign of caspase-3 activation, occurred 6 h after treatment with Py/Zn. Proteolysis of poly (ADP-ribose) polymerase (PARP), a substrate of caspase-3, was also observed 6 h after treatment with Py/Zn. We also confirmed the elevation of caspase-3 activity as an index of the cleavage of amino acid sequences recognized by activated caspase-3. An inhibitor of caspase-3 attenuated the appearance of the DNA ladder. Taken together, these results indicate that the activation of caspase-3 is partly responsible for the induction of apoptosis in Py/Zn-treated HL-60 cells.

Key words zinc; caspase-3; apoptosis; HL-60 cell

The effect of zinc (Zn) on apoptosis is of great interest and has been studied in *in vitro* and *in vivo* experimental models.^{1,2)} Zn has both pro-apoptotic effects and anti-apoptotic effects. While Zn attenuated the induction of apoptosis by glucocorticoid at 0.5–5.0 mM,³⁾ Zn induced apoptosis at 80–200 μM ⁴⁾ in mouse thymocytes. On the contrary, Zn at concentrations of 10 to 50 μM attenuated manganese-induced apoptosis, but higher concentrations of Zn (50–100 μM) increased cell death in human Burkitt lymphoma B cells.⁵⁾ Zn had no effect on interleukin-3-mediated apoptosis in murine B lymphoid cells but enhanced CD95-mediated apoptosis in human T lymphoid cells.⁶⁾ Thus, Zn has opposite effects on the induction of apoptosis, but the mechanism by which Zn affects apoptosis remains unclear.

Caspases are a conserved family of cysteine proteases that play pivotal roles in apoptosis.⁷⁾ Caspases are present as proenzymes that are readily cleaved and activated during apoptosis, providing the cell with a means to rapidly amplify its apoptotic response.⁸⁾ Three fundamental caspase-activating pathways in mammalian cell apoptosis have been identified: (1) a pathway in which mitochondrial functional integrity is altered, (2) a pathway in which extracellular death receptors are coupled to cytoplasmic death domains, and (3) a pathway in which caspase is activated in the endoplasmic reticulum.^{9–11)} Regardless of the apoptotic-activating pathway, caspase-3 appears to be the point of intersection for caspase-mediated cell death leading to the conserved apoptotic morphology. As a primary executor caspase, caspase-3 is responsible for many of the death foci related to apoptosis, including the induction of DNA damage and poly (ADP-ribose) polymerase (PARP) cleavage.^{12,13)}

The effect of Zn on apoptosis induction has been mainly investigated in cells treated with apoptotic stimuli, including some antitumor agents and geranylgeraniol. Inhibitory effects of Zn on the induction of apoptosis were observed with inhibition of apoptotic stimuli-mediated activation of caspase-3.^{14–16)}

Previously, we found that an influx of Zn into the cell,

which was mediated by a Zn ionophore (Py, pyrithione) and Zn, induced apoptosis *via* activation of caspases in human leukemia HL-60 cells.¹⁷⁾ Although a broad-spectrum inhibitor of caspases attenuated Py/Zn-induced apoptosis, the involvement of caspase-3 in Py/Zn-induced apoptosis is obscure. In the present study, we investigated the activation of caspase-3 in Py/Zn-treated cells and estimated the involvement of caspase-3 in Py/Zn-induced apoptosis.

MATERIALS AND METHODS

Materials Zinc sulfate and sodium pyrithione (Py) were purchased from Sigma (St Louis, MO, U.S.A.). Zinc sulfate (10 mM) and Py (100 μM) were dissolved in sterile water, and were stored at -20°C before use. Z-DEVD-fmk, an inhibitor of caspase-3, was obtained from Calbiochem-Novabiochem (San Diego, CA, U.S.A.) and dissolved in EtOH at 20 mM. Volume of vehicle used in this experiment did not exceed 1% of the culture medium.

Cell Culture HL-60 cells (human promyelocytic leukemia cells) were cultured in RPMI 1640 containing 10% fetal bovine serum, and maintained in a 5% CO_2 humidified atmosphere at 37°C .

DNA Fragmentation Assay DNA ladder formation was analyzed as described previously.¹⁸⁾ Briefly, Cells were incubated in the lysis buffer containing 10 mM Tris-HCl (pH 8.0), 10 mM EDTA, 0.5% (w/v) SDS and 0.1% (w/v) RNase A for 60 min at 50°C . Phenol/chloroform-extracted DNA was subjected to a 1.8% agarose gel electrophoresis and stained with ethidium bromide.

Western Blot Analysis Cells were lysed in the lysis buffer [1% Nonidet P-40, 20 mM Tris-HCl (pH 8.0), 137 mM NaCl, 10% glycerol, 1 mM phenylmethylsulfonylfluoride and 1 mM EDTA] by sonication. Equal amounts of samples were subjected to SDS-polyacrylamide gel electrophoresis and then transferred to polyvinylidene difluoride membranes (Millipore Co. Ltd., Bedford, MA, U.S.A.). The membrane was blocked with 10 mM Tris-HCl (pH 7.5), 100 mM NaCl

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and 0.05% Tween 20 containing 5% (w/v) nonfat milk, and then the membrane was incubated with anti-PARP Ab (BD PharMingen, San Diego, CA, U.S.A.) or anti-caspase-3 Ab (BD PharMingen) following the incubation with a horseradish peroxidase-labeled antibody as a secondary antibody. The bands reacted with the antibody were revealed by ECL-based detection (Amersham Biosciences Inc., Piscataway, NJ, U.S.A.).

Caspase Assay Activity of caspase-3 was quantified using caspase colorimetric assay kit according to the manufacturer's protocol (MBL, Nagoya, Japan).

Statistical Analysis Data were analyzed by Student's *t*-test. The acceptable level of significance was set at $p < 0.05$.

RESULTS

We previously reported that the treatment of HL-60 cells with Py ($1 \mu\text{M}$) plus Zn ($25 \mu\text{M}$) resulted in DNA fragmentation, a typical feature of apoptotic cells, and we found that a broad-spectrum inhibitor of caspase attenuated Py/Zn-induced apoptosis.¹⁶⁾ To confirm the activation of caspase-3 during apoptosis induced by Py ($1 \mu\text{M}$) plus Zn ($25 \mu\text{M}$) in HL-60 cells, we examined caspase-3 activity in Py/Zn-treated cells. As shown in Fig. 1, the activity of caspase-3 was elevated by treatment with Py/Zn. Caspase-3 is present as an inactive 32 kDa precursor and is activated upon cleavage by an upstream caspase, which yields subunits of 17 and 19 kDa, following proteolysis of PARP.^{18,19)} Next, we investigated the processing of caspase-3 and PARP proteolysis in Py/Zn-treated HL-60 cells. The cleavage of caspase-3 into 17 and 19 kDa fragments occurred 6 h after Py/Zn-treatment (Fig. 2A). PARP proteolysis was also observed 6 h after treatment with Py/Zn, which paralleled the appearance of DNA fragmentation (Figs. 2B, C). These data indicate that the activation of caspase-3 may be responsible for the induction of apoptosis in Py/Zn-treated HL-60 cells. Finally, to clarify the involvement of caspase-3 in Py/Zn-induced apoptosis, we investigated whether an inhibitor of caspase-3, Z-DEVD-fmk, inhibited apoptosis induced by Py/Zn treatment. As shown in Fig. 3, Z-DEVD-fmk attenuated DNA fragmentation induced by Py/Zn. Taken together, these results indicate that Py/Zn induces apoptosis through a caspase-3-dependent pathway.

DISCUSSION

We previously found that an acute increase in the intracel-

Fig. 1. Caspase-3 Activity in Py/Zn-Treated HL-60 Cells

HL-60 cells were treated with Py ($1 \mu\text{M}$) and Zn ($25 \mu\text{M}$) for 6 h. Then, caspase-3 activity was determined as described in Materials and Methods. Data are means $\pm$ S.D. ($n=4$). *Significant difference from the vehicle-treated cells ($p < 0.05$). Data are representative of three independent experiments.

lular Zn level using a Zn ionophore, Py, induced apoptosis *via* a caspase-dependent pathway in HL-60 cells.¹⁷⁾ In this study, we speculated that this acute increase in Zn levels may induce apoptosis partly *via* activation of caspase-3.

Zn is a familiar inhibitor of caspase-3. In 1997, Perry *et al.* used recombinant caspase-3 to show that Zn directly inhibits caspase-3 in cell-free system.¹⁴⁾ Treatment with Py/Zn increases the amount of labile Zn in the cytosol.^{20,21)} Based on these reports, Py/Zn seems to increase the amount of intracellular labile Zn levels followed by inhibition of caspase-3 activity. However, the treatment of cells with Py/Zn stimu-

A. Activation of Caspase-3

B.

C. DNA fragmentation

Fig. 2. Time-Course Study of the Processing of Caspase-3

Cells were treated with Py ($1 \mu\text{M}$) and Zn ($25 \mu\text{M}$) for the indicated time periods. Then, the cells were harvested, and processing of caspase-3 (A), PARP proteolysis (B), and appearance of DNA (C) were examined as described in Materials and Methods. Data are representative of three independent experiments.

Fig. 3. Effect of DEVD on Py/Zn-Induced Apoptosis in HL-60 Cells

After 3 h of treatment with DEVD ($100 \mu\text{M}$), the cells were treated with Py ($1 \mu\text{M}$) and Zn ($25 \mu\text{M}$) for an additional 6 h. Then, the cells were harvested, and a DNA fragmentation assay was performed. Data are representative of three independent experiments.

lated caspase-3 activity. If the Py/Zn-induced labile Zn interacts directly with caspase-3, caspase-3 may remain in an inactive form. One possible explanation is that the labile Zn may not be associated with activation of caspase-3. Although we did not examine the relationship between intracellular labile Zn and caspase-3 activation, a Zn chelator attenuated Py/Zn-induced apoptosis and PARP proteolysis, a sign that caspases, including caspase-3, have been activated.¹⁶⁾ Moreover, an inhibitor of caspase-3 decreased the appearance of DNA fragmentation (Fig. 3), suggesting that the activation of caspase-3 is at least partly responsible for the induction of apoptosis by Py/Zn in HL-60 cells. Another possible explanation is that the labile Zn may activate caspase-3 in an indirect manner. Indeed, we indicated that activation of p38 mitogen-activated protein kinase (p38MAPK) may be located upstream of the activation of caspases in Py/Zn-treated HL-60 cells.¹⁷⁾ SB203680, an inhibitor of p38MAPK, partly decreased caspase-3 activity in Py/Zn-treated HL-60 cells (Vehicle, OD400 nm=0.070±0.001; Py/Zn OD400 nm=0.225±0.004; Py/Zn plus SB203680 OD400 nm=0.137±0.003), suggesting that Py/Zn may activate p38MAPK following activation of caspase-3. Taken together, these results indicate that Py/Zn-induced apoptosis may be triggered by activation of caspase-3.

This is the first report to indicate that caspase-3 is activated in Py/Zn-treated HL-60 cells. Although additional research is needed to determine the mechanism of caspase-3 activation, our data provide new insight into the apoptosis-inducing property of Zn.

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