Caspase-3 Activation by Lysosomal Enzymes in Cytochrome c-independent Apoptosis in Myelodysplastic Syndrome-derived Cell Line P39

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ABSTRACT

In most cases, apoptosis is considered to involve mitochondrial dysfunction with sequential release of cytochrome c from mitochondria, resulting in activation of caspase-3. However, we found that etoposide induced apoptosis in P39 cells, a myelodysplastic syndrome-derived cell line, without the release of cytochrome c. Furthermore, in etoposide-treated P39 cells, no changes in mitochondrial membrane potential (ΔΨm) were detected by flow cytometry. Flow cytometry using a pH-sensitive probe demonstrated that lysosomal pH increased during early apoptosis in P39 cells treated with etoposide. A reduction in the ATP level preceded the elevation of lysosomal pH. In addition, specific inhibitors of vacuolar H^+-ATPase induced apoptosis in P39 cells but not in HL60 cells. Although etoposide-induced activation of caspase-3 was followed by DNA ladder formation in P39 cells, E-64d, an inhibitor of lysosomal thiol proteases, specifically suppressed etoposide-induced activation of caspase-3. Western blotting analysis provided direct evidence for the involvement of a lysosomal enzyme, cathepsin L. These findings indicate that lysosomal dysfunction induced by a reduction in ATP results in leakage of lysosomal enzymes into the cytosolic compartment and that lysosomal enzyme(s) may be involved in activation of caspase-3 during apoptosis in P39 cells treated with etoposide.

INTRODUCTION

Anticancer drugs that damage DNA can induce apoptosis (1–3) via perturbation of inner mitochondrial membrane permeability (4–7). MPT involving a sudden increase in mitochondrial membrane permeability is a central coordinative event of apoptosis (8–10). Apoptosis usually involves the release of cytochrome c from mitochondria. In the cytosol, cytochrome c combines with Apaf-1 in the presence of ATP to activate caspase-9, which in turn activates effector caspasess such as caspase-3. The activation of caspase-3 and subsequent degradative events are probably triggered by the release of cytochrome c from the intermembrane mitochondrial space (11). P39/Tsugane, a myelomonocytoid cell line derived from a patient with MDS, is useful for studying the differentiation of leukemia cells, the pathogenesis of MDS, and response to experimental chemotherapy (12). These cells readily undergo apoptosis in response to various agents and may be a valuable model for assessment of apoptosis in MDS. The epipodophyllotoxin derivative etoposide, an inhibitor of DNA topoisomerase II (13, 14), is widely used to treat acute leukemia and lymphoma (15, 16). The cytotoxicity of etoposide closely correlates with the number of induced DNA strand breaks.

MATERIALS AND METHODS

Chemicals. All chemicals were obtained from Nacalai Tesque (Kyoto, Japan), unless indicated otherwise. Bafilomycin A1 and concanamycin A1 were purchased from Wako Life Science (Osaka, Japan). E-64d, an inhibitor of thiol protease, was obtained from the Peptide Institute, Inc. (Osaka, Japan). FITC-dex was purchased from Sigma (St. Louis, MO). The caspase-3 substrate Ac-DEVD-AFC was obtained from Enzyme System Products (Livermore, CA). Etoposide, an epipodophyllotoxin derivative, was kindly provided by Bristol-Myers Squibb K.K. (Tokyo, Japan).

Cells and Cell Culture. P39/Tsugane, a myelomonocytoid cell line derived from a patient who had leukemia associated with MDS (12), was obtained from the Japanese Cancer Research Resources Bank (Setagaya, Tokyo, Japan) and maintained in RPMI 1640 (Nissin Bio Medical Laboratory, Kyoto, Japan) supplemented with 10% heat-inactivated FCS ( Irvine Scientific, Santa Ana, CA). 2 mm l-glutamine, 100 units/ml penicillin G, and 100 μg/ml streptomycin in an atmosphere of 5% CO2 in humidified air at 37°C. In all experiments, exponentially growing cells were used. To induce apoptosis, cells were seeded at a concentration of 1 x 10⁶ cells/ml and incubated in the presence of etoposide.

Cell Numbers and Morphology. Viable cells were counted by trypan blue exclusion in all experiments. Morphological assessment was made on cyto-centrifuge cell preparations stained with May-Grünewald-Giemsa solution (Muto Pure Chemicals, Tokyo, Japan). Apoptotic cells were identified on the basis of morphological features such as fragmented nuclei and condensed chromatin. For treatment with specific inhibitors of V-ATPase, bafilomycin A and concanamycin A1, P39 cells and HL60 cells (1 x 10⁶ cells/ml) were cultured with either drug at various concentrations for 5 h. Morphological changes and chromatin condensation associated with apoptosis were assessed by acridine orange staining with fluorescence microscopy.

Conventional Gel Electrophoresis for Detecting DNA Fragmentation. Cells (1 x 10⁶) were harvested via centrifugation and incubated at 50°C overnight in a 100 μl of lysis buffer [100 mM NaCl, 100 mM Tris-HCl (pH 8.0), 25 mM EDTA, 0.5% SDS, and 100 μg/ml proteinase K]. The samples were precipitated with an equivalent volume of 2 mM sodium iodide and 2 volumes of 2-propanol. After centrifugation at 14,000 x g for 15 min and rinsing with...
70% ethanol, the pellets were dissolved in 10 mM Tris-HCl (pH 8.0) and 1 mM EDTA containing 1 μg/ml RNase A. The samples were incubated at 37°C for 1 h, and the DNA concentration was estimated by measuring the absorbance (A) at 260 nm. DNA (5 μg/lane) was electrophoresed on 1.5% agarose gels and visualized with ethidium bromide staining.

Flow Cytometric Detection of ΔΨm. Changes in mitochondrial membrane potential (ΔΨm) were studied by the use of DiOC6-1,000. DiOC6-1,000 is incorporated into mitochondria in strict nonlinear dependence to ΔΨm and emits exclusively within the spectrum of green light (19). After etoposide treatment, 1 × 10⁷ cells were treated continuously with 40 mM DiOC6-1,000 in the culture medium for 15 min at 37°C (20). After treatment with DiOC6-1,000, the cells were washed twice with PBS, resuspended in 1 ml of PBS, and analyzed with a flow cytometer (FACScan; Becton Dickinson, San Jose, CA). The data were recorded in a FL1 photomultiplier, and 10,000 cells per sample were acquired in histograms using the data analysis program CELL Quest.

Flow Cytometric Detection of the Change in Lysosomal pH. To measure vacuolar pH, etoposide-treated cells were incubated with 0.65 mg/ml FITC-dex in a culture medium for 30 min at 37°C. After treatment with FITC-dex, the cells were washed twice with PBS, resuspended in 1 ml of PBS, and analyzed with a flow cytometer equipped with a single 488 nm argon laser. The filter in front of the FL1 photomultiplier transmitted at 530 nm and had a bandwidth of 30 nm. The data were recorded with a FL1 photomultiplier. A total of 10,000 cells/sample were acquired in histograms, using the data analysis program CELL Quest (Becton Dickinson). Dead cells and debris were excluded from analysis by electronic gating of forward and side scatter measurements.

Measurement of Caspase-3 Activity. To analyze caspase-3 activity, etoposide-treated cells (1 × 10⁷ cells) were washed twice with PBS and resuspended in 100 μl of assay buffer [0.1 M HEPES (pH 7.4), 2 mM DTT, 0.1% 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid, and 1% sucrose]. The cell suspensions were kept in liquid nitrogen and thawed at 37°C until the ice had melted in each tube. The freeze-thaw procedures were repeated three times, the cell lysates were centrifuged at 18,500 × g for 15 min, and the supernatants were obtained. Fifty μl of apoptotic extract were diluted with 450 μl of freshly prepared assay buffer. Reaction was initiated by the addition of 20 μM DEVD-AFC to 50 μl of the sample at 37°C, and product formation was measured using a Shimadzu FC 5300 spectrophotometer with excitation at 400 nm and emission at 505 nm.

Immunoblotting. To detect cytochrome c, 1 × 10⁷ cells were harvested by centrifugation at 1,000 x g for 10 min at 4°C. After washing twice with ice-cold PBS, the cell pellet was resuspended in 100 μl of ice-cold buffer A [20 mM HEPES-KOH (pH 7.5), 10 mM KCl, 1.5 mM MgCl₂, 1 mM sodium EDTA, 1 mM sodium EGTA, 1 mM DTT, and 0.1 mM phenylmethylsulfonyl fluoride] containing 250 mM sucrose. After cooling on ice for 15 min, the cells were frozen in liquid nitrogen, thawed once, and disrupted by douncing eight times in a microhomogenizer with a Teflon-coated pestle. Cytosolic fractions were obtained by centrifugation at 50,000 × g for 30 min in a Beckman tabletop ultracentrifuge. Cytosolic fractions and the pellets left after taking cytosolic fractions were subjected to immunoblotting analysis as described above, using anti-cytochrome c polyclonal antibodies (anti-cytochrome c; Santa Cruz Biotechnology, Santa Cruz, CA). To examine the involvement of lysosomal enzyme(s), 1 × 10⁷ cells were harvested by centrifugation at 1,000 x g for 10 min at 4°C. After washing twice with ice-cold PBS, the cell pellet was resuspended in 100 μl of ice-cold Tris-HCl (pH 7.4) containing 250 mM sucrose. After cooling on ice for 15 min, the cells were frozen in liquid nitrogen, thawed once, and disrupted by douncing eight times in a microhomogenizer with a Teflon-coated pestle. Cytosolic fractions were obtained by centrifugation at 50,000 × g for 30 min in a Beckman tabletop ultracentrifuge and subjected to immunoblotting analysis as described above, using anti-cathepsin L monoclonal antibodies (antihuman cathepsin L antisemur; RD Laboratorien, Mühldorf, Germany).

To detect caspase-3, cells were lysed in a nonionic detergent buffer [1% Triton X-100, 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 5 mM EDTA, 11 mM sodium vanadate, 1 mM phenylmethylsulfonyl fluoride, and 10 μg/ml leupeptin]. For the time course study, whole cell lysates were prepared by boiling with electrophoresis SDS sample buffer [2% SDS, 10% glycerol, 5% 2-mercaptoethanol, 0.001% bromophenol blue, and 62.5 mM Tris-HCl (pH 6.8)] for 3 min. Whole cell lysates were separated by SDS-PAGE and transferred onto polyvinylidene difluoride membranes (Immobilon; Millipore, Bedford, MA). The membrane blots were blocked with 5% skim milk in T-PBS for 1 h at 37°C and then incubated with the primary antibody (anti-CPP32; Santa Cruz Biotechnology) in T-PBS for 1 h at room temperature. Next, the membranes were incubated with horseradish peroxidase-conjugated goat antimouse IgG or horseradish peroxidase-conjugated goat antirabbit IgG polyclonal antibodies in T-PBS for 30 min at room temperature. After washing, enhanced chemiluminescence assay was performed, and positive bands were detected on X-ray films. Quantitative analysis of the immunosignals was carried out using an Atto Densitograph system (Atto Corp., Tokyo, Japan).

RESULTS

Etoposide-induced Apoptosis in P39 Cells. Apoptotic morphological changes were first observed 3 h after etoposide treatment and markedly increased 4 h after treatment (Fig. 1A). DNA ladder formation, a characteristic of apoptosis, began to be detected 4 h after the treatment (Fig. 1B). Caspase-3 activation is well-characterized biochemical marker of apoptosis. Because caspase-3 is activated by cleavage of the M₉, 32,000 precursor into the M₇, 20,000 NH₂-terminal fragment and M₅, 11,000 COOH-terminal fragment (21), activation of caspase-3 in etoposide-treated P39 cells was monitored by immunoblotting analysis, using a monoclonal antibody against the M₇, 20,000

![Image](331x128 to 538x489)

Fig. 1. Apoptosis of P39 cells induced by etoposide. A, P39 cells (1 × 10⁷ cells/ml) were treated with 5 μg/ml etoposide at 37°C for the indicated times. Morphological assessment was made on cytocentrifuge cell preparations stained with May-Grunwald-Giemsa solution. Apoptotic cells were identified on the basis of morphological features such as fragmented nuclei and condensed chromatin. The columns show mean values ± SD obtained from three independent experiments. B, DNA ladder formation was detected in P39 cells treated with etoposide. The cells were lysed, and extracted DNA was analyzed by conventional electrophoresis as described in “Materials and Methods.” M₀, size marker DNA (4X174/HaeIII digest).
fragment of caspase-3 (Fig. 2A). The cleavage of procaspase-3 was detected 3 h after etoposide treatment. Caspase-3 activation was also assessed by using DEVD-AFC, a tetrapeptide substrate for caspase-3 conjugated with AFC. Caspase-3-like enzyme activation occurred after 3 h (Fig. 2B). DNA ladder formation was simultaneously detected on conventional electrophoresis (Fig. 1B). These results indicated that the caspase-3-like enzyme is involved in etoposide-induced apoptosis.

No Involvement of MPT and No Release of Cytochrome c on Apoptotic Stimulation. Mitochondrial transmembrane potential (ΔΨm) is reduced very early in apoptosis, and this decline of ΔΨm is appropriate to opening permeability transition pores, which may result in mitochondrial swelling and rupture of the outer mitochondrial membrane with release of cytochrome c from the intermembrane space of the mitochondria into the cytoplasm (22). To study key events in etoposide-induced apoptosis in P39 cells and in HL60 cells, both cell types were treated with 5 μg/ml etoposide, and the involvement of MPT and the release of cytochrome c were assessed. The fluorescence intensity of DiOC6 did not change after processing with etoposide. These results indicated that ΔΨm did not change in P39 cells treated with etoposide. In contrast, DiOC6 fluorescence decreased in etoposide-treated HL60 cells (Fig. 3). Cytochrome c release was assessed by immunoblotting analysis of the cytosolic fractions and the pellets left after taking cytosolic fractions obtained from etoposide-treated P39 cells and HL60 cells. Fig. 4 showed that cytochrome c in the cytosol of HL60 cells increased after treatment with etoposide. The amount of cytochrome c in mitochondria showed a corresponding decrease. In contrast, there was little change in cytochrome c in either the cytosol or mitochondrial fractions in etoposide-treated P39 cells.

Changes in Lysosomal pH in Etoposide-treated P39 Cells. We assessed lysosomal pH in etoposide-treated P39 cells to confirm the involvement of lysosomes in etoposide-induced apoptosis. Lysosomal pH was measured by flow cytometry with FITC-dex, a pH-sensitive fluorescent probe that can enter lysosomes via fluid-phase endocytosis (23, 24). Fig. 5 shows the flow cytometric distribution of P39 cells treated with etoposide. Such cells showed a time-dependent increase in fluorescence intensity up to 90 min (Fig. 5), suggesting that lysosomal pH was increased by etoposide treatment. To examine the involvement of lysosomal enzymes, we assessed the inhibition by E-64d of caspase-3-like enzyme activation in etoposide-treated cells by a fluorometric assay with the substrate DEVD-AFC.

Inhibition of Caspase-3 Activation by E-64d in Etoposide-treated P39 Cells. In HL60 cells, pretreatment with E-64d hardly suppressed the etoposide-induced activation of caspase-3-like enzyme, indicating that E-64d itself does not suppress the activation of caspase-3-like enzyme. As shown in Fig. 6A, E-64d inhibited etoposide-induced apoptosis dose dependently. Pretreatment of P39 cells with E-64d suppressed etoposide-induced activation of caspase-3 by 52% as compared with no pretreatment (Fig. 6B). These results indicated that leakage of lysosomal cysteine protease(s) into the cytosolic compartment is involved in activation of caspase-3-like enzyme.

Detection of Cathepsin L by Immunoblotting Analysis. We used Western blotting to examine changes in expression levels of
cathepsin L proteins during apoptosis of P39 cells. The concentration of the active form of cathepsin L increased by about 2 times 1 h after etoposide treatment. The concentration gradually increased, and after 4 h, it was equivalent to about 10 times the baseline concentration (Fig. 7).

Apoptosis Induced by V-ATPase Inhibitors. To study the mechanism of lysosomal function in etoposide-induced apoptosis, we examined the effects of bafilomycin A and concanamycin A1, specific inhibitors of V-ATPase, on the lysosomal pH of P39 cells and HL60 cells. Both cells were incubated with either inhibitor at various concentrations for 5 h. Apoptosis was evaluated on the basis of morphological features such as chromatin condensation and nuclear fragmentation. Both bafilomycin A and concanamycin A1 induced apoptosis in both cell types. After bafilomycin A treatment, the rate of apoptosis was about 2-fold higher in P39 cells than in HL60 cells (Fig. 8A). After treatment with concanamycin A1, the rate of apoptosis was about 10-fold higher in P39 cells than in HL60 cells (Fig. 8B). In bafilomycin A-induced apoptosis, lysosomal pH increased in P39 cells but not in HL60 cells according to the results of flow cytometry using FITC-dex (Fig. 8C).

DISCUSSION
Recent studies suggest that at least two different pathways lead to activation of caspases and induction of apoptosis; these pathways can be distinguished by accumulation of cytosolic cytochrome c (25). The mechanism of cytochrome c-dependent apoptosis is well defined (26–28). Caspase-3 is believed to be activated by an Apaf complex consisting of Apaf-1 (human homologue of CED-4 protein), Apaf-2 (cytochrome c), and Apaf-3 (procaspase-9) (29, 30). Cytochrome c released from mitochondria during apoptosis induction plays a required role in the recruitment of caspase-9 to Apaf-1, leading to activation of caspase-9 and eventual processing of procaspase-3 (27, 31–33). Interestingly, our results demonstrated that etoposide-induced apoptosis in P39 cells had no discernible effects on cytochrome c release or mitochondrial membrane potential (ΔΨm). Although caspase-3 was activated during etoposide-induced apoptosis in P39 cells, our findings suggest that apoptosis in P39 cells treated with etoposide is not accompanied by mitochondrial dysfunction or subsequent release of cytochrome c. However, the mechanism of caspase-3 activation in cytochrome c-independent apoptosis remains to be clarified. Our findings suggest that during etoposide-induced apoptosis...
apoptosis, caspase-3 is activated by a pathway independent of cytochrome c in P39 cells.

The following results indicate that lysosomal enzymes might participate in the activation of caspase-3 during apoptosis in etoposide-treated P39 cells: (a) the cytosolic ATP level decreased 30 min after etoposide treatment (data not shown); (b) lysosomal pH began to increase 60 min after etoposide treatment; (c) the lysosomal enzyme cathepsin L was released in a time-dependent manner after etoposide treatment; (d) E-64d, an inhibitor of lysosomal thiol proteases, blocked etoposide-induced apoptosis and suppressed activation of caspase-3 in P39 cells; and (e) specific inhibitors of V-ATPase induced apoptosis more effectively in P39 cells than they did in HL60 cells. As for the mechanism by which DNA damage decreases cytosolic ATP, DNA damage may activate poly(ADP-ribose) polymerase, a nuclear enzyme that uses NAD as a substrate. Increased consumption of NAD decreases the ATP level (34, 35). In P39 cells, loss of ATP elevates lysosomal pH, which may cause leakage of the lysosomal enzymes. Lysosomes normally maintain an internal pH of about 4.7 via a membrane-bound H\(^+\)-ATPase pump (36, 37). The normal function and proper orientation of this proton pump in the lysosomal membrane are essential for maintaining an acidic pH, which is required for optimal activity of lysosomal hydrolases (38).

Previous studies indicated that a V-ATPase is expressed in the plasma membrane of some tumor cells (39). Another report suggested that intracellular acidification in neutrophils in tissue culture is an early event in the apoptosis program (40). However, we focused on the lysosomal membrane in this study. Concana
cymycin A1 and bafilomycin A are potent selective inhibitors of V-ATPase in vitro (41, 42). Nishihara et al. (43) reported that concanamycin A1 and bafilomycin A induced apoptosis in WEHI 231 cells and suggested that inhibition of V-ATPase activity triggers apoptosis. Nonetheless, the cause and mechanism of apoptotic cell death induced by V-ATPase inhibitors remain to be defined. Our results indicate that P39 cells are much more sensitive to V-ATPase inhibitors than are HL60 cells. Therefore, the lysosomal membrane of P39 cells might be influenced by reduced ATP levels, leading to leakage of lysosomal cysteine protease(s) into the cytosolic compartment.

E-64d, an inhibitor of lysosomal thiol proteases, suppressed caspase-3-like enzyme activation in etoposide-treated P39 cells. These findings suggest that lysosomal dysfunction induced by decreased apoptosis.
ATP levels results in leakage of lysosomal cysteine protease(s) into the cytosolic compartment and that lysosomal enzyme(s) might be involved in the activation of caspase-3 during apoptosis in etoposide-treated P39 cells. In addition, although a recent study reported that leakage of lysosomal cysteine protease(s) into the cytosolic compartment may be involved in the activation of caspase-3-like protease (44), it should be noted that some critical features of the models used are based on the data acquired from cell-free systems. Several other recent studies suggest that lysosomal enzymes are involved in the apoptosis mechanism and that there is an apoptotic pathway regulated by lysosomal enzymes (44–47). FITC-dex, a fluorescent fluid-phase marker of vacuoles and endocytotic pathways, has been used as an indicator of cellular lysosome content and a tool for dynamic measurement of acute and chronic effects of lysosomotropic agents (24, 48–51). Furthermore, our results have demonstrated that the use of FITC-dex is a valid technique for measuring dynamic changes in the pH of lysosomes for the investigation of cytochrome c-independent apoptosis.

Apoptosis of hematopoietic cells in the marrow of the patients with MDS has been suggested as a mechanism for the development of peripheral cytopenias (52, 53). Aul et al. (53) have indicated that extensive apoptosis of hematopoietic cells in the marrow of patients with MDS might underlie disturbed hematopoiesis, which is the hallmark of these disorders (54). However, the cause of primary MDS remains poorly understood. As one of the mechanisms of MDS, we conclude that lysosomal membrane stability might be damaged in patients with MDS, leading to the release of lysosomal enzymes followed by caspase-3 activation.

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2883

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