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Caspase Activation in MCF7 Cells Responding to Etoposide Treatment

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ABSTRACT

Studies of the biochemical mechanisms evoked by conventional treatments for neoplastic diseases point to apoptosis as a key process for elimination of unwanted cells. Although the pathways through which chemotherapeutics promote cell death remain largely unknown, caspase proteases play a central role in the induction of apoptosis in response to a variety of stimuli including tumor necrosis factor, fas ligand, and growth factor deprivation. In this article, we demonstrate the induction of caspase protease activity in MCF7 human breast carcinoma cells exposed to the topoisomerase inhibitor, etoposide. Caspase protease activity was assessed by incubating cell lysates with the known caspase substrates, acetyl-L-aspartic-L-glutamic-L-valyl-L-aspartic acid 4-methyl-7-aminocoumarin or acetyl-L-tyrosyl-L-valyl-L-aspartic acid 4-methyl-7-aminocoumarin. We observed maximal cleavage of acetyl-L-aspartic-L-glutamic-L-valyl-L-aspartic acid 4-methyl-7-aminocoumarin within 6 hr following etoposide addition, a time that precedes cell death. In contrast, acetyl-L-tyrosyl-L-valyl-L-aspartic acid 4-methyl-7-aminocoumarin was resistant to cleavage activity. This substrate cleavage specificity implies that a caspase-3-like protease is activated in response to DNA damage. Consistent with the lysate protease activity, an intracellular marker of caspase activation, poly-ADP ribose polymerase (PARP), was cleaved in a concentration- and time-dependent manner after etoposide-treatment. PARP cleavage followed caspase activation and reached maximum cleavage between 12 and 16 hr. Incubation of the cells with the peptidic caspase inhibitor z-valine-alanine-asparagine-CH_2F prevented caspase activation, inhibited PARP cleavage, and inhibited cell death. Thus, etoposide killing of MCF7 cells requires a caspase-3-like protease.

Emerging evidence suggests that chemo- and radiotherapies induce apoptosis in neoplastic cells. However, numerous tumors show resistance to these conventional therapies and may suffer from a defect in activation of apoptotic pathways. Although the precise mechanisms through which DNA-damaging agents cause cell death remain unknown, tumor suppressor proteins, such as p53, may play a central role in the responsiveness of human tumors to anticancer treatments. The clinical importance of p53 in cancer therapy is underscored by the presence of dysfunctional p53 in cancers that are refractory to antineoplastic treatments. In contrast, cancers that respond well to chemotherapeutics and radiation seem to utilize functional p53 to eliminate targeted cells by the induction of apoptosis (Lowe, 1995). In view of this, elucidation of apoptotic pathways activated by antineoplastic agents is of critical importance in the search for more effective cancer therapies.

Apoptosis results in numerous cellular changes, such as membrane blebbing, nuclear condensation, and cell shrinkage (Ueda and Shah, 1994). Diverse treatments can induce cell death in various model systems. These include the removal of growth factors, such as granulocyte/macrophage colony-stimulating factor from eosinophils (Stern *et al.*, 1992) or interleukin 2 from T cells (Duke and Cohen, 1986). Apoptosis is also induced by the addition of tumor necrosis factor/fas to MCF7 or Jurkat cells (Tewari and Dixit, 1995; Schlegel *et al.*, 1996), or by the addition of the cytotoxic T cell serine protease, granzyme B (Darmon *et al.*, 1995). In addition, ectopic expression of genes, such as *bax*, in MCF7 cells also induces apoptosis (Sakakura *et al.*, 1996). These different treatments and model systems suggest that a common cellular pathway is responsible for the ultimate phenotype of death.

Consistent with this hypothesis, the caspase family of aspartate-specific cysteine proteases is emerging as the central executioner of apoptosis. Caspase-3 is activated in a variety of cell types during apoptosis. These include cytotoxic T cells treated with fas ligand (Schlegel *et al.*, 1996) or granzyme B

ABBREVIATIONS: zVAD-FMK, z-valine-alanine-asparagine-CH_2F; AMC, aminocoumarin; Ac-DEVD-AMC, acetyl-L-aspartic-L-glutamic-L-valyl-L-aspartic acid 4-methyl-7-aminocoumarin; Ac-YVAD-AMC, acetyl-L-tyrosyl-L-valyl-L-aspartic acid 4-methyl-7-aminocoumarin; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]propanesulfonate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HL, human leukemic cells; PAGE, polyacrylamide gel electrophoresis; PARP, poly-ADP ribose polymerase; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid.

(Darmon *et al.*, 1995), human leukemic cells (HL-60; Zhou *et al.*, 1997), U-937 cells treated with 1-β-D-arabinofuranosylcytosine (Datta *et al.*, 1996), and postconfluent osteosarcoma cells (Nicholson *et al.*, 1995). Further, caspase-3 knockout mice suffer from severe developmental abnormalities attributed to the disturbed regulation of apoptosis (Kuida *et al.*, 1996).

In an effort to elucidate a pathway leading to death after activation of p53 by DNA-damaging agents, we have looked at the induction of the caspases in the MCF7 human breast carcinoma cell line (Hain *et al.*, 1996). We observed that the topoisomerase II inhibitor, etoposide, elicits activation of caspase proteases before MCF7 cell death. Biochemical and pharmacological characterization of this protease activity is consistent with the properties of caspase-3. Inhibition of the etoposide-induced caspase activity with the peptide inhibitor zVAD-FMK also blocks subsequent apoptosis in these cells. These studies suggest a critical role for this subclass of the caspase family in MCF7 cell apoptosis induced by etoposide.

Experimental Procedures

Materials. All reagents were purchased from Sigma (St. Louis, MO) unless otherwise noted.

Cell culture. MCF7 cells were obtained from the American Type Culture Collection (Rockville, MD) and maintained in Dulbecco's minimal essential medium supplemented with 10% fetal calf serum. Stock cultures were split (1:5) twice weekly. Before an experiment, the cells were cultured to 100% confluence and then incubated for 18 hr in the Dulbecco medium supplemented with 1% fetal calf serum (GIBCO, Grand Island, NY).

Protease assay. Cysteine protease activity was assessed as described previously (Fernandes-Alnemri *et al.*, 1995). The confluent, serum-fasted cells, in six-well plates, were treated with etoposide. The cells were washed twice with cold PBS and then scraped into 250 μl of cold protease assay buffer made up of 25 mM HEPES, pH 7.5, 5 mM EDTA, 2 mM dithiothreitol, and 0.1% detergent composed of CHAPS and sonicated to lyse the cells. The lysates were used immediately or, in the case of time-course studies, stored frozen at −20° until use. Twenty-five microliters of extract was assayed with 25 μl of 100 μM substrate, either Ac-DEVD-AMC or Ac-YVAD-AMC (Peptides International, Louisville, KY) in a 96-well microtiter plate at room temperature. The release of AMC was measured with a fluorescent plate reader (Cytofluor II, Perceptive Biosystems, Framingham, MA) over time, with excitation at 380 nm and emission at 460 nm as described previously (Thornberry *et al.*, 1994). The protease inhibitor, zVAD-FMK, was purchased from Enzyme Systems (Dublin, CA).

PARP Cleavage. PARP cleavage in cell lysates was assessed by Western blot analysis. Confluent, serum-fasted MCF7 cells were treated as described. The cells, in six-well plates, were washed twice with PBS, scraped into 1 ml of PBS, and centrifuged at 12,000 × *g*. The cell pellet was disrupted in 100 μl of lysis buffer (20 mM HEPES, pH 7.4, 1% Triton X-100, 50 mM sodium chloride, 1 mM EGTA, 5 mM α-glycerophosphate, 30 mM sodium pyrophosphate, 100 μM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, and 10 μg/ml leupeptin). When nonadherent cells were floating in the medium, these cells were collected by centrifugation and combined with the adherent cells before lysis. The lysates were denatured by boiling in SDS-PAGE sample preparation buffer (Laemmli, 1970). Twenty-five microliters of each sample was electrophoresed through a 12.5% SDS-polyacrylamide gel, and then blotted onto Immobilon-*p* (Millipore Corporation, Bedford, MA). The blot was probed with a rabbit polyclonal antibody raised to amino acids 13–27 of PARP (Genosys Biotechnologies, Houston, TX), followed by a goat antirabbit secondary antibody conjugated to horseradish peroxidase (Jackson Immunoresearch, West Grove, PA). The signal was visualized with enhanced chemiluminescence (Amersham, Arlington Heights IL).

Determination of cell death. Cell death was quantified using the DNA-binding dye, YOPRO-1 (Molecular Probes, Junction City, OR) (Idziorek *et al.*, 1995). Confluent, serum-fasted MCF7 cells in 24-well plates were treated with either UV or etoposide for 16 hr. YOPRO-1 was added to a final concentration of 1.0 μM, and the cells were incubated at 37° for 60 min. YOPRO-1 uptake was assessed with a fluorescent plate reader set for excitation at 460 nm and emission at 585 nm.

Results

We first determined whether caspases are activated by etoposide in MCF7 cells. In this experiment, 100 μM etoposide was added to confluent, serum-fasted MCF7 cells for 0, 3, 6, 12, 16, and 22 hr. After treatment, caspase protease activity was assessed in cell lysates by measuring hydrolysis of fluorogenic caspase substrates, Ac-DEVD-AMC and Ac-YVAD-AMC. Fig. 1 illustrates the temporal induction of caspase protease activity in MCF7 cells responding to 100 μM etoposide. After addition of etoposide, cleavage of Ac-DEVD-AMC was evident within 2 hr and maximal within 6 hr. Activity returned to control levels by 16 hr. In contrast, Ac-YVAD-AMC remained uncleaved throughout the time course and ruled out nonspecific substrate hydrolysis. Induction of the protease activity was also concentration dependent. In parallel with cell death (data not shown), 10 μM etoposide stimulated cleavage of Ac-DEVD-AMC with maximal activity occurring at an etoposide concentration of 100 μM (Fig. 2).

To confirm that DNA damage induced the cleavage of endogenous caspase substrates, we next monitored the cleavage of PARP. In this experiment, MCF7 cells were treated with 0, 5, 10, 50, 100, and 200 μM etoposide for 16 hr. Cell lysates were then prepared and proteins resolved by SDS-PAGE.

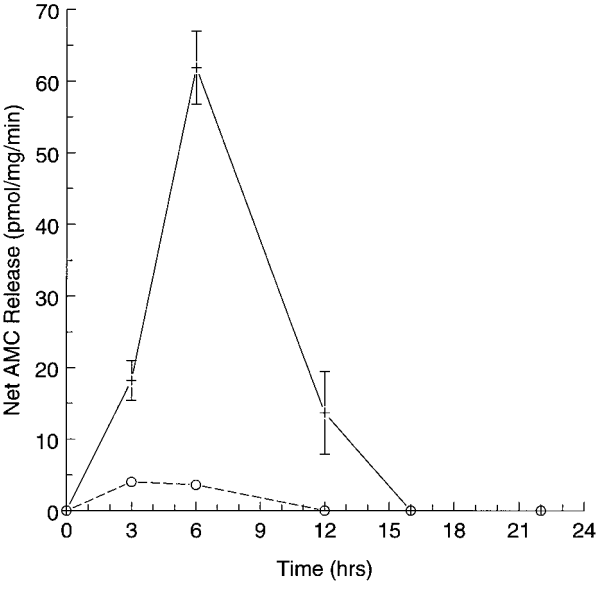

Fig. 1. Time course of etoposide-induced caspase activity in MCF7 cells. Confluent, serum-fasted MCF7 cells were treated with 100 μM etoposide for 0, 3, 6, 12, 16, and 22 hr. Protease activity at each time point was assessed against the substrates Ac-DEVD-AMC (+) and Ac-YVAD-AMC (○). The data are expressed as the mean ± standard deviation of five separate experiments.

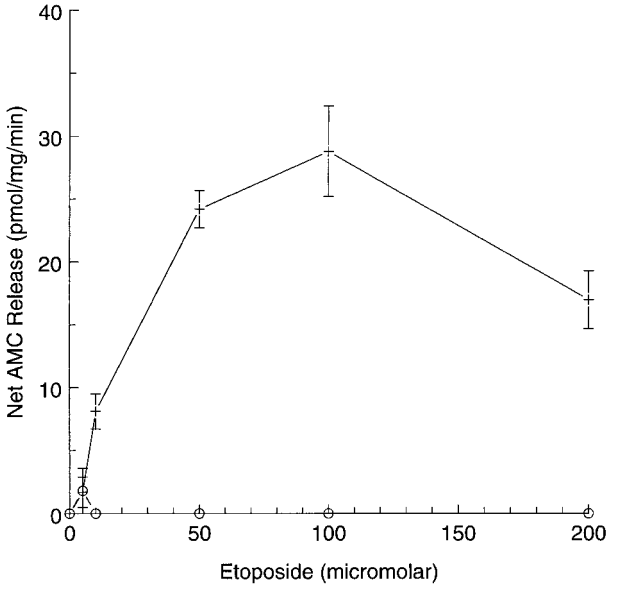

Fig. 2. Concentration-dependent induction of caspase activity by etoposide. Confluent, serum-fasted MCF7 cells were treated with 0, 5, 10, 50, 100, or 200 μM etoposide for 6 hr. Protease activity was assessed against the substrates Ac-DEVD-AMC (+) and Ac-YVAD-AMC (○). The data are expressed as the mean ± standard deviation of five separate experiments.

PARP cleavage products were detected by immunoblotting with a polyclonal antibody specific for the carboxyl-terminal 28 kDa cleavage product. In agreement with measurements of protease enzymatic activity, cleavage of PARP was detected with as little as 10 μM etoposide and saturated with 100 μM etoposide (Fig. 3A). Time-course experiments showed PARP cleavage within 12 hr of addition of 100 μM etoposide (Fig. 3B).

The caspase inhibitor, zVAD-FMK, is cell permeant and blocks apoptosis in a variety of model systems (Zhu *et al.*, 1995). We used this inhibitor to examine whether activation of caspases is required for cell death elicited by etoposide. First, we determined whether zVAD-FMK inhibited the caspase-3-like protease activity present in lysates of apoptotic MCF7 cells. In this experiment, MCF7 cells were treated with 100 μM etoposide for 6 hr, and a concentration curve was established for inhibition of the caspase enzymatic activity in cell lysates by zVAD-FMK. Fig. 4 illustrates the concentration-dependent inhibition of MCF7 cell caspase activity by zVAD-FMK. We observed inhibition of caspase activity with an IC_{50} value of approximately 40 μM and complete inhibition at 100 μM.

Based on the ability of zVAD-FMK to inhibit the protease activity from apoptotic MCF7 cells, we examined the ability of this inhibitor to block cleavage of cellular PARP. MCF7 cells were preincubated with 100 μM zVAD-FMK for 2 hr and then incubated for 16 hr with 100 μM etoposide. After treatment, portions of cell lysates were resolved by SDS-PAGE, and PARP cleavage products were detected by probing the immunoblot with an antibody to the 28-kDa fragment of PARP. As shown in Fig. 3, 100 μM etoposide induced the cleavage of PARP (Fig. 5, *lane 3*). Consistent with the inhibition of a caspase-3-like protease, pretreatment of the cells with 100 μM zVAD-FMK prevented etoposide-induced PARP cleavage (Fig. 5, *lane 4*).

Finally, we asked whether the stimulation of caspase activity and subsequent PARP cleavage were required for cell death in this model system. To accomplish this, MCF7 cells were pretreated with 0–100 μM zVAD-FMK for 2 hr and then incubated for 16 hr with 100 μM etoposide. Cell death was again assessed by the addition of YOPRO-1. Consistent with the blockade of protease activity and PARP cleavage, etoposide-stimulated cell death was inhibited by preincubation of the cells with 100 μM zVAD-FMK (Table 1).

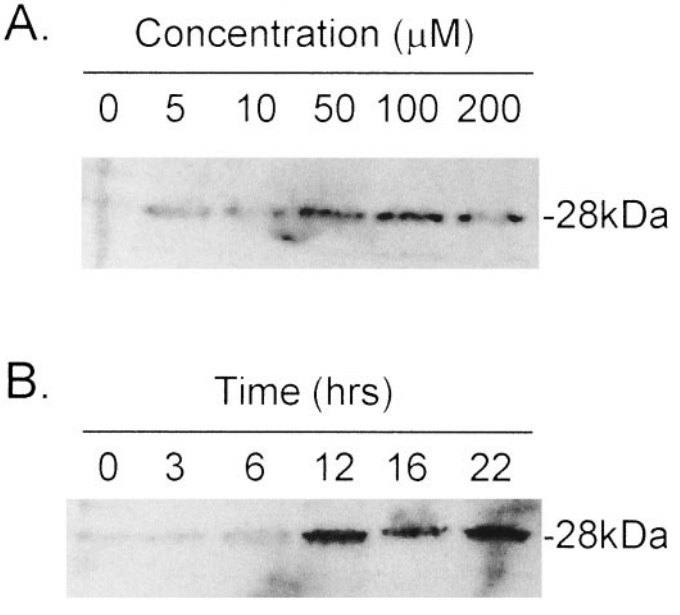

Fig. 3. Etoposide-induced cleavage of PARP. Confluent, serum-fasted MCF7 cells were treated with 0–200 μM etoposide for 16 hr (A) or with 100 μM etoposide for 0–22 hr (B). Cell lysates were resolved by SDS-PAGE and blotted onto Immobilon-*p*, and PARP cleavage was visualized with an antibody against the 28-kDa fragment of PARP. These data are representative of three separate experiments.

Discussion

Accumulating evidence indicates that chemotherapeutic agents kill neoplastic cells by the induction of apoptosis. Although the pathways involved in cell death induced by antineoplastic agents are largely unknown, the tumor suppressor p53 has been implicated in that tumors with mutated p53 often respond poorly to conventional therapies (Lowe, 1995). The role of caspase proteases in p53-mediated apoptosis is ambiguous. Recent reports demonstrated that the p53 null cell line, HL-60, utilized caspases in responding to etoposide (Martins *et al.*, 1997) and camptothecin (Shimizu and Pommier, 1997). In contrast, another report illustrates the requirement for caspase activation in p53-mediated apoptosis after UV irradiation (Fuchs *et al.*, 1997). These apparently contradictory results highlight the need to consider that diverse stimuli lead to caspase activation through both p53-dependent and p53-independent pathways. Our results substantiate the idea that caspases are activated in cells with fully functional p53 and support the hypothesis that multiple sensors of cellular insults converge on the central apoptotic machinery, the caspase proteases.

Several lines of evidence support a role for caspases in etoposide-induced MCF7 cell death. First, cell death was preceded by a rapid increase in a protease activity that specifically cleaved the peptide substrate Ac-DEVD-AMC.

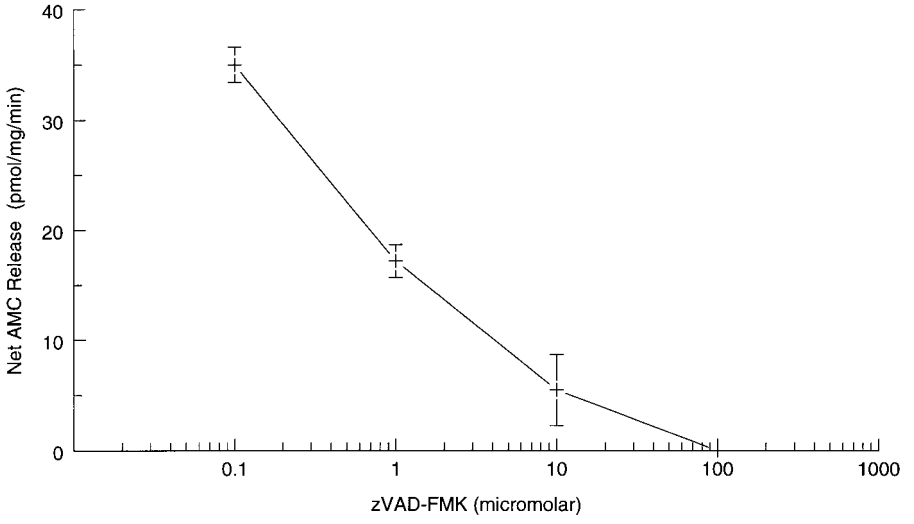

Fig. 4. zVAD-FMK inhibition of caspase activity in MCF7 cell lysates. Confluent, serum-fasted MCF7 cells were treated with 100 μM etoposide for 6 hr. Cell lysates were prepared and 0–100 μM zVAD-FMK was preincubated in the cell lysate for 15 min before measurement of protease activity against the Ac-DEVD-AMC substrate. The data are expressed as the mean ± standard error of net release of AMC. These data are representative of three separate experiments.

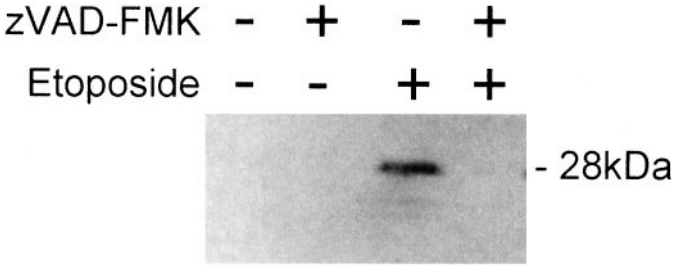

Fig. 5. zVAD-FMK inhibition of etoposide-stimulated PARP cleavage. Confluent, serum-fasted MCF7 cells were pretreated with dimethylsulfoxide (*lanes 1* and *3*) or 100 μM zVAD-FMK (*lanes 2* and *4*) for 2 hr, followed by treatment with dimethylsulfoxide (*lanes 1* and *2*) or 100 μM etoposide (*lanes 3* and *4*) for 16 hr. Cell lysates were resolved by SDS-PAGE, blotted onto polyvinylidene difluoride membranes and probed with an antibody to PARP. This experiment is representative of three separate experiments.

TABLE 1
zVAD-FMK blocks etoposide-induced apoptosis

Experiment	YOPRO Uptake
Control	3033 ± 361
Etoposide	5802 ± 708
Etoposide + zVAD-FMK	3143 ± 291

DEVD is the cleavage sequence derived from PARP and is the preferred substrate sequence of apoptotic caspases, typified by caspase-3. Consistent with this and in agreement with previous results (Kaufman, 1989), we observed cleavage of endogenous PARP in etoposide-treated cells. This confirmed that a caspase-3-like protease is active within etoposide-treated MCF7 cells. Finally, we demonstrated the requirement for caspase activation in etoposide-induced cell death with the caspase inhibitor, zVAD-FMK. Consistent with inhibition of caspase activity, pretreatment of MCF7 cells with zVAD-FMK prevented etoposide-induced PARP cleavage and blocked cell death. Taken together, these data provide strong evidence that etoposide-stimulated apoptosis in MCF7 cells is mediated by the induction of a caspase-3-like protease activity.

A number of recent studies define the substrate and inhibitor specificities for individual members of the caspase family (Margolin *et al.*, 1997; Talanian *et al.*, 1997; Zhou *et al.*, 1997). Although there is some overlap in substrate specificities within the family, several notable distinctions allow biochemical dissection of protease activities in complex mixtures. In general, death effector proteases, typified by caspase-3, readily cleave the peptide Ac-DEVD-AMC, whereas the inflammatory cytokine-processing caspases, such as caspase-1, recognize the sequence Ac-YVADAMC. The activity we observed in apoptotic MCF7 cells is similar to caspase-3-like enzymes, in that it preferentially cleaved Ac-DEVD-AMC. However, Ac-DEVD-AMC also serves as a substrate for the caspases-6, 8, and 10. Precise definition of caspase activities in etoposide-treated MCF-7 cells will require additional pharmacological probes.

The involvement of caspase proteases in chemotherapy-induced cell death provides a potential biochemical marker that predicts tumor cell responsiveness to chemotherapy. Defects in pathways linking DNA damage or cell cycle blockade to caspase activation may account the for lack of responsiveness in many human tumors and provide a focus for defining tumor chemosensitivity in future studies.

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