Distinct Steps in DNA Fragmentation Pathway during Camptothecin-induced Apoptosis Involved Caspase-, Benzyloxy carbonyl- and N-Tosyl-1-Phenylalanylchloromethyl Ketone-sensitive Activities

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ABSTRACT

Monocytic-like leukemia U-937 cells rapidly undergo morphological changes and DNA fragmentation that is typical of apoptosis following treatment with DNA topoisomerase I inhibitor (20-S-camptothecin lactone (CPT)). The tripeptide derivative benzyloxy carbonyl-Val-Ala-Asp(OMe)-fluoromethyl ketone blocks Asp-Glu-Val-Asp-ase (DEVDase) activity and prevents the occurrence of high molecular weight and oligonucleosome-sized DNA fragments associated with apoptosis in CPT-treated cells. In contrast, N-tosyl-1-phenylalanylchloromethyl ketone (TPCK) does not prevent DEVDase activity and high molecular weight DNA fragmentation but completely abrogates the appearance of oligonucleosome-sized DNA fragmentation. These results suggest that caspase 3-like activities are involved with high molecular weight DNA fragmentation pathway, whereas TPCK-sensitive activities are involved in oligonucleosome-sized DNA fragmentation pathway in CPT-treated cells. Electron micrographs reveal that caspase inhibition by benzyloxy carbonyl-Val-Ala-Asp(OMe)fluoromethyl ketone also abrogates the typical morphological changes associated with apoptosis, whereas TPCK does not delay these morphological changes that are typical of apoptosis. Caspase inhibition slows passage of the cells through G2 and causes a transient accumulation of these cells at the G2/M phase of the cell cycle following CPT treatment. In a cell-free system, when purified nuclei are incubated with apoptotic cytosolic extracts obtained from CPT-treated U-937 cells, TPCK causes a similar effect in abrogating the oligonucleosome-sized DNA fragmentation but does not affect DEVDase activity. Addition of either benzyloxy carbonyl-Val-Ala-Asp(OMe)-fluoromethyl ketone or acetyl-Asp-Glu-Val-Ala-Asp-aldehyde completely inhibits DEVDase activity in these extracts. However, acetyl-Asp-Glu-Val-Ala-Asp-aldehyde does not affect the occurrence of oligonucleosome-sized DNA fragmentation in the cell-free system, whereas the benzyloxy carbonyl derivatives benzyloxy carbonyl-Val-Ala-Asp-carboxyl group, benzyloxy carbonyl-Val-Ala-Asp-free hydroxyl group, benzyloxy carbonyl-Val-Ala-Asp-free hydroxyl group, and benzyloxy carbonyl hydrazide abolishes it markedly. Taken together, these observations show the pivotal role of DEVDase activity in triggering the apoptotic process and high molecular weight DNA fragmentation, whereas TPCK- and benzyloxy carbonyl-sensitive activities are involved in the oligonucleosome-sized DNA fragmentation pathway induced by CPT.

INTRODUCTION

Programmed cell death (apoptosis) is a genetically regulated mechanism that occurs during physiological processes such as embryonic development, maturation of the immune cells, tissue remodeling, and maintenance of tissue homeostasis and occurs because of physical or chemical stress (1–3). Deregulation of apoptosis accounts for many pathological conditions, including cancer, immune suppression, and neurodegeneration (4–8). Chemotherapeutic drugs such as DNA topoisomerase I and II inhibitors induce apoptosis in various cell lines (9–13). Cells undergoing apoptosis show distinctive morphological changes, including cell shrinkage, convolution of the plasma membrane (ultimately producing apoptotic bodies), nuclear condensation, and DNA fragmentation (14). Cleavage of chromatin into nucleosomal fragments was once considered the biochemical hallmark of apoptosis (15). There is now evidence of morphological apoptosis associated with high molecular weight DNA fragmentation without internucleosomal DNA fragmentation (16, 17). The ced-3-like cysteine protease family or caspase family plays a central role in programmed cell death (18). The pivotal role of caspase 3 activation (19–21) in mediating drug-induced apoptosis in several cell lines, including U-937 cells, has been reported recently (22–26). Caspase 3 activation is a crucial step that triggers cytoplasmic changes, nuclear disintegration, and DNA fragmentation pathway. Besides its proteolytic effect on various proteins and enzymes involved in chromatin organization and DNA metabolism, caspase 3 also activates DNA fragmentation-promoting factors such as DFF (27). Several studies have also revealed that TPCK3-, N-tosyl-L-lysylchloromethyl ketone-, and dichloro-iso-cumarin-sensitive activities are involved in DNA fragmentation pathway following apoptosis induced by DNA topoisomerase inhibitors (23, 28–36). In this study, we investigated the effect of zVAD-fmk and TPCK on the occurrence of morphological changes associated with apoptosis and the appearance of both high molecular weight and oligonucleosome-sized DNA fragmentation in CPT-treated monocytic U-937 cells. We showed that higher order and internucleosomal DNA cleavage in the apoptotic process are independent steps involved caspase, benzyloxy carbonyl- and TPCK-sensitive activities.

MATERIALS AND METHODS

Chemicals. CPT and TPCK were purchased from Sigma Chemical Co. (St. Louis, MO). The fluorogenic peptide derivatives Ac-DEVD-AMC and DABCYL-YVADAPV-EDANS and the peptide derivatives Ac-DEVD-CHO, zVA-OH, zV-OH, and z-NH₂ were purchased from Bachem Bioscience Inc. (King of Prussia, PA). The caspase inhibitor zVAD-fmk was purchased from Enzyme Systems Products (Livermore, CA). Pig liver carboxyl-ester hydrolase (carboxylesterase; EC 3.1.1.1) was obtained from Boehringer Mannheim Canada (Laval, Quebec, Canada) and used in a cell-free system to convert zVAD-fmk into zVAD-COOH. All other chemicals were of reagent grade and purchased either from Sigma and ICN or from other local sources.

1 The abbreviations used are: TPCK, N-tosyl-1-phenylalanylchloromethyl ketone; zVAD-fmk, benzyloxy carbonyl-Val-Ala-Asp(OMe)-fluoromethyl ketone; CPT, 20-S-camptothecin lactone; Ac-DEVD-AMC, acetyl-Asp-Glu-Val-Asp-amino-4-methylcoumarin; DABCYL-YVADAPV-EDANS, 4-(4-dimethyl-aminophenylazo)benzoyl-Tyr-Ala-Asp-Ala-Pro-Val-5-[(2-aminoethyl amino)naphthalene-1-sulfonic acid; Ac-DEVD-CHO, acetyl-Asp-Glu-Val-Asp-aldehyde; zVA-OH, benzyloxy carbonyl-Val-Ala-Asp-free hydroxyl group; zV-OH, benzyloxy carbonyl-Val-Ala-Asp-free hydroxyl group; z-NH₂, benzyloxy carbonyl hydrazide; zVAD-COOH, benzyloxy carbonyl-Val-Ala-Asp-free carboxyl group; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; TAE, transverse alternating pulsed field electrophoresis; PMSF, phenylmethylsulfonyl fluoride; 3066-40, Nonsider P-40; DEVDase, Asp-Glu-Val-Asp-ase.

3 The abbreviations used are: TPCK, N-tosyl-1-phenylalanylchloromethyl ketone; zVAD-fmk, benzyloxy carbonyl-Val-Ala-Asp(OMe)-fluoromethyl ketone; CPT, 20-S-camptothecin lactone; Ac-DEVD-AMC, acetyl-Asp-Glu-Val-Asp-amino-4-methycoumarin; DABCYL-YVADAPV-EDANS, 4-(4-dimethyl-aminophenylazo)benzoyl-Tyr-Ala-Asp-Ala-Pro-Val-5-[(2-aminoethyl amino)naphthalene-1-sulfonic acid; Ac-DEVD-CHO, acetyl-Asp-Glu-Val-Asp-aldehyde; zVA-OH, benzyloxy carbonyl-Val-Ala-Asp-free hydroxyl group; zV-OH, benzyloxy carbonyl-Val-Ala-Asp-free hydroxyl group; z-NH₂, benzyloxy carbonyl hydrazide; zVAD-COOH, benzyloxy carbonyl-Val-Ala-Asp-free carboxyl group; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; TAE, transverse alternating pulsed field electrophoresis; PMSF, phenylmethylsulfonyl fluoride; 3066-40, Nonsider P-40; DEVDase, Asp-Glu-Val-Asp-ase.
Cell Culture and Drug Treatments. The human U-937 cell line, obtained from the American Type Culture Collection (Manassas, VA), was grown in suspension culture at 37°C under 5% CO₂ in a humidified atmosphere in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum, 2 mM glutamine, 100 units/ml penicillin, and 100 µg/ml streptomycin (Life Technologies, Inc., Grand Island, NY). Exponentially growing cells were used throughout all experiments at a concentration of 5 × 10⁵ cells/ml. Cells were treated with CPT at a concentration of 1.0 µM in the absence or presence of zVAD-fmk or TPCK. Following a 30-min incubation at 37°C, cells were pelleted by centrifugation, and the supernatant was discarded and replaced with fresh medium with or without zVAD-fmk or TPCK. Cell number and viability were estimated by trypan blue dye exclusion assay using a hemocytometer and by MTT-based colorimetric assay according to the manufacturer (Boehringer Mannheim).

Analysis of DNA Fragmentation by Agarose Gel Electrophoresis. To visualize the oligonucleosome-sized DNA fragments, at specified times after drug treatment, cellular DNA was extracted by a salting-out procedure as described previously (37). Electrophoresis was done in 1.6% agarose gel. Gels were subjected to a 30-min run at 170 V and treated cells were prepared as described below. Caspase activities were measured by continuous fluorescence monitoring in a dual-luminescence fluorometer (model LS 50B; Perkin-Elmer) using an excitation wavelength of 360 nm and emission wavelength of 490 nm for the substrate DABCYL-YVADAPV-EDANS and an excitation wavelength of 380 nm and emission wavelength of 460 nm for Ac-DEVD-AMC. Reactions were carried out in cuvettes, and the temperature was maintained at 37°C using a water-jacketed sample compartment. The assay mixture contained 100 mM HEPES (pH 7.4), 20% glycerol, 5 mM DTT, 5 mM EDTA, and 200 µM fluorogenic peptide, Vmax

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\frac{V}{V_0} \times 100
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where \( V \) is the initial velocity in the presence of inhibitor and \( V_0 \) is the initial velocity without inhibitor.

Electron Microscopy. Cells were centrifuged at 400 × g for 10 min and washed in ice-cold PBS. Cell fixation was performed in 0.1 M Millonig's phosphate buffer (pH 7.4; 292 millimoles) containing 2.5% glutaraldehyde, staining was performed with 2% uranyl acetate, and dehydration was performed with several ethanol treatments. Sections (500–700 Å) were mounted on copper grids and stained in lead citrate. Samples were examined (JFE Enterprises, Brookville, MD) by transmission electron microscopy using a Zeiss EM10 CA microscope.

Analysis of DNA Content by Flow Cytometry. Cells were centrifuged at 1000 × g for 2 min and washed in ice-cold PBS. Cell pellets were fixed in 70% ethanol for 2 h at 4°C. After incubation, cells were pelleted by centrifugation and resuspended in a solution of 70% ethanol containing 150 µg/ml RNase A (Sigma) and incubated for 30 min at room temperature. Cells were then pelleted by centrifugation and resuspended in PBS. Propidium iodide (50 µg/ml) was added before cytometry analysis. DNA content and cell cycle distribution were analyzed using a Becton Dickinson FACStar Plus flow cytometer.

Reconstituted Cell-free System. To obtain the cytosolic extracts, control and CPT-treated U-937 cells (1 µM; 4 h after CPT treatment) were pelleted by centrifugation, washed twice in ice-cold PBS, and resuspended in lysis buffer containing 10 mM HEPES (pH 7.4), 80 mM KCl, 20 mM NaCl, 5 mM MgCl₂, 5 mM EGTA, 1 mM DTT, 1 mM PMSF, 0.15 units/ml aprotinin, 10% glycerol, and 0.3% NP-40 at a density of 10⁵ cells/ml, as described previously (38, 39). After incubation at 4°C for 10 min with gentle agitation, samples were centrifuged at 10,000 × g for 10 min, and supernatants were used as cytosolic extracts. Nuclei were prepared from control U-937 cells according to a previously described protocol, with minor modifications (40). Briefly, cells were resuspended at a density of 1.0 × 10⁶ cells/ml in a lysis buffer containing 10 mM Tris-HCl (pH 7.4), 2.5 mM KCl, 2.5 mM MgCl₂, 0.5 mM PMSF, 1 mM DTT, 1 mM EDTA, 0.5 M sucrose, and 0.05% NP-40 and incubated on ice for 10 min. After centrifugation at 800 × g for 10 min at 4°C, the pellets were washed twice in lysis buffer without NP-40; resuspended in 50 mM Tris-HCl (pH 7.5), 5 mM MgCl₂, 1 mM DTT, 0.5 mM PMSF, and 2.1 mM sucrose; and then layered over a cushion of the same buffer. After centrifugation at 30,000 rpm in a Beckman SW-55 rotor for 30 min at 4°C, the pelleted purified nuclei were resuspended in the cytosol extraction buffer without NP-40. Isolated nuclei were then incubated with cytosolic extracts for 30 min at 37°C in the absence or presence of inhibitors. Carboxylesterase (20 µg/ml) was added to convert zVAD-fmk into its active form zVAD-COOH in the reconstituted cell-free system. Reactions were stopped by addition of 0.2% SDS and 1.0 mg/ml proteinase K and incubation for 30 min at 50°C. DNA was then extracted by the salting-out procedure, and DNA fragments were visualized by ethidium bromide staining following conventional agarose gel electrophoresis and TAVE.

RESULTS

Effect of zVAD-fmk and TPCK on CPT-induced High Molecular Weight and Oligonucleosome-sized DNA Fragmentation. DNA fragmentation in apoptotic cells has at least two distinct components. DNA cleavage starts at the sites of attachment of the domains to the nuclear matrix to generate high molecular weight fragments of ~200–300 and 45–50 kbp (41). Subsequently, DNA is cleaved within

![Fig. 1. The effect of TPCK upon oligonucleosome-sized and high molecular weight DNA fragmentation induced by CPT. U-937 cells were treated with CPT (1.0 µM; 30 min) in the absence (top) and presence (bottom) of 10 µM TPCK. At indicated times after drug treatment (Lane numbers, times in h), DNA fragmentation was analyzed by standard agarose gel electrophoresis (left) and TAVE (right). Following electrophoresis, DNA was visualized by ethidium bromide staining.](https://cancerres.aacrjournals.org)
internucleosomal linkers, producing mono- and oligonucleosome-sized DNA fragments, consistent with the characteristic DNA laddering that can be visualized by standard agarose gel electrophoresis (15). We monitored and visualized the oligonucleosomal and higher molecular weight DNA fragmentation by standard agarose gel electrophoresis and TAFE, respectively. U-937 cells were treated with 1.0 µM CPT for 30 min and at selected times following drug treatment, total DNA was extracted or agarose-embedded DNA was prepared. In Fig. 1 (top left), the oligonucleosome-sized DNA fragmentation was visualized as early as 4 h after drug treatment with intensification 8 h following drug treatment. Kinetics of high molecular weight DNA fragments (Fig. 1, top right) showed a predominance of 45–50-kbp fragments. The higher molecular weight DNA fragments of >600 kbp seen in each lane are probably not involved as initial phase of DNA fragmentation taking place in programmed cell death but result from DNA-protein complex formation stabilized by DNA topoisomerase inhibitors that would ultimately signal initiation of apoptosis (42, 43). These results confirm the CPT induction of oligonucleosome-sized DNA fragmentation pathway during apoptosis (35). Our results support the involvement of TPCK-sensitive activities in oligonucleosome-sized DNA fragmentation pathway induced by CPT. In contrast, zVAD-fmk, an inhibitor of various caspases, completely abrogates the occurrence of high molecular weight DNA fragmentation. At 100 µM, zVAD-fmk delayed the appearance of both the characteristic DNA ladder (Fig. 2, top left) and the 45–50-kbp DNA fragments (Fig. 2, top right). However, 300 µM zVAD-fmk completely abrogated the oligonucleosome-sized and the high molecular weight DNA fragmentation (Fig. 2, bottom). High concentration of zVAD-fmk is usually required to favor its cellular uptake and its conversion into active free carboxyl form (zVAD-COOH) by cellular carboxylesterase activities (44). Taken together, these data suggest that caspase activities are involved more specifically in high molecular weight DNA fragmentation pathway. Moreover, high molecular weight DNA fragmentation appears to be an obligatory step to oligonucleosome-sized DNA fragmentation in these cells that is dependent upon TPCK-sensitive activities.

![Fig. 2. The effect of zVAD-fmk upon oligonucleosome-sized and high molecular weight DNA fragmentation induced by CPT. U-937 cells were treated with CPT (1.0 µM; 30 min) in the absence or presence of 100 µM (top) and 300 µM zVAD-fmk (bottom). At indicated times after drug treatment (Lane numbers, times in h), DNA fragmentation was analyzed by standard agarose gel electrophoresis (left) and TAFE (right). Following electrophoresis, DNA was visualized by ethidium bromide staining.](image)

![Fig. 3. The effect of TPCK and zVAD-fmk on caspase activities in CPT-treated U-937 cells. At the indicated times following CPT treatment (1.0 µM; 30 min) in the absence or presence of TPCK (10 µM) and zVAD-fmk (300 µM), DEVD-AMC hydrolysis was measured by fluorospectrometry. Enzyme activities were measured as initial velocities and expressed as relative intensity/min/mg. Data points, means of three independent determinations; bars, SD.](image)

![Fig. 4. The effect of TPCK and zVAD-fmk on cell proliferation and metabolic activity after CPT treatment. Control (○) and CPT-treated (△) cells with 10 µM TPCK (♦) or 300 µM zVAD-fmk (◆) were assessed for viability by trypan blue dye exclusion assays (left) and MTT-based colorimetric assays (right). Data points, means of four (left) and three (right) independent determinations; bars, SD.](image)
Effect of zVAD-fmk and TPCK on Caspase Activities. Many caspases are involved in the apoptotic process, and their activities are undoubtedly associated with the process of cell death itself (45–47). Recent studies have reported that caspase 3 is the major death-associated protease involved following DNA-damaging agents like DNA topoisomerase inhibitors (22–26), and caspase 3 activation coincides with apoptosis induction in U-937 cells (36). We monitored the effect of zVAD-fmk and TPCK on caspase activities in CPT-treated cells using the specific fluorogenic peptide substrates DABCYL-YVADAPV-EDANS and Ac-DEVD-AMC (Fig. 3). DEVDase activity measured by DEVD-AMC hydrolysis in CPT-treated U-937 cells increased significantly after drug treatment with peak activity ~4 h after drug treatment. We observed a slight difference in DEVD-AMC hydrolysis activity in cytosolic extracts obtained from CPT-plus TPCK-treated cells, showing that 10 μM TPCK has a weak effect on DEVDase activity. However, 10 μM TPCK does not prevent high level of DEVDase activity following CPT-treatment. This observation is consistent with those of others who reported that low concentration TPCK does not prevent cleavage of specific caspase substrates (35). In contrast, in cytosolic extracts obtained from CPT-plus zVAD-fmk-treated cells, no DEVDase activity is detected (Fig. 3). No increase in caspase 1-like activity was detected also using the peptide substrate DABCYL-YVADAPV-EDANS in all extracts, suggesting that caspase 1 and 8 are unlikely to be involved in CPT-induced apoptosis (data not shown).

Effect of zVAD-fmk and TPCK on Cell Viability, Morphology, and Growth. To evaluate the effect of zVAD-fmk and TPCK on cell survival after CPT treatment, kinetics of cell growth and survival were measured by trypan blue dye exclusion and MTT-based colorometric assays. As shown in Fig. 4, control U-937 cells grow exponentially throughout the 24-h incubation time, whereas CPT-treated cells die rapidly. Addition of TPCK does not prevent CPT-treated cells from dying. Interestingly, addition of zVAD-fmk maintains substantial membrane permeability and MTT-based metabolic activity in CPT-treated cells. These results suggest that zVAD-fmk, which prevents caspase activation and high molecular weight DNA fragmentation, momentarily protects cells from CPT-induced death. In contrast, TPCK, which prevents oligonucleosome-sized DNA fragmentation without interfering with DEVDase activity and high molecular weight DNA fragmentation, does not protect cell from dying. To investigate further the effect of zVAD-fmk and TPCK on CPT-induced apoptosis, electron micrographs were analyzed to evaluate their effects on cellular morphology. Fig. 5 shows that the nuclei of healthy U-937 are irregular in shape, with gulfs and protrusions. CPT-treated cells show segregation of chromatin into hypercondensed domains along the nuclear envelope and cytoplasm blebbing. Cotreatment of the cells with TPCK failed to prevent the morphological changes typical of apoptosis to occur. In contrast, zVAD-fmk treatment inhibited completely the characteristic morphological events associated with apoptosis, including chromatin condensation and cell shrinkage in CPT-treated cells. In view of the results obtained in DNA fragmentation analysis, these micrographs suggest that the observed morphological changes associated with apoptosis are an event that is independent of the oligonucleosome-sized DNA fragmentation in the apoptotic process but coincides with the appearance of DEVDase activity and high molecular weight DNA fragmentation.

To investigate further the effect of zVAD-fmk on cell proliferation...
in CPT-treated cells, DNA content and cell cycle distribution were measured by flow cytometry. The CPT-treated cells show massive subdiploid DNA content with an increase from 2% (at 0 h) to 80% (at 16 h) of apoptotic cells (Fig. 6, left). TPCK seems to reduce substantially the level of the subdiploid DNA peaks (60% at 16 h), which reflects its inhibitory effect on oligonucleosome-sized DNA fragmentation (Fig. 6, middle). The observation also indicates that cells containing high molecular weight DNA fragmentation could be distributed in the subdiploid DNA population. No oligonucleosome-sized DNA fragments are detected by agarose gel electrophoresis in cells treated with CPT and TPCK at 16 h (data not shown). In contrast, the subdiploid DNA peaks are fairly similar in CPT- plus zVAD-fmk-treated cells ranging from 2% (at 0 h) to 8% (at 16 h; Fig. 6, right). Moreover, passage of these cells through G2 is slowed, an observation that confirms the reported reversible inhibitory effects of CPT on DNA synthesis and DNA replication (48). Moreover, these cells accumulate at the G2/G1 phase of the cell cycle. Cell cycle phase distributions shown in Fig. 6 (right) are 49% G2/M, 29% S, and 19% G2 at 0 h and 68% G2/M, 20% S, and 3% G2 at 16 h after CPT and zVAD-fmk treatment. These results suggest that caspase inhibition in these cells may restore or unmask G1 cell cycle checkpoints following CPT treatment.

**Effect of zVAD-fmk and TPCK in a Reconstituted Cell-free System.** To further investigate the observations made in vivo, a reconstituted cell-free system was used in which isolated nuclei obtained from control cells were incubated in the presence of control or activated cytosolic extracts obtained 4 h after CPT treatment. Incubation was done in the presence of DEVDase activity, whereas Ac-DEVD-CHO and zVAD-COOH (zVAD-fmk with carboxylesterase) completely abrogate DEVDase activity. At 10 µM and 100 µM, TPCK has a weak inhibitory effect on DEVDase activity, whereas 300 µM TPCK inhibits >60% of DEVDase activity (Fig. 7, top). When added to purified nuclei, these activated cytosol extracts triggered oligonucleosome-sized DNA fragmentation in the reconstituted cell-free system (Fig. 7, bottom). However, these extracts did not digest plasmid DNA, suggesting a requirement of nuclear factors to cause DNA fragmentation (results not shown). TPCK, at concentrations that do not interfere significantly with DEVDase activity, abolishes the occurrence of oligonucleosome-sized DNA fragmentation. These results suggest that TPCK-sensitive activities are involved in oligonucleosome-sized DNA fragmentation pathway. Under the same conditions, high concentrations of the tetrapeptide aldehyde derivative Ac-DEVD-CHO does not affect DNA fragmentation in the reconstituted cell-free system, showing that inhibition of DEVDase activities in this cell free-system is not sufficient to abolish DNA fragmentation. These observations suggest that other DNA fragmentation-promoting factors are already activated in these extracts and are not inhibited by Ac-DEVD-CHO. Surprisingly, zVAD-COOH abolished the oligonucleosome-sized DNA fragmentation under the same conditions, whereas Ac-DEVD-CHO had no effect. To delineate more precisely the inhibitory effect of zVAD-COOH upon DNA fragmentation, we tested whether the benzoylcarbonyl derivatives zVA-OH, zV-OH, z-NHNH2, and carboxylesterase alone show similar effects. Interestingly, we observed that...
benzyloxycarbonyl compounds cause a marked reduction of the oligonucleosome-sized DNA fragmentation, even at the lowest concentration tested (Fig. 7, bottom). Taken together, these observations show that the benzyloxycarbonyl group inhibits oligonucleosome-sized DNA fragmentation without affecting DEVDase activity.

In summary, our results show that caspase activation initiates the apoptotic processes, including morphological changes associated with CPT-induced apoptosis and a high molecular DNA fragmentation pathway that must involve activation of DNA fragmentation-promoting factors. Blocking DEVDase activity in CPT-treated cells causes a transient G_1/G_0 arrest of these cells. Moreover, the oligonucleosome-sized DNA fragmentation during apoptosis involved TPCK- and benzyloxycarbonyl-sensitive activities that act downstream of DEVDase activity.

**DISCUSSION**

Antitumor agents, including the DNA topoisomerase I inhibitor CPT, can trigger cell growth arrest or rapidly activate apoptosis and DNA degradation in various cell lines (48, 49). The HL-60 and U-937 cell lines are cell systems that are commonly used to understand the molecular events that trigger p53-independent apoptosis following DNA damage. These cell lines are highly sensitive to DNA topoisomerase inhibitors and undergo rapid apoptosis after short-term drug treatment (9, 11, 36). Although apoptotic cells undergo similar cellular and nuclear changes following physiological and nonphysiological induction, it is clear now that the mechanisms by which these changes occurred may vary depending on the stimuli that trigger apoptosis but also on the intrinsic cellular context to which those stimuli are applied. Previous studies by others have shown that DNA fragmentation in thymocytes and rat liver nuclei involves a cleavage into high molecular weight DNA fragments of ~200–300 and 30–50 kbp, followed by subsequent cleavage into internucleosomal DNA fragments (29, 50, 51). According to the cell line used, apoptosis takes place with cleavage of DNA into high molecular weight fragments prior to or without oligonucleosome-sized DNA fragmentation (52). Thus, inter-nucleosomal DNA fragmentation represents a form of DNA cleavage that is dispensable though prevalent in many cell types undergoing apoptosis. This suggests that the morphological changes associated with apoptosis, characterized by cell shrinkage, cytoplasm blebbing, and chromatin condensation, are not necessarily followed by internucleosomal DNA fragmentation and would, instead, imply higher-order DNA cleavage into 45–50-kbp fragments (42, 43). Our results with CPT-induced apoptosis in the presence or absence of inhibitors are consistent with those observations and show that blocking the inter-nucleosomal DNA fragmentation does not affect morphological changes associated with apoptosis, whereas blocking caspase activity and higher-order DNA fragmentation completely abrogates those morphological changes that are typical of apoptosis.

Apoptotic cell death induction by various stimuli involves a cytotoxic proteolytic cascade that is associated with a pathway of endonuclease activation that is responsible for DNA cleavage. Ced3/Ice-like cysteine proteases or caspases have been identified and proposed to play a pivotal role in executing cell death (18, 45, 47), and more recently, caspase 3 was reported to activate DFF, a protein associated with DNA fragmentation pathway in human cells (27). In murine, caspase 3 cleaved and inactivated ICAD, the murine counterpart of DFF45, a protein that binds and inhibits CAD, a protein with DNase activity (53, 54). The human CAD-like homologue has not yet been identified, and whether TPCK and benzyloxycarbonyl interfere with these activities is unknown. Active serine-like proteases have also been proposed to play an essential role in apoptosis induced by DNA topoisomerase inhibitors (23, 28–35). Tumor necrosis factor and UV light also induce expression of a M, 24,000 serine protease that activates DNA fragmentation (30), and more recently, others have reported partial purification of cytoplasmic serine proteinase potently involved in apoptosis (55). Here, we showed that the two stages of DNA fragmentation can be separated experimentally by using specific inhibitors. Our results in cultured cells and cell-free system show that, downstream of DEVDase activity and high molecular weight DNA fragmentation, TPCK- and benzyloxycarbonyl-sensitive activities are involved in oligonucleosome-sized DNA fragmentation pathway. In treated cells, inhibition of caspase activity by zVAD-fmk prevents the morphological changes and DNA fragmentation that are typical of apoptosis. However, in cell-free system using activated cytosolic extracts, we observed that, although Ac-DEVD-CHO and zVAD-COOH inhibit caspase activity, their effects upon DNA fragmentation were strikingly different. Ac-DEVD-CHO was unable to prevent DNA fragmentation, whereas zVAD-COOH and other benzyloxycarbonyl derivatives markedly abrogate the oligonucleosome-sized DNA fragmentation. These observations suggest that, besides its effect on caspase activity, zVAD-fmk must inhibit other benzyloxycarbonyl-sensitive activities involved with DNA fragmentation in vivo. Interestingly, benzyloxycarbonyl-protected peptide derivatives are routinely synthesized because of the protective effect conferred by benzyloxycarbonyl upon endopeptidase degradation (56). The cellular targets of TPCK and benzyloxycarbonyl in these extracts are not identified yet but may be part of the DFF/CAD-like system. Further studies to purify and characterize these activities are underway.

Blocking caspase activities and, therefore, other benzyloxycarbonyl-sensitive activities by zVAD-fmk in CPT-treated U-937 cells prevents apoptosis and causes an accumulation of these cells at the G_1/G_0 phase of the cell cycle. Current anticancer drugs, including DNA topoisomerase inhibitors, can perturb the orderly progress of DNA replication and cell division (57). Mammalian cells possess complex mechanisms to regulate the progression of the cell cycle, and cell cycle checkpoints are key cell cycle events that tightly control transition of cells from one phase of the cycle to the next (58–60). G_1 checkpoints are activated by DNA damage and incompletely replicated DNA to prevent cells from undergoing replication. Although the molecular events associated with the G_1 arrest in these cells are not characterized in this study, the observation would suggest that blocking caspase activity following drug-mediated DNA damage may restore effective cell cycle checkpoints in these cells, perhaps by preventing caspase-dependent cleavage of cell cycle regulatory proteins.

**REFERENCES**

12. Del Bino, G., and Daryczewicz, Z. Camptothecin, teniposide, or 4'-(9-acridinylamino)-3-methanesulfonyl-m-anisidide, but not mitoxantrone or doxorubicin, in-

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