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Merbarone, a Catalytic Inhibitor of DNA Topoisomerase II, Induces Apoptosis in CEM Cells through Activation of ICE/CED-3-like Protease

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ABSTRACT

Merbarone (5-[*N*-phenyl carboxamido]-2-thiobarbituric acid) is an anticancer drug that inhibits the catalytic activity of DNA topoisomerase II (topo II) without damaging DNA or stabilizing DNA-topo II cleavable complexes. Although the cytotoxicity of the complex-stabilizing DNA-topo II inhibitors such as VP-16 (etoposide) has been partially elucidated, the cytotoxicity of merbarone is poorly understood. Here, we report that merbarone induces programmed cell death or apoptosis in human leukemic CEM cells, characterized by internucleosomal DNA cleavage and nuclear condensation. Treatment of CEM cells with apoptosis-inducing concentrations of merbarone caused activation of c-Jun NH_2-terminal kinase/stress-activated protein kinase, c-*jun* gene induction, activation of caspase-3/CPP32-like protease but not caspase-1, and the proteolytic cleavage of poly(ADP-ribose) polymerase. Treatment of CEM cells with a potent inhibitor of caspases, Z-Asp-2.6-dichlorobenzoyloxymethyl-ketone, inhibited merbarone-induced caspase-3/CPP32-like activity and apoptosis in a dose-dependent manner. These results indicate that the catalytic inhibition of topo II by merbarone leads to apoptotic cell death through a caspase-3-like protease-dependent mechanism. These results further suggest that c-Jun and c-Jun NH_2-terminal kinase/stress-activated protein kinase signaling may be involved in the cytotoxicity of merbarone.

DNA topoisomerase II (topo II) is a critical intracellular target of several clinically important anticancer agents including epipodophylotoxins (etoposide, teniposide), bis(2,6-dioxo) piperazines (ICRF-187, ICRF-193), and merbarone. The cytotoxicity of etoposide (VP-16) and teniposide (VM-26) generally correlates with DNA damage after stabilization of DNA-topo II cleavable complexes; the number of complexes formed is dependent upon the activity or amount of topo II (Nitiss and Beck, 1996). Although cleavable complex formation is associated with lethality, the molecular events by which these drugs cause cell death are not fully understood; even less is known about the cytotoxic mechanisms of the catalytic inhibition of topo II.

Apoptosis, or programmed cell death, is a genetically regulated process occurring naturally in response to a variety of signals, and it is characterized by plasma membrane blebbing, cell shrinkage, nuclear condensation, chromosomal DNA fragmentation, and formation of apoptotic bodies (Wyllie, 1997). Although the initiation stage of apoptosis depends on the type of inducer, the effector and the execution stages are common to several apoptotic stimuli. Numerous studies have mapped the effectors of programmed cell death (White, 1996). Induction of apoptosis by tumor necrosis factor and Fas ligand (Cohen, 1997), VP-16, and camptothecin (CPT; Mashima et al., 1995; Seimiya et al., 1997) involves the activation of a cascade of cysteine proteases that are homologs of the interleukin 1β-converting enzyme (ICE; now termed caspases). To date, 13 caspases in mammalian cells have been identified and are classified into three groups: 1) the caspase-1 family, 2) the caspase-2 family, and 3) the caspase-3 family (Humke et al. 1998 and references therein). Caspases are activated during apoptosis by proteolytic cleavage (Thornberry and Molineaux, 1995). Caspase-3, previously called CPP32/Yama/Apopain, shares a closer homology with Ced-3, the death protease of *Caenorhabditis elegans* (Nicholson et al., 1995), and it is the commonly activated caspase. Once activated, caspases cleave a variety of substrates, including poly(ADP-ribose) polymerase (PARP), DNA-activated protein kinase, the 70 kDa polypeptide subunit of U1 small nuclear ribonucleoprotein complexes, and

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ABBREVIATIONS: ICE, interleukin 1β-converting enzyme; CED-3, *Caenorhabditis elegans* cell death protein; PBS, phosphate-buffered saline; PARP, poly(ADP-ribose) polymerase; DAPI, 4,6-diamino-2-phenylindole; JNK/SAPK, c-Jun NH_2-terminal kinase/stress-activated protein kinase; ERK, extracellular signal-regulated kinase; MAP, mitogen-activated protein; Ac-DEVD-MCA, acetyl-Asp-Glu-Val-Asp-methylcoumaryl-7-amide; Ac-YVAD-MCA, acetyl-Tyr-Val-Ala-Asp-methylcoumaryl-7-amide; Z-Asp, Z-Asp-2,6-dichlorobenzoyloxymethyl-ketone; topo II, topoisomerase II.

GDP dissociation inhibitor for the Ras-related Rho family GTPases, protein kinase C δ (Thornberry, 1997).

c-Jun NH_2-terminal kinase/stress-activated protein kinase (JNK/SAPK) is a member of the mitogen-activated protein (MAP) kinase family that includes extracellular signal-regulated kinase 1/2 and p38 (Davis, 1994). Ten JNK isoforms were identified in human brain by molecular cloning (Gupta et al., 1996). These protein kinases correspond to alternatively spliced isoforms derived from JNK1 (46 kDa), JNK2 (55 kDa), and JNK3 (48 kDa) genes (Gupta et al., 1996). Activation of JNKs requires phosphorylation at Thr-183 and Tyr-185 by a dual specificity kinase MKK4 (mitogen activated protein kinase kinase 4; (Lin et al., 1995). In turn, JNK phosphorylates the transcription factor c-Jun at Ser63 and Ser73 within its N terminal transactivation domain, and augments its transcriptional activity. In addition to phosphorylating c-Jun, JNK has also been shown to phosphorylate the transcription factors ATF2 and Elk1 (Karin et al., 1997).

Recent reports have indicated that JNK may be required for some form of apoptosis induced by UV-C and γ-irradiation, and DNA-damaging drugs (Chen et al., 1996; Zanke et al., 1996). JNK1 positively regulated VP-16- and CPT-induced apoptosis in human myeloid leukemia U937 cells by activating Z-Asp-2,6-dichlorobenzoyloxymethyl-ketone (Z-Asp)-sensitive ICE/CED3-like proteases (Seimiya et al., 1997), and the abrogation of JNK1 activation by 2-deoxyglucose inhibits apoptosis induced by these agents in U937 cells (Haga et al., 1998). Furthermore, correlations between c-*jun* expression and apoptosis have been noticed in this laboratory (Kim and Beck, 1994).

Here, we report that merbarone, a non-DNA damaging topo II inhibitor, activates JNK1 and JNK2, as well as c-*jun* gene expression in human leukemic CEM cells. At apoptosis-inducing concentrations, merbarone activates caspase-3-like proteases but not caspase-1. The inhibition of caspase-3-like protease activity by Z-Asp, a potent caspase inhibitor, abrogated merbarone-induced apoptosis. These results indicate the involvement of caspase-3-like proteases in apoptosis induced by merbarone in CEM cells, and suggest that JNK/SAPK signaling may also be involved in this phenomenon.

Materials and Methods

Cells and Growth Inhibition Assay. Human leukemic cell CEM cells were grown in SMEM (BioWhittaker, Walkersville, MD), supplemented with 10% fetal bovine serum (Sigma Chemical Co., St. Louis, MO) and 2 mM L-glutamine (Life Technologies, Gaithersburg, MD) at 37°C in a humidified chamber in air with 5% CO_2. Merbarone was generously provided by Dr. Randall K. Johnson (SmithKline Beecham, King of Prussia, PA) and Dr. Ven Narayanin (Investigational Drug Branch, National Cancer Institute, Bethesda, MD). The concentration of merbarone used in this study (200 μM) corresponds to 10 times the concentration of drug that inhibits 50% of the growth (IC_{50}) as assayed in a 48-h growth inhibition assay as described previously (Kusumoto et al., 1996). This concentration of the drug was used in all experiments unless otherwise mentioned.

Nuclear Staining Assay. After 24-h treatment with merbarone, CEM cells were collected by centrifugation at 2000*g* for 5 min, and washed twice with ice-cold phosphate-buffered saline (PBS) before being fixed in a solution of methanol/acetic acid (3:1) for 30 min. Fixed cells were placed on slides and stained with 1 μg of 4,6-diamino-2-phenylindole (DAPI)/ml for 10 min, and then washed with PBS. The nuclear morphology of cells was observed by fluorescence microscopy with a ×40 objective. At least 200 cells were scored in three randomly chosen fields for the incidence of apoptosis. The percentage of apoptotic cells was calculated as a ratio of the number of apoptotic cells with fragmented nuclei and condensed chromatin to the total number of cells.

DNA Fragmentation Assay. At the indicated times after treatment, 3×10^6 cells were pelleted and washed twice with PBS and lysed on ice for 30 min in 400 μl of lysis buffer (10 mM Tris-HCl, pH 7.4; 20 mM EDTA, pH 8.0; 0.2% Triton X-100). After centrifugation, the supernatants were extracted with 400 μl of phenol/chloroform/isoamyl alcohol (25/4/1). Supernatants were then mixed with 60 μl of 5 M NaCl and 1 ml of 100% ethanol, and incubated overnight at −20°C. DNA samples were pelleted by centrifugation at 11,000*g* for 20 min. The pellets were resuspended in 20 μl TE + Rnase A (0.1 mg/ml) and incubated for 2 h at 37°C. The samples were then loaded into a 1% agarose gel in TAE buffer with a 123 base pair DNA ladder (Gibco BRL, Gaithersburg, MD) as molecular size marker. After electrophoresis, the gel was stained with ethidium bromide (2 μg/ml) for 1 h, washed, and photographed. The negative image of the stained gel is shown in the figures.

Northern Blot Analysis. Total RNA was isolated from CEM cells by guanidinium thiocyanate-phenol-chloroform extraction (Chomezynski and Sacchi, 1987), fractionated by electrophoresis on 1.2% agarose gels containing 7% formaldehyde, and transferred to Hybond N-nylon membrane (Amersham, Arlington Heights, IL) in 150 mM ammonium acetate. The prehybridization and the hybridization steps were performed as described previously (Kim and Beck, 1994). The c-*jun* cDNA probe (1 kb *Pst*I-*Eco*RI) was generously provided by Dr. M. Roussel (St. Jude Children's Research Hospital, Memphis, TN). The GAPDH cDNA probe was generously provided by Dr. A. Jacquemin-Sablon (Institut Gustave Roussy, Villejuif, France).

Western Blot Analysis. The protein samples were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto nitrocellulose membranes. The membranes were blocked with 5% nonfat dry milk in Tris-buffered saline containing 0.05% Tween 20 and incubated with primary antibodies. Rabbit anti-JNK1 (C-17) was obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA) and the secondary IgG antibodies were purchased from Amersham. Phosphorylation of c-Jun and JNKs was detected with anti-phospho-c-Jun Ser63/73 and anti-phospho-JNKs Thr183/Tyr185 antibodies essentially following the immunoblotting protocol provided by the manufacturer (New England Biolabs, Inc., Beverly, MA). The anti-poly(ADP-ribose) polymerase antibody was obtained from Upstate Biotechnology, Inc., (Lake Placid, NY). The proteins were visualized using the ECL system (Amersham). The intensity of autoradiograms was quantified using a GS-700 imaging densitometer (Bio-Rad, Hercules, CA). Appropriate controls for the linearity of the autoradiography were performed in all the experiments. The blots were exposed to X-ray film in such a way that the intensity of the bands was in the linear range.

ICE-like Protease Assay. After merbarone treatment, all steps were performed at 4°C. Cells were washed twice with ice-cold PBS and once in buffer A (50 mM Tris-HCl, pH 7.4; 50 mM beta glycerophosphate; 1 mM EGTA; 5 mM $MgCl_2$; 1 mM dichlorodiphenyltrichloroethane; 1 mM phenylmethylsulfonyl fluoride; 10 μg pepstatin A/ml; 10 μg aprotinin/ml; and 10 μg leupeptin/ml). Cells were then lysed for 15 min on ice in buffer A plus 100 μg digitonin/ml. After centrifugation for 10 min at 12,000*g*, caspase activity in the supernatant (cytosol) was determined in 50 μl reactions using the ICE-specific substrate tetrapeptide reporter, acetyl-Tyr-Val-Ala-Asp-4-methyl-coumaryl-7-amide (Ac-YVAD-MCA) (Peninsula Laboratories, Inc., Belmont, CA; Thornberry et al., 1992) or the CPP32-specific substrate, acetyl-Asp-Glu-Val-Asp-4-methyl-coumaryl-7-amide (Ac-DEVD-MCA) (Peptides International, Inc., Louisville, KY). Briefly, 10 μg of protein extract were incubated with 100 μM substrate peptide in 100 mM HEPES, pH 7.5; 10% sucrose; 10 mM dichlorodiphenyltrichloroethane; and 0.1% 3-[(3-cholamidopropyl)dimethylammonio]propanesulfonate. Fluorescence was

measured after 2 h at 37°C with excitation at 360 nm and emission at 460 nm using a fluorescence spectrophotometer (PerSeptive Biosystems, Inc., Framingham, MA).

Results

Merbarone induces apoptosis in CEM cells. To characterize the cell death induced by the catalytic inhibitor of topo II, merbarone, we examined the morphology of CEM cells with a fluorescent DNA-binding agent DAPI. Within 24 h of treatment with 200 μM merbarone, CEM cells exhibited condensed and fragmented nuclei (Fig.1). Internucleosomal DNA cleavage, a hallmark of apoptosis (Wyllie, 1997), was evident by 12 h (Fig. 1C). Merbarone induces apoptosis in a dose-dependent manner (Fig. 1D). These results indicate that merbarone induced apoptosis in CEM cells.

Merbarone Activates JNK/SAPK. JNK/SAPK is activated in response to a diverse group of extracellular signals that induce apoptosis, such as UV and γ-irradiation, inflammatory cytokines, and other cellular stresses (Chen et al., 1996; Zanke et al., 1996). Activation of JNK/SAPK occurs via phosphorylation at Thr183 and Tyr185 by the dual specificity enzyme SEK/MKK4 (Lin et al., 1995). Activation of endogenous JNK during merbarone treatment of CEM cells was examined by Western blot analysis using a phospho-JNK/SAPK (Thr183/Tyr185) rabbit polyclonal antibody that detects the dually phosphorylated isoforms of all JNKs/SAPKs at the Thr187 and Tyr185 residues, but not extracellular signal-regulated kinase 1/2 or p38 MAP kinases. As shown in Fig. 2, 200 μM merbarone caused persistent JNK1/2 activation. JNKs were activated as early as 90 min, peaked around 3 to 6 h, and gradually decreased, but remained higher than the 0-h time point. JNK1 activation was not due to an increase in JNK1 protein expression, and preceded the appearance of the internucleosomal DNA cleavage (Fig. 1C). Merbarone induced activation of JNKs in a dose-dependent manner (Fig. 2C).

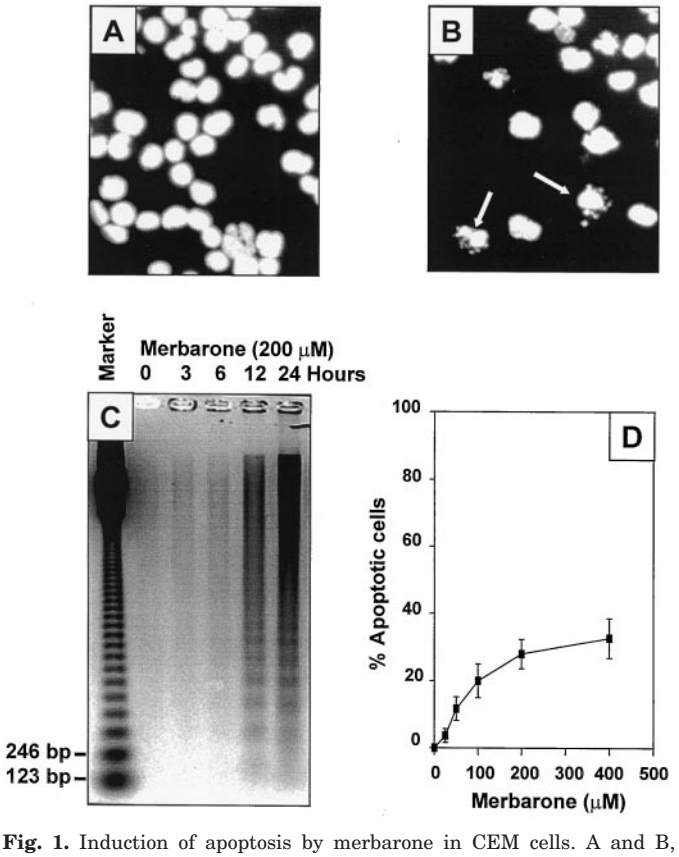

Fig. 1. Induction of apoptosis by merbarone in CEM cells. A and B, nuclear condensation. CEM cells were treated either with DMSO as control (A) or merbarone (200 μM; B) for 24 h. Cells were harvested, washed with ice-cold PBS, fixed in methanol-acetic acid (3:1), and stained with DAPI as described in *Materials and Methods*. Arrows, apoptotic cells with condensed or fragmented nuclei. C, internucleosomal DNA cleavage. 3×10^6 cells were treated with merbarone (200 μM) for the indicated times. Cellular DNA was extracted and analyzed by 1% agarose gel electrophoresis as described in *Materials and Methods*. Marker, 123 base pair ladder marker. D, treated cells with increasing concentrations of merbarone for 24 h were stained with DAPI and the percentage of apoptotic cells was calculated as described in *Materials and Methods*.

Merbarone Induces c-*jun* Gene Expression. Once activated, JNKs phosphorylate c-Jun at Ser63 and Ser73 within its NH_2-terminal activation domain, which augments its transcriptional activity and expression (Karinet al., 1997). Work from this laboratory (Kim and Beck, 1994) and others (Seimiya et al., 1997) had shown an association between c-*jun* proto-oncogene expression and apoptosis induced by DNA-topo II covalent complex stabilizing agents, VM-26 and VP-16. We therefore examined c-*jun* expression and activation during treatment of CEM cells with merbarone. As shown in Fig. 3, Northern blot analysis revealed that treatment of CEM cells with 200 μM merbarone induced transient expression of c-*jun* mRNA with maximum at 6 h, followed by down-regulation by 12 h (Fig. 3, A and B). C-*jun* mRNA expression also increased in a dose-dependent manner (Fig. 3C).

The phosphorylation of c-Jun at Ser63 and Ser73 was determined by Western blot analysis using phospho-specific c-Jun (Ser63) and c-Jun (Ser73) rabbit antibodies that do not cross-react with the corresponding phosphorylated form of JunB or JunD. Consistent with activation of JNKs/SAPKs (Fig. 2) and c-*jun* gene induction (Fig. 3), merbarone induced c-Jun protein expression and phosphorylation at Ser63 and Ser73 in a time- and dose-dependent manner (Fig. 4). Although c-Jun protein levels increased during merbarone treatment, its phosphorylation at Ser63 and Ser73 was transient with maximum at 12 h.

Merbarone Stimulates Caspase-3-like Protease Activity. Recent studies suggested that activation of caspases plays a critical role in the execution stage of apoptosis (Thornberry, 1997). In addition, JNK1 activates caspase-3-like proteases in U937 cells during VP-16- and CPT-induced apoptosis (Seimiya et al., 1997). Because JNK is activated during merbarone-induced apoptosis, we asked whether caspases are activated also. Cytosolic extracts from CEM cells treated with merbarone were incubated with the fluorogenic peptide substrates Ac-YVAD-MCA and Ac-DEVD-MCA. The first substrate detects caspase-1-like activity, whereas the second detects caspase-3-like activity. Treatment of CEM cells with 200 μM merbarone was accompanied by 4- to 5-fold increase in activity that cleaved Ac-DEVD-MCA within 6 to 12 h (Fig. 5A) and preceded the internucleosomal DNA cleavage (Fig. 1C). No increase in caspase activity that cleaves Ac-YVAD-MCA (caspase-1) was observed during merbarone treatment (Fig. 5A). Ac-DEVD-MCA cleavage also increased in a dose-dependent manner (Fig. 5B). These results indicate that caspase-3-like protease activity is stimulated during merbarone-induced apoptosis.

Merbarone Causes the Cleavage of PARP. Activation of caspase-3-like protease leads to the cleavage of several proteins, among them PARP (Tewari et al., 1995). PARP is thought to play a multifunctional role in several cellular processes such as DNA repair and recombination, and maintenance of chromosomal stability, but not in apoptosis (Wang

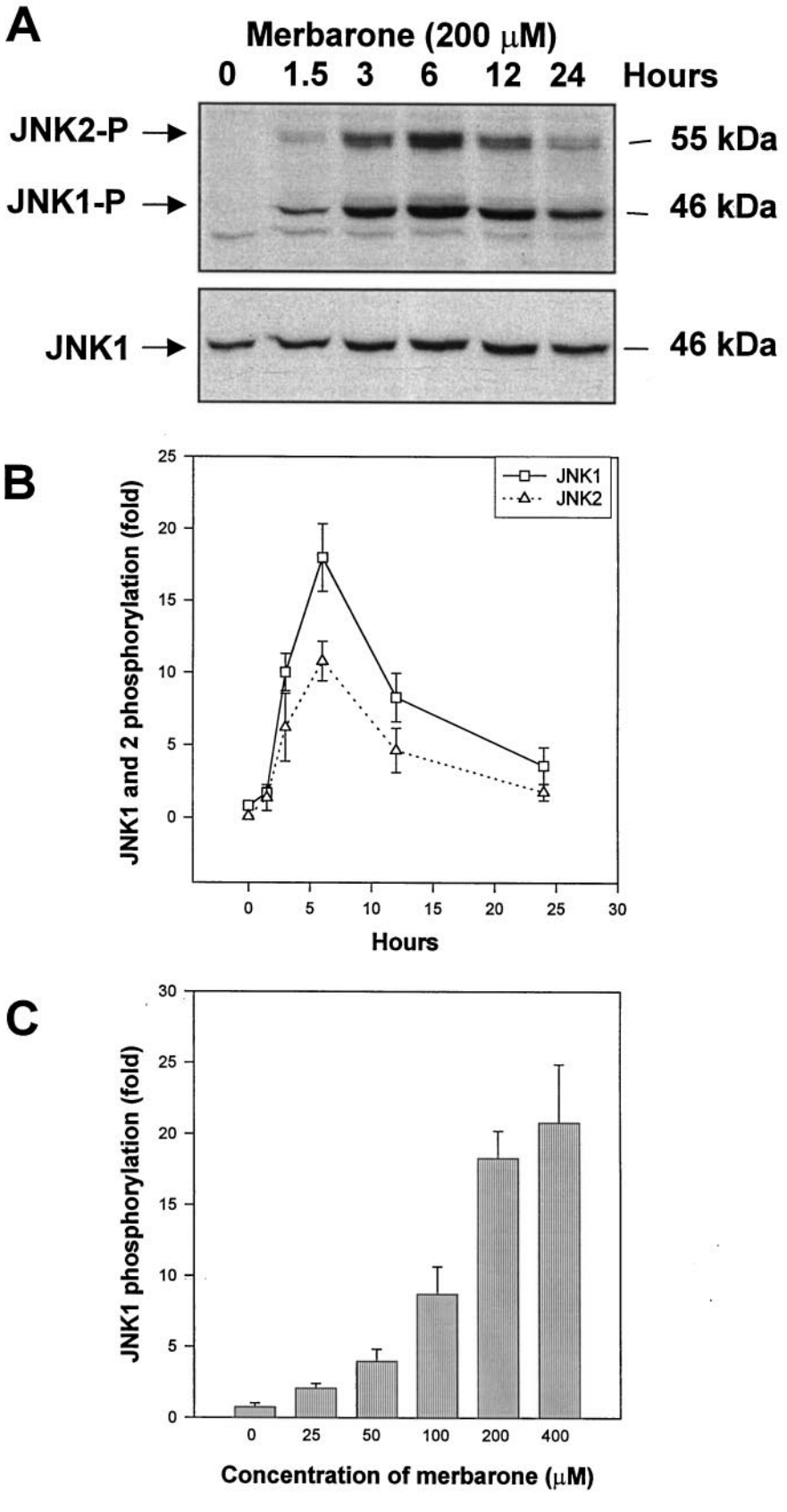

Fig. 2. Phosphorylation of JNKs after merbarone treatment. A, Western blot analysis of JNK1 and JNK2 phosphorylation at various times after treatment of CEM cells with merbarone (200 μM). Relative levels of JNKs phosphorylation at Thr-183 and Tyr-185 were assessed using a phospho-specific rabbit antibody. See *Materials and Methods* for details. B, quantitation of JNK1 and JNK2 phosphorylation levels in (A), relative to the value for the 0-h time point. C, dose-response of JNK1 activation by phosphorylation at 6 h after merbarone treatment. Points, mean for three independent experiments; columns, S.D..

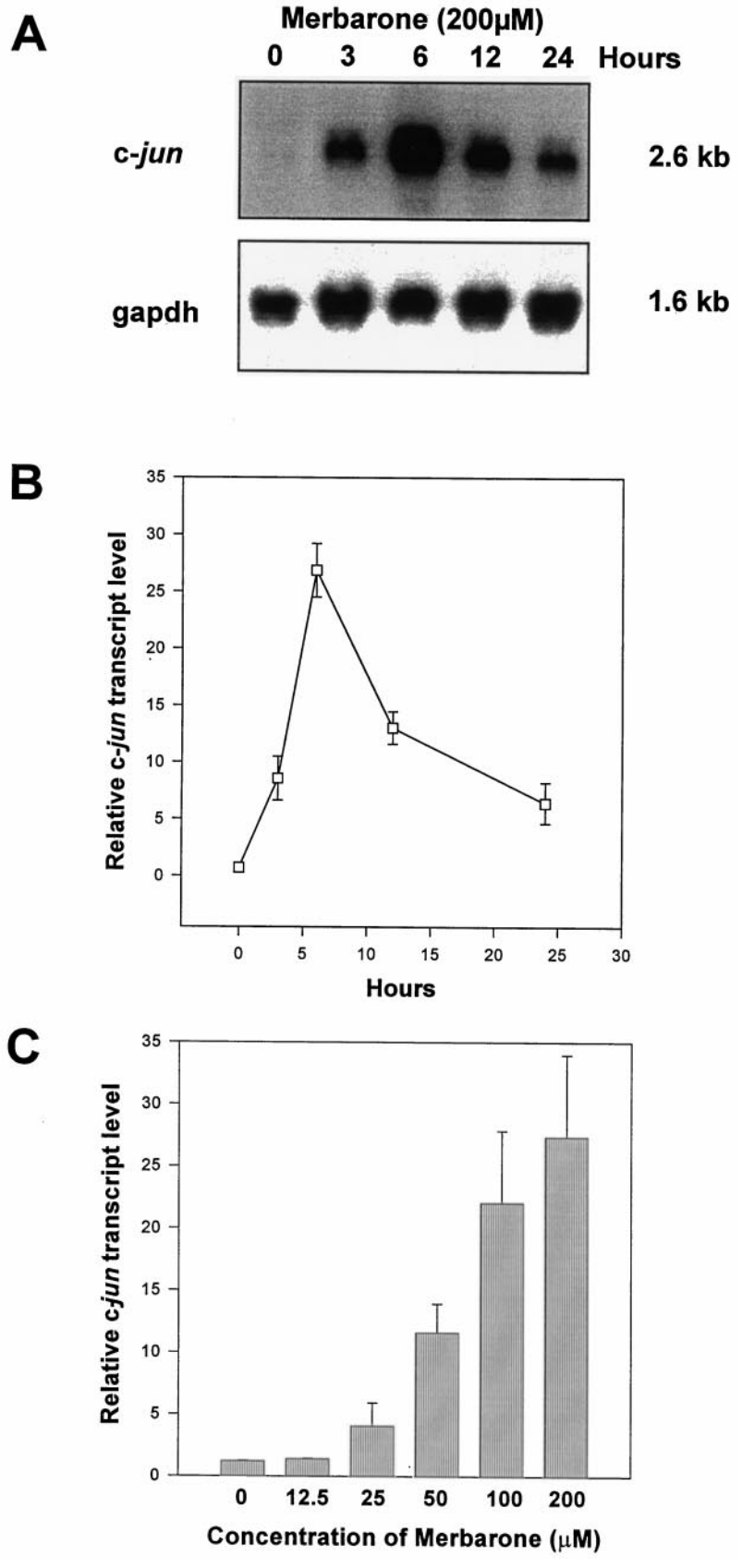

Fig. 3. Effects of merbarone treatment on c-*jun* gene expression in CEM cells. A, expression of c-*jun* mRNA after merbarone (200 μM) treatment. Total RNA (20 μg) was extracted at the indicated times after merbarone treatment, and hybridized to ^{32}P-labeled cDNA probe. Kb, kilobases. B, quantitation of c-*jun* mRNA levels after merbarone treatment. The levels of c-*jun* transcripts were quantified by densitometric scanning. C, dose-response of c-*jun* mRNA induction at 6 h after merbarone treatment. Points, mean for three independent experiments; columns, S.D.. See *Materials and Methods* for details.

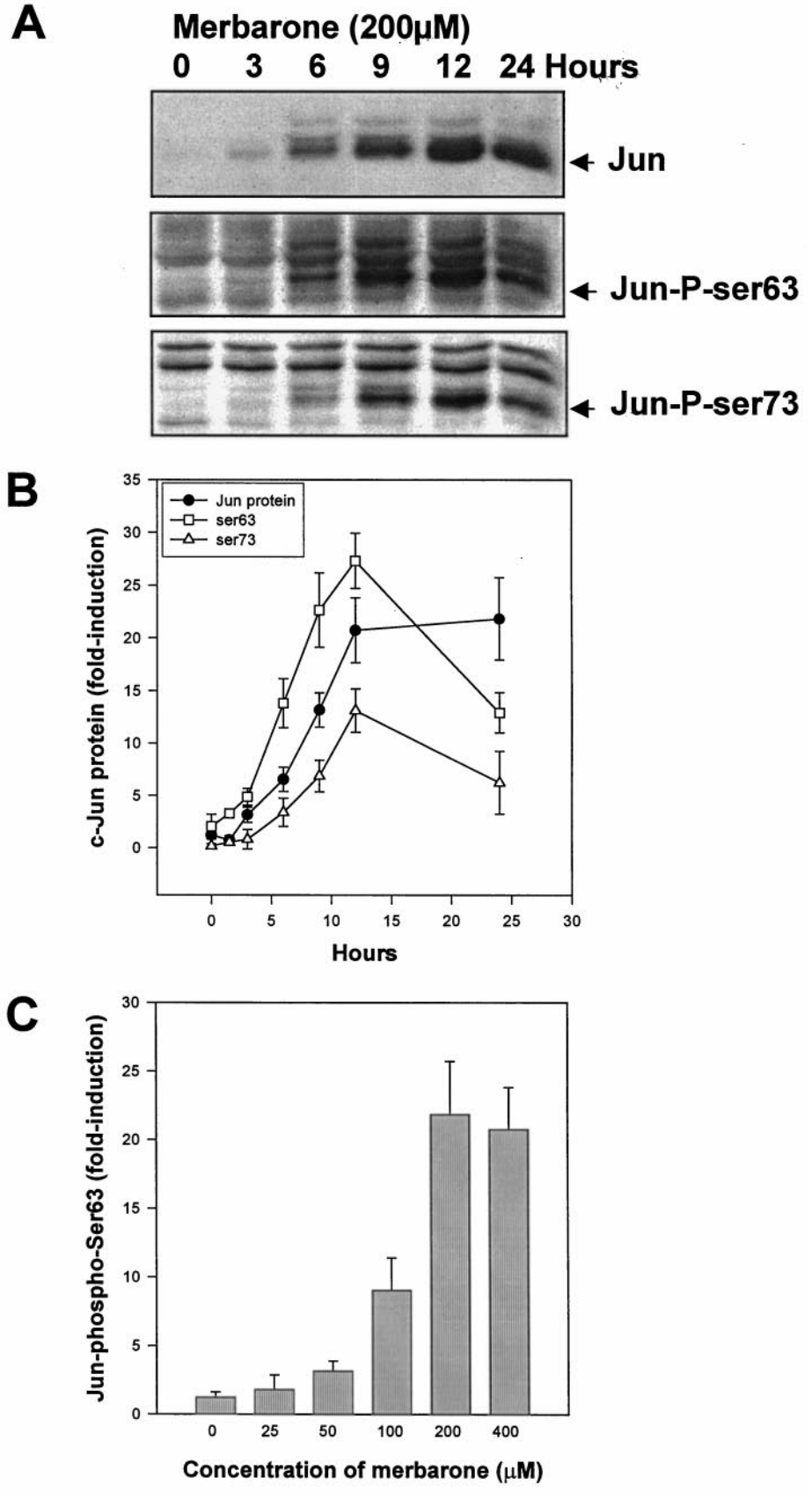

Fig. 4. Production of c-Jun protein and its phosphorylation at Ser63 and Ser73 after merbarone treatment. A, 200 μg of whole cell extracts were prepared after merbarone treatment and analyzed by Western blot as described in *Materials and Methods*. B, quantitation of c-Jun protein bands in (A), relative to the value for the 0-h time point. C, dose-response of c-Jun protein increase and phosphorylation at 12 h after merbarone treatment. Points, mean for three independent experiments; columns, S.D..

et al., 1997). Here we use the cleavage of PARP as an indicator of caspase activation. Treatment of CEM cells with 200 μM merbarone induced the cleavage of PARP, with accumulation of the 89-kDa fragment evident within 12 h (Fig. 6).

Inhibition of Merbarone-Induced Apoptosis and Caspase-3-like Protease by Z-Asp. In order to determine whether the activation of caspase-3-like proteases is required for merbarone-induced apoptosis, we examined the effect of Z-Asp, a potent caspase inhibitor (Mashima et al., 1995), on caspase-3-like activation and apoptosis induced by merbarone in CEM cells. Z-Asp suppressed apoptosis and caspase-3-like activity induced by merbarone in a dose-dependent manner (Fig. 7, A and B). These results indicate clearly that the activation of caspase-3-like proteases is associated with merbarone-induced apoptosis.

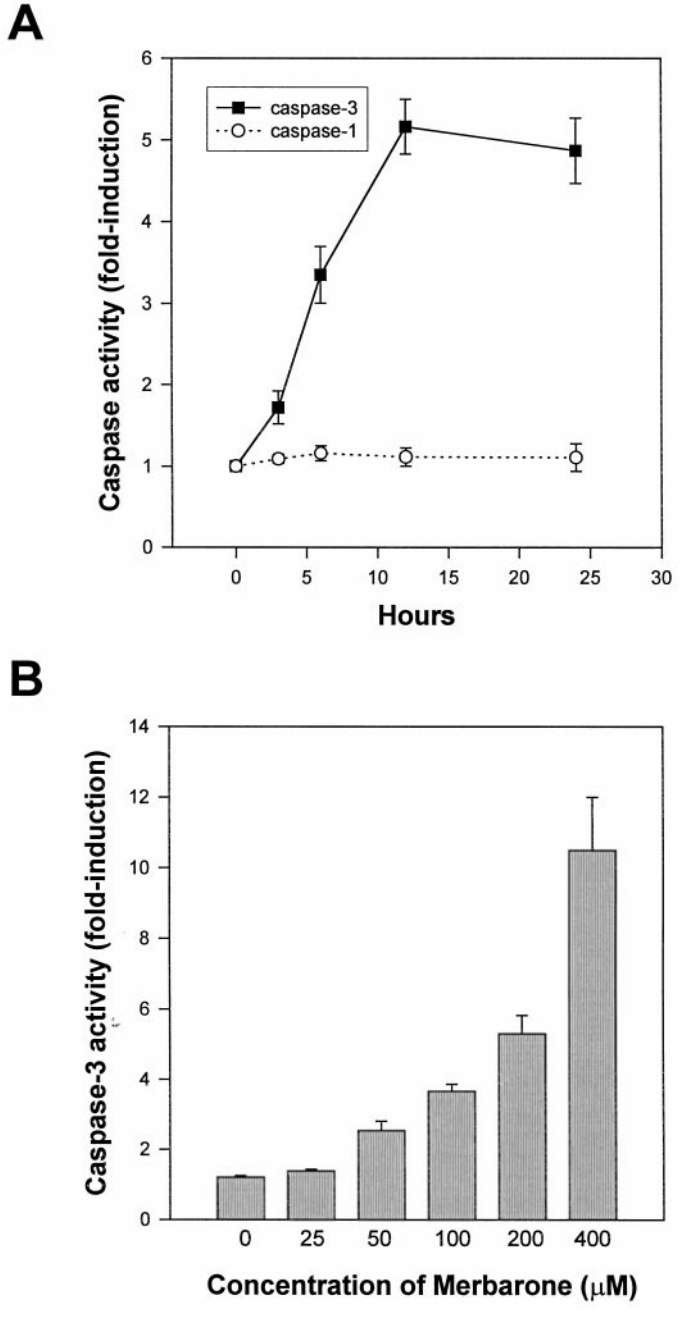

Fig. 5. Caspase activity in CEM cells after treatment with merbarone. A, caspase-1- and caspase-3-like protease activity after treatment of CEM cells with merbarone (200 μM). B, dose-response activation of caspase-3-like activity after merbarone treatment at 12 h. Points, mean for three independent experiments, are expressed relative to untreated controls; columns, S.D..

Discussion

Merbarone is a barbiturate derivative (Cooney et al., 1985) and a member of the class of topo II-targeted agents that include fostriecin (Boritzki et al., 1988), aclarubicin (Jensen et al., 1993), and the bisdioxopiperazines (Tanabe et al., 1991; Ishida et al., 1991; Roca et al., 1994), which inhibit topo II catalytic activity without stabilizing topo II-DNA covalent complexes. Merbarone inhibits chromosome condensation and sister chromatid segregation in nonsynchronized human leukemic CEM cells (Chen and Beck, 1993). This agent also induced G_2/M blockade (Chen and Beck, 1995). Although the cytotoxicity of topo II-DNA stabilizing agents such as VP-16 and VM-26 has been well known to be related to topo II-mediated DNA damage and subsequent signaling events (reviewed in Nitiss and Beck, 1996), the cell killing mechanism(s) of the catalytic inhibition of topo II has yet to be elucidated. The present study was designed to better understand these mechanisms.

JNK/SAPK is a member of the MAP kinase-related family (Gupta et al., 1996; Karin et al., 1997), and is positively involved in apoptosis induced by a variety of cellular stresses, such as nerve growth factor withdrawal (Xia et al., 1995), heat shock, UV- and γ-irradiation (Chen et al., 1996; Zanke et al., 1996), and VP-16 and camptothecin (Seimiya et al., 1997). Furthermore, 2-deoxyglucose was found to block both chemotherapeutic drug-induced apoptosis and JNK activation in U937 cells (Haga et al., 1998). JNK is not thought to be linked to FasL/Fas- and TNF-α/TNFR1-mediated apoptosis (Liu et al., 1996; Lenczowski et al., 1997). JNK activity can determine the physiological response of the cell and can function as common kinase shared by different cellular signaling (Chen et al., 1996). For example, immediate and transient JNK activation led to cell proliferation and differentiation, whereas sustained JNK activation led to apoptosis during UV-C- and γ-irradiation (Chen et al., 1996). Because topo II-DNA stabilizing agents such as VP-16 activated JNK and c-Jun during apoptosis, we suspected that JNK and c-Jun might be associated with apoptosis induced by merbarone in CEM cells. The JNK activation during merbarone treatment of CEM cells reported here was immediate and persistent. JNK activation was detected as early as 90 min,

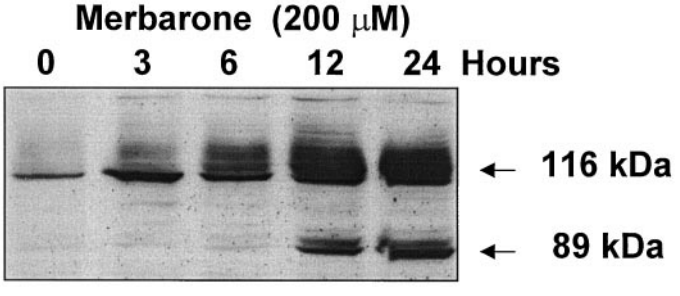

Fig. 6. Cleavage of PARP after treatment of CEM cells with merbarone (200 μM). At indicated times, 100 μg of whole cell protein extracts were prepared and analyzed by Western blot as described in *Materials and Methods*. The experiment shown is one of three independent experiments, all with similar results.

reaching a maximum by 3 to 6 h, and remained persistently activated during the 24 h of the experiment. The induction of JNK was also associated with induction of c-Jun expression. The merbarone concentration (200 μM) may seem high, but this level kills 20–30% of CEM cells in 24 h, and corresponds to 10 times the concentration of the drug that inhibits 50% of the growth of these cells in 48 h, as described previously (Kusumoto et al., 1996). The results described here indicate for the first time the association of JNK signaling and apoptosis induced by merbarone in CEM cells.

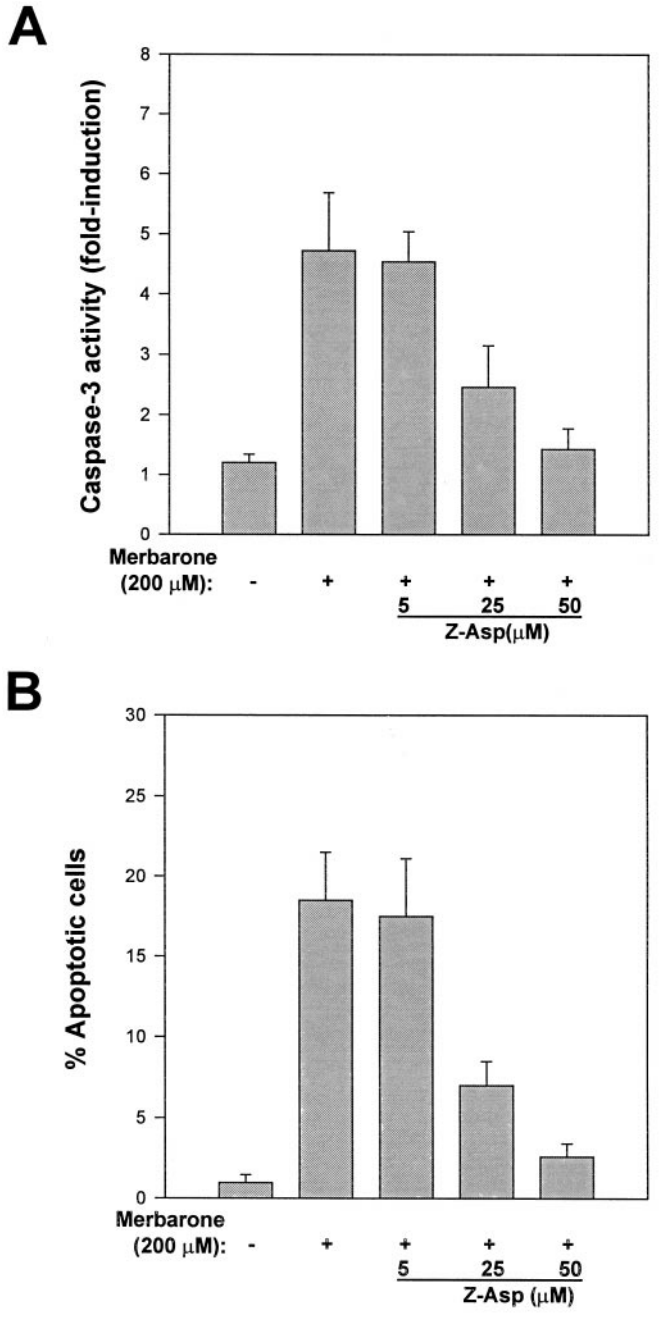

Fig. 7. Effects of caspase-3 inhibitor Z-Asp on caspase-3-like activity and apoptosis-induced by merbarone in CEM cells. A, abrogation of merbarone-induced caspase-3-like activity by Z-Asp. Cells were treated with merbarone (200 μM), Z-Asp (50 μM), or with increasing concentrations of Z-Asp followed by merbarone (200 μM) treatment. Caspase-3-like activity was assayed as described in *Materials and Methods*. B, inhibition of merbarone-induced apoptosis by Z-Asp. Cells were treated as described above. At 24 h, treated cells were harvested, and the percentages of apoptotic cells with condensed or fragmented nuclei were determined by DAPI staining as described in *Materials and Methods*. Columns, mean for three independent experiments; bars, S.D..

The requirement of c-Jun in the induction of apoptosis in different systems has been demonstrated by the use of dominant-negative mutants of c-Jun, neutralizing antibody, or antisense oligonucleotides (Colotta et al., 1992; Ham et al., 1995). Furthermore, Seimiya et al. (1997) indicated that during VP-16- and CPT-induced apoptosis in U937 cells, JNK1 activates ICE/CED-3-like proteases via c-Jun and AP-1 transcription factor. In fact, ICE/CED-3-like proteases, such as ICE and CPP32, are regulated at the transcriptional as well as the post-translational level. For example, the interleukin regulatory factor-1 induced the transcription of ICE/CED-3-like proteases during DNA damage-induced apoptosis of mitogen-activated T-lymphocytes (Tamura et al., 1995), and activation of STAT1 signaling can cause induction of ICE gene expression and apoptosis (Chin et al., 1997).

Whether induction of JNKs and c-Jun leads to caspase-3-like protease activation during apoptosis induced by merbarone in CEM cells is still under investigation. ICE/CED-3-like proteases are essential mediators of apoptosis induced by a variety of stimuli (Thornberry, 1997). Furthermore, caspase-3 knockout mice suffer severe developmental abnormalities attributed to the disturbed regulation of apoptosis (Kuida et al., 1996). Pretreatment of CEM cells with Z-Asp, a potent inhibitor of caspases (Mashima et al., 1995), and the subsequent inhibition of merbarone-induced caspase-3-like activity and apoptosis indicated clearly the requirement of caspase-3-like proteases during apoptosis induced by merbarone.

The present data indicate that merbarone, a catalytic inhibitor of topo II that does not cause frank breaks in DNA, induced apoptotic cell death characterized by internucleosomal DNA fragmentation; cell death was preceded by activation of c-Jun and JNKs, caspase-3-like protease, but not caspase-1, and the cleavage of PARP. Furthermore, recent work from this laboratory revealed that the Fas/Fas ligand (FasL) pathway is not induced by merbarone, but it is activated by DNA-damaging agents, including topo II inhibitors that stabilize DNA-topo II covalent complexes such as VP-16, VM-26, and doxorubicin (Mo & Beck, 1999). Together, these data indicate that the catalytic inhibitors of topo II may induce apoptosis by a mechanism(s) distinct from DNA-damaging topo II inhibitors.

Acknowledgments

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