

DNA Damage Signals Induction of Fas Ligand in Tumor Cells

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ABSTRACT

Many anticancer agents exert their cytotoxicity through DNA damage and induction of apoptosis. Fas ligand (FasL), a key component of T lymphocytes, has been shown to be induced by some of those agents. To address what is an early signal for this induction, we constructed a FasL promoter-luciferase reporter gene to investigate effects of DNA topoisomerase (Topo) II inhibitors on FasL promoter activity. Transient transfection assays in HeLa and other tumor cell lines demonstrated that induction of FasL promoter activity in response to Topo II inhibitors such as VM-26 mimicked endogenous FasL expression under the same conditions. The ability of these agents to induce FasL expression correlated with their ability to cause DNA damage. For instance, complex-stabilizing Topo II inhibi-

tors such as etoposide, teniposide, and doxorubicin, which cause DNA damage, strongly induce FasL expression; by contrast, non-DNA-damaging catalytic Topo II inhibitors such as ICRF-187 and merbarone do not do this. In support of the notion that DNA damage triggers FasL induction, we found that DNA-damaging irradiation also induced FasL promoter activity in a dose-dependent manner. Finally, the catalytic Topo II inhibitor ICRF-187 suppressed VM-26-induced-FasL expression. This suppression correlated with the ability of this drug to inhibit VM-26-induced DNA strand breaks. Together, our results suggest that DNA damage in response to agents such as etoposide and teniposide might serve as an early signal to induce FasL expression.

DNA topoisomerases (Topos) are nuclear enzymes that regulate DNA topology and are required for DNA replication and transcription (Nelson et al., 1986; Brill et al., 1987). These enzymes are also implicated in chromosome segregation, DNA repair, cell cycle progression, and RNA processing (Rose and Holm, 1993; Holloway, 1995; Sekiguchi and Shuman, 1997). Eukaryotic cells express two forms of topoisomerases (D'Arpa et al., 1988; Tsai-Pflugfelder et al., 1988). The type I form (Topo I) is an ATP-independent enzyme that catalyzes DNA relaxation via transient single-stranded DNA breaks (D'Arpa et al., 1988). By contrast, the type II form (Topo II) is an ATP-dependent enzyme that catalyzes knotting-unknotting and catenation-decatenation reactions by the breakage, strand-passage, and reunion of double-stranded DNA (Tsai-Pflugfelder et al., 1988). Because of their essential role in DNA replication and cell growth, as well as their high level of expression in proliferating cells, these enzymes are ideal targets for cancer chemotherapy (Heck and Earnshaw, 1986; Liu, 1989). Topo II inhibitors are among the most useful anticancer drugs for many types of cancer (Liu, 1989; Osheroff et al., 1994).

Mechanistically, the catalytic cycle of Topo II features a

minimum of four distinct steps: 1) DNA binding by the enzyme, 2) DNA cleavage, 3) strand passage, and 4) religation and enzyme turnover (Osheroff et al., 1994). Several well-characterized Topo II inhibitors include doxorubicin, teniposide (VM-26), and etoposide (VP-16). These drugs appear to bind to the Topo II-DNA complex and inhibit the religation of the broken DNA strands, thus inducing protein-associated DNA strand breaks through stabilization of the covalently linked Topo II/DNA-cleavable complexes. Hence, they have been known as cleavable complex-stabilizing inhibitors or Topo II poisons. A consequence of these drug actions is interference with transcription, DNA synthesis, and mitosis, eventually leading to cell death by apoptosis (Fisher, 1994). By contrast, other Topo II inhibitors, such as the bis-dioxopiperazine derivatives and merbarone, do not stabilize DNA-enzyme cleavable complexes, although they also target the enzyme and inhibit its activity; these are catalytic inhibitors of the enzyme. For instance, dioxopiperazine derivatives are believed to bind to Topo II at a stage when religated double-stranded DNA is still locked in the enzyme, thereby inhibiting the enzymatic activity because the bound enzyme cannot initiate a new round of catalysis (Sehested and Jensen, 1996). Based on their action on the enzyme, the catalytic Topo II inhibitors are generally considered to be non-DNA-damaging agents. In addition, compared with the complex-stabilizing inhibitors of Topo II, the catalytic Topo II

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ABBREVIATIONS: Topo, DNA topoisomerase; FasL, Fas ligand; PARP, poly(ADP-ribose)polymerase; Z-VAD.fmk, Z-Val-Ala-DL-Asp-fluoromethylketone; CHO, Chinese hamster ovary.

inhibitors have been shown to antagonize the actions of the cleavable complex-stabilizing Topo II inhibitors (Sehested et al., 1993). Thus, pretreatment with ICRF-187, a dioxopiperazine derivative, reduced VP-16- or daunorubicin-induced DNA breaks and apoptosis (Sehested et al., 1993), presumably because the former competes with VP-16 and daunorubicin for the enzyme, making the enzyme less available for VP-16 and daunorubicin and, thus, decreasing DNA damage.

It has been shown recently that some of the Topo II inhibitors induce Fas ligand (FasL) expression (Friesen et al., 1996). FasL is a 37-kDa protein that belongs to the type II protein superfamily and is expressed predominantly in activated T cells. The Fas/FasL system plays a pivotal role in the regulation of a variety of immunological processes by activating the apoptotic pathway (Ju et al., 1995; Nagata and Golstein, 1995). Binding of Fas to FasL triggers activation of a series of proteases, including interleukin-converting enzymes, and DNA fragmentation, leading to cell death by apoptosis (Lowin et al., 1994). This type of cell death induced by Fas/FasL interactions has been implicated in the elimination of excess activated T cells in the peripheral circulation and in the killing of tumor targets by immune cytotoxic effector cells (Ju et al., 1995).

Although much of attention has been directed to FasL expression in response to T cell activation (Rouvier et al., 1993; Herr et al., 1997), less is known about its regulation by anticancer agents, particularly at the transcriptional level. In this study, we asked what is an early signal for FasL induction in response to Topo II inhibitors.

Materials and Methods

Cell Culture and Transfection. All cell culture media (BioWhittaker, Walkersville, MD) were supplemented with 10% fetal bovine serum (Sigma Chemical Co., St. Louis, MO), 2 mM L-glutamine, 100 U of penicillin/ml, and 100 mg of streptomycin/ml (Life Technologies, Gaithersburg, MD). Human leukemic CCRF-CEM (CEM) cells (Kusumoto et al., 1996) were grown in supplemented (Eagle's) minimum essential medium. Jurkat and Chinese hamster ovary (CHO) cells were obtained from American Type Culture Collection (Rockville, MD) and grown in RPMI 1640; HeLa cells (American Type Culture Collection) were grown in Dulbecco's modified Eagle's medium. Cells were incubated at 37°C in a humidified chamber supplemented with 5% CO₂.

The FasL promoter-luciferase reporter plasmid was introduced into CEM cells by electroporation using the Gene Pulser II apparatus with an extender (Bio-Rad, Hercules, CA) according to the manufacturer's protocol. Briefly, about 5 million exponentially growing cells were harvested, washed twice with phosphate-buffered saline, and resuspended in 400 μ l of supplemented (Eagle's) minimum essential medium without fetal bovine serum. Twenty micrograms of plasmid was used per electroporation. The same amount of pSV- β -galactosidase reporter (Promega, Madison, WI) was cointroduced into the cells in each electroporation experiment to normalize for transfection efficiency. After electroporation, cell suspensions were placed at room temperature for 10 min and then transferred to a T-25 flask with 12 ml of culture medium and incubated overnight at 37°C before drug treatment.

For transfection of adherent cells (HeLa and CHO), plasmid DNA was introduced into cells by the calcium phosphate method (Jordan et al., 1996). Briefly, 20 μ g each of FasL promoter-luciferase construct and pSV- β -galactosidase were used per 100-mm dish containing ~50% confluent cells. After the addition of the transfection solution containing the plasmid DNA to medium, cells were incu-

bated overnight at 37°C. Cells were then subcultured in 12-well plates with 2 ml of medium per well and grown at 37°C overnight before treatment with drugs or radiation.

Chemicals. VM-26, VP-16, doxorubicin, ICRF-187, and merbarone have been described previously (Kusumoto et al., 1996). Bisbenzimidazole (Hoechst 33342) was purchased from Sigma Chemical. Z-Val-Ala-DL-Asp-fluoromethylketone (Z-VAD.fmk) was purchased from Bachem (Torrance, CA). [¹⁴C]Thymidine was purchased from Amersham (Arlington Heights, IL).

Polymerase Chain Reaction Cloning. Genomic DNA was isolated from CEM cells by a standard method (Ausubel et al., 1989). Polymerase chain reactions (PCRs) for amplification of the FasL promoter region were performed according to a standard method (Ausubel et al., 1989) using a commercial kit (AmpliTag; Perkin-Elmer, Foster City, CA). Primers for PCR were FasLp5.7, 5'-CTC-GAGTTCTGATATTTCAAACAGAATAG (sense) (Holtz-Heppelmann et al., 1998), containing the restriction enzyme *Xho*I site, and FasLp3.1, 5'-AAGCTTATGGCAGCTGGTGAGTCAGG (antisense), containing a *Hind*III site for unidirectional cloning into pGL2-Basic (Promega). PCR products were first cloned into PCR2.1 (Invitrogen, Carlsbad, CA) and then subcloned into pGL2-Basic. To verify the cloned DNA fragment, nucleotide sequences were determined by Sequenase kit version 2 (Amersham).

Immunoblot Analysis. Cellular proteins were extracted with lysis buffer (Keane et al., 1996) from exponentially growing cells. Protein concentration was determined using the Bio-Rad protein assay kit. Protein samples were separated in 9% SDS-polyacrylamide gels and transferred to a nitrocellulose membrane using a semidry transfer apparatus (Hoefer Scientific, San Francisco, CA). The membrane was blocked in 5% dry milk in TBS (50 mM Tris, pH 7.4, 0.87% NaCl) and then incubated with specific antibodies for 1 h at room temperature or overnight at 4°C with gentle shaking. After three washes with TBS, secondary antibodies conjugated with horseradish peroxidase (Jackson ImmunoResearch, West Grove, PA) were added to the membranes and incubated for 1 h under the same conditions. After a final three washes with TBS, immunoblots were developed with an enhanced chemiluminescence (ECL) detection method (Amersham). To normalize for protein loading and transfer, anti- β -tubulin antibody (Oncogene Research, Cambridge, MA) was used on the same membrane. Antibodies were purchased from Upstate Biotechnology (polyclonal anti-PARP; Lake Placid, NY), Transduction Laboratory (monoclonal anti-FasL antibody; Lexington, KY), or Santa Cruz Biotechnology (polyclonal anti-FasL antibodies; Santa Cruz, CA).

Drug Treatment and Irradiation. After transfection, adherent cells were trypsinized, subcultured into 12-well plates with 2 ml of medium per well, and grown overnight. Drugs were then added to the medium at concentrations as indicated in *Results*. Cells were harvested for luciferase assays 24 h after drug treatment. For UV irradiation, medium was removed immediately before treatment; cells were then exposed to UV at a defined energy level using Stratagene (Stratagene, La Jolla, CA), and fresh medium was added back after the UV treatment. Cells were then grown for another 24 h, harvested for luciferase assays, and lysed in 100 μ l of 1 \times luciferase assay buffer (Promega). Suspension cells, after electroporation, were grown in 12 ml of medium in T-25 flasks overnight and then divided into 12-well plates with 2 ml of culture medium per well and treated with drug as above. Luciferase activity was assayed in a luminometer (Turner Designs, Sunnyvale, CA), and normalized by β -galactosidase activity for each treatment.

Detection of Apoptosis and Cytotoxicity Assays. Apoptosis was determined by nuclear staining with Hoechst dye. HeLa cells were treated with drugs or UV irradiation for 24 h, trypsinized as usual, and incubated in a fixing solution (methanol/acetic acid, 3:1) for 15 min at room temperature before transferring to a glass slide. After briefly drying the slides, cells were stained with Hoechst dye (1 μ g/ml) and examined under a fluorescent microscope (Zeiss, Thorn-

wood, NY). Any cells displaying shrunken nuclear structures with intense staining were scored as apoptotic cells, and the percentage of apoptosis was determined from a total of 200 cells per treatment. Cytotoxicity assays were done using trypan blue exclusion as suggested by the manufacturer (Life Technologies, Gaithersburg, MD).

Alkaline Elution. Alkaline elution assays for single-stranded DNA breaks were carried out essentially as described by Beere et al. (1996). Briefly, HeLa cells were labeled with 0.1 μ Ci of [14 C]thymidine/ml for 24 h and then treated with drugs for 1 h before harvesting of them for alkaline elution assays. To test the effect of ICRF-187 on VM-26-induced DNA strand breaks, labeled HeLa cells were pre-treated with 100 μ M ICRF-187 for 1 h, followed by an additional 1-h treatment with VM-26 (10 μ M) before harvesting for alkaline elution assays.

Results

Up-Regulation of FasL Protein and FasL Promoter Activity in Different Types of Tumor Cells Treated with Topo II Inhibitor VM-26. It was reported previously that anticancer agents can induce FasL expression (Friesen *et al.*, 1996). Consistent with these results, we observed up-regulation of FasL in VM-26- or doxorubicin-treated CEM and Jurkat cells by Western blot (Fig. 1A). To further investigate FasL induction in response to anticancer drugs, we cloned the FasL promoter (~1200 bp) from CEM cells and verified by DNA sequencing that it was identical with the published sequence (Holtz-Heppelmann *et al.*, 1998). A FasL

promoter-luciferase reporter construct was then made in pGL2-Basic. Different drug concentrations were chosen in this experiment for different cell lines because initial dose variation experiments showed that at these concentrations, the highest level of FasL promoter activity was achieved for a particular cell line. After introduction of this construct into CEM cells by electroporation, followed by VM-26 treatment at 1 μ M, we found a moderate (~2-fold) induction of FasL promoter activity (Fig. 1B). To test whether this is a cell-specific phenomenon, we introduced the same construct into HeLa and CHO cells, respectively; treatment of these cells with VM-26 at 10 μ M resulted in FasL induction in both cell lines. Interestingly, the induction level was much greater than that in CEM cells, apparently due in part to a higher transfection efficiency for both HeLa and CHO cells. As shown in Fig. 1B, treatment of these cells for 24 h with 10 μ M VM-26 yielded about a 12-fold increase in FasL promoter activity compared with the DMSO control; similarly, an approximate 6-fold induction was observed in CHO cells. No more than a 1.5-fold (CEM cells) or 2.3-fold (HeLa and CHO cells) increase in luciferase activity was detected for the empty vector pGL2-Basic under these conditions. Standard deviations are less than 1. Because HeLa cells seem to produce higher levels of FasL promoter activity than the other cell lines, we used this cell line for subsequent experiments.

To test the effect of drug concentration on endogenous FasL protein in HeLa cells, we treated HeLa cells for 24 h with VM-26 at 0, 0.1, 0.5, 1, 5, and 10 μ M, respectively. As shown in Fig. 2A, HeLa cells expressed FasL protein in the absence of drug, but the amount of FasL protein increased with the concentration of VM-26. We also examined FasL

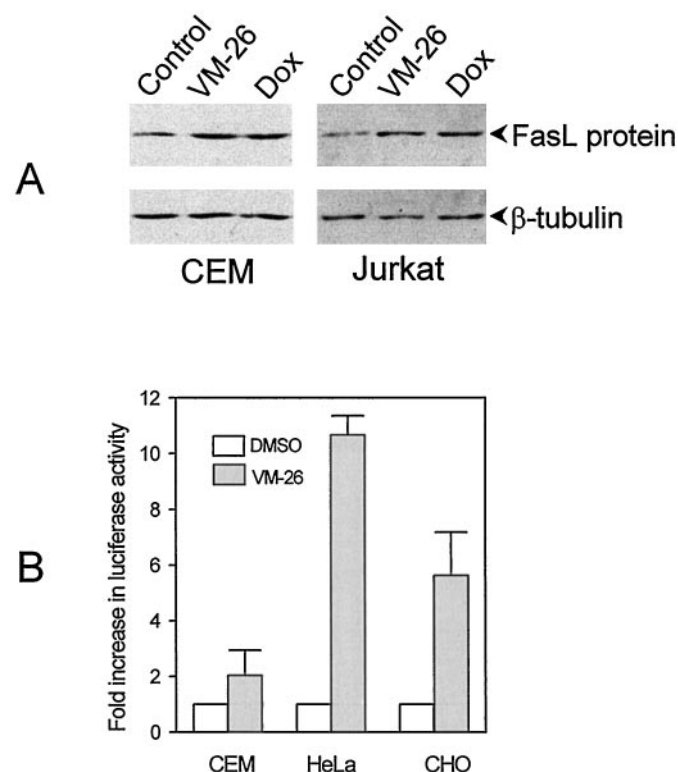


Fig. 1. Induction of FasL expression by Topo II inhibitors VM-26 and doxorubicin (Dox). A, representative Western blot showing expression of FasL in CEM and Jurkat cells treated with VM-26 (1 μ M) or doxorubicin (0.2 μ M) for 24 h. B, VM-26-induced FasL promoter activity in CEM (1 μ M), HeLa (10 μ M), and CHO (10 μ M) cells. Cells were transfected with the FasL promoter-luciferase construct, treated with drugs, and harvested for luciferase assays as described in *Materials and Methods*. FasL promoter activity is expressed as luciferase activity relative to the control [DMSO (dimethyl sulfoxide)] activity set at one. Values are means \pm S.D. of three independent experiments.

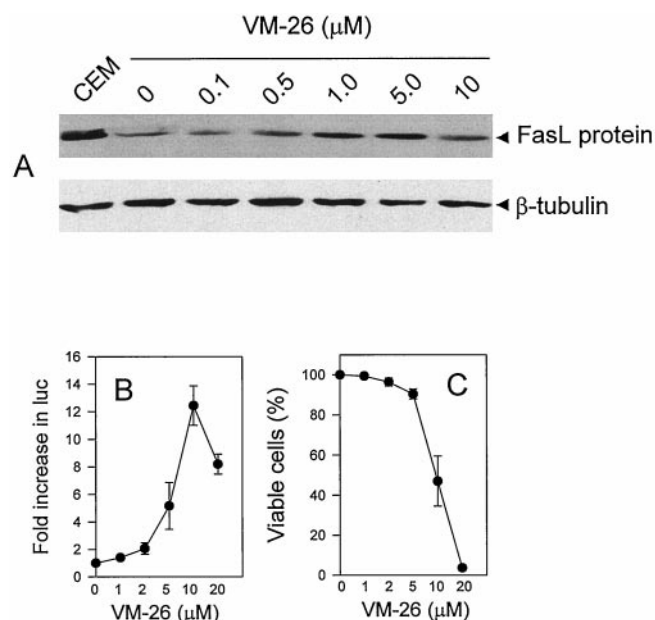


Fig. 2. Effect of VM-26 concentration on endogenous FasL protein (A), FasL promoter activity (B), and cell killing (C) in HeLa cells. A, expression of endogenous FasL protein was examined by Western blotting. Untransfected HeLa cells were treated with VM-26 as above and harvested for protein analysis after 24 h. CEM cells without drug treatment served as a positive control. B, FasL promoter activity in response to VM-26 at indicated concentrations. HeLa cells were transfected with the FasL promoter-luciferase construct and treated with VM-26 for 24 h before harvesting for luciferase assays. C, VM-26 induced cytotoxicity as determined by trypan blue staining. Data in B and C are means \pm S.D. of three independent experiments.

promoter activity under similar conditions (Fig. 2B). At 2 μM , the FasL promoter activity increased about 2-fold, and the maximal induction was observed at 10 μM and then seen to decrease at 20 μM ; concentrations of VM-26 of 10 μM or higher led to substantial cell death (Fig. 2C).

Induction of FasL Promoter Activity by DNA-Damaging Agents. To test whether FasL induction has any specificity, we examined other Topo II inhibitors (doxorubicin, VP-16, merbarone, and ICRF-187). Both doxorubicin and VP-16, like VM-26, stabilize DNA-protein complexes and cause DNA damage and strand breaks (Liu, 1989). These drugs induced FasL promoter activity (Fig. 3, A and B), although the ability to do so varied among them. However, the catalytic Topo II inhibitors ICRF-187 and merbarone, which do not directly damage DNA (Sehested et al., 1993), caused no significant induction of FasL promoter activity (Fig. 3, D and E). ICRF-187 induced less than 2-fold increase in FasL promoter activity at up to 300 μM (Fig. 3E), although cytotoxicity to HeLa cells of ICRF-187 at 300 μM (Fig. 3F) is slightly higher than that of VM-26 at 5 μM (Fig. 2C). Thus, it appears that induction of FasL is associated with DNA damage. To test this, we asked whether other DNA-damaging agents with different modes of action can induce FasL expression. As expected, UV irradiation induced FasL promoter activity. The minimal dose that caused FasL induction was 2

mJ/cm² when assays were carried out 24 h after UV treatment; peak activity was observed at 10 mJ/cm² (Fig. 3C). Like UV irradiation, γ irradiation also induced FasL promoter activity in a dose-dependent manner at the range of 0 to 10 Gy (data not shown).

FasL-Inducing Agents Cause Apoptosis, but FasL Induction Is Not a Consequence of Apoptosis. In addition to FasL induction, the DNA-damaging agents caused apoptosis, as indicated by cleavage of poly(ADP-ribose)polymerase (PARP), a commonly used indicator of apoptosis. At the concentration or energy level that resulted in highest level of FasL promoter activity, we observed significant amount of cleaved PARP (85 kDa) (Fig. 4). By contrast, no PARP cleavage was detected for HeLa cells treated with the catalytic Topo II inhibitors, ICRF-187 and merbarone. Consistent with these data, we also observed that about 50% of cells treated with 10 μM VM-26 for 24 h exhibited shrunken nuclei as revealed by Hoechst dye staining, a feature of apoptosis, whereas no significant apoptosis was seen for cells treated with either ICRF-187 or merbarone.

The results from Figs. 1 through 4 suggest that there is an association of apoptosis and induction of FasL promoter activity, but whether the FasL induction is due to apoptosis or other signals before apoptosis is not clear. Consequently, we followed the time courses of FasL induction related to apo-

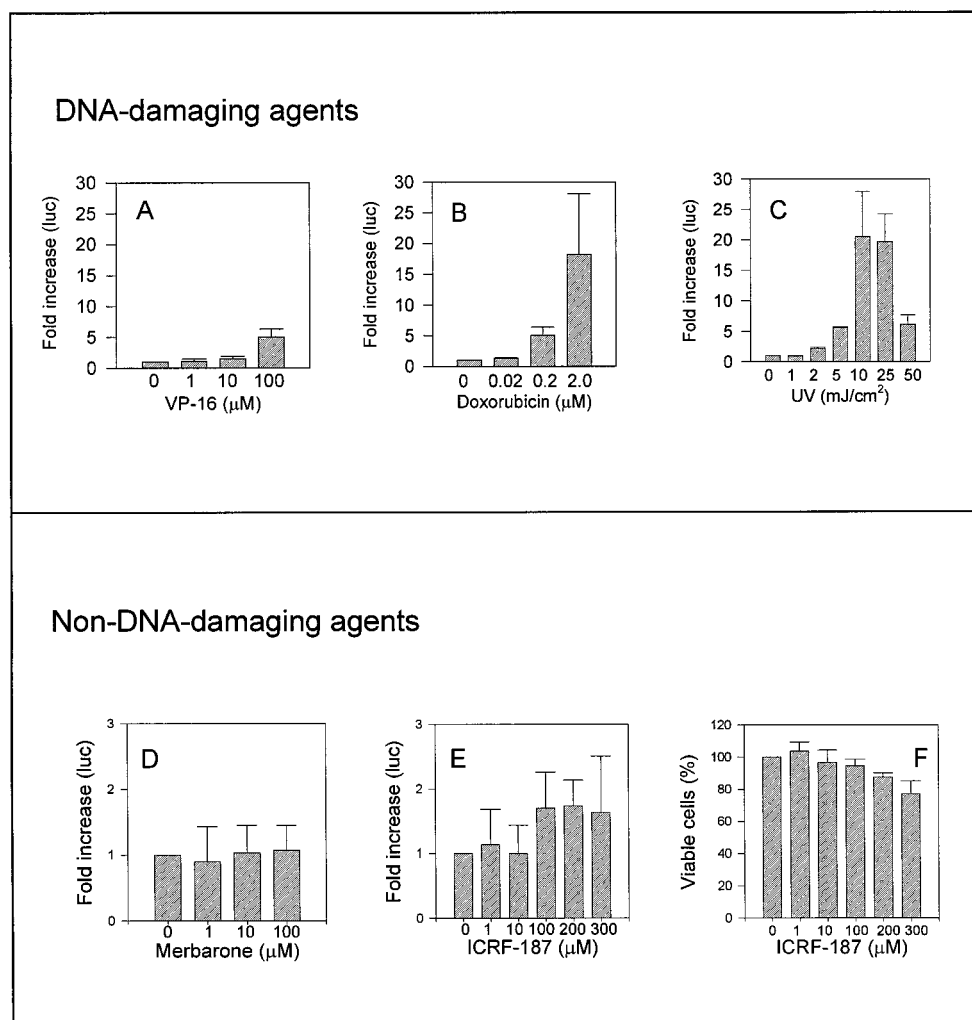


Fig. 3. Induction of FasL promoter activity by different classes of Topo II inhibitors and UV irradiation. HeLa cells were transfected with the FasL promoter-luciferase construct, treated with drugs or UV-irradiated at indicated doses, and harvested for luciferase assays as detailed in *Materials and Methods*. Cytotoxicity assays for ICRF-187 were done by trypan blue staining. Data are means \pm S.D. of three separate experiments.

ptosis to determine the temporal order of these two events. We chose 10 μM for VM-26 and 10 mJ/cm^2 for UV irradiation because both conditions gave the highest level of FasL induction from previous experiments. We detected FasL induction in VM-26-treated cells at 12 h, when we did not detect significant apoptosis. Similarly, UV treatment also induced FasL expression before detection of apoptosis (Fig. 5). This suggests that FasL induction occurs earlier than apoptosis.

However, because there was a stage of overlap between apoptosis and FasL induction, we could not determine whether apoptosis was involved in the late stages of FasL induction. Therefore, we questioned whether any FasL induction could be detected when apoptosis is blocked. Z-VAD.fmk is a broad-spectrum apoptotic inhibitor used to block apoptosis mediated by a variety of agents. At either 50 or 100 μM , this inhibitor blocked apoptosis by VM-26 but had no effect on FasL induction (Fig. 6). These data suggest that FasL induction is an early event in response to drug treatment and is independent of apoptosis.

Catalytic Topo II Inhibitor ICRF-187 Suppresses VM-26-Induced DNA Strand Breaks and FasL Promoter Activity. What, then, triggers FasL induction as a result of drug treatment? Because our results demonstrated that only DNA-damaging agents induced FasL expression, we asked whether DNA damage caused by these agents signals FasL induction. To address this question, we took advantage of the fact that bisdioxopiperazine derivatives can suppress DNA-protein complex formation by cleavable complex-stabilizing agents such as VM-26, thus relieving DNA damage (Sehested et al., 1993; Jensen and Sehested, 1997). We pretreated HeLa cells with ICRF-187 for 1 h and then added VM-26 (10 μM). We found that ICRF-187 at 20 μM inhibited VM-26-stimulated FasL induction by 25% compared with the control (Fig. 7). When the concentration of ICRF-187 was increased to 100 μM , FasL promoter activity was decreased by 50% (Fig. 7). Even when cells were treated with both drugs at the same time, we still observed a significant inhibition of FasL induction, although at a lower level (about 10% inhibition at 100 μM ICRF-187). To confirm that this suppression was due to reduction of VM-26-induced DNA strand breaks by ICRF-187, we performed alkaline

elution assays and found that pretreatment with ICRF-187 suppressed VM-26-induced DNA strand breaks, which was consistent with results of others (Sehested et al., 1993; Beere et al., 1996). Together, our data suggest that DNA damage caused by VM-26 might trigger the induction of FasL.

Discussion

FasL has been implicated in apoptosis and the cytotoxic effect of T lymphocytes. Expression of FasL, however, is not restricted to T lymphocytes. In particular, up-regulation of FasL has been found in some tumor cells (Hahne et al., 1996; Niehans et al., 1997), and we demonstrated in this study that

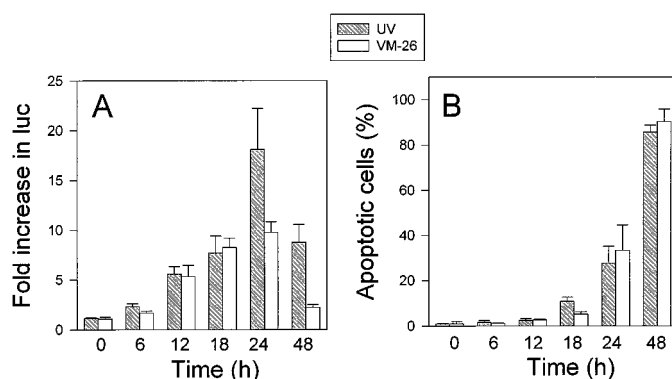


Fig. 5. Temporal induction of FasL promoter activity (A) and apoptosis (B) by VM-26 and UV irradiation. HeLa cells were transfected with the FasL promoter-luciferase construct, treated with 10 μM VM-26 or UV-irradiated (10 mJ/cm^2), and then harvested at indicated time points for luciferase assays or nuclear staining with Hoechst dye. All data are means \pm S.D. of three independent experiments.

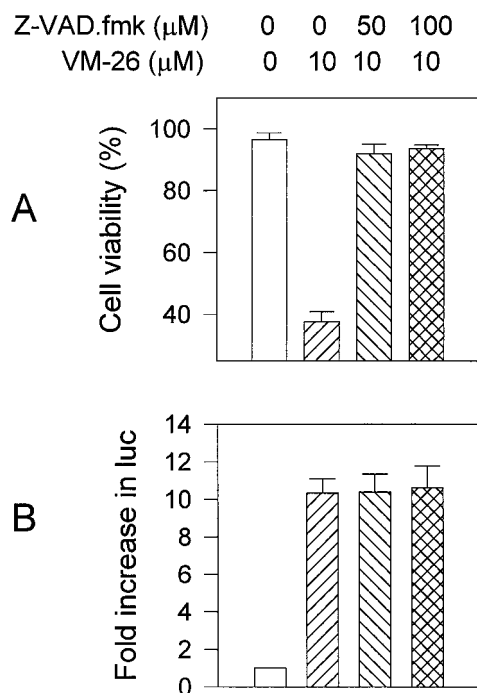


Fig. 6. Effect of Z-VAD.fmk on VM-26-induced cytotoxicity (A) and FasL promoter activity (B). HeLa cells were treated with Z-VAD.fmk at 50 or 100 μM for 1 h. VM-26 (10 μM) was then added to the cultures, and cells were incubated for another 24 h before harvesting for cytotoxicity assays (trypan blue exclusion) or luciferase assays. Z-VAD alone has no significant effect on either FasL promoter activity or cytotoxicity of HeLa cells. All data are means \pm S.D. of three independent experiments.

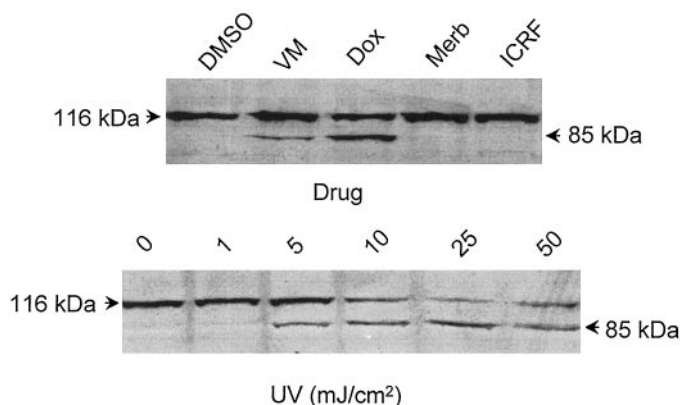


Fig. 4. Effect of Topo II inhibitors and UV irradiation on PARP cleavage. HeLa cells were treated with VM-26 (VM; 10 μM), doxorubicin (Dox; 2 μM), merbarone (Merb; 100 μM), or ICRF-187 (ICRF; 100 μM) for 24 h before harvesting for protein or UV irradiated at indicated energy levels as detailed in *Materials and Methods*, and then incubated for 24 h before protein extraction. Shown is a representative Western blot as revealed by anti-PARP antibodies. DMSO, dimethyl sulfoxide.

nonhematopoietic tumor cells such as HeLa also express FasL. It is well known that a variety of stimuli induce expression of FasL. Because of the important role of FasL in apoptosis and regulation of immunological processes, its expression in response to these stimuli, particularly T cell receptor activation, has caught much attention (Latinis et al., 1997; Holtz-Heppelmann et al., 1998). On the other hand, induction of FasL by anticancer drugs has been reported recently, but the underlying mechanisms behind this phenomenon are far from clear. Accordingly, to better understand FasL gene regulation in response to anticancer agents, we asked here about the role of different types of clinically important anticancer drugs, Topo II inhibitors, in FasL expression by examining their effect on a FasL promoter-luciferase reporter. We found that induction of FasL promoter activity mimicked the expression of the endogenous FasL gene as result of Topo II inhibitor treatment (Figs. 1 and 2), supporting the notion that the FasL promoter reporter is a good indicator of FasL expression in response to these agents, as demonstrated in T cell receptor activation (Latinis et al., 1997) as well as drug induction (Kasibhatla et al., 1998).

Several anticancer drugs have been shown previously to induce FasL expression, and among them are Topo II inhibitors. To extend these observations, we tested several Topo II inhibitors representing different classes and mechanisms of inhibition of this enzyme. Our results indicated that FasL induction is drug specific. For instance, although cleavable complex-stabilizing Topo II inhibitors, such as VM-26, are strong inducers of FasL expression, catalytic Topo II inhibitors have little or no activity in this system. Interestingly, the level of FasL induction appeared to correlate with the ability of the drugs to induce DNA damage. Consistent with these results, we found that DNA-damaging agents, UV irradiation or γ -irradiation, also induced FasL expression, although these agents differ from the Topo II inhibitors in the way in which they cause DNA damage. Importantly, our results

indicate that there is a relationship between DNA damage and FasL induction. In support of this notion, our results with ICRF-187, a catalytic Topo II inhibitor that has been shown in this study and by others (Sehested et al., 1993; Beere et al., 1996) to suppress complex inhibitor-induced DNA damage, demonstrated that this agent also inhibited VM-26-induced FasL expression. Together, these results suggest that DNA damage caused by these agents triggers FasL induction.

The mechanism or mechanisms by which ICRF-187 inhibits DNA damage by complex-stabilizing Topo II inhibitors are not fully understood, but it is believed to involve the different stages of the Topo II catalytic cycle at which these two classes of inhibitors target the enzyme (Osheroff et al., 1994). ICRF-187 binds to Topo II at a stage when religated double-stranded DNA is still locked on the enzyme so that it inhibits enzymatic activity. Due to the importance of this enzyme in cell cycle progression, inhibition of the enzymatic activity by catalytic Topo II inhibitors leads to a block of cell cycle progression at G2/M. Because this portion of the drug-bound enzyme no longer enters the catalytic cycle, the drug reduces the amount of active Topo II required for formation of new DNA-protein complexes targeted by complex-stabilizing inhibitors such as VM-26. Therefore, ICRF-187 treatment may make less target available for complex-stabilizing agents, thereby leading to less DNA damage. From the clinical perspective, inhibition of VM-26-induced FasL expression by ICRF-187 raises the possibility that FasL expression can be modulated by these drug combinations.

Although Topo II inhibitors induce FasL expression, whether such induction of FasL plays a role in drug-induced apoptosis is controversial (Friesen et al., 1996; Eischen et al., 1997; Fulda et al., 1997; Villunger et al., 1997). However, another aspect of drug-induced FasL expression is its impact on the immune system. FasL induced by anticancer drugs has been shown to be functional; it can kill T cells in vitro (Strand et al., 1996). If this type of FasL induction occurs in vivo, it would imply that surviving tumor cells could use FasL as a weapon against T lymphocytes after drug treatment. This may be more likely the case for those tumors that lack Fas expression. Support for this hypothesis comes from our preliminary results showing that the induction of FasL is independent of Fas status; in other words, Topo II inhibitors can induce FasL expression in Fas-deficient cells (Y.-Y. Mo and W. T. Beck, unpublished data). Therefore, under such conditions, the induction of FasL is a "side effect" of the antitumor drug. Melanomas in some patients express elevated FasL (Hahne et al., 1996), and up-regulation of FasL has also been observed in human lung carcinoma (Niehans et al., 1997). Mechanisms of FasL up-regulation in these tumor cells are not clear, but it could be a consequence of exposure to anticancer agents or irradiation that cancer patients have usually received for therapy. Moreover, UV irradiation has been shown to induce FasL expression at least in two cases (Leverkus et al., 1997; Gutierrez-Steil et al., 1998).

Our results suggest that DNA damage caused by complex-forming Topo II inhibitors triggers FasL induction, but little is known about factors or intermediate events that link DNA damage and FasL induction; evidence suggests that activation of nuclear factor κ B and JNK pathways are involved in the induction of FasL (Kasibhatla et al., 1998). In addition, DNA-PK has been implicated in modulating induction of p53

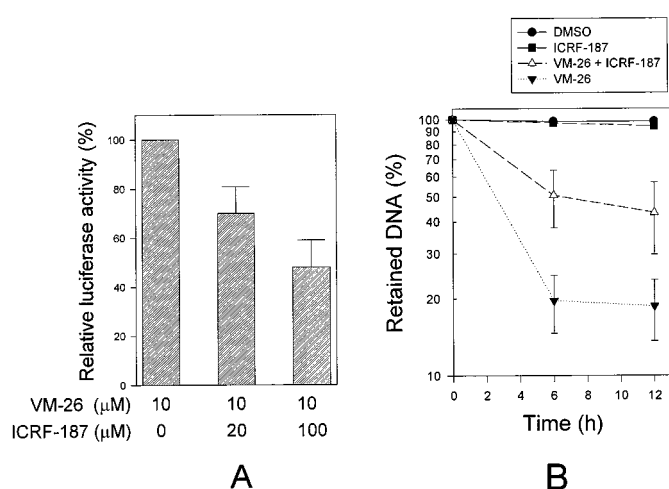


Fig. 7. Effect of ICRF-187 on VM-26-induced FasL promoter activity and DNA strand breaks. **A**, ICRF-187 suppresses VM-26-induced FasL promoter activity. HeLa cells were transfected with the FasL promoter-luciferase construct and pretreated with ICRF-187 at 20 or 100 μ M for 1 h before addition of VM-26 (10 μ M). Cells were incubated for another 24 h and then harvested for luciferase assays. **B**, alkaline elution assays for DNA strand breaks induced by VM-26. HeLa cells were labeled with [14 C]thymidine and analyzed for DNA damage. See *Materials and Methods* for details. All data are means \pm S.D. of three independent experiments.

by phosphorylation in response to DNA damage and thus impairing the ability of MDM2 to inhibit p53-dependent transactivation (Shieh et al., 1997). Understanding these events and factors will provide insight into the signaling pathway that stimulates FasL expression in response to DNA-damaging agents.

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