Topoisomerase II and tubulin inhibitors both induce the formation of apoptotic topoisomerase I cleavage complexes

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Abstract

Topoisomerase I (Top1) is a ubiquitous enzyme that removes DNA supercoiling generated during transcription and replication. Top1 can be trapped on DNA as cleavage complexes by the anticancer drugs referred to as Top1 inhibitors as well as by alterations of the DNA structure. We reported recently that Top1 cleavage complexes (Top1cc) are trapped during apoptosis induced by arsenic trioxide and staurosporine. In the present study, we generalize the occurrence of apoptotic Top1cc in response to anticancer drugs, which by themselves do not directly interact with Top1: the topoisomerase II inhibitors etoposide, doxorubicin, and amssacrine, and the tubulin inhibitors vinblastine and Taxol. In all cases, the Top1cc form in the early phase of apoptosis and persist throughout the apoptotic process. Their formation is prevented by the caspase inhibitor benzoylcarbonyl-Val-Ala-DL-Asp(OMe)-fluoromethylketone and the antioxidant N-acetyl-L-cysteine. We propose that the trapping of Top1cc is a general process of programmed cell death, which is caused by alterations of the DNA structure (oxidized bases and strand breaks) induced by caspases and reactive oxygen species. [Mol Cancer Ther 2006; 5(12):3139–44]

Introduction

Topoisomerase I (Top1) is an essential enzyme in higher eukaryotes as it removes DNA supercoiling generated during transcription and replication (1–3). Top1 relaxes DNA supercoiling by forming DNA single-strand breaks that are produced as Top1 forms a covalent bond between its active site tyrosine (Y723) and a 3'-DNA phosphate. These Top1-linked breaks allow controlled rotation of the broken DNA around the intact strand (2). Immediately after the DNA is relaxed, Top1 spontaneously religates the breaks and restores intact duplex DNA. Under normal conditions, the covalent Top1-cleaved DNA intermediates, referred to as Top1 cleavage complexes (Top1cc), are constitutively transient and almost undetectable because the DNA religation (closing) step is much faster than the DNA cleavage (nicking) step. A variety of common DNA alterations, including base oxidation, methylation, mismatches, carcinogenic adducts, abasic sites, and strand breaks, can trap Top1cc by misaligning the DNA ends within the Top1cc and preventing DNA religation (4, 5). The alkaloid camptothecin and its therapeutic derivatives topotecan and irinotecan also trap Top1 by intercalating specifically inside the Top1cc (2, 6).

Recently, we and others showed the stabilization of Top1cc in cells undergoing apoptosis (7–10). These apoptotic Top1cc have been detected in various human cell types exposed to arsenic trioxide (7), staurosporine (8), or UV irradiation (7). The Top1cc formed during apoptosis likely participate in the cell death program because down-regulation of Top1 by small interfering RNA reduces apoptotic DNA fragmentation induced by arsenic trioxide (8) and staurosporine (9). In the present study, we asked whether the formation of Top1cc is a general process of apoptotic cell death induced by anticancer chemotherapeutic agents. We tested the formation of Top1cc during apoptosis induced by topoisomerase II (Top2) and tubulin inhibitors, which by themselves do not directly interact with Top1. Top2 inhibitors initiate apoptosis by trapping Top2 cleavage complexes (Top2cc), thereby generating DNA double-strand breaks (11–13). Top2 inhibitors, such as etoposide (VP-16), doxorubicin, and amssacrine (m-AMSA), are among the most potent inducers of apoptosis and are commonly prescribed drugs to treat a variety of cancers (14). The tubulin inhibitors vinblastine and Taxol (paclitaxel) are anticancer drugs that initiate apoptosis by binding specifically at the interface of the tubulin heterodimer, thereby interfering with microtubule growth (6, 15, 16).

The present study shows that VP-16, doxorubicin, m-AMSA, and vinblastine induce Top1cc that form in the early phase of apoptosis and persist throughout the apoptotic process. In response to VP-16, the formation of apoptotic Top1cc is prevented by the caspase inhibitor benzoylcarbonyl-Val-Ala-Asp(OMe)-fluoromethylketone (Z-VAD-fmk) and the antioxidant N-acetyl-L-cysteine. We propose that the trapping of Top1cc is a general process of apoptotic cell death, which is caused by alterations of the DNA structure (oxidized bases and strand breaks) induced by caspases and reactive oxygen species (ROS).
Materials and Methods

Cell Culture, Drugs, and Chemical Reagents

The human leukemia (CEM and HL-60) and colon carcinoma (HCT116) cell lines (American Type Culture Collection, Manassas, VA) were cultured as described (8). VP-16, doxorubicin, m-AMSA, vinblastine, and N-acetyl-L-cysteine were obtained from Sigma (St. Louis, MO). The caspase peptide inhibitor z-VAD-fmk was obtained from Bachem (Torrance, CA). Taxol (paclitaxel) was a kind gift from Dr. Tito Fojo (National Cancer Institute, NIH, Bethesda, MD). [2-14C]thymidine was from Perkin-Elmer Life Sciences (Boston, MA).

Detection of Cellular Topoisomerase Cleavage Complexes

Topoisomerase cleavage complexes were detected using the in vivo complex of enzyme bioassay as described (8, 10, 17). Briefly, cells were lysed in 1% Sarkosyl and homogenized. The cell lysates were centrifuged on cesium chloride step gradients at 165,000 x g for 20 h at 20 °C. Twenty fractions (0.5 mL) were collected and diluted (v/v) into 25 mmol/L potassium phosphate buffer (pH 6.6). The DNA-containing fractions (fractions 7–11) were pooled (except in Fig. 1D) and applied to polivinylidene difluoride membranes (Immobilon-P, Millipore, Billerica, MA) using a slot-blot vacuum manifold. Topoisomerase cleavage complexes were detected by immunoblotting using the C21 Top1 mouse monoclonal antibody (1:1,000 dilution; a kind gift from Dr. Yung-Chi Cheng, Yale University, New Haven, CT) or a Top2a mouse monoclonal antibody (clone Ki-S1; 1:1,000 dilution) from Chemicon International (Temecula, CA).

DNA Fragmentation Assay

Apoptotic DNA fragmentation was quantified by filter elution assay as described (18). Briefly, cells were incubated with [2-14C]thymidine (0.02 μCi/mL) for 2 days and chased overnight in radioisotope-free medium. After drug treatment, cells were loaded onto a protein-absorbing filter (Metricon membrane filter, 0.8 μmol/L pore size, 25 mm diameter; Pall Corp., East Hills, NY), washed with PBS, and lysed in 0.2% sodium Sarkosyl, 2 mol/L NaCl, and 0.04 mol/L EDTA (pH 10.0). The filters were then washed with 0.02 mol/L EDTA (pH 10.0). DNA was depurinated by incubation of filters in 1 mol/L HCl at 65 °C and then released from the filters with 0.4 mol/L NaOH at room temperature. Radioactivity was counted by liquid scintillation spectrometry in each fraction (wash, lysis, EDTA wash, and filter). DNA fragmentation was measured as the fraction of disintegrations per min in the lysis fraction plus EDTA wash relative to the total intracellular disintegrations per min.

Caspase-3 Activity

Caspase-3 activity was measured as described (19). Briefly, cells were incubated in lysis buffer [150 mmol/L NaCl, 50 mmol/L Tris-HCl (pH 8.0), 0.1% SDS, 1% NP40,
Results and Discussion

The Top2 Inhibitors VP-16, Doxorubicin, and m-AMSA Induce Top1cc During Apoptosis

Trapped Top1cc and Top2cc can be detected in genomic DNA after cesium chloride gradient centrifugation and immunoblotting with Top1 and Top2 antibodies (8, 10, 17, 20), respectively. In human leukemia CEM cells exposed to VP-16, we detected high levels of Top1cc (Fig. 1A, middle) with kinetics that coincided with the occurrence of apoptosis measured by DNA fragmentation (Fig. 1A, top). Apoptotic DNA fragmentation and Top1cc were both detected after 4 h of VP-16 exposure. Similar levels of Top1cc persisted throughout the apoptotic process as most of the cellular DNA became fragmented (Fig. 1A). The induction of Top1cc induced by VP-16 was coincident with the activation of caspase-3, as shown by the ability of cell lysates to cleave DEVD-AFC, a fluorogenic substrate that mimics the target site of caspase-3 and closely related caspases (Fig. 1B, top), and the decrease of the 32-kDa proform of caspase-3 with the appearance of its cleaved 19- and 17-kDa active fragments (Fig. 1B, middle). Kinetics of caspase-3 activation was further confirmed by the caspase-dependent cleavage of poly(ADP-ribose) polymerase (Fig. 1B, bottom). VP-16-induced Top1cc were also observed in the human colon carcinoma HCT116 cells undergoing apoptosis (Fig. 1C and D). Thus, the induction of Top1cc by VP-16 can be observed in different human cell types as they undergo apoptosis.

VP-16 is a specific Top2 inhibitor (21). It is well established that VP-16 does not trap directly Top1cc. Accordingly, short exposure to VP-16 (for up to 2 h) trapped only Top2cc (Fig. 1A, bottom). However, Top1cc were induced by 4 h of VP-16 exposure (Fig. 1A, middle). These Top1cc persisted after the removal of VP-16 as cells were committed to apoptosis, whereas trapping of Top2cc was only transient (Fig. 2A; ref. 21). Thus, we conclude that trapping of Top2cc by VP-16 initiates apoptosis and subsequently induces the formation of Top1cc. Consistently, two other Top2 inhibitors, doxorubicin and m-AMSA, also induced Top1cc in apoptotic leukemia HL-60 cells (Fig. 2B).

Western Blotting

Western blotting analyses on whole-cell extracts were done as described (8) using the rabbit antihuman caspase-3 (1:5,000 dilution; BD PharMingen, San Diego, CA) and poly(ADP-ribose) polymerase (1:5,000 dilution; Roche, Indianapolis, IN) antibodies.

Figure 2. Induction of apoptotic Top1cc by the Top2 inhibitors, VP-16, m-AMSA, and doxorubicin. A, CEM cells were treated with 200 μmol/L VP-16 for 4 h, washed, and cultured in VP-16-free medium for the indicated times. Top, apoptotic DNA fragmentation. Columns, mean of triplicate samples; bars, SD. Middle and bottom, Top2cc reverse, whereas Top1cc persist following VP-16 removal. B, human leukemia HL-60 cells were treated with 1 μmol/L doxorubicin or 1 μmol/L m-AMSA for 20 h. Top, apoptotic DNA fragmentation. Columns, mean of triplicate samples; bars, SD. Bottom, Top1cc are induced by both doxorubicin and m-AMSA. The DNA-containing fractions were pooled and probed at three concentrations (10, 3, and 1 μg DNA) by immunoblotting with antibodies against Top1 or Top2a.

Figure 3. The caspase inhibitor Z-VAD-fmk and the antioxidant N-acetyl-L-cysteine (NAC) prevent VP-16-induced apoptosis and Top1cc. CEM cells were preincubated with z-VAD-fmk (100 μmol/L for 30 min) or N-acetyl-L-cysteine (30 μmol/L for 30 min) before the addition of 200 μmol/L VP-16 for 8 h. Top, apoptotic DNA fragmentation. Columns, mean of triplicate samples; bars, SD. Bottom, detection of Top1cc. The DNA-containing fractions were pooled and probed at three concentrations (10, 3, and 1 μg DNA) by immunoblotting with an antibody against Top1.
To further examine the relationship between Top1cc and apoptosis, we investigated whether inactivating the apoptotic pathways would affect the generation of Top1cc by VP-16. Inhibition of apoptotic DNA fragmentation by the caspase peptide inhibitor z-VAD-fmk (Fig. 3, top) prevented the induction of Top1cc by VP-16 (Fig. 3, bottom). Together, these findings show the appearance of high levels Top1cc in the early phase of apoptosis induced by Top2 inhibitors.

**VP-16-Induced Top1cc Are Linked to the Generation of Oxygen Radicals**

Because Top1cc can be induced by ROS (22) and oxidative DNA lesions (4, 8–10) and because VP-16 and doxorubicin generate ROS and oxidative DNA lesions (23, 24), we tested whether the apoptotic Top1cc induced by Top2 inhibitors resulted from oxidative fragmentation. Quenching ROS with the antioxidant N-acetyl-L-cysteine prevented VP-16-induced Top1cc and apoptosis (Fig. 3). Similarly, the induction of apoptotic Top1cc by arsenic trioxide and staurosporine has been shown to involve oxidative DNA lesions (8–10). Thus, we propose that Top2 inhibitors induce ROS that produce oxidative DNA lesions (oxidized bases and strand breaks), thereby generating Top1cc in apoptotic cells. These ROS can be generated by permeabilized mitochondria during apoptosis (9).

**The Tubulin Inhibitors Vinblastine and Taxol Also Induce Apoptotic Top1cc**

Next, we examined the occurrence of Top1cc during apoptosis induced by the anticancer drugs vinblastine and Taxol, which induce apoptosis following inhibition of microtubule growth (6, 15, 16). In CEM cells undergoing apoptosis, both vinblastine (Fig. 4A) and Taxol (Fig. 4B) also induced Top1cc. Inhibition of apoptosis by the caspase inhibitor z-VAD-fmk prevented the formation of Top1cc in vinblastine-treated cells (Fig. 4A).

Although it has been reported that Top2α participates in chromatin condensation (25) and in the excision of DNA loops (26) during apoptosis, we did not detect Top2cc in apoptotic cells following exposure to vinblastine (Fig. 4C) and Taxol (Fig. 4D). Similarly, apoptotic Top1cc formed in the absence of Top2cc in response to arsenic trioxide (10). It is therefore possible that the catalytic activity of Top2α (rather than its stabilization on DNA as Top2cc) contributes to chromatin fragmentation. However, we cannot exclude that the levels of Top2cc formed during apoptosis are below the threshold of detection of our assays.

**Conclusion**

Altogether, our findings indicate that the formation of Top1cc is a general process of apoptotic cell death. Table 1 summarizes the various agents that have been identified as producing apoptotic Top1cc. In addition to Top2 and
As Top1 can be directly trapped by DNA breaks and endonuclease G, (30) may contribute to the trapping of apoptotic Top1cc in the parasite Leishmania donovani.

The formation of Top1cc depends on caspase activation and ROS. It is well established that caspases activate endonucleases during apoptosis (30). The DNA breaks produced by apoptotic nucleases, such as CAD/DFF40 and endonuclease G, (30) may contribute to the trapping of Top1cc as Top1 can be directly trapped by DNA breaks (5, 31). In addition, caspases are also involved in the generation of ROS (32, 33). In fact, z-VAD-fmk prevented VP-16-induced oxidative DNA lesions (23). Activation of caspases could therefore serve to generate the ROS that lead to apoptotic Top1cc.

It is therefore plausible that Top1, which is ubiquitous in mammalian cells (34), participates in apoptosis by generating Top1-associated DNA breaks. These breaks may contribute to the cleavage of DNA in high molecular weight fragments (9, 28). Top1cc could also engage the apoptotic machinery in trans as Top1cc are potent initiators of apoptosis (35). Apoptotic Top1cc could therefore serve to amplify the apoptotic process engaged by Top2cc and tubulin inhibition (see Fig. 5) as well as by a variety of other agents, including arsenic trioxide (10), staurosporine (8, 29), UV irradiation (7), tumor necrosis factor–related apoptosis ligand, Fas ligand, and BCL-2 homology domain-3 mimetics (Table 1; ref. 27). The amount of Top1 associated with chromatin may determine the intensity of apoptotic and eventually therapeutic responses to anticancer drugs, such as Top2 and tubulin inhibitors.

References

Table 1. Agents known to induce apoptotic Top1cc

<table>
<thead>
<tr>
<th>Agents that induce apoptotic Top1cc</th>
<th>Cellular target(s)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>VP-16</td>
<td>Stabilization of Top2cc, probably by intercalating within the Top2cc</td>
<td>Present study</td>
</tr>
<tr>
<td>Doxorubicin</td>
<td>Stabilization of Top2cc, probably by intercalating within the Top2cc</td>
<td>Present study</td>
</tr>
<tr>
<td>m-AMSA</td>
<td>Stabilization of Top2cc, probably by intercalating within the Top2cc</td>
<td>Present study</td>
</tr>
<tr>
<td>Vinblastine</td>
<td>Binding at the interface of the tubulin heterodimer</td>
<td>Present study</td>
</tr>
<tr>
<td>Taxol</td>
<td>Binding at the interface of the tubulin heterodimer</td>
<td>Present study</td>
</tr>
<tr>
<td>Colcemid</td>
<td>Inhibits tubulin polymerization</td>
<td>(28)</td>
</tr>
<tr>
<td>TRAIL</td>
<td>Binds to and activates the plasma membrane receptors DR4 and DR5</td>
<td>(27)</td>
</tr>
<tr>
<td>Fas ligand</td>
<td>Binds to and activates the plasma membrane receptor Fas</td>
<td>(27)</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Binds to and activates the plasma membrane receptor TNFR1</td>
<td>(27)</td>
</tr>
<tr>
<td>Antimycin</td>
<td>BH3 mimetic that bind to and inhibit the antiapoptotic effect of BCL-XL at the mitochondrial level</td>
<td>(27)</td>
</tr>
<tr>
<td>BH3I-2</td>
<td>BH3 mimetic that bind to and inhibit the antiapoptotic effect of BCL-XL at the mitochondrial level</td>
<td>(27)</td>
</tr>
<tr>
<td>Arsenic trioxide</td>
<td>Induces the intracellular accumulation of ROS</td>
<td>(10)</td>
</tr>
<tr>
<td>Staurosporine</td>
<td>Inhibitor of protein kinases; Chk1, Chk2, PDK1, and PKC</td>
<td>(8, 29)</td>
</tr>
<tr>
<td>UV irradiation</td>
<td>Generation of pyrimidine dimers and 4,6-photoproducts</td>
<td>(7)</td>
</tr>
</tbody>
</table>

Abbreviations: TNF-α, tumor necrosis factor; TRAIL, tumor necrosis factor–related apoptosis ligand; DR4, death receptor 4, DR5, death receptor 5; TNFR1, tumor necrosis factor receptor 1; BH3, BCL-2 homology domain-3; PDK1, phosphoinositide-dependent kinase 1; PKC, protein kinase C; Chk1, checkpoint kinase 1; Chk2, checkpoint kinase 2.

1 During the preparation of this article, O. Sordet et al., submitted for publication.


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