Topoisomerase I-DNA Complexes Contribute to Arsenic Trioxide-induced Apoptosis*

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Topoisomerase I is an essential enzyme that relaxes DNA supercoiling by forming covalent DNA cleavage complexes, which are normally transient. Topoisomerase I-DNA complexes can be trapped by anticancer drugs (camptothecins) as well as by endogenous and exogenous DNA lesions. We show here that arsenic trioxide (a potent inducer of apoptosis that induces the intracellular accumulation of reactive oxygen species and targets mitochondria) induces cellular topoisomerase I cleavage complexes. Bcl-2 overexpression and quenching of reactive oxygen species, which prevent arsenic trioxide-induced apoptosis, also prevent the formation of topoisomerase I-DNA complexes, whereas enhancement of reactive oxygen species accumulation promotes these complexes. The caspase inhibitor, benzylxycarbonyl-Val-Ala-Asp(OMe) fluoromethyl ketone partially prevents artifactual trioxide-induced topoisomerase I-DNA complexes and apoptosis, suggesting that activated caspases further maintain intracellular levels of reactive oxygen species that induce the formation of topoisomerase I-DNA complexes. Down-regulation of topoisomerase I expression decreases arsenic trioxide-induced apoptotic DNA fragmentation. Thus, we propose that arsenic trioxide induces topoisomerase I-DNA complexes that participate in chromatin fragmentation and programmed cell death during apoptosis.

Arsenic trioxide (As2O3) is one of the most successful treatments for acute promyelocytic leukemia (APL) (1). The cellular responses to As2O3 include apoptosis, inhibition of proliferation, differentiation, and inhibition of angiogenesis (2). As2O3-induced apoptosis was initially described in APL cells (3, 4). However, As2O3 also induces apoptosis in wide range of different cell types (5, 6). As2O3 forms reversible bonds with protein thiol groups. Intracellular levels of GSH titrate intracellular As2O3 by forming transient As(GS)2 complexes and represent a major determinant of As2O3-triggered cell death, with increased intracellular levels of GSH having an inhibitory effect (7–9). A key underlying mechanism for As2O3-induced apoptosis is the dissipation of the mitochondrial transmembrane potential (Δψm), an event inhibited by Bel-2 overexpression (3, 10–12), which is followed by the release of soluble intermembrane proteins into the cytosol, including apoptosis inducing factor, caspase activation, DNA fragmentation, and the classic morphologic changes of apoptosis (10–15). In response to As2O3, the Δψm dissipation essentially results in the intracellular accumulation of reactive oxygen species (ROS), such as hydrogen peroxide (H2O2), hydroxyl radical (HO·), and superoxide radical (O2·−). Inhibition of GSH peroxidase (16) and activation of flavoprotein-dependent superoxide-producing enzymes (such as NADPH oxidase) (13) by As2O3 have been implicated in ROS accumulation. These As2O3-induced ROS generate oxidative DNA lesions, mainly 8-oxoguanine and 8-hydroxy-2′-deoxyguanosine (17–20). As2O3-induced apoptosis has also been related to Bel-2 down-regulation (4, 21), Bax up-regulation (22), histone H3 phosphoacetylation at the caspase-10 gene (15), NFκB inhibition (23), c-Jun NH2-terminal kinase activation (24), and enhanced translocation of PML or promyelocytic leukemia/retnicic acid receptor α chimeric proteins to nuclear bodies (25).

DNA topoisomerase I (Top1) is an essential nuclear enzyme (26, 27), which relaxes DNA supercoiling ahead of replication and transcription complexes by inducing transient single-strand breaks at many sites in the genome, thereby allowing rotation of the DNA double helix around the intact phosphodiester bonds opposite the enzyme-mediated DNA cleavages. Once the DNA has been relaxed, Top1 religates the breaks and regenerates intact duplex DNA. Biochemically, each Top1-mediated break results from the reversible transfer of a DNA phosphodiester bond to the enzyme catalytic tyrosine (human Tyr-723) (27). Under normal conditions, Top1 cleavage complexes are very transient and remain at very low levels; the DNA religation step is much faster than the cleavage step. These transient Top1 cleavage complexes can be stabilized by camptothecins. These drugs, commonly referred to as “Top1 poisons” or “Top1 inhibitors,” specifically bind at the Top1-DNA interface and trap the cleavage complexes by preventing the DNA religation step (28–30). Stabilized Top1 cleavage complexes can be further converted into DNA damage after collisions of replication forks and transcription complexes, initiating cellular responses that include DNA repair (31) and/or apoptosis (30) (see discover.nic.nih.gov/pommier/pommier. html). Recently, Top1 cleavage complexes have been detected after formation of endogenous and exogenous DNA lesions (32). These include oxidative lesions (8-oxoguanine, abasic sites, and strand breaks), UV-induced base modifications, guanine alkylation, polycyclic aromatic carcinogenic adducts, base mis-
matches, and incorporation of genotoxic anticancer agents (including cytosine arabinoside or gemcitabine) (33–38).

The present study provides the first evidence that As$_2$O$_3$ induces the formation of Top1-DNA complexes. The Top1 poisoning effect of As$_2$O$_3$ is indirect because As$_2$O$_3$ does not induce the formation of Top1 cleavage complexes when incubated with recombinant Top1. These Top1-DNA complexes occur in association with As$_2$O$_3$-induced apoptosis, and we propose that they are due to oxidative DNA lesions generated by As$_2$O$_3$-induced ROS and caspase activation. Finally, we provide evidence for the participation of Top1 in chromatin fragmentation during As$_2$O$_3$-induced apoptosis.

**EXPERIMENTAL PROCEDURES**

Drugs and Chemical Reagents—As$_2$O$_3$, etoposide (VP-16), N-acetyl-L-cysteine (NAC), lipoic acid (LA), and DL-buthionine-(SR)-sulfoximine (BSO) were obtained from Sigma; camptothecin (CPT) was obtained from the Drug Synthesis and Chemistry Branch, NCI (Bethesda, MD); and the caspase peptide inhibitor benzyloxycarbonyl-Val-Ala-DL-Asp(OMe)-fluoromethyl ketone (Z-VAD-fmk) was from Bachem (Torrance, CA). Stock solutions were prepared in dimethyl sulfoxide (Me$_2$SO) (CPT, VP-16, Z-VAD-fmk) or ethanol (LA) and stored at $-80^\circ$C. Further dilutions were made in culture medium just before use. The final concentration of Me$_2$SO or ethanol in culture medium never exceeded 0.1% (v/v), which was nontoxic to the cells. As$_2$O$_3$ was diluted in H$_2$O and stored at 4°C. NAC and BSO were diluted in culture medium prior to use. [2-¹⁴C]Thymidine was obtained from PerkinElmer Life Sciences.

**FIG. 1. Arsenic trioxide induces cellular Top1-DNA complexes.**

A, human leukemia NB4 cells were left untreated (Control) or treated with either CPT (1 h, 1 µM) (positive control for Top1), etoposide (VP-16, 1 h, 100 µM) (positive control for Top2), or arsenic trioxide (48 h, 2 µM). Cesium chloride fractions were collected from the bottom of the gradients, and the DNA-containing fractions 7–11 were subjected to Top1 and Top2 immunoblotting. A, a 3'-25P-end-labeled 161-bp DNA fragment was incubated with recombinant human Top1 (rhTop1) in the absence (lane 2) or presence of CPT (0.1 µM (lane 3) and 1 µM (lane 4)) or As$_2$O$_3$ (1 µM (lane 5) and 10 µM (lane 6)). Lane 1, DNA alone. Top1-mediated DNA cleavage fragments are indicated by the arrowheads.

**FIG. 2. Apoptotic cells form Top1-DNA complexes.**

A and B, concentration and time dependence of Top1-DNA complexes in NB4 cells treated with the indicated concentrations of As$_2$O$_3$ for 72 h (A) or with 2 µM As$_2$O$_3$ for the indicated times (B). C, apoptotic DNA fragmentation quantified by filter elution (mean ± S.D. of triplicate samples) in NB4 cells treated with increasing concentrations As$_2$O$_3$ for the indicated times. D–F, NB4 cells were treated with 2 µM As$_2$O$_3$ for indicated times. D, top panel, DEVD-AFC peptide cleavage (caspase-3) activity (mean ± S.D. of triplicate samples). Bottom panel, Western blot analysis of procaspase-3 in whole cell extracts. E, Western blot analysis of Top1 and PARP in whole cell extracts. Numbers are molecular masses in kilodaltons. * indicates cleavage products. F, quantitative analysis by flow cytometry of annexin V-fluorescein isothiocyanate (FITC)-positive cells.
Arsenic Induces Apoptotic Top1-DNA Complexes

Fig. 3. Inhibition of apoptosis by Bel-2 overexpression prevents arsenic trioxide-induced Top1-DNA complexes. Parental (U937) and Bel-2-overexpressing (U937/Bcl2) cells were left untreated (-) or treated with 4 μM As2O3 for 48 h (+) or the indicated times. A, top panel, DEVD-AFC peptide cleavage activity shown as mean ± S.D. of triplicate samples. Bottom panel, Western blot analysis of Top1 in whole cell extracts. Numbers are molecular masses in kilodaltons. * indicates cleavage products. B, apoptotic DNA fragmentation was quantified by filter elution (mean ± S.D. of triplicate samples). C, detection of Top1-DNA complexes in the DNA-containing fractions (fractions 7–11).

Sciences. Recombinant human Top1 was purified from TN5 insect cells (HighFive; Invitrogen) using a Baculovirus construct for the NH2-terminal truncated human Top1 cDNA as described previously (39, 40).

Cell Culture—The human leukemic cell lines NB4 (APL-derived cells, kindly provided by Dr. M. Lanotte, INSERM U496, Hospital Saint-Louis, Paris, France) and U937 (American Type Culture Collection, Manassas, VA) and a U937-derivative cell clone containing the full-length bcl-2 cDNA (U937/Bcl2, kindly provided by Dr. J. Breard, INSERM U461, Chatenay-Malabry, France) were grown in suspension in RPMI 1640 medium with glutamax-I (Invitrogen) supplemented with 10% (v/v) fetal bovine serum (Gemini Bio-Products, Woodland, CA) and antibiotics (100 units/ml penicillin, 100 μg/ml streptomycin) in an atmosphere of 95% air and 5% CO2 at 37 °C. The murine leukemia cell line P388 and its camptothecin-resistant, Top1-deficient subline, P388/CPT45 (35), were gifts from M. R. Mattern and R. K. Johnson (GlassSmithKline).

Detection of Covalent Top1-DNA Complexes—Top1-DNA complexes were detected using the in vivo complex of enzyme bioassay (41). Briefly, 106 cells were lysed in 1% Sarkosyl and homogenized with a Dounce homogenizer. Cell lysate was layered on cesium chloride step gradients and centrifuged at 165,000 × g for 20 h at 20 °C. Twenty fractions (0.5 ml each) were collected from the bottom, diluted into an equal volume of 25 mM potassium phosphate buffer, pH 6.6, and applied to polyvinylidene difluoride membrane (Immobilon-P, Millipore, MA) by using a slot-blot vacuum manifold. Topoisomerase-DNA complexes were detected by immunoblotting using the C21 Top1 mouse monoclonal antibody (a kind gift from Dr. Yung-Chi Cheng, Yale University, New Haven, CT) or a Top2 monoclonal antibody (clone Ki-S1) from Chemicon International (Temecula, CA).

Top1-mediated DNA Cleavage Assay—Approximately 50 fmol of 3′-α-32P-end-labeled 161-bp DNA fragment (39) was incubated with 5 ng of recombinant human Top1 with or without drugs at 25 °C in 10 μl of reaction buffer (10 mM Tris-HCl, pH 7.5, 50 mM KCl, 5 mM MgCl2, 0.1 mM EDTA, 0.1% SDS, and 3.3 volumes of Maxam Gilbert loading buffer (80% formamide, 10 mM sodium hydroxide, 1 mM sodium EDTA, 0.1% xylene cyanol, and 0.1% bromphenol blue, pH 8.0)). Reactions were stopped by adding 0.5% SDS and 3.3 volumes of Maxam Gilbert loading buffer (80% formamide, 10 mM sodium hydroxide, 1 mM sodium EDTA, 0.1% xylene cyanol, and 0.1% bromphenol blue, pH 8.0). Aliquots were separated in 16% denaturing polyacrylamide gels (7.5% urea) in TBE (45 mM Tris, 45 mM boric acid, 1 mM EDTA) for 2 h at 40 Vcm. Imaging and quantitation were performed by using a PhosphorImager (Amersham Biosciences).

DNA Fragmentation—DNA fragmentation-related apoptosis was quantified using the previously reported filter elution assay (42). Briefly, cells were incubated with 0.02 μCi/ml [2-14C]thymidine and cultured at 37 °C for 2 days. Then cells were chased in isotope-free medium overnight, resuspended in fresh medium, and treated. One million treated or untreated labeled cells were loaded onto a protein-absorbing filter (Metrical® Membrane Filter, 0.8-μm pore size, 25 mm diameter; Pall Corp.). Cells were washed with phosphate-buffered saline and lysed in 0.2% sodium Sarkosyl, 2 mM NaCl, 0.04 mM EDTA, pH 10.0. The filters were then washed with 0.02 mM EDTA, pH 10.0. DNA was depurinated by incubation of filters in 1 mM HCl at 65 °C and then released from the filters with 0.4 M 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/min in the lysis fraction plus EDTA wash relative to the total intracellular disintegrations/min. For sub-G1 analysis, DNA content was assessed by staining ethanol-fixed cells with propidium iodide and monitoring by FACScan (BD Biosciences). The numbers of cells with sub-G1 DNA were determined with a CellQuest program (BD Biosciences).

Caspase-3 Activity—Caspase-3 activity was measured as described (43). Cells were incubated in lysis buffer (150 mM NaCl, 50 mM Tris-HCl, pH 8.0, 0.1% SDS, 1% Nonidet P-40, 0.5% sodium deoxycholate) for 30 min at 4 °C and centrifuged (10,000 × g, 20 min, 4 °C). Twenty micrograms of protein from the resulting supernatant was incubated in buffer assay (100 mM HEPES, pH 7.0, 1 mM EDTA, 0.1% CHAPS, 10% glycerol, 20 mM diithiothreitol) in the presence of 100 μM of the fluorogenic peptide substrate Z-DEVD-AFC (Calbiochem). 7-Amino-4-trifluoromethylcoumarin (AFC) released from the substrate was excited at 400 nm to measure emission at 505 nm. Fluorescence was monitored continuously at 37 °C for 20 min in a dual luminescence fluorimeter (SPECTRAMax® GEMINI XS, Molecular Devices). Enzyme activities were determined as initial velocities expressed as relative intensity/min/mg.

Western Blotting—Cells were lysed at 4 °C in buffer containing 1% SDS, 1 mM sodium vanadate, 10 mM Tris-HCl, pH 7.4, supplemented with protease inhibitors (Complete; Roche Diagnostics). Viscosity of the sam-
samples was reduced by brief sonication, and 20 μg of protein (Bio-Rad Protein Assay; Bio-Rad) were incubated in loading buffer (125 mM Tris-HCl, pH 6.8, 10% β-mercaptoethanol, 4.6% SDS, 20% glycerol, and 0.003% bromphenol blue), separated by SDS-polyacrylamide gel, and transferred to polyvinyldene difluoride membrane (Immobilon-P, Millipore, MA). After blocking nonspecific binding sites for 1 h by 0.2% casein

**FIG. 4.** Relationship between reactive oxygen species production and Top1-DNA complexes formation in arsenic trioxide-treated NB4 cells. A–C, NB4 cells were left untreated (Control) or preincubated with either 10 mM NAC or 0.1 mM LA for 30 min before addition of 2 μM As2O3 for 48 h or the indicated times. D–F, NB4 cells were left untreated (control) or treated with As2O3 (0.5 μM), BSO (0.1 mM), or the As2O3/BSO combination (2 h of preincubation with BSO) for 72 h or the indicated times. A and D, reactive oxygen species measured by flow cytometry (FL2-H). B and E, apoptotic DNA fragmentation was quantified by filter elution (mean ± S.D. of triplicate samples). C and F, detection of Top1-DNA complexes in the DNA-containing fractions (fractions 7–11).

**FIG. 5.** Involvement of caspases in the formation of Top1-DNA complexes induced by arsenic trioxide. NB4 cells were left untreated or treated with As2O3 (2 μM), Z-VAD-fmk (100 μM), or the As2O3/Z-VAD-fmk combination (30-min preincubation with Z-VAD-fmk) for 48 h or the indicated times. A, top panel, DEVD-AFC peptide cleavage activity (mean ± S.D. of triplicate samples). Bottom panel, Western blot analysis of Top1 in whole cell extracts. Numbers are molecular masses in kilodaltons. * indicates cleavage products. B, detection of Top1-DNA complexes in the DNA-containing fractions (fractions 7–11). C, apoptotic DNA fragmentation was quantified by filter elution (mean ± S.D. of triplicate samples).
in TPBS (phosphate-buffered saline, Tween 20 0.1%), the membrane was incubated for 2 h at room temperature with primary antibody. Antibodies used include rabbit antihuman procaspase-3 (Pharmingen) and Top1 (local source). After three washes in TPBS, the membrane was incubated with horseradish peroxidase-conjugated goat anti-rabbit antibody (Amer- sham Biosciences) for 1 h at room temperature and then washed three times in TPBS. Immunoblot was revealed using enhanced chemiluminescence detection kit (Pierce) by autoradiography.

Flow Cytometry Analyses—Dihydrothididine (Sigma) was used as a substrate for measuring the production of intracellular hydrogen peroxide (H₂O₂) and other ROS. Expression of phosphatidylserine in the external layer of the plasma membrane was determined by studying the fixation of annexin V-fluorescein isothiocyanate in the presence of Ca²⁺ in cells that remain nonpermeant to propidium iodide (Annexin-V-FLUOS staining kit; Roche Diagnostics). All these analyses were performed using a FACScan cytometer (BD Biosciences).

Top1 Silencing by RNA Interference—A human U6 promoter-driven DNA vector stably expressing siRNA hairpins targeting human Top1 mRNA was constructed. The vectors were obtained by inserting the DNA vector stably expressing siRNA hairpins targeting human Top1 into pBS/U6 plasmid (44), respectively. Top1 cDNA sequence as well as a negative control sequence together with the U6 promoter were then subcloned into pREP4/Top1 and pREP4/negative vector, respectively, accord- ing to the LipofectAMINETM 2000 protocol (Invitrogen). Transfected HCT116 cells were transfected with the Top1 RNA-interfering construct pRE4/Top1 and pRE4/negative vector, respectively, according to the LipofectAMINE™ 2000 protocol (Invitrogen). Transfected HCT116 cells were cultured in Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum and 600 μg/ml hygromycin B. After selection for 10–14 days, HCT116 colonies were monitored for Top1 expression by Western blotting. Silencing Top1 expression was confirmed by confocal microscopy analysis using rabbit antihuman Top1 (local source) and according to the method described previously (43).

RESULTS

Arsenic Induces Apoptotic Top1-DNA Complexes—The presence of Top1-DNA complexes in genomic DNA can be detected after cesium chloride gradient centrifugation and fractionation from tissue culture cells and tumor samples (41). In As₂O₃-treated human promyelocytic NB4 cells, immunoblotting revealed the presence of Top1 in the DNA-containing fractions (fractions 7–11) (Fig. 1A). No Top2-DNA complexes could be detected in these same fractions (Fig. 1A). As expected, Top1-DNA complexes were also observed in NB4 cells exposed to the Top1 inhibitor camptothecin, and Top2-DNA complexes were only observed in cells treated with etoposide (VP-16) (Fig. 1A). However, unlike camptothecin, As₂O₃ was not able to generate Top1 cleavage complexes in normal DNA in the presence of recombinant human Top1 in vitro (Fig. 1B), indicating that As₂O₃ does not directly poison Top1. Thus, cellular Top1-DNA complexes are secondary to intracellular modifications induced by As₂O₃.

As₂O₃-induced Top1-DNA Complexes Are Formed in Cells Undergoing Apoptosis—Given that As₂O₃ is a known inducer of apoptosis in human leukemic cells (2), we tested whether the cellular Top1-DNA complexes resulted from the engagement of apoptotic pathway(s) induced by As₂O₃. Top1-DNA complexes were detected in a concentration- and time-dependent manner (Fig. 2A and B) concomitantly with apoptotic DNA fragmentation (Fig. 2C). Also, the formation of the Top1-DNA complexes induced by As₂O₃ was coincident with the activation of caspase-3, as demonstrated by the decrease of the M₇₃,000 proform of caspase-3 (Fig. 2D, bottom panel) and the ability of cell lysates to cleave DEVD-AFC, a fluorogenic peptide substrate that mimics the target site of caspase-3 and closely related caspases (Fig. 2D, top panel). Kinetics of caspase-3 activation was further confirmed by the caspase-dependent cleavage of the nuclear enzymes Top1 (46) and poly(ADP-ribose)-polymerase (PARP) (47) (Fig. 2E). Furthermore, NB4 cells exhibited phosphatidylserine on the outer plasma membrane leaflet at the time Top1-DNA complexes were detected (Fig. 2F). Thus, the induction of Top1-DNA complexes by As₂O₃ in NB4 cells is closely associated with apoptosis.

To examine further the relationship between Top1-DNA complexes and apoptosis, we examined the formation of these complexes in As₂O₃-sensitive and As₂O₃-resistant cells. We and others (10, 12) had found previously that overexpression of full-length Bcl-2 protects U937 leukemia cells against apoptosis induced by As₂O₃. Bcl-2 overexpression prevented caspase-3 activation and caspase-mediated Top1 cleavage (Fig. 3A) and markedly decreased DNA fragmentation in U937 cells (Fig. 3B). Inhibition of As₂O₃-induced apoptosis by Bcl-2 overexpression also inhibited the formation of Top1-DNA complexes (Fig. 3C). Collectively, these findings indicate that the formation of Top1-DNA complexes is related to apoptosis in NB4 cells following As₂O₃ treatment.

![Fig. 6. Relationship between caspase activation and ROS production in NB4 cells treated with arsenic trioxide.](image-url)
Intracellular Reactive Oxygen Species Production Is Required for As$_2$O$_3$-induced Top1-DNA Complexes—Generation of intracellular hydrogen peroxide (H$_2$O$_2$) and other reactive oxygen species (ROS) is a major determinant for As$_2$O$_3$-induced apoptosis (2). Because Top1 cleavage complexes can be produced by oxidative DNA lesions (see Introduction), we investigated whether modulation of ROS levels by changing the cellular GSH content would affect the generation of Top1-DNA complexes by As$_2$O$_3$.

Treatment of NB4 cells with As$_2$O$_3$ together with NAC (8) or LA, which up-regulate GSH (7, 48), prevented both As$_2$O$_3$-induced ROS production (Fig. 4A) and apoptotic DNA fragmentation (Fig. 4B). By using the same experimental conditions, NAC and LA also abrogated As$_2$O$_3$-induced Top1-DNA complexes (Fig. 4C). Conversely, treatment of NB4 cells with a nontoxic concentration of As$_2$O$_3$ (0.5 μM) (Fig. 2C) together with BSO, which induces GSH depletion (49), potentiated both the accumulation of intracellular ROS (Fig. 4D) and apoptosis (Fig. 4E) following As$_2$O$_3$ exposure. Under these conditions, BSO also potentiated the formation of Top1-DNA complexes (Fig. 4F). Altogether, these findings suggest that ROS lead to the formation of Top1-DNA complexes following As$_2$O$_3$ exposure.

Involvement of Top1 in DNA fragmentation during arsenic trioxide-induced apoptosis. A–D, Top1 was silenced in HCT116 cells using U6 promoter-driven DNA vectors stably expressing siRNA hairpins targeting human Top1 (siRNA-Top1) or a negative control sequence (Control). A, Western blot analysis of Top1 and actin in whole cell extracts. Numbers are molecular masses in kilodaltons. B, confocal microscopy analysis of Top1 (red) and actin (green). C and D, HCT116 cells were treated for 24 h with the indicated concentrations of As$_2$O$_3$, and DNA content was analyzed by flow cytometry. C, sub-G$_1$ cells are indicated by the brackets. D, the results (mean ± S.D. of three independent experiments) for the 30 μM concentration of As$_2$O$_3$ are presented as the percentages of cells with sub-G$_1$ DNA. E, parental (●) and Top1-deficient (P388/CPT45, ○) P388 cells were treated with 3 μM As$_2$O$_3$ for the indicated times. Apoptotic DNA fragmentation was quantified by filter elution (mean ± S.D. of triplicate samples).

Proposed mechanism for the induction of Top1-DNA complexes during As$_2$O$_3$-induced apoptosis. ROS generated by As$_2$O$_3$ produce oxidative DNA lesions that trap Top1 on DNA. Activation of caspases further maintains the level of intracellular ROS.
Involvement of Caspases in Enhancing \( \text{As}_2\text{O}_3 \)-induced ROS and Top1-DNA Complexes—We next investigated the role of caspases in the generation of Top1-DNA complexes. Combined treatment with \( \text{As}_2\text{O}_3 \) and the broad spectrum caspase inhibitor Z-VAD-fmk completely inhibited caspase-3 activation and the caspase-dependent cleavage of Top1 (Fig. 5A). However, caspase inhibition partially prevented the formation of Top1-DNA complexes (Fig. 5B), suggesting that caspase-3 is only partially involved in promoting these complexes. \( \text{As}_2\text{O}_3 \)-induced apoptotic DNA fragmentation was also inhibited by Z-VAD-fmk (Fig. 6C).

We next examined the relationship between ROS production and caspase activation in \( \text{As}_2\text{O}_3 \)-treated cells. First, prevention of \( \text{As}_2\text{O}_3 \)-induced ROS production by NAC or LA blocked caspase-3 activation and caspase-dependent cleavage of Top1 (Fig. 6A), whereas enhancement of \( \text{As}_2\text{O}_3 \)-induced ROS production by BSO led to caspase-3 activation (Fig. 6B). These results indicate that caspase-3 activation is downstream from the production of ROS by \( \text{As}_2\text{O}_3 \). Inhibition of caspase activation by Z-VAD-fmk decreased the intracellular level of ROS produced by \( \text{As}_2\text{O}_3 \) (Fig. 6C), suggesting that caspase activation contributes to the generation of ROS by \( \text{As}_2\text{O}_3 \). Altogether, these findings suggest that caspases indirectly participate in the formation of Top1-DNA complexes by further increasing the intracellular level of ROS induced by \( \text{As}_2\text{O}_3 \).

Involvement of Top1-DNA Complexes in Chromatin DNA Fragmentation during \( \text{As}_2\text{O}_3 \)-induced Apoptosis—To evaluate the involvement of Top1 in chromatin fragmentation during apoptosis, we used silencing of Top1 by siRNA. As shown in Fig. 7, A and B, expression of Top1 was suppressed in HCT116 cell expressing siRNA hairpins targeting human Top1 (siRNA-Top1) compared with cells expressing a negative control sequence (control). Silencing of Top1 significantly reduced \( \text{As}_2\text{O}_3 \)-induced DNA fragmentation in HCT116 cells (Fig. 7, C and D). Also, apoptotic-related DNA fragmentation was reduced in P388/CPT45 cells lacking Top1 (35) compared with P388 cells expressing Top1. Together, these findings indicate that Top1 cleavage complexes contribute to apoptotic DNA fragmentation.

DISCUSSION

This study demonstrates the formation of Top1 cleavage complexes in cells undergoing apoptosis after \( \text{As}_2\text{O}_3 \) treatment. We found that Top1 cleavage complexes coincided with caspase-3 activation, caspase-mediated cleavage of Top1 and PARP, externalization of phosphatidylserine on the outer plasma membrane, and DNA fragmentation (see Fig. 2). Induction of Top1 cleavage complexes is likely to be dependent on \( \text{As}_2\text{O}_3 \)-induced apoptosis as overexpression of full-length Bcl-2 prevents both apoptosis and the formation of these complexes (Fig. 3). Bcl-2 protein negatively regulates mitochondrial depolarization, a pivotal event in \( \text{As}_2\text{O}_3 \)-mediated apoptosis in many cell types (3, 10–12). Although As2O3 can alter proteins by forming bonds with the thiol group of amino acids (2), we found that \( \text{As}_2\text{O}_3 \) cannot induce cleavage complexes in normal DNA in the presence of recombinant Top1 (Fig. 1). Thus, Top1 cleavage complexes are secondary to intracellular modifications induced by \( \text{As}_2\text{O}_3 \) during apoptosis rather than a simple interaction between \( \text{As}_2\text{O}_3 \) and normal Top1-DNA complexes.

We propose that \( \text{As}_2\text{O}_3 \)-induced Top1 cleavage complexes are related to the generation of oxidative DNA lesions (17–20). Indeed, NAC or LA prevents the induction of Top1-DNA complexes by \( \text{As}_2\text{O}_3 \) (Fig. 4C), and enhancement of \( \text{As}_2\text{O}_3 \)-induced ROS by BSO (Fig. 4D) potentiates the formation of the Top1-DNA complexes (Fig. 4F). It is therefore likely that oxidative DNA modifications are involved in the formation of Top1 cleavage complexes by \( \text{As}_2\text{O}_3 \). In fact, cellular exposure to \( \text{H}_2\text{O}_2 \) was recently shown to induce Top1-DNA complexes (50). Thus, we propose (Fig. 8) that \( \text{As}_2\text{O}_3 \)-induced ROS damage DNA, which in turn generate Top1 cleavage complexes.

ROS also target mitochondria and induce the dissipation of the \( \Delta\text{m}_{\text{et}} \), which is followed by the release of cytochrome c and the downstream activation of caspase-9 and caspase-3 (11–15). Because Bel-2 overexpression prevented the formation of Top1 cleavage complexes (Fig. 3C), mitochondrial lesions are likely to initiate the formation of Top1 cleavage complexes. We found that in NB4 cells, the peptide Z-VAD-fmk partially suppressed both Top1 cleavage complexes and DNA fragmentation induced by \( \text{As}_2\text{O}_3 \) (Fig. 5, B and C), suggesting that caspase activation amplifies the generation of Top1-DNA complexes. Although in myeloma cells, \( \text{As}_2\text{O}_3 \) can use caspase-dependent and -independent pathways to apoptosis (51), these enzymes appear essential in APL-derived cells (16). Two mechanisms could be invoked for activation of Top1 cleavage complexes by caspases. First, caspases could cleave/activate Top1 as the reported caspase-3-dependent cleavage of the 100-kDa native Top1 protein into ~70-kDa COOH-terminal fragments (46) occurs concomitantly with the formation of Top1 cleavage complexes (Fig. 2). However, our data suggest that the cleavage of Top1 is unlikely related to its trapping because Top1 cleavage complexes could form (Fig. 5B) under conditions where the cleavage of Top1 is not detected (Fig. 5A). Second, caspases could be involved for the generation of ROS. Indeed, Ricci et al. (52) recently reported that after cytochrome c release, the activation of caspase-3 feeds back on the permeabilized mitochondria to generate ROS as caspase-3 processes complexes I and II in the electron transport chain. Consistently, we found that Z-VAD-fmk decreases the intracellular level of ROS produced by \( \text{As}_2\text{O}_3 \) (Fig. 6C), suggesting that caspases are involved in the generation of ROS. Thus, we propose that activation of caspases could serve to amplify the generation of ROS that leads to Top1-DNA complexes during \( \text{As}_2\text{O}_3 \)-induced apoptosis (Fig. 8).

Finally, this study provides the first evidence for a functional role of Top1 in apoptosis. Silencing of Top1 (Fig. 7A) significantly suppressed the induction of apoptotic DNA fragmentation by \( \text{As}_2\text{O}_3 \) (Fig. 7, C and D). Also, Top1-deficient cells (P388/CPT45) (35) exhibited significantly less \( \text{As}_2\text{O}_3 \)-induced apoptotic DNA fragmentation than the parental cells (P388) (Fig. 7E). The interpretation of this result could however, be questioned because P388/CPT45 cells were selected by continuous exposure to camptothecins (53) and other molecular changes within the apoptotic pathways may also contribute to apoptotic defects in these cells. Nevertheless, Top1 could participate in \( \text{As}_2\text{O}_3 \)-induced apoptosis by directly generating DNA strand breaks as an apoptotic nuclease. Top1-DNA complexes could also engage the apoptotic machinery in trans, as trapping of Top1 by camptothecins is among the most efficient inducers of apoptosis (30). Thus, apoptotic Top1-DNA complexes therefore could serve to amplify the apoptotic process engaged by \( \text{As}_2\text{O}_3 \), as well as other agents including UV (54) and staurosporine (55).

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