DNA Damage-mediated Apoptosis Induced by Selenium Compounds*

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Nai Zhou, Hai Xiao, Tsai-Kun Li, Alam Nur-E-Kamal, and Leroy F. Liu‡

From the Department of Pharmacology, University of Medicine and Dentistry of New Jersey-Robert Wood Johnson Medical School, Piscataway, New Jersey 08854

Selenium (Se) compounds, which are the most extensively studied cancer chemopreventive agents, induce apoptotic death of tumor cells. In the current study, we show that selenite-induced apoptosis involves DNA damage. We showed that selenite-induced apoptosis as evidenced by cleavage of poly(ADP-ribose) polymerase was reduced in NIH 3T3 cells treated with ATM small interfering RNA, suggesting the involvement of the DNA damage regulator ATM. Consistent with ATM/ATR involvement, selenite was also shown to stimulate Ser139 phosphorylation of the ATM/ATR substrate H2AX. Selenite-induced apoptosis was shown to involve DNA topoisomerase II (Top II) as selenite-induced apoptosis was reduced in Top II-deficient HL-60/MX2 cells and in HL-60 cells co-treated with the Top II catalytic inhibitor ICRF-193. Using purified human recombinant Top II, selenite was shown to induce reversible Top II cleavage complexes in vitro. In the aggregate, these results suggest that selenite-induced apoptosis, which involves ATM/ATR and Top II, is likely to be caused by DNA damage.

Cancer chemoprevention has received increasing attention in recent years. Many agents, including naturally occurring and synthetic compounds, have been demonstrated to exhibit cancer preventive activity (1). Of particular significance is the realization that many dietary components have cancer chemopreventive activity (1). Selenium (Se) (a micronutrient) and its natural precursors, selenocysteine, which is incorporated into selenoproteins upon conversion to the amino acid selenocysteine, is used for the synthesis of selenoproteins. In addition, triphenylselenium chloride has excellent chemopreventive activity but does not play a role in the nutritional requirement for Se (7). Another favorable explanation is that Se compounds affect carcinogen activation and metabolism through inhibition of phase I enzymes and induction of phase II enzymes (2–4). This mechanism has been well documented to be important for the chemopreventive activity of many thiol-reactive chemopreventive agents (8–11). However, Se compounds can also suppress carcinogenesis post-initiation of carcinogen treatment in animal models, suggesting that additional mechanisms for cancer chemoprevention may exist (2). The ability of Se compounds to inhibit growth and induce tumor cell apoptosis has been widely demonstrated and suggested to be a potential mechanism for cancer chemoprevention (2). The potency and relative efficacy of Se derivatives as chemopreventive agents in vivo has been shown to parallel their effect on growth inhibition and apoptosis (4). It has been speculated that Se-induced apoptosis can deplete carcinogen-initiated cells and suppress clonal expansion of a transformed population (2). Se compounds are well known for their ability to react with thiols (3). Modification of protein thiols is presumably responsible for enzyme inhibition observed in cells treated with Se compounds (3). However, it is unclear whether modification of protein thiols by Se compounds is the cause of apoptotic cell death.

Top II is an important nuclear enzyme and a drug target (12–15). Its cleavage/religation activity is essential for many vital functions of DNA, such as chromosome condensation and segregation, DNA replication, and transcription (12, 13). However, its delicate act on DNA makes it highly vulnerable to many xenobiotics and physiological stresses that can abort the cleavage/religation reaction to generate Top II-DNA covalent complexes, often referred to as cleavage or cleavable complexes.

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‡ To whom correspondence should be addressed: Dept. of Pharmacology, UMDNJ-Robert Wood Johnson Medical School, 675 Hoes Lane, Piscataway, NJ 08854. Tel.: 732-235-4592; Fax: 732-235-4073; E-mail: liu@umdnj.edu.

2 The abbreviations used are: Top, topoisomerase; hTop IIa, human Top IIa isozyme; ATM, ataxia telangiectasia mutated; ATR, the ATM and Rad3-related protein; PARP, poly(ADP-ribose) polymerase; siRNA, small interfering RNA; SCGE, single cell gel electrophoresis; CPT, camptothecin.
Topoisomerase II Poisoning by Selenium Compounds

(14, 15). Top II-DNA cleavage complexes have been shown to be highly effective in triggering tumor cell apoptosis (16). Recent studies have demonstrated that many thiol alkylators can induce Top II cleavage complexes (17). The involvement of cysteine modification by thiol alkylators has been suggested, because cysteineless mutant Top II is completely resistant to Top II cleavage complex formation induced by thiol alkylators (17). Covalent modification of a cysteinyl thiol(s) on the Top II cleavage complex presumably stabilizes and hence accumulates the Top II cleavage complex (17).

The ability of Se compounds to modify protein thiols has prompted us to test whether they induce Top II cleavage complexes. Our current studies have demonstrated that Se compounds induce Top II cleavage complexes in vitro. In addition, our studies in cells have demonstrated that selenite activates DNA damage signals and induces ATM (18, 19)-dependent apoptosis, suggesting that DNA damage plays a major role in selenite-induced apoptosis.

EXPERIMENTAL PROCEDURES

Materials—Sodium selenite (Na 2SeO 3) was purchased from Sigma. VM-26 was a generous gift from Bristol-Myers Squibb Co. The mitoxantrone-resistant variant of HL-60, HL-60/MX2, was obtained from ATCC. HL-60/MX2 cells are known to be deficient in Top II because of a lowered nuclear Top II content (about 2-fold reduction) and absence of Top IIβ (41). Human Top IIα was purified from yeast over-expressing hTop IIα using the published procedure (20). The ATM siRNA was obtained by annealing the sense (5′-CAUACUACUUCAGAGUACU-3′) and antisense (5′-AAUUUGCUUUAGUGAAGUUA-3′) RNAs, which were purchased from Dharmacon Research, Inc. Cellfectin was purchased from Invitrogen. ATM and actin antibodies were obtained from Santa Cruz Biotechnology, Inc. Poly(ADP-ribose) polymerase (PARP) antibodies and anti-phospho histone H2AX antibodies were purchased from Trevigen Inc. Co.

Alkaline Single Cell Gel Electrophoresis (SCGE) Assay—SCGE or comet assay was performed as described (21). Briefly, cells (5 × 10 5) were pelleted and resuspended in 1 ml of ice-cold phosphate-buffered saline buffer. From this suspension, 50 μl was mixed with 0.5 ml of pre-warmed 0.7% low melting point agarose. 0.1 ml of this mixture was then loaded onto a fully frosted slide that was pre-coated with 0.7% agarose. A very thin layer was formed quickly by rapid application of a coverslip. The slide was kept at 4°C for 10 min. The slides were then submerged in pre-chilled lysis solution (1% N-laurylsarcosine, 1% Triton X-100, 5% NaCl, and 10 mM EDTA, pH 10.5) for 1 h at 4°C. After washing with electrophoresis buffer (0.3 M NaOH and 1 mM EDTA), slides were then subjected to electrophoresis for 10 min at 2 V/cm. After electrophoresis, slides were stained with Sybr Gold (Molecular Probes), denatured, and nuclei were visualized under a microscope. The images of nuclei were captured by a CCD camera and analyzed with the NIH Image program as described (21). DNA damage was then calculated using a macro written for the NIH image protocol (21).

DNA Fragmentation Assay by Agarose Gel Electrophoresis—2.5 × 10 5 cells in each sample were treated with different compounds for 4 h at 37°C. Cells were then pelleted and lysed in 0.4 ml lysis buffer (10 mM Tris-HCl, pH 8.0, 20 mM EDTA, and 0.2% Triton-X100) and incubated on ice for 20 min. After centrifugation to remove nuclei, soluble chromosomal DNA including both high molecular weight DNA and nucleosomal DNA fragments were extracted with phenol/chloroform, precipitated with ice cold 70% (v/v) acetone, and 2 volumes of ethanol and stored at −20°C overnight. DNA was pelleted by centrifugation, rinsed with 70% ethanol, and then resuspended in TE (Tris-EDTA) buffer containing 100 μg/ml RNase A. After 2 h of incubation at 37°C, DNA samples were electrophoresed in 1.5% agarose gel, stained with ethidium bromide, and visualized under UV light.

Top II-mediated DNA Cleavage Assay—The reaction mixture (20 μl each) containing 40 mM Tris-HCl, pH 7.5, 100 mM KCl, 10 mM MgCl 2, 0.5 mM EDTA, 30 μM bovine serum albumin, 20 ng of 3′-end 32P-labeled YeG DNA, 10 ng of purified hTop IIα, and drug was incubated at 37°C for 20 min. The reactions were terminated by addition of 5% SDS and 1 mg/ml proteinase K, followed by incubation for an additional 60 min at 37°C. DNA samples were electrophoresed in 1% agarose gel containing 0.5× TPE (Tris-phosphate-EDTA) buffer. Gels were dried onto Whatman 3MM chromatographic paper and autoradiographed at ~80°C using Eastman Kodak Co. XAR-5 film.

Immunoblotting Analysis—Cells were lysed with 2× SDS sample buffer. Proteins in cell lysates were separated in 10% SDS-PAGE gel and electrophoretically transferred onto a nitrocellulose membrane. All membranes were stained with Ponceau to confirm equal protein loading. The membrane was blocked with 5% milk for 1 h. Immunoblotting was performed using various antibodies. The secondary antibodies were incubated for 1 h at room temperature. Bound secondary antibodies were detected using the ECL Western procedure (Pierce). siRNA—ATM siRNA was produced by annealing an equimolar amount of sense and antisense RNAs according to the manufacturer’s recommended protocol. For transfection, subconfluent NIH 3T3 cells in serum-free Dulbecco’s modified Eagle’s medium were transfected with cellfectin alone, cellfectin complexed with 145 ng of ATM sense RNA, or cellfectin complexed with different amounts of ATM siRNA (15, 45, or 145 ng) for 4 h. Medium was then changed to Dulbecco’s modified Eagle’s medium containing 10% bovine serum, and incubation was continued for another 24 h. Cells were collected and lysed in SDS sample buffer. After SDS-PAGE, immunoblotting was performed using ATM and actin antibodies.

RESULTS

Selenium Induces DNA Damage as Revealed by Alkaline SCGE—The alkaline SCGE or comet assay under alkaline conditions (21) was used to measure chromosomal DNA strand breaks in cells exposed to selenite. Chromosomal DNA strand breaks, which were measured using the comet tail moment, were increased in HL-60 cells exposed to selenite (500 μM for 30 min) as compared with control untreated HL-60 cells (Fig. 1). VM-26 (teniposide), a Top II-specific poison that efficiently induces Top II-mediated DNA damage (22), was used as a control and was also shown to increase the comet tail moment (Fig. 1). These results indicate that selenite, like VM-26, induces chromosomal DNA strand breaks.

Selenium Induces Ser-139 Phosphorylation of H2AX in HL-60 Cells—Activation of nuclear kinases ATM/ATR represents one of the earliest DNA damage signals (23, 24). Activated ATM/ ATR kinases are known to phosphorylate, among other targets, Ser-139 of histone H2AX as revealed by immunoblotting with anti-phospho histone H2AX antibodies, suggesting that selenite, like VM-26, induces activation of ATM/ATR kinases. Identical treatments with selenite and VM-26 were also performed in Top II-deficient HL-60/MX2 cells (Fig. 2, lower panel). As shown in Fig. 2 (lower panel), Ser-139 phosphorylation of H2AX induced by VM-26 was greatly reduced (about 10-fold) in Top II-deficient HL-60/MX2 cells as compared with HL-60 cells.

FIG. 1. Selenite induces strand breaks in chromosomal DNA as revealed by alkaline SCGE. HL-60 cells were treated with VM-26 (1 μM) or selenite (500 μM) for 30 min. Drug-treated cells were processed immediately for analysis by alkaline SCGE or comet assay under alkaline conditions (21). The extent of the DNA breakage induced by drug treatment was quantified by determining the tail moment (21). The tail moment of cells without drug treatment is expressed as 100%. For each drug concentration, 30 cells were scored for tail moment from random sampling. The error bars represent the S.D.

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H2AX. HL-60 and HL-60/MX-2 cells were treated with sodium selenite and the ECL Western procedure (Pierce). Phosphorylation of H2AX was determined by immunoblotting using anti-phospho histone H2AX antibodies and the ECL Western procedure. Cells were then lyzed directly with SDS gel sample loading buffer. Ser-139 phosphorylation of H2AX was determined by immunoblotting using anti-phospho histone H2AX antibodies and the ECL Western procedure (Pierce).

Similar reduction of Ser-139 phosphorylation, albeit to a much lesser extent (about 3-fold reduction), induced by selenite was also observed in HL-60/MX2 cells as compared with HL-60 cells, suggesting that selenite, like VM-26, may induce Top II-mediated DNA damage. However, unlike VM-26, selenite also induces non-Top II-mediated DNA damage.

Selenite-induced Apoptosis Is Reduced in NIH 3T3 Cells Treated with ATM siRNA—We have also tested whether selenite-induced apoptosis involves the nuclear kinase ATM, which is known to mediate apoptosis induced by many DNA damaging agents (23, 26). siRNA technology (27) was used to create ATM deficiency in NIH 3T3 cells. As shown in Fig. 3A, ATM siRNA induced dose-dependent reduction of ATM in NIH 3T3 cells as revealed by immunoblotting using anti-ATM antibodies. Apoptosis induced by selenite was then monitored by PARP cleavage using anti-PARP antibodies. PARP cleavage was caspase has been used extensively as an apoptosis end point. As shown in Fig. 3B, PARP cleavage induced by selenite was greatly reduced in ATM siRNA-treated NIH 3T3 cells, suggesting that selenite-induced apoptosis in NIH 3T3 cells requires the nuclear kinase ATM. The involvement of ATM in selenite-induced apoptosis suggests the possible involvement of nuclear DNA damage.

Selenite-induced Apoptosis Is Reduced in Top II-deficient Cells—To test whether selenite-induced apoptosis may involve the formation of Top II cleavage complexes in the nucleus, HL-60 and HL-60/MX2 (Top II-deficient) cells were treated with selenite. Apoptosis was monitored by DNA fragmentation assay using agarose gel electrophoresis. In this assay, chromosomal DNAs released from nuclei because of digestion by apoptotic nucleases were monitored by agarose gel electrophoresis. These soluble DNAs included both high molecular weight DNA and smaller nucleosomal DNA fragments. As shown in Fig. 4A, selenite (100 μM) treatment of HL-60 but not HL-60/MX2 cells caused release of both high molecular chromosomal DNA and nucleosomal DNA fragments from nuclei after 4 h of treatment, suggesting that selenite-induced apoptosis could involve Top II. Selenite (20 μM) also induced high molecular weight DNA and nucleosomal DNA fragments in HL60 but not HL-60/MX2 cells after prolonged (24 h) incubation (data not shown). VM-26 and camptothecin (CPT) (28) were used as positive and negative controls, respectively (Fig. 4A). Like selenite, VM-26 induced release of both high molecular weight chromosomal DNA and nucleosomal DNA fragments from HL-60 but not HL-60/MX2 cells (Fig. 4A). However, CPT, which is known to induce Top I but not Top II cleavage complexes, induced the release of high molecular weight DNA and nucleosomal DNA fragments from both HL-60 and HL-60/MX2 cells (Fig. 4A).

Selenite-induced Apoptosis Is Antagonized by a Top II Inhibitor ICRF-193—To test the possibility of Top II involvement, ICRF-193, a Top II catalytic inhibitor, was employed to antagonize the formation of Top II cleavage complexes (29, 30). Unlike Top II poisons, ICRF-193 specifically inhibits the catalytic activity of Top II without significantly elevating the level of cleavage complexes (30–32). ICRF-193 is known to antagonize the action of Top II poisons (e.g. VM-26) in terms of both cytotoxicity and the formation of the cleavage complex (30, 32). The mechanism for this interference is not completely known but may be related to the ability of ICRF-193 to trap Top II in an ATP-bound circular clamp conformation, which limits access of Top II to chromosomal DNA (32). As shown in Fig. 4B, ICRF-193 (100 μM) effectively reduced selenite-induced apoptosis as evidenced by the disappearance of the nucleosomal DNA ladder. To demonstrate the specificity of this effect, ICRF-193 was shown to abolish the formation of the nucleosomal DNA ladder induced by VM-26 (a Top II poison) but not by CPT.

Selenite-induced Apoptosis Is ATM-dependent. A. ATM siRNA reduces ATM protein level in NIH 3T3 cells. siRNA was used to create ATM deficiency in NIH 3T3 cells as described (27). The level of ATM in NIH 3T3 cells treated with ATM siRNA was determined by immunoblotting using ATM antibodies. The level of actin was also determined by immunoblotting using actin antibodies. B. Selenite-induced PARP cleavage is reduced in ATM-deficient NIH 3T3 cells. Sub-confluent NIH 3T3 cells were treated with cellfetin, complexed with either 145 ng of ATM sense (ATM+) or 145 ng of ATM siRNA (ATM−) (27). After siRNA treatment, cells were treated with 200 or 500 μM selenite for 4 h. Cells were then collected and lysed in SDS sample buffer. Immunoblotting was then performed using PARP antibodies. Bands corresponding to PARP were quantified by densitometric scanning.

Selenite-induced apoptosis is affected by the level of Top II cleavage complexes. A, selenite-induced apoptosis is reduced in a Top II-deficient cell line. HL-60 and its Top II-deficient variant HL-60/MX2 cells were treated with selenite (100 μM), VM-26 (2 μM), or CPT (2 μM). To detect apoptosis, 2.5 × 10⁶ cells in each sample were treated with different drugs for 4 h at 37 °C. Cells were then pelleted, and nucleosomal DNA laddering assay was performed. B, ICRF-193 antagonizes selenite-induced apoptosis. HL-60 cells were treated with 100 μM ICRF-193 prior to addition of VM-26 (2 μM), CPT (2 μM), or selenite (100 μM). Treatment conditions and DNA fragmentation assay were performed as described for A.
cleavage was observed using 10 μM H9262 cleavage is unusual. Above 1 μM, selenite did not increase the amount of DNA cleavage significantly (Fig. 5). This is not because of the complete titration of Top II into cleavage complexes at 1 μM selenite, because much more extensive DNA cleavage was observed using 10 μM VM-26 (see Fig. 6B) under the same conditions. Such a dose response has also been observed previously (17) with some of the thiol-reactive napthoquinones. It has been suggested that higher concentrations of napthoquinones inactivate Top II and thereby reduce the amount of Top II available for DNA cleavage (17). It seems possible that higher concentrations of selenite may also inactivate Top II and reduce the amount of Top II cleavage complexes.

**DISCUSSION**

The cellular effects of selenite appear quite complex and are concentration-dependent. At lower concentrations, the major effect of selenite appears to be related to its role as a micronutrient (2–4). At intermediate concentrations, selenite appears to exert its chemopreventive activity (2–4). At higher concentrations, selenite induces oxidative stress and may become toxic (35, 36). Previous studies have demonstrated that selenite induces single-strand DNA breaks, reflecting increased reactive oxygen species production in selenite-treated cells (35, 37). Consistent with previous studies, we have demonstrated that selenite induces single-strand DNA breaks in HL-60 cells as revealed by alkaline SCGE assay. Previous studies (38, 39) have also demonstrated that sele-

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**Fig. 5. Selenite induces reversible Top II-mediated DNA cleavage in vitro.** Top II cleavage assay was performed as described using hTop II (32, 33). The concentrations of VM-26 and disulfiram, a known thiol alkylator (17), were 5 and 100 μM, respectively. The concentrations of sodium selenite were indicated on top of each lane. For EDTA reversal, 50 mM EDTA (final concentration) was added to each reaction following the first incubation, and the reactions were further incubated for another 30 min at 37 °C prior to termination with SDS and proteinase K. The concentrations of VM-26, disulfiram, and selenite in the EDTA reversal experiment were 5, 100, and 10 μM, respectively.

(a Top I poison) (Fig. 4B). These results suggest that Top II cleavage complexes may contribute in part to selenite-induced apoptosis.

**Se Compounds Induce Reversible Top II Cleavage Complexes in Vitro.** To demonstrate a direct role of Se compounds in inducing Top II cleavage complexes, purified hTop IIα was used in a standard in vitro DNA cleavage assay in the presence of selenite (33, 34). As shown in Fig. 5, selenite is effective in inducing the cleavage of 32P-labeled DNA in the presence of hTop IIα. DNA cleavage was observed at selenite concentrations as low as 0.5 μM. Selenite-induced DNA cleavage was largely reversed by a second incubation with excess EDTA (Fig. 5). The reversibility of selenite-induced DNA cleavage is indicative of the formation of topoisomerase cleavage complexes (33, 34). In addition to selenite, we also have tested selenocystamine, selenomethionine, and selenate. Selenocystamine is as potent as selenite. However, selenate and selenomethionine were much less active (data not shown). These results are consistent with the known activity of these Se compounds on apoptosis (35). We also tested the effect of selenite on Top IIβ. Selenite induced Top IIβ- and Top IIα-mediated DNA cleavage with about equal efficiency (data not shown).

It is noted that the dose response of selenite-induced DNA cleavage is unusual. Above 1 μM, selenite did not increase the amount of DNA cleavage significantly (Fig. 5). This is not because of the complete titration of Top II into cleavage complexes at 1 μM selenite, because much more extensive DNA cleavage was observed using 10 μM VM-26 (see Fig. 6B) under the same conditions. Such a dose response has also been observed previously (17) with some of the thiol-reactive napthoquinones. It has been suggested that higher concentrations of napthoquinones inactivate Top II and thereby reduce the amount of Top II available for DNA cleavage (17). It seems possible that higher concentrations of selenite may also inactivate Top II and reduce the amount of Top II cleavage complexes.

**Reversal of Selenite-induced Top II Cleavage Complexes by Glutathione—Selenite is known to react with protein thiols (3). It seems plausible that modification of a thiol(s) on Top II by selenite is responsible for the formation of Top II cleavage complexes. To test this possibility, glutathione, which contains a reactive cysteinyl SH group, was used to inhibit and to reverse the Top II cleavage complex induced by selenite. As shown in Fig. 6A, co-incubation of selenite with an increasing amount of glutathione resulted in a gradual reduction of DNA cleavage in the presence of hTop IIα. In addition, a second incubation in the presence of glutathione (0.5 mM) reversed DNA cleavage induced by selenite (Fig. 6B). This result suggests that selenite is chemically reactive with thiol compounds under our assay conditions, consistent with the notion that selenite may poison Top II by reacting with a thiol(s) on Top II.

The use of cysteineless mutant Top II has suggested the involvement of a cysteine residue(s) on Top II that may be critically modified by specific thiol-reactive agents (17). We have tested whether selenite can poison wild type but not cysteineless mutant yeast Top II. Unfortunately, both wild type and cysteineless mutant yeast Top II are resistant to selenite-induced Top II poisoning.2

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2 N. Zhou and L. F. Liu, unpublished results.
nite elevates the p53 level, suggesting a potential involvement of the key DNA damage regulator ATM. ATM, the major phosphatidylinositol 3-kinase-like protein kinase, is involved in integrating the DNA damage signals with the cell cycle and apoptosis (23, 40). Many DNA damaging agents, especially those that generate DNA double-strand breaks, are known to activate ATM kinase and induce ATM-dependent apoptosis (29, 26, 40). Histone H2AX, one of the target proteins of ATM, is phosphorylated by activated ATM at Ser-139 (25). Using antibodies against a Ser-139 phosphorylated H2AX peptide, we have demonstrated that selenite induces Ser-139 phosphorylation of H2AX, suggesting the involvement of ATM in selenite-induced DNA damage signaling. However, the ATM-related phosphatidylinositol 3-kinase, ATR, can also phosphorylate Ser-139 of H2AX in response to replication arrest (41). Consequently, our results cannot distinguish between ATM and ATR activation upon selenite treatment. Indeed, we have shown that selenite efficiently induces Ser-139 phosphorylation of H2AX in ATM−/− cells, suggesting that selenite activates ATR kinase.3

The specific role of ATM in selenite-induced apoptosis was examined using the siRNA technology (27). We have demonstrated that selenite-induced apoptosis as monitored by PARP cleavage is largely dependent on ATM in NIH 3T3 cells. The involvement of ATM and p53 suggests that DNA damage may be important in selenite-induced apoptosis.

A surprising finding is that selenite-induced apoptosis is reduced in Top II-deficient HL-60/MX2 cells compared with parental HL-60 cells. Mitoxantrone-resistant HL-60/MX2 cells are known to be deficient in Top II because of a lowered nuclear Top II content (about 2-fold reduction) and absence of Top IIβ (42, 43). Although the role of each Top II isozyme in apoptosis induced by Top II poisons is still unclear, our results suggest the possible involvement of Top II in selenite-induced apoptosis. Previous studies have suggested that Top IIβ rather than Top IIα cleavage complexes are rapidly converted into DNA double-strand breaks through proteasomal degradation, suggesting a preferential role of Top IIβ in DNA double-strand break-mediated apoptosis (44). It is possible that the lack of Top IIβ in HL-60/MX2 cells is primarily responsible for their resistance to selenite-induced apoptosis.

HL-60/MX2 cells may contain additional mutations in addition to the Top II mutations, which could complicate the interpretation of our selenite results. Thus, we employed a different approach to demonstrate the involvement of Top II in selenite-induced apoptosis using the Top II catalytic inhibitor ICRF-193 (31, 32). ICRF-193 is known to antagonize Top II cleavage complex formation (45). The use of ICRF-193 to antagonize Top II has also led to reduced apoptosis in HL-60 cells, again suggesting a possible involvement of Top II in selenite-induced apoptosis. However, although ICRF-193 has been shown to antagonize Top II cleavage complex formation in cells (45), it is not certain whether ICRF-193 may inhibit other enzymes. Consequently, additional studies are necessary to firmly establish the involvement of Top II in selenite-induced apoptosis. It should be noted that although apoptosis is specifically monitored in our current studies, selenite-induced cell killing is likely to include other cell death mechanisms.

Our in vitro studies using purified hTop IIα have demonstrated that Se compounds, like Top II poisons such as VM-26 and doxorubicin (14), can indeed induce reversible Top II cleavage complexes. However, we have been unable to demonstrate directly the presence of Top II cleavage complexes in selenite-treated cells using either the band depletion or the in vivo complex of enzyme assays (46, 47). It is possible that neither method is sensitive enough to detect the small amount of Top II cleavage complexes induced by selenite in cells.

The molecular mechanism underlying the Top II poisoning effect of Se compounds is unclear. However, the fact that glutathione can reverse the Top II cleavage complexes induced by selenite suggests the involvement of thiol modification in Top II cleavage complex formation. Previous studies have demonstrated that thiol modification of Top II cleavage complexes represents a new mechanism for Top II poisoning (17). Thiol modifiers (e.g. naphthoquinones and organic disulfides) are known to induce Top II cleavage complexes that are qualitatively different from those induced by VM-26 and other Top II-directed anticancer drugs in terms of the ATP effect and isozyme specificity (17, 34). All clinically useful Top II-directed anticancer drugs such as VP-16 (etoposide) and doxorubicin are known to induce Top II cleavage complexes in a reaction, which is 10- to 100-fold stimulated by ATP (34). This result has led to the suggestion that the molecular target for these anticancer drugs is the ATP-bound circular clamp form of Top II (34). However, thiol modifiers are known to induce Top II-mediated DNA cleavage in a reaction that is insensitive to ATP, suggesting that the molecular target for thiol modifiers is the open clamp form of Top II (17). Most, if not all, clinically useful Top II-directed anticancer drugs are also known to act preferentially on Top IIα isozyme (17). However, thiol modifiers do not show significant Top II isozyme specificity (17). In terms of the ATP effect and Top II isozyme specificity, Se compounds behaved like other thiol modifiers in this in vitro DNA cleavage assay (data not shown).

We have also attempted to determine the role of cysteine modification in selenite-induced poisoning of human Top II by mutating cysteine residues on hTop IIα (a total of 13 residues). Our preliminary studies using site-directed mutagenesis have ruled out Cys-862 and Cys-427 to be the critical cysteine residue(s). Studies are in progress to identify the critical cysteine residue (s) on hTop IIα that may be modified by Se compounds.

Previous studies have demonstrated that not all Top II enzymes can be poisoned by thiol-reactive agents. For example, Drosophila Top II, unlike hTop II, cannot be poisoned by thiol-reactive agents at any tested concentrations (17). Similarly, not all thiol-reactive agents can poison hTop II. For example, arsenite, which is known to react with protein sulfhydryls (48), cannot poison hTop II at any tested concentrations.4 These studies suggest that the specific cysteine residue(s) that is critical in reacting with thiol-reactive agents may be embedded in a particular chemical environment that determines its reactivity toward different thiol-reactive agents. In this regard, it is interesting to note that hTop II can be similarly poisoned by other thiol-reactive dietary compounds such as isothiocyanates, curcumin, and retinoic acid.5

At present, it is unclear whether Top II is a target for the cancer chemopreventive activity of Se compounds. It is well known that tumor cells express high levels of Top IIα and are selectively sensitive to Top II-directed anticancer drugs (14, 15). Se compounds, being able to induce Top II cleavage complexes, could selectively eliminate carcinogen-initiated cancerous cells by this mechanism. Consistent with this explanation, selenite has been demonstrated to exhibit anticancer activity against L1210 cell-inoculated mice (49). On the other hand, the concentrations of selenite used in our current studies (100 to 500 μM) are two orders of magnitude higher than the blood concentration of people taking Se supplementation (50). The observed effect on DNA damage and apoptosis could also be

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3 P. Darouei and L. F. Liu, unpublished results.

4 H.-M. Wang and L. F. Liu, unpublished results.

5 N. Zhou and L. F. Liu, unpublished results.
related to the toxic effect of selenite. Clearly, further studies are necessary to establish the potential role of Top II in cancer chemoprevention.

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