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Ubiquitin/26S Proteasome-mediated Degradation of Topoisomerase I As a Resistance Mechanism to Camptothecin in Tumor Cells[1]

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ABSTRACT

Camptothecin (CPT) induces down-regulation of topoisomerase I (TOP1) via an ubiquitin/26S proteasome pathway. Studies using a panel of breast and colorectal cancer cell lines as well as primary nontransformed and oncogene-transformed cells have demonstrated that CPT-induced down-regulation exhibits a high degree of heterogeneity. In general, nontransformed cells are much more proficient in CPT-induced TOP1 down-regulation than their transformed counterparts. Among the breast and colorectal cancer cell lines, there was a general correlation between the extent of CPT-induced TOP1 down-regulation and CPT resistance. The breast cancer cell line ZR-75-1, the most sensitive to CPT, was completely defective in CPT-induced TOP1 down-regulation, whereas the breast cancer cell line BT474, the least sensitive to CPT, exhibited effective CPT-induced TOP1 down-regulation. The 26S proteasome inhibitor MG132 was shown to inhibit CPT-induced down-regulation of TOP1 in BT474 cells and selectively sensitized BT474 but not ZR-75-1 cells to CPT-induced cytotoxicity and apoptosis. In the aggregate, these results suggest that CPT-induced down-regulation of TOP1 could be an important parameter for determining CPT sensitivity/resistance in tumor cells. Analysis of the levels of TOP1 cleavable complexes, SUMO-1-TOP1 conjugates, and ubiquitin-TOP1 conjugates in ZR-75-1 and BT474 cells has suggested that the heterogeneity of CPT-induced down-regulation of TOP1 in tumor cells is at least in part attributable to altered regulation of a process(es) downstream from the TOP1 cleavable complex.

INTRODUCTION

CPTs[3] (*e.g.,* irinotecan and topotecan) represent a new class of anticancer drugs that are being rapidly developed into the clinic. Although their antitumor activity has been well documented in both animal and clinical studies, very little is known about the cellular parameters controlling the sensitivity/resistance of tumor cells to CPTs (1, 2).

The antitumor activity of CPTs is attributable to their specific interference with the breakage/reunion reaction of TOP1 (3, 4). CPT specifically inhibits the religation step of the breakage/reunion reaction of TOP1, resulting in accumulation of a reversible covalent reaction intermediate, the TOP1-CPT-DNA covalent complex, often referred to as the cleavable or cleavage complex (5, 6). The major cytotoxic mechanism of CPT, which is S-phase-specific, is triggered by a collision event between the replication machinery and the CPT-stabilized cleavable complex (7, 8). The replication inhibitor aphidicolin has been shown to effectively abolish CPT cytotoxicity (7, 8). However, at higher concentrations, CPT can also kill non-S-phase tumor cells through apoptosis (9–12). In this case, aphidicolin has no effect on CPT cytotoxicity (10).

The parameters controlling cellular sensitivity/resistance to CPT are largely unknown. Studies of a panel of colorectal cancer cell lines have demonstrated a large degree of variation in their sensitivity/resistance to CPT (9). This variation in CPT sensitivity/resistance is not the result of variations in cellular accumulation of CPT or the cellular level of TOP1 cleavable complexes (9). It has been suggested that events downstream from the cleavable complex may determine CPT sensitivity/resistance (9). Studies of a panel of breast cancer cell lines have led to a similar conclusion (11). A 700-fold variation in sensitivity to CPT has been observed for a panel of breast cancer cell lines (11). No single parameter has been predictive for CPT sensitivity, including expression and activity of TOP1, doubling time, expression of MDR-1, p53 status, and expression of the apoptotic proteins Bcl-2 and Bax (11).

Many downstream events of the TOP1 cleavable complexes have been identified, including G_2 cell cycle arrest, stabilization of p53, increased *c-jun* and *c-fos* mRNA, nuclear factor κB activation, and Chk1 and RPA phosphorylation, all of which are events related to general DNA damage responses (12). However, recent studies have identified two downstream events from the TOP1-CPT-DNA cleavable complexes that are CPT/TOP1 specific and are not related to a general DNA damage response (13–15). In one study, CPT was shown to induce specific down-regulation of TOP1 via an ubiquitin/26S proteasome pathway (15). CPT-induced down-regulation of TOP1 was not preventable by the replication inhibitor aphidicolin (15). In the other studies, CPT was also shown to stimulate SUMO-1 (and also SUMO-2/3) conjugation to TOP1 (13, 14).[4] Again, CPT-induced elevation of SUMO-1-TOP1 conjugates was not preventable by the replication inhibitor aphidicolin (14). In the present study, we studied these two cellular processes in a panel of colorectal and breast cancer cell lines. We found that SUMO conjugation induced by CPT was proficient in all of these tumor cells. However, the extent of CPT-induced down-regulation of TOP1 was highly variable among these tumor cells. In addition, there was a correlation between the extent of TOP1 down-regulation and CPT sensitivity/resistance in these tumor cells. The 26S proteasome inhibitor MG132 was shown to inhibit CPT-induced down-regulation of TOP1 and to sensitize tumor cells proficient in CPT-induced down-regulation of TOP1. These results suggest that CPT-induced down-regulation of TOP1 may be an important parameter determining sensitivity/resistance of tumor cells to CPT. Additional analysis suggested that the heterogeneity of CPT-induced down-regulation of TOP1 in tumor cells is at least in part attributable to altered regulation of a process(es) downstream from the TOP1 cleavable complex.

MATERIALS AND METHODS

Cells. The human fibroblast cell line WI38 and its SV40 T-antigen-transformed variant 2RA; colon cancer cell lines HT29, KM12, colo205, SW480, and HCT116; and the Chinese hamster lung cell line V79 were obtained from American Type Culture Collection (Rockville, MD). Breast cancer cell lines ZR-75-1, MCF7, SKBr3, BT20, T47D, MDA-MB-231, MDA-MB-435, MDA-MB-468, and BT474, were kindly provided by Dr. K-V. Chin (Cancer Institute

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[3] The abbreviations used are: CPT, camptothecin; TOP1, topoisomerase I; SUMO-1, small ubiquitin-related modifier-1; PFGE, pulsed-field gel electrophoresis; HMW, high molecular weight.

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of New Jersey, New Brunswick, NJ). FBCL (primary human skin fibroblasts) cells were kindly provided by Dr. Beppino Giovenella (Stehlin Foundation for Cancer Research, Houston, TX). GM05659C (primary human skin fibroblasts) were obtained from Coriell Cell Repositories, Camden, NJ). The mouse fibroblast CB17 cell line and its E1A/Raf-transformed variant CB17/ER1 and CB17/ER4 were kindly provided by Dr. Stuart Lutzker (Cancer Institute of New Jersey, NJ). All breast and colon cancer cells, as well as WI38, 2RA, and FBCL cells were grown in RPMI supplemented with 10% heat-inactivated fetal bovine serum. GM05659C cells were grown in MEM containing 2× concentrations of amino acids and vitamins and 20% fetal bovine serum. V79, CB17, CB17/ER1, and CB17/ER4 cells were grown in DMEM supplemented with 10% fetal bovine serum. All media were supplemented with L-glutamine (2 mM), penicillin (100 units/ml), and streptomycin (100 μg/ml). All cells were cultured in a 37°C incubator with 5% CO_2.

Immunoblotting of TOP1 and TOP1-SUMO-1 Conjugates. Cells (10^6/sample) were treated with CPT (25 μM in 1% DMSO) for various times at 37°C. Subsequently, cells were either lysed directly with 2× SDS-PAGE sample buffer or with 0.2 N NaOH containing 2 mM EDTA (for monitoring trapped covalent TOP1-DNA complexes). For reversal of TOP1 cleavable complexes, cells were placed in fresh CPT-free tissue culture medium for 30 min (for monitoring total cellular levels of TOP1) prior to lysis. To detect TOP1-SUMO-1 conjugates, cells were lysed using the alkali lysis procedure. Cell lysates were then neutralized with one-tenth volume of a neutralization solution containing 10% NP-40, 1 M Tris (pH 7.4), 0.1 M $MgCl_2$, 0.1 M $CaCl_2$, 10 mM DTT, 1 mM EGTA, and 100 μg/ml each of leupeptin, pepstatin, and aprotinin in 2 M HCl. Neutralized cell lysates were incubated with *Staphylococcus aureus* nuclease S7 (60 units/reaction) for 20 min on ice. Reactions were terminated by the addition of SDS-PAGE sample buffer [final concentrations, 50 mM Tris-HCl (pH 6.8), 15% sucrose, 12 mM EDTA, 3% SDS, and 10% β-mercaptoethanol]. Immunoblotting analysis of cell lysates was carried out using TOP1 antiserum from scleroderma patients as described previously using the ECL Western procedure (Pierce; Ref. 15). The intensity of each band in the autoradiogram was quantitated by densitometric scanning.

Immunoprecipitation of Ubiquitin-TOP1 and SUMO-1-TOP1 Conjugates. BT474 and ZR-75-1 cells were treated with 1 μM MG132 for 1 h followed by 25 μM CPT cotreatment for 30 min. Cells were then lysed directly using the alkali lysis procedure described in "Materials and Methods." Cell lysates were briefly sonicated and treated with S7 nuclease (60 units/reaction) for 30 min on ice. After the addition of 100 μl of radioimmune precipitation buffer (1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, and protease inhibitors in 1× PBS), cell lysates were centrifuged for 20 min at 4°C. Subsequently, supernatants were mixed with 5 mg of protein A-Sepharose beads and anti-TOP1 antibodies and incubated for 2 h at 4°C in a rotating shaker. After incubation, beads were washed three times (5 min each) with radioimmune precipitation buffer and once with rinse buffer [20 mM Tris (pH 8.0), 150 mM NaCl, 10 mM $MgCl_2$, 1 mM DTT, and 1 mM phenylmethylsulfonyl fluoride]. The protein A-Sepharose beads were then resuspended in 30 μl of 2× SDS sample buffer and boiled for 10 min. The samples were analyzed by SDS-PAGE and immunoblotted with anti-TOP1 antibodies. Same blots were stripped and reprobed with antiubiquitin antibodies for detection of ubiquitin-TOP1 conjugates.

CPT Cytotoxicity Assay. Logarithmically growing cells were treated with various concentrations of CPT for 1 h. CPT was then removed by washing the plates with CPT-free medium four times. Washed cells were allowed to grow in CPT-free medium for 4 days. Cell survival was determined by counting cells using the Coulter counter after trypsinization.

PFGE for Monitoring CPT-induced HMW DNA Fragmentation. Logarithmically growing BT474 and ZR-75-1 cells (5×10^5 cells/sample) were treated with CPT with or without MG132. Cells were then trypsinized and resuspended to a density of 10^7 cells/ml in RPMI 1640 supplemented with 10% FCS, L-glutamate (2 mM), penicillin (100 units/ml), and streptomycin (10 μg/ml). Following mixing with an equal volume of 1.5% (w/v) low-melting point agarose premelted in the same culture medium (37°C), the mixture (100 μl each) was loaded into agarose plug makers.

Cell lysis in the plugs was accomplished by placing plugs into new Eppendorf tubes containing 500 μl of TEN buffer [50 mM Tris (pH 8.0), 100 mM EDTA, 100 mM NaCl] with 1% SDS and 0.5 mg/ml proteinase K. After incubation at 50°C for 24 h, the integrity of nuclear DNA in the plugs was analyzed by PFGE. PFGE (4 V/cm) was performed in the cold room (4°C) for 45 h with circulation and a switching time of 40 s. Following electrophoresis, gel was stained with 1 μg/ml ethidium bromide.

RESULTS

Heterogeneity of CPT-induced Down-Regulation of TOP1 in Normal and Tumor Cells. Previous studies have demonstrated that CPT-induces down-regulation of TOP1 in peripheral blood mononuclear cells (16–19), mouse FM3A cells (15), and human KB cells (16, 20). Down-regulation of TOP1 was shown to be mediated by an ubiquitin/26S proteasome pathway (15). In the present studies, we examined CPT-induced down-regulation of TOP1 in both nontransformed human cells and a panel of colorectal and breast cancer cell lines (Fig. 1, *IA–IC*). As shown in Fig. 1, CPT induced rapid down-regulation of TOP1 in nontransformed human cells, including WI38 (primary human lung fibroblasts, 11th passage), FBCL (primary human skin fibroblasts), and GM05659C (primary human skin fibroblasts) cells. Within 4 h, the cellular level of TOP1 was reduced to <20% (Fig. 1, *ID*). Surprisingly, the extent of CPT-induced down-regulation of TOP1 was not as dramatic in most cancer cells examined (Fig. 1, *IB* and *IC*). Except for BT474 breast cancer cells, the extent of CPT-induced down-regulation of TOP1 was <50% for all breast and colorectal cancer cells examined (Fig. 1, *ID*). In striking contrast to ZR-75-1 cells, which were completely defective in CPT-induced down-regulation of TOP1, BT474 breast cancer cells exhibited a near-normal extent of CPT-induced down-regulation of TOP1 (Fig. 1, *IB* and *ID*).

Our results suggest that tumor cells in general are less proficient in TOP1 down-regulation. To test this possibility, we compared the extent of CPT-induced down-regulation of TOP1 in WI38 and 2RA (the SV40 T-antigen-transformed counterpart of WI38). As shown in Fig. 1, *IIA*, CPT-induced down-regulation of TOP1 was much less effective in 2RA cells than in WI38 cells. Within 4 h, the cellular level of TOP1 was reduced to <20% in WI38 cells, whereas the cellular level of TOP1 in 2RA was reduced only slightly (90% of control; Fig. 1, *IIB*). The ER1 and ER4 cell lines, which are variants of the mouse fibroblast CB17 cotransformed with E1A and Raf oncogenes, respectively, also exhibited reduced extent of CPT-induced down-regulation of TOP1 compared with their untransformed CB17 cells (Fig. 1, *IIA*).

Correlation between Extent of CPT-induced Down-Regulation of TOP1 and CPT Sensitivity. The possibility that TOP1 down-regulation may contribute to CPT sensitivity/resistance was tested by comparison between the extent of TOP1 down-regulation and CPT cytotoxicity in various breast cancer cells. As shown in Fig. 1, *III*, ZR-75-1 cells, which were completely defective in TOP1 down-regulation, were the most sensitive to CPT. BT474 cells, which were proficient in TOP1 down-regulation, were the most resistant to CPT. Other breast cancer cells were intermediate in both CPT sensitivity and the extent of TOP1 down-regulation (Fig. 1, *IV*) for the correlation. The correlation coefficient was 0.94. A similar correlation ($r = 0.97$) was observed for the panel of colorectal cancer cells (compare data shown in Fig. 1 with the cytotoxicity data in Ref. 9). For example, KM12 cells, which are least sensitive to CPT among the colorectal cancer cells, are most efficient in CPT-induced TOP1 down-regulation.

To further examine the role of TOP1 down-regulation in CPT sensitivity/resistance, the 26S proteasome inhibitor MG132 was used to inhibit TOP1 down-regulation. As shown in Fig. 2, *IA* and *IB* (see the immunoblotting results), MG132 effectively abolished CPT-induced down-regulation of TOP1 in BT474 and V79 cells. Interestingly, CPT cytotoxicity in these cells was increased in the presence of

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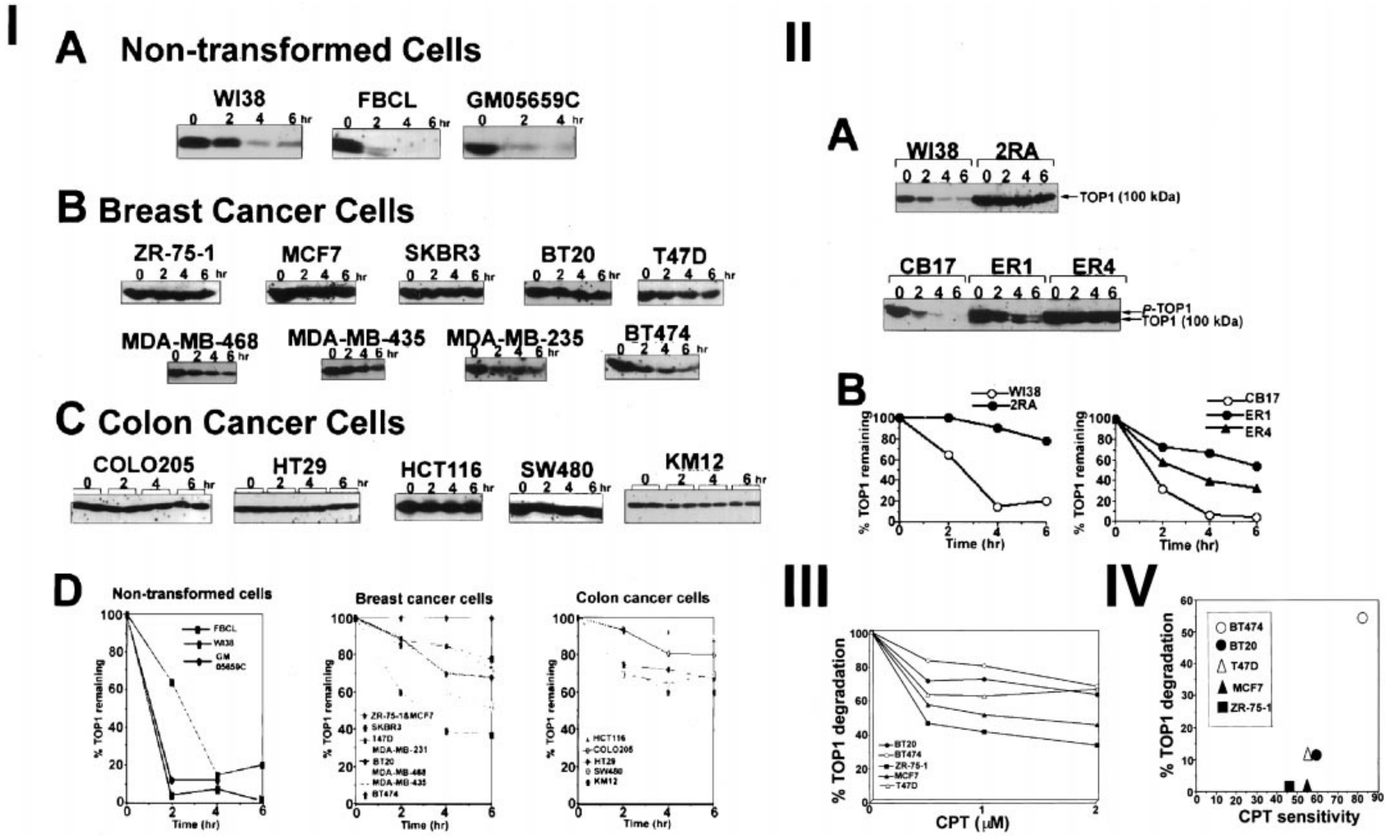

Fig. 1. Heterogeneity of CPT-induced down-regulation of TOP1 in normal and tumor cells. Various normal and tumor cell lines were treated with CPT (25 μM) for different times (0, 2, 4, and 6 h) as indicated. Subsequently, cells were replenished with CPT-free fresh medium and incubated at 37°C for another 30 min to reverse the formation of TOP1 cleavable complexes. After the medium was removed from plates, cells were immediately lysed with 2× SDS-PAGE sample buffer. SDS gel electrophoresis and immunoblotting with antibodies against human TOP1 were performed as described in "Materials and Methods." *I*, CPT-induced down-regulation of TOP1 in primary human skin fibroblasts (WI38, FBCL, and GM05659C; *IA*); in breast cancer cell lines (ZR-75-1, MCF7, SKBr3, BT20, T47D, MDA-MB-468, MDA-MB-435, MDA-MB-231, and BT474; *IB*); and in colorectal cancer cell lines (Colo 205, HT29, HCT116, SW480, and KM12 cells; *IC*). Colo205, HT29, and KM12 samples were loaded in duplicate. The intensity of TOP1 bands in the autoradiograms in *A–C* was quantitated by densitometric scanning and plotted as a function of time of CPT treatment (*ID*). *II*, CPT-induced down-regulation of TOP1 is altered in SV40 T-antigen-transformed cells. *IIA*, CPT-induced down-regulation of TOP1 in primary human lung fibroblast WI38 cells and their SV40 T-antigen-transformed counterpart 2RA cells, as well as in the mouse fibroblast CB17 and their E1A/Raf-transformed variant CB17/ER1 and CB17/ER4 cells. Cells were treated with CPT (25 μM) for different times. Subsequently, cells were replenished with CPT-free medium and incubated at 37°C for another 30 min. Cells were then lysed with 2× SDS-PAGE sample buffer. The amount of TOP1 remaining in the gel was determined by immunoblotting with antibodies against TOP1. *IIB*, the intensity of TOP1 bands in the autoradiograms shown *IIA* was quantitated by densitometric scanning and plotted as a function of time of CPT treatment. *III*, hypersensitivity of breast cancer cells to CPT. Various breast cancer cells were treated with CPT (0.5, 1, and 2 μM) for 1 h. Cell survival was determined by cell counting using a Coulter counter as described in "Materials and Methods." *IV*, correlation between CPT sensitivity (percentage of cells killed by 0.5 μM CPT in 1-h treatment; see *III*) and the extent of TOP1 down-regulation (percentage of TOP1 degraded in cells treated with 25 μM CPT for 2 h).

MG132 ($P = 0.004$ and 0.005 for BT474 and V79 cells, respectively), suggesting that CPT-induced down-regulation may contribute to CPT resistance. As a control experiment, MG132 was shown not to affect CPT cytotoxicity in ZR-75-1 cells (Fig. 2, *IIC*).

CPT can kill cells through apoptosis (10, 12). HMW DNA fragmentation has been used as an early end point for apoptotic cell death (12). To test whether TOP1 down-regulation affects apoptotic cell death, we used PFGE to monitor the production of HMW DNA fragments (12). As shown in Fig. 2, *IIA*, HMW DNA fragments (~50 kb in size) were observed in ZR-75-1 cells treated with CPT (10 μM) for 6 h. Under the same treatment conditions, no HMW DNA fragments were observed in BT474 cells (Fig. 2, *IIA*). As shown in Fig. 2, *IIC*, prolonged treatment (24 h) with a higher concentration of CPT (50 μM) induced a small amount of HMW DNA fragments in BT474 cells. Cotreatment with 1 μM MG132 greatly increased the amount of HMW DNA fragments, indicative of extensive apoptosis (Fig. 2, *IIC*). By contrast, cotreatment with 1 μM MG132 with CPT (5 μM) did not increase the amount of HMW DNA fragments in ZR-75-1 cells (Fig. 2, *IIB*). These results show that inhibition of 26S proteasome, which abolishes CPT-induced down-regulation of TOP1, sensitizes BT474 but not ZR-75-1 cells to CPT-induced apoptosis.

Step(s) Downstream from the TOP1 Cleavable Complex May Contribute to Altered TOP1 Down-Regulation in ZR-75-1 Breast Cancer Cells. As suggested from the above experiments, CPT hypersensitivity of ZR-75-1 cells could result at least in part from a defect in TOP1 down-regulation in these cells. The defect of TOP1 down-regulation could occur at a step either prior or subsequent to the formation of the TOP1-CPT-DNA cleavable complex. As shown in Fig. 3*A*, the TOP1 level is ~2-fold higher in ZR-75-1 cells than in BT474 cells. However, the 2-fold difference in the TOP1 level is unlikely to be solely responsible for the observed difference in TOP1 down-regulation in these breast cancer cells because TOP1 down-regulation was completely absent in ZR-75-1 cells. A band-depletion assay showed that the 100-kDa TOP1 band decreased with increasing CPT concentrations in both ZR-75-1 and BT474 cells with a similar dose response (Fig. 3*B*). Treatment of the lysates shown in Fig. 3*B* with *Staphylococcus* nuclease S7 resulted in the reappearance of most of the 100-kDa TOP1 plus the slower-migrating TOP1-SUMO-1 conjugates (Fig. 3*C*), suggesting that the formation of TOP1 cleavable complexes in response to CPT is not significantly different in ZR-75-1 and BT474 cells. In the aggregate, these results suggest that altered regulation of a process(es) downstream from the TOP1 cleavable

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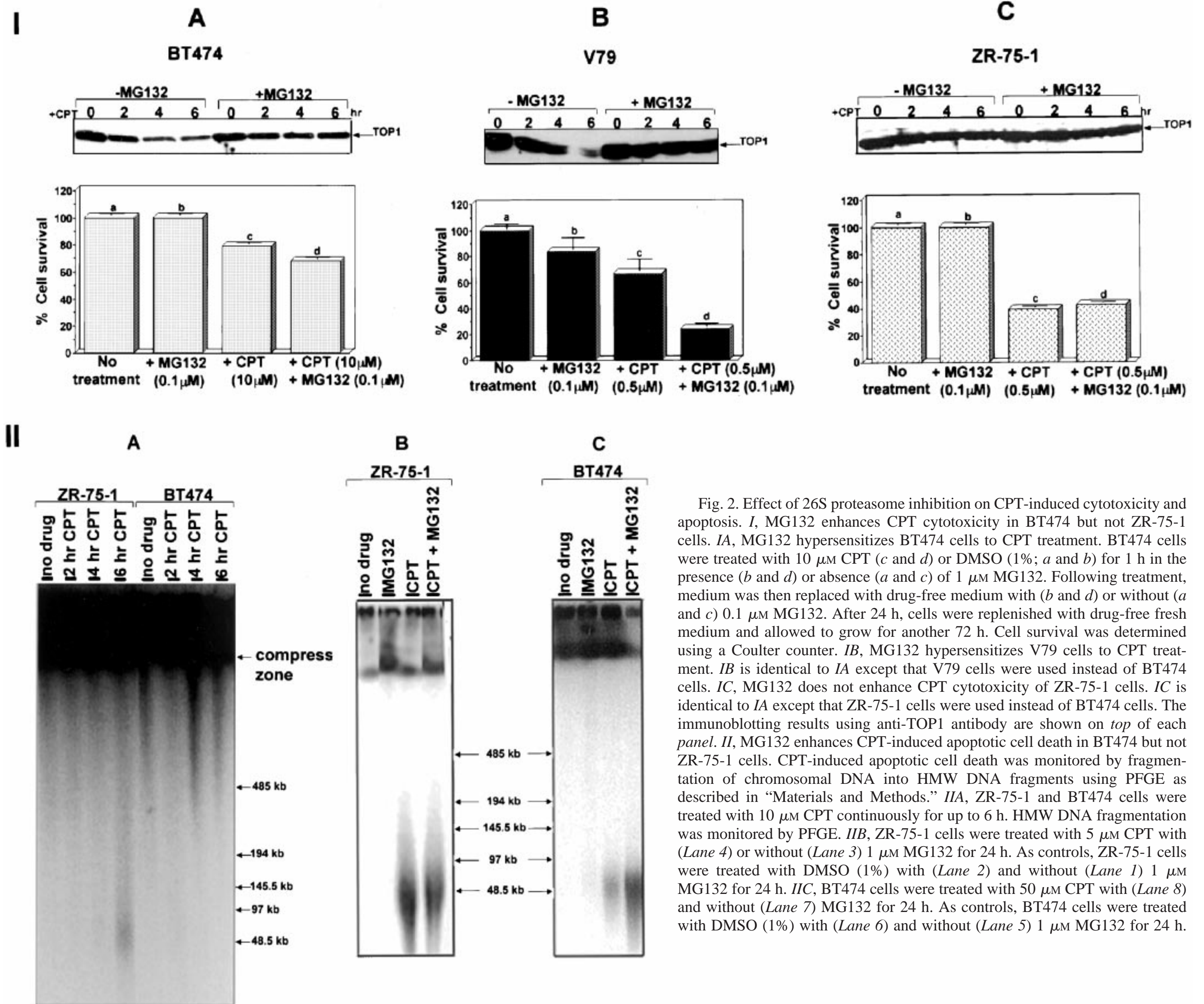

Fig. 2. Effect of 26S proteasome inhibition on CPT-induced cytotoxicity and apoptosis. *I*, MG132 enhances CPT cytotoxicity in BT474 but not ZR-75-1 cells. *IA*, MG132 hypersensitizes BT474 cells to CPT treatment. BT474 cells were treated with 10 μM CPT (*c* and *d*) or DMSO (1%; *a* and *b*) for 1 h in the presence (*b* and *d*) or absence (*a* and *c*) of 1 μM MG132. Following treatment, medium was then replaced with drug-free medium with (*b* and *d*) or without (*a* and *c*) 0.1 μM MG132. After 24 h, cells were replenished with drug-free fresh medium and allowed to grow for another 72 h. Cell survival was determined using a Coulter counter. *IB*, MG132 hypersensitizes V79 cells to CPT treatment. *IB* is identical to *IA* except that V79 cells were used instead of BT474 cells. *IC*, MG132 does not enhance CPT cytotoxicity of ZR-75-1 cells. *IC* is identical to *IA* except that ZR-75-1 cells were used instead of BT474 cells. The immunoblotting results using anti-TOP1 antibody are shown on *top* of each *panel*. *II*, MG132 enhances CPT-induced apoptotic cell death in BT474 but not ZR-75-1 cells. CPT-induced apoptotic cell death was monitored by fragmentation of chromosomal DNA into HMW DNA fragments using PFGE as described in "Materials and Methods." *IIA*, ZR-75-1 and BT474 cells were treated with 10 μM CPT continuously for up to 6 h. HMW DNA fragmentation was monitored by PFGE. *IIB*, ZR-75-1 cells were treated with 5 μM CPT with (*Lane 4*) or without (*Lane 3*) 1 μM MG132 for 24 h. As controls, ZR-75-1 cells were treated with DMSO (1%) with (*Lane 2*) and without (*Lane 1*) 1 μM MG132 for 24 h. *IIC*, BT474 cells were treated with 50 μM CPT with (*Lane 8*) and without (*Lane 7*) MG132 for 24 h. As controls, BT474 cells were treated with DMSO (1%) with (*Lane 6*) and without (*Lane 5*) 1 μM MG132 for 24 h.

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complex may contribute to the observed difference in CPT-induced TOP1 down-regulation in breast cancer cells. Thus, we examined the two known processes, conjugation with SUMO and ubiquitination, which occur downstream from the CPT-induced TOP1 cleavable complex (13, 14). As shown in Fig. 3*D*, SUMO-1-TOP1 conjugates were formed rapidly and reached a steady state within 5 min in both ZR-75-1 and BT474 cells treated with CPT. We also examined deconjugation of SUMO-1-TOP1 conjugates in ZR-75-1 cells and BT474 cells because in some cases SUMO-1 deconjugation has been shown to precede ubiquitination (13, 14). As shown in Fig. 3*D*, SUMO deconjugation occurred at equal rates in both ZR-75-1 and BT474 cells with >50% deconjugation occurring within 30 min. These results indicate that SUMO conjugation and deconjugation processes are not significantly altered in ZR-75-1 cells compared with BT474 cells.

Defective TOP1 down-regulation in ZR-75-1 cells could be attributable to a defect in the ubiquitin-TOP1 conjugation reaction. To test this possibility, the formation of ubiquitin-TOP1 conjugates in cells treated with CPT was examined. Ubiquitin-TOP1 conjugates were immunoprecipitated with anti-TOP1 antibody from cell lysates, and immunoblotting analysis was performed using either anti-TOP1 antibody or antiubiquitin antibody (Fig. 4). CPT was shown to elevate the level of ubiquitin-TOP1 conjugates in both ZR-75-1 and BT474 cells (Fig. 4). Interestingly, cotreatment with MG132 significantly elevated the level of ubiquitin-TOP1 conjugates in BT474 cells (Fig. 4, *II* and *IV*). By contrast, MG132 had little effect on the level of ubiquitin-TOP1 conjugates in ZR-75-1 cells (Fig. 4, *VI* and *VIII*). These results suggest that ubiquitination of TOP1 cleavable complexes occurs in both ZR-75-1 and BT474 cells. However, precise quantitation of the ubiquitination process is technically difficult because of the size heterogeneity of the ubiquitin-TOP1 conjugates (see smears in Fig. 4).

DISCUSSION

The present studies demonstrated that CPT-induced down-regulation of TOP1 is highly heterogeneous in different tissue culture models. In general, transformed and tumor cells appeared to be impaired or defective in CPT-induced down-regulation of TOP1. This *in vitro* result is consistent with results obtained in trials of 72-h infusion of 9-aminocamptothecin in patients with either solid tumors or leukemia (18, 19). During the drug infusion, normal peripheral blood cells (obtained from patients with solid tumors) exhibited

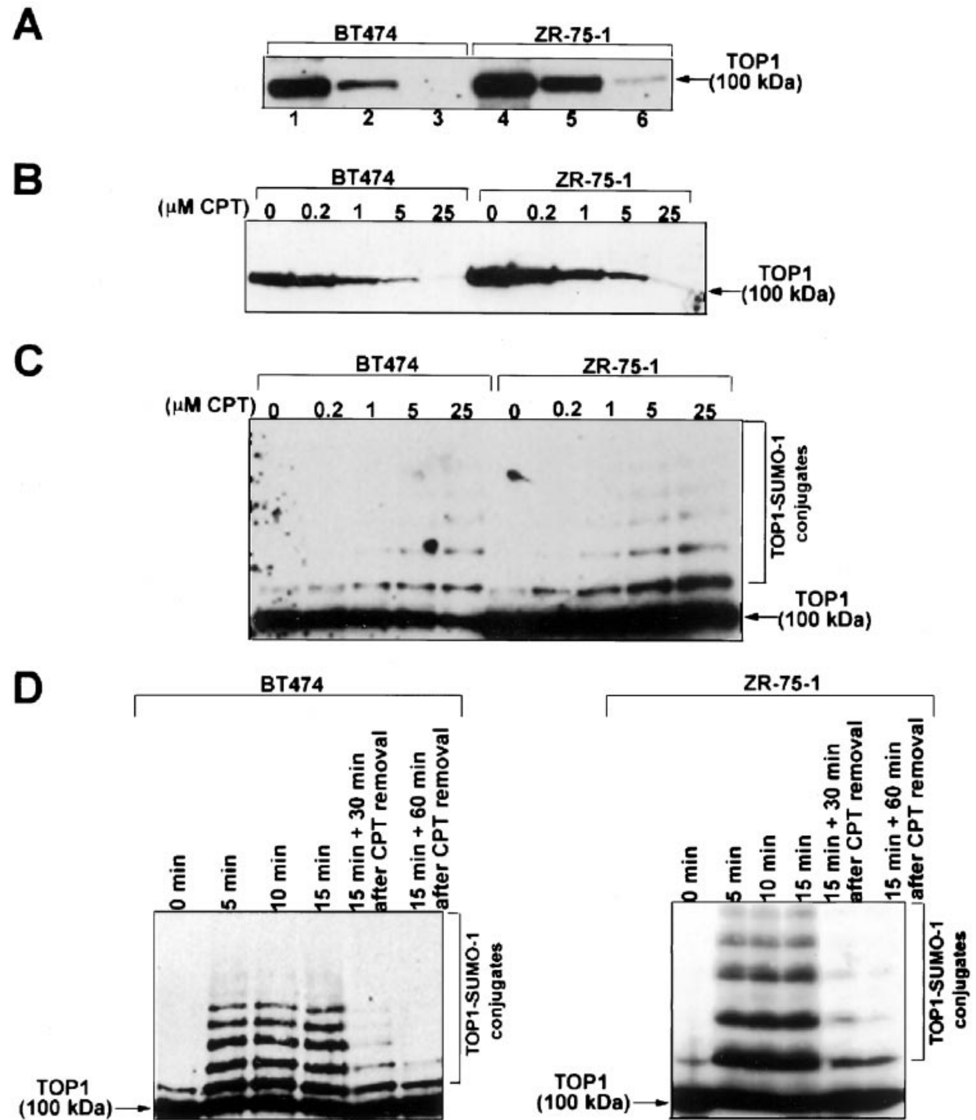

Fig. 3. CPT-induced formation of TOP1 cleavable complexes and SUMO-1-TOP1 conjugates in breast cancer cells. *A*, TOP1 levels in ZR-75-1 and BT474 cells. Approximately 10,000 cells from each cell line were lysed by the alkali procedure and analyzed by immunoblotting using antihuman TOP1 antibodies (*Lanes 1* and *4*). Lysates were also serially diluted 3-fold and analyzed by immunoblotting (*Lanes 2*, *3*, *5*, and *6*). *B*, trapping of covalent TOP1-DNA complexes in breast cancer cells. The amount of CPT-induced TOP1 cleavable complexes was determined by a band-deletion assay. Briefly, ZR-75-1 and BT474 cells were treated with different concentrations of CPT for 15 min and then lysed with 200 mM NaOH containing 2 mM EDTA. Lysates were neutralized as described in "Material and Methods." The amount of TOP1 trapped as covalent TOP1-DNA complexes, which was reflected in band depletion, was determined by immunoblotting with antihuman TOP1 antibodies. *C*, same as in *B* except that the neutralized lysates were treated with *Staphylococcus* nuclease S7 to release TOP1 and SUMO-1-TOP1 conjugates from covalent TOP1-DNA complexes. *D*, CPT-induced SUMO-1-TOP1 conjugates in breast cancer cells. The same set of cells were treated with 25 μM CPT for the indicated times and then incubated in CPT-free medium for either 30 or 60 min to allow reversal of the cleavable complexes. Cells were then lysed using the alkaline lysis procedure. The alkaline lysates were neutralized and treated with *Staphylococcus* nuclease for 20 min on ice. Samples were used for Western blotting to monitor TOP1-SUMO-1 conjugates as described in "Materials and Methods."

down-regulation of TOP1 protein (19), whereas TOP1 protein levels were unchanged in leukemic cells (obtained from patients with leukemia; Ref. 21). Together, these results could suggest that CPT-induced down-regulation of TOP1 occurs in normal cells but is impaired to a different and variable degree in tumor cells. It is unclear whether this difference between normal and tumor cells could contribute to the antitumor activity of CPT.

Breast and colorectal cancer cells have been shown to exhibit greatly different intrinsic sensitivities to CPT (9, 11). However, the reason for this differential CPT sensitivity remains unclear (11). It has been determined that the levels of cleavable complexes in these cancer cells are quite similar (9, 11). Consequently, alteration of a cellular process(es) downstream from the formation of TOP1 cleavable complexes has been suggested to be responsible for the differential CPT sensitivity of these cancer cells (11, 16, 20). Three cellular processes that occur immediately downstream from and are unique to TOP1 cleavable complexes in CPT-treated cells have been identified (13–15, 22). These processes include chromatin structural alteration, as revealed by linking number changes in episomal DNA (22); ubiquitin/26S proteasome-mediated down-regulation of TOP1 (15); and SUMO-1 (SUMO-2/3) conjugation to TOP1 (13, 14). In the present studies, we did not evaluate chromatin structural alteration in these tumor cells because of the technical difficulty. SUMO-1 is a 15-kDa protein that has been shown to have 18% sequence identity to ubiquitin (23, 24). It has been proposed that SUMO-1 conjugation is required for cellular trafficking (23, 24) or to antagonize ubiquitination to prevent its target destruction (23, 24). However, we showed that SUMO-1 conjugation to TOP1 occurs efficiently in all of these tumor cells. Interestingly, ubiquitin/26S proteasome-mediated down-regulation of TOP1 was shown to vary significantly in these tumor cells. Most interestingly, the extent of CPT-induced down-regulation of TOP1 appeared to show a correlation with CPT resistance in these tumor cells. Stronger support for a causal relationship between CPT-induced down-regulation of TOP1 and CPT resistance came from our studies using the 26S proteasome inhibitor MG132, which both abolished TOP1 down-regulation and sensitized BT474 cells to CPT cytotoxicity as well as CPT-induced apoptosis. TOP1 down-regulation has been suggested to be an effective cellular response to counteract the effect of TOP1 cleavable complexes (15). When TOP1 is down-regulated, fewer TOP1 cleavable complexes will be present in cells. Consequently, cells capable of down-regulating TOP1 are expected to be more resistant to the lethal effect of CPT. It is also possible that TOP1 down-regulation is a necessary step for repair because proteolysis of TOP1 in the TOP1 covalent complex is likely

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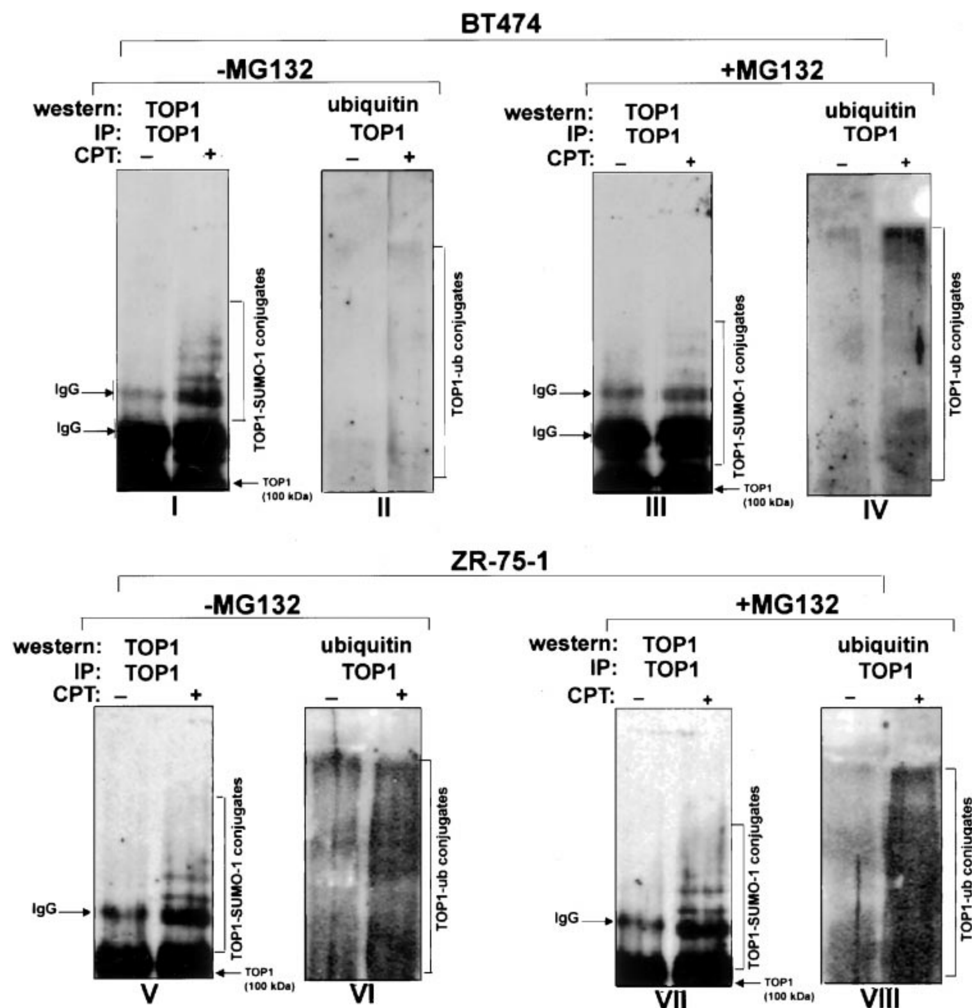

Fig. 4. CPT-induced formation of ubiquitin-TOP1 conjugates in tumor cells. *I*, *II*, *III*, and *IV*, CPT induces ubiquitin conjugation to TOP1 in BT474 breast cancer cells. To analyze ubiquitin-TOP1 conjugates, BT474 cells were treated with 25 μM CPT for 30 min with or without 1 μM MG132. TOP1 in cell lysates was immunoprecipitated as described in "Materials and Methods." Immunoprecipitates were analyzed by 5% SDS-PAGE and then immunoblotted using anti-TOP1 antibodies (*I*). The same filter was stripped and reprobed with antiubiquitin antibodies to detect ubiquitin-TOP1 conjugates (*II*). Immunoprecipitates obtained from MG132-treated cells (control experiment without CPT treatment) were analyzed similarly by immunoblotting with either anti-TOP1 (*III*) or antiubiquitin (*IV*) antibodies. *V*, *VI*, *VII*, and *VIII*, CPT induces ubiquitin conjugation to TOP1 in ZR-75-1 breast cancer cells. Immunoprecipitation and immunoblotting were performed exactly the same as described above except that ZR-75-1 cells were used instead of BT474 cells. *V*, *VI*, *VII*, and *VIII* correspond to *I*, *II*, *III*, and *IV*, respectively.

to reveal the strand break that is then repaired by DNA repair machinery.

The heterogeneity of TOP1 down-regulation in various tumor cells is quite substantial. At one end of the spectrum, ZR-75-1 breast cancer cells are completely defective in CPT-induced down-regulation. At the other, BT474 cells exhibit a near-normal level of CPT-induced down-regulation of TOP1. Studies of ZR-75-1 and BT474 cells have suggested that the difference in the rate of TOP1 down-regulation may occur at a step(s) downstream from the cleavable complexes because the difference in the amount of TOP1 cleavable complexes was only 2-fold between these cells. Our preliminary studies have suggested that both SUMO-1-TOP1 conjugation and ubiquitin-dependent down-regulation of TOP1 are steps downstream from the formation of cleavable complex upon CPT treatment. Although we have confirmed that ubiquitin conjugation to TOP1 is for the purpose of TOP1 destruction via 26S proteasome, the role of SUMO-1 conjugation to TOP1 is yet unclear. As mentioned above, it has been proposed that SUMO-1 conjugation to substrates antagonizes ubiquitination and prevents their destruction via 26S proteasome (23, 24). Because we observed that many tumor cells are defective in TOP1 destruction, we analyzed these two processes in different tumor cells. We showed that the process of SUMO-1 conjugation to TOP1 occurs efficiently in both cells. Similarly, the process of ubiquitin conjugation to TOP1 also appears to be proficient in both cells. However, we were unable to quantitate the amount of ubiquitin-TOP1 conjugates in these cells.

It is possible that the difference in the rate of TOP1 down-regulation in breast cancer cells occurs at one or more steps downstream from the TOP1 cleavable complex. One possible candidate step that could be altered in ZR-75-1 cells is the release of the covalently bound TOP1 from DNA by the catalytic activity of tyrosine DNA phosphodiesterase (25). Tyrosine DNA phosphodiesterase could be required to remove either ubiquitinated TOP1 from DNA prior to 26S proteasome-mediated degradation of TOP1 or the residual TOP1 peptide from DNA after 26S proteasome-mediated degradation of the covalent TOP1-DNA complexes. Alternatively, there may be a defect in either 26S proteasome activity or the recruitment of 26S proteasome to ubiquitin-TOP1 conjugates that are covalently linked to DNA. Regardless of the process(es) that is defective in ZR-75-1 cells, its identification may be particularly important because its regulation may affect CPT sensitivity/resistance in tumor cells.

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