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DNA Damage and Cell Killing by Camptothecin and Its Derivative in Human Leukemia HL-60 Cells

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Camptothecin (CPT) has been recognized as a topoisomerase I (Topo I) inhibitor. However, the mechanism of cytotoxicity of this agent remains unknown. In the present study, we analyzed the kinetics of Topo I-mediated DNA single-strand breaks and internucleosomal DNA cleavage produced by CPT and its derivative, 7-ethyl-10-hydroxycamptothecin (SN-38), in HL-60 cells. DNA single-strand breaks were detected using alkaline sucrose gradient centrifugation when HL-60 cells were incubated with 10 μM CPT or 10 μM SN-38 for 30 min. These DNA single-strand breaks were rapidly repaired after drug removal, while the cytotoxic action of these drugs was sustained. Treatment of HL-60 cells with CPT or SN-38 for 3 h produced extensive degradation of DNA. Agarose gel electrophoresis showed a ladder of DNA fragments consisted of multimers of approximately 200 base pairs, characteristic of apoptosis. Interestingly, this type of DNA fragmentation was also induced within 4 h after repair of DNA single-strand breaks, and subsequently loss of cell viability was observed. When zinc ion, a potent inhibitor of endonuclease, was added to drug-free medium after treatment with CPT or SN-38, internucleosomal DNA cleavage was abolished. Furthermore, addition of zinc ion reduced the loss of cell viability. These data suggest that Topo I-mediated DNA single-strand breaks may be necessary but are not sufficient for cell death, and the endonuclease involved in induction of internucleosomal DNA cleavage may play an important role in HL-60 cell death induced by Topo I inhibitor.

Key words: Camptothecin — DNA damage — HL-60

CPT[2] is a plant antitumor agent which was isolated from *Camptotheca acuminata,* a tree native to south China, by Wall *et al.*[1] It was found to have strong antitumor activity against mouse L1210 leukemia, rat Walker carcinosarcoma, and some other experimental tumors.[1,2] However, preclinical and clinical trials revealed that the drug has severe side effects such as hemorrhagic cystitis and myelosuppression.[3] Recently, semisynthetic CPT derivatives have been developed as candidate antitumor agents.[4,5] For example, 7-ethyl-10-[4-(1-piperidino)-1-piperidino]carbonyloxycamptothecin (CPT-11) (Fig. 1), is now under clinical trial in Japan, and preliminary results are encouraging.[6]

Previous experiments revealed that CPT inhibits Topo I through the formation of Topo I-DNA cleavable complexes.[7,8] These Topo I-DNA cleavable complexes appear to be responsible for DNA single-strand breaks that can be detected using alkaline elution assay or alkaline sucrose gradient centrifugation.[9-11] Previous studies have demonstrated a correlation between CPT-induced DNA single-strand breaks and cytotoxicity.[12-14] Although there is strong evidence that CPT-mediated cell killing involves inhibition of Topo I, other observations call into question the direct relationship between Topo I-mediated DNA single-strand breaks and cell death. For example, CPT has been shown to be specifically toxic to cells in S phase, although the levels of Topo I and drug-induced DNA single-strand breaks appear relatively constant throughout the cell cycle.[9,15,16] The fact that CPT-induced DNA single-strand breaks were rapidly reversed in cells which proceeded to die, led us to investigate further the mechanism of CPT cytotoxicity.

In the present study, we analyzed the kinetics of Topo I-mediated DNA single-strand breaks and endonucleolytic DNA cleavage produced by CPT or its derivative, SN-38, an active metabolite of CPT-11 in human leukemia cell line HL-60. We also investigated in detail the effect of zinc ion, an inhibitor of endonuclease, on CPT-induced internucleosomal DNA cleavage, in order to examine whether the production of endonuclease-mediated DNA fragmentation is an essential step for Topo I inhibitor-induced HL-60 cell death.

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[2] Abbreviations: CPT, camptothecin; Topo I, DNA topoisomerase I; PBS phosphate-buffered saline; SN-38, 7-ethyl-10-hydroxycamptothecin; CPT-11, 7-ethyl-10-[4-(1-piperidino)-1-piperidino]carbonyloxycamptothecin; TKM buffer, 50 mM Tris-HCl (pH 7.5)-25 mM KCl-5 mM $MgCl_2$; TKE buffer, 50 mM Tris-HCl (pH 7.5)-25 mM KCl-1 mM EDTA; DTT, dithiothreitol; PMSF, phenylmethylsulfonyl fluoride; SDS, sodium dodecyl sulfate.

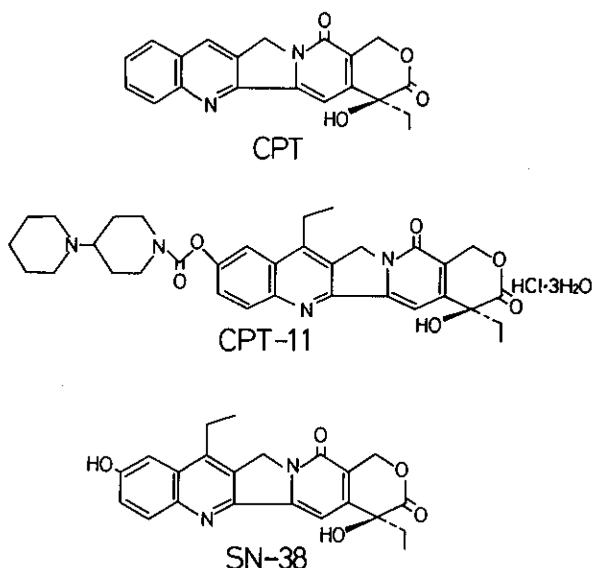


Fig. 1. Chemical structures of CPT, CPT-11, and SN-38.

MATERIALS AND METHODS

Materials CPT, CPT-11 and SN-38 were kind gifts of Yakult Honsha Co., Ltd. (Tokyo). Daunorubicin was supplied by Meiji Seika Co., Ltd. (Tokyo). SN-38 and CPT were dissolved at appropriate concentrations in dimethyl sulfoxide. [methyl-^{3}H]Thymidine (5 Ci/mmol) was purchased from Amersham Japan (Tokyo). Plasmid DNA pBR322 was purchased from Takara Shuzo Co., Ltd. (Kyoto).

Cell culture and measurement of cell growth HL-60 human myelogenous leukemia cells and U937 monoblastic leukemia cells were grown at 37°C in RPMI 1640 medium containing 10% heat-inactivated fetal bovine serum in an atmosphere containing 5% CO_2. Exponentially growing cells were exposed to drugs for the indicated time periods. Viable cells were counted on a hemocytometer by a trypan blue dye exclusion procedure.

Preparation of crude nuclear extract The nuclei were prepared by the method of Kunkel *et al.*[17] with slight modifications. Briefly, nuclei were obtained from 1×10^8 cells in early log-phase culture. Cells were washed twice with cold PBS, and pelleted by centrifugation at 800*g* for 5 min. Nuclei were isolated after incubation in TKM buffer containing 0.25 *M* sucrose and 0.25% Triton X-100. Nuclei were pelleted by centrifugation at 800*g* for 5 min, and nuclear pellets were then washed once with TKM buffer containing 0.25 *M* sucrose. Nuclear protein was eluted for 60 min at 4°C in TKE buffer containing 0.5 *M* NaCl, 1 m*M* DTT, and 1 m*M* PMSF. A solution of nuclear protein was obtained by centrifugation at 100,000*g* for 60 min. Protein concentration was determined by the method of Bradford.[18]

Topo I assay Topo I activity was determined in terms of the relaxation of supercoiled pBR322 DNA, essentially as described by Liu and Miller.[19] The reaction mixtures contained 0.3 μg of pBR322 DNA, 50 m*M* Tris-HCl (pH 7.5), 72 m*M* KCl, 5 m*M* $MgCl_2$, 1 m*M* EDTA, 5 m*M* DTT, 50 μg/ml bovine serum albumin and crude extract. The reaction mixture (20 μl) was incubated at 37°C for 30 min, and the reaction was terminated by the addition of 5 μl of dye solution consisting of 2.5% SDS, 10% Ficoll, and 0.01% bromphenol blue. The mixtures were applied to 1% agarose gel, and electrophoresed at 50 V for 10 h. The gel was stained with ethidium bromide and photographed under UV light illumination. One unit of activity was defined as the minimum amount of crude nuclear extract for full relaxation of 0.3 μg of pBR322 DNA.

Alkaline sucrose gradient centrifugation HL-60 cells were labeled with 0.5 μCi/ml [^{3}H]thymidine for 24 h. Unincorporated [^{3}H]thymidine was washed out, and the cells were resuspended in fresh medium at a concentration of 5×10^5 cells/ml. The cell suspension was incubated in the presence of drug at 37°C for the indicated time periods. Drug treatments were terminated by rinsing the cells with PBS at 0–4°C. The cells were lysed at 4°C for 12 h in 0.2 ml of lysing solution (0.45 *M* NaOH, 0.55 *M* NaCl, 10 m*M* EDTA, and 1% Sarkosyl) overlaid on a 5%–20% alkaline sucrose gradient, containing 0.1 *M* NaOH, 0.9 *M* NaCl, and 10 m*M* EDTA. The gradients were centrifuged at 38,000 rpm for 150 min in a Hitachi RPS40T swinging rotor. Forty 10-drop fractions were collected from the top on filter disks using a Buchler densiflow apparatus. The filters were washed with 10% TCA and 90% ethanol, and dried. Radioactivity was counted in a toluene-based scintillation fluid. λ-DNA (48.5 kb) as a molecular weight marker was centrifuged in parallel with cell lysates. The collected fractions were analyzed by gel electrophoresis to identify the marker along the gradient.

DNA fragmentation assay After drug treatment, HL-60 nuclei were prepared as described above. Nuclear pellets were resuspended in TKM buffer containing 0.25 *M* sucrose and lysed with 1% SDS in the presence of proteinase K (500 μg/ml) and 20 m*M* EDTA at 37°C for 12 h. DNA was extracted by standard phenol/chloroform/isoamyl alcohol extraction procedures as described previously,[20] and treated with 100 μg/ml of RNase A at 37°C for 60 min. DNA samples were electrophoretically separated on 1.2% agarose gels. After electrophoresis at 50 V for 7 h, DNA was visualized by ethidium bromide staining. DNA fragmentation was quantitated by scanning photographic negatives with a densitometer and integrating the area under the curves. Integrated areas

were divided at the 9.8-kb molecular weight marker position into high- and low-molecular weight DNA, and the percentage of DNA fragmentation was determined by dividing the area of low-molecular-weight DNA by the total area.

Digestion of isolated HL-60 nuclei with micrococcal nuclease Freshly isolated HL-60 nuclei were resuspended in 50 m*M* Tris-HCl buffer (pH 7.5), containing 0.25 *M* sucrose, 5 m*M* $MgCl_2$ and 25 m*M* KCl. The nuclear suspension was adjusted to 1 m*M* $CaCl_2$ and incubated with 0.01 unit/ml micrococcal nuclease (Sigma Chemical Co.) at 37°C for 5 min. The enzyme reaction was terminated by the addition of 20 m*M* EDTA. Nuclei were pelleted by centrifugation at 800*g* for 10 min, then DNA was extracted and analyzed by agarose gel electrophoresis as described above.

Endogenous endonuclease assay Endonuclease assay was performed as described previously.[21] Freshly isolated nuclei from HL-60 cells were resuspended in 50 m*M* Tris-HCl buffer (pH 7.5), containing 0.25 *M* sucrose and 25 m*M* KCl. Endogenous endonuclease activity was determined after incubation of the nuclei suspension at 37°C in the presence of various combinations of divalent cations (i.e., Mg^{2+}, Ca^{2+} or Zn^{2+}). DNA was then extracted and electrophoresed as described above.

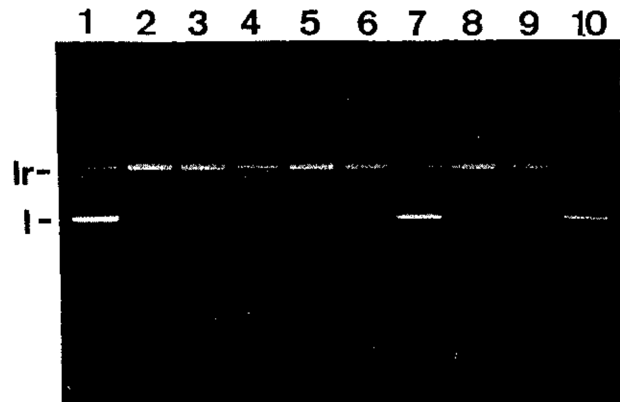


Fig. 2. Effect of CPT-11, SN-38, and CPT on DNA topoisomerase I extracted from HL-60 cells. The enzymatic activity was assayed in terms of the relaxation of supercoiled pBR322 DNA. I and Ir represent supercoiled DNA and relaxed DNA, respectively. Lane 1, substrate pBR322 DNA only. pBR322 DNA was treated with no agent (lane 2); with 100 μM or 1.0 m*M* CPT-11 (lanes 3–4, respectively); with 0.1, 1.0, or 10 μM SN-38 (lanes 5–7, respectively); and with 0.1, 1.0, or 10 μM CPT (lanes 8–10, respectively) in the presence of 1 unit of topoisomerase I.

RESULTS

Effect of CPT-11, SN-38 and CPT on Topo I (Fig. 2) CPT and SN-38 inhibited relaxation of supercoiled pBR322 DNA by the catalytic action of Topo I isolated from HL-60 cells at 1 μM. CPT-11 only slightly inhibited it at 1 m*M*. These data indicate that the inhibitory activity of SN-38 on Topo I was about 1,000 times higher than that of CPT-11.

DNA Single-strand breaks induced by CPT or SN-38 As indicated in Fig. 3A, most of the DNA from control cells sedimented at the bottom of the alkaline sucrose gradient under our experimental conditions. Treatment of HL-60 cells with 10 μM CPT for 30 min resulted in a marked shift of the peak to lower-molecular-weight fractions (Fig. 3B). This shift of peak position to lower-molecular-weight fractions was reversed when HL-60 cells treated with CPT were reincubated in drug-free medium for 30 min (Fig. 3C). Similar results were obtained using SN-38 (data not shown). These data suggest that CPT and SN-38 induce reversible DNA single-strand breaks.

Internucleosomal DNA cleavage induced by CPT or SN-38 To examine the delayed effect of CPT on DNA integrity, HL-60 human leukemia cells were incubated with 1 μM CPT for varying lengths of time. Treatment with 1 μM CPT did not induce a ladder of DNA fragments during the first 120 min, but such a ladder was clearly observed after incubation for 180 min (Fig. 4). This fragmentation of DNA preceded by at least 3 h the initiation of cell death determined by the trypan blue dye exclusion procedure (Fig. 5). After 12 h, most of the DNA was in the form of multiples of about 200 bp units, characteristic of oligonucleosomes. This type of DNA fragmentation strongly resembled the ladder pattern of micrococcal nuclease digestion of HL-60 cell nuclei (Fig. 4, lane 1). When HL-60 cells were incubated with different doses of CPT for 3 h, the degree of DNA fragmentation increased at the higher concentration (Fig. 6A). SN-38 showed almost the same activity to induce DNA fragmentation as CPT (Fig. 6B). The activity to induce DNA fragmentation of CPT and SN-38 was compared with that of daunorubicin, a clinically useful anthracycline antibiotic. CPT and SN-38 could induce more extensive DNA fragmentation than daunorubicin (Fig. 6B). We also observed that CPT induced similar internucleosomal DNA cleavage in U937 cells (data not shown).

Induction of internucleosomal DNA cleavage after repair of DNA single-strand breaks The decrease in sedimentation rate of DNA in alkaline sucrose gradient was reversed wheh HL-60 cells treated with CPT were reincubated in drug-free medium for 30 min (Fig. 3C). However, 4 h after incubation in drug-free medium, a nucleosomal ladder of DNA fragments became evident (Fig. 7). SN-38 also induced internucleosomal DNA cleavage after repair of DNA single-strand breaks (data not shown).

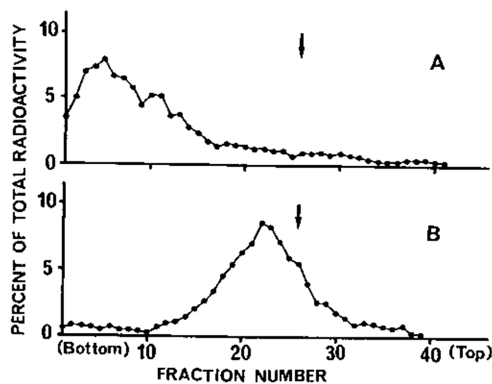
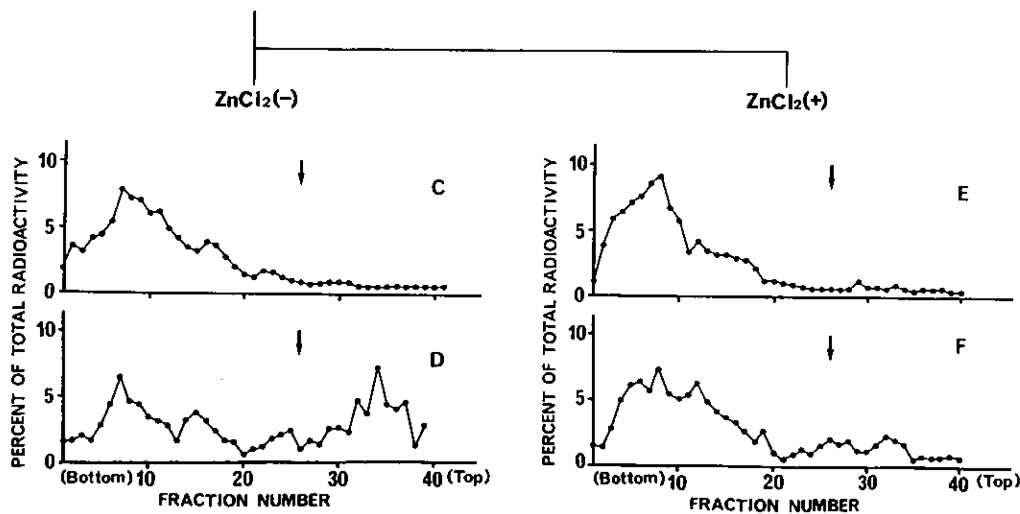


Fig. 3. Effect of CPT and zinc ion on the sedimentation profile of HL-60 cell DNA in an alkaline sucrose gradient. HL-60 cells previously labeled with [^{3}H]thymidine were untreated (A: control), or treated with 10 μM CPT for 30 min (B). HL-60 cells treated with 10 μM CPT for 30 min were rinsed, and incubated in drug-free medium for 30 min or 4 h in the absence (C, D, respectively) or presence of 1 mM $ZnCl_2$ (E, F, respectively). The arrow indicates the peak position of λ-DNA (48.5 kb).



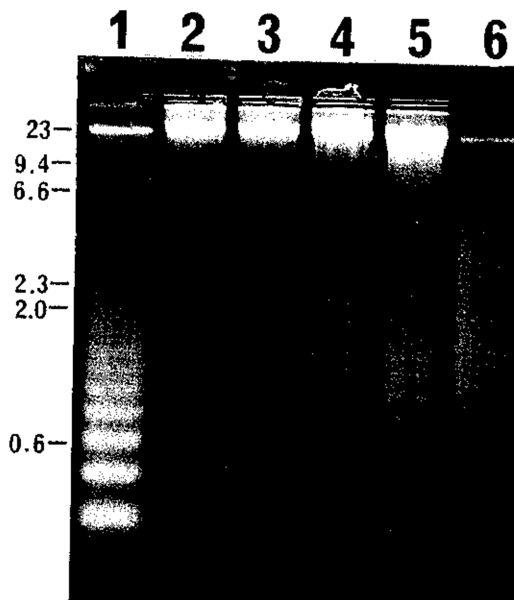


Fig. 4. Internucleosomal DNA cleavage induced by CPT. Isolated HL-60 nuclei were treated with micrococcal nuclease (lane 1). HL-60 cells were treated with 1 μM CPT for 0, 2, 3, 12, or 24 h (lanes 2–6, respectively). Cellular DNA was extracted and subjected to agarose gel electrophoresis as described in "Materials and Methods." Molecular weight markers expressed in kilobase pairs are indicated on the left.

Inhibition of internucleosomal DNA cleavage and cell death by zinc ion It is assumed that internucleosomal DNA cleavage is induced by endogeneous endonuclease. Zinc ion is known to be a potent inhibitor of endonuclease in the nuclei.[21,22] We therefore investigated the effect of $ZnCl_2$ on Topo I inhibitor-induced DNA fragmentation. After treatment of HL-60 cells with 10 μM CPT for 30 min, HL-60 cells were incubated with 1 mM $ZnCl_2$ in drug-free medium. As shown in Fig. 7, $ZnCl_2$ at 1 mM inhibited internucleosomal DNA

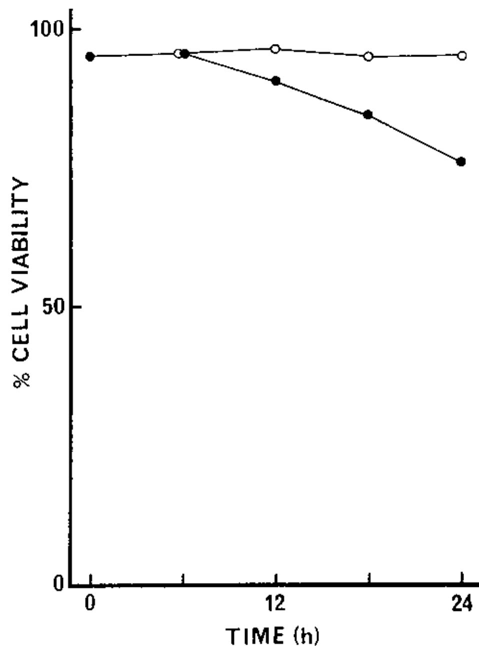


Fig. 5. Effect of CPT on viability of HL-60 cells. HL-60 cells were incubated with no agent (○), or 1 μM CPT (●). Viability was determined by trypan blue dye exclusion. Results are mean values from three separate experiments.

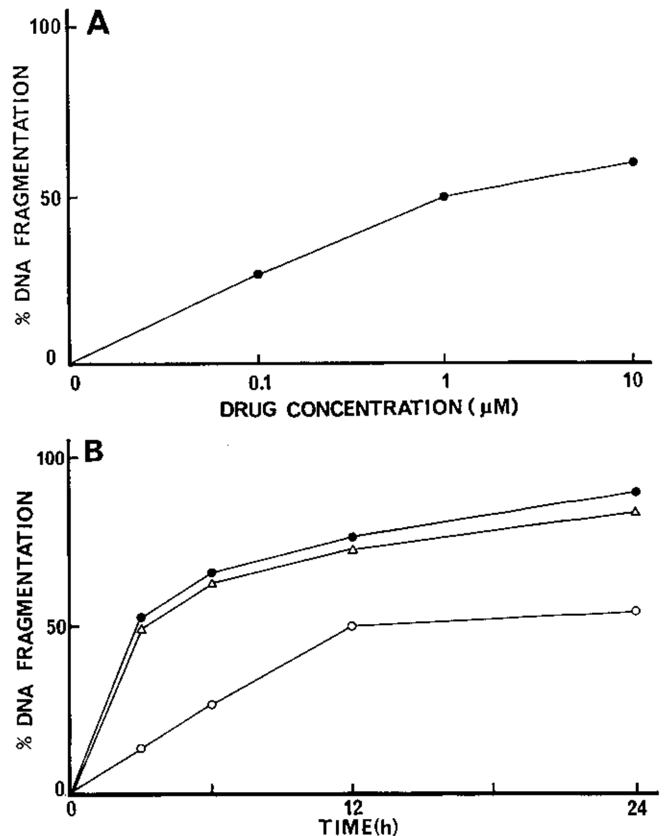


Fig. 6. (A) Dose-response curve of CPT-induced internucleosomal DNA cleavage. HL-60 cells were incubated with different doses of CPT for 3 h. (B) Internucleosomal DNA cleavage induced by CPT, SN-38, or daunorubicin. HL-60 cells were incubated with 1 μM CPT (△), 1 μM SN-38 (●) or 1 μM daunorubicin (○). We analyzed the effects of the three drugs at the concentrations giving 75% cell viability during 24 h incubation. Cellular DNA was extracted and subjected to agarose gel electrophoresis. The percent of DNA fragmentation was determined from a densitometric scan of the photographic negative of the gel as described in "Materials and Methods."

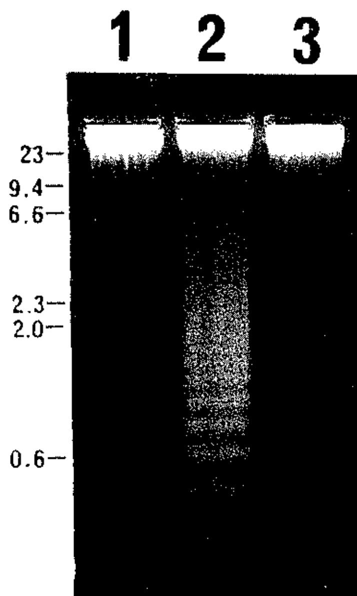


Fig. 7. Induction of nucleosomal pattern of DNA fragmentation after repair of DNA single-strand breaks and inhibition of DNA fragmentation by zinc ion. Lane 1, untreated HL-60 cells (control). HL-60 cells treated with 10 μM CPT for 30 min were rinsed, and incubated in drug-free medium in the presence (lane 3) or absence of 1 mM $ZnCl_2$ (lane 2) for 4 h. Molecular weight markers (values expressed in kilobase pairs) are indicated on the left.

celeavage. The sedimentation profile of HL-60 cells incubated with zinc ion in drug-free medium after treatment with CPT or SN-38 was almost identical to that of the normal control in the alkaline sucrose gradient (Fig. 3F). On the other hand, in the absence of $ZnCl_2$, DNA fragments smaller than λ-DNA (48.5 kb), which were thought to be a nucleosomal ladder, were evident (Fig. 3D). These data (Fig. 3D and 3F) were compatible with the results obtained using agarose gel electrophoresis (Fig. 7). We also observed that addition of zinc ion 60 min after CPT treatment abrogated internucleosomal DNA cleavage. In contrast, addition of zinc ion 90 min

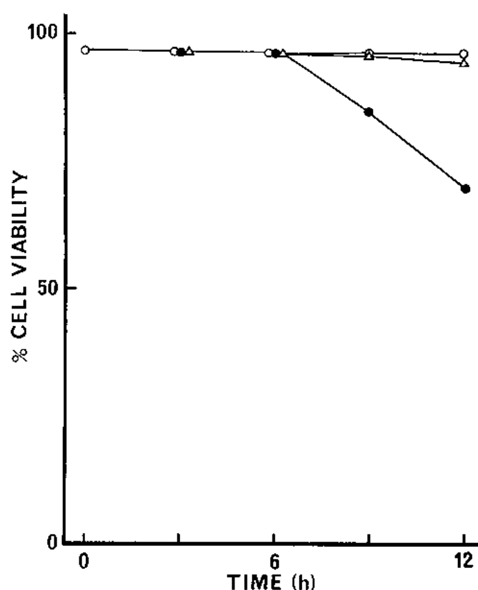


Fig. 8. Effect of zinc ion on viability of CPT-treated HL-60 cells. HL-60 cells treated with 10 μM CPT for 30 min were rinsed, and incubated in drug-free medium in the presence (△) or absence of 1 mM $ZnCl_2$ (●). Incubation with 1 mM $ZnCl_2$ only (○). Viability was determined by trypan blue dye exclusion. Results are mean values from three separate experiments.

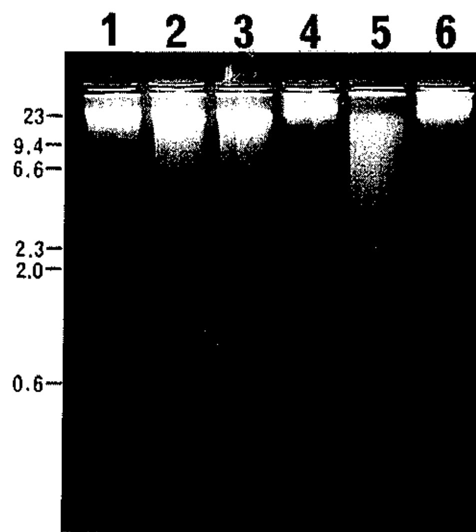


Fig. 9. Analysis of endogenous endonuclease activity in HL-60 nuclei. Lane 1, DNA extracted from untreated HL-60 nuclei (control). Nuclei were incubated for 4 h with 1 mM $CaCl_2$ alone (lane 2), without 1 mM $CaCl_2$ and 5 mM $MgCl_2$ (lane 3), with 5 mM $MgCl_2$ alone (lane 4), with 5 mM $MgCl_2$ and 1 mM $CaCl_2$ (lane 5) or with 1 mM $ZnCl_2$, 1 mM $CaCl_2$ and 5 mM $MgCl_2$ (lane 6). Cellular DNA was extracted and subjected to agarose gel electrophoresis as described in "Materials and Methods." Molecular weight markers (values expressed in kilobase pairs) are indicated on the left.

after drug treatment failed to prevent this DNA fragmentation (data not shown).

We next examined whether $ZnCl_2$ would also inhibit CPT-induced cell death. After 12 h incubation with 1 mM $ZnCl_2$ in drug-free medium, there was no significant decrease in the viability of HL-60 cells treated with 10 μM CPT for 30 min. On the other hand, after 12 h incubation without 1 mM $ZnCl_2$, a marked decrease in viability was observed (Fig. 8). Similar results were obtained in the case of SN-38 (data not shown).

Endogenous endonuclease activity in the nuclei of HL-60 cells To assess the possibility that endogenous endonuclease might produce internucleosomal DNA fragmentation, we examined the endonuclease activity in the HL-60 nuclei. Figure 9 shows that incubation of nuclei from HL-60 cells in the presence of 1 mM $CaCl_2$ and 5 mM $MgCl_2$ activated an endonuclease which cleaved at internucleosomal sites. Incubation of nuclei with $MgCl_2$ alone did not activate this endonuclease, whereas incubation with $CaCl_2$ alone was found partially to activate the endonuclease. Internucleosomal DNA cleavage in the presence of both of these ions was completely abolished by 1 mM $ZnCl_2$. These data suggest that HL-60 nuclei contain a Ca^{2+}/Mg^{2+}-dependent endonuclease which can cleave at internucleosomal sites.

DISCUSSION

In the present study, we examined the internucleosomal DNA cleavage produced by CPT and its derivative, SN-38, in relation to the Topo I-mediated DNA single-strand breaks induced by these drugs in HL-60 cells. We observed that CPT and SN-38 rapidly induced DNA single-strand breaks. These DNA single-strand breaks were repaired within 30 min after drug removal, whereas the cytotoxic action of these drugs was sustained. On the other hand, treatment of HL-60 cells with CPT or SN-38 for 3 h produced a ladder of DNA fragments which consisted of multimers of approximately 200 bp. This DNA fragmentation strongly resembled the ladder pattern of micrococcal nuclease digestion of HL-60 cell nuclei, which suggests that CPT and SN-38 may induce endonuclease-mediated DNA fragmentation. Interestingly, the nucleosomal pattern of DNA fragmentation was also induced within 4 h after resealing of DNA single-strand breaks, and subsequently loss of cell viability was observed. These data strongly suggest that internucleosomal DNA cleavage is more closely associated with CPT-induced cell death than are Topo I-mediated DNA single-strand breaks.

Internucleosomal DNA fragmentation has also been observed in glucocorticoid-treated thymocytes or lymphocytes undergoing apoptosis.[21–24] Recent studies have shown that various kinds of anticancer agents induce a nucleosomal pattern of DNA fragmentation.[25, 26] Induction of internucleosomal DNA cleavage is not a specific action of Topo I inhibitor. However, it was not clear whether this type of DNA fragmentation was a cause or a consequence of the cytotoxicity of CPT.

We have demonstrated for the first time that addition of zinc ion after formation of cleavable complexes blocked CPT-induced internucleosomal DNA cleavage, using agarose gel electrophoresis and alkaline sucrose gradient centrifugation. Most importantly, zinc ion markedly reduced cell death. These data suggest that induction of internucleosomal DNA cleavage, which might be induced by endogenous endonuclease, is not an accompanying effect but a critical process for cell death induced by Topo I inhibitor. Recent studies have shown that interaction between the cleavable complexes and the replication fork triggers CPT-induced cell death.[27] The results obtained in the present study suggest that the zinc ion affects a late process following formation of cleavable complexes, and such a process involved in endogenous endonuclease activation is also important. Recently, similar prevention of DNA fragmentation by zinc ion has been reported in glucocorticoid-induced thymocyte killing,[21] TNF-mediated cytolysis,[28] and etoposide-treated HL-60 cells.[29] Endonuclease-mediated DNA fragmentation might be an important process for cell death induced by various cytotoxic agents.

We found that HL-60 nuclei contain Ca^{2+}/Mg^{2+}-dependent endonuclease. In addition, the protein synthesis inhibitor cycloheximide itself induced internucleosomal DNA cleavage in HL-60 cells (A. Yoshida, unpublished observation). These results are consistent with a previous report.[30] Therefore, it is hypothesized that not newly synthesized endonuclease, but rather endogenous endonuclease may be responsible for internucleosomal DNA cleavage in HL-60 cells. It seems that the some controlling elements including a co-factor(s) (e.g., Ca^{2+}) may be important. However, further study is needed to clarify the mechanisms of activation of endonuclease by CPT in HL-60 cells.

Previous studies, including those at our laboratory, have found DNA damage and other cellular metabolic changes induced by short-term treatment with various kinds of anticancer agents.[31, 32] Different cytotoxic agents have distinct cellular targets. Thus, it seems that different mechanisms are involved in endonuclease activation, although distal processes following formation of internucleosomal DNA cleavage may be common. As a next step, we should examine late processes induced by anticancer agents in relation to the initial DNA damage as described above.

In conclusion, our results indicate that Topo I-mediated DNA breaks may be necessary but are not sufficient for cell death. We have demonstrated that the inhibition of endonuclease by zinc ion blocked internucleosomal DNA cleavage, and most importantly, it also reduced loss of cell viability. This internucleosomal DNA cleavage may play an important role in HL-60 cell death induced by Topo I inhibitor.

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