Effect of Topoisomerase Inhibitors on the Formation of Replication Protein A-Foci in Nuclei Reconstituted in *Xenopus* Egg Extract

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Some DNA topoisomerase inhibitors induce cleavage of DNA strands and lead to cell death. Therefore the inhibitors are utilized for chemotherapy against cancer. The treatment, however, can cause severe side effects such as genetic modification of normal cells. Camptothecin (CPT) and etoposide (VP16) belong to this class of anticancer agents that inhibit DNA topoisomerase I and II, respectively. In the present study, we investigated whether treatment with CPT and VP16 induces DNA breaks in nuclei reconstituted in *Xenopus* egg extract by observing focus formation of replication protein A (RPA), which is thought to accumulate on loci of DNA lesions prior to DNA repair. Treatment with CPT caused formation of discrete RPA foci at a later stage of incubation of demembranated *Xenopus* sperm nuclei with egg extracts. The RPA foci increased with the amount of CPT. However, at 30 min, before or just starting the initiation of DNA replication, the discrete RPA foci in CPT-containing extract were not obvious, suggesting that DNA replication is necessary for the focus formation induced by CPT. On the other hand, discrete RPA foci were induced by VP16 even at 30 min.

Key words —— camptothecin, etoposide, topoisomerase inhibitor, DNA damage, replication protein A

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INTRODUCTION

DNA topoisomerases are enzymes that modify higher-structure of DNA molecules by cleaving and rejoining DNA strands. This class of enzyme introduces or removes DNA superhelical tension, ties or unties DNA knots, and catenates or decatenates DNA molecules, and most are essential for living cells. DNA topoisomerases are categorized into two groups. The type I enzymes alter DNA topology by a single-strand DNA passage mechanism after introduction of single-strand DNA cleavage, while the type II enzymes hydrolyze ATP and introduce double-strand DNA cleavage.[1)]

Camptothecin (CPT) and etoposide (VP16) are inhibitors of eukaryotic DNA topoisomerases and they or their derivatives are utilized as anticancer chemotherapeutic agents.[2)] Although the target of CPT is DNA topoisomerase I, which is classified into the type I topoisomerase, and the target of VP16 is a type II topoisomerase, DNA topoisomerase II, both CPT and VP16 are known to inhibit the rejoining of DNA molecules by trapping topoisomerase and DNA complex after induction of DNA cleavage. The drugs therefore cause DNA breakages that will trigger tumor cell death. However, this effect can also cause severe side effects such as normal cell death and genetic modifications of normal cells, which result in the increased risk of secondary tumors.

Replication protein A (RPA) is a eukaryotic single-strand DNA binding protein complex composed of three subunits.[3)] In addition to its function in DNA replication, recombination, and transcription, RPA has also been suggested to associate with the damaged loci on UV-irradiated DNA,[4,5)] and with the loci of double-strand DNA breaks. In the cells after ionizing radiation, RPA forms foci and most of the RPA foci are localized in the sites of DNA repair.[6,7)] In addition, BLM and WRN were reported to be co-localized with DNA damage-induced RPA foci.[8,9)] BLM and WRN are eukaryotic DNA helicases encoded by the causative genes for Bloom and Werner syndromes, both of which are associated with genetic instability.[10,11)] These findings suggest that RPA foci form at sites of DNA repair. The cell-free system established using the extract derived from *Xenopus* eggs is useful for biochemical and cytological analysis of the dynamic processes involved in the cell cycle,[12,13)] because cell cycle events such as the formation of nuclei, initiation of DNA replication, and condensation of chromatin take place

efficiently and highly synchronously in the extract. The cellular response to DNA damage is believed to be a dynamic linkage of processes, such as the recognition of damage, cell cycle arrest, selection of an appropriate repair process, and restoration of normal cell cycle events. To understand the molecular basis behind these dynamic cellular responses after treatment with topoisomerase inhibitors, we have employed a cell-free system using *Xenopus* egg extracts.

MATERIALS AND METHODS

Preparation of *Xenopus* Egg Extract and Demembranated Sperm —— Metaphase-arrested cell-free extracts derived from *Xenopus* eggs were prepared according to the method described by Chong *et al*.[14] In brief, freshly laid *Xenopus* eggs were dejellied in a 2% cysteine solution, pH 7.6, containing 1 mM EGTA and rinsed with 15 mM Tris HCl, pH 7.6, 110 mM NaCl, 2 mM KCl, 1 mM $MgSO_4$, 2 mM $NaHCO_3$, 0.5 mM Na_2HPO_4, and 2 mM EGTA, and with 50 mM HEPES KOH, pH 7.6, 50 mM KCl, 5 mM $MgCl_2$, 2 mM β-mercaptoethanol, and 5 mM EGTA. The cytoplasmic fraction was obtained after centrifugation of the eggs ($10000 \times g$, 10 min). The fraction was supplemented with cytochalasin B (10 μg/ml) and was cleared by further centrifugation ($84000 \times g$, 20 min). The extract was supplemented with 1% glycerol and frozen until use. Immediately prior to the experiments, the frozen extract was thawed and supplemented with 0.3 mM $CaCl_2$, 250 μg/ml of cycloheximide, 25 μM phosphocreatine, and 15 μg/ml of creatine phosphokinase.

Xenopus demembranated sperm nuclei were prepared as described previously.[14]

Immunofluorescence Microscopy —— Demembranated sperm nuclei (10000 sperm heads) were incubated in 10 μl of *Xenopus* egg extract at 23°C for specified periods. During the incubation, camptothecin (25 μM) (Sigma) and etoposide (20 μg/ml) (Sigma) were added to the reaction mixture as indicated. Ice-cold extraction buffer (EB; 50 mM KCl, 50 mM HEPES KOH, pH 7.6, 5 mM $MgCl_2$) containing 3.7% formaldehyde (90 μl) and EB containing 0.5% Triton X-100 (90 μl) were added to the extract after incubation, and the mixture was put on ice for 2 min. Then, the sample was supplemented with 10 μl of 37% formaldehyde and left on ice for 10 min. The reconstituted nuclei in the extract were passed through EB containing 30% sucrose and fixed on glass coverslips by centrifugation ($400 \times g$, 20 min). The samples fixed on the coverslips were rinsed three times with 0.05% Tween 20 in phosphate-buffered saline (PBS; 137 mM NaCl, 2.68 mM KCl, 8.04 mM Na_2HPO_4, 1.47 mM KH_2PO_4) and incubated with rabbit anti-*Xenopus* RPA antisera diluted in 0.05% Tween 20-PBS supplemented with 10% skim milk for 2 hr at room temperature. The coverslips were rinsed three times with 0.05% Tween 20-PBS and incubated with FITC-labeled donkey anti-rabbit IgG (Jackson Immuno Research Laboratories Inc., U.S.A.). They were rinsed once with 0.05% Tween 20-PBS, incubated for 5 min in 0.05% Tween 20-PBS containing propidium iodide (20 μg/ml) (Wako), and rinsed three times with 0.05% Tween 20-PBS and once with PBS. The coverslips were mounted on a slide glass with PermaFluor Aqueous Mountant (Shandon Inc., U.S.A.).

The prepared samples were observed using a Laser Scanning Confocal Imaging System, MRC-1024 (Bio-Rad).

RESULTS

Induction of PRA Focus Formation by CPT

When demembranated *Xenopus* sperm were incubated with *Xenopus* egg extract, rounded nuclei derived from *Xenopus* sperm appeared 30 min after incubation, and enlargement of nuclei was observed with further incubation (Fig. 1, panels b and d). The nuclei that were formed after 30-min incubation in the extract containing CPT were smaller than those formed in the absence of CPT (panel f). Although enlargement of nuclei was slightly delayed in the presence of CPT, the size of the nuclei was almost the same as that of nuclei incubated in the absence of CPT after 60 min (panels d and h). Accumulation of RPA, which was resistant to detergent treatment, was observed in almost all nuclei after 30-min incubation either in the presence or absence of CPT. Enlarged images of representative nuclei showed the formation of numerous fine RPA foci distributed evenly in the nuclei (panels a and e). While RPA foci were rarely observed in the nuclei incubated in the egg extract without CPT after 60 min incubation (panel c), accumulation of RPA was observed in the nuclei that were incubated in the egg extract containing CPT (panel g). The RPA foci formed after 60-min incubation in the presence of CPT were

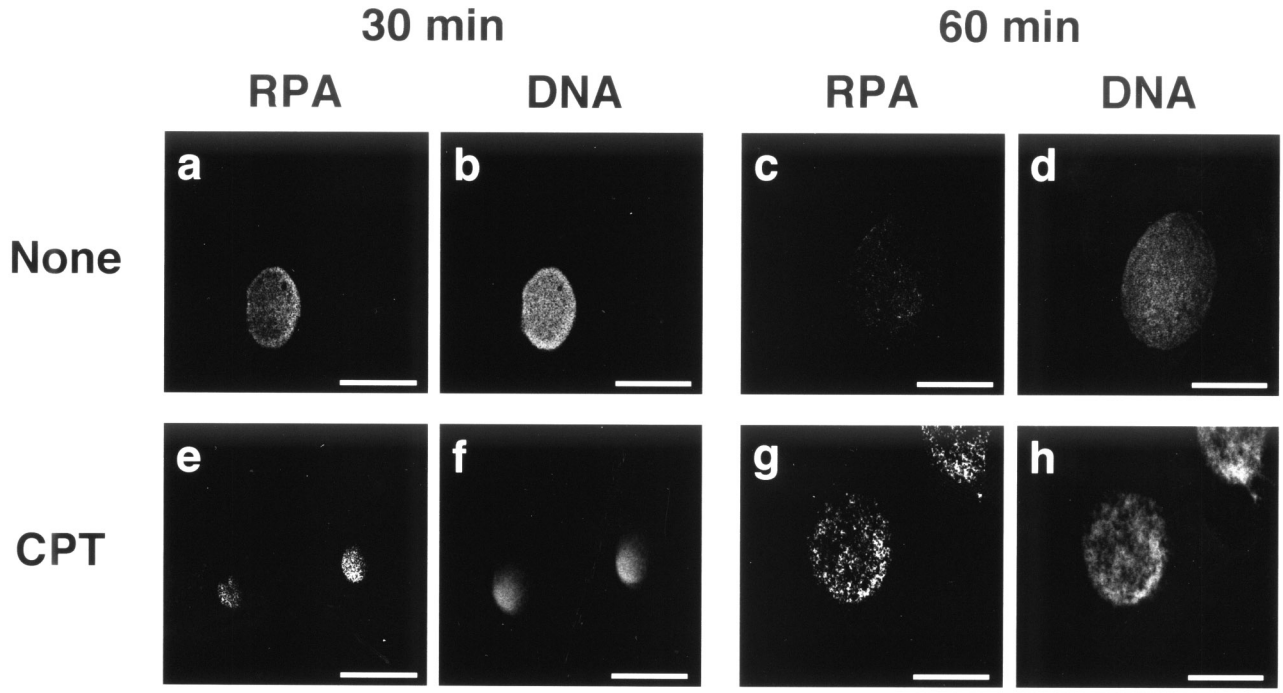

Fig. 1. Focus Formation of RPA in Nuclei Incubated with *Xenopus* Egg Extract Containing CPT

Demembranated *Xenopus* sperm nuclei were incubated at 23°C for 30 min or 60 min in *Xenopus* egg extract with or without CPT. Localization of detergent-insoluble RPA (a, c, e, g) and DNA (b, d, f, h) were visualized. Scale bars indicate 20 μm.

more discrete than those in the nuclei after 30-min incubation.

Effect of CPT Concentration on RPA Focus Formation

We examined the effect of CPT concentration on RPA focus formation. Nuclei were incubated in egg extracts containing various concentrations of CPT for 60 min, and the number of RPA foci in each nucleus was counted. We classified the nuclei into three categories: nuclei containing more than 20, fewer than 20, and no discrete RPA foci. Percentages of nuclei classified into the three categories are shown in Fig. 2. The results showed that the number of RPA foci-positive nuclei and the number of foci in a nucleus increased depending on the concentration of CPT, indicating that the discrete RPA foci were induced by CPT treatment.

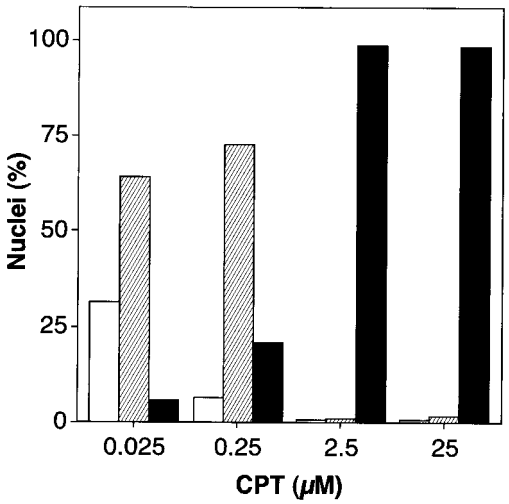

Fig. 2. Increase in the Number of RPA Foci in Response to CPT Concentration

Demembranated sperm nuclei and egg extract were mixed and incubated for 60 min in the presence of the indicated amounts of CPT. Open, shaded, and closed columns indicate the percentage of nuclei containing no, fewer than 20 and more than 20 discrete RPA foci, respectively. The data obtained in the absence of CPT are not presented because of the difficulty in scoring RPA foci due to the diminution of RPA signals.

Induction of RPA Focus Formation by VP16

We next examined the behavior of RPA in the nuclei formed in the extract containing the topoisomerase II inhibitor VP16. Demembranated sperm nuclei were incubated for 30 or 60 min in the egg extract containing VP16, and the accumulation of RPA in the reconstituted nuclei was examined. Similar to the results obtained with the CPT-containing extract, discrete PRA foci were observed after 60-min incubation in the extract containing VP16 (data not shown). In addition, the RPA foci after 30-

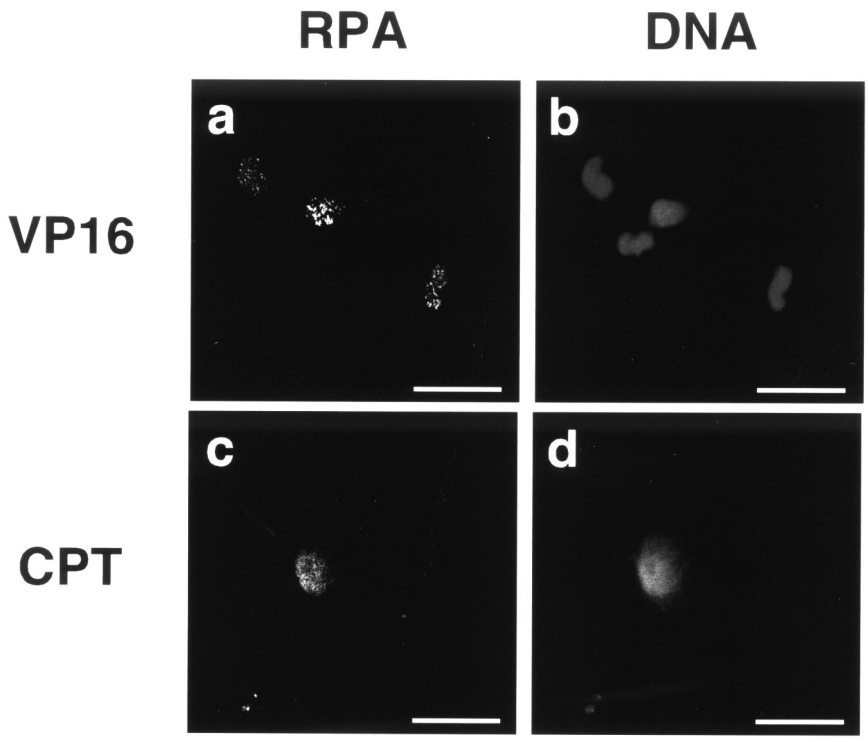

Fig. 3. Focus Formation of RPA in Nuclei Incubated with *Xenopus* Egg Extract Containing VP16

Demembranated *Xenopus* sperm nuclei were incubated at 23°C for 30 min in *Xenopus* egg extract containing VP16 (20 μg/ml) or CPT (25 μM). Localization of detergent-insoluble RPA (a, c) and DNA (b, d) were visualized. Scale bars indicate 20 μm.

min incubation in the presence of VP16 were more discrete than those formed in the presence of CPT (Fig. 3). The foci were similar to those formed after 60-min incubation in the presence of CPT.

DISCUSSION

DNA topoisomerases catalyze the concerted cleavage and rejoining of DNA strands and introduce necessary topological and conformational changes in DNA. This class of enzyme is crucial for many cellular processes such as DNA replication, repair, and recombination, and therefore inhibitors of the enzymes are utilized as anticancer or antibacterial agents. Numerous topoisomerase inhibitors have been reported and categorized into two groups: one directly suppresses biochemical activities of topoisomerases; and the other traps the complex of topoisomerase and DNA after cleavage formation (cleavage complex), which leads to DNA breakage.[15]

CPT is an inhibitor of DNA topoisomerase I and is known to trap DNA topoisomerase I cleavage complexes, resulting in enzyme-linked breaks in the substrate DNA.[16–18] The formation of double-strand breaks by CPT is thought to depend on DNA replication on the basis of several lines of evidence.[19–23]

In this study, we reproduced discrete RPA foci in a cell-free system using *Xenopus* egg extract containing CPT, which were reported to be formed in response to DNA lesions in cultured cells. We also examined the behavior of RPA under the same conditions except for the absence of CPT. The results indicate that fine RPA foci were evenly distributed in the reconstituted nuclei after 30-min incubation but the signal of RPA was diminished during further incubation. The fine, evenly distributed PRA foci are thought to reflect the accumulation of RPA at the initiation site of DNA replication.[24,25] The diminution of the RPA signals in nuclei that were incubated for 60 min in the extract without CPT appears to be due to the completion of DNA replication because the incorporation of precursor of DNA reached a plateau 60 min after incubation (data not shown). The fact that the discrete RPA foci were observed after 60-min incubation in the presence of CPT but not at 30 min when DNA replication starts indicates that the discrete RPA foci were formed *via* DNA replication. Experiments using cultured cells indicated that CPT induced DNA double-strand breaks when

the replication fork collided with the CPT cleavage complex. Thus it appears likely that the discrete RPA foci are loci at which RPA is accumulated on DNA double-strand breaks but not on single-strand breaks. In this context, it is not surprising that VP16 induced discrete RPA foci formation after 30-min incubation because VP16 traps the cleavage complex of topoisomerase II which induces double-strand breaks.

The delay of enlargement of nuclei in the presence of CTP or VP16 indicates the involvement of DNA topoisomerases I and II in the decondensation of highly condensed sperm chromatin. However, the size of nuclei that were incubated for 60 min in the extract containing CPT was similar to the size of nuclei that were incubated without CPT, indicating that topoisomerase I is not essential for the decondensation, probably because DNA topoisomerase II or another topoisomerase replaces the function of topoisomerase I. In this study, we confirmed RPA focus formation in a cell free-system using *Xenopus* egg extract in response to inhibitors of topoisomerases I and II. This cell-free system is expected to be useful for the investigation of the molecular basis of the formation, recognition, and repair of DNA lesions induced by the topoisomerase inhibitors.

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