ICRF-193, a catalytic inhibitor of DNA topoisomerase II, delays the cell cycle progression from metaphase, but not from anaphase to the G1 phase in mammalian cells

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Received 31 January 1997; revised version received 4 March 1997

Abstract **We have shown previously that ICRF-193, a catalytic inhibitor of DNA topoisomerase II (topo II), delays cell cycle progression in HeLa S3 cells. We report here that the delay of the transition in M phase is observed when HeLa S3 cells were treated with ICRF-193 during metaphase, but not thereafter. ICRF-193 also delayed the degradation of cyclin B in the transition from M to G1 phase, while in Chinese hamster ovary (CHO) cells the drug did not delay the progression in M phase. Since HeLa S3 and CHO cells are 'stringent' and 'relaxed' in mitotic control, respectively, it is suggested that under topo II inhibition, the M phase checkpoint operates through an inability for chromosome segregation.**

Key words: Checkpoint in M phase; Topoisomerase II inhibitor; ICRF-193

1. Introduction

Cells have a system termed 'checkpoint control' by which an event does not begin until previous events have been completed. The function of cell cycle arrest is well understood, based on studies of *rad* 9 mutants in budding yeast [1], whereby UV- or X-irradiated wild type cells are arrested in G2 phase and do not restart cell cycle progression until after DNA damage has been repaired. In contrast, irradiated *rad* 9 mutants do not remain in G2, but enter mitosis with unrepaired damage, and suffer mortality. In mammalian cells, loss of p53 function, a tumor suppressor protein often associated with an increased frequency of gene amplification and aneuploidy, results in defects in the G1 and G2 checkpoints [2,3]. Cells from patients with ataxia telangiectasia, a human recessive disorder, exhibit increased sensitivity to ionizing radiation but are unresponsive to the normally associated inhibition of DNA synthesis and do not demonstrate G2 arrest [4]. Inhibitors of microtubule formation block cell cycle progression at prometaphase, and improper attachment of kinetochore spindles to chromosomes delays the progression from metaphase to anaphase [5]. Defects in the driving force of the mitotic spindle also delay the transition to telophase [6].

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Topo I and II are enzymes which control DNA topology by introducing transient single and double strands breaks, respectively, into DNA. In DNA replication and transcription, these enzymes resolve the torsional constraints which accumulate ahead of macromolecular synthesis [7]. Topo II functions as a decatenase in mitotic chromosome condensation, decondensation and segregation [8,9]. In mammalian cells, it has also been shown to play an important role in chromosome condensation, as determined using *Xenopus* oocyte extracts [10,11].

Our recent work revealed that ICRF-193, a non-cleavable complex forming type of topo II inhibitor [12–14], allows cell cycle progression without chromosome segregation, leading to the accumulation of multiploid cells [13,15]. We further found that ICRF-193 delays transition from S to M phase and from M to G1 phase in HeLa S3 cells [15]. In the present study, we examined the phase of mitosis in which the checkpoint control functions with topo II inhibition.

2. Materials and methods

2.1. Drugs

ICRF-193 and CPT-11 were kindly provided by Zenyaku Kogyo Co., Ltd. (Tokyo) and Yakult Honsha Co., Ltd. (Tokyo), respectively. TN-16 was purchased from Wako Pure Chemical Industries Ltd. (Osaka).

2.2. Cell culture

HeLa S3 and a Chinese hamster ovary (CHO) cell line, AA8, were seeded in 60 mm dishes, and grown in Dulbecco's modified Eagle's (DME) medium containing 10% calf serum under a humidified atmosphere of 5% CO_2 in air.

2.3. M phase synchronization

Cells were seeded at 1 or 2×10^6 cells/140 mm dish. The next day, TN-16 was added (final concentration 0.3 μM), after which HeLa S3 cells were cultured for 5 h, and CHO cells for 3 h [15]. Round and floating mitotic cells were then collected and washed with serum free DME.

2.4. Mitotic figures

To examine the mitotic index, cells were suspended in 75 mM KCl, and left to stand for 20 min at 0°C. An equal volume of methanol-acetic acid (3:1) (MeA) was then added to the suspension, with mild agitation before centrifugation. After washing with MeA solution, the cells were resuspended in MeA solution and dispensed onto glass slides. After drying, the samples were stained with Giemsa solution. Mitotic cells were counted under a microscope. To determine the population of cells in M phase, staining of DNA with propidium iodide and of microtubules with anti-tubulin α antibody was also performed.

2.5. Immunoblotting

HeLa S3 cells were pelleted at 3000 rpm for 5 min at 4°C, and lysed

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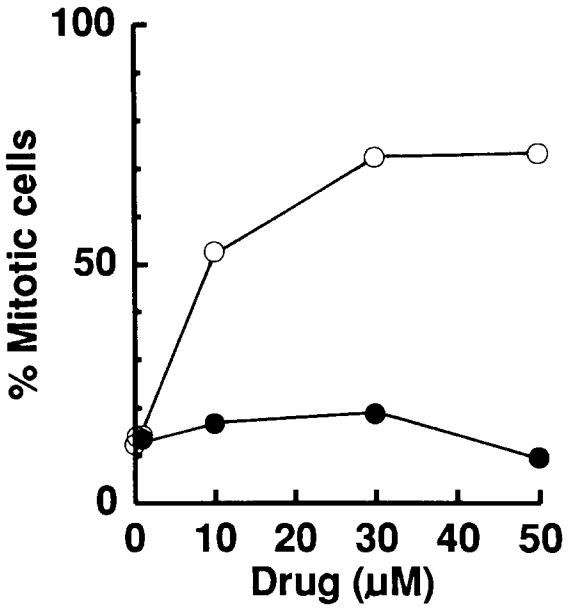

Fig. 1. Inhibition of the exit from M phase in HeLa S3 cells by ICRF-193 but not CPT-11, in a dose dependent manner. Mitotic cells were collected in the presence of TN-16, and after its removal were incubated with various concentrations of ICRF-193 (○) or CPT-11 (●). Percentages of mitotic cells were counted 2 h after release from metaphase. The synchrony of mitotic cells was 80% at the beginning.

on ice for 1 h by resuspension in cold lysis buffer (50 mM Tris-HCl buffer, 350 mM NaCl, 0.1% (v/v) Nonidet P-40, 5 mM Na_2EDTA, 50 mM NaF, pH 7.4) containing protease inhibitors (2 mM phenylmethylsulfonyl fluoride and 20 μg/ml each of antipain, aprotinin, chymostatin, leupeptin and pepstatin A) and then centrifuged at 15000 rpm for 15 min at 4°C. The supernatants were used for immunoblotting after addition of 3×SDS-PAGE loading buffer and boiling. The samples were subjected to electrophoresis on 7.5% polyacrylamide gels and electroblotted onto nitrocellulose membranes, which were then blocked with 5% skim milk and incubated with anti-mouse cyclin B1 antibody (Oncogene Science). After washing with TBST (Tris-buffered saline-Tween), the membranes were treated with HRP-conjugated goat anti-mouse IgG. The blots were visualized by enhanced chemiluminescence (ECL), using the ECL Western blot detection system (Amersham).

3. Results

We previously clarified ICRF-193 to be a non-cleavable complex forming type of topo II inhibitor [12–14]. The drug inhibits the release of topo II from DNA after one round of the catalytic cycle through inhibition of the enzyme's ATPase activity, and stabilizes it in a protein closed clamp which cannot be dissociated with 1 M NaCl [14]. Since it is difficult to test directly whether or not ICRF-193 inhibits topo II activity in situ within the cells, we examined the amount of topo II in this closed protein clamp. The intracellular topo II formed a salt stable complex in the presence of ICRF-193.

In previous work, we showed that cell cycle progression from S to M and M to G1 phases is delayed by ICRF-193 in HeLa S3 but not in CHO cells [15]. To further examine this delay in progression, we also tested the effect of ICRF-193 on the degradation of cyclin B in M to G1 transition, which normally occurs concomitant with a decrease of cdc 2 kinase activity, the master key enzyme [16]. Fig. 1 shows the effects of increasing concentrations of ICRF-193 on the progression from M to G1 phase in HeLa S3 cells, expressed as the percentage of mitotic cells remaining 2 h after release from TN-16, a mitotic inhibitor. 10 μM or higher concentrations of ICRF-193 significantly inhibited the transition. Since topo I does not play a role in chromosome dynamics such as condensation or segregation, the effect of CPT-11, a topo I inhibitor, was examined as a control. The drug did not inhibit the M phase transition. At 30 μM, ICRF-193 delayed the exit from M phase for over 5 h (Fig. 2A). Downes et al. [17] have shown that caffeine can overcome ICRF-193 effects. We confirmed their data, but found that caffeine does not prevent the ICRF-193-induced delay in progression through the M phase (Fig. 2A). As shown in Fig. 2B, reduction in cyclin B levels was also delayed by ICRF-193. To determine whether ICRF-193 treatment exerts the same effects on HeLa S3 cells in anaphase or telophase, they were exposed to 30 μM ICRF-193 at various times after removal of TN-16, and the percentage of mitotic cells was examined (Fig. 3). The addition of ICRF-193 to mitotic cells after 0 or 15 min resulted in complete inhibition of progression, with most cells in M phase after 2 h. With exposure after 30 min or later, cell cycle progression was only slightly delayed and the cells did exit from M phase. No inhibition was observed when cells were exposed to the drug after 60 min. Morphological assessment revealed no cells in anaphase at 15 min. At 30 min, however, about 12% of cells were in anaphase, and at 45 min, cells in ana-

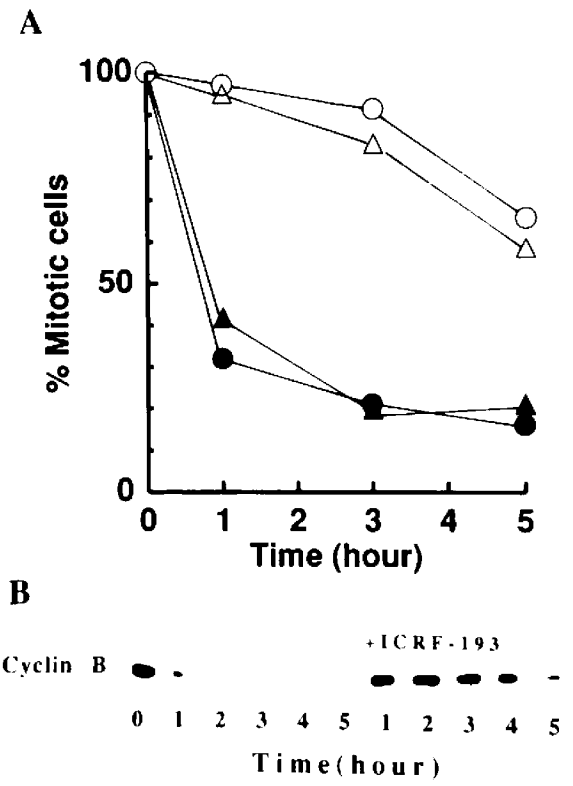

Fig. 2. ICRF-193-induced delay of the cell cycle progression (A) and degradation of cyclin B (B) in M phase. A: After removal of TN-16, mitotic HeLa S3 cells were exposed to no drug (●), 30 μM ICRF-193 (○), 2 mM caffeine and 30 μM ICRF-193 (△) or 2 mM caffeine (▲). Percentages of mitotic cells were determined by counting cells having no nuclear envelope. The amount of cyclin B (B) was examined by immunoblot analysis using anti-cyclin B antibody at the indicated times after release from TN-16.

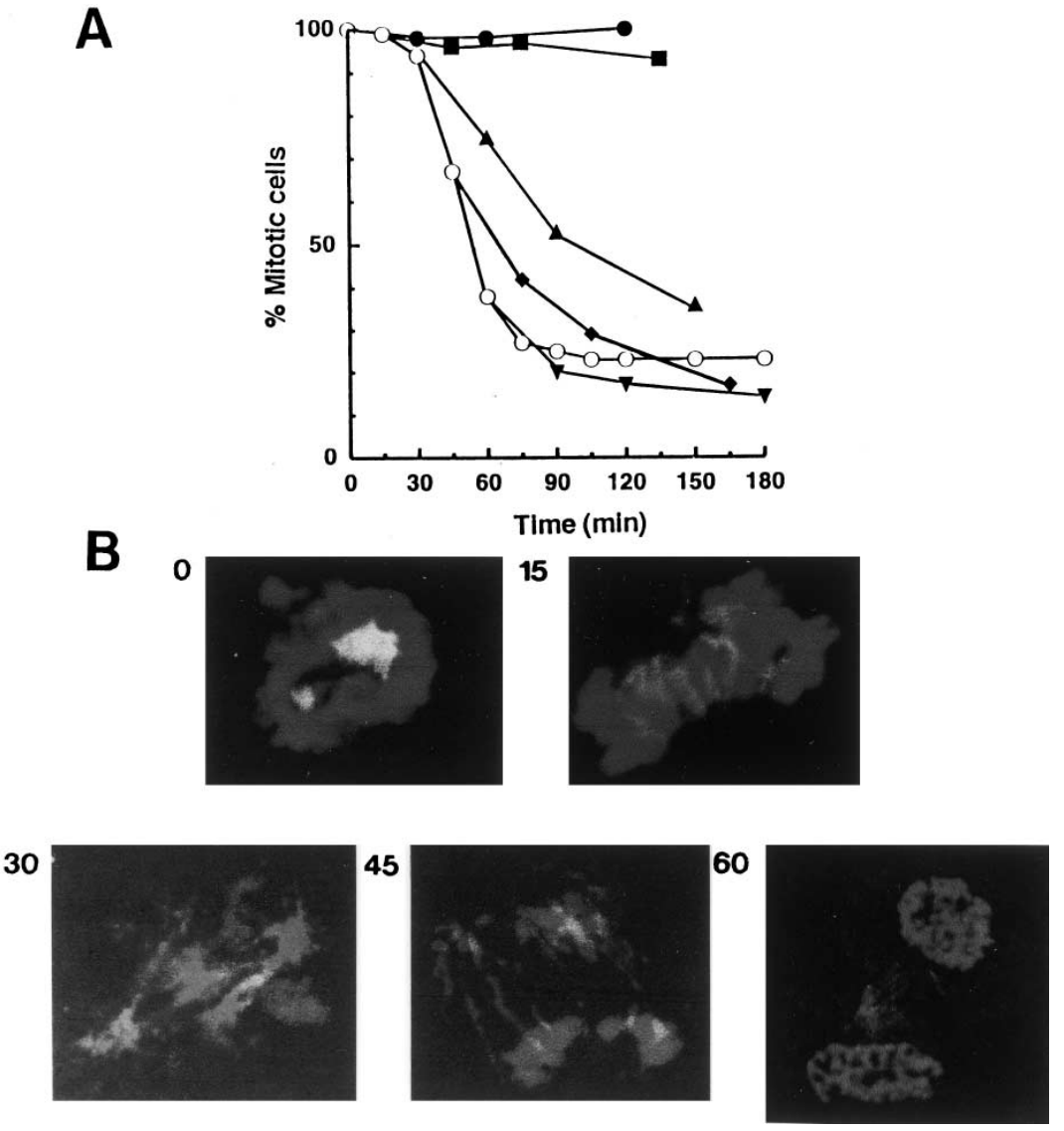

Fig. 3. ICRF-193 delays the cell cycle transition in metaphase, but not in anaphase. A: HeLa S3 cells were exposed to no drug (○) or to 30 μM ICRF-193 at 0 (●), 15 (■), 30 (▲), 45 (◆) and 60 (▼) min after removal of TN-16 and incubated further. Percentages of mitotic cells were examined at the times indicated. B: Cells were fixed at 0, 15, 30, 45 or 60 min after removal of TN-16 and prepared for indirect immunofluorescence with anti-tubulin α antibody (light color) and propidium iodide (gray color).

phase and telophase accounted for 71% of the population (Fig. 3B). These results indicate that the inhibition of topo II by ICRF-193 affects the transition in metaphase, but not in anaphase or telophase.

Differing from HeLa S3 cells, CHO cells were not delayed by ICRF-193 in the progression from M to G1 phase (data not shown).

4. Discussion

In the present study we confirmed our previous findings that the inhibition of topo II by ICRF-193 delays transition of cells from the M to the G1 phase, in contrast with the results of Downes et al. [16]. Furthermore, we demonstrated that the inhibitor-induced delay occurs when cells were exposed to ICRF-193 during metaphase, but not thereafter. Since ICRF-193 inhibits chromosome segregation, this indicates that an inability for chromosome segregation causes the delay in progression. Downes et al. [16] earlier showed caffeine to abrogate the ICRF-induced delay in the transition from the S to the M phase. Using HeLa S3 cells, we confirmed their observation (data not shown), but further found that caffeine did not reverse the delay of transition during M phase. Caffeine has been reported to potentiate UV-, X-ray- or drug-induced lethal damage and inhibit post-replicational repair by abrogating G2 arrest and leading to abortive mitosis [18]. Although caffeine is an inhibitor of phosphodiesterase, it is not known why the drug overcomes G2 arrest and potentiates cell killing induced by DNA damaging agents [19].

As reported previously [15], neither transition from the S to M phase nor progression through M phase was affected by ICRF-193 in CHO cells. HeLa S3 and CHO cells have also been shown to have different responses to inhibitors of the mitotic spindle assembly, such as colcemid and nocodazole, and DNA synthesis inhibitors such as hydroxyurea and aphidicolin [20,21]; only HeLa S3 cells are blocked at M phase by

the presence of colcemid. In contrast, CHO cells continue cell cycle progression without cell division. The difference is that protein synthesis is down-regulated under inhibition of DNA synthesis in HeLa S3 cells, so that they become arrested in S phase, while protein synthesis continues in CHO cells and unbalanced growth ensues. Our present observations offer further evidence that the checkpoint control functions under inhibition of topo II activity in HeLa S3, but not in CHO cells.

In the M phase, inhibitors of microtubule assembly block cell cycle progression at prometaphase by improper attachment of kinetochore spindles to chromosomes, or reduced microtubule dynamics [22–25]. Such improper attachment of kinetochores impairs the force driving the mitotic spindle and delays transition from metaphase to anaphase and telophase [6,21]. The fact that ICRF-193 delayed the progression from metaphase to anaphase, but not from anaphase to G1 phase, suggests that the delay is related to an inability for chromosome segregation through inactivation of topo II activity. Although it remains to be clarified how topo II is involved in chromosome segregation, based on findings of yeast genetic studies, it is very likely that the enzyme removes catenated dimer DNAs remaining in the M phase so that sister chromatids can segregate [26]. Since both inhibitors of microtubule assembly and topo II cause chromosome disaggregation, this might be a signal for the M phase checkpoint.

McIntosh [22] proposed that cells possess a signal inhibiting anaphase onset, which is turned off when proper attachment of microtubules to kinetochores takes place at the metaphase plate. Gorbsky [27] described one candidate for the inhibitory signal: a phosphoepitope, associated with kinetochores, whose expression is reduced when chromosomes are aligned with the metaphase plate. Such an inhibitory signal would not be expected to disappear when cells in metaphase progress into anaphase in the presence of ICRF-193 because microtubules do not connect properly to the DNA of kinetochores resulting in a disordered DNA structure containing catenated dimers. To test this possibility, detailed analysis of the connections between kinetochores and microtubules in mitotic cells exposed to ICRF-193 is necessary.

Acknowledgements: This study was supported by Grants-in-Aid for Cancer Research from the Ministry of Education, Science, Sports and Culture of Japan and by the Suzuken Memorial Foundation.

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