Mammalian DNA Topoisomerase I Mediates the Enhancement of Radiation Cytotoxicity by Camptothecin Derivatives

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ABSTRACT

The role of DNA topoisomerase I as a biochemical mediator of radiosensitization in cultured mammalian cells by camptothecin derivatives was studied. We found in Chinese hamster DC3F cells, camptothecin enhanced the cytotoxicity of radiation in a schedule-dependent manner. At 4 μM, a sensitizer enhancement ratio of 1.45 was observed when radiation was used concurrently with or immediately after drug treatment. By comparison, no enhancement was obtained if radiation preceded camptothecin treatment. Consistently, in human breast cancer MCF-7 cells, sensitizer enhancement ratios of 1.43, 1.38, and 1.05 were observed when radiation was used concurrently with, immediately after, or prior to treatment with 20(S)-10,11-methylenedioxy camptothecin (MDCamp).

Three studies indicated that an intact stereospecific interaction between camptothecin derivatives and DNA topoisomerase I is essential in the induction of radiosensitization: (a) higher concentrations of camptothecin were required to radiosensitize the camptothecin-resistant DC3F/C-10 cells; (b) a newly identified topoisomerase I-targeting Hoechst 33342 also radiosensitized DC3F cells; and (c) 20(S)-methylene dioxy camptothecin, but not its nontoxic 20(R)-stereoisomer, radiosensitized MCF-7 cells by obliterating the "shoulder" of the radiation survival curve.

The mechanism of radiosensitization was investigated in DC3F cells. We found that camptothecin only minimally enhanced the cytotoxic effect of radiation in G0/G1-phase cells obtained by a mitotic shake-off technique as well as in plateau-phase cells arrested by growing to confluence.

Our data suggest a potential development of topoisomerase I drugs as radiosensitizers in treating human malignancies.

INTRODUCTION

The combination of chemotherapy and radiation therapy has become the treatment of choice for a number of advanced human malignancies (1, 2). Although systemic chemotherapy ideally may control metastatic disease, radiation therapy provides effective local control at the primary tumor sites (1, 2). A number of chemotherapeutic drugs are known to be able to synergistically enhance the cytotoxic effect of radiation (3). Various mechanisms have been suggested for the expression of such an effect. We show that an intact stereospecific interaction between DNA topoisomerase I and camptothecin derivatives is essential in the induction of radiosensitization by camptothecin derivatives in cultured mammalian cells (25, 26), and the ability to overcome MDR1-mediated drug resistance mechanisms (27–29) are among a few features of camptothecin derivatives that may contribute to their therapeutic advantages.

Recently, enhancement of the cytotoxic effect of radiation by camptothecin derivatives has been investigated (30–37). With some controversies (36, 37), most data have suggested the existence of a synergistic cell-killing effect between ionizing irradiation and camptothecin derivatives (30–35). The involvement of DNA topoisomerase I has been shown to be the cellular target of a number of antineoplastic drugs (36, 37), including camptothecin derivatives (30–35). The involvement of DNA topoisomerase I in both proliferating and quiescent tumor cells than in normal cells suggests that topoisomerase I-targeting drugs may possess a selective cytotoxic advantage against slowly growing as well as rapidly proliferating tumors (19–24). S-phase-specific cytotoxicity (18), selective cytotoxicity against tumorigenic over nontumorigenic cells (25, 26), and the ability to overcome MDR1-mediated drug resistance mechanisms (27–29) are among a few features of camptothecin derivatives that may contribute to their therapeutic advantages.

In the present study, we characterized the mechanism of radiosensitization induced by camptothecin derivatives in cultured mammalian cells, particularly the involvement of DNA topoisomerase I in mediating such an effect. We show that an intact stereospecific interaction between DNA topoisomerase I and camptothecin derivatives is essential for such a reaction. In addition, we demonstrated that camptothecin derivatives radiosensitized cells in a highly schedule-dependent manner. Synergistic enhancements of radiocytotoxicity with SERs of more than 2.0 were observed when drug treatment was used concurrently with or immediately preceding radiation, but not following radiation. The finding of radiosensitization by a different class of topoisomerase I-targeting drugs, such as Hoechst 33342, further asserts the potential clinical development of topoisomerase I drugs in augmenting radiation therapy against human malignancies.

MATERIALS AND METHODS

Drugs and Materials. Camptothecin lactone (NSC 94600) was obtained from the Drug Synthesis and Chemistry Branch, National Cancer Institute.

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1 The abbreviations used are: SER, sensitization enhancement ratio; MDCamp, 10,11-methylenedioxy camptothecin; SHMT, single-hit multitarget; MI, mitotic index; LQ, linear quadratic.

1529

Downloaded from cancerres.aacrjournals.org on April 14, 2017. © 1997 American Association for Cancer Research.
Camptothecin Derivatives Enhanced the Cytotoxicity of Radiation in a Schedule-dependent Manner in Mammalian Cells. The induction of radiation sensitization of camptothecin derivatives was first tested in exponentially growing Chinese hamster DC3F cells. Three different schedules of 4-h camptothecin incubation in relation to irradiation were used. As shown in Fig. 1, camptothecin radiosensitized DC3F cells in a schedule-dependent manner. At 4 μM, a concentration that resulted in 16% cell survival, a SER of 1.45 was obtained when radiation was used concurrently with or immediately after camptothecin treatment. By comparison, no enhancement (SER, 1.0) was observed when radiation preceded camptothecin incubation (Fig. 1).

To look for treatment combinations that optimize the radiosensitization effect, clonogenic survival assays were conducted with DC3F cells treated concurrently with radiation and camptothecin with various concentrations and drug incubation times. As illustrated in Table 1, a short 0.5-h camptothecin incubation, compared to 1- and 2-h incubations, was less cytotoxic and affected an as-good SER of 1.46.

RESULTS

![Graph showing induction of radiosensitization in DC3F cells by camptothecin (Camp).](Image)

Fig. 1. Induction of radiosensitization in DC3F cells by camptothecin (Camp). Clonogenic survival assays of exponentially growing DC3F cells were conducted as described in “Materials and Methods.” Cells were exposed to different treatment schedules of radiation (XRT) and 4 μM camptothecin incubation. Ionizing radiation amounts of 0.3, 6.9, and 12 Gy were used alone (□), prior to a 4-h drug incubation (●), in the middle of a 4-h drug incubation (●), or at the end of a 4-h drug incubation after camptothecin was removed by washing with drug-free medium (〇). Points, means of triplicates; bars, SD.
Camptothecin was added within 4 h following the radiation of cells on the slope of the radiation survival curve was observed when comparable SER of 1.28 (Fig. 4).

Clonogenic survival assays. As shown in Fig. 4, concurrent 0.5 h of 4 μM camptothecin by varying concentrations of camptothecin by A more than 30-fold resistance to the cytotoxicity of camptothecin ating radiosensitization remains unclear. The camptothecin-resistant lung fibroblast cell line in the presence of 1 μM camptothecin (38, 39).

DNA topoisomerase I, was selected from the DC3F Chinese hamster DC3F/C-10 cell line, which harbors a camptothecin-resistant mutant (Fig. 3, D compared to A). In contrast, no such effect was observed in our DC3F/C-lO cells compared to their parental cells with Hoechst 33342. As shown in Fig. 6, a SER of 2.26 was demonstrated to poison mammalian DNA topoisomerase I in a similar fashion as camptothecin derivatives both in a purified enzyme system and in cultured cells (13, 14). We investigated the possibility of Hoechst 33342 being a reasonable radiosensitizer to mammalian cells. Chemoradiation clonogenic survival assays were conducted in DC3F cells with Hoechst 33342. As shown in Fig. 6, a SER of 2.26 was observed for DC3F cells treated concurrently with radiation and a 2-h incubation of 40 μM Hoechst 33342. No radiation sensitization was observed when cells were exposed to concurrent radiation and a 2-h incubation of low-dose (4 μM) Hoechst 33342 (Fig. 6).

**A Stereospecific Interaction between Camptothecin Derivatives and DNA Topoisomerase I Is Essential for the Induction of Radiosensitization.** Camptothecin derivatives interact with DNA topoisomerase I molecules in a stereospecific manner. Only the 20(S)-camptothecin derivatives, but not their 20-(R)-stereoisomers, are DNA damaging and cytotoxic (42, 43). We investigated the importance of such stereospecific interaction in directing the radiosensitization induced by camptothecin derivatives in MCF-7 cells with clonogenic survival assays. The obtained data were fitted with the SHMT model from programs developed by Albright (40). As illustrated in Fig. 5, only the cytotoxic 20(S)-MDCamp, but not its noncytotoxic 20(R)-stereoisomer, radiosensitized MCF-7 cells. SERs of 2.82, 1.17, and 0.96 were acquired with MCF-7 cells treated with 0.2 μM, 0.02 μM of 20(S)- and 0.2 μM of 20(R)-MDCamp, respectively (Table 3). Drug treatment alone caused cell survivals of 33.7, 51.7, and 100%, respectively (Table 3). As shown in Tables 2 and 3, the cell survivals from drug treatment alone appear to be inversely correlated with the values of their individual SERs.

**A Novel Topoisomerase I-targeting Hoechst 33342 Also Radiosensitized Mammalian Cells.** Hoechst 33342 has recently been demonstrated to poison mammalian DNA topoisomerase I in a similar fashion as camptothecin derivatives both in a purified enzyme system and in cultured cells (13, 14). We investigated the possibility of Hoechst 33342 being a reasonable radiosensitizer to mammalian cells. Chemoradiation clonogenic survival assays were conducted in DC3F cells with Hoechst 33342. As shown in Fig. 6, a SER of 2.26 was observed for DC3F cells treated concurrently with radiation and a 2-h incubation of 40 μM Hoechst 33342. No radiation sensitization was observed when cells were exposed to concurrent radiation and a 2-h incubation of low-dose (4 μM) Hoechst 33342 (Fig. 6).

**Effect of Cell Cycle Distribution on the Induction of Radiosensitization by Camptothecin.** The sensitivity to ionizing radiation varies for cells in different phases of the cell cycle (3). Cell cycle distribution is also known to be a major factor involved in the
radiosensitization induced by various chemotherapeutic agents (3, 4). We investigated the effect of cell cycle distribution on the induction of radiosensitization by camptothecin in plateau-phase DC3F cells. As illustrated in Table 4, both the cell density and percentage of G1 phase cells peak around days 6 and 7 (6.9 × 10^3 cells/mm², 52%; and 6.1 × 10^3 cells/mm², 55%, respectively). In our experiments, cells were permitted to grow for 7 days before they were exposed to chemoradiation treatments. As shown in Fig. 7A, only minimal radiosensitization with a SER of 1.06 (SER of 1.16 in a separate experiment) was observed in the 7-day plateau-phase DC3F cells treated concurrently with radiation and a 2-h incubation of 4 μM camptothecin. Camptothecin alone resulted in 59% cell survival in the 7-day plateau phase cells, compared to approximately 32% cell survival in an asynchronized population (see Tables 1 and 2), is consistent with the S phase-specific cytotoxicity of camptothecin.

G1-phase cells obtained from a mitotic shake-off technique were also studied using clonogenic survival assays. The synchrony of isolated cells was examined by MI. The cell cycle distribution, as the shake-off cells progressing through the cell cycle, was monitored by flow cytometry. Four h after mitotic shake-off, approximately 70–75% of cells were in G1, compared to 35–40% of G1 cells in the exponentially growing cultures. Fig. 7B shows a small radiosensitization effect, with a SER of 1.12 (SER of 1.19 in a separate experiment), was induced by 0.5 h of 4 μM camptothecin. Interestingly, the

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Fig. 3. SHMT survival curves for MCF-7 cells treated with radiation (XRT) and 0.5 h of 0.1 μM 20(S)-MDCamp. Programs developed by Albright (40) were used for curve fitting with the SHMT model for experiments shown in Fig. 2. A, radiation alone; B, concurrent 20(S)-MDCamp and radiation; C, radiation followed by 20(S)-MDCamp. Points, means of triplicates; bars, SD.

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Fig. 4. Induction of radiosensitization in mutant DNA topoisomerase I-containing DC3FC-10 cells by camptothecin (Camp). Clonogenic survival assays of exponentially growing DC3FC-10 cells were conducted as in Fig. 1. Cells were exposed to radiation (XRT) alone (△), concurrent radiation and 0.5 h of 4 μM camptothecin (■), or concurrent radiation and 0.5 h of 40 μM camptothecin (○). Points, means of triplicates; bars, SD.
cell survival of 20–30% with camptothecin alone in this G\textsubscript{1}-phase-enriched population appears to disagree with the S-phase killing mechanism of camptothecin (44, 45).

**DISCUSSION**

Recently, conflicting conclusions regarding the induction of radiosensitization by camptothecin derivatives have been reported (30–37). Inhibition of radiation-induced DNA repair was proposed as the underlying mechanism by some investigators to explain their observation of a synergistic effect between camptothecin derivative and ionizing irradiation (30, 33, 35). On the other hand, some researchers claimed to observe only minimal camptothecin derivative-induced radiosensitization, which they believed simply to be an effect caused by cell cycle redistribution (36, 37). In either cases, DNA topoisomerase I, the major cytotoxic target of camptothecin derivatives, was proposed to play a pivotal role in inducing radiosensitization in cells.

In the present study, we demonstrate that mammalian DNA topoisomerase I mediates the enhancement of radiation cytotoxicity by camptothecin derivatives in cultured Chinese hamster lung fibroblast DC3F cells as well as in human breast cancer MCF-7 cells. Interestingly, the induction of radiosensitization by camptothecin derivatives was shown to be schedule dependent. In contrast to an apparent induction of radiosensitization when camptothecin was used prior to or concurrently with radiation, we found no enhancement of radiotoxicity in mammalian cells when camptothecin was added even within 5 min after the completion of ionizing radiation (Figs. 1 and 2). With low molecular weights and planar chemical structures, camptothecin derivatives diffuse into cells rapidly (27, 46, 47). Also, because the possibility of accomplishing radiation-induced DNA damages within 5 min is an unlikely task for mammalian cells, this observation appears to oppose the notion that DNA repair inhibition is the main radiosensitization mechanism induced by camptothecin.
The DC3F/C-10 cells contain a camptothecin-resistant mutant DNA topoisomerase I (38). An amino acid mutation from Gly505 to Ser in a highly conserved region that is located 220 amino acids away from the presumed catalytic Tyr725 of topoisomerase I has been shown to be responsible for its resistance to camptothecin (39). If an intact DNA topoisomerase I is required in the radiosensitization pathway induced by camptothecin derivatives, a "compromised" radiosensitization response from camptothecin-treated DC3F/C-10 cells would be expected. Indeed, our results demonstrated that when the DC3F/C-10 cells were treated concurrently with radiation and a 0.5-h drug incubation, nearly no radiosensitization with a SER of 1.03 was induced by 4 \mu M of camptothecin (Fig. 4). And a comparable SER of 1.28 was obtained only when a 10-fold higher dose (40 \mu M) of camptothecin was used concurrently with radiation (Fig. 4).

Camptothecin derivatives interact with DNA topoisomerase I molecules in a stereospecific manner. In contrast to their 20(R)-stereoisomers, only the 20(S)-stereoisomers are DNA damaging and cytotoxic (42, 43). Such interaction is evident by the fact that 20(S)-10,11-MDCamp is approximately 10,000-fold more potent in stimulating mammalian DNA topoisomerase I-mediated DNA cleavage in a purified enzyme system than its noncytotoxic 20(R)-stereoisomer.3 We found that 20(S)-10,11-MDCamp, but not its 20(R)-stereoisomer, radiosensitized MCF-7 cells (Fig. 5). This observation establishes a prerequisite role of such a stereospecific interaction between DNA topoisomerase I and camptothecin derivatives in mediating radiosensitization in cells.

The DNA minor groove-binding Hoechst 33342 represents a different class of DNA topoisomerase I drugs (13, 14). It interrupts the breakage/rejoining reaction of mammalian DNA topoisomerase I by trapping reversible topoisomerase I-cleavable complexes in a fashion similar to that of camptothecin derivatives (13, 14). In addition, Hoechst 33342 induces sequence-specific topoisomerase I-mediated DNA cleavage (13). Therefore, it would be of great interest to know whether Hoechst 33342 also radiosensitizes mammalian cells. As evidenced by the induction of radiosensitization with a SER of 2.26 by 40 \mu M Hoechst, we showed that Hoechst 33342 is as potent a radiosensitizer as camptothecin derivatives (Fig. 6). The known impaired membrane permeability of the positively charged Hoechst 33342 may likely contribute to its being relatively ineffective compared to camptothecin derivatives in radiosensitizing cells at lower drug concentrations (Fig. 6). This finding suggests a general application of topoisomerase I-targeting drugs in radiosensitizing mammalian cells.

As shown in Figs. 3 and 5, a complete obliteration of the "shoulder" of the two-component SHMT survival curves was observed when cells were exposed to camptothecin derivatives prior to or concurrently with ionizing radiation. Mammalian cell survival curves are the net result of single-hit killing, which yields an initial direct exponential decline in cell survival rate, and multihit injury, which yields a curve that bends with increase in dose (2). Camptothecin derivatives interfere with the breakage/rejoining reaction of topoisomerase I by stabilizing a reversible topoisomerase I-drug-DNA ternary complex, A. Y. Chen, C. Yu, and L. F. Liu, unpublished results.

\footnotesize{\textsuperscript{3} A. Y. Chen, C. Yu, and L. F. Liu, unpublished results.}

\textbf{Fig. 6.} Induction of radiosensitization by DNA topoisomerase I-targeting Hoechst 33342. Clonogenic survival assays of exponentially growing DC3F cells were conducted as in Fig. 4. Cells were exposed to radiation (XRT) alone (Δ), concurrent radiation and 0.5 h of 4 \mu M Hoechst 33342 (□), or concurrent radiation and 0.5 h of 40 \mu M Hoechst 33342 (○). Points, means of triplicates; bars, SD.

\textbf{Fig. 7.} Effect of cell cycle distribution on the radiosensitization by camptothecin (Camp). A, plateau-phase cell experiment. Plateau-phase DC3F cells were prepared as described in "Materials and Methods." Clonogenic survival assays of the day 7 plateau-phase cells treated with radiation (XRT) alone (Δ), or concurrent radiation and 2 h of 4 \mu M camptothecin (○) were performed as in Fig. 4. B, mitotic shake-off cell experiment. Mitotic shake-off DC3F cells were prepared as described in "Materials and Methods." After a 4-h incubation in 37°C following mitotic shake-off, 70–75% of cells were in G1 phase. Clonogenic survival assays of cells treated with radiation alone (Δ), or concurrent radiation and 0.5 h of 4 \mu M camptothecin (■) were performed. Points, means of triplicates; bars, SD.
tered the cleavable complex. The formation of the cleavable complex specifically prevents the rejoicing step of the breakage/rejoining cycle of the topoisomerization reaction (9, 10). The drug-stabilized cleavable complex, with a concealed single-strand DNA break, may potentially be a "sublethal" DNA damage. Interaction with cellular processes such as DNA replication, RNA transcription, and DNA repair may transform such "potentially sublethal" DNA damages into "sublethal" DNA damages (45). It is plausible that such "sublethal" DNA damages could then be converted into "lethal" DNA damages with the addition of radiation-induced DNA damages. Accordingly, the obliteration of the "shoulder" of survival curves by camptothecin derivatives in our experiments may be explained.

Cell cycle distribution is also known to be a major factor involved in the radiosensitization induced by various chemotherapeutic agents (3, 4). Camptothecin derivatives have been suggested to exert an augmented radiosensitization in cells that were growth inhibited (G0/G1 cells) to maximize repair of potentially lethal damage (33). We found that camptothecin only minimally enhanced the cytotoxic effect of radiation in plateau-phase cells arrested by growing to confluency, as well as in synchronized G1-phase cells obtained by mitotic shake-off technique. This finding may indicate a possible therapeutic advantage of camptothecin derivatives by being able to selectively radiosensitize actively proliferating cancer cells. Additional investigations using different cell synchronization methods to help characterize the induction of radiosensitization in various subphases in the cell cycle are needed.

In summary, we have demonstrated in cultured mammalian cells that camptothecin derivatives enhance radiation cytotoxicity in a schedule-dependent manner. Drug incubation has to be prior to or concurrent with, but not after, ionizing irradiation. The induction of radiosensitization requires a drug-sensitive DNA topoisomerase I that can interact stereospecifically with 20-(S), but not 20-(R), camptothecin derivatives. Hoechst 33342, a different class of DNA topoisomerase I-targeting drug, also induces radiosensitization in cultured mammalian cells with comparable SERs. On the basis of our preliminary studies with two cell cycle synchronization techniques, only minimal radiosensitization was induced by camptothecin in G1 phase cells. More studies are needed to further address questions regarding the existence of cell cycle phase specificity, the potential existence of selective radiosensitization in cancer cells, the schedule-dependent phenomenon in other experimental systems including animal models, the radiosensitization under hypoxic condition, as well as the molecular mechanism of radiosensitization induced by DNA topoisomerase I drugs. We believe that a full understanding of the mechanism underlying the radiosensitizing effect of topoisomerase I drugs not only is pivotal to the imminent clinical application of such combination, but also may provide important clues that may aide in the future development of more effective radiosensitizers. With the demonstrated excellent SERs, our data suggest a potential development of this specific group of radiosensitizers, the DNA topoisomerase I drugs, in treating human malignancies.

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REFERENCES


Table 4 Cell densitya and percentage of G1-phase cellsa during selection of plateau-phase DC3F cells

<table>
<thead>
<tr>
<th>Day</th>
<th>Cell density (cells/mm²)</th>
<th>Percentage of G1 phase cells</th>
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<tr>
<td>1</td>
<td>2.5 x 10⁶</td>
<td>35</td>
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<td>2</td>
<td>1.1 x 10⁶</td>
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<td>4</td>
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<td>6</td>
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<td>55</td>
</tr>
<tr>
<td>7</td>
<td>6.9 x 10⁶</td>
<td>52</td>
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a Cell density = (Number of cells in a 100-mm Petri dish/Surface area of a 100-mm Petri dish in mm² (78.5 mm²).
b Percentage of G1-phase cells denotes an estimation of the percentage of area under the curve of the G1 population based on the flow cytometry DNA histogram.

4 A. Y. Chen and L. F. Liu, unpublished results.


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