Heterozygous Disruption of the DNA Topoisomerase I Gene Confers Cellular Resistance to Camptothecin in Human Cells

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DNA topoisomerase I (Top1) is a ubiquitous nuclear enzyme that plays essential roles in various cellular processes, such as transcription or replication. Agents that target Top1, involving camptothecin and its derivatives, are among the most effective anticancer drugs used in the clinic. Previous work has suggested that the level of Top1 expression correlates with the cytotoxicity of camptothecin, but no direct evidence has been provided thus far in the context of human cells with a strictly isogenic genetic background. In this study, we perform heterozygous disruption of the Top1 gene (*TOP1*) by gene targeting in a human pre-B cell line, Nalm-6, which is karyotypically stable and normal for p53 status. We show that the heterozygous loss of the *TOP1* gene does confer cellular resistance to camptothecin, to an extent comparable to that observed in the absence of functional p53 protein. Such a tolerance was not observed with other agents that target DNA topoisomerase II. Our results provide direct evidence that human cells with decreased Top1 levels are significantly more resistant to killing by camptothecin than are otherwise isogenic cells.

Key words DNA topoisomerase I; camptothecin; p53; gene targeting; Nalm-6

DNA topoisomerase I (Top1) is a ubiquitous and abundant nuclear enzyme that alters the topological structure of DNA and chromosomes through a transient DNA single-strand break (nick) and subsequent religation of the nick.^{1–4)} The enzyme has been implicated in many aspects of DNA metabolism, including DNA replication and transcription.^{3,4)} Top1 has been of considerable interest to human medicine, because it is an important target for cancer chemotherapy.⁵⁾ Top1-targeting agents, as exemplified by camptothecin and its derivatives, are widely used as anticancer drugs in the clinic.^{6–8)} These agents are referred to as “Top1 poisons,” since they convert the essential enzyme into a highly cytotoxic DNA-damaging agent through the formation of “cleavage complex” (also called “cleavable complex”), in which Top1 protein is covalently linked to the 3′-end at the nick.¹⁾ Increasing evidence suggests that Top1 cleavage complexes can be converted into irreversible DNA lesions in a replication-dependent or -independent fashion.^{8–13)} The replication-dependent cytotoxic DNA lesions, which arise from collisions between replication forks and Top1 cleavage complexes, involve DNA double-strand breaks and Top1 covalent complexes.^{9–11,13,14)}

In addition to Top1, cells express DNA topoisomerase II (Top2), which is also a nuclear enzyme involved in many aspects of DNA metabolism and is an important target for cancer chemotherapy.⁵⁾ Indeed, Top2-targeting agents, involving etoposide, doxorubicin and mitoxantrone, are among the most effective and widely used anticancer drugs in cancer chemotherapy.^{15,16)} These agents are referred to as “Top2 poisons,” as they induce chromosomal double-strand breaks by trapping Top2 cleavage complexes.^{17,18)} Mammalian cells possess two genetically distinct Top2 isoforms, Top2 α and Top2 β ,^{1,19)} which are both the target of these Top2-targeting agents. Very recently, we have characterized a novel synthetic benzo[c]phenanthridine alkaloid, NK314, that exhibits strong antitumor activity and found that this agent acts as an α -isoform-specific Top2 poison in living mammalian cells.²⁰⁾

Previous work has suggested that the level of Top1 expres-

sion correlates with the cytotoxicity of camptothecin.^{6,21,22)} In fact, in mouse embryonic stem cells, increased camptothecin resistance was successfully employed to select for correct gene disruption at the *Top1* locus.²¹⁾ In the context of human somatic cell lines, however, there has been no such evidence because of the lack of a series of mutant cell lines in a strictly isogenic genetic background. In this study, we, for the first time, generate human cells with a genetically engineered *TOP1* allele. Specifically, we generate human *TOP1* +/– cells by gene targeting to perform phenotypic analysis of the mutant. Our results have significant implications for our understanding of camptothecin tolerance as well as for the future cancer treatment using Top1-targeting drugs.

MATERIALS AND METHODS

Cell Culture The human pre-B cell line Nalm-6 and its derivatives were cultured in ES medium (Nissui Seiyaku Co., Tokyo, Japan) supplemented with 10% calf serum (Hyclone, Logan, UT, U.S.A.) and 50 μ M 2-mercaptoethanol at 37 °C in a humidified atmosphere containing 5% CO₂. *TP53* –/– Nalm-6 cells have previously been described.²³⁾

Topoisomerase Inhibitors Camptothecin was purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.). Etoposide and NK314 were synthesized at Nippon Kayaku (Tokyo, Japan). Camptothecin and etoposide were dissolved in dimethyl sulfoxide and NK314 in distilled water. All the drugs were stored frozen in aliquots at –20 °C.

Southern and Western Blot Analysis Southern blot analysis was performed as described previously.²⁴⁾ Briefly, 10 μ g of genomic DNA were digested with *Bgl*II, electrophoresed in a 0.8% agarose gel, and transferred to a Hybond-N⁺ membrane (GE Healthcare Bio-Sciences, Piscataway, NJ, U.S.A.), followed by Southern hybridization with a ³²P-labeled probe. Western blot analysis was carried out as described previously.²⁵⁾ Anti-Top2 α , -Top2 β , and -Ku70 antibodies were purchased from BD Transduction Laboratories (Bedford, MA, U.S.A.). Anti-Top1 antibody was purchased

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from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.) and anti-actin antibody from Sigma-Aldrich. Levels of expression were quantified using a Fuji image analyzer LAS-1000UVmini and a MultiGauge software (Fuji Film Co., Tokyo, Japan).

Gene Targeting in Human Nalm-6 Cells The targeting vector pTOP1-Hyg was constructed by using a simplified construction method based on the MultiSite Gateway® Technology (Invitrogen, Carlsbad, CA, U.S.A.) as described.^{26,27} For this purpose, human *TOP1* genomic fragments were obtained by polymerase chain reaction (PCR) amplification with ExTaq DNA polymerase (Takara Bio Inc., Otsu, Japan) from Nalm-6 genomic DNA using primers Top1-5armF (5'-GCCAAGGGAAGCCATTTTCGATG-3') and Top1-5armR (5'-GCAGCTGGGCAGAGTACCTCACAC-3') for the 5'-arm, and Top1-3armF (5'-CCACCATATGAGCCTCTTCCAGAG-3') and Top1-3armR (5'-CTTCCTCTGCTTTGGGGCTCAGC-3') for the 3'-arm (note that all the primers contain an *attB* sequence at their 5'-end, though not indicated). The targeting vector was linearized with the I-SceI restriction enzyme, and transfected into wild-type Nalm-6 cells, as previously described.^{23,24,26–28} Briefly, 4×10^6 cells were electroporated with 4 μ g of the linearized vector, cultured for 22–24 h, and replated at a density of $0.5–1 \times 10^6$ per 90-mm dish into agarose medium containing 0.4 mg/ml hygromycin B (Wako Pure Chemical, Osaka, Japan). After a 2–3 week incubation at 37°C, hygromycin-resistant colonies were isolated, and genomic DNA was prepared from each clone and subjected to Southern blot analysis.

Sensitivity Assays Clonogenic survival assays using Nalm-6 and its derivative cell lines were performed as described previously.²⁴ Briefly, exponentially growing cells were plated at $10^2–10^5$ cells/dish into 60-mm dishes containing 5 ml of agarose medium with various concentrations of topoisomerase inhibitors. After a 2–3 week incubation at 37°C, visible colonies were counted, and the percent survival was determined by comparing the number of surviving colonies to untreated controls. Statistical significance was determined using the paired Student's *t*-test.

RESULTS AND DISCUSSION

Generation of *TOP1* +/− Human Cells We have recently shown that the human pre-B cell line Nalm-6 is highly proficient for gene targeting by homologous recombination, which, in general, barely occurs in cultured mammalian cells.²³ To generate *TOP1* +/− Nalm-6 cells, we transfected linearized targeting vector pTOP1-Hyg (Fig. 1A) into wild-type cells. Targeted disruption of one allele of the *TOP1* gene (*i.e.*, heterozygous gene disruption) was confirmed by Southern and Western blot analysis (Figs. 1B,C). Of note, Top1 levels in the *TOP1* +/− cells were decreased to *ca.* 70% that of wild-type cells. The heterozygous gene disruption did not affect expression levels of Top2 proteins (Top2 α and Top2 β).

Growth Properties of *TOP1* +/− Cells We compared the growth rate of *TOP1* +/− cells with that of wild-type cells and found no significant difference in growth rate between the two cell lines (Fig. 2A; the doubling-times of wild-type and *TOP1* +/− cells were both *ca.* 20 h). Additionally, there was little or no difference in plating efficiency between wild-type and *TOP1* +/− cells (Fig. 2B). These results show

Fig. 1. Targeted Disruption of the Human *TOP1* Gene

(A) Scheme for *TOP1* gene targeting. The targeting vector was designed to replace exon 9 with a hygromycin-resistance (*Hyg^r*) gene. The figure is not drawn to scale. Triangles represent *loxP* sequences. DT-A, a gene encoding diphtheria toxin A fragment. (B) Southern blot analysis of BglII-digested genomic DNA from wild-type (+/+) and *TOP1* heterozygous (+/−) cells using the probe shown in (A). (C) Western blot analysis for Top1, Top2 α , Top2 β and Ku70 of wild-type (+/+) and *TOP1* heterozygous (+/−) cells. Whole cell extract from 1×10^5 cells was loaded on a 7.5% SDS-polyacrylamide gel. Levels of expression were quantified using an image analyzer. Ku70 served as a loading control.

Fig. 2. Growth Properties of *TOP1* +/− Nalm-6 Cells

(A) Growth curves of wild-type and *TOP1* heterozygous cells. Data are the mean $\pm$ S.D. of three independent experiments. All error bars fall within the symbols. (B) Plating efficiencies of wild-type (+/+) and *TOP1* heterozygous (+/−) cells. Data are the mean $\pm$ S.D. of eleven independent experiments.

that heterozygous disruption of *TOP1* in Nalm-6 causes little or no growth defects under ordinary culture conditions.

Increased Camptothecin Resistance in *TOP1* +/− Cells

We next performed clonogenic survival assays in the presence of camptothecin to examine the sensitivity of *TOP1* +/− cells to this drug. As shown in Fig. 3A, the mutant cells showed increased resistance to camptothecin relative to wild-type cells, and the differences were statistically significant (*e.g.*, $p < 0.005$ at 4 nM). Additionally, concentrations that achieve a 90% inhibition in survival were statistically different (4.1 ± 0.2 nM for wild-type vs. 5.2 ± 0.2 nM for *TOP1* +/−, $p < 0.05$). By contrast, the mutant cells did not show increased resistance to the Top2 poisons etoposide and NK314 (Figs. 3B,C). These observations clearly show that decreased levels of Top1 expression result in cellular resistance to camptothecin. The data also confirm that NK314 does not target Top1 in living cells.²⁸

Recently, Burgess *et al.* reported that murine cells with decreased Top1 expression showed increased sensitivity to Top2 poisons.²⁹ In addition, Miao *et al.* used siRNAs to reduce Top1 expression *ca.* 5-fold in human cell lines, and showed that those Top1 knockdown cells were *ca.* 2-fold more sensitive to Top2 poisons.³⁰ In the present study, we did not observe an increased sensitivity to etoposide in *TOP1* +/− cells. It is possible that since our mutant cell line retained as high as *ca.* 70% that of wild-type Top1 levels, func-

Fig. 3. Sensitivity of Human *TOP1* $+/-$ Cells to Camptothecin, Etoposide, and NK314

Wild-type and *TOP1* heterozygous cells were treated with the indicated concentrations of camptothecin (A), etoposide (B), and NK314 (C) and allowed for colony formation, as described in Materials and Methods. Data are the mean $\pm$ S.D. of three to four independent experiments (* $p < 0.05$, ** $p < 0.005$). Where absent, error bars fall within symbols.

Fig. 4. Sensitivity of Human *TP53* $-/-$ Cells to Camptothecin, Etoposide, and NK314

Wild-type and *TP53*-knockout cells were treated with the indicated concentrations of camptothecin (A), etoposide (B), and NK314 (C) and allowed for colony formation, as described in Materials and Methods. Data are the mean $\pm$ S.D. of three to four independent experiments (* $p < 0.05$). Where absent, error bars fall within symbols.

tional compensation by Top2 was not prominent enough to confer increased sensitivity to etoposide. We note, however, that our *TOP1* $+/-$ cells were slightly but reproducibly more sensitive to NK314 than wild-type cells (e.g., $p < 0.05$ at 125 nM). In this regard, it is intriguing that the study by Miao *et al.* suggested that Top2 α (and not Top2 β) could compensate for the reduced Top1 function.³⁰⁾ Thus, reduced Top1 levels may lead to increased sensitivity to those agents that target Top2 α , not Top2 β .

Increased Camptothecin Resistance in *TP53* $-/-$ Cells

The increased camptothecin resistance we observed in the *TOP1* $+/-$ mutant prompted us to employ *TP53* knockout human cells to evaluate the significance of this tolerance, as *TP53* is the most frequently mutated gene in human cancers and is reported to impact on the cellular sensitivity to various genotoxic anticancer agents.^{31,32)} We previously generated and characterized *TP53* $-/-$ Nalm-6 cells, which in fact exhibited increased resistance to ionizing radiation, with a concomitant decrease in the number of dying cells.³³⁾ We thus examined the sensitivity of *TP53* $-/-$ cells to camptothecin. As shown in Fig. 4A, the mutant cells showed significantly increased resistance to camptothecin relative to wild-type cells ($p < 0.05$ at 4 nM). It should be noted that the level of the tolerance was similar or comparable to that observed in the *TOP1* $+/-$ mutant (see Fig. 3A). Consistent with our previous report,³³⁾ *TP53* $-/-$ cells did not show increased resistance to the Top2 poison etoposide, though this seemed not to be the case for NK314 (Figs. 4B, C).

Concluding Remarks and Future Perspectives In the present study, we have conducted genetic analyses using gene-knockout human cell lines to investigate cellular sensitivity to camptothecin. Indeed, this is the first report on the generation of a human mutant cell line with a genetically deleted *TOP1* allele. The heterozygous disruption of the human *TOP1* gene resulted in a significant decrease in Top1 levels and conferred cellular resistance to the Top1 poison

camptothecin. Our results confirm the notion that the level of Top1 expression correlates with camptothecin cytotoxicity, and do provide direct supporting evidence in the context of human somatic cells. Most importantly, the level of camptothecin resistance of *TOP1* $+/-$ cells was similar or comparable to that observed with a *TP53* $-/-$ mutant, implying that the level of Top1 expression is a more critical determinant of cellular sensitivity to Top1 poisons than is the p53 status. Our results also suggest that cells with decreased levels of Top1 expression in a p53-negative background would be even more resistant to camptothecin, though this may await further work with a double-knockout cell line lacking both Top1 and p53.

Intriguingly, we previously reported that loss of non-homologous end-joining (NHEJ), a major repair pathway for repair of DNA double-strand breaks, confers camptothecin resistance in vertebrate cells.³⁴⁾ Additionally, we have recently found that human cells lacking both p53 and DNA ligase IV (a critical NHEJ protein) are more resistant to camptothecin than either single mutant (our unpublished results), thus highlighting the notion that the repair of Top1-mediated DNA damage is an important determinant for the cellular responses to Top1 poisons and for the selectivity of these agents for cancer cells. Collectively, it is conceivable that multiple factors (e.g., Top1, p53, and NHEJ proteins) impact on cellular sensitivity to camptothecin. It will thus be interesting to examine whether one or more of these factors may be compromised in certain cancer cells that have acquired resistance to Top1 poisons. Finally, it should be emphasized that since the human cell mutants described herein have been created by targeted gene disruption, the isogenicity between the cell lines is otherwise completely retained. Hence, these human cell mutants will be valuable for studying the contribution of topoisomerases to the cytotoxicity of potential anti-cancer agents.

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