Original Papers

Circadian Rhythm of Anti-Cancer Drug Target Topoisomerase I As a Proposal Model for Chronotherapy In Vitro

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Abstract

Many behavioral processes are under circadian regulation in almost all organisms from bacteria to mammals. Although substantial evidences support the idea that circadian clock could modulate the efficacy of anticancer therapy, the mechanism(s) has not been clear. Here we demonstrated that expression of topoisomerase I (top1) mRNA and its promoter activity underwent circadian oscillation after serum-rich medium treatment in HCT 116 cells. Moreover, the formation of cleavage complex composed of top1 and top1 poison, camptothecin (CPT), detected by nicking assay was also oscillated. Colony formation assay indicated that cytotoxicity of 8h-CPT treatment was significantly higher at 4-h after serum-shock than at 20-h ($p = 0.0289$). In addition, dexamethasone treatment also induced circadian rhythm of top1 in HCT 116 cells. These results raise the possibility that the circadian rhythm of top1, which would be induced by some types of drugs such as dexamethasone, could be considered for chemotherapy in clinic.

Key Words: topoisomerase I (top1), topoisomerase II (top2), camptothecin (CPT)

(Received May 10, 2010; Accepted July 4, 2010)

Introduction

Many physiological, biochemical and behavioral processes are under circadian regulation, which is generated by an internal time-keeping mechanism referred to as biological clock in almost all organisms from bacteria to mammals^{1,2}. Circadian rhythm is controlled by genetically determined as networks of transcription-translation feedback loops involving sets of clock genes³⁻⁵.

The substantial evidences that circadian clock could modulate the morbidity and efficacy of anticancer therapy have been proposed⁶⁻⁹. The observations that the medication schedule adjusted to circadian rhythm prove to be increased tolerance of leukemic mice to arabinosyl cytosine in 1972⁶. Levi described more than 30 different anticancer drugs with different pharmacological actions exhibit strong circadian rhythm dependent effects on drug-induced toxicity in mice and rats⁹. These findings led to the idea of chronotherapy to optimize the efficacy of anticancer drug on patients with some types of malignancy by the modification of delivery of drugs at appropriate

times of the day. Although the chronotherapeutic approach seems to be a nice idea and also has demonstrated encouraging results⁹, it has not been popular in clinic, probably the precise mechanism of the therapy based on circadian rhythm is not still clear.

Recently, Gorbacheva et al¹⁰ demonstrated that wild-type and circadian mutant mice demonstrate striking differences in their response to the anticancer drug cyclophosphamide (CY). While the sensitivity of wild-type mice varies greatly, depending on the time of drug administration, *Clock* mutant and *Bmal1* knockout mice are highly sensitive to treatment at all times tested. On the contrary, mice with loss-of-function mutations in *Cryptochrome* (*Cry1^{-/-}Cry2^{-/-}* double knockouts) were more resistant to CY compared with their wild-type littermates. Thus, both time-of-day and allelic-dependent variations in response to chemotherapy correlate with the functional status of the circadian CLOCK/BMAL1 transactivation complex. However, the reports describing mechanism(s) of relationship between anticancer drug effect(s) and circadian rhythm are very few. In addition, the precise analysis of the molecular target for anticancer drug in circadian rhythm has not been performed yet.

DNA topoisomerases have been recognized as key targets for some of the most active anticancer drugs presently used in the clinic. In general, the drugs con-

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vert the enzymes into a cellular poison by stabilizing the topoisomerase cleavage complexes, which are the catalytic intermediate for topoisomerization reactions¹¹. Two of the cellular DNA topoisomerases are targeted by anticancer drugs. In the case of topoisomerase II (top2), the cleavage complexes consist of DNA double-strand breaks linked at each of the 5'-DNA termini to a top2 molecule^{12,13}, whereas in the case of top1, the cleavage complexes are DNA single-strand breaks with the enzyme linked to the 3'-DNA termini¹⁴⁻²². For the list of top1 inhibitors, the only class of inhibitors approved for clinical use are the CPT derivatives^{20, 23-28}.

The aim of the present study was to elucidate the relationship(s) between top1, one of the important targets for chemotherapy, and circadian rhythm. Here we demonstrated that expression of topoisomerase I (top1) mRNA and promoter activity belonged to circadian rhythm after serum-rich medium treatment in HCT 116 cells. We also found that the formation of cleavage complex composed with top1 and top1 poison, camptothecin (CPT) detected by nicking assay and cytotoxicity of camptothecin was also dependent on circadian rhythm of top1 in HCT116 cells.

Materials and Methods

Cells and Cell Culture, Chemicals and Enzymes.

Human colon carcinoma HCT 116 cells were cultured in RPMI medium 1640 (Life Technologies, Gaithersburg, MD) containing 5% heat-inactivated fetal calf serum and 2 mM glutamine in a 5% CO₂ incubator at 37°C. No antibiotic was added to the medium. The cells were trypsinized and passaged once a week. Camptothecin (CPT) was purchased from Sigma (St. Louis, MO). Stock solutions of drugs were prepared in dimethyl sulfoxide at a concentration of 10 mM. Dexamethasone was also purchased from Sigma (St. Louis, MO). Stock solutions of drugs were prepared in dimethyl sulfoxide at a concentration of 1 mM. Further dilutions were made in distilled water immediately before use.

Production of Circadian Rhythm by Treatment with High Concentration of Serum. The treatment of cultured HCT 116 cells with 50% concentrations of fetal bovine serum (FBS) for 2-h and the replacement of medium without serum was performed to produce circadian rhythm in these cells by the method as previously described²⁹.

Production of Circadian Rhythm by Treatment with Dexamethasone. The treatment of cultured HCT 116 cells with 100nM concentrations of dexamethasone for 2 h and the replacement of medium with 5% concentrations of fetal bovine serum (FBS) was performed to produce circadian rhythm in these cells by the method as

previously described³⁰.

RT-PCR. Total RNA was prepared by Trizol (Life Technologies, Inc., Gaithersburg, MD) from HCT116 cells. cDNA was synthesized with 2 µg of total RNA and random hexadeoxynucleotide primer (Life Technologies, Inc., Gaithersburg, MD) in 20 µl of a solution containing reverse transcriptase. After synthesis of cDNA, it was diluted 1:4 with water and stored at -20°C until use. Each PCR was performed with cDNA derived from 20 ng of RNA. PCR reactions were carried out in a total volume of 20 µl containing cDNA, dGTP, dATP, dTTP, and dCTP at a concentration of 200 µM, 4 µM of each primer, and 0.25 unit of ExTaq polymerase (Takara Shuzo, Otsu, Shiga, Japan). The PCR primer sequences were as follows; *Per1* sense primer 5'-GCC AGT GAC TTT CCA ACA-3' and antisense primer 5'-GAG GCA CAT TTA CGC TTA GTG-3' corresponding to 333 bp (residues 1626 to 1958). *Per2* sense primer 5'-GAT GCC AAG GTT TGT GGA GTT CCT G-3' and antisense primer 5'-GCT CTG TGA GCT CCT GAA TGC TG-3' corresponding to 724 bp (residues 670 to 1393). *Per3* sense primer 5'-GAC AGC AGC CAC AGT GAA-3' and antisense primer 5'-ACA TGA ATC AGA TAG GGG GAT G-3' corresponding to 596 bp (residues 115 to 710). *Cry1* sense primer 5'-ATC CGC TGC GTC TAC AT-3' and antisense primer 5'-ATG ACT TCT ACT CCA GCT TCA G-3' corresponding to 283 bp (residues 677 to 960). *Cry2* sense primer 5'-GTG TTC CCA AGG CTG GTT CTC C-3' and antisense primer 5'-GTA GGT CTC GTC GTG GTT CTC C-3' corresponding to 324 bp (residues 317 to 640). *Clock* sense primer 5'-AGG GTA TTT GCC ATT TGA-3' and antisense primer 5'-GCC AAG TTC TCG TCG TC-3' corresponding to 282 bp (residues 890 to 1171). *Bmal1* sense primer 5'-AGA CAA TGA GGG GTG TAA CC-3' and antisense primer 5'-AAA CTG AAC CAT CGA CTC CG-3' corresponding to 338 bp (residues 908 to 1245). *CKIε* sense primer 5'-ATG AAC GGA TCA GCG AGA AGA-3' and antisense primer 5'-GAA GAG GTT GCG GAA GAG-3' corresponding to 158 bp (residues 785 to 942). *Top1* sense primer 5'-AGA CCT CGA GAT GAG GAT GA-3' and antisense primer 5'-AAA TAC TGG CTC ATC TGG G-3' corresponding to 515 bp (residues 412 to 926); *G3PDH*, sense primer 5'-CCC CTG GCC AAG GTC ATC CAT GAC AAC TTT-3' and antisense primer 5'-GGC CAT GAG GTC CAC CAC CCT GTT GCT GTA-3' corresponding to 513 bp. The PCR products were electrophoresed on 2%-agarose gel. The PCR cycling parameters were as follows: 10 min at 94°C followed by 35 cycles of 30 s at 94°C, 30 s at 57°C, and 1 min at 72°C, and a final cycle at 72°C for 10 min. The PCR products were electrophoresed on 2%-agarose gel.

Quantitative Real-Time PCR. For quantification, cDNA

was used as template in real-time PCR assay (Bio-Rad, Hercules, CA), according to the manufacturer's protocol. The housekeeping *G3PDH* gene served as the reference and was amplified parallel to *top1* gene for every sample. The oligonucleotide primers for the detection of *top1* and *G3PDH* genes were the same as those described above. The constituents of each PCR (15 μ l) were 1 μ l of cDNA (or distilled H₂O), 0.5 μ l of primer 15 pmol/liter each, 6.0 μ l of distilled H₂O, and 7.5 μ l of TaqMan Universal PCR Mastermix (Perkin Elmer, Waltham, MA).

Cloning of promoter region of human *top1* gene. The human *top1* promoter region (-875 to +153 from the transcription initiation site)³¹⁻³³ was amplified by subjecting human genomic DNA to the PCR using specific primer. The amplified human *top1* promoter region was then cloned into the XhoI and BglII restriction site of pGL3basic vector (Promega, San Luis Obispo, CA). The primer sequence used for the cloning of promoter region of *top1* gene was: sense primer 5'-TTT CTC GAG TGT AAT ACA GGG CC-3' and antisense primer 5'-TTT AGA TCT AGG TAA GCG GCG GTG ACT GTG C-3'. The PCR cycling parameters using LA Taq polymerase (Takara Shuzo, Otsu, Shiga, Japan) were: 10 min at 94°C followed by 35 cycles of 30 s at 94°C, 30 s at 57°C, and 1 min at 72°C, and a final cycle at 72°C for 10 min. And then, for visualization of promoter activity assay, the promoter region of *top1* including multi-cloning site of the pGL3basic vector were cloned into the SacI and HindIII restriction site of pTurboGFP-PRL-dest1 vector (Evrogen Joint Stock Company, Moscow, Russia).

Cloning of promoter region of human *Per1*. Human *Per1* promoter region (-1376 to +39 from the transcription initiation site) was amplified by subjecting human genomic DNA to the PCR using specific primer. The amplified human *per1* promoter region was then cloned into the BglII and HindIII restriction site of pGL3basic vector (Promega, San Luis Obispo, CA). The primer sequences used for cloning of the promoter region of *Per1* gene were: sense primer 5'-TTT AGA TCT GAA TCC GGT GCA CTG TCA CT-3' and antisense primer 5'-TTT AAG CTT TCC TCA CAG GAG GCG TGC GG-3'. The PCR cycling parameters using LA Taq polymerase (Takara Shuzo, Otsu, Shiga, Japan) were: 10 min at 94°C followed by 35 cycles of 30 s at 94°C, 30 s at 57°C, and 1 min at 72°C, and a final cycle at 72°C for 10 min.

Cloning of cDNA of *Clock* and *Bmal1*. cDNA of human *Clock* and *Bmal1* was amplified by subjecting human placenta cDNA to PCR using specific primer. The amplified human *Clock* and *Bmal1* cDNA was then cloned into the EcoI and NotI restriction site of pcDNA3.1(+) vector (Invitrogen, Carisbad, CA). The primer sequence used for the cloning of the cDNA were

as follows; *Clock* sense primer 5'-TTT CTC GAG CTA CTG TGG TTG AAC CTT GG-3' and antisense primer 5'-TTT AAG CTT TCC TCA CAG GAG GCG TGC GG-3'. *Bmal1* sense primer 5'-TTT GAA TTC ATG GCA GAC CAG AGA ATG G-3' and antisense primer 5'-TTT CTC GAG TTA CAG CGG CCA TGG CAA G-3'. The PCR cycling parameters using Pyrobest polymerase (Takara Shuzo, Otsu, Shiga, Japan) to amplify the cDNA of *Clock* were: 10 min at 94°C followed by 35 cycles of 30 s at 94°C, 30 s at 52°C, and 2 min at 72°C, and a final cycle at 72°C for 10 min. For amplification of cDNA of *Bmal1*, the PCR cycling parameters were the same as above, except using LA Taq polymerase (Takara Shuzo, Otsu, Shiga, Japan).

***Top1* and *Per1* Transcriptional Assays.** The method used was a modification of previously described one³⁴. Briefly, one day before transfection, HCT116 cells were seeded (2×10^5 /well) in 12-well plates containing RPMI1640 supplemented with 5% FBS. Cells were transiently transfected with 10 ng of each reporter construct in pGL3basic (Promega, San Luis Obispo, CA) and 1.0 mg (total) of expression construct in pcDNA3.1 (Invitrogen, Carisbad, CA), using LipofectAmine-Plus reagent (Invitrogen, Carisbad, CA) according to the manufacturers instructions. The total amount of DNA/well was adjusted by adding pcDNA3.1 vector (Invitrogen, Carisbad, CA). At 48 h after transfection, cell extracts were prepared with use of 100 μ l of passive lysis buffer (Promega, San Luis Obispo, CA), and 20 μ l of the extracts were applied to assays of firefly luciferase by GloMaxTM 96 microplate luminometer (Promega, San Luis Obispo, CA). Luciferase activity was normalized to protein concentration.

Visualization of Promoter Activity. Cells were transiently transfected with the *top1* reporter construct in pTurboGFP-PRL-dest1 vector (Evrogen Joint Stock Company, Moscow, Russia) by LipofectAmine-Plus reagent (Invitrogen, Carisbad, CA) according to the manufacturers instructions, and seeded into 35 mm glass dish. After 2-h incubation with the medium containing 50% of FBS, the GFP signal were detected by the fluorescence microscopy, IX81 (Olympus, Tokyo, Japan). The images were captured at every 30 min for 48 hrs. The intensity of was measured by the equipped software, Metamorph (Molecular Devices Corporation, Sunnyvale, CA).

Preparation of Nuclear Extracts. The method used is a modification as previously described^{35,36}. Briefly, log-phase cultures containing 5×10^9 cells were washed twice at 4°C by using nucleus buffer (150 mM NaCl/1 mM KH₂PO₄/5 mM MgCl₂/1 mM EGTA) and recovered by centrifugation at $200 \times g$ for 10 min. Cell pellets were resuspended in nucleus buffer containing 0.03% Triton

X-100. After incubation at 4°C for 10 min, nucleus pellets were recovered by centrifugation at 350 × g for 10 min. After washing with ice-cold nucleus buffer, pellets were recovered by centrifugation at 350 × g for 10 min. Salt extraction of the nuclear pellet was achieved by adjusting the final NaCl concentration to 0.35 M and gentle mixing at 4°C for 30 min. After centrifugation at 12,000 × g for 30 min, supernatants containing salt-soluble material were collected as nuclear extracts that were immediately used for DNA nicking assay.

DNA Nicking Assay. The method used is a modification as previously described³⁶. Briefly, each reaction (10 ml final volume) in 10 mM Tris-HCl, pH 7.5/50 mM KCl/5 mM MgCl₂/0.1 mM EDTA/1 mM ATP/15 mg/ml BSA contained 0.25 mg of supercoiled pHOT1-DNA (TopoGEN, Columbus, OH). The pHOT1-DNA was reacted with nuclear extract at the indicated time for 30 min at 25°C, and terminated by adding 0.5% SDS and 0.5 mg proteinase K (final concentrations) for 1 h at 50°C. After adding 1 ml of 10 × loading buffer (20% Ficol 400/0.1 M TrisHCL, pH 7.8/100 mM NaCl/1 mM Na₂EDTA, pH 8.0/1% SDS/0.25% bromophenol blue), samples were loaded onto 1% agarose gels in 1× TBE (90 mM Tris/64.6 mM boric acid/2.5 mM EDTA, pH 8.3) containing 0.05 mg/ml ethidium bromide. DNA was visualized by transillumination with ultraviolet light (300 nm). The quantification of the percentage of nicked DNA was performed by the equipped software; PDQuest (Bio-Rad, Hercules, CA).

Comet assay. The alkaline comet assay was performed. Briefly, drug-treated HCT116 cells were pelleted and resuspended in ice-cold PBS. A 50-μL sample of resuspended cells was then mixed with an equal volume of prewarmed 1% low-melting point agarose. The agarose-cell mixture was placed on a slide precoated with 0.5% agarose and spread gently with a coverslip. After 10 min at 4°C, the slides were immersed in precooled (4°C) lysis solution [2.5 mol/L NaCl, 100 mmol/L Na₂EDTA, 10 mmol/L Tris-HCl (pH 10), 10% DMSO, 1% Triton X-100, and 1% laurosylsarcosinate] for 80 min in a dark chamber. After soaking with electrophoresis buffer (0.3 mol/L NaOH and 1 mmol/L EDTA) for 20 min, the slides were subjected to electrophoresis for 15 min. Finally, the cells were stained with ethidium bromide, and individual cells were viewed using an Olympus BX51 UV fluorescence microscope. DNA damage was quantified in at least 50 randomly selected comets per slide by calculating the area being expressed by the percentage of DNA in the tail x the tail length divided by that in control sample.

Colony formation assay. The treatment of cultured HCT 116 cells with 50% concentrations of fetal bovine serum

(FBS) for 2 h and the replacement of medium without serum was performed to produce circadian rhythm in these cells. Then, cells were treated with CPT for 8 h at the indicated time depending on top1 activity regulated by circadian rhythm. After removal of the drug by washing twice with PBS, 500 of cells were seeded in triplicate in 60-mm culture dishes and cultured RPMI1640 medium containing 5% heat-inactivated fetal bovine serum. The colonies were fixed, stained with Toluidine Blue, and counted 10 days after seeding cells.

Results

Circadian Rhythm of Top1 mRNA (Fig.1). First, we examined whether expression of *top1* undergoes circadian oscillation in a colon carcinoma cell line, HCT116 cells, after the treatment of serum-rich medium. Analyses of mRNA levels by conventional PCR (Fig. 1A) and real time PCR (Fig. 1B) showed that the levels of *top1* mRNA markedly decreased at 8-12 h after the serum-shock and increased to reach a peak at 20 h, then decreased again during 32-44 h and slightly increased at 48 h. Other known circadian clock genes such as *Period1* (*Per1*), *Period2* (*Per2*), *Period3* (*Per3*), *Cryptochrome1* (*Cry1*), *Cryptochrome2* (*Cry2*), *Clock*, *Bmal1* and *Casein kinase Iepsilon* (*CKIε*) were appropriately oscillated (Fig. 1A and B). Moreover, the serum-induced oscillation of *top1* mRNA was also observed in other cell lines such as MCF7, SW 480 and KB3-1 cells (Fig.1C).

Circadian Rhythm of Top1 Promoter Activity by Transactivation of Clock and Bmal1 (Fig.2). The observation of *top1* mRNA circadian rhythm prompted us to follow whether promoter activity of *top1* was subject to circadian rhythm. To observe *top1* promoter activity in a single cell, turbo-GFP /*top1* reporter construct was transfected into HCT116 cells, which were observed with fluorescence microscopy after the serum-shock. As shown in Fig. 2A, the GFP signal showed clear-cut oscillation after the serum shock; it was decreased at 8 h and increased to become a peak during 20-28 h, and then decreased again, which was similar to the circadian profile of *top1* mRNA (Fig. 1). When signal intensity was measured in multiple cells, they showed similar pattern of circadian fluctuations (Fig. 2B). Next, to explore the involvement of transcription factors, Clock and/ or Bmal1, in the *top1* expression, transcriptional assay was performed using *per1* reporter construct as a positive control in HCT 116 cells. Although co-transfection of either or both of Clock and Bmal1 raised *per1*-Luc reporter activity, only co-transfection of both factors showed significant increase in *top1* promoter activity (2.4-fold, *p* < 0.05) when compared with those without transfection or with transfection of either Clock or Bmal1 (Fig. 2C). These data indicated that circadian rhythm of *top1*

expression was regulated by transcription factors, which would involve Clock and Bmal1 binding to E-box at promoter of *top1*.

Dependence of Cytotoxicity of Camptothecin, Top1 Poison, On Circadian Rhythm of Top1. To estimate the oscillation of functional *top1* protein levels, we performed a nicking assay using nuclear extracts from HCT116 cells, *top1* poison camptothecin (CPT), and pHOT1-DNA detected as a substrate. The ability of nuclear extract to form nicked DNA showed apparent os-

cillation; peak at 4 h and 28 h, and trough at 20 h and 44 h (Fig. 3A and B). The DNA single strand breaks (SSBs) by *top1* was further confirmed in a single cell by alkaline comet assay. HCT116 cells were treated with 10 nM or 30 nM of CPT at 4 h or 20 h after serum-rich treatment of HCT 116 cells. The alkaline comet assay revealed that the amount of SSBs at 4 h was significantly higher than those at 20 h in both 30 nM and 100 nM of CPT, respectively ($p < 0.01$) (Fig. C). These data indicated that *top1* protein levels also show serum-induced oscillation. Next, we examined whether cytotoxicity of CPT undergo

Fig. 1 **Circadian rhythm of *top1* mRNA.** (A & B) Expression of *top1*mRNA and clock genes were analyzed by conventional PCR (A) and real time PCR in HCT116 cells (B). (C) Circadian rhythm of *top1* mRNA detected by conventional PCR in MCF7, SW480 and KB 3-1 cells.

Fig. 2 **Circadian rhythm of *top1* promoter activity. Upregulation of *top1* by *Bmal1* and *Clock*.** (A) Timelapse microscopic analysis of circadian rhythm of *top1* promoter fluorescence in nucleus. (B) Circadian fluorescence profiles of 7 individual cell nuclei after treatment of serum-rich medium. (C) Transcriptional regulation of *top1*-Luc and *Per1*-Luc reporter activity after transfection of *Clock* and/ or *Bmal1*.

circadian oscillation in HCT 116 cells. The effects of 8h-treatment with CPT that started at 4 h or at 20 h after serum-shock on colony formation were assayed. We examined two time points because nuclear extracts at 4 h revealed the highest proportion of nicked DNA by CPT and those at 20 h showed the lowest proportion of nicked DNA (Fig. 3A & B). Dose-response curve at each condition revealed that IC₅₀ of 8h-CPT treatment at 4 h (8.61 ± 1.40) was significantly lower than that at 20 h (64.44 ± 0.085 , $p = 0.017$) after serum-shock (Fig. 3C). The data of colony formation assay at 10 nM of CPT were shown in Fig. 3D. The number of colony at 4 h-treatment by CPT was significantly lower than that at 20 h-treatment (E ; $p = 0.0289$). These data strongly suggested that cytotoxicity of CPT was dependent on *top1* circadian rhythm in HCT 116 cells.

Production of Top1 Circadian Rhythm by Dexamethasone. In clinical application serum-rich treatment for patients are very difficult. Therefore, we

next chose dexamethasone to create circadian rhythm, since dexamethasone can be medicated into human and has been reported to produce circadian rhythm in several types of cell lines³⁰). As expected, the levels of *top1* mRNA markedly increased at 8 h and the decrease at 28 h after treatment of 100 nM dexamethasone with the coordination of clock genes such as *Per1*, *Per2*, *Per3*, *Cry1*, *Cry2*, *Clock*, *Bmal1* and *CK1 ϵ* (Fig. 4).

Discussion

The present study reports circadian rhythm of *top1* as one of the important targets for anticancer drugs, has an influential effects on cytotoxicity of CPT, *top1* poison in HCT116 cells. The mechanism and importance of circadian rhythm of eukaryotic *top1* are based on the following observations: (i) Circadian rhythm of mRNA and promoter activity of *top1* (Fig. 1 and 2), (ii) Upregulation of *top1* by transfection of both *Clock* and *Bmal1* through their binding to E-box at promoter of *top1* (Fig. 2), (iii)

Fig. 3 Effect of circadian rhythm of *top1* on cytotoxicity of *top1* poison, camptothecin (CPT). (A) Circadian rhythm of DNA single strand breaks detected by nicking assay (upper panel). Percent nicked DNA was computed after FluorImager analysis (Molecular Dynamics) by using the imagequant software. Each point was plotted in a graph (lower panel). (B) Dependence of cytotoxicity of CPT on circadian time of *top1* detected by comet assay in HCT116 cells. Pictures of cells treated with 0, 30, and 100 nM of CPT were shown in left panel. The amount of DNA SSBs revealed by tail-moment at 4 h (white bar) and at 20 h (dark bar) after treatment of cells with serum-rich medium was shown in right panel. (C) Dependence of cytotoxicity of CPT on circadian time of *top1* in HCT116 cells. Dose-response curves were detected by colony formation assay (upper panel). Photographs of colonies stained with Toluidine Blue (right & lower panel). Cumulative data (mean $\pm$ s. d.) showing the significant ($p = 0.0289$) difference of proportion of survival cells treated at 4h (white bar) and at 20h (dark bar) after treatment of cells with serum-rich medium (left & lower panel).

Dependence of cytotoxicity of CPT on circadian rhythm of top1 nicking ability, which is also under the regulation of circadian rhythm (Fig. 3), (iv) Dexamethasone produces circadian rhythm as well as serum-rich medium treatment of HCT116 cells (Fig. 4).

In spite of the studies of the relationship between circadian rhythm and chemotherapy *in vivo* and in clinic^{8,9)}, the researches of circadian rhythm of anti-cancer drug targets *in vitro* are very rare. Development of new anti-cancer drugs has been intensively performed. Further, to improve the efficacy of anticancer drugs in patients with malignancy, huge oncologists are investigating treatment schedule and drug delivery system for anticancer drug. Aim of chronotherapy is one of the most active treatment schedule, based on the adjustment of drug delivery to the 24 h rhythm generated by circadian rhythm. More than 30 different anticancer drugs with different pharmacological actions may associated with circadian rhythm dependent effects on drug-induced toxicity in mice and rats⁹⁾. They also demonstrated the efficacy of the treatment of topotecan, doxorubicin and cisplatin was dependent on circadian rhythm in rats bearing a transplanted plasmacytoma³⁸⁾. Although the chronotherapeutic approach seems to be a nice idea and also has demonstrated encouraging results⁹⁾, it has not been popular in clinic. Thus, the present study identifies that cytotoxicity of CPT is dependent on circadian rhythm of top1 with circadian rhythm of nicking ability of top1. To best of our knowledge, these findings are the first evidences that a target molecule top1 against anti-cancer drug indicates circadian rhythm in human cell lines.

DNA topoisomerases have been recognized as key targets for some of the most active anticancer drugs

presently used in the clinic. Only one previous report describing the relationship between circadian rhythm, top1 and effect of top1 poison in mice is as follows³⁹⁾. The experiments using 773 male mice and irinotecan revealed that the efficacy of irinotecan was dependent on treatment time-related, resulting a four to eight-fold larger G2-M phase arrest following irinotecan administration at 19 or 23 HALO in comparison to the other times of drug administration, apparently representative of the repair of more extensive DNA damage. Our present study revealed not only the confirmation of circadian rhythm of *top1* in mice by the present evidences of circadian rhythm of top1 mRNA and promoter activity, but also demonstrated that the effects of top1 cytotoxicity was dependent on circadian rhythm of top1 nicking ability in human colon carcinoma HCT116 cells. The principal pharmacological action of top1 is that the cleavage complexes are DNA single-strand breaks with the enzyme linked to the 3–DNA termini by top1. For the list of top1 inhibitors, the only class of inhibitors approved for clinical use are the CPT derivatives^{20,23–28)}. Thus, the present data provide that the principal pharmacological mechanism of CPT mediated by top1 is regulated by circadian rhythm in human colon carcinoma HCT 116 cells.

Recent exhausted studies have defined the system of the central feedback loop regarding the time-keeping system at the molecular level using animal models, genetic analysis and molecular approaches^{3–5)}. Among them, two bHLH-PAS-containing transcription factors, CLOCK and BMAL1, form heterodimers that bind to E-box enhancer elements in the promoters of target genes driving the transcription of three period genes³¹⁾ and two cryptochrome genes (Cry1 and Cry2) are very important^{40–43)}.

Fig. 4 Induction of circadian rhythm by dexamethasone. HCT 116 cells were grown to subconfluence in a medium containing 5% fetal bovine serum for 6 days. At time 0, cells were shifted to a medium containing 100 nM of dexamethasone. Then, the serum-rich medium was replaced 2 h after its addition with serum-free medium. The cells were harvested at every 4 h for 48 h. Conventional PCR (Fig. 4A) and Quantitative RT-PCR (Fig. 4B).

Because, these two molecules are indispensable for keeping feedback loop of circadian rhythm⁵⁾. After the PER and CRY proteins have been translated in the cytoplasm, they form heterocomplexes that translocate into the nucleus and inhibit their own transcription⁴⁾. CRY plays a crucial role in this negative feedback process by interacting directly with the CLOCK/BMAL1 heterodimers⁴³⁾. Eukaryotic top1 contains E-box at the promoter region of top1 (-319 to -314 from the transcription initiation site)³⁴⁾. The present study also revealed that both Bmal1 and Clock could be required for upregulation of top1 in HCT116 cells. Taken together with the previous findings and our data, E-box at promoter region of top1 may be essential for circadian rhythm of top1.

In clinic, Levi, his colleagues and the International Organization for Cancer Chronotherapy has contributed to the chemotherapy based on circadian rhythm⁹⁾. This type of chemotherapy contributed to the improvement of the prognosis of patients with some types of malignancies. For example, it reportedly improved the prognosis of patients with metastatic colorectal carcinoma⁴⁴⁾. The chemotherapy based on circadian rhythm seems to be nice, however the profile of circadian rhythm of each cell in one human carcinoma is not still clear. Our present observations of top1 circadian rhythm *in vitro* raised the further questions that one of the major problems for its clinical use is how and when anticancer drugs could be adjusted on circadian time of their target molecules in clinic. Dexamethasone as well as serum-rich medium treatment of cells can produce circadian rhythm in some types of cell lines³⁰⁾. Therefore, we tested whether dexamethasone could produce circadian rhythm of top1, resulting in circadian rhythm of top1 by 100 nM of dexa-

methasone in HCT116 cells. This result raised the future possibility that the treatment of dexamethasone before medication of top1 poison could enhance the efficacy of chemotherapy in clinic, for obtaining better efficacy of chemotherapy.

In conclusion, we demonstrated that circadian rhythm of a target for chemotherapeutic agent, top1, has an effect on cytotoxicity of top1 poison, CPT. Our proposal model for clinical use of circadian rhythm of target molecule(s) for chemotherapy is shown in Fig. 5. Actually, clock genes expression levels were differed from tumor to tumor, further, from cell to cell by the analysis of clock gene expression in 90 human solid carcinomas (unpublished data, Y. T. & T. I.). Therefore, each cell in human solid carcinomas may have an independent clock, respectively. This hypothesis is also sustained by the fact that almost molecules in human solid carcinomas reveal the heterogenic expression in human solid carcinomas. Therefore, the importance of chemotherapy with the concept of circadian time may be that any carcinoma cell in one tumor has same clock in expression level of target for anticancer drug. One of the possible ideas based on the present data is that transfer of circadian rhythm by dexamethasone could produce circadian rhythm of circadian-related genes as well as top1. If this proposal model could be came out in clinic, the efficacy of chemotherapy depending on circadian time would be more improved in clinic (Fig. 5).

Fig. 5 Proposal therapeutic model for chemotherapy based on circadian rhythm of topoisomerase I. To adjust clocks in carcinoma cells in human solid carcinoma, the transfer of circadian rhythm in each carcinoma cell by dexamethasone could produce circadian rhythm of circadian-related genes as well as top1 in every carcinoma cell. If this proposal model could be came out in clinic, the efficacy of chemotherapy depending on circadian time would be more improved in clinic.

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