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Circadian regulation of mouse topoisomerase I gene expression by glucocorticoid hormones

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Topo I, topoisomerase I
CPT, camptothecin
CORT, corticosterone
CPT-11, 7-ethyl-10-[4-(1-piperidino)-1-piperidino] carbonyloxycamptothecin
ICR, Institute of Cancer Research
RU486, 11β-(4-dimethyl-amino)phenyl-17β-hydroxy-17-(1-propynyl)estra-4,9-dien-3-one

ABSTRACT

Because glucocorticoid hormones modulate various biological processes, the endogenous rhythm of their secretion is thought to be an important factor affecting the efficacy and/or toxicity of many drugs. Topoisomerase I (Topo I) is a nuclear target of the anticancer drug camptothecin (CPT). In this study, we demonstrate that Topo I expression in tumor-bearing mice and the efficacy of CPT on the tumor are affected by the 24-h variation in circulating glucocorticoid levels. A single administration of corticosterone (CORT) to the tumor-bearing mice resulted in a significant increase in Topo I mRNA levels not only in the tumor masses but also in other healthy tissues such as liver and skeletal muscle. The CORT-induced increase in Topo I mRNA was suppressed by pretreating the mice with RU486, a glucocorticoid receptor antagonist. Significant 24-h oscillations in the Topo I mRNA levels were observed in the tumor and healthy liver without exogenous CORT, and were eliminated by adrenalectomy of the mice. This result suggests that endogenous glucocorticoid hormones are involved in the circadian regulation of Topo I gene expression. Furthermore, the anti-tumor efficacy of the Topo I inhibitor CPT-11 on the tumor-bearing mice was enhanced by administering the drug at the time when the Topo I activity was increased. Our present results demonstrate that glucocorticoid is involved in the 24-h oscillation mechanism of Topo I gene expression and suggest that monitoring the circadian rhythm in Topo I activity is useful for choosing the most appropriate time of day to administer of Topo I inhibitors.

1. Introduction

In mammals, the master circadian pacemaker is located in the suprachiasmatic nucleus (SCN) of the anterior hypothalamus. It is responsible for adapting endogenous physiological functions to cyclic environmental cues such as light, temperature and social communication. The master clock, in turn, synchronizes subsidiary oscillators in other brain regions and

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peripheral tissues through neural and/or hormonal signals [1,2]. These subsidiary oscillators coordinate a variety of biological processes, producing 24-h rhythms in physiology and behavior. Daily rhythm of glucocorticoid secretion from the adrenal cortex is regulated by the hypothalamus–pituitary–adrenal (HPA) axis, which in turn is controlled by the SCN [3]. Because glucocorticoids are involved in the regulation of a variety of physiological functions, such as lipid metabolism and immunity function, the circadian changes in circulating glucocorticoid levels are thought to affect the efficacy and/or toxicity of many drugs.

CPT-11 (7-ethyl-10-[4-(1-piperidino)-1-piperidino]carbonyloxycamptothecin) is a hemisynthetic, water-soluble derivative of the camptothecin (CPT), which is isolated from the Chinese tree, *Camptotheca acuminata*. CPT-11 shows significant anti-tumor activity against a variety of solid tumors, including colorectal and cervical cancers [4–6]. The anti-tumor activities of CPT and its analogs depend on their binding potencies to the transient cleavable complex formed between DNA and topoisomerase I (Topo I), a nuclear enzyme involved in DNA replication machinery. Filipski et al. [7] have recently reported that the efficacy and/or toxicity of CPT-11 varies according to its administration time. Although an interaction has been suggested between glucocorticoid receptor signaling and Topo I [8], the relevance of 24-h variation in glucocorticoid secretion in the anti-tumor activity of CPT-11 remains unclear.

In this study, we found that Topo I gene in tumor cells was expressed in response to glucocorticoid stimuli. The Topo I mRNA levels in the tumor cells implanted in mice fluctuated following the 24-h oscillation in circulating glucocorticoid levels. Because a significant 24-h variation in the Topo I enzyme activity was also observed in the implanted tumor cells, we investigated the relevance of the rhythmic variation in the Topo I activity for the anti-tumor efficacy of CPT-11.

2. Materials and methods

2.1. Materials

CPT-11 was kindly supplied by the Yakult Co. Ltd. (Tokyo, Japan). The drug was dissolved in a sterilized boiled water (80 °C). Corticosterone 21-acetate (CORT) and RU486 were purchased from Sigma Chemical Co. (St. Louis, MO). For use, the two compounds were dissolved in a sterilized saline containing 1% DMSO.

2.2. Animal and cell experiments

Male ICR mice (5 weeks of age) were purchased from Charles River Japan Inc. (Kanagawa, Japan). They were housed under a standardized light/dark cycle condition (light on 07:00–19:00 h) at room temperature of 24 ± 1 °C and a humidity of 60 ± 10% with food and water ad libitum. The animals were adapted to the light/dark cycle for 2 weeks before the experiments. During the dark period, a dim red light was used to aid treatment of the mice. Two murine tumor cells lines (sarcoma 180 and B16 melanoma) were commercially obtained from the Dainippon Pharmaceutical Co. Ltd. (Osaka, Japan). Lewis lung carcinoma cells were supplied by the Cell Resource Center for Biomedical Research, Tohoku University (Sendai, Japan). The tumor cells were maintained in DMEM supplemented with 10% fetal bovine serum at 37 °C in a humidified 5% CO_2 atmosphere. A 50-μl volume containing 1.5×10^6 viable tumor cells was injected in the right hind footpads of each mouse. The tumor volume was estimated according to the following formula: tumor volume (mm^3) = $4\pi(XYZ)/3$, where 2*X*, 2*Y*, and 2*Z* are the three perpendicular diameters of the tumor. After the tumor size reached ~200 mm^3, the tumor-bearing mice were bilaterally adrenalectomized as described previously [9–11]. The adrenals were removed via a dorsal approach using aseptic technique under sodium pentobarbital anesthesia. The adrenalectomized mice were given 0.9% NaCl to drink during the duration of the experiment. Sham adrenalectomy was conducted by the same procedure to expose the adrenals without their removal. To explore the influence of CORT on the mRNA levels of Topo I in the implanted tumor cells or in healthy liver and skeletal muscle, adrenalectomized tumor-bearing mice were given subcutaneous injections of CORT at 09:00 h. Because the levels of Topo I mRNA in those tissues significantly increased from 4 to 8 h after the glucocorticoid stimuli (data not shown), mRNA levels of Topo I in the implanted sarcoma 180 tumor cells or healthy tissues (liver and skeletal muscle) were assessed at 4 h after CORT treatment. To examine the influence of glucocorticoid receptor antagonist on the CORT-induced increase in Topo I mRNA levels, adrenalectomized tumor-bearing mice were pretreated with RU486 (20 mg/kg, s.c.) 30 min before CORT injection (1.0 mg/kg, s.c.). To investigate the relevance of 24-h variation in circulating glucocorticoid levels for the expression of Topo I gene, blood samples were drawn by orbital sinus collection at 09:00, 13:00, 17:00, 21:00, 01:00 and 05:00 h. Thereafter, total RNA was extracted from the implanted tumor cells and healthy liver and skeletal muscle. To explore the temporal variations in protein abundance of Topo I and its enzymatic activity, tumor masses were collected at six time points outlined above.

2.3. Quantitative RT-PCR analysis

Total RNA was extracted from the implanted sarcoma 180 tumor masses or healthy tissues (liver and skeletal muscle) by means of a TRIzol reagent (Invitrogen, Carlsbad, CA). The cDNA of mouse *Topo I* (GenBank accession no. X70958) and *GAPDH* (GenBank accession no. M32599) were synthesized and amplified with use of a superscript one-step RT-PCR system (Invitrogen). The *sequences* of *Topo I* specific primers were as follows: sense, 5′-TCT AAG CAT AGC AAC AGT-3′; antisense, 5′-ATT TGA TGC CTT CTG GAT-3′. The *sequences* of *GAPDH* specific primers were as follows: sense, 5′-GAC CTC AAC TAC ATG GTC TAC A-3′; antisense, 5′-ACT CCA CGA CAT ACT CAG CAC-3′.

To evaluate the quantitative reliability of RT-PCR, we performed a kinetic analysis of amplified products to ensure that signals were derived only from the exponential phase of amplification. From each sample after the first 25 cycles of amplification, we drew a 5-μl aliquot for electrophoresis, and submitted the tubes to one more cycle of PCR. This procedure was repeated for a total of 30 cycles. The PCR products were

run on 3% agarose gels. After staining with ethidium bromide, the gel was photographed on Polaroid-type film. The density of each band was analyzed with NIH image software on a Macintosh computer.

The exponential phase of *GAPDH* amplification in all experimental conditions occurred between the 26th and the 28th cycles, and the exponential phases of all target genes (clock genes and *Topo I* occurred between the 27th and the 30th cycles). The amplification efficiencies of the *GAPDH* and clock or *Topo I* genes were comparable. The amplification products were therefore collected and quantified at the 27th or 28th cycle. The ratio of the amplified target to the amplified internal control (calculated by dividing the value of *Topo I* by that of *GAPDH*) was compared among groups.

2.4. Measurement of CORT concentration

Plasma samples were obtained by centrifugation at 1500 × *g* for 3 min and stored at −20 °C until assay. Concentrations of CORT in plasma were assessed with use of Corticosterone [^{125}I] RIA system (Amersham Biosciences UK Ltd., Little Chalfont, Buckinghamshire, UK).

2.5. Immunoblot analysis

Tumor masses were homogenized with an ice-cold lysis buffer [20 mM Tris–HCl (pH 7.8), 150 mM NaCl, 5 mM EDTA, 0.5% NP40, 2 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, and 10 μg/ml antipain]. After removal of insoluble materials by centrifugation at 12,000 × *g* for 10 min, the resulting supernatants were used for experiments. Lysates containing 100 μg of total protein were mixed with an equal volume of 2× sample buffer [125 mM Tris–HCl (pH 6.8), 10% 2-mercaptoethanol, 4% SDS, 10% sucrose, and 0.004% bromophenol blue]. Protein concentrations in the all lysates of tumor masses were measured with a BCA Protein Assay kit (Pierce, Rockford, IL). The lysate samples (50 μg) were separated on 6% SDS polyacrylamide gels and transferred to polyvinylidene difluoride membranes. The membranes were reacted with antibodies against Topo I (Santa Cruz Biotechnology Inc., Santa Cruz, CA). Specific antigen/antibody complexes were visualized by using horseradish peroxidase-conjugated secondary antibodies and Super Signal Chemiluminescent Substrate (Pierce Biotechnology Inc., Rockford, IL).

2.6. Measurement of Topo I activity in sarcoma 180 tumor masses

Tumor masses were homogenized with 150 mM KCl/potassium phosphate buffer (pH 7.5). After centrifugation, the pellets were washed once with the phosphate buffer and resuspended with 0.35% Triton/phosphate buffer (pH 7.5). The cell suspension was centrifuged at 10,000 × *g* for 4 min at 4 °C. The pellets were resuspended in 100 μl of extraction buffer [1 M Tris–HCl (pH 8.0)/5 M NaCl/14 M 2-mercaptoethanol/10 mg/ml bovine serum albumin]. After 30 min, the cell suspension was centrifuged and the supernatant was stored at −80 °C. Topo I activity was measured by the relaxation of supercoiled plasmid DNA using a Topoisomerase I assay kit (TopoGEN Inc., Columbus, OH). The relaxed plasmid substrate DNA and the reaction product with the supercoiled DNA in buffer without any enzyme fraction were used as markers. The amount of DNA was quantified using a NIH image analysis program on a Macintosh. Topo I activity was calculated from the ratios of relaxed DNA to total DNA (relaxed DNA + supercoiled DNA).

2.7. Measurement of anti-tumor effect

Tumor-bearing mice received intraperitoneally (i.p.) injections containing a single daily dose of CPT-11 (50 mg/kg), every other week at 17:00 or 05:00 h. Control mice were injected with vehicle alone. Injections of CPT-11 were begun 3 days after inoculations with tumor cells. In all mice, tumor volumes were measured every 4 days throughout the experiment. The tumor volume was estimated by the same method described above (Section 2.2).

2.8. Statistical analysis

The significance of the 24-h variation in each parameter was tested by ANOVA. The statistical significance of differences among groups was analyzed by ANOVA and the Tukey multiple comparison test. A 5% level of probability was considered to be statistically significant.

3. Results

3.1. The influence of glucocorticoid on levels of Topo I mRNA in implanted tumor cells

In the first set of experiments, we investigated whether glucocorticoid can affect the levels of Topo I mRNA in sarcoma 180 tumor cells implanted in mice. To exclude the influence of endogenous glucocorticoids, the tumor-bearing mice were adrenalectomized. A single subcutaneous injection of CORT resulted in the accumulation of Topo I mRNA in a dose-dependent manner (Fig. 1A). Significant accumulations of the mRNA from the Topo I gene were found in the tumors after injections of 0.5 and 1.0 mg/kg CORT ($P < 0.05$ and $P < 0.01$, respectively). Treatment of mice with 1.0 mg/kg CORT resulted in a 4.0-fold increase in the mRNA levels of Topo I gene. Similar dose-dependent accumulations of Topo I mRNA were also observed in the healthy quiescent tissues such as liver and skeletal muscle (Fig. 1B). The CORT-enhanced accumulation of Topo I mRNA was significantly suppressed by pre-treatment with a glucocorticoid receptor antagonist RU486 (from 3.4- to 2.5-fold; Fig. 1C). These results suggest that glucocorticoid has the ability to induce the Topo I mRNA probably via glucocorticoid receptor not only in malignant tumor masses, but also in healthy quiescent tissues.

3.2. Adrenalectomy-caused elimination of 24-h oscillation in the Topo I mRNA levels in implanted tumor cells

We next investigated how the 24-h variation in circulating glucocorticoid levels affects the expression of Topo I mRNA in the implanted tumor cells. As in nocturnally active rodents,

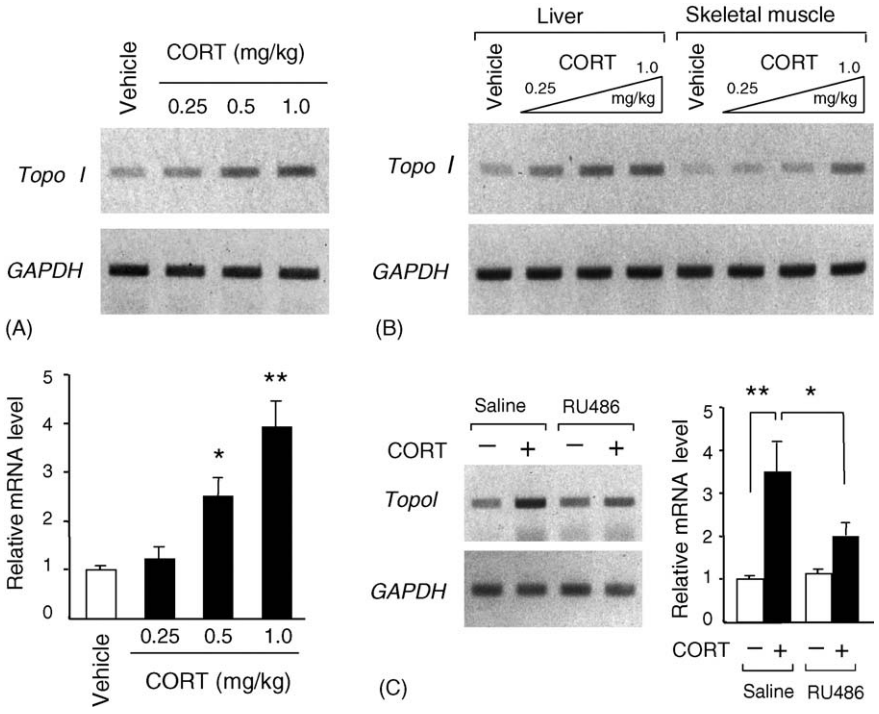

Fig. 1 – Influence of CORT on the mRNA levels of Topo I in sarcoma 180 tumor cells implanted in mice. (A) Dose-dependent increase in the mRNA levels of Topo I in the implanted sarcoma 180 cells by CORT. Indicated dosage of CORT (0.25, 0.5, 1.0 mg/kg) or vehicle (1% DMSO in saline) was administered subcutaneously into tumor bearing and adrenalectomized mice. The mRNA levels of Topo I were measured at 4 h after CORT injection. Each value represents the mean ± S.E. of four mice. $^{**}P < 0.01$, $^{*}P < 0.05$, compared with vehicle-treated mice. (B) Dose-dependent increase in the mRNA levels of Topo I in healthy liver and skeletal muscle of mice by CORT. Indicated dosage of CORT (0.25, 0.5, 1.0 mg/kg) or vehicle (1% DMSO in saline) was administered subcutaneously into the tumor bearing and adrenalectomized mice. The mRNA levels of Topo I were measured at 4 h after CORT injection. (C) Inhibition of CORT by RU486 induced increase in the mRNA levels of Topo I in the implanted sarcoma 180 cells. The tumor-bearing and adrenalectomized mice were pretreated with RU486 (20 mg/kg, s.c.) at 30 min before CORT injection (1.0 mg/kg, s.c.). Each value represents the mean ± S.E. of four mice. $^{**}P < 0.01$, $^{*}P < 0.05$, compared between the two groups.

plasma CORT levels in the tumor-bearing mice showed a significant 24-h variation with higher levels around the late light phase ($P < 0.01$, Fig. 2A). However, there was no significant 24-h variation in plasma CORT levels in plasma of adrenalectomized tumor-bearing mice (Fig. 2A). We therefore used these animals to investigate the influence of the varying glucocorticoid levels on Topo I mRNA expression in the implanted tumor cells. A significant time-dependent variation in the Topo I mRNA levels was observed in the tumor cells implanted in control (sham-operated) mice ($P < 0.01$, Fig. 2B). Higher levels of Topo I mRNA were found from the late light phase to the mid dark phase. By contrast, there was no significant time-dependent variation in the Topo I mRNA levels in the tumor cells implanted in adrenalectomized mice; the mRNA levels remained at trough levels for the control mice throughout the day (Fig. 2B). The adrenalectomy-caused elimination of the oscillation in Topo I mRNA expression was also found in healthy liver (Fig. 2C). Because a single administration of exogenous CORT rapidly induced the expression of Topo I mRNA in adrenalectomized mice, these results suggest that the oscillation in the Topo I mRNA levels is caused by circadian change in the circulating glucocorticoid levels.

3.3. *Time-dependent variation in Topo I activity in the tumor masses*

Topo I is one of the most important target molecules in cancer chemotherapy. We therefore explored whether there is a time-dependent variation in the protein abundance of Topo I and its enzyme activity in the tumor cells. As shown in Fig. 3A, the protein abundance of Topo I showed a 24-h oscillation, with higher levels from the dark phase to the early light phase. Similarly, a significant rhythmic variation was also observed in Topo I enzyme activity ($P < 0.05$, Fig. 3B). The rhythmic pattern resembled the overall increases and decreases in Topo I protein abundance.

3.4. *Influence of dosing time on the anti-tumor effect of CPT-11*

The daily rhythmic variations in Topo I activity observed in the tumor may affect the efficacy of Topo I inhibitors. We therefore examined whether the anti-tumor efficacy of CPT-11 varied according to its administration time. In our preliminary experiments, significant time-dependent variations in Topo I activity were also observed in both Lewis lung

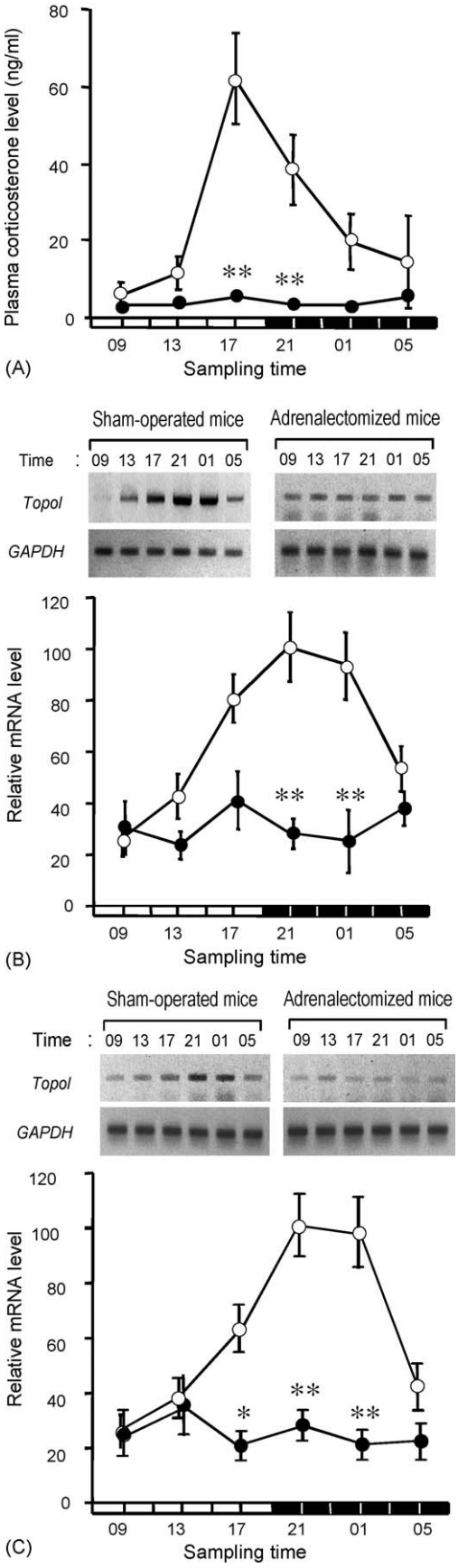

Fig. 2 – Influence of adrenalectomy on the 24-h oscillation in the mRNA levels of Topo I in implanted sarcoma 180 cells. (A) Temporal profiles of CORT concentrations in plasma of sham-operated (open circle) or adrenalectomized mice (closed circle). Each value represents the mean ± S.E. of four to six mice. $^{**}P < 0.01$, compared with the value for sham-operated group at the corresponding times. (B and C) Temporal profiles of mRNA levels of Topo I in the implanted tumor cells (B) and healthy liver (C) in sham-operated (open circle) or adrenalectomized (closed circle) mice. For plot of mRNA, the mean peak value of sham-operated mice is set at 100. GAPDH mRNA was used as internal control for transcripts. Each value represents the mean ± S.E. of three to four mice. The photographs (top) show representative RT-PCR products of Topo I in sarcoma 180 tumor cells (B) or healthy liver (C), $^{**}P < 0.01$, $^{*}P < 0.05$, compared with the value for sham-operated group at the corresponding times.

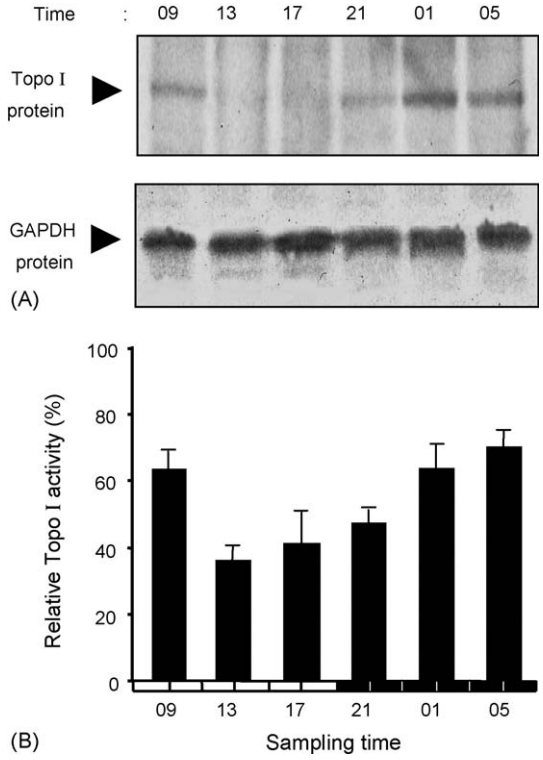

Fig. 3 – Twenty-four-hour variations in Topo I protein abundance and its enzyme activity in implanted sarcoma 180 tumor cells. (A) Temporal expression profile of Topo I protein in tumor masses. GAPDH protein was used as internal control. (B) Time-dependent difference in Topo I activity in tumor masses. Each value represents the mean ± S.E. of three to four mice. The enzymatic activity exhibits a significant 24-h variation ($P < 0.05$).

carcinoma and B16 melanoma cells implanted in mice (data not shown). Therefore, we used these cells to test the influence of dosing time on the ability of CPT-11 to suppress tumor growth. Since there was no significant time-dependent difference in the growth of these implanted tumor cells, we used the mean value of the tumor volume between 17:00 and 05:00 h as the control. Although the growth of all three

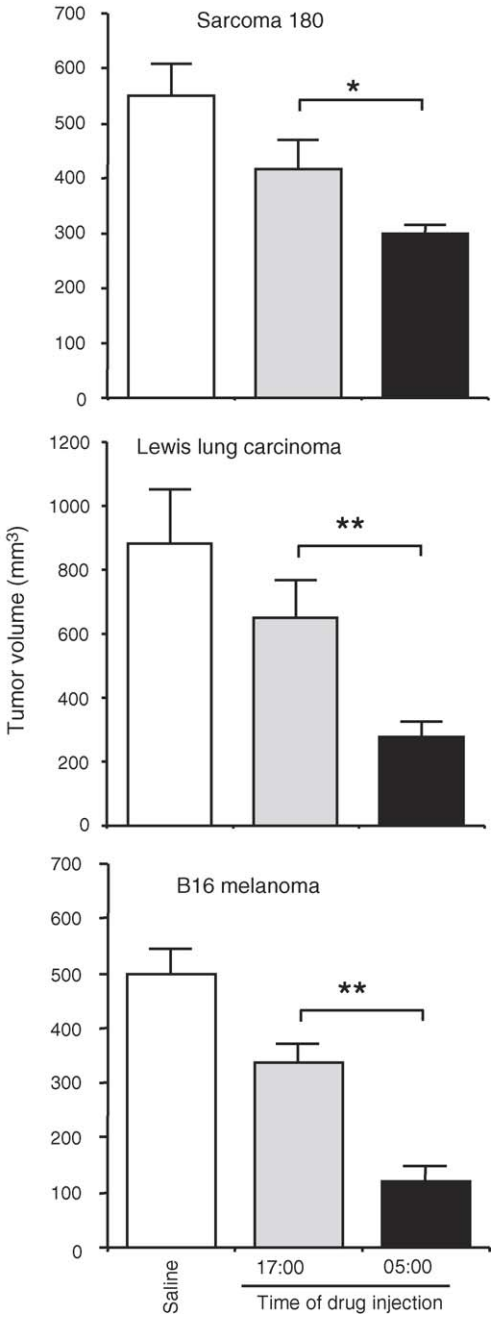

Fig. 4 – Influence of dosing time on the ability of CPT-11 to inhibit tumor growth. CPT-11 (50 mg/kg) or vehicle was administered intraperitoneally at 17:00 or 05:00 h every other week. Each value represents the mean ± S.E. of six to eight mice. $^{**}P < 0.01$, $^{*}P < 0.05$, for comparison between two dosing times.

different types of tumor cells was significantly suppressed by administration of CPT-11, the anti-tumor effect was more potent in mice injected with the drug at 05:00 h than with that at 17:00 h (Fig. 4). These results suggest that the anti-tumor effect of CPT-11 could be enhanced by optimizing the dosing schedule to the time when the Topo I mRNA expression has increased in response to circulating glucocorticoid levels.

4. Discussion

The oscillation in the transcription of specific clock genes plays a central role in the generation of 24-h rhythms in various biological processes [1,12]. Although clock genes are expressed throughout the whole body, the oscillation in the expression of clock genes in different tissues is coordinated by neural and/or hormonal signals which are driven by the central SCN pacemaker [13,14]. Our present study demonstrates that the expression of Topo I mRNA in tumor masses of mice oscillates in response to the 24-h changes in circulating glucocorticoid levels. The oscillating expression of Topo I mRNA appears to result in a 24-h variation in its enzymatic activity. Twenty-four-hour variation in circulating glucocorticoid levels is observed not only in nocturnally active rodents, but also in diurnally active humans; it is thought to serve as a humoral signal for synchronization of the peripheral clock genes. The 24-h variation in the levels of Topo I mRNA in the implanted tumor cells was disappeared by adrenalectomy. However, the rhythmic expression patterns of the clock genes, such as *Clock*, *Bmal1*, *Per2* and *Cry1*, in the tumor cells implanted in the adrenalectomized mice did not differ significantly from those in the control (sham-operated) mice (Supplemental Data). This finding suggests that adrenalectomy can eliminate the 24-h oscillation in Topo I mRNA levels in the tumor without affecting the rhythmicity of clock gene expression. Therefore, it is unlikely that the molecular components of the circadian clock are involved in the glucocorticoid-dependent regulation of Topo I gene expression.

Glucocorticoids exert their actions on gene expression by activating the cytoplasmic receptors: glucocorticoid forms a heterodimer complex with the receptor, enters the nucleus, and promotes the transcription of genes through binding to the responsive element [15–17]. CORT-induced accumulation of Topo I mRNA in the implanted tumor cells was significantly prevented by pre-treatment of the mice with the glucocorticoid receptor antagonist, RU486. The result suggests that glucocorticoid and its receptor are involved in the accumulation of Topo I mRNA transcripts. However, when cultured sarcoma 180 tumor cells were exposed to 60 ng/ml CORT for 8 h, no significant accumulation of the Topo I mRNA was observed (data not shown). Therefore, glucocorticoid may promote indirectly the transcription of Topo I gene in the implanted tumor cells in a circadian manner. However, it remains unknown how glucocorticoid can promote and cause oscillation in the expression of Topo I.

Twenty-four-hour variations in biological functions, such as gene expression and protein synthesis are thought to be important factors affecting the efficacy and/or toxicity of many drugs. Our present results suggest that circadian variations in Topo I activity affected the pharmacological efficacy of CPT-11: CPT-11 was more potent in mice injected with the drug during the late dark phase than during the late light phase. Calvet et al. [18] have recently demonstrated that qualitative and/or quantitative changes of Topo I in tumor cells alter the cytotoxic effect of CPTs. Therefore, the dosing time-dependent difference in the anti-tumor effect of CPT-11 may be caused by oscillation in Topo I activity in tumor cells.

In conclusion, the present findings show that the time-dependent variation in circulating glucocorticoid levels can induce 24-h oscillation in Topo I mRNA levels in solid tumors in mice. The anti-tumor effect of CPT-11 can be expected by administering the drug at the time when Topo I activity has been increased. Circadian changes in Topo I activity can be determined by monitoring the 24-h rhythm of glucocorticoid secretion. Optimizing the dosing time of CPT-11 may not only decrease its side effects [19], but also increase its anti-tumor effect. Our present results suggest a way to achieve better chronopharmacotherapy for the treatment of cancers with CPT-11.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bcp.2005.12.034.

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