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Biomodulation by Hyperthermia of Topoisomerase II-Targeting Drugs in Human Colorectal Cancer Cells

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We examined whether heat stress could enhance the sensitivity of human colon cancer WiDr cells to topoisomerase II-targeting anticancer agents, etoposide (VP-16) and teniposide (VM-26), and also determined the most effective timing for the drug administration after exposure to hyperthermia. Both topoisomerase II contents and topoisomerase II activity were significantly increased in WiDr cells 3 to 12 h after heat stress at 43°C for 1 h, in comparison with those immediately after the heat stress. Cytotoxicity by VP-16 was most significantly enhanced 3 to 12 h after exposure to 43°C for 1 h, but no synergistic effect was observed when the drug was administered immediately after the heat stress. A combination of VM-26 with heat stress, but not that of a topoisomerase I-targeting camptothecin derivative (CPT-11), or vincristine, showed a synergistic cytotoxic effect on WiDr cells. VP-16 alone induced cellular accumulation at the G2+M phase, whereas the combination of VP-16 and heat stress further increased the cell population at the G2+M phase, and decreased S-phase cells. A possible application of the combination of VP-16 and hyperthermia in clinical use is discussed.

Key words: Biomodulation — Hyperthermia — Topoisomerase II — Etoposide

DNA topoisomerase II, which catalyzes conformational changes of DNA, is closely involved in processes such as DNA replication, transcription, chromosomal condensation, and decondensation.[1)] Topoisomerase II-targeting anticancer agents interfere with the breakage-rejoining reaction catalyzed by this enzyme, and intermediate forms of drug-enzyme-DNA cleavable complexes are accumulated in tumor cells.[2,3)] Of the topoisomerase II-targeting agents, the podophyllotoxin derivatives VP-16[4] and VM-26 are prominent anti-tumor drugs and potentiation of the action of these drugs without disturbance of normal cellular function might be very useful in cancer therapy. Cellular sensitivity to VP-16/VM-26 in human epidermoid cancer KB cells is closely correlated with cellular levels of topoisomerase II.[4–7)] Furthermore, enhanced expression of topoisomerase II after hyperthermic treatment at 42°C results in sensitization of KB cells to the cytotoxic effects of VP-16/VM-26.[8)] Topoisomerase II itself thus appears to be a heat-responsive protein.[9)]

An attractive clinical application of hyperthermia is to combine its use with administration of chemotherapeutic agents, to enhance synergistically the cytotoxicity of the drugs against tumor cells. For example, heat has been shown to potentiate the cytotoxicity of several drugs including *cis*-platinum,[10–12)] and local anesthetics,[13)] and to sensitize cells to radiation, probably by inhibiting one or several critical processes involved in repair of DNA damage.[14,15)] Hyperthermia enhances VP-16 cytotoxicity in a multidrug-resistant mutant leukemia cell line[16)] and prior hyperthermic treatment at 42°C enhances the expression of topoisomerase II, followed by potentiation of the cytocidal effect of VP-16 on human epidermoid carcinoma cells.[8)] However, another study found that hyperthermia (41.8°C) fails to enhance drug cytotoxicity to human T cell lymphoblasts.[17)] Also, human and rodent cell lines were shown to be protected from DNA damage and cytotoxicity when heat was applied before administration of m-AMSA, an inhibitor of topoisomerase II.[9,18,19)] It remains to be established why anti-tumor activity by topoisomerase II-targeting agents is potentiated by hyperthermia in some systems but not in others.

In this study, we investigated when expression of topoisomerase II was most prominently enhanced after heat treatment, and examined whether the cytotoxicity of topoisomerase II-targeting anticancer agents to human colon cancer cells could be biomodulated by hyperthermia.

MATERIALS AND METHODS

Cell culture WiDr human colon cancer cells were cultured in Dulbecco's minimal essential medium (Nissui Seiyaku Co., Tokyo) containing 10% fetal bovine serum

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[4] Abbreviations: VP-16, etoposide; VM-26, teniposide; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; HSP, heat shock protein; kDNA, kinetoplast DNA; cDNA, complimentary DNA; GST, glutathione S-transferase.

(Hyclone, Logan, UT), L-glutamine (0.292 mg/ml), kanamycin (100 μg/ml), and penicillin (100 units/ml) as reported by Hidaka *et al.*[20] and Matsuo *et al.*[5, 8]

Chemicals VP-16 was obtained from Nihon Kayaku Co., Tokyo; VM-26 was obtained from Bristol Myers Co., Kanagawa; CPT-11 was obtained from Yakult Co., Tokyo; vincristine was obtained from Sigma Chemical Co., St. Louis, MO.

Heat and drug treatment and cell survival assays Cell survival was assayed in terms of colony formation under hyperthermia in the absence or presence of anti-cancer drugs.[21] We seeded 400 WiDr cells into 35-mm dishes and these were incubated at 37°C for 22 h. The dishes were placed in an incubator regulated at the elevated temperature (43°C), and then returned to the 37°C incubator. It took 5 min to raise the temperature of the media in the dish from 37°C to 43°C. The cells were exposed to anticancer drugs for 1 h immediately or at an interval after heat treatment. The medium was replaced with fresh medium at 37°C, and the dishes were incubated at 37°C for 10 days to allow colony formation. Each experiment was performed at least three times, and the data were analyzed statistically using Student's *t* test (two-sided).

Western blots Crude nuclear extracts were prepared from 1×10^8 WiDr cells in the early logarithmic phase at various intervals after heating, as reported previously.[5] Equivalent amounts of nuclear extract were separated by SDS-PAGE (7.5% gels). Protein fractions from the gels were electrophoretically transferred onto nitrocellulose membranes in 25 m*M* Tris-HCl (pH 8.3), 92 m*M* glycine and 20% methanol for 2 h at 20 V. The membranes were further incubated with antibody against human DNA topoisomerase II (1:2000) for 1 h at room temperature, followed by rinsing with phosphate-buffered saline (pH 7.4), and were then developed according to the manufacturer's protocol (Vectastain ABC-GO kit; Vector Laboratories, Burlingame, CA).

Northern blots Northern blot analysis was performed as reported previously.[5, 6, 8] Total RNA was extracted from WiDr cells as described[6] and aliquots of 20 μg were electrophoresed in 1% agarose and 2.2 *M* formaldehyde gels and transferred onto Nylon membranes (Schleicher and Schuell, Dassel, Germany). A ^{32}P-labeled cDNA fragment with a specific activity of 10^8 cpm/mg of DNA was prepared by random priming. Hybridization was allowed to proceed in Hybrisol I (Oncor, Gaithersburg, MA) for 24 h at 42°C. The membranes were washed at room temperature in 2× standard saline-citrate (1×, 150 m*M* NaCl:15 m*M* sodium citrate, pH 7.0) and 0.1% SDS, then visualized by autoradiography on Kodak XAR film. The mRNA level was quantified using a Fuji BAS 2000 bio-imaging analyzer (Fuji Photo Film Co., Tokyo).

Assays of topoisomerase II activity The topoisomerase II catalytic activity in nuclear extracts was assayed in terms of the ATP-dependent decatenation of kDNA according to the method reported by Matsuo *et al.*[5, 8] and Takano *et al.*[6] The standard reaction for DNA topoisomerase II assay contained 50 m*M* Tris-HCl (pH 7.5), 85 m*M* KCl, 10 m*M* $MgCl_2$, 5 m*M* dithiothreitol, 0.5 m*M* EDTA, bovine serum albumin (0.03 mg/ml), and 1 m*M* ATP. The decatenation reaction of catenated DNA was carried out with nuclear extract containing 17.5 μg/ml of protein and 0.1 μg of kDNA in a final volume of 10 μl at 30°C for 15 min. The reaction was terminated by addition of 2 μl of stop buffer containing 5% Sarkosyl and 0.0025% bromophenol blue in 25% glycerol. Samples were then electrophoresed through 1% agarose gels. After staining with ethidium bromide, gels were photographed under UV illumination.

Cell cycle analysis We tested the cell cycle in 4 groups separated according to the heating condition with or without VP-16 administration. Exponentially growing cells (1×10^6 cells) were seeded in Petri dishes (35 mm) and incubated for 22 h at 37°C, then those which were scheduled for heating were exposed to hyperthermic conditions at 43°C for 1 h. Fifty μ*M* VP-16 was administered for 1 h to the cells scheduled for drug exposure, at 3 h after heating. After exposure to VP-16 for 1 h, the drug-containing medium was removed and replaced with fresh drug-free medium (37°C), and the cells were further incubated at 37°C for 24 h. Cells were collected 4 h and 28 h after the heating procedure and the cell cycle was analyzed (Fig. 5). Collected cells were stained using a Cycle TEST (Becton-Dickinson, Bedford, MA) kit for the isolation and staining of cell nuclei: The cells were disaggregated with trypsin and a non-ionic detergent, processed by enzymatic digestion of RNA, then stained with propidium iodide. The fractions were analyzed by cytofluorometry (FACS, Becton-Dickinson) and the DNA content was estimated from a DNA histogram in comparison with that of non-heated cells.

RESULTS

To investigate the influence of hyperthermia on the cytotoxicity of VP-16, we employed heat treatment at 43°C for 1 h, which is commonly used for clinical hyperthermic treatment. This thermal dose alone reduced cell survival to about 90% of the initial fraction of human colon cancer WiDr cells. The cytotoxicity of VP-16 was not potentiated when exponentially growing WiDr cells were exposed to VP-16 for 1 h just after heat treatment at 43°C for 1 h. In contrast, when WiDr cells were exposed to VP-16 for 1 h at 3 and 6 h after heat treatment, the cytotoxic effect was significantly enhanced (Fig. 1-A, B and C). This delayed potentiation of VP-16 by hyper-

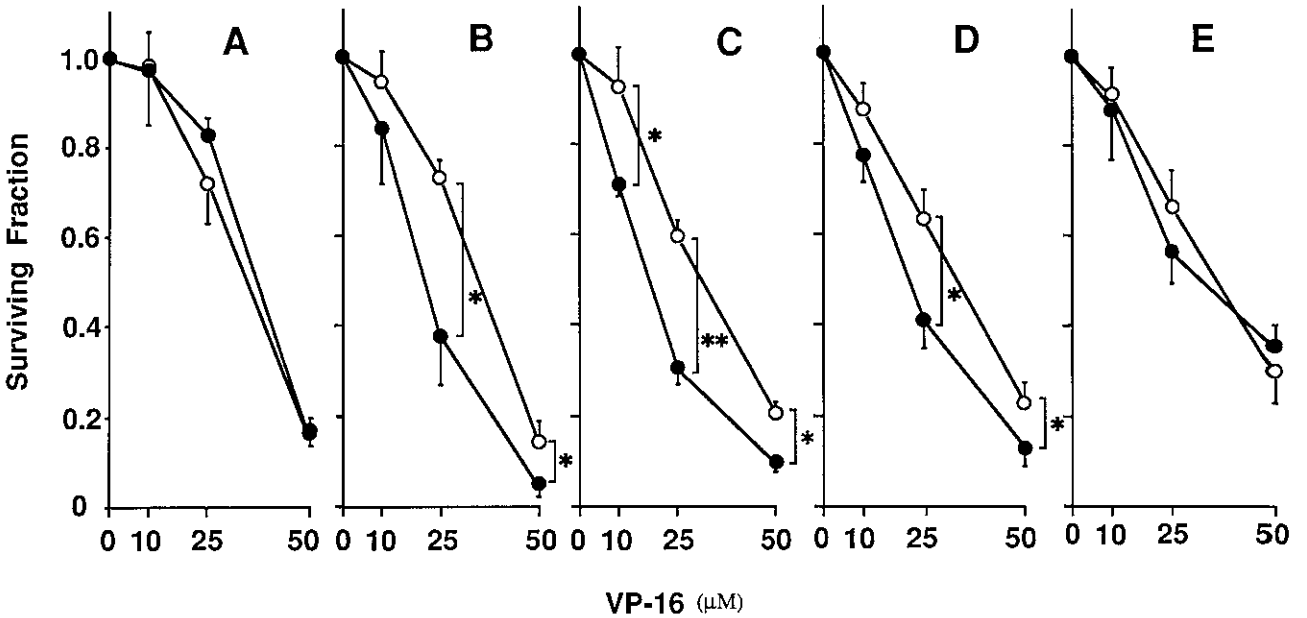

Fig. 1. Effects of hyperthermia on cytotoxicity by VP-16. WiDr cells were exposed to 43°C for 1 h, followed after 0–24 h by incubation at 37°C and exposure to graded doses (10 μM, 25 μM and 50 μM) of VP-16 for 1 h at 37°C. Cell survival was determined by colony formation assay. WiDr cells were exposed to VP-16 immediately (A), 3 h (B), 6 h (C), 12 h (D) or 24 h (E) after heat treatment. ○, non-heat-treated cells; ●, heat-treated cells. The surviving fraction in the absence of VP-16 was normalized as 1.0. Each point with vertical bars represents the mean and standard deviation of three independent experiments. The values were analyzed using the two-sided Student's t test (* $P<0.05$, ** $P<0.01$). The values of IC_{50} (μM) (non-heat-treated cells/heat-treated cells) are as follows: A, 35.1/37.5; B, 34.8/21.1; C, 31.5/18.0; D, 33.0/21.3; E, 32.4/36.3.

thermia was observed at least until 12 h after heating, whereas no such potentiation was observed 24 h later (Fig. 1-D and E). Treatment of WiDr cells at 43°C for 1 h potentiated the cytotoxic action of VP-16 at 3 h or later, but not immediately after heat treatment, suggesting delayed potentiation of the topoisomerase II-targeting agent by hyperthermia.

We examined whether hyperthermia induced the potentiation of other anticancer agents such as the topoisomerase II-targeting drug VM-26 (Fig. 2-A, B and C), the topoisomerase I-targeting drug CPT-11 (Fig. 2-D, E and F), and vincristine (Fig. 2-G, H and I). When WiDr cells were exposed to VM-26 after heat treatment at 43°C for 1 h, the cytotoxic effect of VM-26 was significantly enhanced when VM-26 was administered 3 h (data not shown) and 6 h after heat treatment (Fig. 2-B). In contrast, no potentiation of VM-26 was observed when VM-26 was added immediately or 24 h after the heat treatment (Fig. 2-A and C). Neither CPT-11 nor vincristine was potentiated by hyperthermic pretreatment (Fig. 2-D, E, F, G, H and I). The delayed enhancement of cytotoxicity of anticancer drugs after heat treatment appeared to be specific for the topoisomerase II-inhibitory anticancer agents.

Since cellular sensitivity to topoisomerase II inhibitors is closely correlated with intracellular topoisomerase II level,[5-8, 15, 22, 23] we investigated the mRNA level of topoisomerase II after heating at 43°C for 1 h (Fig. 3). This heat shock stress increased the cellular level of topoisomerase II mRNA about 2- to 4-fold during incubation for 3, 6, and 12 h at 37°C after exposure to the elevated temperature of 43°C for 1 h, in comparison with that at 0 h, when topoisomerase II mRNA expression was decreased slightly. However, topoisomerase II mRNA expression had reverted to almost the same level as the control 24 h after heat treatment at 43°C for 1 h. No significant increase in topoisomerase I or GST-π mRNA expression was observed after heating. A remarkable increase in mRNA of a representative heat shock protein, HSP-70, was observed in WiDr cells from just after heat treatment until 3 h, and the level had reverted to the control level 12 h later at 37°C (Fig. 3).

To examine whether cellular contents and catalytic activity of topoisomerase II were increased in heat-treated cells, we performed Western blot analysis with an anti-topoisomerase II antibody and examined the decatenation activity of kinetoplast DNA. Western blots with anti-topoisomerase II antibody showed that the topoisom-

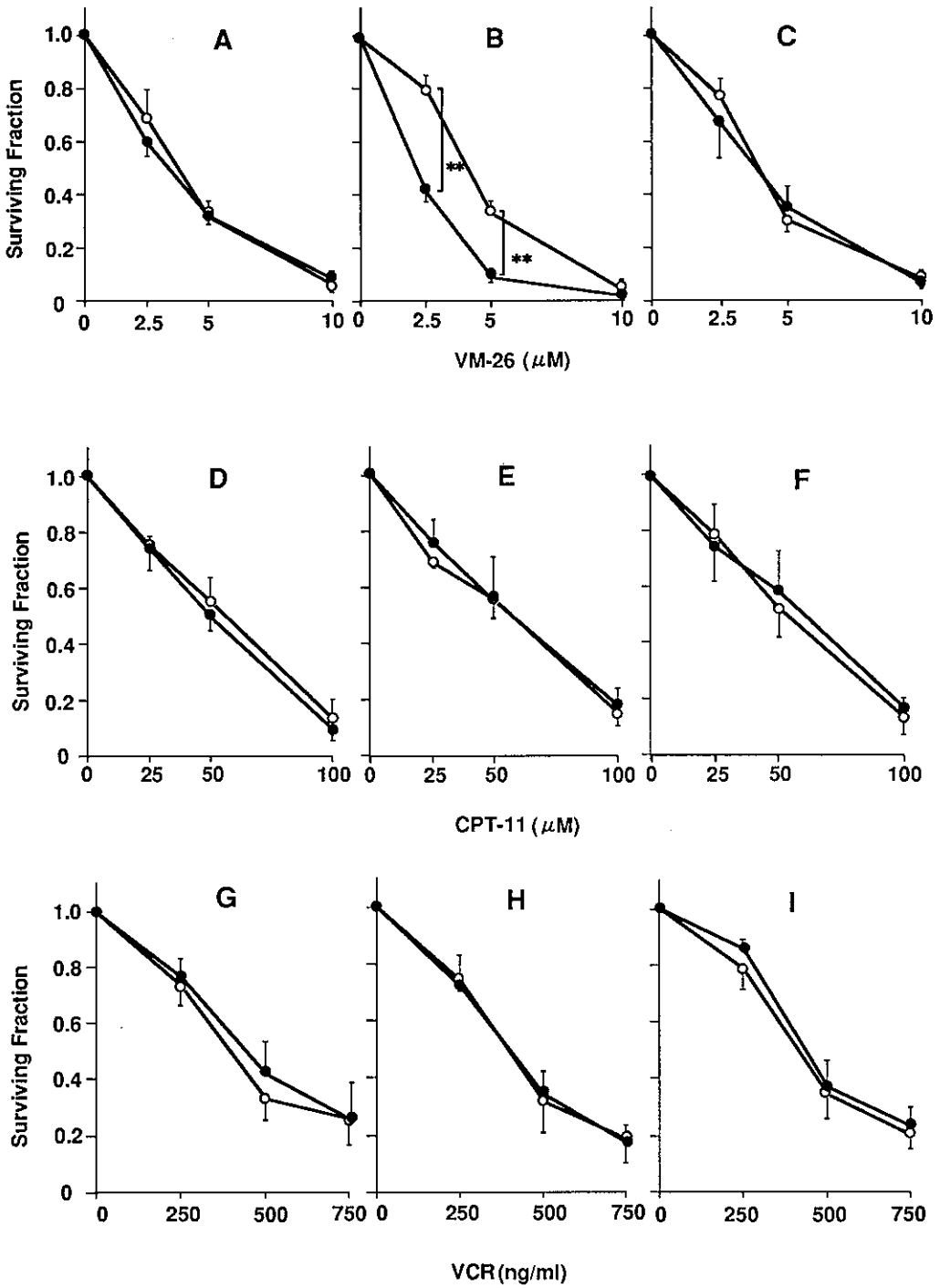

Fig. 2. Effect of hyperthermia on cytotoxicity by VM-26 (A–C), CPT-11 (D–F) and vincristine (VCR) (G–I). WiDr cells were exposed to 43°C for 1 h, followed by incubation for 0–24 h at 37°C and exposure to various doses of each drug for 1 h at 37°C. Cell survival was determined by colony formation assay. WiDr cells were exposed to drugs immediately (A, D, G), or 6 h (B, E, H) or 24 h (C, F, I) after heat treatment at 43°C for 1 h. ○, non-heat-treated cells; ●, heat-treated cells. Each point represents the mean and standard deviation of three independent experiments. The surviving fraction in the absence of any drug was normalized as 1.0. The values were analyzed using the two-sided Student's t test (** $P<0.01$). The values of IC_{50} of VM-26 (μM), CPT-11 (μM) and vincristine (ng/ml) (non-heat-treated cells/heat-treated cells) are as follows: VM-26 (A, 3.78/3.42; B, 4.2/2.22; C, 4.0/3.9), CPT-11 (D, 56.4/50.4; E, 55.9/55.8; F, 51.6/59.4), vincristine (G, 401/450; H, 390/401; I, 409/435).

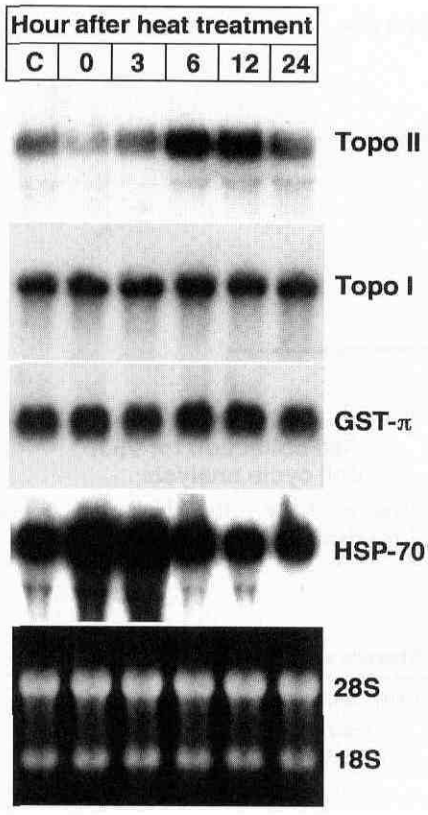

Fig. 3. Expression of topoisomerase I and II, HSP-70 and GST-π mRNAs in WiDr cells at various periods after exposure to 43°C for 1 h. WiDr cells were heated at 43°C for 1 h, followed by the indicated periods of incubation at 37°C. RNA was then extracted and Northern blot hybridization was performed. C shows the control without heat treatment. Ribosomal RNAs loaded on the gels are indicated. Topo II, Topoisomerase II; Topo I, Topoisomerase I; GST, glutathione S-transferase; HSP, heat shock protein.

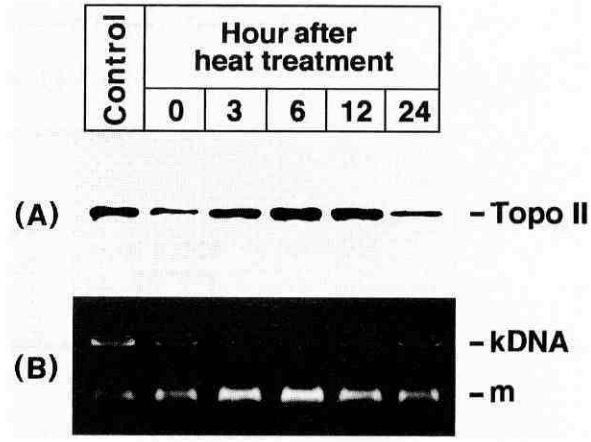

Fig. 4. Western blots (A) and decatenation activity (B) of topoisomerase II in WiDr cells exposed to heat treatment at 43°C. In (A), WiDr cells were exposed to the elevated temperature of 43°C for 1 h, followed by incubation for the indicated periods at 37°C. Nuclear extracts containing 4.25 μg of protein were analyzed by Western blotting with anti-topoisomerase II antibody. In (B), decatenation activity by topoisomerase II was compared in nuclear extracts from non-heat-treated and heated cells after incubation for the indicated periods at 37°C. WiDr cells were exposed to the elevated temperature of 43°C for 1 h, followed by incubation for 0–24 h at 37°C. The reaction mixture was incubated with nuclear extract containing 17.5 μg/ml of protein. m, free minicircles.

erase II content was increased by 2- to 3-fold 3 to 6 h after heating, relative to that just after heat treatment (Fig. 4-A). Decatenation of kDNA by crude nuclear extracts from heat-treated cells was found to be much higher than that of untreated cells, and the activity remained at an increased level at 6 h after hyperthermic treatment at 43°C for 1 h (Fig. 4-B).

The cellular level of topoisomerase II varies during the cell cycle.[24-26] Thus, the enhanced levels of topoisomerase II and its mRNA observed here in the heat-stressed KB cells might have been due to accumulation of cells at a specific phase of the cell cycle. We examined the effects of heat stress in the absence or presence of VP-16 on the cell cycle of WiDr cells. The time schedule of cell cycle analysis is shown in Fig. 5. When WiDr cells were exposed to hyperthermia at 43°C for 1 h, the proportion of cells at S phase was increased 4 h after heat stress compared with non-heated cells, suggesting that those exposed to heat treatment had a longer S phase. The proportion of heated cells in S phase recovered almost to the same level as in non-heated cells at 28 h after heat treatment (Table I). In comparison with non-heated and non-drug-exposed cells, those exposed to VP-16 showed higher percentages of G2+M-phase cells 28 h after cessation of heat treatment. When non-heated cells were treated with VP-16, the cell population at G2+M by 24 h after drug exposure was about 31%, which was 6-fold higher than that of non-drug-exposed cells. In addition, when VP-16 was administered 3 h after heat treatment at 43°C for 1 h, the population accumulated at the G2+M phase by 24 h after drug exposure was significantly increased to more than 10-fold that of non-heated and non-VP-16-exposed cells.

DISCUSSION

In the present study, we demonstrated that hyperthermia enhanced the cytotoxicity towards cultured human colon cancer cells of topoisomerase II-targeting anticancer agents such as VP-16 and VM-26, but not CPT-11 or vincristine (Figs. 1 and 2). This hyperthermia-induced

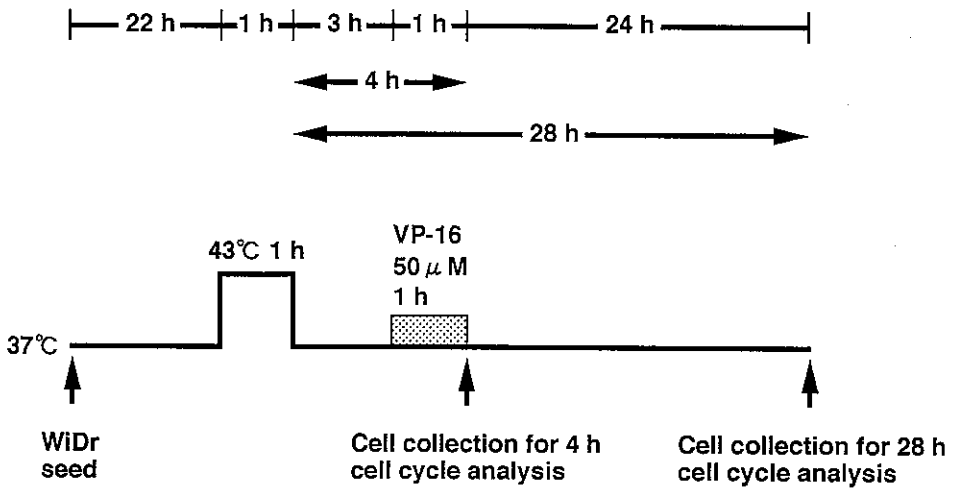

Fig. 5. Schematic time schedule in cell cycle analysis. Cytofluorometric analysis of WiDr cells, which were treated with or without heat and/or VP-16 according to this time schedule, was conducted and the data are given in Table I.

Table. I Effects of Heat Stress at 43°C on the Cell Cycle in the Absence and Presence of VP-16[a)]

Cell cycle phase	Time after heat treatment (h)	Heat and VP-16 administration condition[b)]			
		Heat (−) VP-16 (−)	Heat (+) VP-16 (−)	Heat (−) VP-16 (+)	Heat (+) VP-16 (+)
G1	4	50.13±3.60	39.73±0.06	49.70±2.55	39.50±3.99
	28	75.83±0.42	73.17±3.19	56.57±9.19	39.73±12.07
S	4	27.20±0.52[d)]	35.63±2.85[c)]	27.77±0.83	38.80±2.63
	28	19.23±3.71	17.07±0.67	12.43±2.15	8.33±1.74
G2+M	4	22.67±4.06	24.63±2.80	22.50±1.91	21.73±6.49
	28	4.93±3.33	9.80±3.40	31.00±7.46	51.97±10.34

a) The data are shown as the percentage (%) of cells at various cell cycle stages under the same heating and drug administration conditions, and are presented as the mean±SD of at least three replicate samples.
b) Cells were seeded into 35-mm Petri dishes and incubated for 22 h in a CO_2 incubator at 37°C. Then, the cells were exposed to heat treatment at 43°C (Heat (+)) or 37°C (Heat (−)), for 1 h. Three h after heat exposure, cells were exposed to 0 (VP-16 (−)) or 50 μM (VP-16 (+)) VP-16 for 1 h. The media were changed to fresh drug-free medium immediately after VP-16 exposure. Cell cycle was assayed 4 h and 28 h after heat treatment. (See schematic time schedule in Fig. 5.)
c) Significantly different ($P<0.05$) from the values for *d*).

enhancement of the effect of VP-16 or VM-26 was observed when cells were incubated for 3 or 6 h at permissive temperature after exposure to 43°C for 1 h, but not when the cells were not further incubated after heat stress. We obtained almost the same results when we used HeLa cells with the same protocol (unpublished data). Cellular topoisomerase II levels limit cellular sensitivity to VP-16/VM-26.[7)] Cellular levels of topoisomerase II and topoisomerase II activity were increased on incubation for more than 3 h at 37°C after heat stress at 43°C. Thus, the colon cancer WiDr cells appeared to be highly sensitive to the combination of VP-16/VM-26 and hyperthermia when incubated for 3–12 h at 37°C after heat stress (Fig. 1 and Fig. 2-A, B and C). No potentiation was observed when the cells were incubated for 24 h after heat stress. Enzymatic activity of topoisomerase II might be inactivated during longer incubation (24 h) at 37°C after heat stress, resulting in a failure of synergy of VP-16 and hyperthermia. Survival curves of untreated WiDr cells at later periods indicated slight resistance to VP-16/VM-26, but not to vincristine or CPT-11, as compared with those of earlier periods (Figs. 1 and 2). Topoisomerase II-targeting drugs such as VP-16 and VM-26 are most effective against cells in the S phase.[27)] In these

experiments, monolayer-cultured cells were trypsinized and 400 cells were then seeded in 35-mm dishes, followed by heat treatment with or without drugs. Trypsinization and re-seeding may induce an incomplete synchronization of cell population,[28] resulting in altered sensitivity to VP-16/VM-26 between earlier periods and later periods.

Hyperthermia modifies the cytotoxic effect of some anticancer drugs such as bleomycin, *cis*-platinum and mitomycin.[10-12] However, there have been conflicting reports concerning the effects of hyperthermia on the cytotoxicity of topoisomerase II inhibitors. Kampinga *et al.*[18] reported that hyperthermia at 45°C prior to drug treatment reduces the cytotoxic effects of m-AMSA, a potent topoisomerase II inhibitor, in HeLa cells. Another report demonstrated that treatment at 45.5°C for 10 min immediately before addition of VP-16 causes about 100-fold protection from the cytotoxic effects of anticancer drugs relative to cells without heat treatment, and also that significant protection of human fibroblasts and human melanoma cells is observed when they are heated at 45°C immediately prior to VP-16 treatment.[19] In contrast, hyperthermia enhances VP-16 cytotoxicity in a multidrug-resistant leukemia cell line.[16] Matsuo *et al.*[8] reported significant enhancement of VP-16 cytotoxicity in human epidermoid cancer KB cells treated at 42°C for 1–3 h prior to VP-16 administration, and also that the cytotoxicity of this agent is not enhanced when KB cells are exposed to 45°C for a short period. Consistent with a previous study by Matsuo *et al.*,[8] our present study demonstrates that synergy of heat stress and VP-16/VM-26 is observed only when the cellular content and activity of topoisomerase II are elevated in cancer cells by hyperthermic treatment. Hyperthermia just before drug administration may not have sufficient time to increase cellular topoisomerase II levels, resulting in failure to induce a potentiation of VP-16/VM-26. The inconsistent synergy of hyperthermia and VP-16/VM-26 among various cancer cell types suggests that cellular responses to heat stress might differ in relation to the induction of topoisomerase II. Determination of the key enzyme, topoisomerase II, in heat-treated cancer cells thus appears to be requisite for the development of successful potentiation of topoisomerase II-targeting agents by hyperthermia.

In this study, we demonstrated that the cell cycle of WiDr was greatly affected by heat and VP-16 treatment (Table I). The heat dose which reduces the cell survival to 20–50% induces a long mitotic delay of 11 h in Chinese hamster cells.[29] The cellular sensitivity to topoisomerase II inhibitors is often increased in proliferating cells and in cycling cells during S phase.[27,30] After exposure to topoisomerase II inhibitors, mammalian cells undergo irreversibly arrest in the G2 phase, followed by cell death.[31,32] In our present study, the hyperthermic treatment at 43°C for 1 h delayed the cell cycle and the cellular population of heated cells in the S phase was significantly increased in comparison with that of non-heated cells. When WiDr cells were exposed to VP-16 for 1 h, the cells were accumulated at the G2+M phase by 24 h after exposure to the drugs, and the accumulation of preheated cells at the G2+M phase was enhanced (Table I). In addition, the accumulation of G2+M-phase cells by 24 h after exposure to VP-16 was increased to a greater extent when VP-16 was administered after heat stress compared to administration of heat or etoposide alone (Table I). Hyperthermic treatment at 43°C for 1 h thus appears to accelerate the VP-16-induced irreversible arrest of WiDr colorectal cancer cells at the G2+M phase. Table I also indicates that the untreated cells showed very different cell cycle populations at 4 h and 28 h. In these experiments, 1×10^6 cells were seeded in 35-mm Petri dishes after trypsinization of the monolayer culture, and cell cycle analysis was performed 27 h (4 h after heat) and also 51 h (28 h after heat) after the trypsinization (see Fig. 5). The colorectal cells at 4 h after heat appeared to be in the exponential growth state, and those at 28 h in the late log-phase or sub-confluent state. The altered cell population at these growth phases might be responsible for the difference between 4 h and 28 h in Table I.

In conclusion, we found delayed enhancement of the cytotoxicity of topoisomerase II inhibitors after exposure of human colon cancer cells to hyperthermia at a temperature of 43°C. The delayed increment of target enzymatic level in the cells after hyperthermic exposure is suggested to be involved in the delayed enhancement of cytotoxicity by topoisomerase II inhibitors. The biomodulation of the cytotoxicity of topoisomerase II-targeting drugs could be a consequence of the different kinetics of topoisomerase II gene expression in response to preheating at different temperatures. The combination of hyperthermia and delayed arterial infusion of topoisomerase II-targeting agent should be clinically useful for the treatment of metastatic colon cancer in the liver. Further *in vivo* study is needed, however, before the clinical application of this combination therapy.

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REFERENCES

1) Liu, L. F. DNA topoisomerase poisons as antitumor drugs. *Ann. Rev. Biochem.*, **58**, 351–375 (1989).
2) D'Arpa, P. and Liu, L. F. Topoisomerase-targeting antitumor drugs. *Biochim. Biophys. Acta*, **989**, 163–177 (1989).
3) Osheroff, N., Zechiedrich, E. L. and Gale, K. C. Catalytic function of DNA topoisomerase II. *Bioassays*, **13**, 269–273 (1991).
4) Ferguson, P. J., Fisher, M. H., Stephenson, J., Li, D., Zhou, B. and Cheng, Y. Combined modalities of resistance in etoposide-resistant human KB cell lines. *Cancer Res.*, **48**, 5956–5964 (1988).
5) Matsuo, K., Kohno, K., Takano, H., Sato, S., Kiue, H. and Kuwano, M. Reduction of drug accumulation and DNA topoisomerase II activity in acquired teniposide-resistant human cancer KB cell lines. *Cancer Res.*, **50**, 5819–5824 (1990).
6) Takano, H., Kohno, K., Ono, M., Uchida, Y. and Kuwano, M. Increased phosphorylation of DNA topoisomerase II in etoposide-resistant mutants of human cancer KB cells. *Cancer Res.*, **51**, 3951–3957 (1991).
7) Takano, H., Kohno, K., Matsuo, M., Matsuda, T. and Kuwano, M. DNA topoisomerases-targeting antitumor agents and drug resistance. *Anti-Cancer Drugs*, **3**, 323–330 (1992).
8) Matsuo, K., Kohno, K., Sato, S., Uchiumi, T., Tanimura, H., Yamada, Y. and Kuwano, M. Enhanced expression of the DNA topoisomerase II gene in response to heat shock stress in human epidermoid cancer KB cells. *Cancer Res.*, **53**, 1085–1090 (1993).
9) Li, G. C. Heat shock proteins: role in thermotolerance, drug resistance, and relationship to DNA topoisomerases. *Ned. Chem. Ind. Monogr.*, **4**, 99–103 (1987).
10) Fisher, G. A. and Hahn, G. M. Enhancement of cisplatinum (II) diamminedichloride cytotoxicity by hyperthermia. *Natl. Cancer Inst. Monogr.*, **61**, 255–267 (1982).
11) Bertino, J. R., Kowal, C. D., Klein, M. E., Dombrowski, J. and Mini, E. The potential for chemotherapy and hyperthermia. *In* "Hyperthermia and Radiation Therapy; Chemotherapy in the Treatment of Cancer," ed. J. M. Valth, pp. 162–170 (1984). Karger AG, Basel.
12) Engelhardt, R. Hyperthermia and drugs. *Rec. Res. Cancer Res.*, **104**, 136–203 (1987).
13) Hahn, G. M. and Li, G. C. Interactions of hyperthermia and drugs: treatments and probes. *Natl. Cancer Inst. Monogr.*, **61**, 317–323 (1982).
14) Corry, P. M., Robinson, S. and Getz, S. Hyperthermic effects on DNA repair mechanisms. *Radiology*, **123**, 475–482 (1977).
15) Jorritsma, J. B. M. and Konings, A. W. T. Inhibition of repair of radiation-induced strand breaks by hyperthermia, and its relationship to cell survival after hyperthermia alone. *Int. J. Radiat. Biol.*, **43**, 505–516 (1983).
16) Toffoli, G., Beviliacqua, C., Franceschin, A. and Boiocchi, M. Effect of hyperthermia on intracellular drug accumulation and chemosensitivity in drug-sensitive and drug-resistant P388 leukemia cell lines. *Int. J. Hyperthermia*, **5**, 163–172 (1989).
17) Cohen, J. D., Robins, H. I. and Schmitt, C. L. Tumoricidal interactions of hyperthermia with carboplatin, cisplatin and etoposide. *Cancer Lett.*, **44**, 205–210 (1989).
18) Kampinga, H. H., Kruk, G. V. D. and Konings, A. W. T. Reduced DNA break formation and cytotoxicity of the topoisomerase II drug 4′-(9′-acridinylamino)methanesulfon-m-anisidide when combined with hyperthermia in human and rodent cell lines. *Cancer Res.*, **49**, 1712–1717 (1989).
19) Dynlancht, J. R., Wong, R. S. L., Albright, N. and Dewey, W. C. Hyperthermia can reduce cytotoxicity from Etoposide without a corresponding reduction in the number of topoisomerase II-DNA cleavable complexes. *Cancer Res.*, **54**, 4129–4137 (1994).
20) Hidaka, K., Endo, H., Akiyama, S. and Kuwano, M. Isolation and characterization of amphotericin B-resistant cell lines in Chinese hamster cells. *Cell*, **14**, 415–421 (1978).
21) Soejima, S., Hidaka, K., Kuwano, M. and Hisatsugu, T. Hyperthermic sensitivity and cholesterol levels of mammalian cell lines in culture. *Cancer Lett.*, **60**, 159–167 (1991).
22) Ross, W. E., Rowe, T., Glisson, B., Yalowich, J. and Liu, L. F. Role of topoisomerase II in mediating epipodophyllotoxin induced DNA cleavage. *Cancer Res.*, **44**, 5857–5860 (1984).
23) Glisson, B. S., Killary, A. M., Merta, P., Ross, W. E., Siciliano, J. and Siciliano, M. J. Dissociation of cytotoxicity and DNA cleavage activity induced by topoisomerase II-reactive intercalating agents in hamster-human somatic cell hybrids. *Cancer Chemother. Pharmacol.*, **31**, 131–138 (1992).
24) Heck, M. M. S., Hittelman, W. N. and Earnshow, W. C. Differential expression of DNA topoisomerase I and II during the eukaryotic cell cycle. *Proc. Natl. Acad. Sci. USA*, **85**, 1086–1090 (1988).
25) Drake, F. H., Hofmann, G. A., Bartus, H. F., Mattern, M. R., Crooke, S. T. and Mirabelli, C. K. Biochemical and pharmacological properties of p170 and p180 forms of topoisomerase II. *Biochemistry*, **28**, 8154–8160 (1989).
26) Woessner, R. D., Mattern, M. R., Mirabelli, C. K., Johnson, R. K. and Drake, F. H. Proliferation- and cell cycle-dependent differences in expression of the 170 kilodalton and 180 kilodalton forms of topoisomerase II in NIH-3T3 cells. *Cell Growth Differ.*, **2**, 209–214 (1991).
27) Estey, E., Adlakha, R. C., Hittelman, W. N. and Zwelling, L. A. Cell cycle stage dependent variations in drug-induced topoisomerase mediated DNA cleavage and cytotoxicity. *Biochemistry*, **26**, 4338–4344 (1987).
28) Paul, J. "Cell and Tissue Culture," pp. 30–33 (1975). Churchill Livingstone, Edinburgh, London and New

York.
29) Westra, A. and Dewey, W. C. Variation in sensitivity to heat shock during the cell-cycle of Chinese hamster cells *in vitro*. *Int. J. Radiat. Biol.*, **19**, 467–477 (1971).
30) Sullivan, D. M., Glisson, B. S., Hodges, P. K., Smallwood-Kentro, S. and Ross, W. E. Proliferation dependence of topoisomerase II mediated drug action. *Biochemistry*, **25**, 2248–2256 (1986).
31) Lock, R. B. and Ross, W. E. Inhibition of p34cdc2 kinase by etoposide or irradiation as a mechanism of G2 arrest in Chinese hamster ovary cells. *Cancer Res.*, **50**, 3761–3766 (1990).
32) Bertrand, R., Kerrigan, D., Sarang, M. and Pommier, Y. Cell death induced by topoisomerase inhibitors. Role of calcium in mammalian cells. *Biochem. Pharmacol.*, **42**, 77–85 (1991).