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Augmentation of antiproliferative activity of CPT-11, a new derivative of camptothecin, by tumor necrosis factor against proliferation of gynecologic tumor cell lines

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The co-operative effects of recombinant human tumor necrosis factor (rH-TNF) and CPT-11, a new derivative of camptothecin, against the proliferation of human gynecologic tumor cell lines were examined *in vitro*. The Ishikawa cells were responsive to rH-TNF, the HHUA cells exhibited a minimal degree of responsiveness to rH-TNF, and the HeLa S3 and Caov-3 cells were unresponsive to rH-TNF. The HHUA, Ishikawa and Caov-3 cells were responsive to CPT-11, and the HeLa S3 cells were relatively sensitive to CPT-11 cytotoxicity. In all four cell lines, rH-TNF at clinically achievable concentrations exhibited synergy with CPT-11. The combination therapy of rH-TNF and CPT-11 will be a new approach against gynecologic cancers.

Key words: Camptothecin analog, DNA topoisomerase I targeted agent, gynecologic tumor cell line, synergistic antiproliferative activity, tumor necrosis factor.

Introduction

Camptothecin, an antitumor alkaloid isolated from *Camptotheca acuminata*,[1] is a potent inhibitor of DNA synthesis and has shown significant antitumor activity against experimental animal tumor models.[2] However, this compound has been a disappointment because of both its low response rate in clinical trials and significant myelotoxicity.[3,4] The recent demonstration that DNA topoisomerase I is the main, if not exclusive, target of camptothecin[5] has revived interest in research on camptothecin analogs as antitumor agents. Efforts have been directed at the synthesis of new camptothecin derivatives with higher antitumor activity, less toxicity, and high aqueous solubility. One of them, 7-ethyl-10-[4-(1-piperidino)-1-piperidino]carbonyloxy camptothecin (CPT-11) (Figure 1) has shown potent antitumor activity against various kinds of murine tumors.[6] Early clinical trials are now underway in Japan.[7,8]

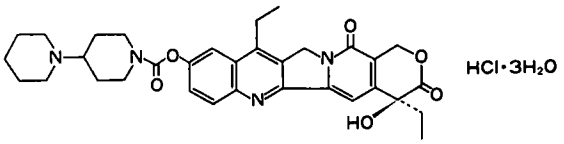

Figure 1. Chemical structure of CPT-11.

The gene encoding tumor necrosis factor (TNF) has been cloned and expressed in *Escherichia coli*, and highly purified recombinant human TNF (rH-TNF) is now available for clinical administration.[9] Since rH-TNF used alone in clinical phase I trials of patients with various malignancies has been relatively inactive,[9] the potential activity of rH-TNF may be apparent when the agent is combined with cytotoxic chemotherapeutic agents. Recent studies have demonstrated that rH-TNF significantly enhanced the cytotoxicity of chemotherapeutic drugs that inhibit the nuclear enzyme topoisomerase II *in vitro*[10] and *in vivo*.[11] In this study, we examined the co-operative effects of CPT-11 and rH-TNF against the proliferation of four gynecologic tumor cell lines.

Materials and methods

Cell lines

Endometrial carcinoma cell lines of HHUA and Ishikawa were gifts from Dr T. Fujimoto, Kyoto

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Prefectural University of Medicine, Kyoto, Japan. HeLa S3 derived from an epitheloid carcinoma of human uterine cervix and Caov-3 derived from a human ovarian adenocarcinoma were obtained from the American Type Culture Collection, Rockville, MD. HHUA and HeLa S3 cell lines were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS). Ishikawa cells were grown in Eagle's minimum essential medium (MEM) with 10% FBS, and Caov-3 cells were grown in Dulbecco's modified MEM with 10% FBS and 4.5 mg/ml glucose. All cell lines in monolayer were passaged once or twice weekly and incubated at 37°C in 5% CO_2.

Agents

CPT-11 was a generous gift from Yakult Co. Ltd. (Tokyo, Japan). rH-TNF was kindly supplied by Dainippon Pharmaceutical Co. Ltd. (Osaka, Japan). The titer of rH-TNF was expressed as U/ml of the Japanese rH-TNF reference (J-PS5K01; National Institute of Health, Tokyo, Japan), which is based on the cytotoxic activity in murine fibroblast L-M cells. A single lot of rH-TNF with a specific activity of 2.9×10^6 U/mg protein was employed in this study.

Antiproliferative assay

Antiproliferative activity was measured by the tetrazolium-based colorimetric assay.[12] The cells were seeded in 96-well microplates and allowed to adhere overnight before drug addition. The cell density used was 1×10^4 cells/well, which was selected in order to maintain the cells in an exponential phase of growth and to obtain a linear relation between absorbance and cell number at the end of the incubation time. Dilutions of CPT-11 and rH-TNF were made in the cell culture medium, and were added to the cells to a final volume of 0.2 ml/well; the cells were incubated for 72 h at 37°C in 5% CO_2. After incubation, dead cells which had detached from the surface of wells were removed by washing with phosphate-buffered saline (PBS), and 0.1 ml of culture medium and 10 μl of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution (5 mg/ml in PBS; Sigma, St. Louis, MO) were added. After 4 h at 37°C for MTT cleavage, the formazan product was solubilized by the addition of 0.1 ml of 0.04 N HCl in isopropanol. The optical density (OD) of each well was measured with a microphotometer (MTP-12; Corona Electric, Ibaragi, Japan), using a test wavelength of 577 nm and a reference wavelength of 630 nm. The relative percentage viability was calculated by the formula [mean OD (drug-treated)/mean OD (drug-untreated)] × 100, where mean OD represents the average from the triplicate determinations (the coefficient of variance was within 7%). The dose-effect data were analysed by linear regression using the median effect equation,[13] and IC_{50}, the dose required to yield 50% inhibition of cell growth, was calculated from the linear regression line.

Evaluation of the combined effect

The expected additive effect of the two agents was calculated by the formula $A \times B \div 100$, where A is the relative percentage viability of cells treated with rH-TNF, and B is the relative percentage viability of those treated with CPT-11. When the combination of rH-TNF and CPT-11 resulted in augmentation of the expected additive effect by more than 20%, the interaction of the two agents was defined as synergistic. The range for additive interaction was taken to be the expected additive effect $\pm$20%. The enhancing effect of rH-TNF on the cytotoxicity of CPT-11 was evaluated in terms of the modification index (MI). MI was calculated by the formula (IC_{50} to CPT-11 alone)/(IC_{50} to CPT-11 in the presence of rH-TNF).

Statistical analysis

All experiments were performed in triplicate and repeated at least three times to ensure reproducibility of the results. The data presented are from representative experiments. The relative percentage viabilities were compared by Student's t-test. Any result with p less than 0.05 was considered significant.

Results

Antiproliferative effects of rH-TNF and CPT-11 as single agents

The dose–response curves of the four cell lines to rH-TNF are shown in Figure 2. The Ishikawa cells showed dose-dependent responsiveness to rH-TNF (IC_{50}: 91 U/ml). The HHUA cells exhibited a

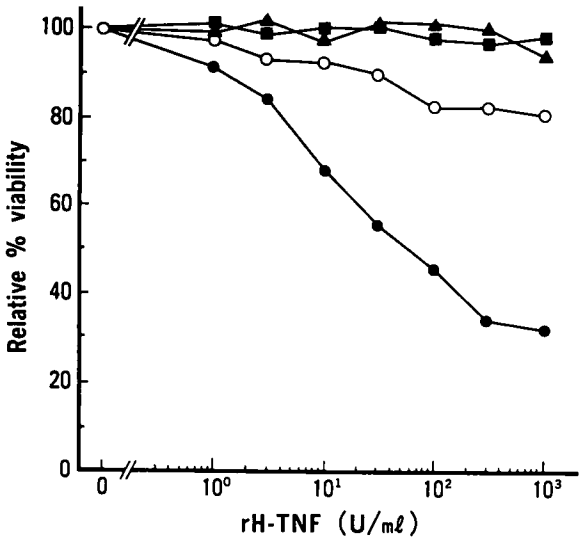

Figure 2. Effects of rH-TNF against proliferation of HHUA (○), Ishikawa (●), HeLa S3 (■), and Caov-3 (▲) cell lines. Cells were incubated for 72 h with rH-TNF at various concentrations. Points are means of three experiments each assayed in triplicate.

minimal degree of responsiveness to rH-TNF; they showed 19% inhibition of their viability at the highest concentration (1000 U/ml) of rH-TNF tested. The HeLa S3 and Caov-3 cells were unresponsive to concentrations of up to 1000 U/ml of rH-TNF. The HHUA, Ishikawa and Caov-3 cells were responsive dose-dependently to CPT-11 (Figure 3). The mean values of IC_{50} in the HHUA, Ishikawa and Caov-3 cells were 6.76, 7.59 and 4.77 μg/ml, respectively. The HeLa S3 cells showed 29% inhibition of their viability at the highest concentration (10 μg/ml) of CPT-11 tested.

Antiproliferative effect of rH-TNF and CPT-11 in combination

Simultaneous incubation of all cell lines with rH-TNF and CPT-11 for 72 h resulted in an inhibition of cell viability (Figure 3). In all four cell lines, rH-TNF at concentrations of 10 and 100 U/ml exhibited synergy with CPT-11 in the range of

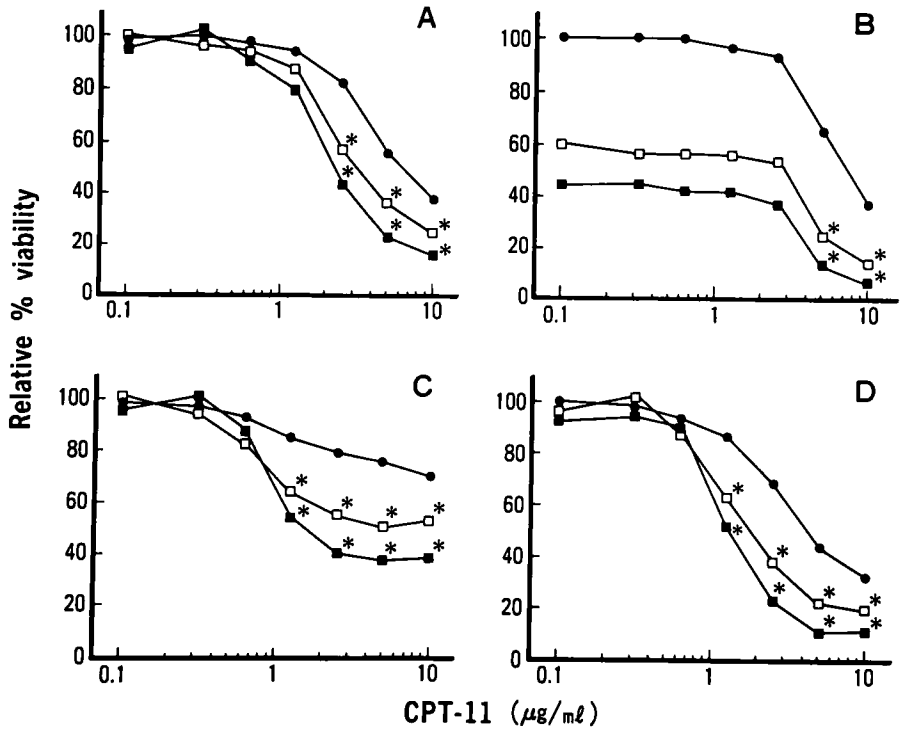

Figure 3. Effects of CPT-11 and rH-TNF, singly and in combination, against proliferation of HHUA (A), Ishikawa (B), HeLa S3 (C), and Caov-3 (D) cell lines. Cells were incubated for 72 h with CPT-11 at various concentrations, in the absence (●) or presence of rH-TNF at concentrations of 10 U/ml (□) or 100 U/ml (■). Points are means of three experiments each assayed in triplicate. *20% or more cell growth inhibition than the expected additive effect.

concentrations where CPT-11 alone exerted more than 10% growth inhibition. For example, in the HHUA cells, an expected additive effect by 100 U/ml of rH-TNF and 2.5 μg/ml of CPT-11 combined could be estimated to correspond to 32% inhibition of the viability, but the actual inhibition was 56%. However, the interaction between rH-TNF at concentrations of 10 and 100 U/ml and CPT-11 at non-toxic concentrations, where CPT-11 alone showed less than 10% cytotoxicity, was only additive. The IC_{50} values of CPT-11 in the presence or absence of rH-TNF at concentrations of 10 and 100 U/ml are shown in Table 1. The MI values indicate that, in all four cell lines, rH-TNF at 10 and 100 U/ml enhanced the cytotoxicity of CPT-11; the mean MI values by rH-TNF at concentrations of 10 and 100 U/ml were 2.94 and 4.75, respectively. The results of the order of addition studies are shown in Table 2. In all four cell lines, the responsiveness to CPT-11 was not affected by preincubation with rH-TNF, whereas sequential treatment with CPT-11 followed by incubation with rH-TNF resulted in significant enhancement of the cell growth inhibition.

Discussion

Topoisomerase inhibitors comprise an important group of anticancer drugs. Camptothecin is a specific inhibitor of mammalian DNA topoisomerase I which catalyses changes in the topological state of duplex DNA by performing single-strand breakage-resealing cycles.[14] CPT-11 is a new derivative of camptothecin, and a good candidate for clinical trials because of higher antitumor activity, less toxicity, and high aqueous solubility.[6–8] Indeed, the HHUA, Ishikawa and Caov-3 cells were highly responsive to CPT-11, and the HeLa S3 cells were relatively sensitive to CPT-11 (Figure 3).

Although TNF has potent antitumor activity against some kinds of tumor cells, about 60% of tumor cell lines are resistant to the growth inhibitory effect of TNF despite the fact that they express similar numbers of TNF receptors as sensitive tumor cells.[15] In this study, the Ishikawa cells were responsive to rH-TNF, the HHUA cells exhibited a minimal degree of responsiveness to rH-TNF, and the HeLa S3 and Caov-3 cells were unresponsive to rH-TNF (Figure 2). Unfortunately the dramatic antitumor effects predicted by the preclinical models have not been apparent in the phase I and phase II clinical studies of rH-TNF.[16–18]

Previous studies have demonstrated that some biological response modifiers (BRMs) can enhance

Table 1. Combination effects of rH-TNF on IC_{50} to CPT-11

Cell line	Concentration of rH-TNF (U/ml)	IC_{50}[a] to CPT-11 (μg/ml)	MI[b]
HHUA	0	6.76	
	10	3.96	1.71
	100	2.69	2.51
Ishikawa	0	7.59	
	10	1.94	3.91
	100	1.15	6.60
HeLa S3	0	21.93	
	10	5.49	3.99
	100	3.12	7.03
Caov-3	0	4.77	
	10	2.22	2.15
	100	1.68	2.84

[a] IC_{50} value was calculated from the dose-effect data shown in Figure 3.
[b] MI was determined as the ratio of IC_{50} to CPT-11 alone to IC_{50} to CPT-11 in the presence of rH-TNF.

Table 2. Effects of rH-TNF or CPT-11 against proliferation of HHUA, Ishikawa, HeLa S3, and Caov-3 cell lines pretreated with CPT-11 or rH-TNF

Cell line	First incubation (12 h)	Second incubation (60 h)	Relative viability (%)[a]
HHUA	Medium alone	CPT-11	61.7 ± 3.2[b]
	TNF	CPT-11	63.0 ± 4.2[b]
	Medium alone	TNF	85.5 ± 6.5[c]
	CPT-11	TNF	59.6 ± 3.9[c]
Ishikawa	Medium alone	CPT-11	70.2 ± 4.3[b]
	TNF	CPT-11	67.0 ± 5.3[b]
	Medium alone	TNF	54.9 ± 3.8[c]
	CPT-11	TNF	20.4 ± 1.8[c]
HeLa S3	Medium alone	CPT-11	84.2 ± 5.4[b]
	TNF	CPT-11	82.0 ± 6.6[b]
	Medium alone	TNF	97.5 ± 3.3[c]
	CPT-11	TNF	60.7 ± 4.6[c]
Caov-3	Medium alone	CPT-11	55.8 ± 3.4[b]
	TNF	CPT-11	57.3 ± 4.6[b]
	Medium alone	TNF	98.0 ± 2.5[c]
	CPT-11	TNF	62.3 ± 4.7[c]

Cells were preincubated for 12 h with 100 U/ml of rH-TNF, 5.0 μg/ml of CPT-11 or culture medium (controls) (first incubation). Medium was aspirated and the cells were washed twice with Hanks' balanced salt solution. The cells were then incubated for 60 h with 5.0 μg/ml of CPT-11 or 100 U/ml of rH-TNF (second incubation).
[a] Mean ± SE of four determinations.
[b] Not significant, $p > 0.1$ by Student's *t*-test.
[c] $p < 0.01$ by Student's *t*-test.

the sensitivity of tumor cells to conventional chemotherapeutic agents, and TNF is one of the promising BRMs for this purpose.[10,11,18,19] In this study, we focused our attention on the modulatory effect of rH-TNF on the cytotoxicity of CPT-11 against the proliferation of gynecologic tumor cell lines. The results shown in Figure 3 indicate that the interaction between rH-TNF and CPT-11 was synergistic in all four cell lines. However, at non-toxic concentrations of CPT-11, the combined use of rH-TNF was ineffective, suggesting that modulation of the CPT-11 effect occurs only at concentrations of CPT-11 which result in at least some diminution of cell viability. Interestingly, a low concentration of rH-TNF (10 U/ml) enhanced about three times the cytotoxicity of CPT-11 (Table 1). Ten U/ml of rH-TNF is a clinically relevant plasma level, 19-fold lower than the peak plasma concentration in patients administered the maximum tolerated dose of rH-TNF (a 30-min infusion of 1×10^6 U/body).[21] Sequential treatment of tumor cells with CPT-11 followed by incubation with rH-TNF was also synergistic, but not vice versa (Table 2). Hence, pretreatment with rH-TNF does not appear to render tumor cells more vulnerable to subsequent killing by CPT-11, but coincident or subsequent treatment with rH-TNF may augment the sensitivity of tumor cells to the cytotoxicity of CPT-11.

The exact mechanism of the synergistic interaction between rH-TNF and CPT-11 remains unclear and several explanations are possible. Camptothecin is known to exert its maximum lethal cytotoxicity against cells in the S phase of the cell cycle.[22,23] Darzynkiewicz *et al.*[24] showed that, in the TNF-resistant human leukemic cell line HL-60, rH-TNF initially induced a transient arrest in the G_2 phase while later it delayed progression through the G_1 phase. Therefore, it is unlikely that rH-TNF enhances the cytotoxicity of CPT-11 by increasing the proportion of cells in the S phase, although the cytokinetic effect of rH-TNF on the four cell lines tested has not been established. Utsugi *et al.*[25] have observed that rH-TNF treatment of murine L929 fibrosarcoma cells produced a rapid and transient increase in specific activity of extractable topoisomerases I and II, resulting in a potentiation of topoisomerase inhibitor-induced DNA strand breakage. Kanzawa *et al.*[26] have reported that the low activity of CPT-11-resistant cells on the intracellular formation of 7-ethyl-10-hydroxy camptothecin (SN-38), which is an active metabolite of CPT-11, and decreased total activity of topoisomerase I in CPT-11-resistant cells could be considered as the resistance mechanisms. Therefore, it is possible that rH-TNF may increase the level of SN-38 or topoisomerase I in tumor cells, resulting in higher sensitivity to CPT-11 cytotoxicity. It is also possible that rH-TNF may render tumor cells more sensitive to CPT-11 cytotoxicity by increasing membrane permeability and thus an influx of CPT-11. These possibilities are presently under investigation.

In summary, we have shown that rH-TNF at clinically achievable levels enhances the cytotoxicity of CPT-11, a new DNA topoisomerase I targeted agent, *in vitro*. Clearly, the combination of rH-TNF with CPT-11 requires further study *in vivo* and should be taken into consideration in the planning of future animal and human trials using rH-TNF for the treatment of gynecologic cancers.

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