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Potentiation of Topoisomerase I and II Inhibitors Cell Killing by Tumor Necrosis Factor: Relationship to DNA Strand Breakage Formation

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Recombinant human tumor necrosis factor (rHuTNF) synergistically potentiates the cytotoxicity of the topoisomerase I inhibitor camptothecin, and the topoisomerase II inhibitors epidoxorubicin, etoposide, mitoxantrone, ellipticine, actinomycin D and 4′-(9-acridinylamino)methanesulfon-*m*-anisidide on A2780 human ovarian cancer cell line. Similar synergy was not observed with a combination of rHuTNF and *cis*-platinum or mitomycin C. When A2780 cells were incubated with rHuTNF simultaneously with camptothecin or mitoxantrone or VP16, increased numbers of DNA single-strand breaks were produced. rHuTNF alone did not induce DNA strand breakage. These data provide evidence that the enhancing effect of rHuTNF is closely related to the DNA damage mediated by topoisomerase-targeted drugs. These observations may have relevance for ovarian cancer treatment.

Key words: TNF — Topoisomerase — DNA breakage — Cytotoxicity — Ovarian cancer

The availability of recombinant cytokines [tumor necrosis factor (TNF), interferon, interleukin 2, etc.] renders combination therapies involving cytokines and classical chemotherapeutic drugs possible. Significant improvements in experimental cancer therapies have been made by using such combinations. Previous work from our and other laboratories showed a synergistic cytotoxic effect when TNF is given simultaneously with DNA topoisomerase II-targeted anti-cancer drugs, such as etoposide (VP16) and mitoxantrone (MITO), in murine and human cancer cell lines *in vitro*[1-7] and *in vivo*.[8-11]

A human ovarian cancer cell line, A2780, was evaluated for sensitivity to human recombinant TNF (rHuTNF) in the range 0.1–10000 U/ml. The dose-response curve is shown in Fig. 1. A2780 cells were very highly responsive to rHuTNF with an IC_{50} (50% inhibitory concentration) of 8.8 U/ml. The IC_{50} of L929 cells, a standard reference line, was 10000 U/ml (Fig. 1). Thus, A2780 cells were 1136-fold more sensitive to rHuTNF than L929 cells.

The effects of rHuTNF, at equiactive concentrations in respect of each drug, on the cytotoxicity of a topoisomerase I inhibitor [camptothecin (CPT)] and topoisomerase II inhibitors [epidoxorubicin (epiDOXO), VP16, MITO, ellipticine (EPT), actinomycin D (DACT), 4′-(9-acridinylamino) methanesulfon - *m* - anisidide (m-AMSA)], were also studied in A2780 cell line.

Table I depicts the IC_{50} values obtained with each drug without and with rHuTNF. As shown in Table I, rHuTNF significantly enhanced the cytotoxicity of the topoisomerase I- and II-targeted drugs.

The non-topoisomerase-targeted drugs *cis*-platinum (CDDP) and mitomycin C (MTC) demonstrated no synergy when combined with rHuTNF, under the same conditions as shown in Table I. The potentiation rate, expressed as the ratio of drug IC_{50} without and with rHuTNF, was 15.2 for CPT, 168.0 for VP16, 62.5 for epiDOXO, 13.08 for DACT, 10.0 for MITO, 2.7 for EPT and 1.8 for m-AMSA.

The dose-response curve for each drug, with the exception of MITO, since we have previously demonstrated a strong potentiation of MITO by rHuTNF,[5,7] is shown in Fig. 2 (from panel A to panel F). When rHuTNF at equitoxic concentrations with respect to CPT dosages (panel A) or to epiDOXO (panel B) or to DACT (panel C) or to VP16 (panel D) was added simultaneously with each drug, a very significant enhancement of cytotoxicity was seen on A2780 cells. This enhancement of efficacy can not be a simple additive effect, because the effect was much greater than the extrapolated value for twice the concentration of each drug alone. For EPT (panel E) or for m-AMSA (panel F) only a simple additive effect was observed, because the effect was approximately the same as the extrapolated value for a double dose of the drug alone.

To define the role of TNF in the augmentation of

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tumor cell killing by topoisomerase I and II inhibitors, the effect of rHuTNF on the production of enzyme-linked DNA strand-breaks induced in A2780 cells by topoisomerase inhibitors was determined. Drug-induced DNA strand-breaks were measured by the alkaline elution technique according to Russo *et al.*[12] rHuTNF alone (1000 U/ml) did not induce DNA strand-breaks. When rHuTNF was incubated simultaneously with either CPT or VP16 or MITO for 60 min, increased numbers of DNA strand-breaks were generated by these three topoisomerases inhibitors (Table II). For VP16 and MITO we have also verified that the potentiating effect is not confined to a single concentration, but spans a 1000-fold concentration range.

We had previously reported that rHuTNF could potentiate the cytotoxicity of MITO.[13] Here we present new evidence suggesting that this potentiating effect is probably a general property of topoisomerase inhibitors (seven drugs tested), rather than a property of a single

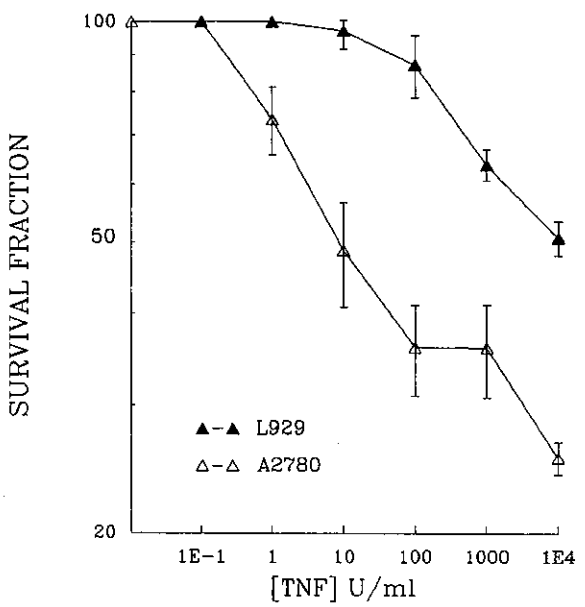

Fig. 1. Effect of rHuTNF on A2780 human ovarian cancer cell lines and on L929 murine fibrosarcoma cell line (Reference line). At 24 h after plating, cells were exposed to different concentrations of rHuTNF for an additional 20 h. Cytotoxicity was evaluated with the crystal violet assay[20] (the values are the average of at least three independent experiments performed in quadruplicate; error bars denote standard errors in this and in the following experiments).

Table I. Effect of rHuTNF on the Cytotoxic Effect of Different Topoisomerase I and II Inhibitors on A2780 Human Ovarian Cancer Cell Line

Drug	IC_{50} (μM)		rHuTNF potentiation ratio[b]
	−rHuTNF	+rHuTNF[a]	
CPT	0.792	0.0520	15.20
VP16	0.420	0.0025	168.00
EpiDOXO	3.500	0.0560	62.50
DACT	0.017	0.0013	13.08
MITO	0.002	0.0002	10.00
EPT	28.900	10.7500	2.70
m-AMSA	15.400	8.3800	1.80
CDDP	1.070	1.9900	NP
MTC	2.520	2.6180	NP

Values represent the average of at least three independent determinations.
a) The rHuTNF concentrations utilized for these experiments were equiactive in respect to mitoxantrone.
b) Degree of potentiation of each different line by rHuTNF expressed as ratio of the mitoxantrone IC_{50} without and with rHuTNF.
rHuTNF was obtained from KNOLL-BASF (Ludwigshafen, Germany). A stock solution of rHuTNF, containing 0.1 mg/ml of protein was stored at −80°C. Specific activity was 8.74×10^6 U/mg protein (48 h L929 bioassay without actinomycin D, as determined in the KNOLL-BASF laboratory). Drugs were diluted in RPMI 1640 with 10% serum to achieve appropriate final concentrations, and immediately used. Cytotoxicity was monitored with the crystal violet assay, as described by Ruff and Gifford[21] with minor modification.[5] The IC_{50} (50% inhibitory concentration) was obtained by linear interpolation. NP = not potentiated.

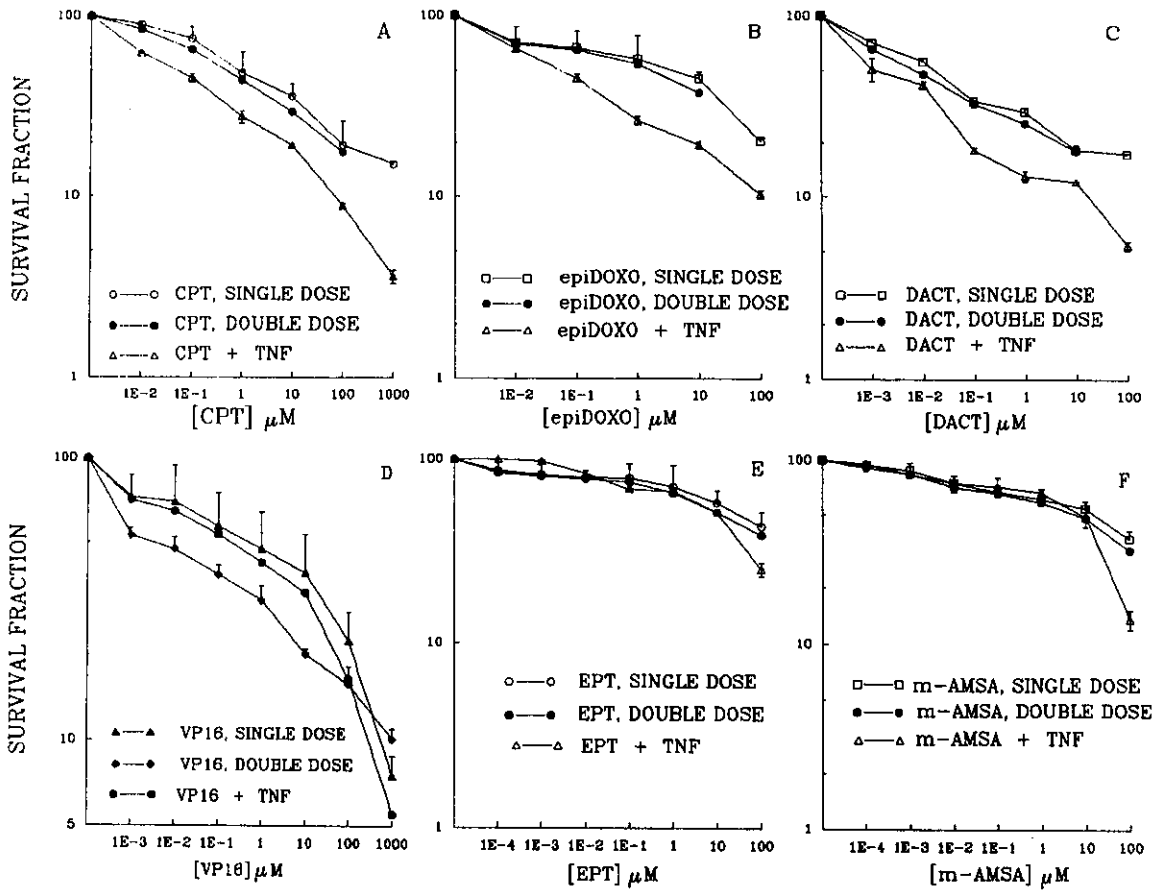

Fig. 2. Effect of rHuTNF on the cytotoxicity of the various DNA topoisomerase I and II inhibitors on A2780 human ovarian cancer cell line. Panel A: Effect of CPT and rHuTNF on A2780 ovarian cancer cell line. A2780 cell line was exposed 24 h after plating to various concentrations of CPT alone or in the presence of rHuTNF at equitoxic concentration for 20 h. The equitoxic dosages of rHuTNF were calculated in the following way: from the results of Fig. 1, we have interpolated the dosages of rHuTNF giving the same survival fraction as the respective dosages of CPT; these equitoxic dosages are as follows: (1)=0.25 U/ml; (2)=0.25 U/ml; (3)=24.38 U/ml; (4)=100.0 U/ml; (5) and (6)=1000 U/ml (1000 U/ml was always used when it was impossible to calculate the equitoxic dosage). Panel B: Effect of epiDOXO and rHuTNF on A2780 ovarian cancer cell line. The equitoxic dosages of rHuTNF are as follows: (1)=1.32 U/ml; (2)=2.63 U/ml, (3)=8.32 U/ml, (4)=20.32 U/ml, (5)=1000 U/ml. Panel C: Effect of DACT and rHuTNF on A2780 ovarian cancer cell line. The equitoxic dosages of rHuTNF are as follows: (1)=1.15 U/ml; (2)=4.68 U/ml; (3), (4), (5), (6)=1000 U/ml. Panel D: Effect of VP16 and rHuTNF on A2780 ovarian cancer cell line. The equitoxic dosages of rHuTNF are as follows: (1)=1.15 U/ml; (2)=1.48 U/ml; (3)=4.7 U/ml; (4)=14.1 U/ml; (5)=59 U/ml; (6) and (7)=1000 U/ml. Panel E: Effect of EPT and rHuTNF on A2780 ovarian cancer cell line. The equitoxic dosages of rHuTNF are as follows: (1)=0.29 U/ml; (2)=0.42 U/ml; (3) and (4)=0.59 U/ml; (5)=1.29 U/ml; (6)=4.07 U/ml; (7)=28.8 U/ml. Panel F: Effect of m-AMSA and rHuTNF on A2780 ovarian cancer cell line. The equitoxic dosages of rHuTNF are as follows: (1)=0.17 U/ml; (2)=0.24 U/ml; (3) and (4)=1.66 U/ml, (5)=2.75 U/ml; (6)=6.03 U/ml; (7)=83.2 U/ml.

drug. Non-topoisomerase inhibitor drugs such as CDDP and MTC appeared devoid of any potentiating effect (Table I).

Similarly, we had previously reported a parallelism between cytotoxicity and DNA breaks for MITO.[13] Here we have shown that this parallelism can be extended to two additional topoisomerase inhibitors CPT and VP16 (Table II). Despite their differences in mechanisms, biochemical requirements and cellular functions, topoisomerases I and II work by forming enzyme-bridged strand-breaks that act as transient gates for the passage of other DNA strands.[14] In the presence of these drugs, an aborted reaction intermediate, termed the "cleavable complex," accumulates. These intermediates are detected

Table II. Effect of rHuTNF (1000 U/ml) on Protein-concealed DNA Strand-breaks Induced by CPT, or VP16 or MITO

Treatment	Concentration (μM)	DNA single-strand breaks (rad equivalents)		
		−rHuTNF	+rHuTNF	
Medium control	—	0.0	0.0	
CPT	10.000	96.7±7.5	191.3±11.3	(97.8)
VP16	0.001	26.0±4.6	32.6±5.8	(25.4)
	0.010	71.7±9.5	104.3±11.1	(45.5)
	0.100	107.9±15.6	251.3±9.5	(132.9)
	1.000	397.0±25.7	402.6±11.5	(1.4)
MITO	0.100	0.0	28.2±4.6	−
	1.000	86.7±13.2	108.7±10.1	(25.4)
	10.000	134.5±10.4	184.5±8.8	(37.2)

A2780 cells were treated with drugs for 1 h at 37°C with or without rHuTNF (1000 U/ml) and then assayed for DNA strand-breaks by alkaline elution, under deproteinizing conditions. Results are expressed in terms of the X-ray dose that would produce an equivalent elution in single-strand-breaks (SSBs) assay, and the corresponding units are designated SSBs rad equivalents. A2780 cells were treated with different dosages of X-rays from a Cs source to obtain a standard curve (data not reported). Numbers in parentheses are percentage increases in DNA SSBs over the number produced by 1 h incubation with the topoisomerase inhibitor alone. All the experiments were performed at least in triplicate. The results are given as average±standard error.

as protein-associated DNA strand breaks in mammalian cells,[12, 15] and are equivalent to the enzyme-DNA-complexes observed in purified systems.[16] These complexes are likely to be lethal to cells if they are not removed. The results of elution experiments clearly demonstrate TNF-induced potentiation of "cleavable complex" formation following treatment with CPT or MITO or VP16. This would increase cellular sensitivity to killing by inhibitors such as CPT, or VP16, or MITO because the ability of these inhibitors to form toxic lesions is probably proportional to the number of cleavable complexes in the cell.

The strong cytotoxic synergy shown by MITO (potentiation rate 10) or by VP16 (potentiation rate 168) (in the range of concentrations achievable in the peritoneal cavity) or by CPT (potentiation rate 15.2) toward a human ovarian cancer cell line suggests potential clinical applications. Interestingly, VP16 and MITO are two agents particularly suitable for intraperitoneal chemotherapy of ovarian cancer[17, 18] and CPT, which is in a preliminary phase II study,[19] was suggested to be a clinically useful chemotherapeutic agent for ovarian cancer.

Finally, it is important to emphasize that rHuTNF clearly enhanced the cytotoxicity of topoisomerase I and topoisomerase II inhibitors at concentrations of rHuTNF that are clinically achievable ($\leq$5000 U/ml[20]). These observations may have relevance for ovarian cancer treatment. The combination therapy could be administered, by the intraperitoneal route, in ovarian cancer patients with advanced epithelial carcinomas and ascites who have relapsed after a standard chemotherapy regimen.

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REFERENCES

1) Alexander, R. B., Nelson, W. G. and Coffey, D. S. Synergistic enhancement by tumor necrosis factor of *in vitro* cytotoxicity from chemotherapeutic drugs targeted at DNA topoisomerase II. *Cancer Res.*, **47**, 2403–2406 (1987).
2) Coffmann, F. D., Green, L. M., Godwin, A. and Ware,

C. F. Cytotoxicity mediated by tumor necrosis factor in variant subclones of Me-180 cervical carcinoma line: modulation by specific inhibitors of DNA topoisomerase II. *J. Cell. Biochem.*, **39**, 95–105 (1989).
3) Utsugi, T., Mattern, M. R., Mirabelli, C. K. and Hanna, N. Potentiation of topoisomerase inhibitor-induced DNA strand breakage and cytotoxicity by tumor necrosis factor: enhancement of topoisomerase activity as a mechanism of potentiation. *Cancer Res.*, **50**, 2636–2640 (1990).
4) Baloch, Z., Cohen, S. and Coffman, F. D. Synergistic interactions between tumor necrosis factor and inhibitors of DNA topoisomerase I and II. *J. Immunol.*, **145**, 2908–2913(1990).
5) Vigani, A., Chiara, S., Miglietta, L., Repetto, L., Conte, PF., Cimoli, G., Morelli, L., Billi, G., Parodi, S. and Russo, P. Effect of recombinant tumor necrosis factor on A2774 human ovarian cancer cell line: potentiation of mitoxantrone cytotoxicity. *Gynecol. Oncol.*, **41**, 52–55 (1991).
6) Billi, G., Venturini, M., Piccini, D., Parodi, S., Rosso, R., Conte, PF. and Russo, P. Synergistic cytotoxic effects of recombinant human tumor necrosis factor and etoposide (VP16) or doxorubicin on A2774 human epithelial ovarian cancer cell line. *Int. J. Gynecol. Cancer*, **2**, 147–151 (1992).
7) Russo, P., Parodi, S., Billi, G., Oliva, C., Venturini, M., Noviello, E. and Conte, PF. Potentiation by tumor necrosis factor of mitoxantrone cytotoxicity to human ovarian cancer cell lines. *Jpn. J. Cancer Res.*, **83**, 684–687 (1992).
8) Alexander, R. B., Isaacs, J. T. and Coffey, D. S. Tumor necrosis factor enhances the *in vitro* and *in vivo* efficacy of chemotherapeutic drugs targeted at DNA topoisomerase II in the treatment of murine bladder cancer. *J. Urol.*, **138**, 427–429 (1987).
9) Das, A. K., Walther, P. J., Buckley, N. J. and Poulton, S. H. M. Recombinant human tumor necrosis factor alone and with chemotherapeutic agents. *Arch. Surg.*, **124**, 107–110 (1989).
10) Krosnick, J. A., Mulé , J. J., McIntsoh, J. K. and Rosemberg, S. A. Augmentation of antitumor efficacy by combination of recombinant tumor necrosis factor and chemotherapeutic agents *in vivo*. *Cancer Res.*, **49**, 3729–3733 (1989).
11) Bahnson, R. R. and Ratliff, T. L. *In vitro* and *in vivo* anti-tumor activity of recombinant mouse tumor necrosis factor (TNF) in a mouse bladder tumor (MTB–2). *J. Urol.*, **141**, 172–175 (1990).
12) Russo, P., Poggi, L., Parodi, S., Pedrini, A. M., Kohn, K. W. and Pommier, Y. Production of protein-associated DNA breaks by 8-methoxycaffeine, caffeine and 8-chlorocaffeine in isolated nuclei from L1210 cells: comparison with those produced by topoisomerase II inhibitors. *Carcinogenesis*, **12**, 1781–1790 (1991).
13) Cimoli, G., Valenti, M., Venturini, M., Conte, P. F. and Russo, P. Augmentation of antineoplastic effects by the combination of recombinant human tumor necrosis factor and mitoxantrone on primary culture of human ovarian cancer cells. *Anticancer Res.*, **12** (1992), in press.
14) D'Arpa, P. and Liu, L. Topoisomerase-targeting antitumor drugs. *Biochim. Biophys. Acta*, **989**, 163–177 (1989).
15) Pommier, Y., Minford, J. K., Schwartz, R. E., Zwelling, L. A. and Kohn, K. W. Effects of the intercalators 4′-(9-acridinylamino)methanesulfon-m-anisidide (m-AMSA, amsacrine) and 2-methyl-9-hydroxyellipticinium (2-Me-OH-E^+) on topoisomerase II-mediated DNA strand cleavage and passage. *Biochemistry*, **24**, 6410–6416 (1984).
16) Minford, J., Pommier, Y., Filipski, J., Kohn, K. W., Kerrigan, D., Mattern, M., Nichols, S., Schwartz, R. E. and Zwelling, L. A. Isolation of intercalator dependent protein-linked DNA strand cleavage activity and identification as topoisomerase II. *Biochemistry*, **25**, 9–16 (1986).
17) O'Dwyer, P. J., LaCreta, F. P., Daugherty, J. P., Hogan, M., Rosenblum, N. G., Rosemblum, J. L. and Comis, R. L. Phase I pharmacokinetic study of intraperitoneal etoposide. *Cancer Res.*, **51**, 2041–2046 (1991).
18) Markman, M., George, M., Hakes, T., Reichman, B., Hoskins, W., Rubin, S., Jones, W., Almandrones, L. and Lewis, J. L., Jr. Phase II trial of intraperitoneal mitroxantone in the management of refractory ovarian cancer. *J. Clin. Oncol.*, **8**, 146–150 (1990).
19) Umesaki, N., Takeuchi, S., Noda, K., Yakushiji, M. and Research Group of CPT-11 in Gynecological Cancer. The clinical effect of DNA topoisomerase inhibitors (CPT-11) on gynecological cancer. *Proc. Int. Symp. on DNA Topoisomerases in Chemotherapy (ISTOP 1991)*, 64 (1991).
20) Salmon, S. E., Young, L., Scuderi, P. and Clark, D. Antineoplastic effects of tumor necrosis factor alone and in combination with γ-interferon on tumor biopsies in clonogenic assay. *J. Clin. Oncol.*, **5**, 1816–1821 (1987).
21) Ruff, M. R. and Gifford, G. E. Tumor necrosis factor. *Lymphokines*, **2**, 235–272 (1981).