Anti-Cancer Drugs, 1, pp. 157–163

Effect of tumor necrosis factor on human tumor cell lines sensitive and resistant to cytotoxic drugs, and its interaction with chemotherapeutic agents

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We investigated the cytotoxic effects of recombinant tumor necrosis factor (TNF) alone and in combination with interferon-γ (IFN-γ) and/or cytotoxic drugs on a variety of human tumor cell lines (U937, IGROV-1, HT29, LoVo, MCF7 and U20S), including cell lines with *in vitro* acquired resistance (LoVo/DX and MCF7/DX selected for resistance to doxorubicin (DX) and characterized by pleiotropic drug resistance; U20S selected for resistance to cisplatin (CDDP)), using MTT assay. U937 and MCF7 were sensitive to the cytotoxic effect of TNF, whereas all the other cells were insensitive up to 1000 U/ml (the maximum tested dose). Surprisingly, TNF was cytotoxic (30–40% cytotoxicity) against two resistant lines (LoVo/DX and U20S/Pt) but not against the parent sensitive lines. Treatment with increasing doses of TNF after 6 h incubation with a subtoxic concentration of IFN-γ produced a synergistic effect in four cell lines (U937, HT29, LoVo/DX and MCF7), whereas in the other five the cell killing of the combination was comparable with that achieved by TNF alone. The combination of subtoxic doses of TNF and increasing doses of drugs targeted at DNA topoisomerase II (i.e. DX, actinomycin D and VP16) produced an additive cytotoxic effect in all cell lines. The same results were obtained combining TNF and CDDP, except in U20S/Pt cells in which TNF synergistically increased CDDP cytotoxicity. The combination of TNF and IFN-γ enhanced cytotoxicity about 20-fold for DX and 6-fold for CDDP, evaluated in terms of the modification index, against LoVo/DX and U20S/Pt cells respectively. These results indicate that TNF alone can be effective on a cell population resistant to conventional cytotoxic drugs. Moreover, the use of TNF in combination with antitumor drugs seems to be a promising therapeutic approach, in particular when IFN-γ is also combined.

Key words: Chemotherapeutic drugs, resistance, tumor necrosis factor.

Introduction

Tumor necrosis factor (TNF) is a polypeptide with a wide range of biological activities.[1] A growth inhibitory or cytolytic effect of TNF was documented in about 25% of tumor cell lines[2] and in tumor cells from cancer patients.[3] *In vivo* studies have demonstrated that its antitumor effects may be either direct or mediated via activation of an inflammatory response.[4,5] Recombinant human TNF has been developed[6,7] and is currently being investigated in phase I and II clinical trials.

The cytotoxic spectrum of TNF alone, however, is somewhat limited, since the sensitivity of tumor cells to TNF may vary even among cells of the same origin.[3] Therefore, with a view to future clinical applications of TNF, preclinical attempts are being made to potentiate its effect by combined use with other drugs, by immunomodulating agents or by different treatment modalities. Although a cytotoxic potentiation of TNF and an enhancement of its antitumor effects by combination with chemotherapeutic agents have already been reported,[8,9] nevertheless more detailed studies *in vitro* and *in vivo* are required. In the present study a panel of human tumor cell lines, sensitive and resistant to cytotoxic drugs, were utilized to evaluate (a) a possible relation between the cell response to TNF and development of drug resistance, (b) the interaction of TNF with IFN-γ and conventional cytotoxic drugs and (c) the role of the combination of TNF and IFN-γ as modulator of drug resistance.

Materials and methods

Cell cultures

The human monoblastoid cell line U937 (ATCC CRL 1593) was cultured in RPMI-1640 medium

This work was partially supported by a grant from the Ministero della Sanitá, Rome, Italy.

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plus 10% heat-inactivated fetal calf serum (FCS). IGROV-1, kindly supplied by Dr Benard (Institut Gustave Roussy, Villejuif, France), was cultured in RPMI-1640 supplemented with 10% heat-inactivated FCS. HT29 (ATCC HTB 38) and LoVo (ATCC CCL 229), adenocarcinomas of the colon, were maintained in Ham's F12 medium supplemented with 10% FCS and vitamins. LoVo/DX was obtained *in vitro* after treating LoVo cells with doxorubicin (DX).[10] MCF7, a human mammary carcinoma (ATCC HTB 22), was grown in DMEM containing 10% heat-inactivated FCS. MCF7/DX cells were selected that were able to grow in the presence of 10 μM DX.[11] U20S, a human osteosarcoma cell line (ATCC HTB 96), was grown in McCoy's 5A medium plus 15% heat-inactivated FCS. U20S/Pt cells were obtained in our laboratory after *in vitro* exposure of U20S to increasing concentrations of CDDP and were grown in the presence of 1 μg/ml CDDP. All cell lines were free of mycoplasms and were kept in 5% CO_2 solution in air at 37°C. Trypsin–EDTA was used to prepare single-cell suspensions.

Drugs

rh-TNF and rh-IFN-γ, obtained from Amersham Corp., had specific activities of $\geq 1 \times 10^7$ units/mg. Solutions of these drugs were stored at 4 °C. Just before the experiments, stock solutions were dissolved in medium to the required concentration. CDDP (Platinex) and VP16 (Vepesid) were obtained from Bristol Italiana Sud S.p.A. Actinomycin D (AcD; Cosmegen) was purchased from Merck Sharp and Dohme, and DX from Farmitalia Carlo Erba. The drugs were diluted with tissue culture medium immediately before use.

Cytotoxic assay

Cell survival was assessed by tetrazolium dye (MTT) assay.[12] In brief, cells were harvested from exponential-phase maintenance cultures, dispensed into 96-well culture plates (Costar Plastics 3799) in 100 μl volumes using a repeating pipet (Multipette 4780, Eppendorf) and treated with 10 μl of drug solution or medium for control wells. Each plate had 8 control wells and 8 wells for each dose. After incubation of the microtiter plates for 96 h, 10 μl of MTT working solution (5 mg/ml) was added to each culture well and cultures were incubated at 37 °C for 4 h. The culture medium was removed from the wells and replaced with 100 μl of DMSO using a multichannel pipet. The absorbance of each well was measured with a microculture plate reader (SLT Labinstruments, Austria) at 550 nm interfaced with an Apple computer. Preliminary experiments were performed to determine the appropriate seeding number of each cell line, after confirming a linear relation between absorbance and number of cells in the growth curve of each cell line: 10^3 cells/well for HT29; 2×10^3 cells/well for LoVo-LoVo/DX, MCF7-MCF7/DX, U20S and U937; 2.5×10^3 cells/well for IGROV-1; 3.5×10^3 cells/well for U20S/Pt. IC_{50} was defined as the drug concentration that produced 50% reduction of absorbance.

Analysis of drug interactions

According to the method of Kern *et al.*,[13] the expected value of cell survival (S_{exp}, defined as the product of the survival observed for drug A alone and the survival observed for drug B alone) and the actual survival observed (S_{obs}) for the combination of A and B were used to construct a synergistic ratio (R) as follows:

$$R = S_{exp}/S_{obs}$$

Synergy was defined as any value of R greater than unity. An R value of 1.0 (additive effect) or less indicated an absence of synergy. For the purpose of comparison, the effects of the single drugs and combinations were evaluated separately in each experiment.

Results

Effect of TNF

The cytotoxic effect of TNF on all the human tumor cell lines considered was examined in a large range of TNF doses (from 0.1 to 1000 U/ml) and the results are summarized in Table 1. Cytotoxic effects of TNF (30–40% cell killing) were observed in four of the cell lines, the other five being insensitive (less than 20% cytotoxicity) to the action of TNF up to 1000 U/ml (the highest dose tested). Moreover, in MCF7/DX cells resistance to DX was also associated with resistance to TNF. In fact the dose of 1000 U/ml of TNF, which inhibited survival in 80% of MCF7 cells, was ineffective on MCF7/DX cells. In contrast, in the treatment of LoVo and U20S cells, the dose of 1000 U/ml of TNF was

Table 1. TNF sensitivity of human malignant cell lines

Cell line	Dose (U/ml)	Percentage cell survival[a]	IC_{40} (U/ml)
U937	10	28 ± 19	0.9
	1	58 ± 29	
	0.1	91 ± 29	
IGROV-1	1000	100	>1000
	100	102 ± 16	
	10	111	
HT29	1000	81 ± 3	>1000
	100	85 ± 6	
	10	92 ± 5	
LoVo	1000	88 ± 9	>1000
	100	92 ± 9	
	10	102 ± 7	
LoVo/DX	1000	61 ± 10	100–1000
	300	59 ± 6	
	100	61 ± 5	
	30	69 ± 7	
	10	77 ± 12	
MCF7	1000	19 ± 3	30
	100	46 ± 9	
	10	72 ± 6	
MCF7/DX	1000	94 ± 3	>1000
	100	93 ± 4	
	10	103	
U20S	1000	97 ± 9	>1000
	100	103 ± 9	
	10	110	
U20S/PT	1000	66 ± 2	~1000
	300	71 ± 7	
	100	86 ± 6	
	30	84 ± 8	

[a] Values of at least three experiments.

effective on the resistant cells (40% and 30% cytotoxicity respectively) but not on the parental cell lines.

Effect of TNF and IFN-γ in combination

In the combined treatments with TNF and IFN-γ, increasing doses of TNF were added to the cells after 6 h preincubation with a fixed subtoxic dose of IFN-γ ($\leq IC_{20}$). Preincubation with IFN-γ was chosen because in preliminary experiments it was observed that the growth of some cells (i.e. HT29) was significantly more inhibited when treated first with IFN-γ and then with TNF. The results of representative experiments for each cell line reported in Table 2 show a different influence of IFN-γ on TNF cytotoxicity on the various cell lines. A comparison of R values, calculated according to Kern *et al.*, indicates that in U937, HT29, LoVo/DX and MCF7, IFN-γ synergistically enhanced the cytotoxic effect of TNF ($R > 2$); in LoVo, MCF7/DX, IGROV, U20S and U20S/Pt the combined treatment was additive ($0.8 > R > 1.3$).

When TNF and IFN-γ were added to the cells simultaneously, the R values decreased from 8.5, 6.3, 1.8 to 5.8, 3.5 and 1.3 in HT29 cells, and at the highest dose of TNF, from 2.8 to 1.2 in LoVo/DX.

Effect of TNF on cytotoxicity of anticancer drugs

The results of combined treatments of TNF and conventional cytotoxic agents (DX, AcD, VP16 and CDDP) expressed as R values are reported in Figure 1. The simultaneous combination of increasing concentrations of each drug and a fixed subtoxic dose of TNF produced an additive effect on all cell lines for all the drugs tested, the range of R values being between 0.8 and 1.1. In the only exception, U20S/Pt cells, the combination of increasing concentrations of CDDP (0.5–8 μg/ml) with a fixed dose of TNF (300 U/ml), caused a synergistic cytotoxic effect ($R = 2.6$) at the highest CDDP dose, as shown by the results of a representative experiment (Table 3).

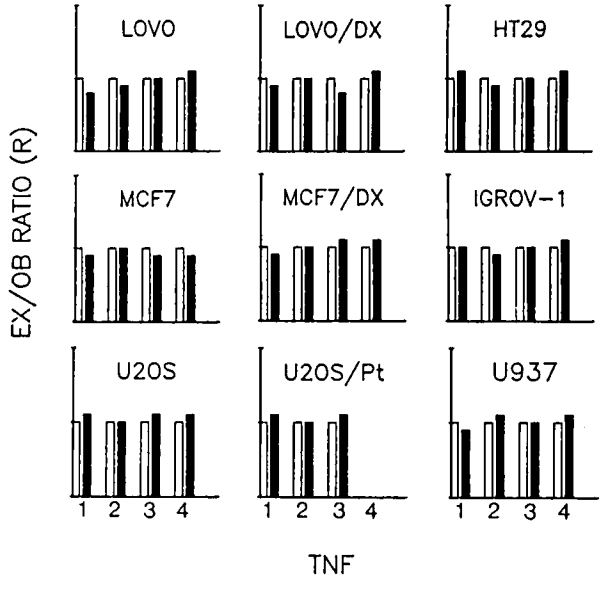

Figure 1. Effect of TNF combined with cytotoxic drugs on a panel of human tumor cell lines.

DX (1), AcD (2), VP16 (3), and CDDP (4) in the absence (□) or in the presence (■) of TNF at the concentration of 10^2 U/ml with the exception of MCF7 (10 U/ml) and U937 (10^{-1} U/ml). The EX/OB ratio is the ratio between the expected to the observed survival value.

Table 2. Cytotoxicity of TNF alone or after 6 h preincubation with a subtoxic dose of IFN-γ and synergistic ratios of the two agents in combination

Cell line	TNF (U/ml)	Percentage survival[a]				R (expected/observed)
		TNF alone	IFN-γ[b] alone	TNF + IFN-γ expected	TNF + IFN-γ observed	
U937	10	23	89	20	5	4
	3	39		35	12	2.9
	1	76		70	13	5.4
IGROV-1	1000	92	93	85	84	1.01
	100	88		82	78	1.05
	10	99		92	83	1.1
HT29	1000	84	80	67	8	8.5
	100	87		70	11	6.3
	10	91		73	41	1.8
LoVo	1000	97	79	77	59	1.3
	100	96		76	58	1.3
	10	100		79	66	1.19
LoVo/DX	1000	55	76	42	15	2.8
	100	63		48	18	2.7
	10	84		64	35	1.8
MCF7	1000	17	109	18	7	2.6
	100	37		40	11	3.6
	10	68		74	55	1.3
MCF7/DX	1000	91	83	75	93	0.8
	100	95		79	90	0.9
	10	103		85	95	0.9
U20S	1000	99	78	77	92	0.8
	100	103		80	102	0.8
	10	109		85	103	0.8
U20S/Pt	1000	64	89	57	66	0.9
	100	80		71	68	0.9
	10	81		72	77	0.9

[a] Standard deviations averaged 10%.
[b] 300 U/ml.

Effect of TNF and IFN-γ on DX and CDDP cytotoxicity

In LoVo/DX and U20S/Pt cells, the simultaneous combination of both the cytokines (TNF and IFN-γ) with DX or CDDP respectively was also examined. The results reported in Tables 4 and 5 were expressed as modification index (MI), defined as the ratio of IC_{50} of the drug alone and IC_{50} of the drug in the presence of TNF and/or IFN-γ. Against LoVo/DX cells (Table 4), DX cytotoxicity was enhanced 3.8-fold by 100 U/ml of TNF,

Table 3. Cytotoxicity of CDDP alone or in combination with TNF on U20S/Pt cells and synergistic ratios of the two agents in combination

CDDP (μg/ml)	Percentage survival[a]				R (expected/observed)
	CDDP alone	TNF[b] alone	CDDP + TNF		
			Expected	Observed	
8	19	70	5	13	2.6
2	58		37	41	1.1
0.5	81		62	57	0.9

[a] Standard deviations averaged 10%.
[b] 300 U/ml.

Table 4. Influence of TNF and/or IFN-γ on cytotoxicity of DX against LoVo/DX cells

Drugs	Dose (μg/ml)	Percentage survival	IC_{50}	MI[a]
DX	4.8	21 ± 5	0.7	
	1.2	41 ± 5		
	0.3	65 ± 2		
	0.075	87 ± 8		
TNF[b] + DX	4.8	13 ± 4	0.18	3.88
	1.2	20 ± 5		
	0.3	44 ± 11		
	0.075	59 ± 10		
IFN-γ[c] + DX	4.8	17 ± 4	0.55	1.27
	1.2	36 ± 9		
	0.3	63 ± 7		
	0.075	76 ± 5		
(IFN-γ + TNF)[d] + DX	4.8	9 ± 4	0.035	20
	1.2	12 ± 5		
	0.3	27 ± 4		
	0.075	40 ± 1		

[a] MI = (IC_{50} DX alone)/(IC_{50} DX in combination).
[b] TNF 100 U/ml = 64 ± 2.
[c] IFN-γ 300 U/ml = 85 ± 4.
[d] TNF 100 U/ml + IFN-γ 300 U/ml = 65 ± 1.

1.3-fold by 300 U/ml of IFN-γ and 20-fold by TNF and IFN-γ combined. Against U20S/Pt cells (Table 5) CDDP cytotoxicity was enhanced 2.3-fold by 300 U/ml of TNF, 1.1- and 1.5-fold by 300 and 1000 U/ml of IFN-γ respectively, and further enhanced 2.9- or 6.3-fold by the combination of TNF and IFN-γ.

Discussion

The results presented in this study indicate a variable activity of TNF against human tumor cell lines of different histotypes. Particular attention was given to the effects of TNF on LoVo, MCF7 and U20S parental cells and their resistant counterparts, to verify whether a relation existed between the response of the cells to TNF and the development of resistance to conventional cytotoxic agents. The results indicated that the sensitivity of MCF7 cells to DX was related to the sensitivity to TNF. This result agreed with that of Dollbaum *et al.*[14] who found a direct positive correlation between TNF and DX toxicity on human mammary carcinomas. Unlike MCF7 cells, LoVo cells were resistant to TNF, and the resistant counterpart was somewhat

Table 5. Influence of TNF and/or IFN-γ on cytotoxicity of CDDP against U20S/Pt cells

Drugs	Dose (μg/ml)	Percentage survival	IC_{50}	MI[a]
CDDP	8	15 ± 8	2.2	
	2	58 ± 6		
	0.5	83 ± 3		
TNF[b] + CDDP	8	4 ± 2	0.95	2.3
	2	35 ± 6		
	0.5	65 ± 5		
IFN[c] + CDDP	8	13 ± 9	2	1.1
	2	55 ± 1		
	0.5	78 ± 10		
IFN[d] + CDDP	8	10 ± 7	1.4	1.5
	2	49 ± 4		
	0.5	58 ± 8		
(IFNγ + TNF)[e] + CDDP	8	3 + 1	0.75	2.9
	2	32 ± 3		
	0.5	58 ± 7		
(IFNγ ± TNF)[f] + CDDP	8	8 ± 5	0.35	6.3
	2	31 ± 1		
	0.5	40 ± 7		

[a] MI = (IC_{50} CDDP alone)/(IC_{50} CDDP in combination).
[b] TNF 300 U/ml = 74 ± 4.
[c] IFN-γ 300 U/ml = 86 ± 8.
[d] IFN-γ 1000 U/ml = 94 ± 3.
[e] TNF 300 U/ml + IFN-γ 300 U/ml = 72 ± 3.
[f] TNF 300 U/ml + IFN-γ 1000 U/ml = 67 ± 2.

sensitive, although in a dose-independent manner. The different behaviors of MCF7/DX and LoVo/DX cells, both multidrug resistant variants, indicated that the correlation between DX and TNF cytotoxicity may not be a general phenomenon and that the development of tumor cell resistance to TNF does not confer resistance on cytotoxic drugs.[15]

Combined treatment with TNF and IFN-γ resulted in a synergistic cytotoxic effect on U937, HT29, LoVo/DX and MCF7 cell lines. It should be noted that a synergistic effect was observed mainly in the cell lines sensitive to TNF and in the the HT29 cell line. The lack of synergism, however, found in IGROV-1, LoVo, MCF7/DX and U20S could be due to the use of doses of TNF too low to detect any interaction between the two cytokines. Moreover, the lack of synergism in LoVo/DX cells after simultaneous exposure to TNF and IFN-γ may be related, as reported for HT29 cells,[16] to the lack of induction of TNF receptor synthesis by IFN-γ.

When TNF was used in combination with CDDP, the combined effect was synergistic only in the U20S/Pt cell line. In all the other cell lines, TNF in combination with DX, AcD, VP16 and CDDP was found to produce an additive effect. In contrast with another study,[9] the combinations in the present study were never synergistic. This discrepancy is still unclear and could be related to the different cell lines and different methods used to analyze the effect of the interaction. However, this additive cell killing might still have a pharmacological interest, since an augmentation of antitumor efficacy by the combination of TNF and chemotherapeutic agents has been observed *in vivo*.[17]

When a simultaneous combination of TNF and IFN-γ was used to modulate the cytotoxicity on LoVo/DX and U20S/Pt cell lines of the drugs studied, an increase of the cytotoxicity of each drug was found (MI = 20 for DX and 6 for CDDP). Thus, the combination TNF–IFN-γ can act as a modulator of resistance to DX or CDDP. A potentiation of CDDP cytotoxicity by TNF and IFN-α, -β, and -γ has already been observed.[18] The present study also documents the ability of the combination of TNF and IFN-γ to modulate DX cytotoxicity. In particular, the capability of TNF–IFN-γ to increase DX cytotoxicity in LoVo/DX cells was even superior to that observed for some revertants such as verapamil, trifluoperazine and cyclosporine A.[19]

In conclusion, TNF alone can be effective on a cell population resistant to cytotoxic drugs. Moreover, the use of TNF in combination with antitumor drugs seems to be promising, particularly when also IFN-γ is combined. Preclinical *in vivo* studies designed to exploit these *in vitro* observations are expected to provide a sound basis for clinical trials employing cytokines and antitumor drugs in the treatment of human cancer.

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(Received 24 September 1990; accepted 8 October 1990)