Cancer Chemother Pharmacol (1990) 26: 1–8

Cancer Chemotherapy and Pharmacology

Cycloheximide-induced modulation of TNF-mediated cytotoxicity in sensitive and resistant ovarian tumor cells

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Received 17 May 1989/Accepted 15 September 1989

Summary. The mechanism of sensitivty and resistance of various ovarian carcinoma lines to recombinant tumor necrosis factor (rTNF)-mediated cytotoxicity has been investigated using a 24-h ^{51}Cr-release assay. The cell line PA-1 is sensitive to TNF in a dose-dependent manner, whereas the cell line SKOV-3 is resistant to TNF even at high concentrations. The simultaneous addition of TNF and cycloheximide (CHX) in the assay converted the resistant SKOV-3 line into a sensitive line, but no detectable change was observed with PA-1. rTNF inhibited DNA, RNA, and protein synthesis of the sensitive PA-1 line, whereas it had no effect on SKOV-3. This finding was not due to differences in the expression of TNF receptors, as both cell lines expressed equivalent numbers of receptors. The addition of CHX to TNF resulted in suppression of DNA, RNA, and protein synthesis in both the sensitive and the resistant cell lines. Pretreatment of the cell line with TNF for 3 h and subsequent washing resulted in significant cytotoxicity of the sensitive PA-1 line and some cytotoxicity against SKOV-3. However, if the cells were pretreated with CHX for 3 h followed by rTNF for 24 h, a significant decrease in cytotoxicity was observed in both cell lines. Under these conditions, there was no significant inhibition of DNA, RNA, or protein synthesis. Pretreatment of cells for 24 h with TNF and 24 h with CHX resulted in augmentation of the cytotoxicity of PA-1 and SKOV-3, whereas pretreatment for 24 h with CHX followed by 24 h with TNF resulted in no cytotoxicity. Cells pretreated with CHX for 24 h showed poor binding of [^{125}]I-TNF and poor internalization, whereas cells pretreated for 24 h with TNF showed marked enhancement of internalization. The sensitivity of freshly derived ovarian carcinoma lines to TNF and CHX demonstrated that TNF-resistant cells became more sensitive if treated with CHX. These results demonstrate the potential use of metabolic inhibitors in increasing the sensitivity of fresh ovarian tumor cells to TNF.

* Supported in part by the Concern Foundation, Los Angeles, and by a gift from Chugai Pharmaceuticals, Japan

Abbreviations: BRM, biological response modifier; CHX, cycloheximide; DNA, deoxyribonucleic acid; HBSS, Hanks' balanced salt solution; IFN, interferon; rIFN-gamma, recombinant interferon-gamma; RNA, ribonucleic acid; rTNF, recombinant tumor necrosis factor; [^{3}H]-leu, [^{3}H]-leucine; [^{3}H]-TdR, [^{3}H]-thymidine; [^{3}H]-UdR, [^{3}H]-uridine

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Introduction

Tumor necrosis factor (TNF) is a cytokine, primarily produced by macrophages or B cells [11, 25], that exerts many activities. TNF has been reported to cause necrosis of tumors transplanted in mice and to show cytotoxic and cytostatic activity in murine and human tumor cells in vitro [3, 6, 8, 12, 13, 20]. Recombinant DNA technology has made it possible to produce large amounts of recombinant TNF (rTNF) for clinical and investigational uses [15, 19]. Recent reports [5, 20, 24] have shown that TNF may not be directly cytotoxic against a variety of fresh human tumor cells. Although the mechanism of TNF-mediated cytotoxicity has not yet been clarified, it has been reported that TNF arrests cells at the G2 phase of the cell cycle and that TNF-mediated cytolysis occurs at the mitotic phase [4]; thus, TNF may take a long time to mediate its cytotoxic activity [7].

In an attempt to improve the cytotoxic activity of TNF, particularly in resistant target cells, simultaneous use of rTNF with various chemotherapeutic agents or biological response modifiers (BRMs) has been considered. The combination of rTNF with actinomycin D or interferon results in augmentation of cytotoxicity [1, 4, 5, 7, 10, 20, 25]. Other reports have also shown that the inhibitor of protein synthesis, cycloheximide (CHX), improves the cytotoxicity of TNF in resistant target cells [9, 10, 18].

The cytotoxic effect of TNF on freshly derived human ovarian tumors has not been investigated. Our recent study [14] showed that some, but not all, fresh ovarian tumor

cells were sensitive to high doses of rTNF in vitro. These results suggest that rTNF alone is not clinically effective in treating ovarian tumors, and the use of high doses of TNF may cause serious side effects [2, 23]; therefore, it might be necessary to apply a relatively low dose of TNF in combination with other therapeutic modalities. Our previous study [14] demonstrated that the combination of suboptimal doses of rTNF and other BRMs (rIFN-2 and OK-432) was not very effective against ovarian tumor cells in vitro. In the present study, the effect of CHX on the antitumor activity of rTNF was examined in vitro against established and freshly derived ovarian tumor cell lines.

Materials and methods

Preparation of ovarian tumor cells. Two in vitro established cell lines, PA-1 and SKOV-3, were used. They are plastic-adherent and are thus maintained in monolayer cultures on plastic in culture medium at 37° C in 5% CO_2. The medium consisted of RPMI-1640 (M. A. Bioproducts) supplemented with 10% fetal calf serum (Gibco), 1% nonessential amino acids (Gibco), 1% L-glutamine (Gibco), 1% penicillin-streptomycin (Gibco), and 1% Fungisone (Flow). For use, the cells were harvested by overlaying the monolayer with a solution containing 0.25% trypsin and 0.02% EDTA (Gibco). After 5 min incubation for PA-1 and 20 min incubation for SKOV-3 at 37° C in 5% CO_2, the adherent cells were removed from the plastic by tapping the flask sharply and the cells were collected. They were then washed three times in Hanks' balanced salt solution (HBSS) and resuspended in culture medium.

rTNF. Purified human rTNF (sp. act. 5.02×10^7 units/mg; lot 3056-55) was a generous gift from Genentech (South San Francisco, USA).

CHX. CHX was purchased from Sigma Chemical Company (Lot 96F-0688). CHX stock solution was dissolved at 1 mg/ml in culture medium containing 20% ethanol and was then diluted in culture medium as needed.

^{51}Cr-release test. Target cells were labeled with Na_2 [^{51}Cr]O_4 (Amersham; 1 mCi/ml), and 0.2 ml ^{51}Cr was then added to 10^6 cells in 2 ml culture medium. After 3 h incubation at 37° C in 5% CO_2, the cells were washed three times in HBSS and suspended at a final density of 5×10^4/ml in culture medium. The assay was set up in triplicate in 96-well, U-bottom microtiter plates (Nunclon). In all, 0.1 ml target-cell suspension and 0.1 ml rTNF and/or CHX solution was added to each well and incubated at 37° C in 5% CO_2. After 24 h incubation, the ^{51}Cr released into 0.1 ml supernatant from each well was determined using a Beckman 5500 gamma counter. For total lysis, 0.1 ml 2% Triton X was added to target cell and 0.1 ml culture medium was used for spontaneous lysis. The mean value of triplicates was used to calculate the percentage of specific lysis as follows:

$$\% \text{ specific lysis} = \frac{(\text{test cpm} - \text{spontaneous cpm})}{(\text{total cpm} - \text{spontaneous cpm}) \times 100}$$

DNA, RNA, and protein synthesis. The effect of rTNF or CHX on DNA, RNA and protein synthesis was examined following the incorporation of [^3H]-thymidine ([^3H]-TdR), [^3H]-uridine ([^3H]-UdR), and [^3H]-leucine ([^3H]-leu) respectively, into cells. The target cells were incubated at a final density of 5×10^4/0.2 ml per well in a 96-well, flat-bottom microtiter plate (Nunclon). To each well, a 0.5-μci sample of each tritiated precursor was added 18 h before cell harvest. The radioactive pulse was extended for 18 h to ensure full assessment of the effect of the particular agent on DNA synthesis. Since the tumor cells were heterogeneous and represented different phases of the cell cycle, we wished to determine the effect of the various agents on other stages of the cell cycle, as TNF may affect cells that are not primarily in the S phase.

After 24 h incubation, the supernatants were aspirated by pipette and 0.1 ml solution containing 0.25% trypsin and 0.02% EDTA was added to each well. After 5 min incubation for PA-1 or 20 min incubation for SKOV-3, the cells were removed from the plastic by pipetting and 0.1 ml culture medium was added to each well. Thereafter, the cells were harvested onto glass-fiber filters using a multiple-cell harvester (MASH II, M. A. Bioproducts). After drying at room temperature, the glass-fiber filters were placed in minivials with 3 ml liquid scientillation fluid (Safety-solve) and the radioactivity was counted in a liquid scintillation counter (Beckman LS7500). All experiments were set up in triplicate, and the mean value was used to calculate the percentage of control uptake as follows:

$$\% \text{ control uptake} = \frac{(\text{test cpm} - \text{BG})}{(\text{control cpm} - \text{BG}) \times 100}$$

Binding and internalization of [^{125}I]-rTNF. rTNF was iodinated using Iodobeads (Pierce) according to the method described in a previous report [26]. This assay was set up in triplicate in 12×75 mm test tubes (Falcon 2058). For assay of specific binding to TNF receptor, 10^6 cells were suspended in 0.5 ml culture medium, the tubes were placed on ice for 10 min, and then [^{125}I]-rTNF was added to achieve a final concentration of 0.5 n*M*. After incubation at 4° C for 2 h, the cells were centrifuged for 60 s at 3,000 *g* and washed twice in ice-cold culture medium. After the washes, a small amount of culture medium remaining in the tubes was removed by paper filter and the radioactivity was measured in a gamma counter. Preliminary experiments indicated that all of the excess unbound [^{125}I]-rTNF was removed under these conditions. Nonspecific binding was determined by adding a 100-fold excess of unlabeled rTNF, and this represented <10% of the total bound radioactivity. Nonspecific binding was subtracted from the total binding. The data obtained from the saturation binding experiments were subjected to a Scatchard analysis. The total number of rTNF binding sites was calculated by inspection of the graph.

The surface-binding and internalization of rTNF in tumor cells in the 24-h incubation with radiolabeled rTNF was assessed according to the modified methods described by Tsujimoto et al. [21]. Briefly, 10^6 cells were incubated with 0.5 n*M* [^{125}I]-rTNF for 24 h at 37° C; this experiment was set up in triplicate. The cells were washed three times in ice-cold culture medium and resuspended in 2 ml 0.05 *M* glycine-HCl buffer (pH 3.0). After incubation for 5 min at 4° C, the cells were centrifuged and the supernatants, collected. The pellet was washed twice in culture medium and the cells were solubilized in 0.1% $NaDoSO_4$. ^{125}I radioactivity found in the glycine buffer represented the surface-bound rTNF, and the activity found in the solubilized fraction gave a measure of the internalized intracellular rTNF. Student's *t*-test was used to determine the statistical significance of the values obtained.

Results

We have previously shown [14] that certain ovarian tumor cells are sensitive to the cytotoxic effect of rTNF, whereas others are resistant. The mechanism of tumor cell resistance to TNF was examined using two established ovarian tumor cell lines, one sensitive (PA-1) and one resistant (SKOV-3) to rTNF. The present studies examined the effect of CHX treatment, the metabolic responses to TNF, and the determination of [^{125}I]-rTNF binding and internalization. In all experiments the cytotoxic activity was determined by a 24-h ^{51}Cr-release assay.

Effect of simultaneous addition of CHX and rTNF

The cytotoxic effect of various doses of rTNF and CHX on ovarian tumors was examined. PA-1 was sensitive to rTNF, and maximal cytotoxicity was obtained with 1000

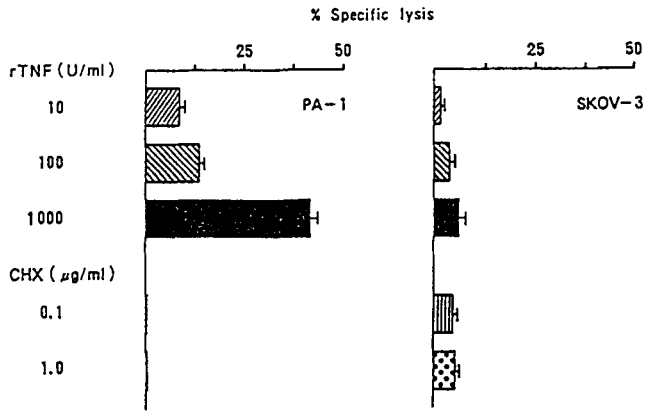

Fig. 1. The cytotoxic effect of rTNF or CHX on PA-1 and SKOV-3 ovarian tumor cell lines. The radiolabeled tumor cells were incubated with various concentrations of rTNF or CHX for 24 h and the percentage of specific lysis was calculated from the radioactivity released into the supernatants as described in Materials and methods

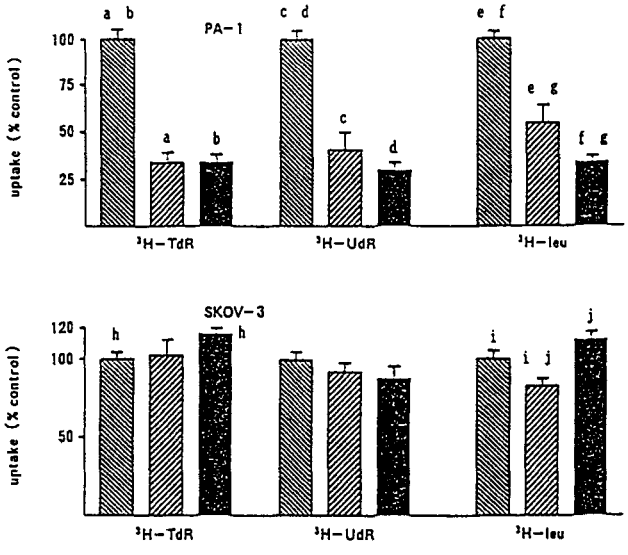

Fig. 2. Effect of rTNF on DNA, RNA, and protein synthesis in PA-1 and SKOV-3 ovarian tumor cell lines. The tumor cells were cultured with culture medium (▧) or 1,000 units/ml rTNF for 24 h (▨). A sample of tumor cells was pretreated with 1,000 units/ml rTNF for 3 h, washed, and incubated for a further 24 h (■). [^{3}H]-TdR, -UdR, or -leu was added to the wells 18 h before cell harvest. After incubation, the cells were harvested onto glass-fiber filters and radioactivity was measured as described in Materials and methods. *a–f:* $P < 0.001$; *g–i:* $P < 0.05$; *j:* $P < 0.01$

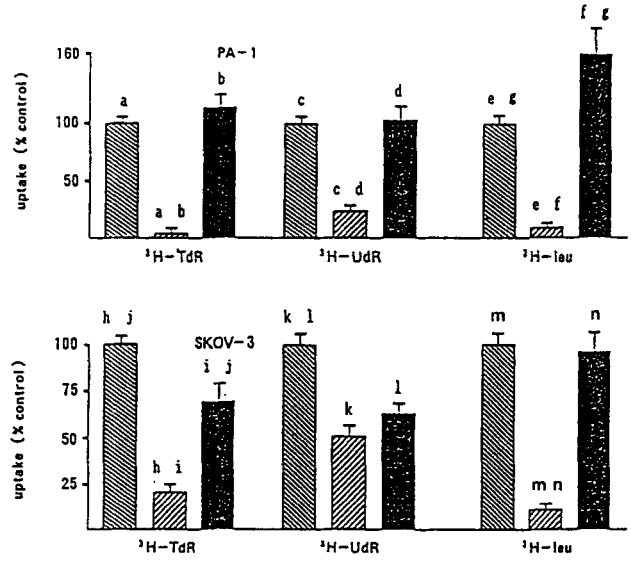

Fig. 3. Effect of CHX on DNA, RNA, and protein synthesis in PA-1 and SKOV-3 ovarian tumor cell lines. The tumor cells were cultured with culture medium (▧) or 1.0 μg/ml CHX (▨) for 24 h. One sample of cells was pretreated with 1.0 μg/ml CHX for 3 h, washed, and incubated for 24 h (■). [^{3}H]-TdR, -UdR, or -leu was added to the wells 18 h before cell harvest. After incubation, the cells were harvested onto glass-fiber filters and radioactivity was measured as described in Materials and methods. *a–f, h, i, k–n:* $P < 0.001$; *g, j:* $P < 0.01$

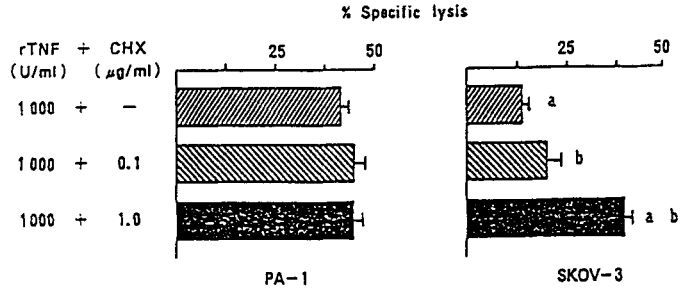

Fig. 4. The effect of rTNF and CHX used together on PA-1 and SKOV-3 ovarian tumor cell lines. The radiolabeled tumor cells were cultured with rTNF and CHX for 24 h and cytotoxicity was assessed as described in Materials and methods. *a, b:* $P < 0.01$

units/ml rTNF. SKOV-3 was resistant at all rTNF concentrations used. Cycloheximide at 0.1 and 1.0 μg/ml was not cytotoxic to either tumor cell line (Fig. 1). rTNF had a marked suppressive effect on DNA, RNA, and protein synthesis of the TNF-sensitive PA-1 cell line, whereas it had no effect on the TNF-resistant SKOV-3 cell line (Fig. 2). Since the dose of rTNF used exerted 35%–40% lysis and suppression of metabolic activities was >75%, these results suggest that rTNF inhibits the metabolic activity of residual cells that are not lysed by rTNF in a 24-h assay. Treatment with CHX abolished protein synthesis in both cell lines (Fig. 3).

When the tumor cells were cultured with an optimal dose of rTNF in the presence of CHX (0.1 or 1.0 μg/ml), the TNF-resistant SKOV-3 cell line was lysed, whereas the magnitude of lysis of similarly treated PA-1 cells was not changed (Fig. 4). The profile of DNA, RNA, and protein synthesis of SKOV-3 was similar to that of PA-1 (Fig. 5).

Both PA-1 and SKOV-3 cell lines expressed surface membrane receptors for TNF as detected by binding of [^{125}I]-rTNF (Fig. 6). The number of receptors was higher in cells treated for 24 h in medium; following treatment with rTNF or CHX, the number of TNF receptors decreased. Thus, the expression of TNF receptors on the target cell membrane of SKOV-3 was not sufficient to render the cells sensitive to lysis by TNF.

The above findings demonstrate that the effect of rTNF on the sensitive PA-1 line resulted in inhibition of metabo-

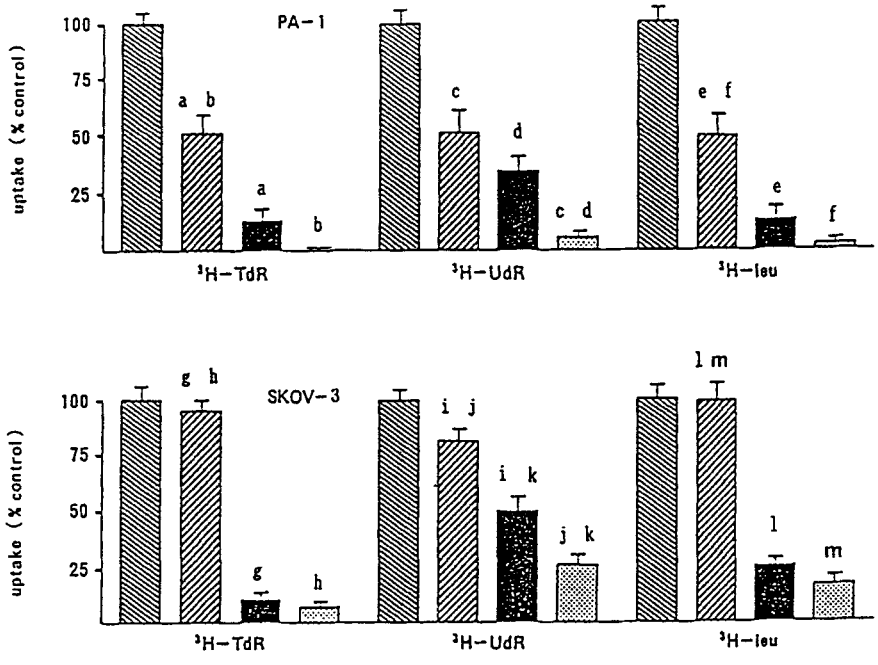

Fig. 5. The effect of rTNF and CHX used together on DNA, RNA, and protein synthesis in PA-1 and SKOV-3 tumor cells. The cells were incubated in culture medium (▧), 1,000 units/ml rTNF (▨), 1.0 μg/ml CHX (■), or 1,000 units/ml rTNF + 1.0 μg/ml CHX (□) for 24 h. [^{3}H]-TdR, -UdR, and -leu were added to the well 18 h before cell harvest. After incubation, the cells were harvested onto glass-fiber filters and radioactivity was measured as described in Materials and methods. *a–j, l, m: P <0.001; k: P <0.05*

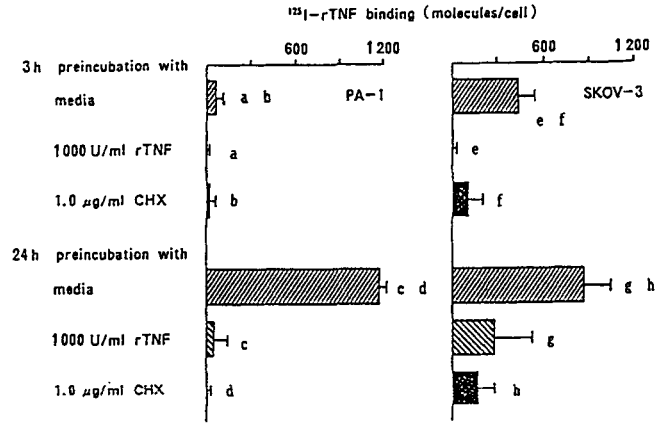

Fig. 6. Effect of CHX or rTNF on ^{125}I-labeled rTNF binding. Tumor cells were incubated in culture medium (▧), 1,000 units/ml rTNF (▨), or 1.0 μg/ml CHX (■) for 3 h or 24 h, then the cells were washed in ice-cold HBSS and the cell concentration was adjusted to 10^6 cells for each tube. The cells were then incubated with ^{125}I-rTNF for 2 h at 4° C and [^{125}I]-rTNF binding was determined as described in Materials and methods. *a, b: P <0.05; c–e, h: P <0.001; f, g: P <0.01*

lic activity, whereas it had no effect on the resistant line. Treatment with CHX rendered the resistant SKOV-3 cell line sensitive to TNF.

Effect of tumor cell pretreatment with rTNF or CHX

Tumor cell pretreatment for 3 h. Pretreatment of tumor cells with rTNF for 3 h followed by 24 h incubation with 1.0 μg/ml CHX resulted in a significant augmentation of lysis in both PA-1 and SKOV-3 cell lines (Fig. 7). In contrast, pretreatment for 3 h with CHX followed by 24 h incubation with rTNF resulted in a decrease of lysis in both PA-1 and SKOV-3 tumor cells (Fig. 8).

Cells pretreated with rTNF for 3 h showed suppressed DNA, RNA, and protein synthesis comparable with the effect seen when TNF was present during the whole 18 h of the assay (Fig. 2). Cells pretreated for 3 h in rTNF and subsequently in CHX for 24 h showed suppression of DNA, RNA, and protein synthesis in both cell lines (Fig. 9). Cells treated for 3 h with CHX followed by 24 h

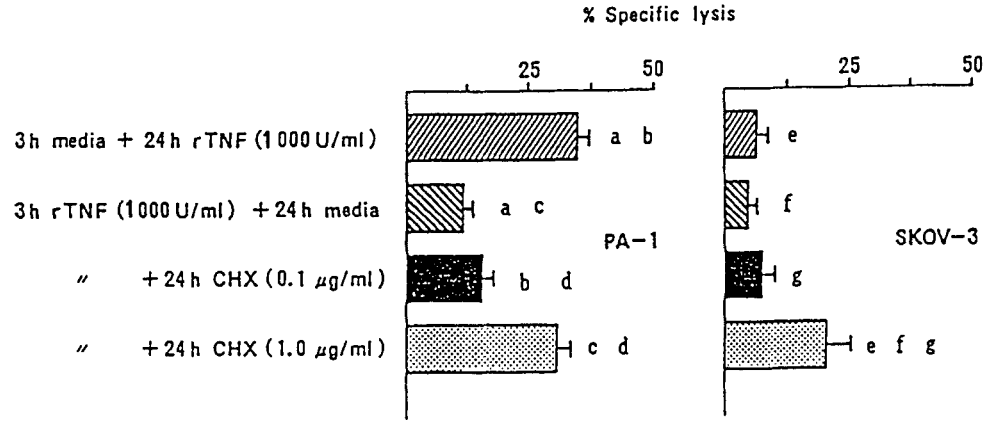

Fig. 7. Effect of 3 h pretreatment of tumor cells with rTNF and subsequent incubation with CHX. The radiolabeled tumor cells were pretreated with 1,000 units/ml rTNF for 3 h, washed, and then incubated in culture medium (▧), 0.1 μg/ml CHX (▨), or 1.0 μg/ml CHX (■) for 24 h. To control for the cytotoxicit of rTNF, the tumor cells were incubated in culture medium for 3 h, washed, and then incubated with 1,000 units/ml rTNF for 24 h. The cytotoxicity was assessed as described in Materials and methods. *a–g: P <0.01*

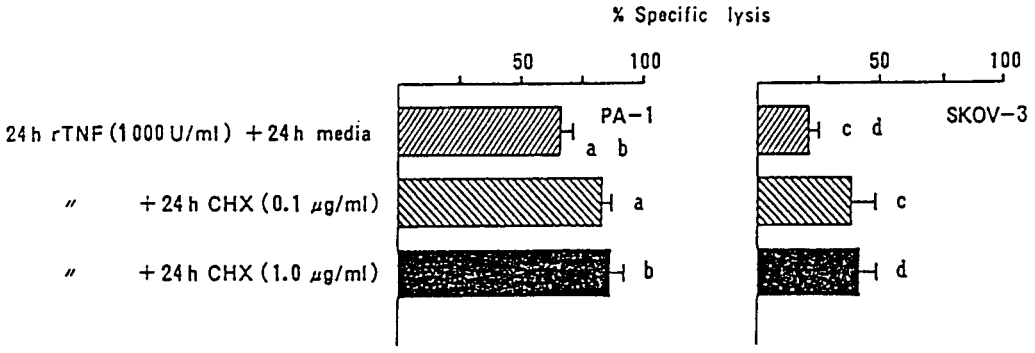

Fig. 8. Effect of 3 h pretreatment of tumor cells with CHX and subsequent incubation with rTNF. The radiolabeled tumor cells were pretreated in culture medium, 0.1 μg/ml CHX, or 1.0 μg/ml CHX for 3 h and washed. TNF was added for 24 h. The cytotoxicity was assessed as described in Materials and methods. *a, c, d:* $P < 0.05$; *b, e:* $P < 0.01$

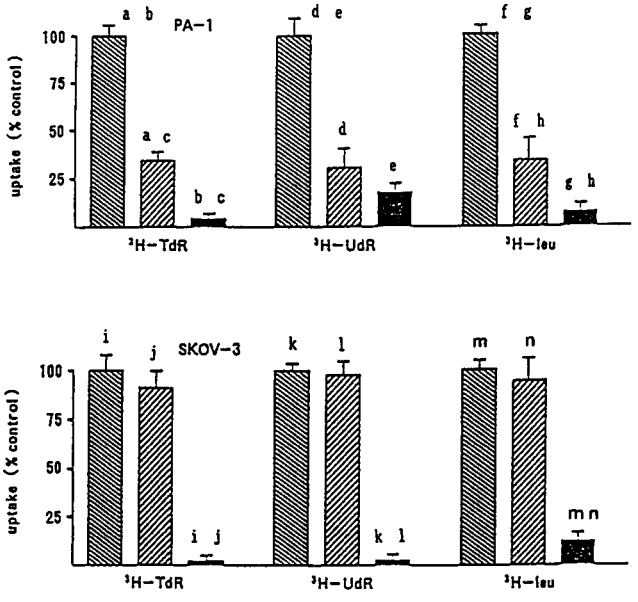

Fig. 9. The effect of 3 h pretreatment with rTNF and subsequent treatment with CHX. The cells were pretreated with 1,000 units/ml rTNF for 3 h and washed. For control, the cells were incubated in culture medium alone for 3 h. Thereafter, the cells were incubated with 1.0 μg/ml CHX for 24 h. [³H]-TdR, -UdR, or -leu was added to the wells 18 h before cell harvest. After incubation, the cells were harvested onto glass-fiber filters and radioactivity was measured according to the method described in Materials and methods. ▨, control; ▨, 3 h pretreatment with 1,000 units/ml rTNF + subsequent incubation in culture medium for 24 h; ■, 3 h pretreatment with 1,000 units/ml rTNF + subsequent incubation with 1.0 μg/ml CHX for 24 h. *a–g, i–n:* $P < 0.001$; *h:* $P < 0.05$

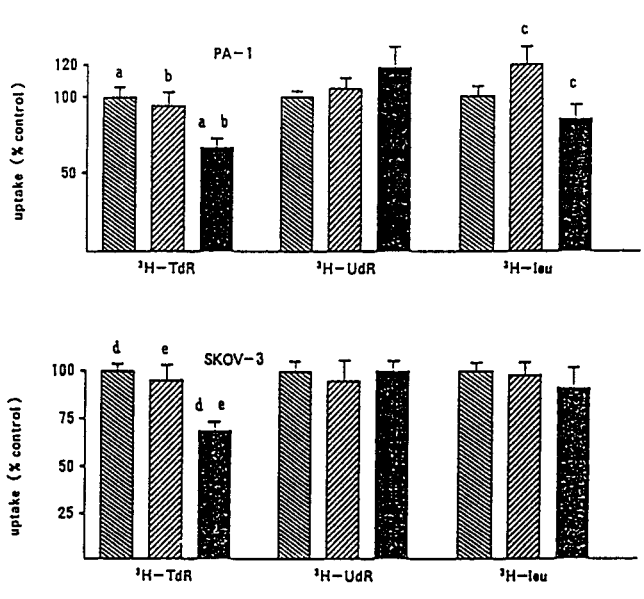

Fig. 10. The effect of 3 h pretreatment with CHX and subsequent treatment with rTNF. The cells were pretreated with 1.0 μg/ml CHX for 3 h and washed. For control, the cells were incubated in culture medium alone for 3 h. Thereafter, the cells were incubated with 1,000 units/ml rTNF for 24 h. [³H]-TdR, -UdR, or -leu was added to the wells 18 h before cell harvest. The cells were harvested onto glass-fiber filters and radioactivity was measured according to the method described in Materials and methods. ▨, control: ▨, 3 h pretreatment with 1.0 μg/ml CHX + subsequent incubation with culture medium for 24 h; ■, 3 h pretreatment with 1.0 μg/ml CHX + subsequent incubation with 1,000 units/ml rTNF for 24 h. *a–e:* $P < 0.01$

incubation in rTNF showed slight suppression of DNA synthesis, whereas neither RNA nor protein synthesis was affected (Fig. 10). Thus, these results demonstrate that there was a correlation between the cytotoxic activity seen and the inhibition of metabolic activities.

Tumor cell pretreatment for 24 h. The sequential treatment of tumor cells with rTNF for 24 h followed by CHX for 24 h resulted in increased susceptibility to lysis in both PA-1 and SKOV-3 cells (Fig. 11). Cells pretreated for 24 h in CHX and subsequently for 24 h in TNF showed no lysis (Fig. 12). However, pretreatment with CHX for 24 h resulted in a significant decrease in binding and internalization (Fig. 13). It is noteworthy that no competitive inhibition was seen in the presence of nonlabeled rTNF, since the ligand was present during the 18-h assay period.

Susceptibility of fresh ovarian carcinoma lines to rTNF and CHX

The effect of rTNF on fresh ovarian carcinoma cell lines was minimal in $^3/_5$ lines studied, whereas it was significant in $^2/_5$ lines (Table 1). The addition of CHX enhanced the TNF-mediated cytotoxic activity, particularly that in the resistant lines. Thus, these results demonstrate that CHX can induce sensitivity in rTNF-resistant ovarian tumor cells.

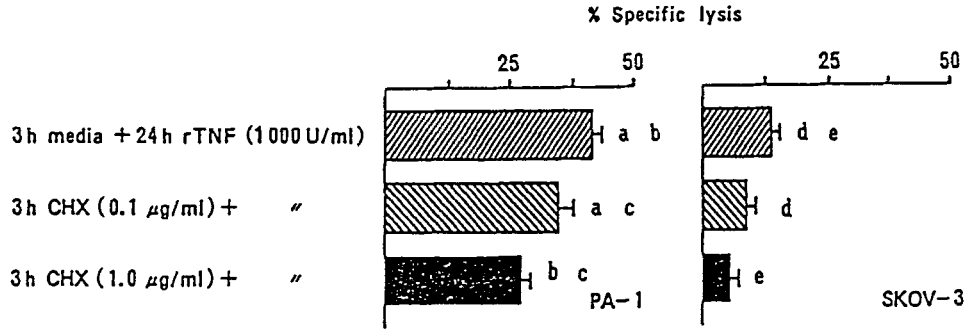

Fig. 11. The effect of 24 h treatment with rTNF and subsequent 24 h treatment with CHX. The cells were treated with 1,000 units/ml rTNF for 24 h and washed. Thereafter, the cells were labeled with ^{51}Cr and incubated in culture medium, 0.1 μg/ml CHX, or 1.0 μg/ml CHX for 24 h. The cytotoxicity was assessed according to the method described in Materials and methods. *a–d: P* <0.05

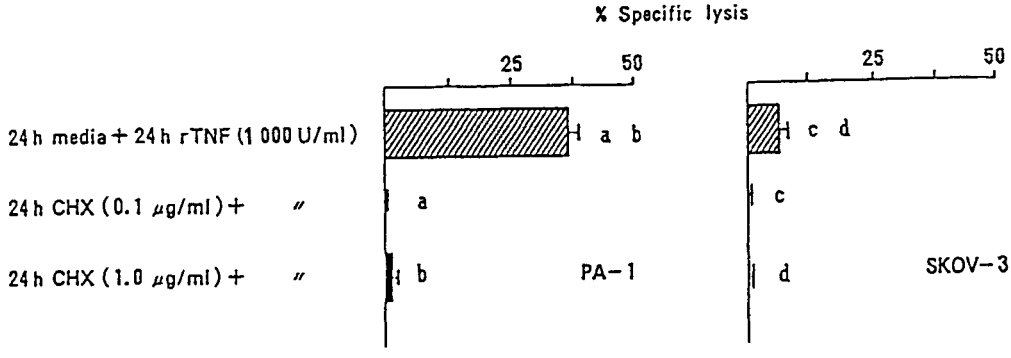

Fig. 12. The effect of 24 h treatment with CHX and subsequent 24 h treatment with rTNF. The cells were treated with culture medium, 0.1 μg/ml CHX, or 1.0 μg/ml CHX for 24 h and washed. Thereafter, the cells were labeled with ^{51}Cr and incubated with 1,000 units/ml rTNF for 24 h. The cytotoxicity was assessed according to the method described in Materials and methods. *a, b: P* <0.001; *c, d: P* <0.01

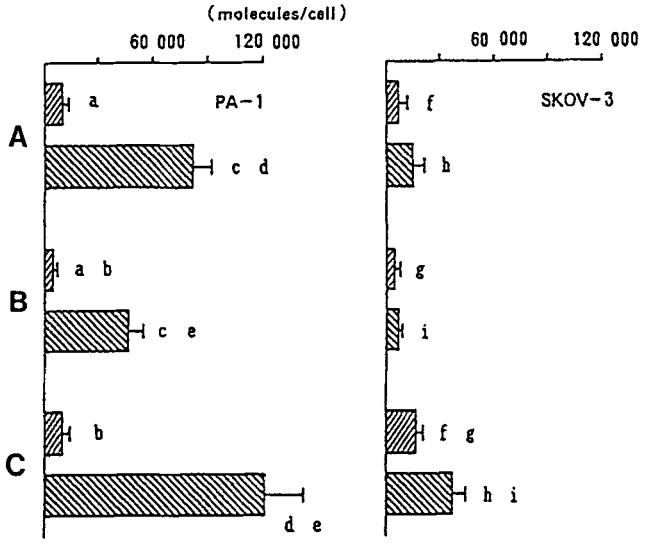

Fig. 13. Effect of CHX or rTNF on surface-binding and internalization of rTNF in the tumor cells. The tumor cells were preincubated with culture medium, 1.0 μg/ml CHX, or 1,000 units/ml rTNF for 24 h at 37° C. The cells were washed three times in ice-cold culture medium and adjusted at a cell concentration of 10^6 cells for each tube. Thereafter, the cells were incubated in culture medium with [^{125}I]-rTNF for 24 h at 37° C, washed in ice-cold HBSS, and resuspended in 0.05 *M* glycine buffer (pH 3.0) for 5 min at 4° C and centrifuged. The supernatant was collected and radioactivity was measured as surface-bound rTNF. The pellet was washed twice and the cells were solubilized in 0.1% $NaDoSO_4$; the resultant radioactivity was a measure of the internalized rTNF. ▧, surface-bound rTNF; ▨, internalized rTNF. *a, b, d, f–h, j: P* <0.05; *c, e, i: P* <0.01. *A*, 24 h media + 24 h [^{125}I]-rTNF (0.5 nM); *B*, 24 h CHX (1.0 μg/ml) + 24 h [^{125}I]-rTNF (0.5 nM); *C*, 24 h rTNF (1,000 U/ml) + 24 h [^{125}I]-rTNF (0.5 nM)

Table 1. Effect of the combination of rTNF and CHX on freshly separated human ovarian tumor cells

Fresh ovarian tumor:		%Specific lysis in 48- (24-h) ^{51}Cr-release assay:		
Patient number (age in years)	Histology	rTNF (1000 units/ml)	CHX (1.0 μg/ml)	rTNF+CHX
1. (38)	Serous	4 (0)	6 (2)	14 (2)
2. (45)	Serous	13 (0)	4 (0)	19 (5)
3. (75)	Endometrioid	10 (4)	6 (5)	31 (14)
4. (52)	Serous	33 (25)	8 (0)	34 (31)
5. (57)	Serous	22 (21)	0 (0)	28 (26)

Discussion

We presented evidence demonstrating that TNF-resistant ovarian carcinoma tumor cells can be rendered sensitive following their exposure to CHX. The time course of exposure was important, as simultaneous exposure or pre-exposure to TNF (3–24 h) followed by exposure to CHX was uniformly associated with increased cytotoxicity. In contrast, pre-exposure of tumor cells to CHX (3–24 h) followed by TNF was associated with decreased cytotoxicity in the sensitive lines and did not result in conversion of the resistant line to a sensitive line.

Previous studies have indicated that most TNF-sensitive target cells require the inhibition of protein synthesis to maximize cytotoxicity. This phenomenon is well docu-

mented in the classic L929 fibroblast cell line, among others [17]. It is not clear why protein-synthesis inhibition is required for TNF-mediated lysis. It has been suggested that some proteins protect the cells and that inhibition of their synthesis renders the cells sensitive to TNF [9]. DNA repair mechanisms may also be implicated in the phenomenon of sensitivity and resistance. Clearly, as reported previously for many target cells [22] and in the present study for the ovarian carcinoma cell lines, the mere expression of TNF receptors is not sufficient to determine sensitivity or resistance.

The sequence of addition of CHX to resistant target cells was a determining factor in their sensitivity to TNF. Pre-exposure of cells to CHX for 3–24 h followed by pretreatment with TNF resulted in decreased cytotoxicity. This decrease correlated with a decrease in the expression of TNF receptors. It has been reported that the TNF receptor has a rapid turnover and is dependent on de novo protein synthesis [16]; thus, the inhibition or decrease of TNF receptor expression results in resistance. This is clearly indicated in the present studies, which demonstrated that exposure of PA-1 to CHX for 24 h resulted in a marked decrease of binding and internalization of [^{125}I]-TNF and a decrease in cytotoxicity. Also, we found that the resistant SKOV-3 cell line binds as much TNF as the sensitive line but internalizes only 50%–60% of the amount of TNF internalized by PA-1. Although these results suggest that internalization may play a role in TNF sensitivity, previous studies with other tumor targets [22] failed to establish such a correlation. It is conceivable that the resistance of different tumor cell lines to TNF may be localized at different steps of the lytic pathway, e.g., receptor binding, internalization, processing, or other intracellular events.

The effect of TNF on the sensitive PA-1 line resulted in inhibition of DNA, RNA, and protein synthesis. However, these findings were not observed in the resistant target SKOV-3. Clearly, CHX inhibits these events and renders SKOV-3 sensitive to TNF. It is not clear whether inhibition of these metabolic activities is a requisite for sensitivity. Nonetheless, such effects might have important clinical implications for the therapeutic use of TNF. These results suggest that the clinical application of TNF in vivo should be followed by drugs that inhibit protein synthesis, such that maximal sensitivity and cytotoxicity can be achieved.

Our studies with fresh ovarian tumor cells showed that sensitivity to TNF is generally amplified by the addition of CHX. Although CHX is used as a chemotherapeutic agent, it has resulted in adverse effects when used at high doses. In the present study, low concentrations of CHX were used (0.1–1.0 μg/ml) that were sufficient to convert resistance to sensitivity; these doses were much lower than those reported by others (10–100 μg/ml). Our studies show that low doses of TNF and CHX are sufficient to kill fresh tumor cells. The clinical application of such combination therapy will depend on the susceptibility of normal tissues to these agents. However, in ovarian cancer, i.p. administration of these agents may reduce the risk of tissue cytotoxicity and maximize the local antitumor effect. Thus, the combination of CHX or other, similar agents with TNF should be useful in the treatment of ovarian carcinoma.

It is noteworthy that our studies showing that pretreatment of tumor cells with CHX followed by TNF results in tumor resistance have strong clinical implications. It may be reasoned that patients who have undergone chemotherapy may carry tumor cells that are insensitive to TNF, as was shown in the present in vitro studies. Furthermore, such patients may bear tumor cells that are multiple-drug-resistant as well as resistant to TNF. Thus, it is important to monitor each patient's tumor cell sensitivity to TNF alone or in combination with other drugs prior to selecting protocols for therapy with TNF.

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