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Common expression of a tumor necrosis factor resistance mechanism among gynecological malignancies

C. Bethan Powell, David G. Mutch, L. Stewart Massad, Ming-Shian Kao, and John Leslie Collins

The Oncology Division of The Department of Obstetrics and Gynecology, Washington University School of Medicine, St. Louis, Mo, USA

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Summary. The efficacy of tumor necrosis factor α (TNFα) as an anticancer agent is limited. This limitation might be related to the expression of a protein-synthesis-dependent resistance mechanism that prevents the lysis of tumor cells by TNFα. To test this possibility eight randomly selected human cell lines, three derived from ovarian carcinomas and five derived from cervical carcinomas, were tested for their in vitro sensitivity to TNFα-mediated lysis. The results of this analysis showed that all eight cell lines are normally resistant to lysis by TNFα. However, in the presence of inhibitors of protein synthesis, seven of them showed a significant increase in TNFα-mediated lysis. Measurement of protein synthesis showed that there is a linear correlation between the level of inhibition of protein synthesis and the level of TNFα-mediated lysis. The fact that seven of eight randomly selected cell lines are resistant to TNFα because they express a protein-synthesis-dependent resistance mechanism suggests that this mechanism of resistance may be common among gynecological cancers. The results also suggest that a therapy involving TNFα and inhibitors of protein synthesis might be useful for the treatment of gynecological malignancies.

Introduction

The disappointment of multidrug chemotherapy for the treatment of gynecological malignancies has stimulated interest in the use of biological response modifiers as anticancer agents. The biological response modifier, tumor necrosis factor α (TNFα) was first described as a factor in the serum of animals treated with *Mycobacterium bovis*, strain BCG, that caused tumor necrosis [3]. The gene encoding human TNFα has been isolated and the availability of TNFα as a recombinant molecule, with properties indistinguishable from that of the native molecule [21, 23], has prompted considerable research on its in vivo and in vitro effects. TNFα has been shown to mediate a variety of biological responses including cytostasis and cytotoxicity of tumor cells [9, 11, 18, 22]. The in vitro cytostatic and cytolytic activity of TNFα for some cancer cells would seem to make TNFα an ideal candidate for the immunotherapy of malignant disease. However, the experience to date with TNFα in clinical trials has shown few responses [2, 6, 12, 20]. This limitation in the efficacy of TNFα as an anticancer agent would appear to indicate that most cancer cells are resistant to the cytostatic or cytolytic activity of TNFα.

We have previously shown that cells that are resistant to lysis by human natural cytotoxic (NC) cells express a protein-synthesis-dependent resistance mechanism, such that when protein synthesis is inhibited they become sensitive to NC-mediated lysis [4, 5, 17]. Because natural cytotoxic cells use TNFα to mediate lysis [15, 16], the resistance mechanism that prevents this lysis actually does so by preventing lysis by TNFα. That resistance to TNFα-mediated lysis of some cell lines is dependent on protein synthesis has now been confirmed in several laboratories [7, 8, 13, 14, 19]. It would therefore appear that an anticancer therapy that combined protein synthesis inhibitors with TNFα might be potentially useful for the treatment of some cancers that are resistant to lysis by TNFα. To begin to explore this possibility we tested the in vitro sensitivity of eight arbitrarily selected human cell lines derived from gynecological malignancies (three ovarian and five cervical carcinoma cell lines) to lysis by TNFα in the presence or absence of inhibitors of protein synthesis. The results of this analysis showed that the sensitivity to lysis by TNFα of seven of the eight cell lines tested was increased in the presence of inhibitors of protein synthesis. These results indicate that the expression of the resistance mechanism is common among gynecological malignancies and that an anticancer therapy combining TNFα and protein synthesis inhibitors might be advantageous.

Offprint requests to: J. L. Collins, Department of Obstetrics and Gynecology, Washington University School of Medicine, 4911 Barnes Hospital Plaza, St. Louis, MO 63 110, USA

Materials and methods

Cell lines. The human ovarian carcinoma cell lines, CaOV-3 (ATCC HTB 75), SK-OV-3 (ATCC HTB 77), and OVCAR-3 (ATCC HTB 161); and the human cervical carcinoma cell lines HT-3 (ATCC HTB 32), Me-180 (ATCC HTB 33), MS751 (ATCC HTB 34), SiHa (ATCC HTB 35), and C-33A (ATCC HTB 32) were obtained from The American Type Culture Collection (Rockville, Md).

CaOV-3, SK-OV-3, ME-180, MS751, SiHa, and C-33A cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (HyClone Lab., Logan, Utah) and 30 mg/ml L-glutamine (Sigma Chemical Co., St. Louis, Mo). Cells were grown at 37° C in a humidified atmosphere of 95% air and 5% CO_2.

HT-3 cells were maintained in McCoy's medium supplemented with 10% fetal bovine serum and 30 mg/ml L-glutamine. Cells were grown at 37° C in a humidified atmosphere of 90% air and 10% CO_2.

OVCAR-3 cells were maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum, 30 mg/ml L-glutamine, and 10 µg/ml recombinant human insulin (Eli Lilly and Co., Indianapolis, Ind.). Cells were grown at 37° C in a humidified atmosphere of 90% air and 10% CO_2.

All cells were maintained as exponential monolayer cultures by passage two times a week. Cells were removed from culture dishes with 0.05% trypsin (Gibco, Grand Island, N. Y.) in phosphate-buffered saline, pH 7.2, containing 0.04% EDTA (Sigma) and 2×10^5 cells plated in 100-mm tissue-culture dishes (Corning Glass Works, Corning, N. Y.).

TNFα. Recombinant human TNFα was obtained as a frozen stock solution in phosphate-buffered saline from the Cetus Corporation (Emeryville, Calif.). A unit of TNFα is defined as the concentration of TNFα that causes 50% lysis of the TNFα-sensitive cell line L929 [1, 3]. We used the number of units of TNFα as determined by the Cetus Corporation. Just before its addition to the assay of cytotoxicity TNFα was thawed and diluted in the medium used for the routine growth of cells.

Anti-TNFα antibody. Immune rabbit serum containing antibodies to human TNFα and nonimmune rabbit serum was provided by the Cetus Corporation. Enough anti-(human TNFα) serum was added to block the lytic activity of 5 units/ml TNFα. An equivalent amount of nonimmune serum was added to control wells.

Inhibitors of protein synthesis. Solutions of actinomycin D (Sigma), cycloheximide (Sigma), and emitine (Sigma) were freshly made in the same medium used for the routine growth of cells.

Measurement of protein synthesis. Cells (3×10^5) were plated in 35-mm tissue-culture plates (Corning) in 1 ml medium used for the routine growth of cells. The next day emitine, at the concentrations indicated, was added to the plates. After 2 h incubation at 37° C, the medium was removed and 1 ml medium containing 10 µCi/ml [^{3}H]leucine (Dupont/NEN, Wilmington, Del.) was added; the cells were then incubated at 37° C for an additional 2 h. After 2 h the medium was removed and the cells solubilized with 2 ml 0.3 M NaOH for 5 min and transferred to centrifuge tubes. The dissolved cellular material was precipitated with 8 ml 15% trichloroacetic acid (Sigma) and the precipitate washed twice with 15% trichloroacetic acid. The precipitate was dissolved in 0.5 ml 0.1 M NaOH and 200 µl added to 5 ml ScintiVerse II (Fischer Scientific, St. Louis, Mo) scintillation fluid for counting.

Assay of cytotoxicity. The cytotoxicity of cells treated with inhibitors of protein synthesis, TNFα, or a combination of protein synthesis inhibitors and TNFα, was determined by the release of radiolabelled ^{51}Cr from target cells plated in 96-well microtiter plates (Corning) as previously described [4]. Briefly, $(1-2) \times 10^6$ cells were incubated with 100 µCi/ml ^{51}Cr (Dupont/NEN) diluted in phosphate-buffered saline pH 7.2, containing 1% bovine serum albumin (Sigma) for 1 h at 37° C. After 1 h the cells were washed four times, counted, and resuspended in the same medium used for the routine growth of cells. Experimental wells contained 10^4 ^{51}Cr-labelled cells and varying concentrations of TNFα alone, inhibitors of protein synthesis alone, or combinations of TNFα and inhibitors of protein synthesis. Control wells (for the determination of the spontaneous release of ^{51}Cr) contained 10^4 cells in medium. Spontaneous release in all experiments ranged from 25% to 37% of the total incorporation of ^{51}Cr. The total incorporation of ^{51}Cr was determined by counting 10^4 cells in a gamma counter. Total ^{51}Cr in all experiments ranged from approximately 1500 to 3300 cpm. The cells were incubated for 16 h at 37° C in a humidified atmosphere of 95% air and 5% CO_2. After incubation the cells were centrifuged at 200 *g* for 10 min and the supernatant fluid was removed for counting of released ^{51}Cr (cpm). The percentage specific lysis of target cells was calculated using the following formula:

$$\text{Specific lysis (\%)} = 100 \times \frac{\text{(mean experimental release)} - \text{(mean spontaneous release)}}{\text{(mean total)} - \text{(mean spontaneous release)}}$$

Triplicate wells were assayed for each experiment and the experiments were repeated at least three times. The standard error of the mean of triplicate wells was in all experiments less than 5%. Representative experiments are shown in Figs. 1–4, and in Table 1.

Results

In order to determine the effect of protein synthesis inhibitors on the TNFα-mediated lysis of cervical and ovarian carcinoma cells, actinomycin D, cycloheximide, or emitine was added to the lytic assays of the following cell lines: ME-180, HT-3, MS751, SiHa, C-33A, CaOV-3, SK-OV-3, and OVCAR-3. The concentration of protein synthesis inhibitors was titrated over a wide range. The concentrations of protein synthesis inhibitors shown in Fig. 1 were those that caused the greatest increase in TNFα-mediated lysis, measured as the maximum difference between the percentage specific lysis in the presence of protein synthesis inhibitors alone, or TNFα alone, and the percentage specific lysis in the presence of protein synthesis inhibitors and TNFα. As shown in Fig. 1, the cell lines ME-180, HT-3, MS751, SiHa, CaOV-3, SK-OV-3, and OVCAR-3 were relatively resistant to lysis mediated by TNFα alone. At 25 units of TNFα/ml the percentage specific lysis of all cell lines was less than 10%. Cells treated only with inhibitors of protein synthesis showed variable degrees of lysis. The level of lysis caused by inhibitors of protein synthesis appears to be dependent on the cell line and to a lesser extent on the inhibitor. For example, at the same concentration of actionmycin D (1 µM) SiHa cells are lysed only 1%, whereas MS751 cells are lysed 27%. This is also true for cycloheximide and emitine, in that there are some cell lines that are sensitive to cycloheximide or emitine while other cell lines are relatively resistant to lysis at the same concentration. Although there are exceptions, in general, cycloheximide alone caused less lysis than either actinomycin D alone or emitine alone. Most importantly, the combination of inhibitors of protein synthesis and TNFα resulted in a level of cell lysis that was synergistic (as determined by isobolographic analysis) when compared to either alone. For most cell lines there was synergy between TNFα and all three inhibitors of protein synthesis and all of the cell lines showed synergy between TNFα and at least one of the protein synthesis inhibitors. The exception was the cell line C-33A. As shown in Table 1, like the other cell lines, C-33A cells are resistant to lysis by TNFα alone; however, in contrast to the other cells, the lysis of C-33A cells is not increased in the presence of TNFα and cyclo-

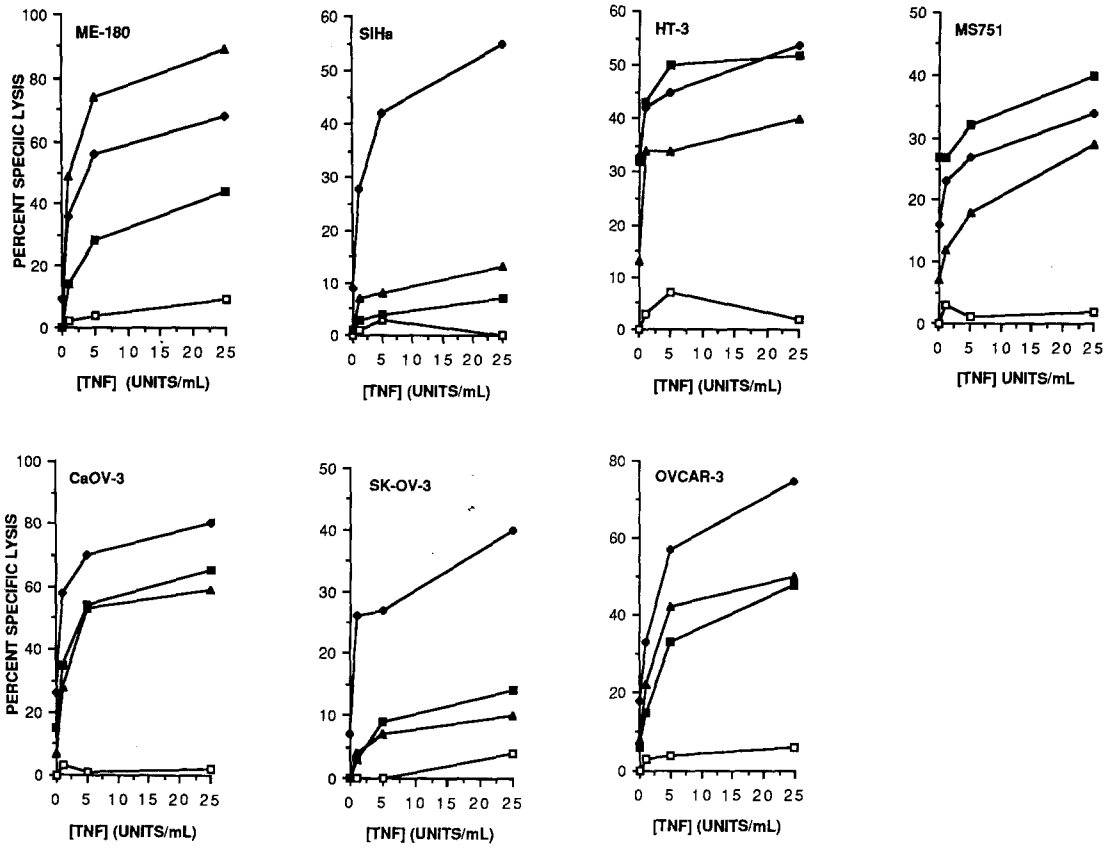

Fig. 1. The percentage specific lysis of ME-180, SiHa, HT-3, MS751, CaOV-3, SK-OV-3, and OVCAR-3 cells as a function of the concentration of tumor necrosis factor α (TNFα) with no protein synthesis inhibitors added (□), with actinomycin D added (■), with cycloheximide added (▲), or with emitine added (◆). The concentration of actinomycin D was 10 μM (SK-OV-3), 1 μM (ME-180, MS751, SiHa, OVCAR-3), 0.1 μM (HT-3), and 10 nM (CaOV-3). The concentration of cycloheximide was 1 mM (SiHa), 0.1 mM (HT-3, SK-OV-3, OVCAR-3), 10 μM (ME-180, MS751), and 0.1 μM (CaOV-3). The concentration of emitine was 0.1 mM (HT-3, SiHa, SK-OV-3, OVCAR-3), 1 μM (ME-180, MS751), and 0.1 μM (CaOV-3)

Table 1. Protein synthesis inhibitors do not increase the lysis of C-33A cells by TNFα

Inhibitor	Concentration (M)	Specific lysis (%) at various concentrations of TNFα (units/ml)			
		0	1	5	25
None	-	0	1	0	0
Actinomycin D	10^{-7}	9	7	9	10
	10^{-6}	24	27	30	29
	10^{-5}	31	39	33	34
Cycloheximide	10^{-5}	4	5	0	8
	10^{-4}	9	14	12	16
	10^{-3}	49	49	52	53
Emitine	10^{-7}	9	8	8	11
	10^{-6}	24	20	24	25
	10^{-5}	46	45	40	49

heximide, actinomycin D, or emitine. This is true even when these protein synthesis inhibitors were used at relatively toxic concentrations.

To confirm that target lysis in the presence of protein synthesis inhibitors is mediated by TNFα, immune rabbit serum containing anti-TNFα antibodies or nonimmune rabbit serum was added at the same time as TNFα. As shown in Fig. 2, anti-TNFα antibodies block the lysis of cells treated with TNFα and emitine.

In order to determine the temporal requirements for protein synthesis relative to the time when TNFα is added, cells were exposed to emitine for various lengths of time before they were exposed to TNFα. Regardless of when emitine was added, the percentage specific lysis was measured 16 h after the addition of TNFα. As shown in Fig. 3, the maximum percentage specific lysis occurred when emitine was added at the same time as TNFα. When emitine was added either before or after the addition of TNFα, the percentage specific lysis was reduced. The longer the addition of emitine was delayed after the addition of TNFα the less the amount of cell lysis. The decrease in cell lysis that occurred when emitine was added after TNFα is directly related to how long the resistance mechanism operates relative to the length of the assay. For example, when emitine was added 6 h after the addition of TNFα, the cells became sensitive to lysis, but that sensitivity could only be measured over the remaining 10 h of the assay. This was shown in a separate set of experiments, in which emitine was added 6 h after the addition of TNFα and lysis was measured 16 h after the addition of emitine. The percentage specific lysis that occurred in these experiments was identical to the value resulting when emitine was added at the same time as TNFα and lysis was mea-

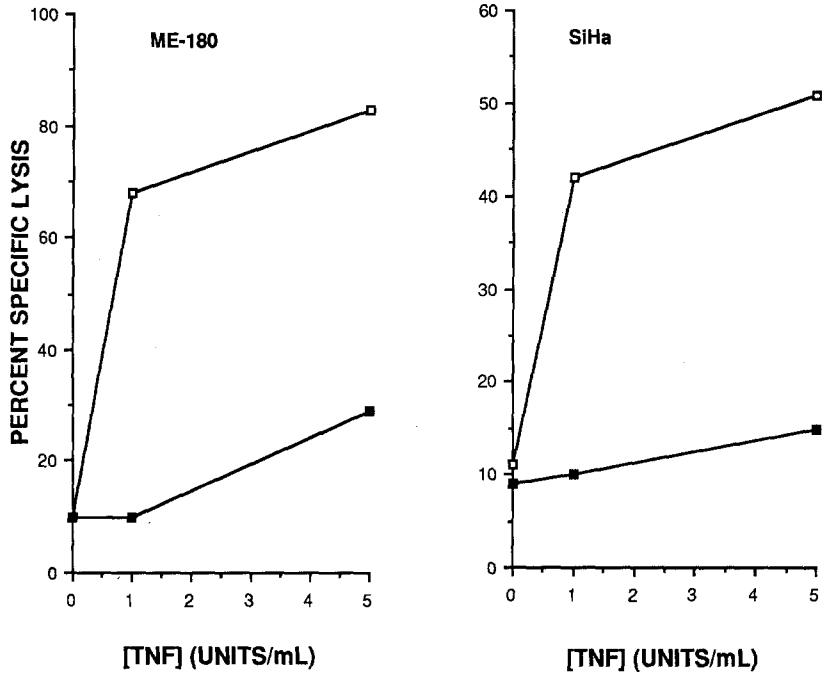

Fig. 2. The percentage specific lysis of ME-180 and SiHa cells as a function of the concentration of TNFα in the presence of 0.1 mM emitine (SiHa) or 1 μM emitine (ME-180). Rabbit immune serum, containing antibodies specific for human TNFα (■), or nonimmune serum (□) was added at the beginning of the lytic assay

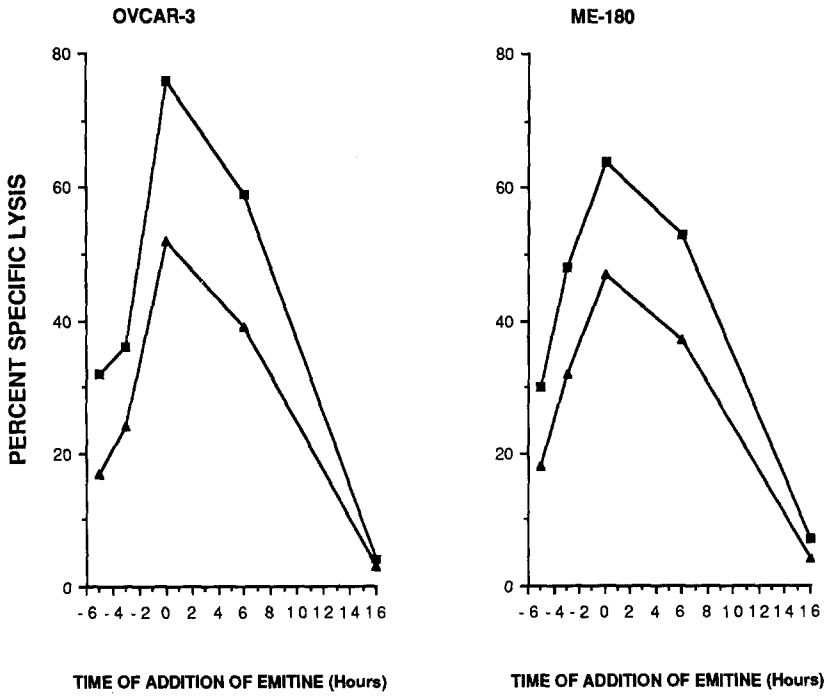

Fig. 3. The percentage specific lysis of OVCAR-3 and ME-180 cells in the presence of 25 units/ml TNFα as a function of the time when 0.1 mM (■) or 10 μM (▲) emitine (OVCAR-3); or when 1 μM (■) or 0.1 μM (▲) emitine (ME-180) was added relative to that of TNFα. TNFα was added at 0 time. Independent of when emitine was added, the percentage specific lysis was determined 16 h after the addition of TNFα

sured 16 h later (data not shown). This indicates that lysis by TNFα does not begin until protein synthesis is inhibited, independent of when TNFα is added. This is consistent with a TNFα resistance mechanism, the maintenance of which is dependent on protein synthesis. As shown in Fig. 3, there was also a time-dependent reduction in lysis when emitine was added before the addition of TNFα; the earlier emitine was added, the less the cell lysis that occurred. This indicates that although *de novo* protein synthesis is not required for lysis, protein synthesis is required to maintain the potential of cells to be lysed by TNFα. In contrast to the resistance mechanism, the reduction in lysis when emitine was added before TNFα is not dependent on the length of the assay since the cells were always exposed to TNFα for 16 h.

In order to determine the relationship between the level of protein synthesis and the level of TNFα-mediated lysis various concentrations of emitine were added to one set of cells, and to a duplicate set of cells emitine was added at the same time as TNFα. The level of protein synthesis (incorporation of [^{3}H]leucine) and cell lysis was measured at 4 h and 16 h, respectively. As shown in Fig. 4, within a given cell line there is a near-linear relationship between the percentage inhibition of protein synthesis in the pres-

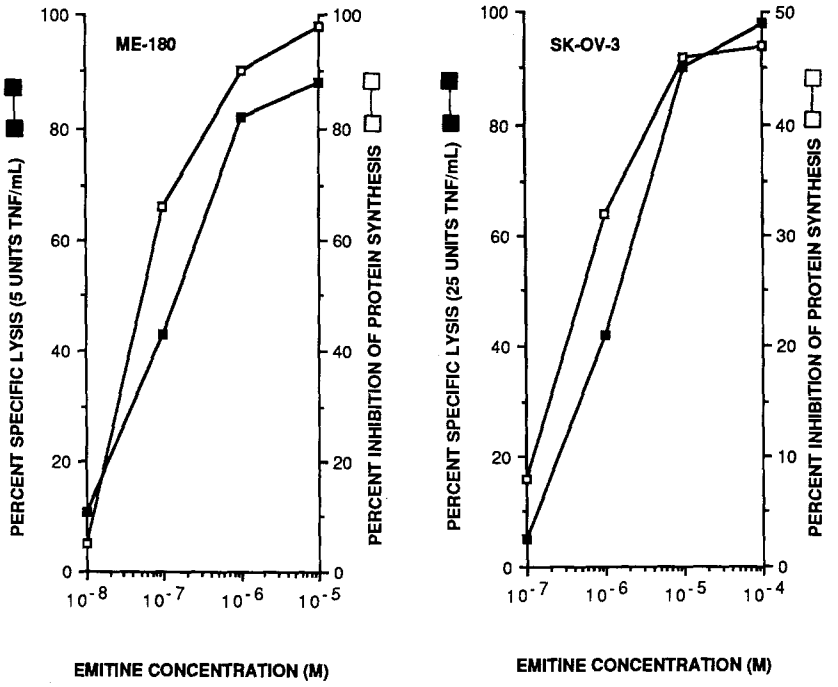

Fig. 4. The percentage specific lysis of ME-180 and SK-OV-3 cells in the presence of TNFα and the percentage inhibition of protein synthesis as a function of the concentration of emitine

ence of emitine and the percentage specific lysis in the presence of emitine and TNFα; the greater the inhibition of protein synthesis the greater the percentage specific lysis. Although the level of protein synthesis is related to the level of cell lysis, there is no absolute correlation between the level of protein synthesis and the level of cell lysis. For example, 82% specific lysis of ME-180 cells occurs when protein synthesis was inhibited by 90%, whereas 90% specific lysis of SK-OV-3 cells occurs when there is only a 44% inhibition of protein synthesis.

Discussion

With the exception of C-33A cells, the experiments presented clearly indicate that the resistance of ME-180, HT-3, MS751, SiHa, CaOV-3, SK-OV-3, and OVCAR-3 cells to lysis by TNFα is dependent on the maintenance of protein synthesis. When protein synthesis is inhibited they become sensitive to lysis by TNFα. The synergy caused by the combination of TNFα and actinomycin D (i.e., an inhibitor of protein synthesis that blocks at the level of transcription) and TNFα and cycloheximide or emitine (i.e., inhibitors of protein synthesis that block at the level of translation) is approximately equal in magnitude. This indicates that the maintenance of the TNFα resistance mechanism is equally dependent on the continued synthesis of both mRNA and protein. Because the inhibitors of protein synthesis had an effect when added at the same time as TNFα, the protein(s) as well as their mRNA(s) must have relatively short half-lives.

Two lines of evidence indicate that the inhibition of protein synthesis increases lysis by TNFα. First, three different inhibitors of protein synthesis: actinomycin D, which blocks DNA-dependent mRNA transcription, and emitine and cycloheximide, which block at different sites in the translation of mRNA into protein, all increased the sensitivity of cells to TNFα-mediated lysis (Fig. 1). The fact that all three inhibitors produced the same effect argues strongly that the increased sensitivity to lysis by TNFα in the presence of actinomycin D, cycloheximide, or emitine is directly related to the ability of these agents to block protein synthesis. Second, we have demonstrated a linear correlation between the level of inhibition of protein synthesis in the presence of emitine and the level of lysis in the presence of emitine and TNFα; as the inhibition of protein synthesis increases, TNFα-mediated lysis also increases (Fig. 4). Although this relationship varies quantitatively for different cell lines, within a given cell line this relationship remains linear over a wide range of emitine concentrations.

That the increase in lysis in the presence of protein synthesis inhibitors and TNFα is also dependent on the ability of TNFα to sensitized cells is shown by the fact that antibody specific for human TNFα blocks the lysis of cells treated with emitine and TNFα (Fig. 2).

From these findings we conclude that some cancer cells must actively resist lysis by TNFα and this resistance is dependent on the maintenance of protein synthesis. The fact that seven of eight arbitrarily selected cell lines show increased lysis by TNFα in the presence of inhibitors of protein synthesis suggests that the expression of this resistance mechanism may be very common, at least among gynecological malignancies. Although we have not, as yet, determined whether C-33A cells express receptors for TNFα, the most plausible explanation for their lack of sensitivity to lysis by TNFα in the presence of inhibitors of protein synthesis (Table 1) is that C-33A cells do not express receptors for TNFα. In the absence of TNFα binding there is no "activation" of the TNFα lytic mechanism, and therefore no lysis can occur even if the resistance mechanism is inhibited. We do not believe that the resistance of C-33A cells to lysis in the presence of TNFα and inhibitors of protein synthesis is due the the inability of the

inhibitors to block protein synthesis. Protein synthesis was inhibited in C-33A cells by more than 90% in the presence of 1 μM emitine (data not shown).

The mechanism of TNFα lysis is unclear, but may be, in part, mediated through the generation of superoxides [24, 25]. Consistent with the role of superoxides in the TNFα-mediated lysis of cells, there is recent evidence demonstrating that the expression of the gene encoding manganous superoxide dismutase, an enzyme capable of scavaging superoxides, confers partial resistance to lysis by TNFα [24, 25]. It therefore seems possible that inhibitors of mRNA and protein synthesis prevent the expression of manganous superoxide dismutase in ovarian and cervical carcinoma cells and thereby render them sensitive to lysis by TNFα.

Resistance of cancer cells to lysis by TNFα has presented a major block to the clinical success of TNFα as an anticancer agent. The experiments described here suggest a strategy for manipulating the TNFα resistance mechanism in cancer cells by inhibiting protein synthesis. If we extrapolate from these in vitro experiments to the autochthanous situation we would predict that the majority of cervical and ovarian carcinomas would normally be resistant to TNFα, and that the inhibition of protein synthesis in these cells would make them sensitive to TNFα. That most gynecological malignancies are resistant to TNFα appears to be true given the poor response rate in clinical trials with TNFα [2, 6, 12, 20]. If we are correct in our assumptions, the way to increase the efficacy of TNFα is to treat cancer patients simultaneously with TNFα and inhibitors of protein synthesis. Given the dramatic increase in sensitivity to lysis by TNFα in the presence of protein synthesis inhibitors it might also be possible to use relatively low doses of TNFα, thereby reducing some of the complications associated with attempts to maintain high serum concentrations of TNFα during therapy [2, 6, 12, 20]. The obvious choice for the inhibitor of protein synthesis would be actinomycin D. Actinomycin D increases the sensitivity of cells to TNFα in vitro and its properties as a chemotherapeutic agent are well known [10].

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