Synergistic Enhancement by Tumor Necrosis Factor of in Vitro Cytotoxicity from Chemotherapeutic Drugs Targeted at DNA Topoisomerase II

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ABSTRACT

Recombinant human tumor necrosis factor (rHTNF) alone had no effect on L929 tumor cells at 100 units/ml for 20 h of continuous exposure. However, under the same conditions, rHTNF markedly enhanced the cytotoxicity of Adriamycin, actinomycin D, 4'-(9-acridinylamino)-methanesulfonyl-m-anisidine, teniposide (VM 26), and etoposide (VP 16), all targeted at DNA topoisomerase II. The rHTNF had a minimally enhancing effect on the cytotoxicity of bleomycin, hydroxyurea, and 1-β-D-arabinofuranosylcytosine and no effect on the cytotoxicity of cis-platinum, mitomycin C, vincristine, and vinblastine, all chemotherapeutic drugs with dose-related cytotoxic effects on L929 cells but mechanisms of action which do not appear to involve topoisomerase II. Treatment with rHTNF first and then topoisomerase-targeted drugs yielded no enhanced cytotoxicity, whereas pretreatment with drug followed by rHTNF yielded marked enhancement of cytotoxicity. Topoisomerases have previously been implicated in cell kill phenomena following treatment with certain chemotherapeutic agents [K. M. Tewey, et al., Science (Wash. DC), 226: 466–468, 1984]. The data suggest that the lethality to the cell from topoisomerase-targeted drug treatment is increased by rHTNF in vitro. We suggest that rHTNF may be a useful adjuvant to this class of drugs which has well-known antitumor activity.

INTRODUCTION

TNF2 is of great interest because of its reported selective cytotoxicity towards certain tumor cells while sparing normal cells. At present, its clinical effectiveness has not been established as a single agent, although it is under extensive investigation. The recent availability of highly purified recombinant human tumor necrosis factor has stimulated interest in resolving the mechanism of action of TNF.

TNF was first described as a serum factor in Bacillus Calmette-Guérin-treated and endotoxin-challenged mice (1). The serum from such animals causes necrosis of meth-A sarcoma tumors growing intradermally in BALB/c mice (1). TNF also has direct cytotoxic effects upon certain tumor cell lines in vitro (2–4) and on some primarily cultured human cancer cells (5). The in vitro cytotoxicity of TNF has most usually been demonstrated using the murine fibrosarcoma cell line L929 as the target (6, 7). The addition of actinomycin D to the incubation medium has been shown to increase the cytotoxicity to the cell monolayers (7, 8), and its inclusion is now standard in TNF assays. It is unknown why actinomycin D is required, but many believe it is due to the inhibition of RNA synthesis (8).

In the absence of actinomycin D or certain other drugs, TNF alone added to the medium has antiproliferative effects on sensitive cell lines but only at much higher doses (1,000- to 10,000-fold) and with longer incubation periods (6, 9–11). We were struck by the vast increase in cell killing observed when rHTNF and actinomycin D were combined. We critically examined the effect of each agent separately and in combination and found that rHTNF appeared to enhance the cytotoxic effect of actinomycin D. This enhancement occurred at concentrations of rHTNF that had no effect on L929 cells in the absence of actinomycin D.

Actinomycin D, besides inhibiting RNA synthesis, has recently been found to interfere with the function of an important nuclear enzyme, DNA topoisomerase II (12). This enzyme is involved in the control of the winding and unwinding of DNA (13). Recently, several widely used chemotherapeutic drugs such as Adriamycin (12), VP 16, and VM 26 (14) have been found to interfere with the function of topoisomerase II. This class of drugs is thus termed "topoisomerase targeted." We found that rHTNF significantly enhanced the cytotoxicity of all topoisomerase-targeted drugs we tested and that rHTNF did not significantly enhance a large number of other drugs whose mechanism of action does not appear to involve topoisomerase II. In these studies, purified rHTNF was used in order to avoid contaminating bioactivities present in sera and because rHTNF has the potential for use as a therapeutic agent in human cancers (11).

MATERIALS AND METHODS

Reagents. Recombinant human TNF was provided by Cetus Corporation (Emeryville, CA). Etoposide (VP 16), teniposide (VM 26), and mAMSA were generously provided by Dr. Leroy Liu (Baltimore, MD). All other chemotherapeutic drugs were purchased from Sigma (St. Louis, MO).

Cells. The murine fibrosarcoma cell line L929 (American Type Culture Collection, Rockville, MD) was maintained by serial passage of monolayer cultures in TC-75 flasks (Corning, Corning, NY) in medium consisting of Eagle's minimal essential medium with L-glutamine (Gibco, Grand Island, NY) containing 10% heat-inactivated horse serum (Gibco, Grand Island, NY), streptomycin (100 μg/ml), and penicillin (100 units/ml) (medium).

Cytotoxicity Assay. The cytotoxicity of L929 cells was monitored with the crystal violet assay as described by Ruff and Gifford (6, 15). The assay measures the number of cells present in a monolayer at the end of a given incubation period (6). Any cell still attached is considered surviving; cytotoxicity is calculated by comparing the number of surviving cells in treated monolayers to the number of cells in identical but untreated monolayers. This is the standard TNF assay in widespread use for measuring TNF levels in sera and other fluids (7, 16).

Five-day-old confluent monolayers of L929 cells were suspended by treatment with 0.25% trypsin solution (Sigma, St. Louis, MO) and collected by centrifugation. The cells were counted and inoculated into 96-well flat-bottomed microtiter plates (Costar, Cambridge, MA) at a seeding density of 3 x 104 cells per well in a volume of 0.1 ml of medium. Cells were incubated 24 h in 5% CO2 at 37°C. Medium was discarded and replaced with 0.2 ml of fresh medium containing various concentrations of drugs and/or rHTNF. This was accomplished by placing plain medium over the cells and performing serial 1:2 to 1:5 dilutions across the microtiter plate using a multichannel pipet (Flow,
Labs., McLean, VA). Cells were then incubated at 37°C in 5% CO₂ for the cytotoxicity incubation period, generally 20 h. At the end of the incubation period the medium was removed by inverting the plate, and the monolayers were stained by immersion in 0.5% crystal violet in 25% methanol for 10 min at room temperature. The plates were rinsed in tap water and dried.

The stained monolayers were dissolved in 0.1 ml/well of 1% SDS at room temperature for 15 min as described by Zacharchuk et al. (17). The absorbance of each well was measured at 540 nm against 1% SDS blanks using a Tiethek Multiskan MC (Flow Labs., McLean, VA).

The percentage of cytotoxicity was calculated by the formula, 100 × (1 - Aexposed/Acontrol), where Aexposed is the absorbance of cells treated with various concentrations of rHTNF and/or drugs, and Acontrol is the absorbance of cells treated with medium only. One unit of TNF activity is required that to achieve 50% cytotoxicity under these standard conditions in the presence of 1 μg/ml actinomycin D.

For order of addition studies the 20-h incubation period was divided into a 7-h first incubation and a 13-h second incubation. After the first incubation, medium was poured off, and the cells were washed with Hanks’ balanced salt solution. After the second incubation, the plates were stained and cytotoxicity determined as above.

RESULTS

In the presence of actinomycin D (1 μg/ml), a striking cytotoxic effect on L929 cells from rHTNF was seen. Under the standard TNF assay conditions [6, 15], L929 cell monolayers in 96-well microtiter plates (3 × 10⁴ cells/well) were exposed for 20 h to serial dilutions of a rHTNF solution. Medium in all wells contained actinomycin D (1 μg/ml). In this assay, cells exposed to both rHTNF and actinomycin D were compared to cells exposed to actinomycin D alone. Hence, any effect of actinomycin D alone upon the cells was not detected. A solution of rHTNF assayed under these conditions was found to contain 118 ± 33 units/ml (mean ± SE) in good agreement with the activity provided by Cetus at 100 units/ml.

In contrast to its striking cytotoxic effect in the presence of actinomycin D, rHTNF alone had a minimal cytotoxic effect on L929 cells. Even with continuous exposure for 48 h at a rHTNF dose approaching 100,000 units/ml the maximum cytotoxicity from rHTNF alone did not exceed 40% (data not shown). This is in marked contrast to the cytotoxic effect of rHTNF in the presence of 1 μg/ml actinomycin D where the cytotoxicity of rHTNF (1 unit/ml) is 50% at 20 h by definition.

Because of this marked discrepancy we wondered what effect actinomycin D alone would have in this assay. We therefore examined the effect of each agent independently and in combination as shown in Fig. 1. In this experiment, cells continuously exposed for 20 h to various combinations of rHTNF and actinomycin D in medium were compared to cells exposed to medium alone. Hence, any effect on cell proliferation or death caused by either agent was detected. In the absence of actinomycin D, rHTNF alone had no effect upon the cells at doses from 0.01 to 100 units/ml, continuous exposure for 20 h. The addition of actinomycin D alone at zero rHTNF concentrations (Fig. 1A, Y-axis) resulted in a dose-dependent increase in cytotoxicity. The combination of both agents resulted in dose-dependent synergy of cytotoxicity, detected as an upward deflection of this dose-response curve in the presence of actinomycin D. When cytotoxicity was plotted as a function of actinomycin D concentration in the presence or absence of a constant dose of rHTNF from 0 to 100 units/ml (Fig. 1B), it was apparent that actinomycin D alone had a significant cytotoxic effect on the cells and that this effect was increased by rHTNF at all concentrations of actinomycin D where the drug alone had a cytotoxic effect upon the cells. It thus appeared that the cytotoxicity in this assay was due to actinomycin D with enhancement by rHTNF.

To determine if rHTNF might enhance the cytotoxicity of other chemotherapeutic drugs, a variety of agents was tested. Dosages were chosen in a range such that the drug alone would have an effect on the cells and the effect of rHTNF (100 units/ml) on this curve could be evaluated. Actinomycin D not only blocks RNA synthesis but also interacts potently with DNA topoisomerase II (12), and we therefore tested a series of other topoisomerase-targeted agents to determine if their cytotoxicity could also be enhanced by rHTNF. As shown in Fig. 2, rHTNF at 100 units/ml significantly enhanced the cytotoxicity of the topoisomerase-targeted drugs Adriamycin and VM 26 at all drug doses that, by themselves, had an effect on the cells. The non-topoisomerase-targeted drugs cis-platinum and mitomycin C demonstrated no synergy when combined with rHTNF under the same conditions as shown in Fig. 1. The cytotoxicity of all topoisomerase-targeted drugs tested including, in addition, VP 16 and mAMSA was significantly enhanced by rHTNF. The maximum observed cytotoxicities for all drugs tested in the presence or absence of rHTNF (100 units/ml) are presented in Table 1.
Cytotoxicity was computed photometrically by comparing the absorbance of treated monolayers to the absorbance of monolayers exposed to medium alone.

**DISCUSSION**

In drug-mediated inhibition studies, the order of addition of agents given in combination can be critical. Therefore, VM 26 or actinomycin D was added to the cells at different times relative to rHTNF incubation. As shown in Table 2, cytotoxicity due to the drug alone was not affected by preincubation with rHTNF (100 units/ml) for 7 h. However, preincubation with drug first and then rHTNF resulted in marked enhancement of cytotoxicity comparable to continuous incubation with both agents. Thus, topoisomerase-targeted drug cytotoxicity enhancement by rHTNF was only seen if drug treatment preceded due to the drug alone was not affected by preincubation with actinomycin D were combined was due to inhibition of RNA synthesis by the drug.

Previous reports (6) describe the effect of actinomycin D as an increase in cytotoxicity when actinomycin D was added to lymphoblasts, a cytolytic lymphokine with striking similarity to TNF (18), was first reported by Eifel et al. in 1975 (19). Ostrove and Gifford (8) noted an increase in [H]uridine uptake in L929 cells treated with rabbit TNF and suggested that the increase in cell killing that they noted when TNF and actinomycin D were combined was due to inhibition of RNA synthesis by the drug.

Previous reports (6) describe the effect of actinomycin D as a simple shifting of the TNF dose-response curve for L929 cytotoxicity using rabbit tumor necrosis factor. In our study, we were thus surprised to find such a marked difference between the rHTNF cytotoxicity curves with and without actinomycin D and that even very large doses of rHTNF alone could not cause nearly the cytotoxicity of the combination.

A careful examination of the effect of each agent upon the cells showed significant cytotoxicity from actinomycin D alone even at very low concentrations and low cytotoxicity from rHTNF alone even at high concentrations. The combination, however, yielded striking synergy with efficacy not attained by either agent alone. Increasing actinomycin D doses also lowered the threshold of rHTNF needed for synergy (Fig. 1A).

Besides inhibiting RNA synthesis, actinomycin D interacts potently with DNA topoisomerase II (12). Recently, topoisomerases have generated intense interest as many of the intercalating chemotherapeutic drugs (12, 20) as well as the nonintercalating epipodophyllotoxins VP 16 and VM 26 (14) have been found to interfere with the function of mammalian DNA topoisomerase II. The topoisomerases are enzymes that can catalyze the formation of various isomeric forms of DNA (reviewed in Ref. 13) by transiently breaking and rejoining the DNA strand. This occurs by means of a transient enzyme-DNA covalent bond associated with a DNA break. The topoisomerase-targeted drugs cause the accumulation of such topoisomerase-DNA linkages (12, 14, 20, 21) presumably by stabilizing this usually transient reaction intermediate.

As with actinomycin D, rHTNF clearly synergistically enhanced the cytotoxicity of all the topoisomerase-targeted drugs tested. Only some of the agents whose mechanism of action do not involve topoisomerase II had their cytotoxicity enhanced by rHTNF, and the effect was much less pronounced and in some cases required very high drug doses. Many of the drugs tested which do not appear to target topoisomerase II do damage DNA in other ways, but enhancement by rHTNF was not seen. This suggests that the enhancing effect of rHTNF is in some way related to topoisomerase-mediated DNA damage but not other forms of DNA damage.

An association between the enhancing effect of rHTNF and topoisomerase-mediated DNA damage is also suggested by the cytotoxicity data using rHTNF with novobiocin and ethidium bromide. Ethidium bromide and novobiocin are inhibitors of DNA topoisomerase II function through different mechanisms. Novobiocin inhibits the ATPase activity of the enzyme (22), and ethidium bromide binds to DNA by intercalation and presumably prevents the enzyme from binding to DNA by steric hindrance or some other mechanism (23). While novobiocin and ethidium bromide may be termed inhibitors of topoisomerase II function they do not cause the formation of enzyme-

**Table 1**

| Drug             | Dose range | Max observed cytotoxicity | rHTNF (100 units/ml) | 100 units/ml | % of increase with rHTNF | p*
|------------------|------------|---------------------------|----------------------|--------------|--------------------------|---
| Adriamycin       | 0.04–500 μM| 51.2 ± 6.0<sup>a</sup>    | 95.5 ± 2.4           | 86           | <0.001                   | <0.001
| Actinomycin D    | 0.003–100 μM| 58.8 ± 2.0               | 99.3 ± 0.3           | 69           | <0.001                   | <0.001
| mAMSA            | 0.03–5 μM  | 33.3 ± 0.9               | 55.0 ± 3.1           | 65           | <0.001                   | <0.001
| VP 16            | 0.3–100 μM | 43.3 ± 1.4               | 68.9 ± 1.6           | 59           | <0.001                   | <0.001
| VM 26            | 0.04–10 μM | 50.8 ± 2.8               | 77.2 ± 0.9           | 52           | <0.001                   | <0.001
| Hydroxyurea      | 0.07–20 μM | 50.2 ± 0.1               | 68.4 ± 0.4           | 36           | <0.012                   | <0.012
| 1-β-Arabinofuranosylcytosine | 0.3–100 μM | 47.6 ± 5.0               | 62.7 ± 4.7           | 31           | NS<sup>b</sup>           | NS<sup>b</sup>
| Bleomycin        | 0.0005–3 units/ml | 70.6 ± 8.6             | 92.9 ± 4.0           | 31           | <0.02                    | <0.02
| Mitomycin C      | 0.04–300 μM| 63.4 ± 3.8               | 71.8 ± 4.2           | 13           | NS<sup>b</sup>           | NS<sup>b</sup>
| cis-Platinum     | 0.1–900 μM | 55.2 ± 3.7               | 60.7 ± 4.7           | 10           | NS<sup>b</sup>           | NS<sup>b</sup>
| Vinblastine      | 0.0002–1 μM| 50.3 ± 6.9               | 55.7 ± 1.0           | 10           | NS<sup>b</sup>           | NS<sup>b</sup>
| Novobiocin       | 3–1000 μM | 89.7 ± 1.3               | 97.0 ± 0.3           | 8            | NS<sup>b</sup>           | NS<sup>b</sup>
| Vincristine      | 0.0005–3 μM| 55.6 ± 1.2               | 57.1 ± 0.9           | 3            | NS<sup>b</sup>           | NS<sup>b</sup>
| Ethisidum bromide| 0–30 μM   | 47.4 ± 0.1               | 47.5 ± 1.0           | 1            | NS<sup>b</sup>           | NS<sup>b</sup>
| Medium only      |            |                          |                      |              |                          |<sup>c</sup>

<sup>a</sup> P values determined by Student's t test.  
<sup>b</sup> Mean ± SD of three determinations.  
<sup>c</sup> NS, P > 0.05.  
<sup>d</sup> By definition.

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**Table 2**

<table>
<thead>
<tr>
<th>First incubation (7 h)</th>
<th>Second incubation (13 h)</th>
<th>Cytotoxicity</th>
</tr>
</thead>
<tbody>
<tr>
<td>VM 26 (5 μM)</td>
<td>rHTNF (100 units/ml)</td>
<td>64.5 ± 0.5&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Actinomycin D (1 μM)</td>
<td>rHTNF (100 units/ml)</td>
<td>81.8 ± 1.8</td>
</tr>
<tr>
<td>Medium only</td>
<td>rHTNF (100 units/ml)</td>
<td>-2.5 ± 1.6</td>
</tr>
<tr>
<td>rHTNF (100 units/ml)</td>
<td>VM 26 (5 μM)</td>
<td>34.1 ± 1.8</td>
</tr>
<tr>
<td>Medium only</td>
<td>VM 26 (5 μM)</td>
<td>33.2 ± 0.2</td>
</tr>
<tr>
<td>rHTNF (100 units/ml)</td>
<td>Actinomycin D (1 μM)</td>
<td>29.8 ± 0.1</td>
</tr>
<tr>
<td>Medium only</td>
<td>Actinomycin D (1 μM)</td>
<td>31.3 ± 0.2</td>
</tr>
</tbody>
</table>

<sup>a</sup> Mean ± SD of three determinations.
linked DNA breaks as do the topoisomerase-targeted drugs. Both novobiocin and etidium bromide demonstrated dose-dependent cytotoxicity to L929 cells, but enhancement by rHTNF did not occur. Enhancement by rHTNF only occurred with drugs known to cause topoisomerase-linked DNA breaks. These observations provide additional evidence that the enhancing effect of rHTNF is closely related to the DNA damage mediated by topoisomerase-targeted drugs and not to some other effect on the cell from drug treatment.

The order of addition studies clearly show that the effect of rHTNF is seen only if drug treatment precedes or is coincidental with rHTNF exposure. Treatment with rHTNF alone followed by washing and exposure to topoisomerase-targeted drugs did not cause enhancement of cytotoxicity to L929 cells. The 7-h incubation period for rHTNF pretreatment is well in excess of that needed for rHTNF receptor binding and internalization (10). Hence, pretreatment with rHTNF does not appear to render L929 cells more vulnerable to subsequent killing by topoisomerase-targeted drugs, but coincident or subsequent treatment with rHTNF augments drug-mediated killing.

The mechanism of the enhancement by rHTNF of topoisomerase-targeted drug cytotoxicity is unknown. Our data suggest that the effect of rHTNF is related to the topoisomerase-mediated DNA damage caused by these drugs. One possibility is that rHTNF may increase the amount of topoisomerase-mediated DNA damage by, for example, increasing the amount of enzyme present. However, since DNA-enzyme cross-links appear rapidly following drug treatment (12, 14, 20, 21) and the effect of rHTNF on cytotoxicity occurs much later, it is unlikely that rHTNF has a significant effect on the amount of enzyme-DNA cross-links. A likely possibility is that the effect of rHTNF is manifested through some other mechanism which renders the cell more sensitive to this unique form of DNA damage. It is also possible that rHTNF may affect uptake of the topoisomerase-targeted drugs or vice versa. We have not investigated this possibility.

It is by no means clear that the in vitro and in vivo antitumor effects of TNF are manifestations of the same bioactivity. Similarly, it is not clear if the ability of rHTNF to enhance the cytotoxicity of topoisomerase-targeted drugs is related in any way to its antiproliferative effect in vitro when used alone. Studies on cell lines insensitive to rHTNF alone using rHTNF in combination with topoisomerase-targeted drugs would be one approach to answering this question. It would be of interest to see if cells not sensitive to rHTNF alone could be so rendered by drug treatment.

While clearly effective in the treatment of murine intradermal meth-A fibrosarcoma (24), early reports using rHTNF alone in other in vivo animal tumor models have been less encouraging (Footnote 3; Ref. 25). Clearly, the combination of rHTNF with topoisomerase-targeted drugs requires further study in in vivo tumor models and should be a consideration in the planning of future human clinical trials using rHTNF.

In summary, we have shown that rHTNF significantly enhances the cytotoxicity of DNA topoisomerase-targeted chemotherapeutic drugs in vitro. The effect appears to be an increase in the lethality to the cell of the drug-mediated DNA lesions which must occur before or during rHTNF treatment. While in vitro cytotoxicity may not correlate with in vivo effects, the data may be relevant to the planning of future animal and human trials using rHTNF for the treatment of cancer.

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