ABSTRACT

We tested the effect of recombinant human tumor necrosis factor (TNF) on the growth of the murine methylcholanthrene induced fibrosarcoma and the human ovarian carcinoma (NIH:OVCAR-3) in mice. The mice received multiple doses (25-250 μg/kg) of TNF starting 7–10 days after s.c. transplantation of tumors when they were easily palpable. TNF was administered i.v. every other day for a total of 6 injections per mouse, or i.p. daily for 7 days. Complete tumor regression was observed in the methylcholanthrene induced tumor bearing mice in 90% of the mice treated with TNF (100 μg/kg), 67% treated with TNF (50 μg/kg), and 34% treated with TNF (25 μg/kg). Tumors which did not completely regress were growth retarded during the course of TNF treatment. All mice given the highest TNF dose are still alive and tumor free (currently over 400 days), whereas the median survival of control mice was 28–39 days. Partial regression was observed in 100% of mice bearing the ovarian carcinoma treated i.p. with 250 μg/kg. Injections of TNF i.v. resulted in higher percentage of cures than i.p. injections at similar dose levels.

These results suggest that tumor necrosis factor represents a likely potent drug against solid tumors and that the method of administration is critical in optimizing its use in cancer.

INTRODUCTION

TNF is a protein that was discovered in the serum of mice or rabbits given injections of Bacillus Calmette-Guérin or Corynebacterium parvum, followed by endotoxin (lipopolysaccharide), 1 to 2 weeks later (1–3). Serum containing TNF causes necrosis of some tumors in vivo and is specifically cytotoxic and/or cytostatic to tumor cells in tissue culture (1–4). The i.v. administration of serum containing TNF into mice bearing the BALB/c sarcoma, Meth A, resulted in apparent destruction of the center of the tumor mass within 24–48 h, leaving a peripheral rim of viable tissue (1). Partially purified preparations of mouse TNF were reported to exhibit antitumor activity against both mouse and human transplanted tumors in nude mice (5). Although it was noted that "sometimes" a complete cure was observed in Meth A, Sarcoma 180, Ehrlich, and MM46 tumors (5), survival data with long-term follow-up on the cures has not been presented.

Partially purified native human TNF as isolated from the human promyelocytic leukemia cell line HL-60 was shown to cause significant necrosis of an established human breast carcinoma upon a single i.v. injection of TNF into nude mice (6) and the necrosis of the murine Meth A sarcoma when injected i.v., i.p., or i.m. (7). Recently, recombinant human TNF produced in Escherichia coli was also shown to cause hemorrhagic necrosis of the Meth A tumor model (7) and of an undifferentiated human colon carcinoma when injected i.t. (8). It was also reported by the latter investigators that complete tumor regression was observed in two of four mice receiving 5 × 10^5 units of TNF.

Studies in our laboratories and those described above (6–8) demonstrated that single injections of human recombinant TNF cause tumor necrosis. However, the tumors usually recover and kill mice. Long-term effects of multiple injections of TNF on survival of mice with tumors have not been reported. In this study we demonstrate that repetitive systemic administration of TNF (a) cured mice of s.c. murine methylcholanthrene induced fibrosarcomas, prolonging their survival by >13 months, and (b) caused partial regression of human ovarian tumors in nude mice. In addition, we show that both tumor cell lines were resistant to the cytotoxic action of TNF in vitro.

These results suggest that the mechanism of growth inhibition and regression of s.c. tumors in vivo by TNF may be due to both the direct effect of TNF on the tumor cells and its indirect effect on the tumor vasculature and/or the host immune system.

MATERIALS AND METHODS

Tumor Necrosis Factor. Highly purified recombinant human tumor necrosis factor expressed in E. coli (lot LYM12 02185B) was kindly provided by Dr. Leo Lin's laboratory at Cetus Corporation and was used for all in vivo studies. The endotoxin level in the TNF preparation, was <1.2 μg/ml as determined by the Limulus amebocyte lysate assay (Associates of Cape Cod, Inc., Woodshole, MA). Upon dilution of the concentrated material in PBS with 2% bovine serum albumin, the mice were killed mice (9) cured mice of s.c. murine methylcholanthrene induced fibrosarcomas, prolonging their survival by >13 months, and (b) caused partial regression of human ovarian tumors in nude mice. In addition, we show that both tumor cell lines were resistant to the cytotoxic action of TNF in vitro.

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Cells and Tumors. Murine (BALB/c) methylcholanthrene induced fibrosarcoma cells (Meth A) were kindly supplied by Dr. Lloyd Old's laboratory at Sloan Kettering, New York, NY. The cells were propagated as ascites in BALB/c nude mice. For s.c. tumors 5 × 10^5 cells were suspended in 0.2 ml of serum free tissue culture medium and injected in the suprascapular region on day 0. Tumors were evaluated by caliper (micrometer) measurements of 3 orthogonal directions (length x width x height). Routinely the tumors reached about 100–200 mm³ in 10 days. Human ovarian carcinoma cells NIH:OVCAR-3 (9) were supplied to Cetus by Dr. T. Hamilton, National Cancer Institute, Bethesda, MD. The cell line is similar propagated as an ascites in BALB/c nude mice. Upon s.c. injection of 1 × 10^5 cells in 0.2 ml of serum free tissue culture medium, a 30-mm³ tumor is formed in about 10–12 days. For both tumor models, TNF treatment began 10 days after tumors were initiated. Tumor volumes were recorded, and animals were weighed and randomized into groups. Tumor volumes and animal weight were monitored every other day or every 3 days for the duration of the experiments.

Mice. Female BALB/c inbred mice 7–10 weeks old, 18–20 g, were obtained from Simonsen Labs, Gilroy, CA. Female nude athymic BALB/c mice, 7–10 weeks old, were obtained from Charles River, Wilmington, MA.

In Vitro Testing of Cell Lines for Response to TNF. Meth A and NIH:OVCAR-3 cells were suspended in growth media, seeded at 1 × 10^5 cells/ml in 96-well microtiter plates, and incubated at 37°C in 5% CO₂ for 18–24 h. Two-fold dilutions of a 10-μg/ml TNF solution were added to the cells and the cultures were incubated for 5 days. The cultures were evaluated for viability either microscopically (trypan blue...
dye exclusion) or by measurement of [3H]thymidine uptake (5 μCi/ml, 78 Ci/mmol) for 18 h. The cells were harvested onto glass fiber filters for scintillation counting. Cell lines that routinely respond to TNF, such as murine fibrosarcoma L929 and human breast carcinoma MCF-7 (6), were used as positive controls for the assay.

RESULTS

The effect of six i.v. injections (given every other day) of TNF on the Meth A tumor starting 10 days after tumor transplant is shown in Fig. 1. An approximate 24-fold increase in tumor volume was observed in control injected mice 18 days after the start of treatment while no increase in tumor volume was observed in mice treated with TNF (50 μg/kg) 18 days after the start of treatment. Statistical analysis comparing the TNF treated (50 μg/kg) and diluent control treated mice 18 days after initiation of treatment showed significant differences in tumor volume ($\chi^2 = 22.38$, $P = 0.0002$) but no significant differences at the start of TNF treatment.

Titration of the dose-response curve of the Meth A tumor to TNF is shown in Table 1. Mice bearing solid tumors were given i.v. TNF injections of 25, 50, and 100 μg/kg, respectively. Retardation of tumor growth as well as regression were observed in all groups of mice receiving TNF. Mice given injections of TNF (25 μg/kg) had tumors with an approximate size of one-half that of the controls 18 days after initiation of treatment, while those receiving TNF (50 and 100 μg/kg) had tumors that were 0–3% of the control size. In this experiment, complete remission was observed in 38% of mice receiving TNF (25 μg/kg), 88% in those mice receiving TNF (50 μg/kg), and 100% in those receiving TNF (100 μg/kg). The survival proportions of the latter two groups are statistically distinguishable from the control groups using Fisher's (one-tailed) exact test ($P < 0.005$). Although significant heterogeneity was observed in the response of the mice receiving TNF (25 μg/kg), the mean life span of all TNF treated groups was significantly higher than that of the controls (Table 1). A repeat of this experiment using 7 mice/group reproduced similar results. Cures were observed in 80, 43, and 29% of mice treated with 100, 50, and 25 μg/kg, respectively.

The 100-μg TNF/kg dose may be interpreted as the 25% lethal dose for the BALB/c mice bearing a Meth A solid tumor since 2 of 8 mice died 1 day after the first injection. In a repeat experiment of 7 mice died 1 day after the first injection.

The effect of daily i.p. injections of TNF on the growth of the human ovarian carcinoma tumors NIH:OVCAR-3 in nude mice is shown in Fig. 2. After 7 days of TNF treatment with 250 μg/kg/injection there was a 2.5-fold decrease in tumor volume. The tumors, however, regained growth upon cessation of TNF treatment with a 4-fold increase in tumor volume over the following 3 days (Fig. 2, day 10). The tumor volume of the diluent control animals increased (5-fold) over the 10 days. Statistical analysis indicated that the tumor volume for the TNF treated animals (250 μg/kg/injection) was significantly smaller than the diluent control animals on days 7 and 14 ($P < 0.005$ and <0.02, respectively). Retardation of tumor growth but not regression was observed in the groups of mice which received 125 and 62.5 μg/kg/injection.

In addition to decreasing tumor volume, i.v. TNF administration of 50 and 100 μg/kg/injection resulted in a slight decrease (3–5%) in body weight ($P < 0.05$) as evaluated on day 28 (Table 1). However, the mice recovered after completion of TNF administration and gained weight during further observation similar to results in controls (data not shown).

When injected i.v., the most pronounced effect of TNF in the Meth A model was to increase median survival time of the mice as shown in Fig. 3. The median survival times of the uninjected and PBS control groups were 39 and 28 days, respectively, while those treated with TNF were 53, >400, and >400 for the 3 doses, respectively. The survival times of the mice treated with TNF (50 μg/kg) and with TNF (100 μg/kg) differed significantly from the survival of the excipient controls ($P = 0.001$ and $P = 0.002$, respectively; Breslow test). All long-term survivors have been tumor free for >13 months.

When administered i.p., TNF was not as effective in the prolongation of survival time as compared to the i.v. route. This observation is demonstrated in Fig. 4. The median survival times of uninjected and PBS control groups were 30 and 28 days, respectively; results for those given TNF injections were 46, 34, and 54 days. Both 25- and 75-μg/kg doses of TNF significantly prolonged survival time ($P = 0.003$ and $P = 0.016$; Breslow test); however, the 50-μg/kg dose did not ($P = 0.082$). The lack of a dose-response correlation is unclear but may be related to the significant variability that was observed in median survival times among controls in this model. Although the i.p. route of TNF administration was less effective in prolonging survival than the i.v. route, complete tumor regression was observed 8 days after the end of TNF treatment in approximately 10% of the mice given injections of 25 μg/kg and 40% in those given injections of 75 μg/kg. This finding suggests that the prophylactic efficacy of TNF is highly dependent on its route of administration. A significantly larger number of cures might have been obtained using the i.p. route if TNF dosage was increased to concentrations greater than 75 μg/kg.

Administration of TNF i.p. into BALB/c mice bearing ascites
TUMOR REGRESSION BY TUMOR NECROSIS FACTOR

Table 1  Efficacy testing of human recombinant TNF against mouse Meth A fibrosarcoma

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment*</th>
<th>Tumor volume (mm³ ± SD)</th>
<th>Change in tumor volume</th>
<th>No. of cures (%)</th>
<th>Median survival time (days)</th>
<th>FBW/BW</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Control, no injections</td>
<td>319 ± 142</td>
<td>4169 ± 2103</td>
<td>3850</td>
<td>0/8 (0)</td>
<td>39</td>
</tr>
<tr>
<td>2</td>
<td>PBS control, 0.1 ml/injection</td>
<td>249 ± 108</td>
<td>5443 ± 3063</td>
<td>5194</td>
<td>0/8 (0)</td>
<td>28</td>
</tr>
<tr>
<td>3</td>
<td>TNF, 25 µg/kg/injection</td>
<td>254 ± 132</td>
<td>2646 ± 3816</td>
<td>2392</td>
<td>3/8 (38)</td>
<td>53</td>
</tr>
<tr>
<td>4</td>
<td>TNF, 50 µg/kg/injection</td>
<td>174 ± 90</td>
<td>143 ± 369</td>
<td>−31</td>
<td>7/8 (88)</td>
<td>&gt;400</td>
</tr>
<tr>
<td>5</td>
<td>TNF, 100 µg/kg/injection</td>
<td>307 ± 201</td>
<td>0</td>
<td>−299</td>
<td>6/6 (100)*</td>
<td>&gt;400</td>
</tr>
</tbody>
</table>

*PBS control and TNF test animals were given i.v. injections every other day for a total of 6 injections, starting on day 10 after tumor cell injection.

Change in tumor size (day 28 − day 10) in mm³.

Number of animals with no tumor/number of animals per group at day 28. Survivors at day 28 were still alive at day 400.

FBW/BW, final body weight/initial body weight at day 28.

Two mice died after receiving the first injection of TNF.

Fig. 2. Effect of systemic repetitive administration of recombinant tumor necrosis factor on the growth of the s.c. human ovarian carcinoma NIH:OVCAR-3 in nude mice. TNF i.p. administration was initiated (arrow*) every day for a total of 7 injections. Treated animals received 0.5 ml containing 250 µg/kg/injection (•), 125 µg/kg/injection (△), 62.5 µg/kg/injection (●), or PBS with 2% bovine serum albumin (○). Bars, SD.

Fig. 3. Survival curves for TNF i.v. administration [25 µg (○), 50 µg (△), 100 µg (●) per kg per injection], excipient control [phosphate buffered saline with 2% bovine serum albumin (○)], and noninjected tumor bearing controls (△) are shown. The experimental protocol is described in “Materials and Methods” and in the legend to Fig. 1 with the following modifications. Each group consisted of 10 mice and all animals received TNF i.p.

of the Meth A fibrosarcoma or nude mice bearing ascites of the NIH:OVCAR-3 carcinoma proved ineffective in alleviating tumor burden or in prolonging survival (data not shown). Similarly, TNF (10 µg/ml) had no effect on the growth of the murine Meth A fibrosarcoma cells and the human ovarian carcinoma cells in vitro (Fig. 5), while it was cytotoxic to the human breast carcinoma cells (MCF-7) at ng levels.

DISCUSSION

Tumor necrosis factor exhibits both cytotoxic and cytostatic activities on a variety of tumor cells but not on many normal cell lines in culture (6, 10–12). The mechanism of tumor regression by tumor necrosis factor in vivo is still unknown. It is possible that tumor necrosis factor acts both directly on the tumor cells and indirectly by activating certain arms of the immune system. Recent reports show that TNF augments the phagocytic and cytotoxic activities of polymorphonuclear neu-
TUMOR REGRESSION BY TUMOR NECROSIS FACTOR

The methylcholanthrene induced fibrosarcoma model is thought to be an unusually responsive tumor to various immunomodulators (20). However, a stringent test of its usefulness in predicting efficacy of anticancer agents is thought to be its use with slightly larger tumors at the onset of treatment, a condition which seems to minimize its nonselective response to various agents (17). We thus used established tumors that ranged in age from 10 to 15 days after cell transplantation (with an approximate size range of 125–600 mm³) for the assessment of the therapeutic effect of TNF on this tumor model. The absence of spontaneous regression of tumors in the diluent (phosphate buffered saline) control animals supports the validity of this model and its nonresponsiveness to nonselective immunostimulation. High doses of endotoxin (10–100 µg/ml) have been reported to produce hemorrhagic necrosis but not tumor regression in the Meth A tumor model (1). Endotoxin levels in the TNF preparations used in this study are low, less than 2 ng/injection, and thus the tumor regression that was observed is highly unlikely to be due to endotoxin contamination of the TNF. One, however, cannot exclude the possibility that small quantities of endotoxin may have locally activated the host immune and/or inflammatory mechanisms which might have potentiated the effect of TNF.

The human ovarian carcinoma NIH:OVCAR-3 model (9) was not as responsive to TNF as the murine methylcholanthrene induced fibrosarcoma. Complete tumor regression by TNF was not achieved in the ovarian tumors in the nude mice. The lack of cures in this model may be due to inherent differences in sensitivities of the tumors to TNF and the growth characteristics and vasculature patterns of the tumors. Both tumor cell lines were found resistant to the cytotoxic action of TNF in vitro and when subcultured as ascites in vivo. These findings suggest that the mechanism of action of TNF in vivo may involve the activation of the host immune system and/or direct action on the local vascular system of tumors. It is tempting to speculate that small quantities of TNF locally affect multiple cellular components of the tumor. TNF may activate macrophages to produce factors that potentiate TNF cytotoxic action; TNF may recruit neutrophils and lymphocytes into the tumor area by activation of the endothelium or may directly affect neutrophils by altering their cell surface and enhancing their ability to adhere to the blood vessel endothelium. In light of the published reports on the interaction of TNF with cells of the immune system (13, 14, 16), any or all of the above scenarios may lead to tumor regression by TNF. Histopathological studies on the kinetics of regression of tumors by TNF would undoubtedly shed some light on this area of research.

The putative receptor for TNF has been identified (18–22). Studies in our laboratories and those of other investigators show that there is no correlation between receptor number and sensitivity to TNF. Thus, differences in receptor number between the murine Meth A tumor cells and the human ovarian carcinoma cells may not necessarily explain the differences we observe in their response to TNF in vivo.

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Cures and Partial Regression of Murine and Human Tumors by Recombinant Human Tumor Necrosis Factor

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