Augmentation of Antitumor Efficacy by the Combination of Recombinant Tumor Necrosis Factor and Chemotherapeutic Agents in Vivo

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ABSTRACT

We evaluated the in vivo antitumor effects of the combination of recombinant human tumor necrosis factor (rhTNF) and three chemotherapeutic agents in an established murine tumor model. C57BL/6 mice bearing a subdermal weakly immunogenic 3-methylcholanthrene-induced sarcoma (MCA-106) received one i.v. dose of cyclophosphamide (Cy) (100 mg/kg), doxorubicin (5 mg/kg), or 5-fluorouracil (75 mg/kg) on either Day 8, 10, or 12. All animals received one i.v. dose of rhTNF (4 or 6 μg/mouse) on Day 10. The most effective time for administration of the chemotherapeutic agent was determined to be 48 h following rhTNF administration of all agents tested. The combined results of four separate experiments evaluating tumor size on Day 28 following tumor inoculation revealed that the groups treated with 4 or 6 μg of rhTNF and Cy (on Day 12) had tumor size reductions of 70 and 94%, respectively, compared to untreated controls (P < 0.005). Mice treated with Cy alone, or with 4 or 6 μg of rhTNF alone had tumor size reductions of 30, 35, and 41%, respectively, compared to untreated controls (P < 0.02). Analysis of cure rates demonstrates that the combination of Cy with 4 or 6 μg tumor necrosis factor cured 35 and 48% of the animals, respectively (P < 0.01), compared to 10, 0, and 14% of mice treated with single agent Cy, 4 μg rhTNF, or 6 μg rhTNF, respectively. The timing of Cy and TNF administration was critical since administration of Cy prior to or concurrent with rhTNF was not effective in reducing tumor area or increasing cure rates over those achieved with either agent alone. Mice treated with doxorubicin alone had an increase in tumor size of 139 ± 29% over untreated controls (P = 0.05) on Day 28 following tumor inoculation and none were cured. In contrast, mice treated with doxorubicin plus 4 or 6 μg rhTNF exhibited early reductions in tumor size such that on Day 28 the average tumor areas were decreased by 66 ± 34% (P < 0.05) and 73 ± 1% (P < 0.02) of untreated controls with cure rates of 29% and 43% (P < 0.02), respectively. However, the combination of 6 μg rhTNF plus doxorubicin led to substantial lethal toxicity with only 29% of mice surviving treatment. 5-Fluorouracil alone resulted in an increase in tumor area of 164% (P < 0.05) over that of untreated controls on Day 28 following tumor inoculation. Mice treated with the combination of 5-fluorouracil plus 4 or 6 μg rhTNF had only small reductions in tumor area by 36% (P < 0.05) and 20% (P < 0.05) of untreated controls, with cure rates of 17 and 0% (P > 0.05). Finally, attempts to treat mice bearing a nonimmunogenic sarcoma (MCA-102) by combination therapy with rhTNF plus Cy failed. This study demonstrates that the administration of certain chemotherapeutic agents, particularly Cy, following rhTNF can result in a combined antitumor effect against a weakly immunogenic sarcoma and serves as a rationale for clinical trials employing the combination of rhTNF and chemotherapy in patients with cancer.

INTRODUCTION

Previous work from our laboratory showed that the systemic administration of rhTNF could mediate in vivo regression of established weakly immunogenic sarcomas in mice. Tumor regression appeared to occur in two phases (1, 2). The characteristic hemorrhagic phase initiating central tumor necrosis occurred within 30 min after systemic injection of rhTNF. This was followed by a regressive phase that was immune mediated, presumably by Lyt2+ cells (3).

We (1) and others (2, 4, 5) have noted that rhTNF is toxic when administered at high doses in vivo, especially to tumor-bearing mice. In addition, although substantial tumor size reductions could be achieved following administration of rhTNF at subtoxic doses, few animals were actually cured of disease (1). Thus, efforts to minimize TNF toxicity while maintaining or increasing therapeutic efficacy would be of substantial benefit.

Significant improvements in experimental cancer therapies have been made by combining biological response modifiers to augment their action and reduce the doses necessary to achieve the same or better antitumor effect. For example, an improvement of the in vivo antitumor effects is seen with combined administration of rhTNF with interleukin 2 (6-8) or with γ interferon (9) compared to the effects of either agent alone. Further, Papa et al. (10) noted significant augmentation of therapeutic efficacy by the combination of Cy plus recombinant interleukin 2 as measured by prolongation of survival of mice bearing advanced lung metastases from weakly immunogenic sarcomas.

In vitro studies have demonstrated an augmentation of antitumor effects by the combination of rhTNF and chemotherapeutic agents and have served as the rationale for the in vivo therapeutic studies reported here. Alexander et al. (11, 12) demonstrated in vitro that rhTNF augmented the cytolytic activity of topoisomerase directed chemotherapeutic agents and that this combination of agents was effective in reducing tumor volume in vivo. Based on these findings and results showing improved antitumor efficacy with multiple cytokines from our laboratory (6, 13, 14), we investigated whether or not the systemic administration of rhTNF in combination with chemotherapeutic agents to mice with established sarcomas resulted in an improvement in antitumor effectiveness compared to that achieved by either agent alone.

MATERIALS AND METHODS

Animals. Female C57BL/6 (B6) mice were at least 12 weeks of age when used in experiments. Mice were obtained from the Small Animal Section, Veterinary Resources Branch, NCI, Bethesda, MD, or from The Jackson Laboratories (Bar Harbor, ME).

Tumors. MCA-102 and 106 tumors, syngeneic to B6 mice, were induced in our laboratory by the intramuscular injection of 0.1 ml of 1% 3-methylcholanthrene in sesame oil (15). Tumors were thawed periodically from cryopreserved stock of first passage tumor and serially passaged s.c. in B6 mice. Tumors were used only between the third and seventh transplant generations. The MCA-102 and 106 sarcomas are nonimmunogenic and weakly immunogenic, respectively (16).

Single cell suspensions were prepared as previously described (1). Fresh tumors were excised, minced with scissors, and stirred for 3 h at room temperature in a mixture of deoxyribonuclease, hyaluronidase, and collagenase (Sigma Chemical Co., St. Louis, MO), filtered through 100-gauge nylon mesh (Nitex; Lawshe Industrial Co., Bethesda, MD)
washed three times in HBSS (Biofluids, Rockville, MD) and resuspended at $1 \times 10^6$ cells/ml.

Chemotherapeutic Agents. Cyclophosphamide (Cytoxan; Mead Johnson, Bristol-Myers Oncology Division, Syracuse, NY), 100 mg per vial lyophilized in 0.9% NaCl, was reconstituted with sterile water for injection and further dilutions were made in HBSS. Doxorubicin (Adriamycin; Adria Laboratories, Erbomant Inc., Colombus, OH), 100 mg per vial lyophilized in 0.9% NaCl was reconstituted with sterile water and further dilutions were made in HBSS. 5-FU (Sigma Chemical Co., St. Louis, MO) was reconstituted with sodium phosphate buffer to 50 mg/ml and further dilutions were made in HBSS (17). All chemotherapeutic agents were of clinical grade.

rhTNF. rhTNF was kindly provided by the Cetus Corporation (Emer-ville, CA) and had a specific activity of $2.2 \times 10^7$ units/mg as determined in vitro by the murine L929 cytolytic bioassay (18). The endotoxin level of the purified preparation was 0.2 mg/mg of rhTNF measured by a standard stimulus assay. rhTNF solutions were prepared by freshly reconstituting each vial containing 0.29 mg of lyophilized rhTNF with 1.0 ml of sterile water and further diluting the solution to the required concentration with HBSS containing 2% pooled B6 mouse serum. The mouse serum served as a nonantigenic protein carrier to reduce the nonspecific adsorption of low concentrations of rhTNF to the walls of the container. Each 1.2-ml vial of excipient contained 10 mg of sodium phosphate buffer and 1.0% mannitol and was also freshly diluted with 2.0% pooled B6 mouse serum for each experiment. Mice received 1.0 ml volumes of rhTNF or excipient as a single i.v. injection into the lateral tail vein.

s.c. Tumor Model. B6 mice were injected in the s.c. tissue on the ventral abdominal surface with a suspension of $5 \times 10^6$ MCA sarcoma cells in 0.05 ml of HBSS. The tumors achieved an average diameter of 5–6 mm over the course of 10 days. All tumor size measurements were recorded as the product of the largest perpendicular diameters of the tumor determined with a vernier caliper. Mice were randomly assigned to groups prior to treatment. All subsequent tumor measurements were made on coded, ear-tagged mice in a blinded fashion (1).

Chemommotherapy Model. On Day 10 after tumor injection, animals received a single i.v. injection of rhTNF or excipient in the lateral tail vein. Doses of rhTNF ranged from 4 to 6 mg/mouse. Forty-eight hours prior to, concurrent with, and 48 h following rhTNF injection, animals received a single i.v. dose of Cy (100 mg/kg), Dox (5 mg/kg), or 5-FU (75 mg/kg) in the lateral tail vein. Animals were followed on a daily basis for survival and tumors were measured three times weekly for the first 2 weeks following rhTNF administration, twice per week until Day 30 and thereafter, weekly. At least six mice were included in each treatment group.

Statistical Analyses. Statistical determinations were made by Wilcoxon rank sum analysis or Fisher's exact test. All $P$ values reported are two tailed.

RESULTS

Effect of Treatment with the Combination of rhTNF and Chemotherapeutic Agents on Established MCA-106 Subdermal Tumors. B6 mice bearing established (10 day) subdermal weakly-immunogenic MCA-106 tumors were treated with either rhTNF alone or in combination with chemotherapeutic agents. Preliminary experiments evaluated timing of administration of the chemotherapeutic agent relative to that of rhTNF. Table 1 demonstrates the effect of three chemotherapeutic agents given at three separate time points, either alone or in combination with rhTNF. Combined results of two separate trials were expressed as an average percentage change in tumor size of treated groups as compared to untreated controls at Day 28 following tumor inoculation. In these experiments, the tumor area was $54 \pm 3$ mm$^2$ by Day 10 when the rhTNF was administered. rhTNF alone at doses of 4 or 6 mg per mouse given on Day 10 had minimal effect on tumor reduction (tumor size of 106 and 83% of untreated controls, respectively). Dox and 5-FU mediated no reduction in tumor size when given alone and Cy had minimal effects reducing tumor to 57, 99, and 61% of the size of untreated controls when given on Days 8, 10, or 12, respectively. In contrast, the combination of rhTNF and Cy mediated profound antitumor effects. For example, Cy administered 48 h following 4 or 6 mg of rhTNF reduced the tumor size to 39 and 5%, respectively, of untreated controls. Doxorubicin given 48 h following 4 or 6 mg of rhTNF resulted in tumor size reductions of 34 and 27%, respectively, compared to untreated controls. Administration of 5-FU in combination with rhTNF had little, if any antitumor effect. The injection of either Dox or 5-FU alone resulted in actual increases in tumor size compared to the untreated controls. The most effective time for administration of the chemotherapeutic agent was 48 h following rhTNF administration for all agents used. For example, the combination of 6 mg rhTNF with cyclophosphamide reduced tumor size over that of untreated controls to 91, 35, or 5% depending on whether the Cy was administered 48 h prior to, concurrently with, or 48 h following rhTNF administration, respectively. Using these data as a guide, further experimental trials were focused on the antitumor efficacy of chemotherapeutic agents administered 48 h following systemic rhTNF.

As shown in Fig. 1, a compendium of four separate experiments, mice treated with Cy alone exhibited a reduction in average tumor area of 30% ± 12 by Day 28 after tumor inoculation compared with untreated controls ($P^2 < 0.001$). Mice treated with 4 or 6 mg of rhTNF alone had a reduction in average tumor area of $35 \pm 9$ ($P^2 < 0.02$) and $41 \pm 9$ ($P^2 < 0.001$), respectively, compared to untreated controls. In contrast, mice treated with the combination of 4 or 6 mg rhTNF plus Cy had substantially greater reductions in tumor size of $70 \pm 9$ ($P^2 < 0.005$ versus Cy and 4 mg rhTNF alone) and 94% ± 3 ($P^2 < 0.005$ versus Cy and 6 mg rhTNF alone).

Mice treated with Dox alone had an increase in tumor size at Day 28 following tumor inoculation over untreated controls of $139\% \pm 29$ ($P^2 > 0.05$) (Fig. 2). Mice treated with Dox plus 4 or 6 mg rhTNF exhibited early reductions in average tumor area such that on Day 28 following tumor inoculation these average tumor areas were decreased by $66 \pm 34$ ($P^2 < 0.05$) and $73% \pm 1$ ($P^2 < 0.02$), respectively, of untreated controls. However, tumors in mice not completely cured of disease were
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Fig. 1. The effect of treatment with rhTNF and cyclophosphamide on size of the MCA-106 sarcoma in B6 mice (compendium of four separate experiments). Cyclophosphamide (100 mg/kg) was administered as a single i.v. injection 48 h following rhTNF administration. RhTNF was given as a single i.v. injection at a dose of 4 or 6 µg on Day 10 following tumor inoculation. Tumor size averaged 51.8 ± 0.9 mm³ at the time of therapy.

Fig. 2. The effect of treatment with rhTNF and doxorubicin on size of the MCA-106 sarcoma in B6 mice (compendium of three separate experiments). Doxorubicin (5 mg/kg) was administered as a single i.v. injection 48 h following rhTNF administration. RhTNF was given as a single i.v. injection at a dose of 4 or 6 µg on Day 10 following tumor inoculation. Tumor size averaged 53.7 ± 2.5 mm³ at the time of therapy.

Fig. 3. The effect of treatment with rhTNF and 5-fluorouracil on size of the MCA-106 sarcoma in B6 mice (compendium of two separate experiments). 5-Fluorouracil (75 mg/kg) was administered as a single i.v. injection 48 h following rhTNF administration. RhTNF was given as a single i.v. injection at a dose of 4 or 6 µg on Day 10 following tumor inoculation. Tumor size averaged 40.7 ± 6.0 mm³ at the time of therapy.

Table 2 Summary of lethal toxicity and curative rates in tumor bearing mice treated with rhTNF and chemotherapeutic agents

| Tumor      | rhTNF (µg) | % survived | % cured | P  
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<tr>
<td>MCA-106</td>
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* Death within 36–48 h of rhTNF administration.
* Animals free of palpable tumor at 90 days.
* Compared to untreated controls.
* Experiments that utilize Cy or Dox represent a compilation of four and three separate experiments, respectively. Cy, Dox, and 5-FU were administered as a single i.v. injection at doses of 100 mg/kg, 5 mg/kg, and 75 mg/kg, respectively.

Note: From treatment of 10-day subdermal MCA-106 sarcoma with rhTNF with or without chemotherapy is shown in Table 2 (results of four combined experiments). Animals treated with Cy alone, or with 4 or 6 µg of rhTNF alone had 10, 0, and 14% cures at 90 days, all of which were not statistically significant from untreated controls. In contrast, those treated with the combination of 4 or 6 µg of rhTNF plus Cy had significantly higher cure rates of 35% (P² < 0.01), and 48% (P² < 0.01), respectively. Animals treated with Dox alone had no cures at 90 days; however, those treated with the combination of 4 or 6 µg of rhTNF plus Dox displayed percentage cures of 29% (P² < 0.02) and 43% (P² < 0.02), respectively. It should be noted that although the curative rates of mice treated with the combination of rhTNF plus Dox appeared as efficacious as those treated with rhTNF plus Cy, animals receiving the former combination or Dox alone appeared severely cachectic and lethargic, regardless of whether or not tumor regression occurred following therapy. In fact, the combination of 6 µg rhTNF plus Dox led to substantial lethality with only 29% of mice surviving treatment. Mice treated with 5-FU alone dem-
onstrated no cures and those treated with 4 or 6 μg of rhTNF plus 5-FU had cure rates of 17% (P² > 0.05) and 0% (P² = 1.00), respectively.

The mean survival time of untreated mice bearing MCA-106 sarcoma was approximately 48 days (Fig. 4). Mice treated with Cy alone, or with 4 or 6 μg of rhTNF alone survived 51, 45, or 63 days, respectively (P² > 0.05). In contrast, animals treated with the combination of 4 or 6 μg rhTNF plus Cy survived a mean of 65 and 74 days, respectively (P² < 0.04 for both). Thus, mice bearing the weakly immunogenic MCA-106 sarcoma treated with the combination of rhTNF and cyclophosphamide had significantly prolonged survival over single-agent treated animals.

Table 2 shows that rhTNF lethal toxicity, manifested by 36–48 h following systemic administration occurred in treated mice as previously reported (1). Acute toxicity appeared as rigor, diarrhea, and lethargy followed by death, with 55% and 61% of mice surviving 4 and 6 μg rhTNF, respectively. With the exception of Dox, the administration of chemotherapeutic agents 48 h after rhTNF had no effect on this toxicity. Preliminary experiments with chemotherapeutic agents (Cy, Dox, and 5-FU) given 48 h prior to and concurrently with rhTNF also did not alter this toxicity (data not shown).

Effect of the Combination of rhTNF and Cyclophosphamide on the Nonimmunogenic MCA-102 Sarcoma. The combination of rhTNF and Cy had no impact on the growth rate of the nonimmunogenic MCA-102 (Fig. 5, top panels). Cy alone reduced the MCA-102 tumor to 85% (P² < 0.04) of the area of untreated controls. Animals treated with 4 or 6 μg of rhTNF plus 5-FU showed an increase in tumor size of 5% (P² > 0.05) and a reduction in tumor size of 12% (P² < 0.05), respectively. MCA-102 bearing mice treated with the combination of Cy plus 4 or 6 μg of rhTNF showed reductions in tumor area as compared to untreated controls of 15% (P² < 0.05) and 12% (P² < 0.05), respectively.

Although mice bearing the nonimmunogenic MCA-102 sarcoma and treated with the combination of rhTNF and Cy showed little if any significant tumor size reduction (Fig. 5), a prolonged survival, albeit small, was observed when compared to controls. As shown in Fig. 5 (bottom panels), untreated mice survived an average of 26 days while those treated with Cy, 4 μg rhTNF, or 6 μg rhTNF alone survived an average of 31, 27, and 30 days (P² > 0.05). Mice treated with 4 or 6 μg rhTNF plus Cy had an average survival of 33 and 36 days, respectively (P² < 0.04 for both as compared to rhTNF or Cy alone). There were no survivors at 90 days in any treatment group.

DISCUSSION

Several laboratories have demonstrated both in vivo and in vitro augmentation of the antitumor effects of TNF when combined with traditional antimetabolic chemotherapeutic agents. For example, Regenass and colleagues noted that the addition of Dox, Cy, and 5-FU to partially purified TNF (derived from lipopolysaccharide and Corynebacterium parvum challenged CD-1 mice) could enhance the number of complete regressions of the subdermal Meth A sarcoma (17). Since this study used a partially purified TNF preparation, which contains other monokines and lymphokines in addition to TNF (19), it was difficult to ascribe the antitumor activity to a single substance. The Meth A murine fibrosarcoma has been serially transplanted for several decades, is a highly immunogenic tumor, and is unusually sensitive to TNF (20, 21).

Alexander et al. (11, 12) demonstrated that rhTNF caused enhanced tumor cytolyis when added to cultured murine L929 fibrosarcoma and MBT 2 transitional cell bladder tumor following the addition of topoisomerase II directed agents such as Dox and actinomycin D. A similar augmentation was observed in vivo in which rhTNF combined with i.p. actinomycin D or VP 16 caused a significant reduction in the volume of a s.c. MBT-2 bladder tumor as compared to each agent alone. In both in vivo and in vitro settings, the greatest antitumor effect was obtained by the addition of chemotherapeutic agent 24 h prior to addition of rhTNF. These investigators suggested that chemotherapeutic agents directed against topoisomerases were most effective when combined with TNF. In contrast, our study using Cy in combination with rhTNF showed that heightened antitumor efficacy occurred only in the cases when rhTNF was administered prior to the chemotherapeutic agent. Further, we did not find that enhancement of the antitumor effect of rhTNF was mediated predominantly by topoisomerase-directed chemotherapeutic agents, since Cy was also effective in our model.

Haranaka et al. (22) achieved augmentation of antitumor efficacy in vivo against the murine Meth A sarcoma with rhTNF combined with mitomycin C, but not with cyclophosphamide. The route of administration and choice of tumor used may explain the discrepancies between this and our findings.

Unlike these studies, we used newly induced sarcomas in early transplant generations. In our study, the combination of rhTNF and chemotherapeutic agents in vivo had greater antitumor efficacy than either agent alone. The mechanism of this augmentation is unclear, however several possibilities exist.
rhTNF causes early hemorrhagic necrosis and may act as a cytoreductive agent, thus reducing the overall tumor burden upon which the chemotherapeutic agent must act. However, early hemorrhagic necrosis is also seen in the treatment of the nonimmunogenic MCA-102 sarcoma and combined effects of rhTNF and chemotherapy were not obtained in this model. Cy was the most effective chemotherapeutic agent used in this study and it is known to reduce suppressor factors in mice which may be important in the dampening of the immunological reaction to the tumor (23). Another potential mechanism may involve an enhancement by Cy of the Lyt2+ cell population known to be effective in the secondary regressive phase of rhTNF activity against the weakly immunogenic MCA-106 sarcoma (3). Lyt2+ cells have been shown to be involved in both rhTNF and rIL-2 mediated tumor regression in vivo (3, 16). Further, recent published data from our laboratory by McIntosh et al. (6) using rhTNF and rIL-2 and Papa et al. (10) using Cy and rIL-2, have shown synergistic antitumor activities by these combinations; both presumably act through Lyt2+ cells.

The combination of rhTNF and Cy or Dox resulted in significant cures of mice bearing the weakly immunogenic MCA-106 sarcoma. Only a very modest prolongation of survival was seen when treating the nonimmunogenic MCA-102 sarcoma and further means to augment the therapeutic response of rhTNF to this tumor are needed. The fact that MCA-102 survival was seen when treating the nonimmunogenic MCA-102 sarcoma and -106 are both equally resistant to the antiproliferative effect(s) of rhTNF in vitro (1) suggests that the augmented therapeutic effect of rhTNF plus chemotherapy on the weakly immunogenic MCA-106, but not on the nonimmunogenic MCA-102 sarcoma, is immune mediated and not the result of direct rhTNF action.

This study illustrates that the administration of certain chemotherapeutic agents following rhTNF can mediate an augmented antitumor effect and may serve as a rationale for clinical trials employing this combination in patients with cancer.

REFERENCES


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