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ORIGINAL ARTICLE

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Intra-arterial administration of TNF-α followed by arterial ablation is an effective therapy for a regionally confined TNF-resistant rat mammary adenocarcinoma

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Abstract Tumor necrosis factor-alpha (TNF-α) is an immunomodulatory cytokine that has exhibited anti-tumor activity in a variety of experimental systems. However, the toxicities associated with systemic administration of TNF-α have limited its clinical utility and have led to the investigation of targeted delivery techniques with the ability to present the TNF-α dose directly to the vascular bed of the tumor. The intra-arterial (IA) administration of TNF-α to patients with liver metastases represents one such approach, and recent work suggests that subsequent ablation of the tumor's arterial supply via embolization may enhance the efficacy of intra-arterial treatments (hepatic chemoembolization). The present study was undertaken to test the hypothesis that IA administration of TNF-α is superior to the intravenous (IV) route for inhibition of tumor growth in a regionally confined rat mammary adenocarcinoma model that provides for ablation of the arterial supply to the tumor following cytokine therapy. Rats bearing hind limb mammary adenocarcinomas received single IA or IV infusions of 8×10^5, 1×10^6, and 1.5×10^6 units of TNF-α via the common femoral artery (CFA) followed 1 h later by ligation of the artery. Control animals received either no treatment or IA infusion of 2% normal rat serum (NRS) followed by ipsilateral CFA ligation. Tumor size was measured every other day after treatment. Tumor growth inhibition occurred in the first 5 to 10 days after treatment. IV administration of TNF-α did not result in visual tumor necrosis or significant reduction in the rate of tumor growth. IA administration of TNF-α resulted in statistically significant diminution of tumor size as compared to untreated controls and animals receiving IA 2% normal rat serum (NRS; $P < 0.05$ at days 6, 8 and 10), regardless of the dose employed. The maximum growth inhibition with IA TNF-α was a 91% reduction in tumor volume that was achieved with a dose of 1×10^6 U TNF-α. These results demonstrate improved anti-tumor activity with the IA administration of TNF-α over the IV route in a regionally confined mammary adenocarcinoma. IA administration of biologic response modifiers like TNF-α may therefore be a useful approach for the hepatic chemoembolization of breast adenocarcinomas metastatic to the liver.

Keywords Breast cancer · Intra-arterial · Tumor necrosis factor

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Introduction

Tumor-necrosis factor-alpha (TNF-α) is a cytokine secreted by macrophages and many other cell types that has pleiotropic effects upon host tissues. TNF-α is normally produced in the context of infectious and inflammatory processes and contributes to the lethality of the systemic inflammatory response syndrome. In vitro studies have shown that recombinant human TNF-α has cytostatic or cytotoxic effects on a variety of human and murine transformed cell lines [1, 2]. Further, experiments

in mice have shown that TNF-α is effective against certain tumors when administered systemically or intra-lesionally. However, not all tumor types are sensitive, and complete tumor regression is not always obtained [3, 4, 5, 6]. In addition, TNF-α possesses significant toxicity in vivo, which limits the total systemic dosage that can be administered [7].

In human cancer trials, the intravenous (IV) administration of TNF-α results in significant toxicity with modest overall response rates [8, 9, 10, 11, 12, 13, 14]. Isolation-perfusion techniques and improved techniques for placement of arterial catheters currently allow the delivery of anti-cancer agents directly to the vascular bed of the tumor, offering a theoretical pharmacokinetic advantage over systemic treatments administered IV [15, 16, 17, 18]. A further anti-tumor effect may be obtained via ablation of the tumor's arterial blood supply immediately following intra-arterial (IA) administration of the cytotoxic agent, a technique known as hepatic arterial chemoembolization [19]. We therefore developed a rat model in which to test the efficacy of IA administered TNF-α when it is followed by embolization of the arterial blood supply [20]. In this model, a rat mammary adenocarcinoma is implanted into the hind limb of the animal with the result that the majority of its blood supply derives from the common femoral artery. This system makes use of a tumor that is relatively resistant to TNF-α, and also provides a means of making careful observations and of obtaining exact tumor measurements. In the present study, we show that IA administration of TNF-α followed by arterial ligation is superior to the IV route. These findings may have application in the treatment of breast and other adenocarcinomas that have metastasized to the liver.

Materials and methods

Animals

One hundred and eighty gram female Fischer F344 rats were obtained commercially and housed in the university vivarium. Food and water were provided ad libitum.

Recombinant human TNF-α

Recombinant human TNF-α was supplied by Genentech (San Francisco, Calif.). Each vial contained 0.5 mg of TNF-α in 1 ml of phosphate-buffered saline (PBS). Specific activity was 5×10^7 U/mg as measured in the in vitro microplate L929 cell assay. There was less than 0.03 U/mg of endotoxin present in this TNF-α preparation.

Tumor

R3230 mammary adenocarcinoma was obtained from Mason Research Laboratories (Madison, Wis.). The R3230 tumor is non-immunogenic and does not metastasize after subcutaneous (s.c.) implantation [21]. The tumor was maintained in vivo by s.c. implantation of a 4-mm cube of fresh tumor into the flank of a healthy Fischer F344 female rat. Animals with localized tumor were prepared by implanting freshly harvested 2-mm cubes of tumor in the right lower hind limb of Fischer F344 female rats. The right hind limb was shaved and a 4–6 mm transverse incision was made on the inner aspect of the distal right thigh. Tumor fragments were placed s.c. over the distal gastrocnemius muscle, and the skin incision was closed with a single 4–0 silk suture. Tumors were selected for treatment when they had reached a diameter of 10–15 mm, which occurred 14–21 days after implantation.

Cell lines

The R3230 cell line was prepared from R3230 tumor tissue. A 5-mm cube of freshly harvested tumor was mechanically dissociated using a pair of scalpel blades, and suspended in 3 ml of PBS. Two milliliters of a 1% collagenase solution (Sigma Chemicals, St. Louis, Mo.) was then added to this suspension and incubated at 37°C for 4 h. The resulting cell suspension was washed in PBS and plated in RPMI-1640 medium supplemented with 10% fetal calf serum (FCS; 10% RPMI).

The L929 cell line (a chemically-induced sarcoma of mouse origin) was obtained from the American Type Culture Collection [22]. The cell line was maintained as an adherent monolayer in 10% RPMI at 37°C in an atmosphere of 5% CO_2 and served as a TNF-sensitive target in cytolytic assays.

In vitro cytolytic assays

R3230 and L929 cells were established as monolayers in 96-well flat-bottomed microtiter plates (Corning, N.Y.) in 10% RPMI. The R3230 and L929 cell lines were established at densities of 8,000 and 10,000 cells/well, respectively. Triplicate wells were then treated with varying concentrations of TNF-α and incubated for 24 h at 37°C in 5% CO_2. After incubation, viable adherent cells were stained with a 1% solution of crystal violet, and excess stain and non-viable free cells were rinsed away with distilled water. Intracellular stain was then solubilized by the addition of 100 µl of a 150 mM HCl-methanol solution to each well. The amount of stain in each well was quantitated in a Titertek Multiscan Plate Reader (Flow Laboratories) which was equipped with a 580-nm Multiscan Interference Filter. The number of viable cells in each well was taken to be proportional to the degree of absorbance measured for that well. Results of the cytotoxicity assays were calculated as follows:

$$\%\ \text{viability} = 1 - \frac{\text{mean absorbance of test triplicates}}{\text{mean absorbance of untreated control triplicates}} \times 100$$

TNF-α infusions

Fischer F344 female rats received closed-chamber halothane inhalational anesthesia supplemented by intramuscular injection of xylazine (5–8 mg/kg; Ayerst Laboratories, New York, N.Y.) and ketamine hydrochloride (75–95 mg/kg; Bristol Laboratories, Syracuse, N.Y.). Animals received TNF-α at doses of 8×10^5, 1×10^6, or 1.5×10^6 U either IA via the common femoral artery of the tumor-bearing limb, or IV via the common femoral vein of the opposite leg. A 0.5-mm polyethylene catheter was used for all cannulations. For IA treatments, the catheter was inserted in the right common femoral artery well above the tumor site, and secured with a 4–0 silk ligature. This catheter was connected via a 27-gauge needle to a 1-cc tuberculin syringe containing the infusate. Each dose of TNF-α was administered in 0.5 ml of solution, which consisted of 2% normal rat serum (2% NRS) in PBS. Infusions were carried out over a period of 25 min at a rate of 1.2 ml/h using a syringe pump (Sage Instruments, Cambridge, Mass.).

One hour after IA administration of TNF-α, the catheter was removed and the right femoral artery ligated proximal and distal to the cannulation site using 4–0 silk. Similarly, 1 h after IV administration of TNF-α, the right femoral artery was ligated in order to control for the effects of arterial ligation upon the tumor. Control

animals received an infusion of 0.5 ml of 2% NRS via the IA route to the tumor-bearing limb, followed 1 h later by arterial ligation, or arterial ligation alone. All incisions were closed with stainless steel staples. Tumors were visually examined each day; size [length×width; L×W] was measured prior to infusion and every other day thereafter. The greatest tumor diameters measured at right angles to one another were multiplied to give the tumor area in mm^2.

Statistical analysis

In order to control for slight variations in tumor size both pre- and post-treatment, we generated a normalized value for the size of each tumor by dividing tumor size (L×W) at day X (i.e., tumor size on day X after treatment) by the tumor size at day zero (i.e., tumor size at time of treatment). The normalized tumor sizes for all animals in any one treatment group were then averaged at each time point. In this manner, we were able to generate a normalized value that represented the average response of a 15-mm diameter tumor to a particular experimental manipulation at a given time after treatment. Comparisons of tumor sizes were made between different treatment groups at post-infusion days 2, 4, 6, 8 and 10. Analysis of statistical significance was performed using the paired Student's *t*-test and the Bonferroni correction for multiple *t*-tests [23]. The data for each treatment group shows tumor size in terms of percentage change from baseline tumor size. All treatment arms utilized 10 rats per group.

In order to determine statistically which treatment or treatments resulted in the greatest overall change in growth rate and biological behavior of the R3230 tumor, the growth curve for each group was converted into log form. The differences among the slopes of these log curves were analyzed for statistical significance using repeated-measures analysis of variance.

Results

In vitro effects of TNF-α on R3230 and L-929 cell lines

We first sought to establish the sensitivity of the R3230 cell line to TNF-α. R3230 and L-929 cells were established as monolayers in microplates and exposed to various levels of TNF-α, as described in the Materials and methods section. The number of viable cells were assessed following a 24-h treatment, and the results of 3 separate studies are summarized in Fig. 1. Compared to the L-929 cell, the R3230 cell line was relatively insensitive to TNF-α at the concentrations tested. The 1.25×10^3 ng level of TNF-α led to a 19% reduction in R3230 cell number, and a 39% reduction in the R3230 cell number was achieved when the dose of TNF-α was increased to 1.25×10^4 ng/well. In contrast, the L-929 cell line was highly sensitive to the lytic effects of TNF-α at all concentrations tested. Indeed, a 65% reduction in cell number was noted at the lowest concentration of TNF-α (1.25×10^1 ng/well).

Growth of R3230 rat mammary adenocarcinoma in the lower extremity of Fischer F344 female rats

Extensive studies were first conducted to establish a tumor inoculum that reproducibly resulted in tumors of a uniform size within 14 days in normal rats. Animals were implanted with tumor cubes of various sizes, and tumor

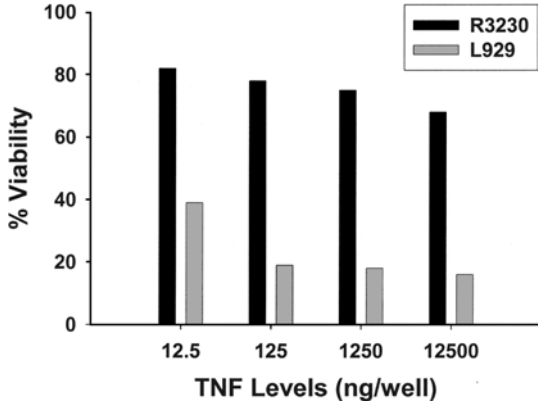

Fig. 1 In vitro sensitivity of the R3230 cell line to TNF-α. R3230 cells were cultured in complete media in the presence of varying concentrations of TNF-α (12.5–12,500 ng/well) and evaluated for viability via mean absorbance at 24 h. These results represent the average of the 3 experiments in which the standard error was < 5%

dimensions were measured every other day following implantation. Results are given in Fig. 2, and reveal that implantation of 2-mm cubes resulted in 10-mm tumors by day 14. Tumors enlarged rapidly over the next 10 days, and by day 24 had achieved a diameter of 40 mm. By day 35, animals began to show signs of anemia and malnutrition. At this point the animals were killed and the experiment halted. Tumors were well-vascularized and showed no evidence of central necrosis by the time they had attained a diameter of 10–15 mm at 2–3 weeks. Based on these experiments, we chose to employ tumors that had attained a diameter of 10–15 mm in the infusion experiments. The R3230 tumor did not metastasize after implantation and remained confined to the lower extremity.

Effects of IA and IV infusions of TNF-α on regionally confined rat mammary adenocarcinomas

In preliminary studies, we tested a variety of TNF-α doses and demonstrated that IV or IA doses of TNF-α

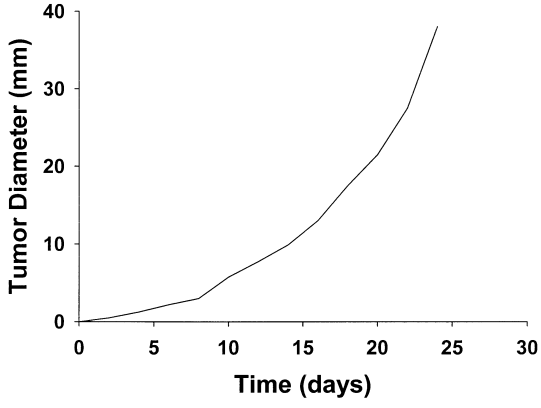

Fig. 2 Growth of the R3230 tumor in vivo. Fischer F344 female rats ($N = 10$) were anesthetized and implanted with 2-mm cubes of freshly harvested tumor in the right lower extremity. Tumor growth was measured every other day until day 35

below 8×10^5 U had little or no effect upon the growth of the R3230 tumor (data not shown). We also found that doses greater than 1.5×10^6 units were lethal in approximately 30% of treated animals. Therefore, tumor-bearing animals were treated with either 8×10^5, 1×10^6, or 1.5×10^6 units of TNF-α given either IV or IA as described in the Materials and methods section.

Ligation of the femoral artery had no adverse effects upon the normal tissues of the lower extremity, as seen in Fig. 3 [20]. No evidence of ischemia or loss of function was observed at any time following ligation. Infusion of 2% NRS followed by arterial ligation led to a slightly reduced rate of tumor growth initially, followed by a rapid return to a growth rate that paralleled the group receiving arterial ligation alone. Visual inspection of these tumors after ligation of the femoral artery revealed no gross ischemic changes or tumor necrosis. Histological studies have confirmed that these control tumors were essentially unaffected by the loss of this arterial blood supply (data not shown). Statistical comparison of these 2 control groups (ligation alone and 2% NRS infusion) using paired *t*-tests at days 2–10 post-infusion revealed no significant differences between the 2 groups with respect to tumor size.

The growth characteristics of tumors that were treated with IV or IA TNF-α are shown in Fig. 4A, B. There were no deaths among the rats in these experimental groups. Occasionally, animals exhibited signs of weakness and lethargy after IA or IV administration of TNF-α. These symptoms resolved within 24 h. In our experience, the greatest changes in tumor size occurred 5–7 days after infusion. For this reason, growth curves have been displayed to 10 days after treatment. IV administration of TNF-α did not result in visual tumor necrosis or significant reduction in the rate of tumor growth. At no point were tumors that had been treated IV with 8×10^5, 1×10^6, or 1.5×10^6 units of TNF-α statistically different in size from control tumors that had received only 2% NRS.

In contrast, IA administration of TNF-α at 8×10^5 units frequently resulted in the partial necrosis of tumors. Greater degrees of tumor necrosis were observed at the 1×10^6 unit dose. After IA administration of 1.5×10^6 units of TNF-α, tumors routinely became dusky and assumed a cyanotic hue within 24 h of treatment. By day 2, the tumors were almost entirely necrotic, and by day 4, the tumors had begun to involute (see Fig. 3). One of the 6 rats receiving 1.5×10^6 units of TNF-α IA was completely cured of its lower extremity tumor. The tumors in the remaining animals treated with this IA dose of TNF-α had decreased in size by an average of 90% by day 10. Regardless of the dose, IA administration of TNF-α resulted in statistically significant diminution of tumor size as compared animals receiving ligation alone or 2% NRS post-infusion ($P<0.05$ at days 6, 8 and 10). As expected, the most significant reduction in tumor size came with the IA infusion of 1.5×10^6 U of TNF-α ($P=0.002$ at day 10 post-infusion).

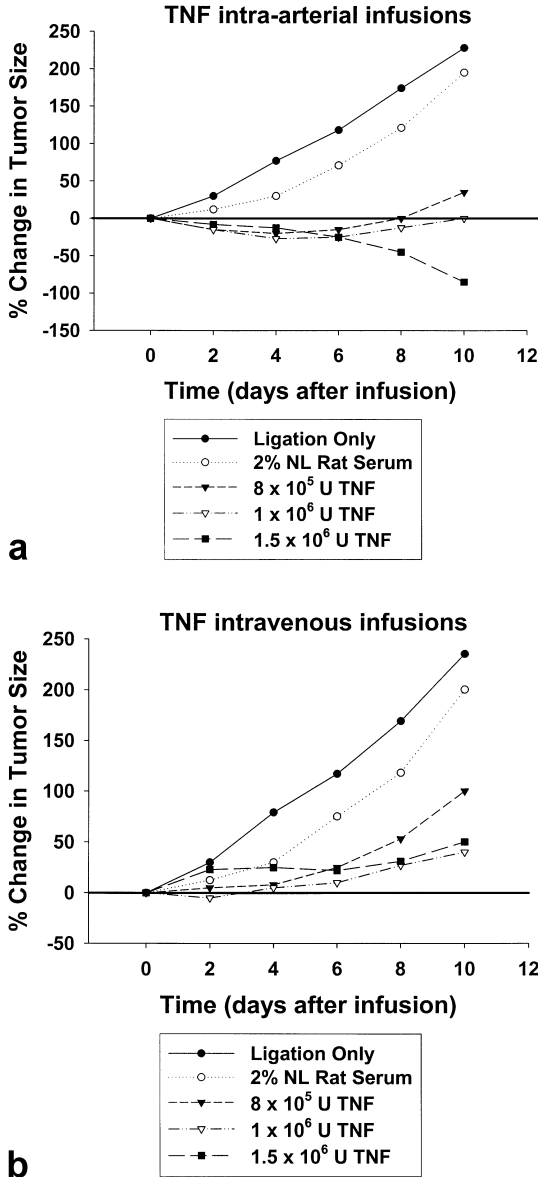

Fig. 3A, B IA administration of TNF-α to a regionally confined rat mammary adenocarcinoma. Rats bearing 2-week-old R3230 tumors (10–15 mm in diameter) were anesthetized and their femoral vessels exposed. Tumors were treated with IA or IV TNF at 8×10^5, 1×10^6, or 1.5×10^6 U/mouse or 2% normal rat serum (NRS) via continuous infusion over 1 h. Control animals underwent ipsilateral CFA ligation alone and received no infusion. Tumors were measured (L×W) every other day. IA administration of TNF-α (but not IV) led to statistically significant inhibition of tumor size at days 6–10 as compared to control-treated mice. Ligation alone (*closed squares*); 2% NRS (*closed triangles*); 8×10^5 U TNF-α (*open circles*), 1×10^6 U TNF-α (*open squares*), or 1.5×10^6 U TNF-α (*open triangles*)

Statistical analysis of tumor growth rates

Fig. 5 shows tumor growth curves in logarithmic form that were generated for controls (ligation alone and 2% NRS infusion) and for animals that had received TNF-α either IV or IA. The slopes of these curves were examined using repeated-measures analysis of variance.

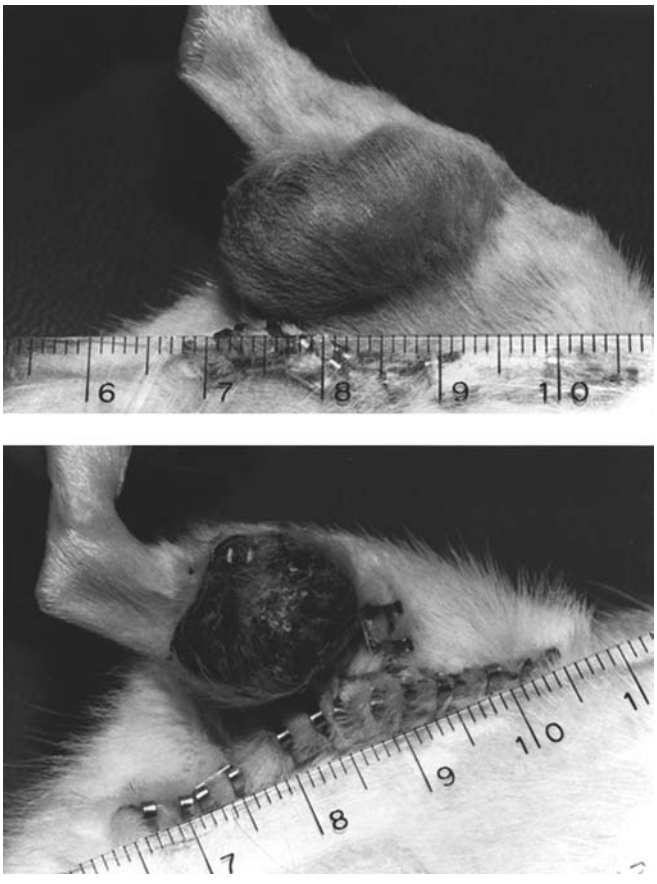

Fig. 4 Gross appearance of a tumor treated with IA TNF. The animals pictured received 1.5×10^6 U TNF-α via the contralateral common femoral vein (*top panel*) or via the ipsilateral common femoral artery (*bottom panel*) followed by ligation of the ipsilateral common femoral vessel. This photo was taken at day 3

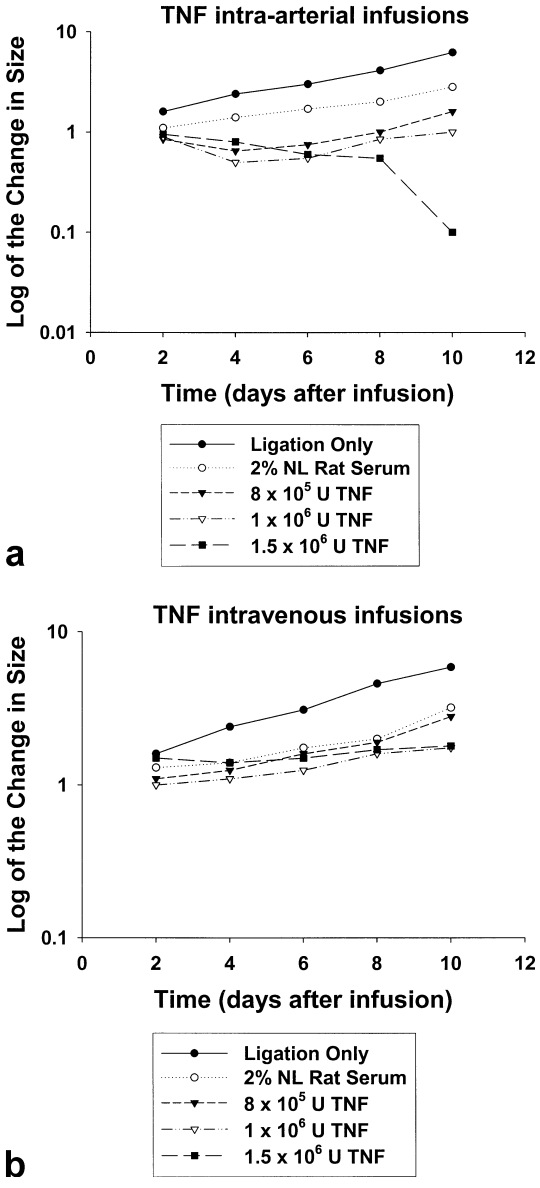

Fig. 5 Logarithmic growth curves for rats receiving TNF-α or normal rat serum infusions via the IA or IV routes. IA administration of TNF-α at 1.5×10^6 U led to a statistically significant alteration in the growth rate of the R3230 mammary adenocarcinoma at day 10 by the Bonferroni correction for multiple *t*-tests

Neither the infusion of 2% NRS nor the IV infusion of TNF-α significantly altered the slope of the tumor growth rate as compared to untreated controls. In contrast, the administration of increasing doses of TNF-α via the IA route resulted in enhanced inhibition of tumor growth and alteration of the slope of the growth rate of the experimental tumors when compared to controls and IV infusion of TNF-α. Using this method of statistical analysis, we found that the IA administration of 1.5×10^6 units of TNF-α significantly altered the growth behavior of the R3230 tumor.

Discussion

In the present study we have compared the efficacy of IA and IV administered TNF-α in a rat hind-limb model of mammary adenocarcinoma. The arterial blood supply to the lower extremity was ligated following the treatment, in order to mimic the technique of arterial chemoembolization. All tested IA doses of TNF-α resulted in a statistically significant decrease in tumor size by day 10 post-infusion, as compared to IV administration of TNF-α, the effects of which were not significantly different from those of control treatments. In fact, at the 1.5×10^6 U IA dose, tumors lost an average of 90% of their mass as the result of necrosis. Analysis of tumor growth rates using repeated measures analysis of variance revealed that the IA administration of 1.5×10^6 U of TNF-α was the only treatment that significantly altered the growth behavior of the R3230 tumor in this experiment. It is also important to note that IA therapy with TNF-α followed by ablation of the arterial blood supply did not result in any apparent physiologic or functional damage to the normal tissues of the tumor-bearing leg.

Treatment of localized neoplasms with IA TNF-α has been reported by several groups over the past 10 years, and the response rates in patients with inoperable melanoma metastases and soft-tissue sarcomas have been impressive [24, 25, 26, 27, 28, 29, 30]. Recent studies using TNF-α in combination with cytotoxic agents with or without interferon gamma (IFN-γ) have confirmed the high response rates, but the overall survival of these patients was not significantly prolonged. Other studies have shown that it is possible to administer TNF-α to hepatic and renal tumors via the IA route [30]. Treatment of unresectable hepatic metastases with TNF-α plus melphalan has produced response rates as high as 75% in the phase II setting [31, 32, 33, 34, 35]. Although breast cancer metastatic to the liver remains a significant health problem, there is very little experience with the treatment of these tumors using intra-arterially administered cytokines. Interestingly, there is a case report of a single breast cancer patient with multiple liver metastases who exhibited a complete response after infusion of TNF-α plus IFN in combination with 5-fluorouracil and cyclophosphamide, and remained disease-free for over 8 years [36].

Hepatic chemoembolization involves the administration of chemotherapeutic agents to liver metastases via arteriographic techniques, after which the arterial blood supply is interrupted via embolization. A phase II trial of hepatic artery chemoembolization for metastatic colorectal cancer exhibited a radiologic response rate of 63%, and the median survival of these patients was 8.6 months [37]. Our results suggest that the combination of IA TNF-α treatments and arterial ligation can significantly alter the growth pattern of a regionally confined rat mammary adenocarcinoma. In some instances the tumors became frankly necrotic, and the animals exhibited a significant reduction in tumor burden for prolonged periods of time. Importantly, the tumor employed in these experiments was shown to be relatively insensitive to TNF-α in vitro and to arterial ligation alone. The mechanism of anti-tumor action is therefore found in the dual vascular insult of ligation and damage to tumor endothelium by TNF-α. The combined treatment is enough to induce necrosis of tumors. Therefore, this therapeutic approach might be considered for any of the many cancers that metastasize to the liver and exhibit resistance to standard chemotherapeutic interventions.

We have shown that IA administration of TNF-α followed by arterial ligation inhibits tumor growth more effectively than IV therapy in an experimental model of a regionally confined malignancy. Future investigations in our laboratory will focus on other tumor types and the potential for combining TNF-α treatments with standard chemotherapeutic agents.

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