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Clinical Cancer Research

Preclinical Investigation of PEGylated Tumor Necrosis Factor α in Dogs with Spontaneous Tumors: Phase I Evaluation

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Abstract

Purpose: Tumor necrosis factor-α (TNF) is a cytokine with potent antitumor activity; however, toxicity and short half-life have limited its utility. Polyethylene glycol (PEG) conjugation of biotherapeutics can decrease immunogenicity while improving bioactivity and half-life. PEGylation of TNF (PEG-TNF) significantly improved half-life and toxicity in mice, resulting in enhanced antitumor activity. This study characterized toxicity, biological effect, and antitumor activity of PEG-TNF in pet dogs with spontaneous cancer.

Experimental Design: A phase I clinical trial enrolled dogs with measurable tumors in which standard therapy had failed or been declined. Physiologic, hematologic, and biochemical parameters were evaluated and tumor biopsies obtained serially. A subset of patients underwent serial dynamic contrast-enhanced magnetic resonance imaging.

Results: Fifteen dogs were enrolled at doses from 20.0 to 30.0 μg/kg. Dose-limiting toxicity at 30.0 μg/kg consisted of vascular leak in one and hypotension/coagulopathy in one, establishing 26.7 μg/kg as the maximum tolerated dose. Mean elimination half-life was 15.3 ± 4.9 hours. Biological activity (transient fever and leukopenia, increased tumor inflammation, and necrosis) was observed at all dosages. A significant increase in tumor blood flow was observed with dynamic contrast-enhanced magnetic resonance imaging. Minor/transient antitumor responses were observed in dogs with melanoma, squamous cell carcinoma, and mammary carcinoma, and a partial response was observed in a dog with angiosarcoma.

Conclusions: Using a clinically relevant, spontaneous large animal model of neoplasia, we have shown that biologically effective doses of PEG-TNF can be administered safely, and that PEG-TNF administration is associated with encouraging biological activity. These results justify the clinical evaluation of PEG-TNF in human cancer. *Clin Cancer Res; 16(5); OF1–11. ©2010 AACR.*

Tumor necrosis factor-α (TNFα) was first identified as a cytokine capable of inducing hemorrhagic necrosis in tumors (1). It exhibits a range of biological effects, including direct tumor cell cytotoxicity, immunomodulation, and endothelial toxicity (2–5). Early enthusiasm for human TNFα (hTNFα) as a systemic anticancer therapy was tempered when multiple studies indicated that hTNFα had a very short circulating half-life (14 minutes to 2.5 hours) and severe side effects, primarily hypotension (6–8).

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An alternative to systemic treatment is localized administration of high concentrations of hTNFα through isolated limb perfusion (9–13). Tumors responding to hTNFα delivered by isolated limb perfusion include melanomas, soft tissue sarcomas, desmoid tumors, and angiosarcomas (14). The major limitation of isolated limb perfusion is that metastatic disease is not affected by this procedure. However, these studies show that hTNFα can be an effective anticancer treatment, provided it is administered in high doses and systemic toxicity is avoided.

The clinical utility of many proteins and cytokines can be limited by their short circulating half-life, and thus they must be frequently administered to achieve therapeutic efficacy. Formulation of therapeutic proteins with polyethylene glycol (PEG) can significantly increase circulating half-life and decrease immunogenicity (15, 16), and increase biological activity as well as potentially increasing intratumor drug accumulation (17, 18). Several therapeutic proteins formulated with PEG have been approved by the Food and Drug Administration and are in widespread use.

Data obtained from preclinical murine models showed that PEGylation of hTNFα (PEG-hTNFα) can decrease

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Translational Relevance

The anticancer efficacy of tumor necrosis factor α (TNFα) seems limited by substantial toxicity and short half-life, as its regional delivery at high concentrations has considerable antitumor activity. Strategies to prolong half-life and mitigate the toxicity associated with systemic TNFα administration would be useful. PEGylated TNF (PEG-hTNFα) has both reduced toxicity and improvements in half-life and increased antitumor activity in murine models, but these models often fail to predict the antitumor efficacy and toxicity observed in human clinical trials. We describe a phase 1 clinical trial of PEG-TNFα in dogs with spontaneous cancer, a species whose response to TNF is very similar to that in humans. The present study shows that biologically active doses of PEG-TNFα can be administered safely to dogs with cancer, and that its administration is associated with prolongation in plasma half-life and modulation of tumor inflammation, necrosis, and vascular permeability. Taken together, these data suggest that PEG-TNFα may be a viable therapy in humans.

toxicity and increase antitumor efficacy in mouse models, when compared with native TNFα (19–23). However, no studies evaluating the tolerability and/or antitumor activity of any PEG-hTNFα formulations in large animals or spontaneous cancer models have been conducted.

The laboratory dog is a common model for the study of the hemodynamic effects of TNFα (24–27). Similar changes in blood pressure, cardiac output, and oxygenation are observed in dogs receiving hTNFα as are seen in humans, at doses below the predicted therapeutic range. In addition, hTNFα induces diverse molecular and biochemical events in dogs similar to those described in humans. These events include induction of endothelial intercellular adhesion molecule-1, and P- and E-selectin expression (28–30), increased circulating endothelial-derived vasoactive factors (27), increased neutrophil adherence (28, 31), and *in vitro* cytotoxicity against canine tumor cells (4, 5). Preliminary studies by our group suggested that, although native hTNFα was capable of inducing the expected hemodynamic changes in normal laboratory dogs (hypotension, hypoxemia), administration of a biologically equivalent dose of PEG-hTNFα resulted in no significant hemodynamic changes with preservation of other effects such as neutrophil margination.[5]

[5] G.S. Rapoport, K.T. Kruse-Elliott, B.W. Nemke, I.D. Kurzman, D.H. Thamm, M.A. Clark, R.L. Stepien, D.M. Vail. Attenuation of tumor necrosis factor-α–associated cardiovascular toxicity by conjugation to polyethylene glycol (Abstr). In: Proc Vet Cancer Soc 23rd Annu Conf Madison, WI. September 26-29, 2003.

Clinical trials in pet dogs with spontaneous cancer are important translational models from rodents to practical applications in human cancer. The dog is an excellent model for the investigation of novel cancer therapeutics owing to its large size, relative outbreeding, similar responses to environmental influences, and biological/physiologic similarity to humans. Moreover, dogs with spontaneous tumors naturally develop therapy resistance and spontaneous metastasis. Additionally, tumor burdens in the spontaneous cancers of dogs are more similar to humans than the tumor volumes found in murine models, which may be important with regard to factors such as hypoxia and clonal variation. The size of canine tumors also allows for serial imaging and tissue collection over time (32, 33).

We hypothesized that the PEGylation of hTNFα would result in decreased toxicity and increased circulating half-life compared with native hTNFα and, thus, exhibit more biological and antitumor activity than native TNFα in tumor-bearing dogs. To test this hypothesis, a pharmacokinetically and pharmacodynamically intensive phase 1 dose escalation study in dogs with spontaneous cancer was done. The end points of this study were (*a*) evaluation of safety, (*b*) determination of plasma and intratumor pharmacokinetics, (*c*) determination of the maximum tolerated dose (MTD), and (*d*) evaluation of clinical and biological efficacy of PEG- hTNFα.

Materials and Methods

Patient population. All dogs in this study were pet dogs presenting as patients to the University of Wisconsin-Madison Veterinary Medical Teaching Hospital or the Colorado State University Animal Cancer Center. Study participation was offered in cases in which standard therapy had failed or had been declined by the dog's owner, or in cases of advanced disease in which no meaningful standard therapy exists. Dogs were treated in accordance with the "NIH Guidelines for Care and Use of Laboratory Animals." Protocol approval was obtained from both the Institutional Animal Care and Use Committees and the Colorado State University Veterinary Teaching Hospital Clinical Review Board. Signed informed consent and consent to necropsy were obtained from all owners.

All dogs had measurable disease at study entry but there were no restrictions on stage of disease or disease burden. Histologic confirmation of diagnosis was obtained in all patients. Staging methods used varied depending on the histologic type and anatomic site of the tumor, and the clinical status. These included, but were not limited to, physical examination, complete blood count, serum biochemistry profile, urinalysis, coagulation profile, and thoracic radiographs. Dogs were eligible for the study provided they had adequate performance status, and hematologic and serum biochemical parameters to undergo therapy. Specifically, (*a*) hepatic transaminases not exceeding 3× normal, total bilirubin not exceeding 1.5× normal; (*b*) creatinine not exceeding 2× normal; (*c*) at least 2,500

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neutrophils/μL, 75,000 platelets/μL, and an hematocrit of at least 28%; (*d*) no evidence of preexisting, nontumor-related cardiopulmonary disease; and (*e*) modified Eastern Cooperative Oncology Group performance status of <2 (0, normal activity; 1, restricted activity, decreased activity from predisease status; 2, compromised, ambulatory only for vital activities; 3, disabled, needs to be force fed, is unable to confine urination and defecation to acceptable areas, and; 4, dead). No dogs had received chemotherapy or radiation therapy within 3 wk before study entry, nor was concurrent antineoplastic therapy used. None received concurrent corticosteroids or nonsteroidal anti-inflammatory drugs, and all patients were free of serious concurrent disease. Tumors were measured by physical assessment (i.e., caliper measurements) or by the serial examination of radiographs, ultrasound, or advanced imaging (e.g., computed tomography).

PEG-hTNFα formulation. Clinical grade recombinant PEG-hTNFα was produced in *Escherichia coli* as previously reported (34) in a Good Manufacturing Practice facility by Phoenix Pharmacologics, Inc. The PEG 20,000 MW was covalently attached to the primary amines of the hTNFα molecule according to the methods described by Tsutsumi et al. (21, 35) and packaged in sterile vials at a concentration of 1 mg/mL.

Treatment evaluations. Dogs were admitted to the Critical Care Units for 24 h following their first PEG-hTNFα treatment. Before treatment, jugular catheters were placed for drug and fluid administration and blood sample collection, and a catheter was placed into the dorsal pedal artery for repeated measurement of arterial blood pressures and blood withdrawal for blood gas analysis. Catheters were removed after 24 h. The dogs were monitored according to the schedule depicted in Table 1. Dogs experiencing significant pyrexia (temperature, >40.0°C) received acetaminophen (10 mg/kg per os) with or without maintenance rates of i.v. crystalloids.

Treatment and evaluation of toxicity. To prevent emesis, 0.5 mg/kg of ondansetron was administered i.v. to all dogs 20 min before the administration of PEG-hTNFα. Dogs received PEG-hTNFα in 5 mL 0.9% NaCl, administered as a 1-min i.v. bolus at 3-wk intervals. The starting dose was 20 μg/kg, and dose escalations were done at 3.33-μg/kg increments according to a standard 3 + 3 design. No intrapatient dose escalations occurred within a cohort. All adverse effects were prospectively graded according to the Veterinary Cooperative Oncology Group Common Terminology Criteria for Adverse Events v1.0 (36). Cardiopulmonary changes that were considered dose-limiting included mean arterial pressures of <60 mm Hg or PaO_2 of <70 mm Hg. Additional evidence of pulmonary toxicity was obtained by the evaluation of thoracic radiographs 24 h after treatment. For the purpose of dose escalation, a dose-limiting toxicity (DLT) was defined as a grade III toxicity in any category other than transaminase elevation, or previously defined dose-limiting cardiopulmonary change. The MTD was defined as the highest dose level in which no more than one of six dogs developed a DLT. All dogs in a cohort were observed for at least 3 wk following treatment before beginning accrual to a higher dose level.

Response assessment. Maximal tumor diameter was recorded before and at 7 and 21 d following the first two PEG-hTNFα treatments, and then before each subsequent treatment. Standard Response Evaluation Criteria in Solid

Table 1. Evaluation schedule following the first treatment

Time point	0	0.5 h	2 h	6 h	12 h	24 h	3-4 d	7 d	21 d*
Temperature, pulse, respiration	X	X	X	X	X	X	X	X	X
Tumor biopsy	X					X	X	X	X
Tumor volume and photograph	X							X	X
Indirect blood pressure	X					X			X
Serum biochemistry profile	X					X	X	X	X
Direct blood pressure	X	X	X	X	X	X			
Arterial blood gas	X	X	X	X	X	X			
CBC (including platelets)	X	X	X	X	X	X	X	X	X
Lactate	X	X	X	X	X	X			
Plasma for TNFα pharmacokinetics	X	X	X	X	X	X	X	X	X
Coagulation profile	X					X	X	X	X
DCE-MRI†	X					X	X	X	
Thoracic radiographs	X					X			X
Quality of life questionnaire	X							X	X
Body weight	X							X	X

*Before receiving the second PEG-hTNFα treatment on day 21.
†Performed in select patients.

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Tumors response criteria were used to assess clinical antitumor activity as follows: complete response (no measurable disease), partial response (>30% but <100% reduction in sums of diameters of all measurable lesions), stable disease (<30% regression or <20% progression), and progressive disease (>20% increase in the measurable disease diameters or the development of a new lesion). Complete or partial responses needed to persist for a minimum of 6 wk to be considered clinically relevant. Thoracic radiographs (and other imaging if indicated) were repeated 21 d following the second PEG-hTNFα treatment. Treatment could be continued on an every-3-wk basis as long as patients experienced stable disease or better without DLT.

hTNFα pharmacokinetics. Plasma hTNFα concentrations were measured at predose and 0.5, 1, 2, 6, 12, 24, 72, and 168 h postdosing using a colorimetric sandwich ELISA assay (Quantikine, R&D Systems), according to manufacturer directions. Manufacturer specifications state that this kit has <1% cross-reactivity with canine TNFα. Calculation of pharmacokinetic parameters was done by noncompartmental analysis as previously described (37) using the Excel 2003 software (Microsoft Corp.).

Intratumor hTNFα concentrations. Frozen tumor biopsy samples in lysis buffer were thawed, weighed, homogenized, sonicated, and centrifuged to recover insoluble protein. Protein was quantified using the BCA method and equal amounts were loaded in duplicate into wells of the ELISA kit described above. ELISA was done as described and hTNFα concentrations were expressed as picograms hTNFα per milligram of tumor tissue.

Dynamic contrast-enhanced MRI. MRI was performed with dogs under general anesthesia in a 1.5-Tesla MR instrument (General Electric Signa LX). After routine anatomic MR imaging, dynamic contrast-enhanced MRI (DCE-MRI) was done by controlled injection of gadolinium diethylenetriaminepentaacetic acid i.v. (Magnevist, Berlex Laboratories; 0.1 mmol/kg at 3 mL/s) while simultaneously repeating three-dimensional spoiled gradient echo T1-weighted scans (30° flip angle, 6- to 10-mm slice thickness) through the tumor volume using a temporal resolution of <12 s for 8 to 10 min. Analysis of DCE-MRI was compartmental based, done by region-of-interest analysis of the entire tumor volume using three-dimensional geometrically constrained region growth and also using an arterial input function derived from a local artery (Perfusion Analyzer, VirtualScopics Inc.). Biomarkers such as transfer rate constant (K_{trans}), instantaneous area under the curve (AUC), volume of extracellular space, and % nonenhancing voxels were derived by "intensity-based" two-compartment modeling (38, 39).

Histologic assessment. Formalin-fixed, paraffin-embedded tumor biopsy samples obtained before and at various times following PEG-hTNFα treatment (see Table 1) were routinely sectioned, paraffin embedded, and stained with H&E for light microscopic evaluation. A single board-certified veterinary pathologist (EJE), blinded to time following treatment and clinical response, semiquantitatively assessed parameters of inflammation and necrosis, according to an adaptation of a previously published scoring system (40).

Immunohistochemistry. The immunohistochemistry staining was done using standard techniques on an automated stainer (Discovery, Ventana Medical Systems). Briefly, 4-μm sections were cut and mounted on positively charged slides. The sections were deparaffinized and then rehydrated with descending alcohol concentrations to Tris-buffered saline with 0.05% Tween 20. Heat-induced epitope retrieval with a proprietary citrate-containing antigen retrieval buffer (pH 6.0; S1699, DAKO Cytomation) at 125°C for 1 min was followed by the blocking of endogenous peroxidase with 3% hydrogen peroxide and incubation with the primary antibody. Tissue sections were incubated overnight in humidified chambers with optimized concentrations of the following primary mouse monoclonal antibodies: mouse anti-canine P-selectin, undiluted (provided by Dr. J. Sirois, University of Montreal, Montreal, Canada); mouse anti-human CD31, 1:50 (M0823, DAKO Cytomation); and rabbit anti-human/mouse activated caspase-3, 1:500 (AF835, R and D Systems). Sections were then incubated with the Envision+ Dual link System-HRP (K4061, DAKO Cytomation) with 3,3′-diaminobenzidine (DAB) as a substrate. Slides were then lightly counterstained with hematoxylin.

Image analysis for P-selectin and microvessel density was done using the KS 400 system software (Carl Zeiss). For each tissue section, five semirandom "hot" zone images were assessed using a Zeiss Axioplan 2 imaging scope coupled with a Zeiss AxioCam HRc camera. Using a threshold feature, DAB-stained pixels were converted to white and unstained pixels to were converted to black, yielding a binary image. P-selectin immunoreactivity or microvessel density was then determined as the number of white pixels over total pixels. For standardization, the camera exposure and the threshold levels were constant for the acquisition and analysis of all images.

Analysis for activated caspase-3 used the same system, but the number of DAB-stained nuclei and the number of total nuclei were calculated. Percent apoptotic cells were then determined by dividing DAB-stained nuclei by total nuclei and multiplying by 100.

Statistical analysis. Changes over time in measured variables were compared with baseline measurements using a paired, two-tailed Student's *t* test. For those variables expressed as a percentage of pretreatment values, a two-tailed one-sample *t* test was used to determine significant deviation from 100%. Changes between dose cohorts were compared using the χ^2 analysis. For all analyses, a *P* value of <0.05 was considered significant.

Results

Patient demographics. There were nine female and six male dogs representing nine breeds enrolled, with a median body weight of 28 kg (17-45 kg) and a median age of 9.5 years (5–14). Nine tumor types were treated.

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Among carcinomas, there were two head and neck squamous cell carcinomas, two apocrine gland carcinomas, and one mammary gland carcinoma. Among sarcomas, there were one each of osteosarcoma, histiocytic sarcoma, fibrosarcoma, and angiosarcoma. There were three melanomas and two mast cell tumors. One dog, which was diagnosed with an atypical cutaneous lymphoma upon initial histopathology, had the diagnosis revised to a nonneoplastic condition upon slide review following treatment. This dog was included in toxicity evaluation but was not included in antitumor response assessment, nor were its tissues used for histology or immunohistochemistry. The median number of prior nonsurgical treatments was 0, with a range of 0 to 3. Five dogs had prior surgery, two had prior radiation therapy, and four had prior chemotherapy. A total of 25 treatments were administered at doses ranging from 20 to 30 μg/kg. The median number of treatments per patient was one, with a range of one to five.

Adverse effects. Fever, diarrhea, and vomiting were the most commonly encountered clinical adverse effects. Diarrhea and vomiting occurred within hours of PEG-hTNFα administration and generally resolved within 24 to 48 hours. Hyporexia often accompanied vomiting and diarrhea, and occasionally persisted for up to 1 week. These adverse effects did not exceed grade 2 in any patient, were not dose related, and tended to be reduced in frequency with repeated dosing. Repeatable and predictable pyrexia was observed at all dose levels, exceeding the reference range by 0.5 to 12 hours postdose and returning to normal by 24 to 48 hours (Fig. 1A). There was no association between peak temperature and administered dose. No dosage reductions were used for adverse gastrointestinal effects or pyrexia. Grade 1 hypocalcemia, hypoglycemia, and hypokalemia, all clinically silent, were observed in several dogs at 24 hours, generally resolving by 72 hours. Other adverse effects are detailed in Table 2. Elevations in alanine aminotransferase and aspartate aminotransferase (AST) were nearly always detected 24 hours postdose, were clinically silent, and were resolving or resolved by 72 hours, and were thus not considered dose limiting. The AST was much more often and more profoundly elevated, and changes in AST usually paralleled increases in creatine kinase. Based on previous experience, these changes were likely related to sedation/anesthesia and biopsy done before drug administration (41).

The 20 μg/kg dose cohort was expanded to six dogs owing to a single episode of severe generalized pain, for which a drug-related adverse effect could not be ruled out. Two of three dogs treated at the 30 μg/kg dose experienced grade 4 adverse effects.

A dog with head and neck squamous cell carcinoma treated at 30 μg/kg experienced grade 4 elevations in hepatic transaminases, grade 3 elevation in total bilirubin, and grade 3 hypoalbuminemia associated with systemic vascular leak, manifested as generalized peripheral edema. This dog was treated aggressively with fluid support, colloids, and plasma and was discharged from the hospital 5 days following drug administration. Fourteen days following treatment, clinical and biochemical signs consistent with hypoadrenocorticism were noted, and the dog was euthanized. Bilateral necrosis of >90% of the adrenal tissue was detected on postmortem examination. Interestingly, this dog's peak and AUC hTNFα concentrations were several times higher than the other dogs treated in the 30 μg/kg cohort (see below).

A dog with multifocal, widely metastatic mast cell tumor developed severe hypotension and grade 4 coagulopathy within 12 hours of treatment at 30 μg/kg, which did not respond to plasma, colloid, and pressor support. Euthanasia was elected 24 hours following treatment. In this dog, a significant increase in plasma histamine was detected following treatment (data not shown), which coincided with development of the clinical signs. We hypothesized that acute and massive mast cell degranulation was responsible for this dog's hypotension and coagulopathy, although another cause could not be ruled out.

Hematologic/metabolic changes. Complete blood counts were obtained at various times following the first PEG-TNFα administration. A profound reduction in peripheral leukocyte count, characterized by marked reductions in neutrophil numbers and moderate reductions in lymphocytes, was observed within 30 minutes after PEG-hTNFα administration, returning to baseline by 6 hours (Fig. 1B). A small but statistically significant reduction in platelet count was observed at 24 hours, which returned to the reference range by 7 days (Table 2; Fig. 1C). These changes were independent of dose cohort.

Significant increases in partial thromboplastin time, independent of dose cohort, were also noted at 24 hours, generally returning to baseline by 7 days (Table 2). Mild but significant hypoalbuminemia was also observed at 24 hours and 7 days, returning to baseline at 21 days. Lowest albumin concentration was significantly correlated with administered dose ($r^2 = 0.38$, $P = 0.014$).

Significant increases in plasma lactate were noted starting at 30 min postdose, generally peaking at 6 hours and returning to baseline by 24 hours (Fig. 1D). These increases were not dose related. A profound increase in serum creatine kinase was noted 24 hours following administration, returning to baseline 3 to 4 days posttreatment (data not shown). This was attributed to anesthesia/biopsy, which often occurred the day of treatment initiation (41).

Importantly, no reductions in mean systolic blood pressure (Fig. 1E) or arterial blood oxygen concentration (Fig. 1F), the most common dose-limiting toxicities of unconjugated hTNFα in humans and dogs, were observed. The sole exception was the dog with diffuse mast cell tumor mentioned above.

Dynamic contrast-enhanced MRI. The seven dogs that underwent serial DCE-MRI were evaluated for the effects of PEG-hTNFα on tumor perfusion and blood flow. The resulting data suggested increased tumor mean K_{trans} and AUC versus baseline measurement, which are functions of increased perfusion and vascular permeability. A significant increase in K_{trans} from baseline was observed 3 days

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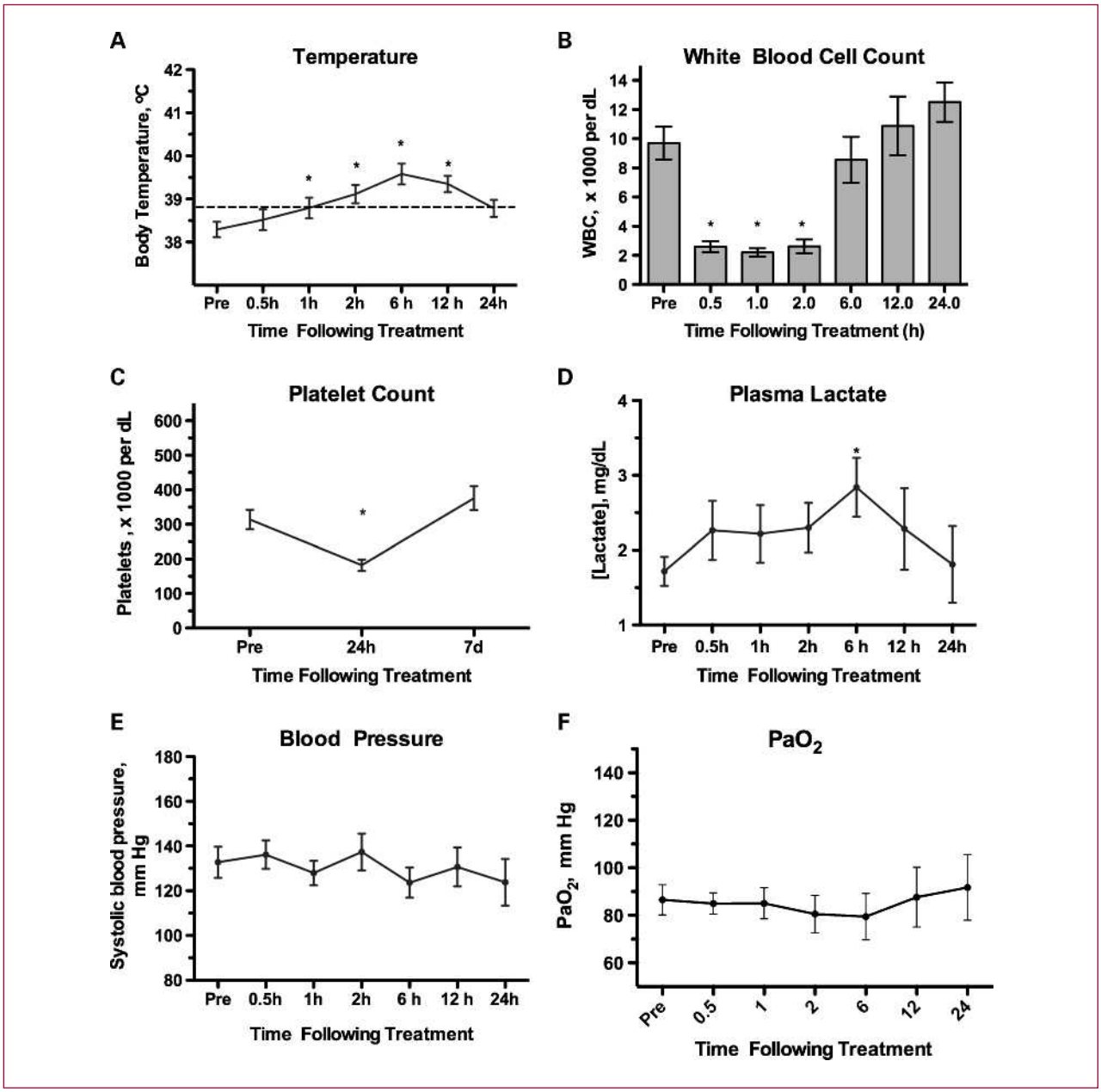

Fig. 1. Clinical and biological effects of PEG-hTNFα in dogs with spontaneous cancer. A, an increase in body temperature was observed within 1 h of treatment, peaking at 6 h, and returning to the reference range within 24 h. Dotted line, the upper limit of normal for body temperature in dogs. B, a profound but transient decrease in total WBC count was observed immediately following PEG-hTNFα administration, rebounding by 6 h. This was characterized by reductions in neutrophils and lymphocytes (data not shown). C, a modest but significant reduction in platelet count was observed at 24 h, returning to the reference range by 7 d. D, plasma lactate was increased following PEG-hTNFα administration, peaking at 6 h. Notably, no significant changes in systolic blood pressure (E) or arterial oxygen saturation (F) were noted following PEG-hTNFα administration. Points and columns, mean measurement; bars, SEM; *, statistically significant ($P < 0.05$) differences versus baseline measurements.

following treatment initiation ($P = 0.0175$; Fig. 2A). We hypothesize that these changes were associated with increased tumor-associated inflammation, as observed histologically (see below). Although there was no overall change in the percent nonenhancing (%NE) tumor value, a measure of poorly perfused and/or necrotic tissue, the one dog experiencing objective tumor regression experienced a dramatic increase in %NE 24 hours following treatment (Fig. 2B), which could have been indicative of more significant vascular collapse and/or necrosis.

Postmortem findings. Postmortem evaluations were done in 10 patients. Hepatic changes were observed in

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2 (20%), consisting of peracute multifocal hepatocellular necrosis in 1 (the dog experiencing presumed mast cell tumor degranulation), and mild multifocal suppurative hepatitis with mild portal fibrosis in 1. Renal changes were observed in 5 (50%), consisting of fibrotic change in 3, subacute infarcts in 1, and primarily lymphoplasmacytic inflammatory change in 3. The hepatic and renal changes ranged from mild to severe, and there was no obvious relationship between dose or number of treatments administered. There were no repeatable findings in other organs.

Histology and immunohistochemistry. Serial biopsy samples, collected before treatment and at 1 day, 3 to 4 days, and 7 days following the first treatment, contained evaluable material in nine patients. There was a significant increase in mean inflammatory scores and mean necrosis scores 1 day following treatment (Fig. 3). Inflammatory infiltrate was characterized by a mixture of neutrophils and lymphocytes. There was a statistically insignificant increase in endothelial P-selectin immunoreactivity 24 hours postdrug administration (data not shown). There were no significant changes in total apoptosis, microvessel density, or endothelial-specific apoptosis.

Table 2. Adverse events associated with PEG-hTNFα administration to tumor-bearing dogs

Abnormality/dosage (μg/kg)	Severity grade			
	I	II	III	IV
ALT/AST				
20	2	1	1	
23.3	1		1	
26.7			3	
30			2	1
Creatinine				
20				
23.3				
26.7	1			
30		1	1	
Albumin				
20	2			
23.3				
26.7	3			
30		2		
Prolonged PT/APTT				
20	4	1		
23.3	2			
26.7	2	1		
30	2			1
Thrombocytopenia				
20	4			
23.3	2	1		
26.7	1	1		
30	2		1	

Abbreviation: ALT, alanine aminotransferase.

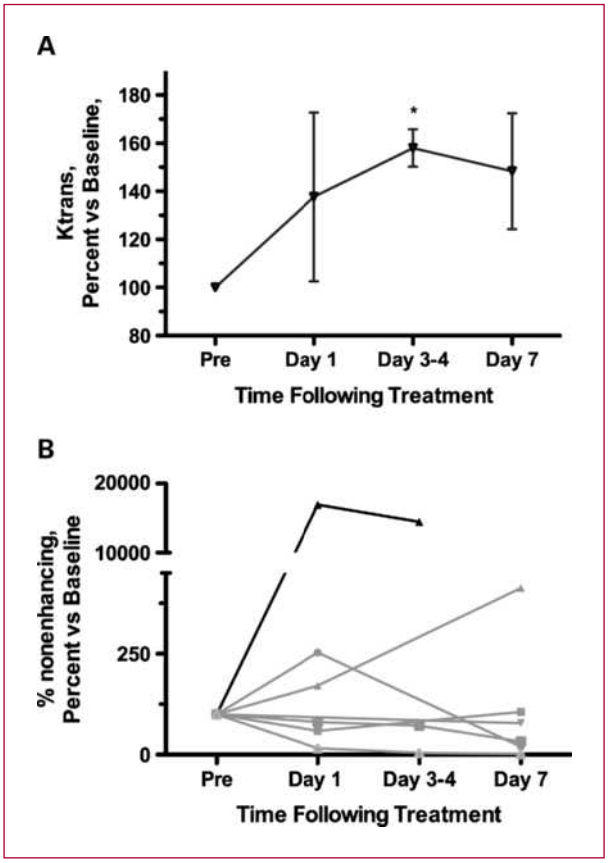

Fig. 2. Changes in tumor perfusion as assessed by DCE-MRI in dogs receiving PEG-hTNFα. A subset of patients was assessed serially for changes in tumor perfusion. A, a significant increase in K_{trans}, indicative of increases in tumor perfusion and/or vascular permeability, is observed 24 h following treatment, achieving statistical significance at day 3 to 4. Points, mean measurement; bars, SEM. *, $P < 0.05$. B, changes in percent nonenhancing tumor following treatment are depicted in individual dogs. A dramatic (200-fold) increase in percent nonenhancing tumor was noted in the one dog in the study experiencing an objective response (black line).

TNF pharmacokinetics. Plasma samples were obtained at the time points indicated in Table 1 following the first treatment, and hTNFα concentrations were determined using a commercial ELISA. Pharmacokinetics were modeled using noncompartmental analysis (Table 3). The mean elimination half-life was 15.3 ± 4.9 hours. This compares favorably with the reported plasma half-lives for native TNFα of 14 minutes to 2.4 hours in humans (8, 42–44) and 21 minutes in dogs (27). There was a clear linear relationship ($R^2 = 0.995$, $P = 0.046$) between dose and plasma $AUC_{0\rightarrow24}$ for the dose levels with at least three evaluable pharmacokinetic profiles (20, 23.3, and 26.7 μg/kg), showing dose proportionality within this dose range. There was no correlation between dose and other measured pharmacokinetic parameters. Interestingly, the

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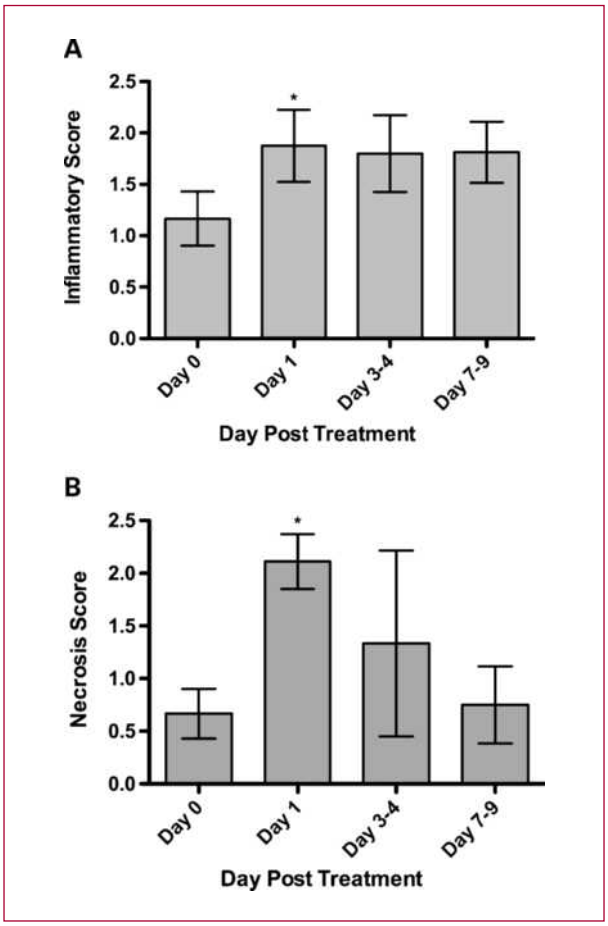

Fig. 3. Histologic assessment of tumor inflammation and necrosis following PEG-hTNFα administration. A subset of patients underwent serial biopsy for histologic assessment of inflammation and necrosis using a semiquantitative scoring system. Significant increases in tumor-associated inflammation (A) and necrosis (B) were observed 24 h following treatment. Columns, mean measurement; bars, SEM. *, $P < 0.05$.

patient developing severe vascular leak and adrenal thrombosis at 30 μg/kg had peak plasma hTNFα concentrations approximately five times greater than the other two dogs in the cohort.

In a subset of patients, hTNFα was measured serially in tumor biopsy samples. Increased hTNFα was detectable in all samples. A mean peak concentration of ~175 pg/mg tissue (19.8-531 pg/mg) was observed after 24 hours. Peak intratumor hTNFα concentrations did not correlate with dosage cohort.

Antitumor response. Thirteen of 15 patients were evaluable for response to therapy. Two dogs (melanoma and head and neck squamous cell carcinoma) experienced >80% reduction in local tumor volume at dosages of 20 and 30 μg/kg, respectively; however, both were transient (<3 weeks) and the dog with melanoma had pulmonary metastases that failed to respond. A dog with metastatic mammary carcinoma experienced a 45% reduction in measurable tumor volume 7 days following treatment, but was euthanized at 12 days due to progressive pain, presumably from diffuse bone metastases identified radiographically and on postmortem examination. Severe and diffuse necrosis and inflammation were found in all tumor tissues from this dog at necropsy, which may have contributed to its increased pain. A dog with s.c. hemangiosarcoma experienced a partial response persisting for 3 months.

Discussion

In this study, the acute and short-term toxicities associated with the administration of escalating doses of PEG-hTNFα to dogs with a variety of tumors were evaluated. Pyrexia and mild, self-limiting gastrointestinal disturbance were the most common adverse effects. Significant increases in creatine kinase and AST, but much less commonly alanine aminotransferase, were observed in 10 patients after 24 hours; however, these were not associated with other evidence of hepatotoxicity and decreased rapidly following treatment. These were attributable to anesthesia and biopsy, which were often done the day of therapy initiation, as others have observed (41). Hypoalbuminemia was observed but was generally mild and transient. Most of these toxicities were not dose related. Two of three dogs developed DLT at 30 μg/kg, establishing 26.7 μg/kg as the MTD. In one of the dogs experiencing DLT, an unexpectedly high peak plasma hTNFα concentration was observed. It must be

Table 3. Plasma pharmacokinetics of PEG-hTNFα following i.v. administration

Dose	*n*	$T_{1/2}\lambda$ (h)	$AUC_{0\rightarrow24}$ (ng/mL × h)	CL (mL/h/kg)
20	6	14.0 ± 4.2 (9.9-19.4)	848 ± 260 (484-1172)	25.9 ± 9.5 (17.1-41.3)
23.3	3	14.3 ± 4.0 (11.5-18.9)	1241 ± 191 (1033-1408)	19.1 ± 3.1 (16.5-22.6)
26.7	3	18.5 ± 4.1 (13.8-21.0)	1762 ± 411 (1463-2230)	15.7 ± 3.3 (12.0-18.2)
30	2	15.4 (8.0-22.8)	977 (700-1254)	33.4 (23.9-42.9)

NOTE: Values represent the mean ± SD for the calculated pharmacokinetic parameters. The range of values is shown below for each parameter in parentheses.

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mentioned that evidence of biological activity was observed at all doses, and thus doses less than the MTD may be biologically effective.

There was one fatality reported in the immediate period following PEG-hTNFα administration at 30 μg/kg. Clinical, clinicopathologic, and postmortem findings suggested acute degranulation from a diffuse metastatic mast cell tumor, resulting in severe hypotension and coagulopathy. Histopathology obtained at necropsy 24 hours postdose showed severe and diffuse tumor necrosis, edema, and hemorrhage, suggesting that the degranulation observed was due at least in part to massive tumor cell death.

Hepatic and renal changes were noted in some patients at necropsy. These were variable between patients and subclinical in all, except the dog experiencing acute hypotension and coagulopathy leading to euthanasia at 24 hours. Again, these effects did not seem to be dose related.

At tolerable dosages of PEG-hTNFα, we observed significant biological effects, both systemically and in tumor tissues. We detected significant and profound reduction in neutrophil counts within 30 minutes of administration, returning to baseline by 6 to 12 hours. The kinetics of this change is inconsistent with leukocyte cytotoxicity and is more consistent with alterations in neutrophil margination and extravasation in response to inflammatory stimuli. There were mild but significant reductions in platelet numbers at 24 hours and increases in clotting times, but these returned to baseline by 7 days and were not dose limiting. Although high doses of native TNFα do not elicit overt disseminated intravascular coagulation or a clinical thrombotic response, modest changes in coagulation parameters have been observed by others (45). A significant increase in plasma lactate was observed 6 hours following treatment, again returning to baseline rapidly after administration. The increases remained in a range considered clinically insignificant; however, an ~3-fold increase in arterial lactate has been noted in swine and rabbits receiving TNF, associated with enhanced glucose uptake into peripheral tissues (46, 47).

A subset of dogs underwent serial biopsy for assessment of tumor necrosis and inflammation, and serial DCE-MRI for assessment of tumor perfusion. An increase in tumor perfusion (increased K_{trans}) was observed at 24 hours, reaching statistical significance at 72 hours. This change paralleled an increase in tumor inflammation and necrosis, assessed histologically. We hypothesize that an increase in tumor-associated inflammation resulted in increased vascular permeability. Supporting this, extensive perivascular edema and hemorrhage were observed histologically in some tumor samples following treatment, although these changes were not quantitative. This finding has important ramifications for future studies, as it suggests that pretreatment with PEG-hTNFα could enhance the uptake and, thus, efficacy of other cytotoxic agents. Indeed, this has been shown using tumor vasculature–targeted TNFα with multiple cytotoxic agents (48, 49) and with native TNFα combined with liposomes (50).

Importantly, major adverse effects associated with the systemic delivery of unconjugated hTNFα, namely hypotension, reduced cardiac output, and hypoxemia, were not observed in canine cancer patients treated with PEG-hTNFα in this study, with the exception of the dog experiencing acute mast cell tumor degranulation. This suggests preservation of proinflammatory and, presumably, antitumor effects with mitigation of the DLTs associated with hTNFα.

Dogs with a variety of tumor histotypes were treated in this study. These included carcinomas, sarcomas, and hematopoietic tumors, and represent a more diverse gamut of tumor types than are often encountered in human phase I clinical trials. Although the primary focus of this study was to characterize the short-term toxicoses associated with PEG-hTNFα administration, preliminary information about antitumor activity was also generated. Encouraging evidence of antitumor activity was observed in the form of three minor or transient responses in dogs with melanoma, head and neck squamous cell carcinoma, and mammary carcinoma. A strong partial response lasting 3 months was observed in a dog with s.c. hemangiosarcoma. Interestingly, melanoma and angiosarcoma are two tumor types reported to respond to hTNFα when delivered through isolated limb perfusion (14). Antitumor activity was observed over the range of dose levels.

In conclusion, we have shown safety, biological activity, and preliminary evidence of antitumor activity of PEG-hTNFα in dogs with spontaneous cancer. Future studies should evaluate select tumor histotypes (e.g., melanoma, head and neck cancer, angiosarcoma) within the context of phase 2 investigations, and the observed alterations in tumor perfusion should justify pilot studies evaluating combinations of PEG-hTNFα with standard cytotoxic agents. Furthermore, these encouraging preclinical results in a relevant, spontaneous, large animal model of cancer in a species with similar sensitivity to TNFα strongly justify clinical evaluation of PEGylated hTNFα formulations in human cancer patients.

Disclosure of Potential Conflicts of Interest

M. Clark, former employee, shareholder, board member, Phoenix Pharmacologics Inc. Phoenix Pharmacologics supplied the test article for these investigations at no cost.

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Preclinical Investigation of PEGylated Tumor Necrosis Factor α in Dogs with Spontaneous Tumors: Phase I Evaluation

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