# A phase I trial of hyperthermia-induced interleukin-12 gene therapy in spontaneously arising feline soft tissue sarcomas

**Farzan Siddiqui,[1] Chuan-Yuan Li,[3] Susan M. LaRue,[1] Jean M. Poulson,[3] Paul R. Avery,[2] Amy F. Pruitt,[5] Xiuwu Zhang,[3] Robert L. Ullrich,[1] Donald E. Thrall,[4] Mark W. Dewhirst,[3] and Marlene L. Hauck[5]**

Departments of [1]Environmental and Radiological Health Sciences and [2]Microbiology, Immunology, and Pathology, Colorado State University, Fort Collins, Colorado; [3]Department of Radiation Oncology, Duke University Medical Center, Durham, North Carolina; and Departments of [4]Molecular and Biomedical Sciences and [5]Clinical Sciences, North Carolina State University College of Veterinary Medicine, Raleigh, North Carolina

## Abstract

**Interleukin-12 (IL-12), a proinflammatory cytokine, shows anticancer properties. Systemically administered IL-12 causes dose-dependent toxicity. To achieve localized intratumoral gene expression, an adenoviral gene therapy vector with IL-12 controlled by a heat-inducible promoter (heat shock promoter 70B) was developed and tested in a phase I clinical trial in cats with spontaneously arising soft tissue sarcoma. A feasibility study was done in 16 cats with soft tissue sarcoma using murine IL-12 and/or enhanced green fluorescent protein adenoviral vectors under cytomegalovirus or heat shock promoter 70 control. Subsequently, we conducted a phase I clinical trial using an adenoviral feline IL-12 construct in 13 cats with soft tissue sarcoma. The soft tissue sarcomas were irradiated (48 Gy/16 fractions) followed by intratumoral injection of adenovirus. Twenty-four hours postinjection, tumors were heated (41°C, 60 min). Tumor expression of feline IL-12 and IFN-γ was determined. Cats were monitored for systemic toxicity. For the murine IL-12 construct, an association was noted between viral dose and murine IL-12 levels within tumor, whereas serum levels were minimal. Mild toxicity was noted at $10^{11}$ plaque-forming units (pfu). With the feline IL-12 construct, high levels of feline IL-12 mRNA were detected in tumor biopsies with low or absent IFN-γ mRNA following gene therapy. Hematologic and hepatic toxicities were noted at the highest viral doses and were associated with detection of IFN-γ mRNA in tumor. It is possible to localize gene expression and limit systemic toxicity of IL-12 using the hyperthermia-induced gene therapy approach. The maximum tolerated dose of the feline IL-12 adenoviral vector was $10^{10}$ pfu/tumor as dose-limiting toxicities were noted at the $4 \times 10^{10}$ pfu dose. [Mol Cancer Ther 2007;6(1):380–9]**

## Introduction

Interleukin-12 (IL-12) is a proinflammatory immunomodulatory cytokine with anticancer properties (1). It achieves its effects via its downstream cytokines, most notably IFN-γ. However, recombinant IL-12 protein is also systemically toxic. We initially developed a vector encoding murine IL-12 placed under the control of a heat-inducible promoter to localize murine IL-12 production in the tumor. The rationale for using this heat shock promoter (hsp) is that hyperthermia can be selectively delivered to either local or regional disease sites, thereby promoting control of IL-12 expression. Heating the tumor leads to activation of the *hsp* and subsequent local IL-12 production.

The feasibility of combining fractionated radiotherapy, hyperthermia, and heat-inducible gene therapy was studied in a nonimmunogenic B16.F10 melanoma line syngenic with C57BL/6 mice (2). It was concluded that hyperthermia-regulated gene therapy in combination with radiation is feasible and therapeutically effective in murine tumors with no apparent systemic toxicity (2, 3). The sequencing of radiation therapy and *IL-12* gene therapy was based on an earlier report (4). In this study, we had shown in a mouse tumor model that the best results are obtained when immunotherapy was administered after fractionated radiation therapy. This study did not include hyperthermia as a component. However, as we were intending to use hyperthermia only as a physical agent for transgene expression in the current study, we decided to use the same treatment sequence (i.e., fractionated radiation therapy followed by hyperthermia-induced gene therapy).

Vaccine- or injection site–associated feline soft tissue sarcomas are a unique clinical entity. They were first recognized by Hendrick and Goldschmidt (5) as soft tissue sarcomas arising at the site of vaccination in cats. The prevalence of sarcoma development after vaccination has been variously reported as between 1 in 10,000 (6) and 1 in 1,000 (7). Cats with injection site sarcomas belong to a younger age group than cats with non–injection site

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**Requests for reprints:** Marlene L. Hauck, Department of Clinical Sciences, College of Veterinary Medicine, North Carolina State University, 4700 Hillsborough Street, Raleigh, NC 27606. Phone: 919-513-8274; Fax: 919-513-6336. E-mail: marlene_hauck@ncsu.edu



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sarcomas (8). Hendrick (9) reported an average interval of 26 months between rabies vaccination and tumor development. In another study (6), the median interval was found to be 11 months between feline leukemia virus vaccination and tumor formation. It is postulated the adjuvants used in vaccines lead to local reactions and chronic inflammation with subsequent tumor development (10). The treatment for this disease involves multimodality therapy including radiation therapy and surgery (8).

These spontaneously arising soft tissue sarcomas provided an excellent model to conduct a phase I trial of hyperthermia-induced *IL-12* gene therapy and to show the feasibility of localized, controlled gene expression. These patients are ideal for this study as the tumors are easily accessible for direct intratumoral injections of the viral construct and amenable to being heated using a microwave applicator designed for superficial hyperthermia.

For the purposes of this phase I study in feline spontaneous soft tissue sarcomas, an adenoviral construct was developed with the feline *IL-12* gene replacing the murine *IL-12* gene. The main aim of this trial was to establish the maximum tolerated safe dose of the viral construct. The hypothesis being tested was that hyperthermia would induce high local intratumoral levels of IL-12 and its downstream effector cytokine, IFN-γ.

## Materials and Methods

### Feasibility Study

In the preliminary study using the murine IL-12 and enhanced green fluorescent protein (eGFP) constructs, our aim was to establish the techniques and assess the feasibility of the proposed gene therapy trial. Client-owned cats with spontaneously arising soft tissue sarcomas presenting for treatment to the Colorado State University and North Carolina State University Veterinary Teaching Hospitals were included in this study. Written, informed consent was obtained from the owners. A minimum of three cats per viral dose level was planned.

### Phase I Clinical Trial

In the phase I clinical trial using the feline IL-12 construct, 13 cats were treated from July 2003 to March 2005. The eligibility criteria were (*a*) the patients had to have a confirmed diagnosis of sarcoma (fibrosarcoma) with or without metastases; (*b*) the tumor had to be superficial, palpable, and amenable to being injected; (*c*) prior surgery and chemotherapy were acceptable; (*d*) no prior radiation therapy was permitted; and (*e*) the health status of the cat had to be sufficient to permit completion of this 3-month study. A minimum of three cats per viral dose level was planned.

The staging studies included (*a*) a complete blood count; (*b*) serum chemistry; (*c*) urinalysis; (*d*) tumor biopsy and histopathology; (*e*) three-view thoracic radiographs; (*f*) abdominal radiographs or ultrasound if the tumor was caudally located; and (*g*) computer-assisted tomography scan for radiation therapy treatment planning. All protocols were approved by the Institutional Animal Care and Use Committees at Duke, Colorado State University, and North Carolina State University.

### Design of Vectors

For construction of recombinant adenovirus vectors, the AdEasy system for recombinant adenovirus production was used. It is a system developed by Dr. T.C. He (11). It is developed as a fast and easy alternative to traditional systems for the production of recombinant adenovirus. The construction of a recombinant adenovirus is a two-step process in which the desired expression cassette is first assembled into a transfer vector (pShuttle) with virus packaging signals, and subsequently transferred into the adenoviral genome plasmid (with no virus packaging signals) by homologous recombination.

The murine *IL-12* gene was obtained as described (3). The two subunits of the gene (*p35* and *p40*) were linked together through the internal ribosome entry site sequence so that the expression of both subunits was expressed from a single promoter.

The feline *IL-12* genes (*p35* and *p40*) were cloned by reverse transcription-PCR (RT-PCR) from the feline kidney CRFK cell line. After sequence verification, the two genes were fused together through a flexible linker that consists of $(Gly_4Ser)_3$. This type of fusion has been shown to work well in the past (12, 13).

The *eGFP* gene was obtained from the commercially available plasmid pEGFP-1 (BD Clontech, Palo Alto, CA).

Murine *IL-12*, *eGFP*, and feline *IL-12* genes were placed under the control of the hsp70B gene promoter that has been shown to have high heat-induced induction and low background expression (3, 14, 15). A second eGFP construct was designed with the constitutively expressing cytomegalovirus (CMV) promoter. The gene expression cassettes were then transferred into the pShuttle plasmid of the AdEasy-1 system. The recombined pAdEasy-1 plasmids containing the individual genes were then transfected into 293 cells for virus packaging according to established procedures (11). A procedure similar to those described by Graham and Prevec (16) was followed to produce adenovirus in large scale. Briefly, 293 cells were infected at a multiplicity of infection of 5 to 10 using a total of $1 \times 10^9$ 293 cells. After 3 to 4 days, the resultant viruses were purified by double CsCl banding. In general, $\sim 3 \times 10^{10}$ to $10 \times 10^{10}$ plaque-forming units (pfu) were yielded at the end of the amplification procedure by use of 293 cells cultured in 20 × 150-mm Petri dishes.

In summary, the preliminary study used three different constructs, Ad hsp murine IL-12, Ad CMV eGFP, and Ad hsp eGFP, whereas the phase I clinical trial used the Ad hsp feline IL-12 vector.

### Radiation Therapy

The cats were immobilized for radiation therapy by administering general anesthesia (1.5–2% isoflurane with 100% oxygen) and treated using a 6MV linear accelerator (Siemens, New York, NY) at Colorado State University and a telecobalt machine at North Carolina State University. The total dose delivered was 48 Gy (prescribed to the 95% isodose line) in 16 fractions over 22 days treating daily.

### Gene Injections

Intratumoral gene injections were delivered on the first Monday after completion of radiation therapy. This led to a gap of 3 to 5 days between the end of radiation therapy and adenoviral injection, depending on when the radiation therapy was completed. Tumor dimensions were determined from caliper and computer-assisted tomography scan measurements. Tumor volume was calculated as (length × breadth × depth × $\pi/6$) $cm^3$. The viral construct (Ad *hsp* feline IL-12) was diluted in normal saline to a volume equal to 30% of the tumor volume. Under computer-assisted tomography guidance, needles were positioned and repositioned in the tumor in a 1 × 1-cm grid pattern until visually parallel, equidistant, and reaching the deep surface of the tumor.

The total volume of injection was divided by the number of injection sites and equal volumes were injected at each site. While injecting, the needle was gradually withdrawn out of the tumor. This led to deposition along the track. Theoretically, each track would form a cylinder with a 1-$cm^2$ cross-sectional area using this injection technique. This protocol is similar in concept to that previously reported by Li et al. (17).

### Hyperthermia

Twenty-four hours were allowed for physical diffusion of the injected construct and adenoviral infection of the tumor cells. Hyperthermia treatment was then administered. Two closed-end catheters (Flexineedles, Best Medical International, Springfield, VA) were placed in the tumor under computer-assisted tomography scan guidance, following the Radiation Therapy Oncology Group guidelines (18). Precalibrated fiberoptic thermometers were placed in these catheters to provide temperature measurements during hyperthermia treatment. Local tumor hyperthermia was delivered using a 433-MHz microwave applicator, as previously described (19). Deionized water was used as a coupling medium. Skin temperature was kept below 42°C using a circulating water bolus. Tumor temperatures were mapped at 3- to 5-mm increments along the catheter track. The target $T_{90}$ was 40.1°C, with an upper limit of 44°C placed on the $T_{50}$, for 60 min. Thermal data were entered into a database to calculate hyperthermia dose parameters.

### Biopsies

Core tissue biopsies were obtained using a 22-mm throw automated biopsy device (Manan Pro-Mag 2.2, Manan Medical Products, Northbrook, IL) with a 14-gauge needle. They were collected from the tumor during the week of gene therapy. In larger tumors, biopsies were done before virus injection and daily thereafter for 4 days. In smaller tumors, four biopsies were not possible. In these subjects, biopsies were collected at baseline and 24 h postheating. All biopsies were done under anesthesia and snap frozen in liquid nitrogen. They were stored at −80°C until further processing. We attempted to minimize the problem of necrotic tumor biopsy samples by collecting two to three grossly viable biopsies on each day in the larger tumors. All samples collected in 1 day were pooled for analysis.

### Serum Samples

Serum samples were collected for evaluation of systemic levels of murine IL-12. The samples were allowed to clot, centrifuged at 2,000 × *g* for 30 min, and the serum frozen at −20°C until analysis.

### Toxicity Monitoring

Blood samples were drawn through the jugular vein for complete blood counts and chemistry panels. These were routinely collected on all 5 days of the week of gene therapy and whenever the patient's clinical condition required further evaluation. Hematologic and hepatic toxicities were graded based on values outlined in Table 1. Dose-limiting toxicities were defined as any grade 4 toxicity according to the National Cancer Institute Common Toxicity Criteria version 2.0 or any hematologic or biochemical abnormality defined as severe toxicity (see Table 1).

### ELISA

Murine IL-12 was measured quantitatively in serum and tumor samples using the Mouse IL-12 ELISA Kit (R&D Systems, Inc., Minneapolis, MN). The tumor biopsies were weighed, homogenized in PBS, and murine IL-12 levels measured in the supernatant and serum according to the instructions in the kit.

### Expression of Green Fluorescent Protein

Biopsies taken after transfection with the adenoviral vector containing eGFP were snap frozen in liquid nitrogen. Sections were cut on a microtome within 24 h and immediately imaged on a fluorescence microscope (MPS microscope, Carl Zeiss, Hanover, MD) for evidence of protein expression (fluorescence).

### Cell Lysis, RNA Isolation, and cDNA Synthesis

For real-time PCR, tumor tissue samples were lysed and RNA isolated using TRIzol Reagent (Invitrogen) per manufacturer's protocol. RNA purity was assessed spectrophotometrically. Total isolated RNA was treated with DNase I (Invitrogen, Carlsbad, CA) to remove any genomic

**Table 1. Toxicity grading**

| System | Normal | Mild toxicity | Moderate toxicity | Severe toxicity |
|---|---|---|---|---|
| Hematologic | | | | |
| WBC ($\times10^3/\mu L$) | 5–17 | 3–5 | 2–3 | <2 |
| Granulocytes ($\times10^3/\mu L$) | 4–10 | 2–4 | 1–2 | <1 |
| Platelets ($\times10^3/\mu L$) | 150–500 | 80–150 | 40–80 | <40 |
| PCV (%) | 28–50 | NA | 15–28 | <15 |
| Hepatic | | | | |
| ALT (IU/L) | 17–59 | 60–150 | 151–400 | >400 |
| Alk. phos (IU/L) | 2–40 | 41–120 | 121–400 | >400 |
| Albumin (g/dL) | 2.8–4.5 | 2.2–2.8 | 1.8–2.2 | <1.8 |
| Bilirubin (mg/dL) | 0–0.2 | 0.2–2.0 | 2.0–2.5 | >2.5 |

NOTE: The hematologic and hepatic toxicity grading criteria used in this study for cats were based on values modified from the National Cancer Institute Common Toxicity Criteria version 2.0.

Abbreviations: PCV, packed cell volume; ALT, alanine aminotransferase; Alk. phos, alkaline phosphatase.

DNA. cDNA synthesis was then carried out using Superscript II RNase H⁻ Reverse Transcriptase (Invitrogen). Each reaction mixture contained 10 μL of RNA solution to which was added 4 μL first strand buffer (5×), 1 μL deoxynucleotide triphosphate (10 mmol/L), 1 μL DTT (0.1 mol/L), 0.25 μL RNase Out (40 units/μL), 0.25 μL SuperScript II (200 units/μL), 2 μL random hexamers (300 ng/μL), and 1.5 μL DNase, RNase–free water, giving a total of 20 μL per reaction mixture. This was incubated at 42°C for 50 min, following which 30 μL of DNase, RNase–free water were added to the mix and the enzymes inactivated by placing the reaction tubes on a 95°C heat block for 5 min. The cDNA was then stored at −20°C until the time of RT-PCR.

### Real-time PCR

Due to lack of standardized commercially available feline IL-12 ELISA kits, real-time RT-PCR was used to detect and quantitatively express the production of feline IL-12, IFN-γ, and glyceraldehyde-3-phosphate dehydrogenase mRNAs.

Sequences for the primers and probes were obtained from the literature (20) and purchased from MWG Biotech (High Point, NC).

The reporter dye attached covalently at the 5′ end was FAM (6-carboxyfluorescin) and the quencher bound to the 3′ end was TAMRA (6-carboxytetramethylrhodamine). Real-time PCR was done using the Applied Biosystems ABI Prism 7000 (Foster City, CA). The amplification protocol was 2 min at 50°C, 10 min at 95°C, 45 to 50 cycles of 15 s at 95°C, and 60 s at 60°C. Cycle threshold values were obtained from the ABI software and the $2^{-\Delta\Delta Ct}$ method was used to determine the relative expression of the genes of interest (21). Briefly, a standard housekeeping gene (e.g., *GAPDH, β-actin*, or *$\beta_2$-microglobulin*) is chosen as an internal control gene. This serves to normalize the amount of cDNA loaded for each reaction. An untreated control is selected as the ''calibrator.'' The relative expression data are then obtained as the fold change in gene expression normalized to the chosen endogenous reference gene and relative to the untreated control.

Before using the $2^{-\Delta\Delta Ct}$ method for relative quantification, we compared its use for the primer and probe sequences and PCR conditions being used in our experimental conditions to published results (21, 22). Briefly, the target cDNA was serially diluted and real-time PCR done. Cycle threshold values for the serially diluted samples are plotted as a function of the log dilution. The slope of the line is used to obtain the efficiency of PCR amplification using the following formula: efficiency = $10^{(-1/\text{slope})} - 1$.

## Results

### Feasibility Studies Using Ad Hsp Murine IL-12, Ad CMV eGFP, and Ad Hsp eGFP

Preliminary studies using eGFP were done to show the feasibility of this approach in 16 cats with fibrosarcomas. The tumors were injected with either the eGFP-containing construct alone (CMV or hsp promoter) or a combination of the eGFP construct and a murine IL-12–containing construct. This was done to assess the feasibility of the proposed clinical trial and to establish the injection technique. One of the concerns before initiation of the trial was whether there would be sufficient numbers of viable cells remaining after the radiotherapy course. Thus, it was important to determine whether viral uptake was possible in this previously irradiated tumor tissue. Detection of murine IL-12 by ELISA or GFP by fluorescence (Fig. 1) in the tumor samples was done to ascertain that there were viable cells infected and these cells were capable of protein production. A total of 16 cats with soft tissue sarcomas were studied in three cohorts at dose levels of $10^9$ (four cats), $10^{10}$ (six cats), and $10^{11}$ (six cats) pfu of adenovirus per tumor. Some cats received both the murine *IL-12* gene and the *eGFP* gene. Results from the murine IL-12 study are shown in Fig. 2, with solid circles representing the tumor murine IL-12 levels in picograms per milliliter and open squares showing the serum levels in picograms per milliliter. Maximum levels of murine IL-12 protein were seen at 48 h post-hyperthermia in the tumor samples. Circulating murine IL-12, as measured in the serum samples, remained low in all cases.

### Toxicity from Preliminary Trials Using Adenovirus Murine IL-12 ± eGFP Construct

No toxicities were noted in the lower-dose groups or with the injection of eGFP. Additional cats were treated at $10^{10}$ pfu to obtain a minimum of three cats that received murine IL-12 alone (no eGFP adenoviral vector was administered concurrently); due to scheduling, a total of four cats were treated at this dose level. Due to insufficient virus dose in two cats, a total of five cats were included in the highest dose group (one cat received 0.75 × 10e11 and one cat received 0.9 × 10e11). At the highest dose group for the

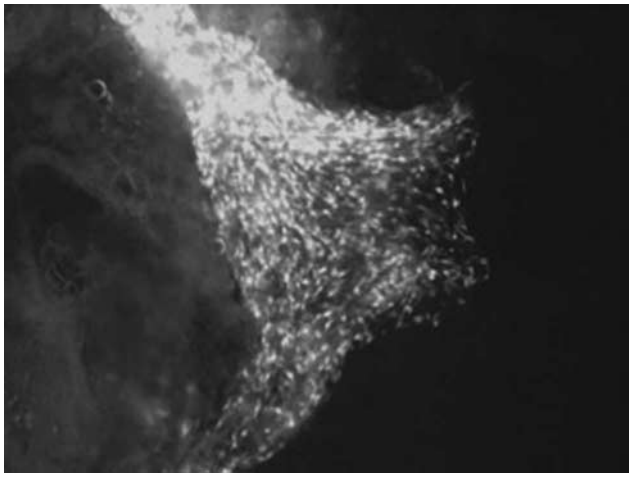

**Figure 1.** Expression of eGFP. After completion of radiation therapy, the tumors were injected with Ad CMV eGFP. The tumors were then biopsied and examined for green fluorescence. Detection of green fluorescence indicated that, postradiation, there were viable cells present in the tumor capable of protein production.

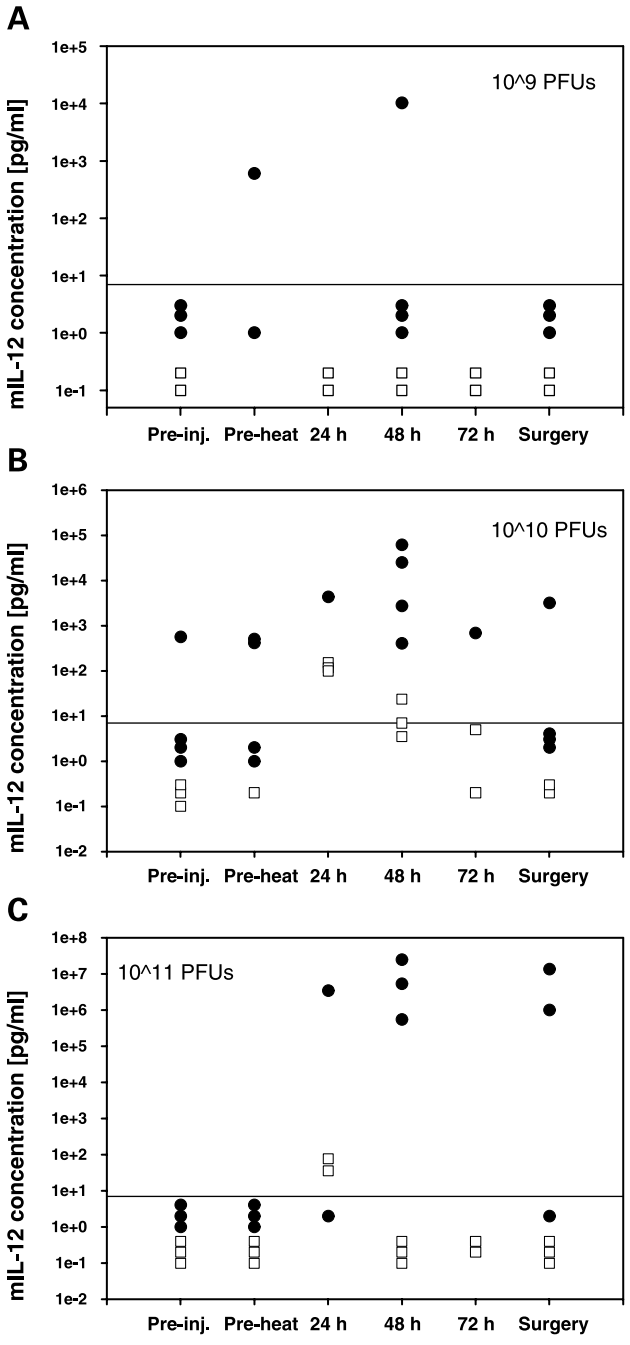


**Figure 2.** Tumor and serum levels of murine IL-12. Murine IL-12 (*mIL-12*) levels were measured in the serum (□) and homogenized tumor samples (●) using ELISA at various time points. The units were picogram per milliliter for the serum and picogram per milligram for the tumor. *Horizontal line,* lower limit of detection of IL-12 in this assay. **A,** $10^9$ pfu/tumor; **B,** $10^{10}$ pfu/tumor; **C,** $10^{11}$ pfu/tumor.

murine IL-12 construct ($10^{11}$ pfu), the only toxicity noted were mild febrile episodes that responded to maintenance fluid therapy and piroxicam (antipyretic) administered orally at a dose of 0.3 mg/kg once.

### Phase I Clinical Trial Using Adenovirus Hsp Feline IL-12 Construct

All cats completed treatment as outlined in the protocol receiving 48 Gy in 16 fractions over 3 weeks followed by gene therapy. One cat, however, had a protocol deviation. This cat had high fever in the 24 h following the gene injection ranging from ~104°F to 105°F. The temperature did not decrease with oral prednisolone and i.v. fluid therapy. Twenty-four hours after treatment, the fever was reduced after administration of i.v. dexamethasone. Hyperthermia treatment was deferred for 24 h in this patient.

The study was designed as a standard phase I trial, with three cats per dose group. The number of cats per dose cohort was adjusted based on the observation of dose-limiting toxicities. An additional cat was treated in the $10^{10}$ pfu cohort due to simultaneous recruitment at both Colorado State University and North Carolina State University. In the highest dose group ($4 \times 10^{10}$ pfu/tumor), almost all cats had clinically nonnormal courses. Refusal to feed, lethargy, pulmonary edema, and hepatic and hematologic toxicities were observed and management in the critical care unit was required for periods of up to 2 weeks. However, no treatment-related mortality was seen and eventually all cats returned home to their owners. After noting two dose-limiting toxicities in cats at $4 \times 10^{10}$ pfu/tumor, the final cat was treated at the maximum tolerated dose of $10^{10}$ pfu.

Table 2 summarizes hyperthermia data for all cats. $T_{50}$ and $T_{90}$ signify the temperatures exceeded by at least 50% or 90% of the measured intratumoral points. $T_{min}$ and $T_{max}$ are the minimum and maximum temperatures recorded during the 60 min of treatment. The target temperatures ($T_{90}$ ≤40°C and $T_{50}$ <44°C for 60 min) were achieved in almost all cases. Four cases had $T_{90}$ >40°C but <41°C.

Table 3 summarizes dose levels, tumor volumes, injection sites, and levels of cytokine production. The tumor sizes varied from 1.1 to 130 $cm^3$, requiring 2 to 38 injection sites. A high degree of variability was noted in the fold increase of feline IL-12 mRNA as detected by RT-PCR. However, there was some indication of its dependence on tumor size within a particular dose cohort. Production of IFN-γ, the downstream cytokine, was also not reflective of the fold increase in feline IL-12 mRNA. Only at the $4 \times 10^{10}$ pfu/tumor dose cohort was IFN-γ mRNA unequivocally detected in four of five cases.

Table 4 summarizes the worst grade of hematologic or hepatic toxicity noted during the week of gene therapy. The viral dose seemed to be associated with detectable IFN-γ levels and consequently with clinical toxicity.

## Discussion

Effective eradication of established tumors and generation of a systemic immune response with a simple gene delivery system are important goals for cancer gene immunotherapy. Cytokine genes are the most widely and extensively studied immunostimulatory agents in cancer gene therapy (1, 23) In several studies, IL-12 was effective in inducing the

**Table 2. Hyperthermia data**

| Cat no. | $T_{50}$ (°C) | $T_{90}$ (°C) | $T_{min}$ (°C) | $T_{max}$ (°C) |
|---|---|---|---|---|
| 1 | 40.4 | 39.0 | 39.0 | 44.6 |
| 2 | 41.1 | 39.4 | 39.4 | 43.2 |
| 3 | 41.0 | 38.6 | 38.6 | 45.3 |
| 4 | 40.7 | 39.9 | 39.9 | 43.0 |
| 5 | 41.5 | 40.6 | 39.8 | 43.5 |
| 6 | 40.8 | 38.9 | 36.6 | 45.2 |
| 7 | 41.3 | 40.1 | 37.0 | 42.4 |
| 8 | 40.9 | 37.5 | 35.4 | 45.7 |
| 9 | 41.5 | 39.5 | 38.3 | 44.2 |
| 10 | 40.9 | 39.9 | 38.7 | 42.2 |
| 11 | 41.5 | 40.4 | 39.2 | 44.0 |
| 12 | 41.5 | 40.2 | 39.5 | 43.2 |
| 13 | 41.1 | 39.8 | 39.5 | 42.7 |

NOTE: The target temperatures were $T_{90} \leq 40°C$ and $T_{50} < 44°C$. These were achieved in 9 of 13 cats. Four had $T_{90} > 40°C$ but $<41°C$. Temperatures as high as 45.7°C were also reached at some isolated points in the tumors for short durations.

eradication of experimental tumors, preventing development of metastases, and eliciting long-term antitumor immunity (24, 25). Depending on the tumor model, IL-12 can exert antitumor activities via T cells (12, 26–29), natural killer cells (30–34), or natural killer–like T cells (35). In addition to its immunostimulatory properties, IL-12 possesses antiangiogenic effects, thus inhibiting tumor formation and metastases (30, 36). Induction of cytokines, such as IFN-γ (26, 37–40) and IFN-inducible protein-l0 (41), which have antiangiogenic properties, has also been implicated as a mechanism of antitumor activity of IL-12.

Local and systemic administration of IL-12 protein has been studied in various murine models (26, 27, 33, 42) and in phase I/II human trials (43, 44). However, systemically administered human recombinant IL-12 is associated with severe toxicity, even at doses as low as 1 μg/kg/d (45). Common toxicities include fever/chills, fatigue, nausea, vomiting, and headache. Routine laboratory changes include anemia, neutropenia, lymphopenia, hyperglycemia, thrombocytopenia, and hypoalbuminemia. Dose-limiting toxicities include oral stomatitis and liver function test abnormalities, predominantly elevated transaminases. IFN-γ has been implicated as the cytokine directly responsible for IL-12 toxicity (46, 47). Pulmonary edema has also been reported in mice (46).

Local and efficient expression of IL-12 may avoid systemic toxicity of recombinant cytokines (28, 48, 49). Intratumoral injections of adenoviral vectors or IL-12 plasmid DNA, naked or in complex with cationic lipid (50), have been studied as a means to deliver therapeutic IL-12 with the rationale that IL-12 will be produced only in the infected tumors, thereby reducing systemic side effects. A few reports have indicated the efficacy of this approach (51–53). However, elevated systemic transgene levels are still observed in many cases as adenovirus can reach the circulation (54–56) and infect other organs such as liver, spleen, and lungs, and the promoters in most previous reports are constitutively active, such as CMV-based promoters. This combination makes it likely that intratumoral injection of this constitutively active adenovirus approach will still result in toxicity (57, 58).

Our approach involved localizing gene expression using intratumoral injection of the adenoviral construct with the gene of interest (*IL-12*) placed under the control of the inducible hsp followed by local tumor hyperthermia to spatially and temporally control IL-12 production. Prior studies in preclinical models (2, 3) have shown that this

**Table 3. Cytokine production data**

| Cat no. | Dose level (pfu/tumor) | Tumor volume ($cm^3$) | Injection sites | IL-12 mRNA (relative fold increase over baseline, 24 h post-HT) | IFN-γ mRNA (relative fold increase over baseline, 24-48 h post-HT) |
|---|---|---|---|---|---|
| 1 | $10^9$ | 130.0 | 38 | 2.7 ± 0.3 | Trace (+/−)* |
| 2 | $10^9$ | 1.1 | 2 | 19,000† ± 5,233 | Trace (+/−) |
| 3 | $10^9$ | 26.0 | 15 | 97 ± 7 | 6.2 ± 0.9 |
| 4 | $10^{10}$ | 2.5 | 2 | 3,028 ± 114 | Trace (+/−) |
| 5 | $10^{10}$ | 1.6 | 2 | 1,685 ± 184 | Trace (+/−) |
| 6 | $10^{10}$ | 12.4 | 16 | 358 ± 30 | Trace (+/−) |
| 7 | $10^{10}$ | 2.3 | 2 | 3,841 ± 643 | Trace (+/−) |
| 8 | $4 \times 10^{10}$ | 5.0 | 10 | 1,318 ± 312 | 42 ± 20 |
| 9 | $4 \times 10^{10}$ | 31.7 | 27 | 7,043‡ ± 1145 | 1.7 ± 0.2 |
| 10 | $4 \times 10^{10}$ | 1.5 | 5 | 29,565 ± 9,208 | Trace (+/−) |
| 11 | $4 \times 10^{10}$ | 37.0 | 20 | 14,658 ± 2,055 | 152 ± 34 |
| 12 | $4 \times 10^{10}$ | 28.8 | 25 | 2,793 ± 489 | 6.2 ± 2.5 |
| 13 | $10^{10}$ | 1.9 | 4 | 1,858 ± 691 | Trace (+/−) |

NOTE: Details of the tumor volumes and number of injections for the cats treated at various dose levels are shown. The IL-12 and IFN-γ mRNA levels were measured using RT-PCR in the tumors at 24 h and 24 to 48 h post-hyperthermia (post-HT) respectively.

*IFN-γ production detectable in some PCR wells as increase in the real-time amplification curve, but not unequivocal.

†Forty-eight hours post-hyperthermia; only one extra tumor biopsy was possible due to small tumor size.

‡Cat febrile after gene injection on Monday for ~12 h.

**Table 4. Hematologic and hepatic toxicity table**

| Cat no. | Dose level (pfu/tumor) | Hematologic* | | | | Hepatic | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | W | G | Plt | PCV | ALT | ALP | Alb | Bili |
| 1 | $10^9$ | S | Mo | N | Mo | N | N | Mi | N |
| 2 | $10^9$ | Mi | Mi | N | Mo | Mi | N | N | N |
| 3 | $10^9$ | N | N | N | N | N | N | N | N |
| 4 | $10^{10}$ | Mi | Mi | Mi | Mo | Mi | N | N | Mi |
| 5 | $10^{10}$ | Mo | Mo | Mi | Mo | Mi | Mi | N | Mi |
| 6 | $10^{10}$ | Mo | Mo | Mi | Mo | Mi | Mi | N | Mi |
| 7 | $10^{10}$ | Mi | Mi | N | N | Mi | Mi | N | Mi |
| 8 | $4 \times 10^{10}$ | Mi | Mi | S | S | Mo | N | Mi | S |
| 9 | $4 \times 10^{10}$ | Mi | Mi | Mi | Mo | N | N | Mi | Mi |
| 10 | $4 \times 10^{10}$ | Mo | Mi | Mi | N | N | N | N | N |
| 11† | $4 \times 10^{10}$ | Mi | Mi | S | S | Mi | N | Mi | Mi |
| 12 | $4 \times 10^{10}$ | N | N | Mi | S | N | N | S | S |
| 13 | $10^{10}$ | Mi | Mi | Mi | N | Mi | N | N | N |

NOTE: Blood investigations were done during the week of gene therapy and hyperthermia and the hematologic and hepatic toxicity graded in all cases for each parameter based on values outlined in Table 2.

Abbreviations: W, WBC; G, granulocytes; Plt, platelets; PCV, packed cell volume; ALT, alanine aminotransferase; ALP, alkaline phosphatase; Alb, serum albumin; Bili, total bilirubin; N, normal; Mi, mild toxicity; Mo, moderate toxicity; S, severe toxicity.

*Drop in granulocyte counts seen in all cases ~48 to 72 h post-hyperthermia.

†Case also had pulmonary edema.

approach virtually eliminates spurious expression of transgenes in sites outside the heated area and also minimizes systemic levels of circulating IL-12. Gene therapy and hyperthermia were added to radiation therapy and surgery, which are the standard of care modalities for this cancer.

Before starting the clinical trial, *in vitro* studies were done to characterize this construct (15). As a prelude to the proposed phase I clinical trial, we did preliminary studies using murine IL-12 and eGFP constructs. This was done as we could measure tumor and plasma levels of murine IL-12, visualize eGFP fluorescence, and assess the efficacy of this approach. High levels of eGFP and murine IL-12 were seen in tumor samples with limited circulating murine IL-12, indicating that dose-dependent differential expression was achieved between tumor and plasma levels, and this could be exploited to achieve a better therapeutic index for a potentially toxic cytokine such as IL-12.

We used real-time PCR to evaluate the levels of cytokine expression because there were no standardized ELISA kits or antibodies available for detection of feline cytokines and RT-PCR is a sensitive technique enabling the detection of mRNA even in small tissue samples. Ideally, we had planned to biopsy the tumors on all days from day 0 through day 3 to assess the dynamics of cytokine production. In the larger tumors, we were able to achieve that aim and even collect two samples on the same day to have more tissue material available for analysis. However, in the smaller tumors, obtaining adequate tumor tissue was not possible. In those cases, we obtained baseline and 24 h post-hyperthermia samples only. Baseline measurements were obtained in all cases and mRNA levels in biopsy samples from the subsequent days were expressed as fold increases over the baseline.

There was a high degree of variability on the fold increase in feline IL-12 mRNA production. There are several factors that could be responsible for this variability. Multiplicity of infection could be affected by dose of virus injected, volume of normal saline that the construct is diluted in, and number of injection sites. Additional factors could include tumor volume, temperatures achieved during hyperthermia, and percent necrosis of the tumor, which influences the percentage of viable target cells. The site of tumor tissue sampled during biopsy could also influence the estimation of transgene expression because such biopsies were randomly acquired within the treated volume. Variations in canine adenovirus receptor status could influence the transfection efficiency. Finally, fevers could influence the overall level and time course of gene expression. There was a correlation between tumor volume and feline IL-12 expression within the different dose levels. Larger tumors showed less fold increases in feline IL-12 mRNA, compared with smaller tumors when injected with the same amount of virus.

There was no correlation seen between the feline IL-12 production in a tumor and the presence of IFN-γ mRNA. We believe that at least two factors contributed to this. First, IFN-γ is produced by lymphocytes. The treatment sequence of this trial used radiation therapy first and there was only 3 to 5 days between the end of radiotherapy and initiation of gene therapy. The cumulative radiation dose (48 Gy) would have eliminated the lymphocyte pool in the tumors (59), and it is unknown how rapidly that pool would reconstitute. Second, the construct was injected equally over the volume of the tumor. The center of the tumors, especially in the larger sized tumors, would more likely be necrotic. Lymphocytes, if present at all, would be mainly concentrated around the periphery. Possible strategies to increase IFN production could be to perform the gene therapy and hyperthermia before radiation therapy or to inject the construct differentially, with more of the virus in the periphery or margins of the tumor and less in the center.

In our dose escalation study, IFN-γ was not detectable at the lower feline *IL-12* gene therapy doses. Its presence was unequivocally detected only at the $4 \times 10^{10}$ pfu dose level. However, it was interesting to note that all cats that had IFN-γ levels detectable in their tumors experienced systemic toxicities. This suggests that the feline IL-12 produced locally in the tumor caused downstream IFN-γ production, enough of which was being absorbed by the bloodstream to cause some degree of systemic toxicity. The advantage of our approach over straight systemic expression, however, is that the local expression level of the cytokine will be orders of magnitude higher than could be achieved following systemic administration of cytokine.

Blood tests were done on all days of the week of gene therapy to determine whether hematologic or hepatic toxicities developed. Toxicities were nonexistent to mild

in the lowest dose group, but were moderate to severe in the highest dose group. At all dose levels, a drop in granulocyte counts was seen 48 to 72 h post-hyperthermia with a rapid recovery seen by the next day. In some cases, the nadir was below the lower limit for normal and it was qualified as a mild toxicity. In other cases, the nadir remained within the reference range. This rapid transient response could possibly be explained by the similar immune-mediated destruction of lymphocytes by cytokines seen in primate models (60, 61). Similarly, there was a transient increase in liver enzymes and/or total bilirubin seen with rapid recovery. At lower doses, thrombocytopenia and granulocytopenia were common, whereas at higher doses, anemia was also seen in three cases. These anemias required blood transfusions and, in those cases, total bilirubin values were elevated posttransfusion. One cat, treated at the $4 \times 10^{10}$ dose, developed pulmonary edema. This complication, along with refusal to feed and poor general condition, necessitated a 2-week stay in the critical care unit. The cat recovered fully, however, and surgical tumor resection occurred ~6 weeks after gene therapy.

Particular attention was paid in this trial to reducing core body temperature if the cats started becoming febrile. They were closely monitored in the critical care unit with hourly rectal temperatures being recorded. If the temperature reached 39°C (102.2°F), the cage was cooled using a fan and i.v. fluids were started. Any elevation in temperature beyond 39.5°C (103.2°F) was aggressively managed with antipyretics and/or i.v. dexamethasone. These measures were effective in four cases in which fevers were observed.

Borrelli et al. (62) reported sustained high levels of green fluorescent protein, placed under the control of the hsp, with repeated induction using heat at 41°C for 2 h. This study was done *in vitro*. One of our cats provided us with the opportunity to determine if this was also possible in a clinical setting. This cat had a fever that started a few hours after gene injection and persisted overnight despite antipyretic efforts. The core body temperature was 39.5°C to 40.5°C for more than 12 h. Local hyperthermia was not delivered on schedule in this case. A biopsy was collected to assess cytokine production, however. Hyperthermia was delivered 48 h after gene therapy, following clinical stabilization. Feline IL-12 mRNA levels were highest following the fever and decreased afterward even though local hyperthermia was administered. In this particular example, therefore, we were not able to prove that hsp induction was repeatable in the same subject. However, this was a single case and the study was not designed to prospectively test this concept. Additional prospectively controlled studies would be warranted to further examine this concept.

In conclusion, this trial served as a proof of principle that it is possible to deliver IL-12 safely using the hyperthermia-induced gene therapy approach. The trial was done in spontaneously arising soft tissue sarcomas in client-owned cats. Although this patient population posed certain challenges as far as tumor tissue sample collection or procedural flexibility was concerned, it represented the heterogeneity expected in an outbred population of aged individuals and the toxicities observed would be expected to be more representative of human subjects.

We were able to establish the maximally tolerated safe dose ($10^{10}$ pfu). This dose is based on standard definitions of maximally tolerated dose (63) and is consistent with those seen in a similar human phase I clinical trials. The toxicities we saw could be related, to a certain extent, with the IFN-γ production in the tumors.

A limitation in our study was the inability to directly measure tumor or serum levels of the feline cytokines. This was due to the lack of appropriate cross-reactive antibodies to feline cytokines. The preliminary study using the murine IL-12 was able to provide some guideline for what we could expect following administration of the feline gene therapy construct.

Based on the results of this study, we recommend that future studies focus on emphasizing the enhancement of IFN-γ levels after transgene therapy. To accomplish this, efforts need to be undertaken to emphasize stimulation of lymphocytes residing in the tumor. This would necessitate a more complete understanding of lymphocyte populations in tumors before and after radiotherapy. Ultimately, evaluation of the potential for improving radiotherapy response and potential effects on metastatic potential could be conducted under the auspices of either phase II or phase III trials.

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