

Enhancing antimelanoma immune responses through apoptosis

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We examined the feasibility of using tumor apoptosis at accessible sites to enhance antimelanoma immune responses in a model of spontaneous canine melanoma. We show that priming peripheral blood mononuclear cells with apoptotic melanoma cells significantly enhanced autologous and allogeneic lymphokine-activated killing of tumor cells. Since various pathways required for intrinsic apoptosis are often inactivated in melanoma, we used Fas ligand (FasL) overexpression to promote extrinsic apoptosis. FasL induced apoptosis in five of six cell lines. Each of the susceptible lines, but not the resistant one, expressed Fas mRNA. In addition, direct intratumoral administration of FasL DNA to tumor-bearing dogs was safe, with no adverse events reported over 7 days of observation. A reduction of tumor burden was seen in three of five dogs treated. The reduction of tumor volume was correlated with Fas expression by the tumors, although one dog with a Fas-negative tumor survived for 82 weeks after treatment. Our data show that overexpression of FasL is suitable to promote apoptosis of Fas⁺ melanomas, and support the notion that priming immune responder cells with apoptotic tumor cells may enhance antitumor responses. The results also suggest that intratumoral administration of FasL offers a safe route for therapeutic gene delivery.

Cancer Gene Therapy (2003) 10, 726–736. doi:10.1038/sj.cgt.7700625

Keywords: melanoma; apoptosis; Fas; Fas ligand; dogs

Melanomas are tumors that arise from melanocytes or their precursor cells and present as pigmented nodules. The location of these tumors in the dog can be used to predict biological behavior. Tumors on the haired skin are generally benign, whereas tumors in the mouth or nail beds are usually malignant. As is true for its human counterpart, canine malignant melanoma is often incurable due to the inability to manage nonresectable and metastatic forms of this disease.¹ New approaches to treat this cancer are warranted, because the existing therapies for metastatic tumors are inadequate. Furthermore, spontaneous malignant melanoma of dogs provides a suitable model system to evaluate novel therapeutic

approaches for human patients with malignant melanoma because the progression of the disease is similar in these species.

Gene therapy has shown some success in the treatment of melanoma in mice, humans, and dogs,^{2–5} and “priming” through induction of apoptosis also has been shown to enhance immune responses against both viral and tumor antigens.^{6–8} Previous data indicate that various pathways that promote intrinsic apoptosis are commonly inactivated in canine melanoma.⁹ Consequently, pro-apoptotic genes that can activate the cell death program irrespective of the genetic make-up of the tumor would be needed to develop a feasible treatment protocol that will re-establish the capacity of tumor cells to undergo apoptosis. In addition, recent data suggest that vigorous inflammatory responses at the site of a primary tumor might promote an antitumor response that can prevent growth of distant metastases.^{10,11} Forced overexpression of Fas ligand (FasL) appears to fulfill these criteria.^{10–14} Ligation of Fas by FasL, or by anti-Fas antibodies that

Received September 7, 2002.

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promote Fas receptor multimerization (probably formation of trimeric receptor complexes), results in formation of a death-inducing signaling complex (DISC), which initiates a cascade of events that culminate in activation of caspases 8 and 9 and lead to apoptosis.¹⁵ Some cells are refractory to DISC formation, but in these cells ligation of Fas can also promote apoptosis by amplification of the signal through mitochondrial "activation".¹⁵ The potent proapoptotic properties of FasL make it a desirable agent for cancer therapy. Our group and others have shown that malignant cells that do not express Fas, or that express Fas mutants with signaling defects and are resistant to FasL-dependent apoptosis *in vitro*, fail to form tumors in mice if they are transduced with membrane-bound FasL.^{10,11,13,16–21} The unusual antitumor properties of FasL, therefore, appear to offer a major advantage in its application for cancer therapy. For this study, we examined the capability to induce apoptosis of canine melanoma cells *in vitro* by overexpression of FasL, and we assessed the safety of *in vivo* administration of FasL in a Phase I clinical trial that included five dogs with spontaneous malignant melanoma (World Health Organization (WHO) stage III or IV).

Methods

Cells and cell lines

The CML-2, CML-13, JEN, SCO, SHA, BEAR, and TLM1 melanoma cell lines have been described previously.^{2,22} Cell lines were also derived from tumor explants from each dog enrolled in the *in vivo* safety trial.²³ The tumors were aseptically minced and mechanically disrupted, digested with trypsin, and separated through a fine mesh to produce a single-cell suspension. These cells were cultured in DMEM containing 10% fetal bovine serum (FBS). Canine peripheral blood mononuclear cells (PBMC) were isolated from anticoagulated whole blood obtained from healthy dogs using discontinuous density gradient centrifugation over Ficoll-Hypaque.²⁴ Paired cell lines and PBMC that were cryopreserved in liquid nitrogen were available from two melanoma-bearing dogs (JEN, BEAR) that participated in a previous gene therapy trial.² To prepare PBMC for cytotoxicity assays, cryopreserved cells were thawed to 37°C, sorted for viability, and cultured overnight as described below. The L1210-Fas cell line is a derivative of the L1210 murine lymphoma cell line engineered to overexpress the Fas receptor. L1210-Fas cells were maintained in RPMI-1640 media supplemented with 10% FBS, 2 mM L-glutamine, 2 mM sodium pyruvate, and 10 mM N-2-hydroxyethyl piperazine-N-N-2-ethane sulfonic acid (HEPES) at a concentration of 250,000 cells/ml.

Chemicals and reagents

Tissue culture materials were obtained from BD-Falcon (Bedford, MA); chemicals were obtained from Sigma (St Louis, MO) unless otherwise specified. Human recombi-

nant interleukin-2 (IL-2) was obtained from Chiron Corp (Emeryville, CA) and Hoffman-La Roche, Inc. through the Biologic Response Modifiers Program, Division of Cancer Treatment, National Cancer Institute (Frederick, MD).

Cell killing assays

Melanoma cell killing mediated by canine PBMC was performed as described.²⁴ For allogeneic cytotoxicity assays, PBMC from healthy dogs were cultured (primed) in the presence of live CML-2 cells, serum-deprived apoptotic CML-2 cells,²⁵ or in the absence of melanoma cells (unprimed) for 20 hours. For autologous cytotoxicity assays, PBMC from each melanoma-bearing dog available for this study (JEN and BEAR) were cultured (primed) in the presence of live cells or serum-deprived apoptotic cells derived from their own tumor, or in the absence of melanoma cells (unprimed) for 20 hours. Primed and unprimed PBMC were then cultured with 5×10^3 ⁵¹Cr-labelled target cells in the presence or absence of IL-2 for 18 hours. For the autologous cytotoxicity assays, the recovery of viable PBMC was limiting; thus, killing was examined only at an effector-to-target ratio of 25:1. Assessment of FasL-dependent cytolysis was performed as described.²¹ Melanoma cell lines were removed from culture flasks by incubating at 37°C in a trypsin-free chelating solution (135 mM NaCl, 5 mM KCl, 20 mM HEPES, and 1.5 mM EDTA, pH 7.4). These cells were cultured with ⁵¹Cr-labelled L1210 cells for 16–20 hours. Percent-specific lysis was calculated using the following formula: $(e-s/m-s) \times 100$, where *e*, *s*, and *m* equal the amount of radioactivity released from melanoma cells incubated with effector cells (experimental lysis), with 100 μ l medium instead of effector cells (spontaneous lysis) or with 100 μ l of 1% Triton X-100 (maximum lysis), respectively. For assays where multiple effector-to-target ratios were used, data were normalized to lytic units. One lytic unit is the number of effector cells required to kill 1×10^3 target cells.

FasL gene delivery

Murine FasL was delivered into canine melanoma cells by adenovirus-mediated transduction (Ad-mFasL).²¹ Cell monolayers were trypsinized and washed once with standard growth medium. Cells were resuspended in 500 μ l of medium containing the indicated ratio of replication-deficient viral particles (plaque-forming units or PFU) of Ad-mFasL per cell. Cells were incubated for 1 hour in a 37°C water bath with periodic mixing and were washed twice with medium prior to replating at a density of 1×10^5 /ml. Human FasL was transfected into the cells using cationic liposomes (Dosper, Roche Diagnostics, Chicago, IL).²⁶ A measure of 3 μ g of the green-fluorescent protein (GFP) expression vector encoding hFasL was mixed with 9 μ g of Dosper in 100 μ l of HEPES-buffered saline and incubated for 15 minutes at 25°C. The empty GFP vector was used as a negative control. These mixtures were added in 1 ml of OptiMEM serum-free medium (Gibco BRL) to melanoma cells (3×10^5) that

had been adhered to six-well plates by an overnight incubation. The cell–DNA–liposome mixtures were incubated for 6 hours at 37°C, followed by the addition of 1 ml of complete media and incubation overnight at 37°C. FasL expression was verified by the ability of transduced or transfected cells to kill L1210-Fas mouse lymphoma cells.

Gene expression

Expression of Fas mRNA was determined by RT-PCR. Total RNA was isolated from melanoma cells and PBMC using the RNAwiz kit (Ambion, Austin, TX) as per the manufacturer's instructions. Total RNA (1 µg) was converted to first-strand cDNA using the First Strand Synthesis kit from Roche. For assessment of Fas expression, primers were designed based on conserved sequences of Fas in humans and mice: 5'-catgccatggG-GACCCAGAATACCAAGTGCAGATG-3' (sense) and 5'-ggactagtGGTGTGCTGGTGAGTGTGCATTC-3' (antisense), where lower case nucleotides represent restriction sites for cloning. Primers were mixed at a concentration of 5 pM/µl with 10 µl cDNA in 10 × PCR buffer (PCR Core Kit, Roche). Amplification was performed using an annealing temperature of 53°C for 30 cycles. The

identification of the amplification product as a partial canine homologue was verified by sequencing. The Genbank accession number is AF536812.

Clinical trial design

The main goal of this trial was to evaluate safety of intralesional administration of the FasL gene, with limited estimates of efficacy as a secondary objective. In order to accomplish this with the smallest number of animals possible, we used a sequential testing strategy aimed at considering early termination in the event that adequate safety was not observed. New participants could only be enrolled in the trial after the results of the previous animal with regard to toxicity (see below) were evaluated. Table 1 outlines the decision-making process for enrollment of each new subject.

Based on the poor outcome for dogs with this disease and the frequency of toxic side effects associated with radiation therapy (part of the standard of care for this disease), we designed the trial to define the toxicity associated with intralesional FasL gene therapy, using the probability that adverse events would occur less than 30% of the time as a benchmark. We calculated the relationship (cumulative probability) of efficacy and the predicted

Table 1 Decision-making process for clinical trial enrollment

Conditional outcome	Decision rule	Rationale
Two dogs show toxic side effects	Consider stopping	The probability of experiencing two dogs positive for toxicity in a row is 0.04 if the toxicity rate is 0.20 and 0.09 if the rate is 0.30 If no other adverse effects are seen and other dogs have been negative prior to two positive dogs, consider continuing; otherwise stop
One of three dogs shows toxic side effects	Consider stopping (after one more dog)	The probability of one in six dogs is 0.393 when the toxicity rate is 0.200 and 0.302 when the rate is 0.30. The probability of one in three when only three dogs are exposed is 0.384 and 0.441 for toxicity rates of 0.20 and 0.30, respectively. Thus, unless other data suggest that the treatment has failed, attempt one more dog. If the result in the third dog is a toxicity, Rule 1 applies, otherwise continue.
If one of four dogs shows toxic side effects	Continue	
If two of five dogs show toxic side effects	Continue	As Rule 1 eliminates the possibility of certain sequences, the only ways two of five dogs can occur (each with a probability of 0.0205 or 0.0309 assuming five trials and toxicity rates of 0.20 and 0.30) are: negative–positive–negative–positive–negative positive–negative–positive–negative–negative positive–negative–negative–positive–negative positive–negative–negative–negative–positive These four combinations would occur over 10% of the time in five dogs with either toxicity rate, so that stopping is considered only if other signs or symptoms suggest failure
If three dogs show toxic side effects	Stop	Only one sequence is possible: positive–negative–positive–negative–positive and this would indicate that the toxicity rate is likely higher than 0.30

number of “safe” events (for 0, 1, 2, ... successes). Success was defined as tumor regression or stable disease (size of tumor less than or equal to the initial size with none or only “acceptable” adverse side effects). Although resulting in a large confidence interval, we determined that a sample size of five to six dogs could provide confidence that the success rate was at least 33%, and possibly as high as 50%, as well as reasonable assurance that the toxicity rate was less than 30%, and provide impetus for additional experimentation.

Toxicity and efficacy measures

Unacceptable occurrence of severe local toxicity or systemic toxicity was evaluated for each dog based on the presence of discomfort (at or near the injection site), pain, necrosis, severe systemic inflammation, acute hepatitis or liver necrosis, renal failure, or myocardial toxicity. Mild inflammatory changes in the complete blood count (CBC) due to the administration of a foreign gene, and “stress leukograms” associated with hormonal influence incited by the hospital environment and the presence of disease (advanced melanoma) were expected and were not considered to be significant in the absence of additional evidence of systemic toxicity. These mild inflammatory changes included neutrophilia ($\leq 25,000/\mu\text{l}$), lymphocytosis ($\leq 10,000/\mu\text{l}$), and/or monocytosis ($\leq 2500/\mu\text{l}$). Stress leukograms would be characterized by neutrophilia ($\leq 30,000/\mu\text{l}$) with lymphopenia ($\leq 1000/\mu\text{l}$). Any change in the CBC beyond these, or in combination with biochemical changes described below that could not be attributed to disease progression, would be considered significant. The activities of alanine aminotransferase (ALT), aspartate aminotransferase (AST), gamma-glutamyl transferase (GGT), and alkaline phosphatase (ALP), along with the levels of total bilirubin, albumin, and cholesterol were used to assess liver function. The following criteria were used as indicators of hepatotoxicity: two-fold elevations of ALT and AST (concurrently), two-fold elevation in ALT concurrent with four-fold elevation in ALP, four-fold elevation in bilirubin concurrent with two-fold elevation in GGT and >2.5 -fold elevation in ALT, or any alteration in enzymes along with decreased albumin and cholesterol. These findings would then trigger an ultrasound-guided liver biopsy, and the histopathological diagnosis would be used to rule out or confirm toxicity, and to enact the stopping rules if applicable. The levels of blood urea nitrogen (BUN), creatinine, phosphorus, and total protein were used to assess renal function. The following criteria were used as indicators of nephrotoxicity: creatinine >1.8 mg/dl, BUN >35 mg/dl, and phosphorus within the reference range. These findings would then trigger evaluation of a new sample within 24 hours. Consistent elevations of BUN and creatinine that could not be attributed to dehydration or disease progression would be considered significant. Hypoproteinemia with severe proteinuria and pyuria was not considered as an indicator of renal damage unless it persisted for more than 2 weeks even with appropriate treatment. Hypopro-

teinemia with severe proteinuria in the presence of renal casts would be considered as a possible indicator of acute renal damage and re-evaluated within 7 days. Persistent changes that could not be attributed to disease progression would be considered significant. The following criteria were used as indicators of myocardial toxicity: auscultation, electrocardiogram, and the activities of creatine phosphokinase (CPK) and AST. The development of murmurs that were not detected during the original physical exam, conduction abnormalities, or concurrent elevations (>2.5 -fold) in CPK and AST (with no changes in ALT) would be considered significant. These findings would trigger radiographic and echocardiographic evaluation of the heart. Evidence of significant change in heart size from baseline, reduced contractility, and other biochemical evidence of heart disease would be considered significant.

These index variables were used to classify a dog as experiencing a toxic result. Thus, if any of the compound parameters for systemic toxicity (liver, kidney, heart, or unacceptable systemic inflammation that could not be attributed to disease progression) showed a significant elevation at either the 3- or 7-day sampling or if an animal died from toxicity attributable to treatment (not due to disease progression), then that dog will be classified as having a toxic reaction. All the data and interpretation by the project investigators and by contract pathologists were subject to review by a Safety Monitoring Committee that included a licensed veterinarian who were not associated with the study. The Safety Monitoring Committee had the power to recommend that stopping rules be activated as needed.

Efficacy was measured based on local effects, including necrosis, inflammation, tumor regression, stable disease, or tumor progression, as well as systemic effects including the disease-free interval and duration of remission.

FasL administration to tumor-bearing dogs

The main criterion for patient inclusion consisted of the presence of measurable oral melanoma (WHO stage I–IV). Five dogs with a provisional diagnosis of oral malignant melanoma (based on clinical signs and cytopathology) were entered into the trial. The characteristics of the research subjects are shown in Table 2. The dog owners were offered participation in the trial as one of the treatment options available that might cause palliation of their dog’s disease. All the procedures associated with this trial were approved by the Institutional Animal Care and Use Committees of AMC Cancer Research Center and Colorado State University, and by the Institutional Review Board (for use of human subjects in research as it pertained to dog owners) of AMC Cancer Research Center. The tumors were staged by imaging studies, histological examination, and evaluation for distant metastasis to regional lymph nodes and lungs (Table 2). Immunostaining was performed by IHC Services (Smithville, TX). Complete blood counts and serum biochemistry profiles were carried out to assess anesthetic risk and to rule out the presence of additional

Table 2 Signalment of dogs enrolled in Phase I clinical trial^a

	Breed	Age (years)	Sex	Tumor location, and WHO stage
Dog 1	Min. Schnauzer	12	FS	Palate, stage III
Dog 2	Mix breed	13	MC	Left mandible, stage III
Dog 3	Mix breed	7	FS	Zygomatic arch extending to occipital bone ^b
Dog 4	Mix breed	11	MC	Palate, stage III
Dog 5	Min Poodle	13	MC	Palate, stage III

^aDogs of any age, breed, and sex with oral melanoma (WHO Stage I–IV) were eligible for enrollment. WHO stage III includes patients where the primary tumor is >4 cm in diameter (T3), or patients with tumors of any size where there are moveable ipsilateral lymph nodes that contain tumor (any T, N1b), but where there are no distant metastases (including distant nodes).

^bA final diagnosis of melanoma was confirmed for dogs 1, 2, 4, and 5. The tumor in dog 3 had morphological features that were consistent with a poorly differentiated, nonpigmented, spindle cell tumor. The differential diagnoses included amelanotic melanoma and osteosarcoma, but the cells did not express markers of melanocytic (Melan A, S100) or osteoblastic (osteocalcin) differentiation, leading to a final diagnosis of poorly differentiated sarcoma.

medical problems. Anesthesia was induced using acepromazine and glycopyrrolate for sedation, followed by catheterization of a cephalic vein and intravenous administration of propofol. Anesthesia was then maintained with inhaled Isoflurane. Routine wedge biopsies of the tumors were obtained. Next, 600 μ g of hFasL-GFP dissolved in 1 mg of cationic liposomes (Lipofectin[®], Life Technologies, Bethesda, MD through an *in vivo* license held by Roche, Palo Alto, CA) was delivered by injection of five to seven sites in and around the tumor in a volume of approximately 1 ml of sterile balanced salt solution (0.9% NaCl) using 22-g needles. Recovery from anesthesia was monitored by certified veterinary technicians and dogs were discharged to their owners on the same day. Narcotic analgesics were used for pain control as needed. Dogs were required to return 3 days after the initial treatment for a full physical examination, complete blood count, and serum biochemistry panels, and 7 days after the initial treatment for a physical exam, complete blood count, serum biochemistry panels, and initiation of standard of care therapy (surgery and/or radiation).

Results

Priming canine PBMC with apoptotic cells enhances antitumor cytotoxicity

We first examined the capacity to induce priming through apoptosis under allogeneic conditions. An effective and reproducible method to induce apoptosis in many cultured cells, including canine melanoma cells, is serum withdrawal for 3–4 days.²⁵ Thus, CML-2 cells were cultured in complete media or in serum-deprived media for 3 days prior to priming. Then, canine PBMC were cultured without priming, or primed by incubation with viable CML-2 cells or apoptotic CML-2 cells for 20 hours, after which primed and unprimed PBMC were cultured with viable ⁵¹Cr-labelled CML-2 cells (targets) to determine the extent of cytotoxicity in the presence or absence of IL-2, adjusting the effector-to-target ratios from 25:1 to 100:1. The results show that, under these

conditions, unprimed canine PBMC exhibited detectable natural killer cell activity toward allogeneic melanoma cells (105 \pm 20.9 lytic units), and that cytolysis was enhanced in the presence of IL-2 (149 \pm 13.9 lytic units), possibly through generation of LAK cells.²⁴ The ability of PBMC to kill CML-2 targets was reduced when these cells were primed using viable CML-2 cells (64 \pm 19.4 lytic units), although the cytolytic activity was restored in the presence of IL-2 (172 \pm 24.4 lytic units). More importantly, the ability of PBMC primed using apoptotic cells to kill the CML-2 target cells was unaffected in the absence of activating cytokines (107 \pm 16.6 lytic units); yet the activity of these cells in the presence of IL-2 was significantly greater ($P < .05$) than that seen for the other conditions tested (274 \pm 34.7 lytic units).

To verify that the priming effects were not simply due to allogeneic reactions (i.e., major histocompatibility complex mismatches between the normal PBMC and the CML-2 cells), we used paired canine PBMC and melanoma cell lines that were available from a previous study.² Viable autologous PBMC were cultured with each cell line under the same conditions described above. The recovery of PBMC from each dog was limiting, so assessment of cytotoxicity was limited to an effector-to-target ratio of 25:1. JEN or BEAR cells were cultured in complete media or in serum-deprived media for 3 days prior to priming. Then, autologous PBMC were cultured without priming, or primed by incubation with the corresponding viable cells or apoptotic cells for 20 hours, after which primed and unprimed PBMC were cultured with viable ⁵¹Cr-labelled targets to determine the extent of cytotoxicity in the presence or absence of IL-2. Figure 1 shows that PBMC from both dogs had only marginal cytolytic activity against the respective autologous melanoma cells, and IL-2 did not enhance cytolysis in either case, possibly due to poor recovery of natural killers cells after cryopreservation. As was seen under allogeneic conditions, the cytolytic activity of these PBMC was not significantly affected by priming with viable tumor cells. In one of the two dogs (BEAR), priming with viable cells significantly increased cytolysis in the presence of IL-2 ($P < .01$). Priming with apoptotic cells alone also did not promote cytolysis of melanoma cells by autologous

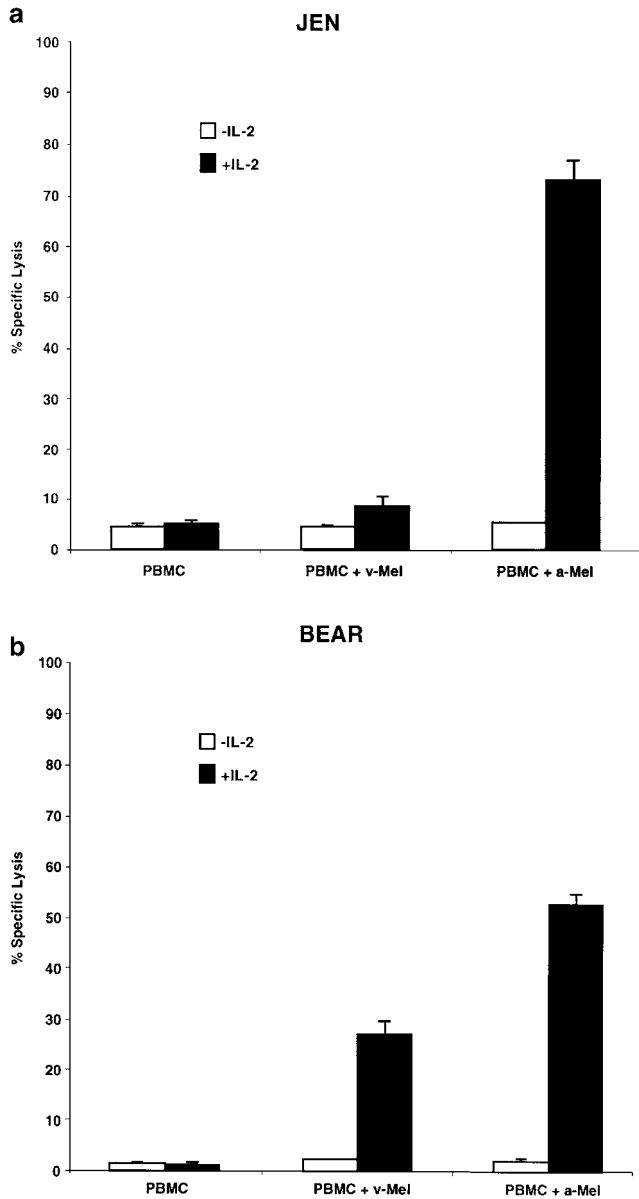


Figure 1 Priming canine PBMC with apoptotic cells enhances antitumor cytotoxicity. PBMC isolated from two dogs with oral melanoma (JEN and BEAR) were incubated alone in complete media, or with viable (v-Mel) or apoptotic (a-Mel) melanoma cells derived from each dog's tumor for 20 hours. The unprimed or primed PBMC (effector cells) were then incubated in the presence of the corresponding autologous melanoma cells (target cells) labelled with ^{51}Cr in the presence or absence of IL-2 for 16 hours. The effector-to-target (E:T) ratio used for these experiments was 25:1; percent-specific cytotoxicity was determined as described in Materials and methods. The cytotoxic activity of PBMC incubated with apoptotic melanoma cells in both dogs, and with viable melanoma cells in the case of BEAR was significantly higher ($P < .005$ and $P < .01$, respectively) than that seen in the control conditions.

PBMC, but PBMC from both dogs primed with apoptotic cells showed significant enhancement of cytolytic activity ($P < .005$) in the presence of IL-2 when compared to every other condition tested.

Induction of apoptosis of canine melanoma cells by FasL

Ligation of Fas with FasL is an effective means to induce apoptosis of many cell types.²⁷ Based on previous data indicating that various molecules that promote apoptosis using the intrinsic pathways are commonly inactivated in canine melanoma,⁹ we examined the capacity to promote apoptosis of these cells by overexpression of FasL as a means to activate the extrinsic pathways.¹⁴ FasL was transduced into six distinct canine melanoma cell lines by infection with replication-deficient adenoviruses encoding murine or human FasL. To ensure that apoptosis was not mediated by the adenovirus vector, cell lines were also transfected with a plasmid encoding human FasL using cationic liposomes,²⁶ as well as with a control adenovirus vector expressing only the GFP tag. Infection of the cell lines was verified microscopically by assessment of GFP fluorescence. As shown previously,^{25,26} melanoma cells transfected with empty vectors showed negligible apoptosis. Each of the transduced (Fig 2a) or transfected (not shown) cell lines expressed detectable levels of FasL as

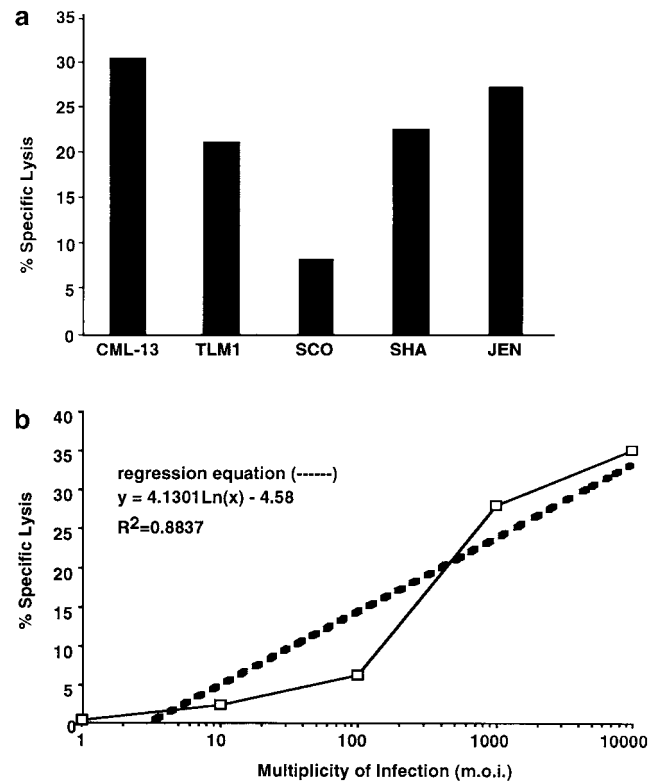


Figure 2 Overexpression of FasL in canine melanoma cells. Adenovirus-mediated transduction of murine FasL was used to overexpress the gene in CML-13, TLM1, Scooter (SCO), Shadow (SHA), and Jenny (JEN) cell lines. A total of 100,000 cells from each line were infected with Ad-m-FasL at an m.o.i. of 1000 viral particles/cell. (a) The expression of FasL was examined in each cell line by its ability to kill Fas-bearing L1210-Fas target cells at an E:T ratio of 0.5:1. (b) Relationship between FasL expression and m.o.i. in the FasL-resistant JEN cell line.

determined by their ability to kill L1210-Fas target cells. However, only five of the six cell lines underwent apoptosis (affecting nearly 100% of the cells) upon expression of FasL as determined by microscopic examination of cellular morphology, whereas one cell line (JEN) was resistant. Increasing the multiplicity of infection (m.o.i.) in this cell line showed that there was a log-linear relationship between m.o.i. and FasL expression to >1000 viral particles per cell (Fig 2b), but the corresponding increase in FasL expression failed to induce apoptosis of this cell line.

Loss of Fas expression confers resistance to FasL-mediated apoptosis

Resistance to FasL-mediated killing could be due to loss of Fas expression by the tumor cells, or to inhibition of signalling pathways that operate downstream of Fas as is the case in the murine Lewis lung carcinoma cell line²⁸ (J Sun and D Bellgrau, unpublished). To investigate the first

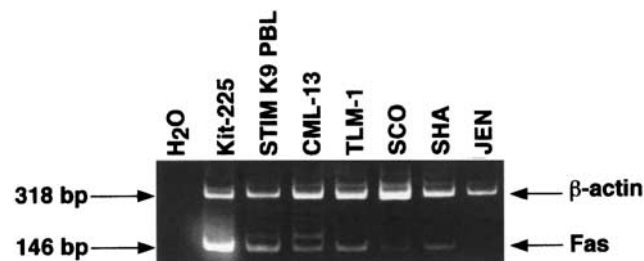


Figure 3 Expression of Fas mRNA by canine melanoma cell lines. Fas expression was examined by RT-PCR as described in Materials and methods. The expression of β -actin was used as a loading control for the reactions and to ensure integrity of the RNA. Normal canine PBMC were harvested without stimulation or were stimulated by PHA followed by restimulation with IL-2 every 3 days for 10 days prior to harvesting. Human Kit-225 human leukemia cells were maintained in culture in the presence of IL-2. The predicted sizes for the Fas and β -actin amplification products were 146 and 318 bp, respectively. The smaller visible bands in the gel represent primer dimers.

possibility, we cloned a fragment of canine Fas that shares ~78% homology with the corresponding Fas fragments of primate, rodent, and ruminant origin. We then used this gene fragment as a marker to analyze Fas gene expression by RT-PCR. Each of four susceptible melanoma lines analyzed had detectable levels of Fas mRNA, but the resistant cell line did not (Fig 3), indicating that Fas expression was required for FasL-mediated apoptosis.

Safety and efficacy of FasL administration to tumor-bearing dogs

The data described above suggest that Fas⁺ melanoma cells are susceptible to FasL-mediated cell killing. Administration of FasL to tumor cells *in vivo* has been shown to mediate both direct (Fas-dependent apoptosis) and indirect (immune-mediated tumor cell killing) tumor cell death.^{10,11,14,18-21} We used a 7-day delay of therapy trial to determine if intratumoral administration of FasL was safe in tumor-bearing dogs. Each of five dogs was given 600 μ g FasL cDNA mixed with 1 μ g cationic liposomes delivered in 1 ml into viable areas of the tumor. Dogs were monitored for 1 hour or until complete recovery and then were re-evaluated at 3 and 7 days after the procedure. There were no adverse events observed over the course of 7 days, and three of five tumors showed measurable regression (Table 3 and Fig 4). Cells isolated from the two tumors showing the most dramatic responses had detectable Fas expression, whereas those isolated from the tumor showing the weakest response and those isolated from a tumor with no measurable response had no detectable Fas mRNA (Table 2 and Fig 5). At that time, each dog was provided standard of care therapy as indicated for each tumor (surgery, radiation, or palliation; Table 4). The median survival for dogs with stage III melanoma that undergo standard of care is as short as 16 weeks, and usually less if the tumor is nonresectable; death is usually disease-related.²⁹⁻³¹ In this group, two dogs achieved complete remission (CR), two dogs achieved partial remission (PR), and one dog had no response. The two dogs that achieved CR died of

Table 3 Clinical response of cancer-bearing dogs treated with FasL^a

	Local toxicity (inflammation at or near injection site)	Systemic toxicity (constitutional signs or changes in hematological parameters)	Measurable response (day 7)	Fas mRNA expression
Dog 1	None	None	Stable disease (no regression or progression)	ND ^b
Dog 2	None	None	58% reduction in tumor volume	Yes
Dog 3	None	None	NA ^c	No
Dog 4	None	None	12.5% reduction in tumor volume	No
Dog 5	None	None	23% reduction in tumor volume	Yes

^aToxicity measures were evaluated immediately after administration of the gene therapy (local reactions), and at 3 and 7 days after treatment (systemic changes). Tumor volumes were measured in three dimensions using graded calipers.

^bND=not done. Biopsy samples were insufficient for analysis and a cell line could not be established.

^cNA=not available. An objective response could not be measured for this dog because there was no visible external tumor, and pretreatment computed tomography images were not obtained.

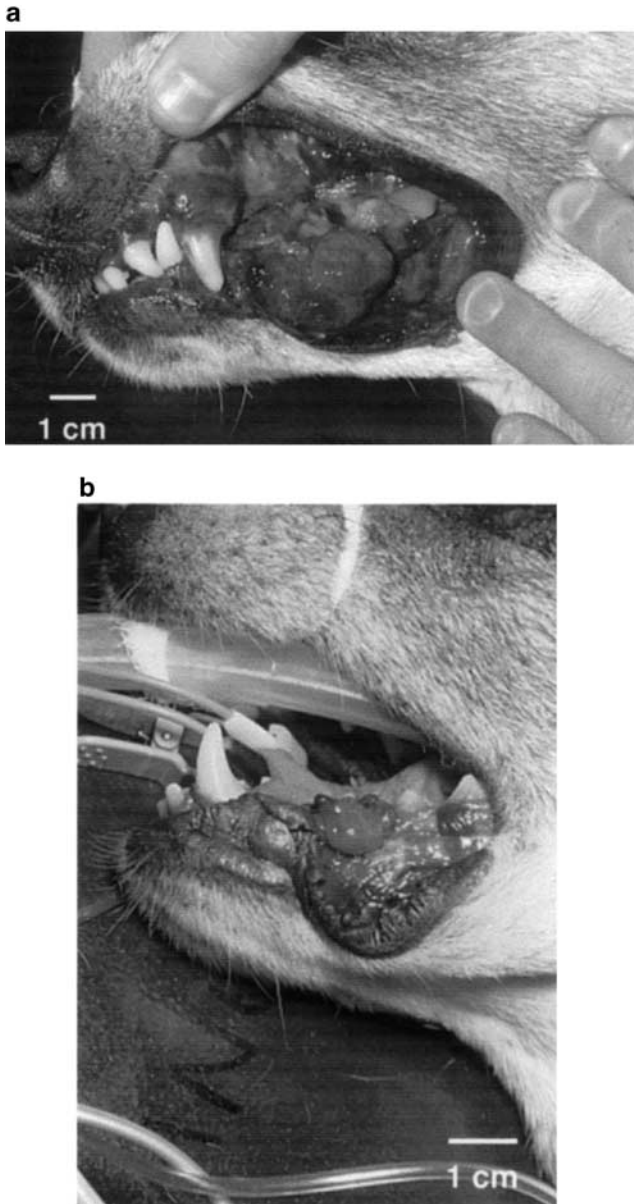


Figure 4 Effect of FasL gene therapy in a dog with oral melanoma. (a) Dog 2 at presentation after the tumor was surgically debulked, and a mass measuring 19 mm × 14 mm × 1 mm was left for administration of gene therapy. (b) Dog 2 at day 7, when the tumor measured 14 mm × 8 mm × 1 mm. The dog was subsequently treated with surgery (hemi-mandibulectomy) and radiation therapy. Bars = 1 cm.

unrelated causes while free of disease at 24 and 44 weeks post-treatment (Table 4). One of the two dogs that achieved PR died of unrelated causes at 13 weeks with stable disease; the other dog that achieved PR showed evidence of progression at 42 weeks. The tumor was again surgically debulked and the dog that remained maintained good quality of life until it died of progressive disease 82 weeks after the initial treatment. While the expression of Fas by the tumors correlated with the initial magnitude of

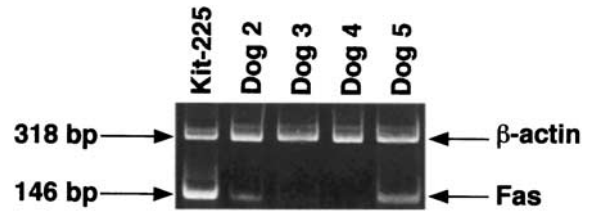


Figure 5 Fas mRNA expression in tumors from dogs treated with FasL gene. Fas expression was examined by RT-PCR as in Figure 2. The expression of β -actin was used as a loading control for the reactions and to ensure integrity of the RNA.

regression, it did not appear to influence response to therapy or survival time. Based on these data, we can say that if the serious adverse event (SAE) rate is truly 30%, the probability that we would observe no SAE is 0.168 and the probability that zero SAE would occur in five dogs if the rate is 40% is 0.078 (7.8% probability that zero of five dogs have a severe reaction).

Discussion

We used malignant melanoma of dogs as a model to evaluate the feasibility of using apoptosis of tumors at accessible sites as a means to enhance antimelanoma immune responses. Malignant melanoma occurs spontaneously in dogs and humans, among other species. Although there are some differences in the initial manifestations of the disease (i.e., the tumor in dogs occurs most commonly in the epithelium of the oral cavity and seems to be unrelated to exposure to ultraviolet light), the progression of disease between humans and dogs is remarkably similar.¹ The disease is highly metastatic in both species, spreading to regional lymph nodes and to the lungs. Also, the response of advanced melanoma WHO stage III or IV) to conventional therapy in these two species is equally dismal. The 5-year survival rate for human patients with disseminated disease is less than 5%;³² the median survival for dogs with stage III melanoma is 14–16 weeks.^{29–31,33} Neither single-agent nor combination chemotherapy is effective to treat malignant melanoma. In dogs, radiation therapy can be palliative and prolong life in cases without metastatic disease.³³ However, in most instances, dogs with oral melanoma (stage I–IV) develop metastatic disease and die within 34–110 weeks of diagnosis. The poor outcomes associated with conventional treatments have prompted investigations of alternative treatments for malignant melanoma, including immunotherapy. Among human cancers, melanoma appears to be uniquely suited to the use of this treatment modality, since this tumor tends to be highly immunogenic.^{4,32} Intriguingly, an immunodominant epitope of the Melan A/MART-1 antigen is conserved in dogs,^{22,34} and as it is in people, its expression may be prognostically significant.³⁵

The use of the biologic response modifiers IL-2 and interferon α (IFN- α) has been evaluated in various human

Table 4 Outcome of cancer-bearing dogs treated with FasL gene therapy^a

	Treatment	Remission	Survival	Cause of death ^b
Dog 1	Radiation	CR	44 weeks (CR)	Diabetic encephalopathy (no tumor)
Dog 2	Hemimandibulectomy+radiation	CR	24 weeks (CR)	Unknown (no tumor)
Dog 3	Palliative (piroxicam)	None	3 weeks (progressive disease)	Euthanasia (disease-related)
Dog 4	Debulking+radiation	PR	82 weeks (recurrence at 42 weeks; progressive disease)	Euthanasia (disease-related)
Dog 5	Debulking	PR	13 weeks (stable disease)	Euthanasia (after trauma, unrelated to disease)

^aFollow-up was carried out by the attending veterinarian and by telephone interviews with the owners.

^bThe cause of death for dogs 1, 3, and 5 was verified by a licensed veterinarian. Dog 2 died while sleeping and a necropsy was not performed. The owners reported no visible tumor recurrence in the oral cavity, no palpable lymph nodes present, and no other signs consistent with metastatic disease (e.g., labored breathing, exercise intolerance, inappetence) at the time of death.

clinical trials, with objective responses observed in 20–30% of patients.⁴ IFN- α is now an FDA-approved adjuvant therapy for patients with resected stage III melanoma. A preclinical study using human recombinant (hr) IL-2 in combination with hr-TNF α documented tumor regression in five of 13 dogs with malignant melanoma, with acceptable toxicity.³⁶ Only one of the five dogs, however, had CR lasting more than 3 years.

Myriad additional approaches are under development for human patients with malignant melanoma, including whole tumor vaccines, synthetic vaccines, peptide vaccines, cytokines, dendritic cell vaccines, and DNA or RNA vaccines.⁴ Preliminary data from these trials suggest variable levels of success ranging from no benefit over conventional therapy to 5-year survival rates as high as 34%. The assessment of immunotherapeutic approaches for dogs with melanoma has been more limited. Three independent trials have examined the delivery of IL-2 or GM-CSF DNA with histoincompatible cells,⁵ in combination with staphylococcal enterotoxin B,² or in autologous tumor vaccines.^{37,38} The objective response rates for these studies were 50, 33, and 20%, respectively. The first two approaches led to median survival times that exceeded the control group or the anticipated survival based on historical data (39 and 24 weeks, respectively).

These data support the use of immunotherapy in the treatment of melanoma. A particularly appealing aspect of this modality is the possibility to use a patient's own immune cells to destroy metastatic or inaccessible tumors. However, a limitation of "traditional" tumor vaccines and treatments directed against specific tumor antigens is that expression of such antigens can be variable in cancer patients. Yet, it may be possible to circumvent this limitation through a treatment approach that induces immunity against antigens that are expressed in the patient's own tumor. We have pursued development of an immunotherapy approach based on the concept that apoptosis and inflammation can be used to enhance immune recognition of endogenous tumor antigens. In principle, induction of tumor cell apoptosis at an accessible site will enhance the load of intact tumor antigens that are processed and presented by APC to

immune effector cells. Moreover, the recruitment of cells that can mount an effective antitumor (type I) immune response could be further enhanced by promoting inflammation as a "danger signal". This in turn might increase the frequency of tumor-specific immune cells that can be activated by systemic immunotherapy to destroy microscopic foci of tumor cells at both the primary site (that might lead to recurrence) and metastatic sites. The mechanistic basis for the use of apoptosis to "prime" the immune system remains incompletely understood. Although it has been suggested that antigens derived from apoptotic cells can silence the immune system as a response that may have adaptive advantages to help avoid autoimmunity,^{39–41} experimental evidence shows that phagocytosis of apoptotic cells by macrophages or dendritic cells can lead to processing and presentation of antigens derived from the apoptotic cells. In two previous studies, it was shown that APC could acquire and present viral antigens from apoptotic cells that led to effective, MHC-restricted CTL responses against viable, virally infected cells,^{6,7} and the principle of priming APCs with apoptotic tumor cells to enhance antitumor immunity was demonstrated in a model of abdominal carcinomatosis in rats.⁸ Syngeneic macrophages were exposed to apoptotic tumor cells or viable tumor cells *in vitro*, followed by injection (of reisolated, purified activated macrophages) into tumor-bearing rats (with abdominal carcinomatosis). Those macrophages that were exposed to apoptotic tumor cells induced a cure in 80% of the animals when combined with systemic administration of IL-2. In contrast, no animals were cured when given macrophages exposed to viable tumor cells even in the presence of IL-2. Furthermore, the cured animals were resistant to subsequent tumor challenge, and spleen cells from these animals showed remarkable cytolytic activity against tumor cells *in vitro*. The use of FasL as a priming agent offers a unique advantage, since it can not only promote apoptosis of susceptible tumor cells, but also has robust proinflammatory properties that promote rejection of Fas-resistant tumors, as well as protective immune responses to subsequent challenge with those tumors.^{10–13,16–18}

For this study, we first examined the effect of priming on canine melanoma cell killing using an established allogeneic system.^{24,42,43} The data indicate that the killing activity of PBMC primed with apoptotic cells alone was similar to that of unprimed cells, but, more importantly, priming PBMC with apoptotic cells increased their capacity to kill viable melanoma cells in the presence of IL-2. These results are consistent with recruitment of additional IL-2-responsive cells upon presentation of antigens derived from apoptotic cells. The data also suggest that IL-2 production may have been the limiting factor for the response. Next, we assessed if these effects were also present in autologous conditions, using paired PBMC and melanoma cell lines established from two tumor-bearing dogs. The data show that under autologous conditions, apoptosis induction still offered a remarkable advantage to prime cytolytic activity of PBMC, albeit without abatement of the requirement for IL-2.

Although apoptosis to promote immunologic “priming” can be easily achieved through various means *in vitro*, the development of this principle into a feasible therapeutic approach would require reliable and reproducible induction of tumor cell apoptosis *in situ* using a treatment that has no or limited toxicity. The observation that various pathways required for intrinsic apoptosis are frequently inactivated in canine melanoma led us to consider the possibility of promoting extrinsic apoptosis in these cells using FasL overexpression. The use of FasL was previously shown to be safe by one of our groups in rodents bearing prostatic cancer xenografts;²¹ moreover, as noted above, Fas expression by the tumors would not be absolutely required for the treatment to be effective. In our experiments, overexpression of FasL induced apoptosis in each of five Fas-positive canine melanoma cell lines. Predictably, a Fas-negative cell line was resistant to FasL-induced apoptosis *in vitro*. In addition, direct intratumoral administration of FasL DNA to tumor-bearing dogs was safe, with no FasL-dependent adverse events reported over 7 days of observation and a reduction of tumor burden seen in three of five dogs treated. This reduction of tumor volume was correlated with expression of Fas by the tumor cells, although four of the dogs, including at least one that had a Fas-negative tumor, had an objective response (CR or PR) after surgery and/or radiation therapy, and the latter dog survived for 82 weeks after the initial treatment. The dog that did not show a response had a poorly differentiated and advanced tumor (bony lysis was evident from the frontal bone and zygomatic arch to the occipital bone) that was unlikely to respond to any available therapy. While it is possible that the expression of FasL at the tumor site may have sensitized tumor cells in the remaining dogs to the effects of radiation, or promoted nonspecific immune or inflammatory responses that contributed to the observed clinical responses, we could not verify these possibilities based on histological examination of the biopsy sections obtained 7 days after injection of FasL DNA. Nevertheless, the data support the notion that priming immune responder cells with

apoptotic tumor cells may enhance antitumor immune responses, and also suggest that overexpression of FasL is suitable to promote apoptosis of Fas⁺ canine melanomas. Moreover, the results also suggest that intratumoral administration of FasL *in vivo* provides a safe route for therapeutic gene delivery.

Acknowledgments

We thank the technical and nursing staffs of the Animal Cancer Center of Colorado State University and Animal Hospital Center for assistance with care and handling of dogs in this study and Drs Erin Dickerson and Valerie Fadok for critical review of the manuscript. This work was supported in part by Grant 1626 from the AKC Canine Health Foundation and Grant 98CA-36 from the Morris Animal Foundation. SRB was supported in part by a fellowship from the University of Colorado Cancer Center.

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